Sergi Bonet · Isabel Casas
William V. Holt · Marc Yeste *Editors*

Boar Reproduction

Fundamentals and New Biotechnological Trends

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Foreword

The field of assisted reproductive technologies continues to be a vibrant and exciting area of scholarly effort. I am pleased to be able to recommend this latest literary contribution to the field which focuses specifically on the boar and is entitled *Boar Reproduction. Fundamentals and New Trends on the Biotechnology of Pig Reproduction*. My colleagues Drs. Sergi Bonet, Isabel Casas, William Holt, and Marc Yeste have all been productive researchers whose output has aided in increasing our body of knowledge in the area of boar reproduction. Along with their direct contributions in this book, these editors have also recruited key expertize to author chapters in order to bring to the readership a truly robust and timely text.

This book shows great breadth and logical sequence in the presentation of materials related to boar reproduction. Initial topics include coverage of classic information regarding the boar reproductive system and both developmental and biological aspects of the spermatozoon. These fundamental topics are next put into context through a current review of both intrinsic and extrinsic factors which can affect reproduction and semen quality in the boar. As in the life of a spermatozoon, a series of chapters follows devoted to gaining an appreciation of the dynamic interactions which occur between the spermatozoon and the female genital tract. Last, the swine industry's active use of assisted reproductive technologies provides a wealth of information which is covered in a concise and informative fashion.

The embodied work should provide great benefit to the reader, whether they are a scientist, educator, professional, manager, or student. It is with the utmost enthusiasm that I endorse this most recent contribution to the field of boar reproduction and spermatology.

Gary C. Althouse
Professor of Reproduction and Swine Health
Marion Dilley and David George Jones
Chair in Animal Reproduction
University of Pennsylvania

Preface

Research into the reproductive biology of domestic animals has made very significant progress in recent years. Moreover, porcine reproductive biotechnology has been and is used as an excellent model for its subsequent applications in humans.

This book aims to provide the scientific community and porcine industry with up-to-date knowledge in the field of boar biology and reproductive biotechnology.

As Director of the 'TechnoSperm' research group (www.technosperm.com, University of Girona) I would like to thank all the authors and editors who have participated in this book with their scientific contributions. Each of them, from their own field of expertize, has contributed knowledge and contrasted it extensively with the most recent data published by other researchers in journals and conferences related to the field of reproductive biology.

TechnoSperm was founded in 1987 and this book is main fruit of the scientific and technical research accumulated by this group over these years, focused principally on swine reproductive biotechnology.

I would like to take this opportunity to express my sincere thanks to all the researchers who, over the years, have formed or are part of TechnoSperm, and also to all researchers of other research groups that have contributed to our scientific and technical training. A very special thanks to Prof. Mercè Durfort Coll (University of Barcelona) and Prof. Josep Egozcue Cuixart († 2006) (Autonomous University of Barcelona), whose passion for Cell and Reproductive Biology I have been fortunate enough to benefit from, since I started my research work.

This book has received the support of ACC10 (Autonomous Government of Catalonia) as part of the program to promote action plans by TECNIO Agents.

Sergi Bonet
Professor of Cell Biology
Director of TechnoSperm
University of Girona

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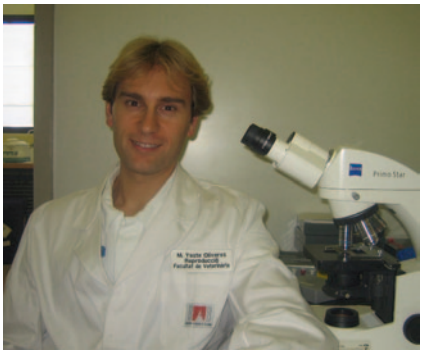
Part III Artificial Insemination (AI) Technologies in Pigs

About the Editors

Sergi Bonet graduated with a Bachelor of Science at the University of Barcelona (1982) and earned his Ph.D. at the Autonomous University of Barcelona (1986). He was Associate Professor at the Autonomous University of Barcelona and he has been Professor of Cell Biology at the University of Girona since 1995. Professor Bonet also served as Secretary-General (1986–2002) and Vice-Chancellor of the Girona University (1995–2002). He started to investigate porcine reproduction in 1987 when he founded 'TechnoSperm', the research group that has led to date. His research has been mainly focused on the epididymal maturation, quality analysis, and cryopreservation of boar spermatozoa, as well as on the histology and physiology of the boar reproductive tract. He has published more than 80 papers in peer-reviewed journals and led more than 60 research projects. In 2010, he was appointed as member of the Royal Academy of Sciences and Arts of Barcelona.

Isabel Casas obtained her Ph.D. in Biology at the University of Girona (2010) after having completed her thesis on the cryopreservation of boar sperm. In 2011, she engaged in a Postdoctoral Fellow at the University College Dublin, and in 2012 in the New Bolton Center from the University of Pennsylvania. Dr. Casas lectured at the University of Girona and assisted as consultant of boar sperm banking for private enterprises. Up to now, she has published 16 papers in peer-reviewed journals and presented more than 20 communications to international congresses. Apart from serving as Editor of this book, she has also authored the Chapter about boar sperm cryopreservation.

William V. Holt obtained his Ph.D. in 1979 at the Royal Veterinary College, when he was appointed Postdoctoral Research Assistant at the Institute of Zoology. Professor Holt was Director of Science of The Zoological Society of London (1999–2001; 2006–2007), where he also led the Reproductive Biology Department (1995–2011). He is currently Visiting Professor of Reproductive Biology at the University of Sheffield and at the Royal Veterinary College (London), and Honorary Professor at the University College London. Professor Holt is also honorary Research Associate at the Smithsonian Conservation Biology Institute (Washington, D.C.). His research has been focused on sperm–oviduct interactions, development of computerised system to measure sperm motility, analyses of sperm populations and sperm cryopreservation not only in pigs but also in other mammalian species. He has published more than 150 papers in peer-reviewed journals. He chaired the British Andrology Society between 2004 and 2008.



Marc Yeste graduated with a Bachelor of Science and Master of Biotechnology degrees and earned his European Ph.D. in 2008. He also obtained the Bachelor in Political and Social Sciences at the Spanish Open University (UNED). Dr. Yeste was Associate Professor of Cell Biology at the Faculties of Medicine and Science at the University of Girona (2007–2010) and Visiting Researcher at the Institute of Zoology (Zoological Society of London, UK) (2006–2007). He is currently Postdoctoral Research Fellow 'Juan de la Cierva' at the Department of Animal Medicine and Surgery of the Autonomous University of Barcelona, where he also lectures Animal Reproduction. Up to know, he has published more than 30 papers in peer-reviewed journals and has co-authored more than 80 communications to International Congresses. His research has been focused on boar sperm preservation in liquid and frozen storage, the effects of boar feed on semen quality, and on the sperm interactions with oviductal and epididymal epithelial cells in in vitro co-culture. Apart from serving as Editor of this book, he has also written the four chapters about the stay of boar sperm in the uterine and oviductal environments.

Part I
Boar Spermatozoa Within the Male Genital Tract

Chapter 1
The Boar Spermatozoon

M. Dolors Briz and Anna Fàbrega

Abstract The microscopic appearance of the boar spermatozoon allows us to appreciate both its inner and outer structural complexity. Both light and electron microscopy may be used to study the structure and ultrastructure of this highly specialized cell and the way it probably works to achieve successful fertilization. Compartmentalization of the spermatozoon is a critically important feature of its structure as it enables this cell to perform the variety of tasks needed to fulfill its role. Different sperm malformations usually affect some cellular components essential for the correct development of the spermatozoon–oocyte interaction in the fertility process. Careful assessment of sperm morphology may sometimes indicate the possible cause of sperm quality and fertility decrease. Moreover, regional specialization of the plasma membrane, related to lipid/protein composition and distribution, allows the underlying cellular molecules to interact independently with their external environment, thereby enabling the efficient performance of the various tasks necessary for successful fertilization.

1.1 Introduction

The practice of artificial insemination (AI) in pigs has undergone a major expansion in the past two decades, mainly due to its greater advantages in comparison to natural mating, and the increased trend toward industrialized large porcine livestock production in contrast with small farms. AI practice only provides acceptable results in terms of fertility and prolificity when boar ejaculates exhibit excellent sperm quality. Sperm morphology is an essential criterion for the assessment of sperm fertilizing ability; thus, proper evaluation of the morphological characteristics, and especially of sperm malformations, represents a fundamental part of a routine seminal analysis of boar ejaculates (see Sect. 11.3.3).

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A careful assessment of sperm morphology may indicate, in some cases, the likely cause of sperm quality and fertility decrease (Bonet et al. 1992, 1995; Briz et al. 1996).

In livestock production, particularly in AI and in selection and multiplication centers, the presence of infertile or subfertile boars has great negative repercussions because, in principle, between 10 and 15 females can be inseminated with the ejaculate of a single male; for this reason, in these porcine production centers the study of sperm morphology of boar ejaculates becomes a routine control and, besides being a male fertility indicator, it is used as a basic parameter for the etiological diagnosis of infertility and subfertility. There is a wide range of factors capable of affecting the sperm morphology of a boar ejaculate, basically, dysfunctions of the reproductive system (testicular, epididymal, or sex gland pathologies), genetic factors (breed, congenital pathologies), environmental factors (temperature, humidity, photoperiod), and/or husbandry factors (nutrition, socialization, sperm handling, frequency of semen collection) (see Chap. 4). Sometimes, it is possible to establish a correlation between the agent responsible for infertility or subfertility and the presence of a high percentage of a specific sperm malformation. In this sense, for example, a high frequency of semen collections without resting periods often results in a fertility decrease produced by the presence of a remarkable rise in immature spermatozoa, of spermatozoa with tails bent at the annulus and of spermatozoa with coiled tails in the ejaculate (Bonet 1987, 1990; Bonet and Briz 1991b; Briz et al. 1993; Pruneda et al. 2005).

The accurate assessment of images obtained by means of light and electron microscopy allows us to appreciate the extreme structural complexity of both the surface and the inside of the boar spermatozoon (Figs. 1.1 and 1.2) (Bonet and Briz 1991a; Briz et al. 1993, 1995; Briz 1994; Bonet et al. 1994a, b, 2000, 2006). As with many other mammalian spermatozoa, the boar spermatozoon is a highly specialized cell with a characteristic surface morphology containing different cellular compartments, as clearly seen through its different ultrafine cross-sections, which can be examined in great detail under transmission electron microscopy (Fig. 1.2). At first glance, the ejaculated boar spermatozoon is a cell with a compact head, an acrosome associated with the nucleus, a well-developed mitochondrial sheath and coarse or outer dense fibers in the tail (Figs. 1.3, 1.4, 1.5, 1.6).

Different sperm malformations usually affect some of the cellular structures (acrosome, nucleus, mitochondria, axoneme, plasma membrane, etc.) (Figs. 1.7, 1.8, 1.9, 1.10, 1.11, 1.12, 1.13, 1.14, 1.15, 1.16) essential for the correct development of the spermatozoon–oocyte interaction in the fertility process (Bonet 1990; Bonet and Briz 1991b; Bonet et al. 1993); if any one of these malformations occurs in an important number of the ejaculated spermatozoa, the male is likely to be subfertile or, in the worst of cases, infertile.

The boar sperm plasma membrane, as in all other animal cell types, is a continuously limiting cell boundary that serves to maintain cell integrity, and which forms a dynamic interface between the cell and its immediate environment. Despite this, and in contrast to many other cell types, both the structure and function of the sperm plasma membrane is highly heterogeneous with different sharply

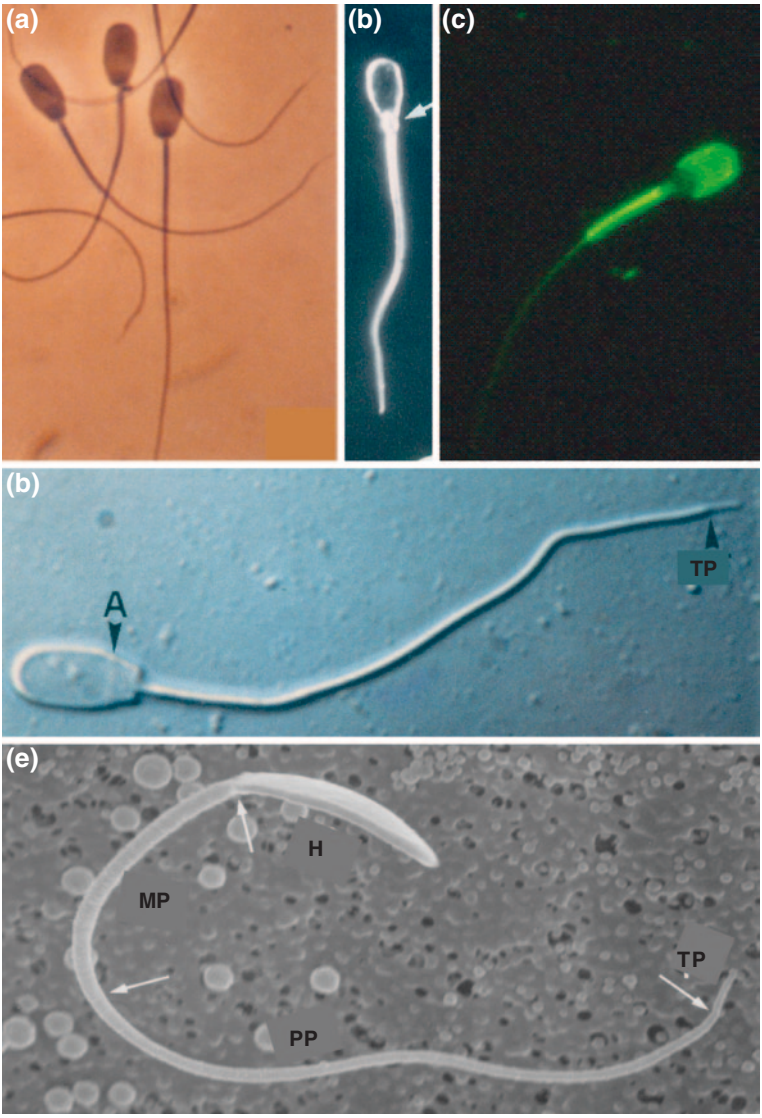


Fig. 1.1 Light (**a**–**d**) and electron (**e**) micrographs showing the general structure of the boar spermatozoon. **a** Mature spermatozoa; positive phase-contrast light microscopy (×1,400). **b** Immature spermatozoon with proximal cytoplasmic droplet (*arrow*); dark-field light microscopy (×1,000). **c** Mature spermatozoon; fluorescence light microscopy (×1,600). **d** Mature spermatozoon; *A*, acrosomal vesicle limit; terminal piece (*TP*); Nomarski interference contrast light microscopy (×2,700). **e** Side view of a mature spermatozoon (*arrows point to the boundaries between several cell regions*); head (*H*); midpiece (*MP*); principal piece (*PP*); terminal piece (*TP*); scanning electron microscopy (×5,800)

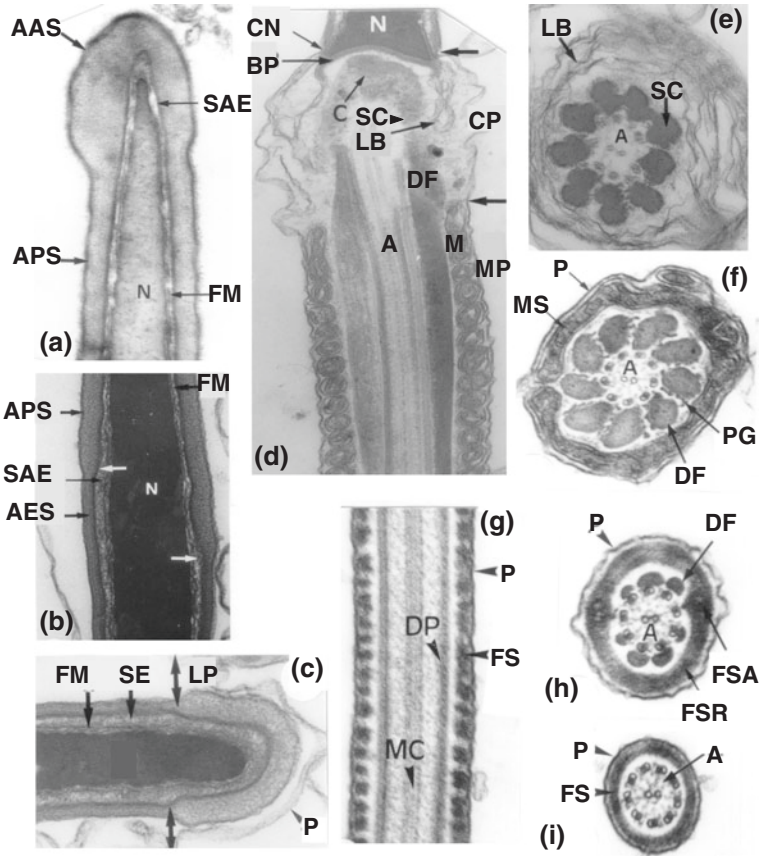
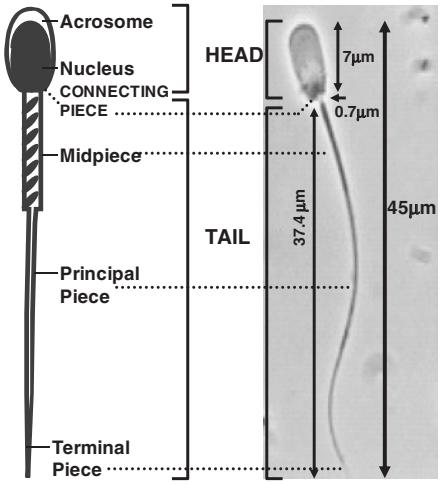


Fig. 1.2 Transmission electron micrographs showing the ultrastructure of the boar spermatozoon in its major parts: head (**a**–**c**), connecting piece (**d**–**e**) and tail (**f**–**i**). **a** Sagittal section through the apical (*AAS*) and principal (*APS*) segments of the acrosome (×80,000). **b** Sagittal section of the acrosome principal (*APS*) and equatorial (*AES*) segments (*white arrows point to the limit between these two segments*) (×80,000). **c** Cross section at the lateral protuberance (*LP*) level of the acrosome equatorial segment (*double arrows indicate the limit of the LP*) (×90,000). **d** Longitudinal section of the connecting piece (*CP*) (*arrows point to the limits of the CP*) (×70,000). **e** Cross section through the connecting piece (×85,000). **f** Cross section through the midpiece (*MP*); notice the nine peripheral microtubule doublets and the central microtubule pair of the axoneme (*A*) (×80,000). **g** Longitudinal section of the principal piece (*PP*) (×80,000). **h** Cross section through the mid-anterior region of the principal piece (×80,000). **i** Cross section through the mid-posterior region of the principal piece (×80,000). axoneme (*A*); basal plate (*BP*); capitulum (*C*); circular neckline (*CN*); dense fibers (*DF*); peripheral microtubule doublet (*DP*); perinuclear fibrous material (*FM*); fibrous sheath (*FS*); fibrous sheath axes (*FSA*); fibrous sheath ribs (*FSR*); laminar bodies (*LB*); mitochondria (*M*); central microtubule pair (*MC*); mitochondrial sheath (*MS*); nucleus (*N*); plasmalemma (*P*); peripheral granules (*PG*); subacrosomal space (*SAE*); segmented columns (*SC*)



DIMENSIONS OF NORMAL MATURE BOAR SPERMATOZOA	Length (µm)	Width / Diameter (µm)
Whole spermatozoon	45.0	-
Head	7.0	3.7 (0.4[a])
Connecting piece	0.7	0.5
Tail	37.4	-
midpiece	9.0	0.7
principal piece	26.2	0.4
terminal piece	2.2	0.2

[a]Thikness

Fig. 1.3 Diagrammatic representation of the major parts of normal mature boar spermatozoa and their typical dimensions. The head contains the cell nucleus and the acrosome and the tail can be divided into three regions: the midpiece, the principal piece, and the terminal piece. Between head and tail there is a short linking segment, the connecting piece (or neck). Every part and region presents characteristic dimensions

defined membrane domains. Extensive biochemical studies have shown that the mammalian sperm surface is organized into lipid domains (with differences in membrane fluidity and lipid composition) significantly different from those in somatic cells and, that most sperm plasma membrane proteins also reveal a high degree of mosaicism (with differences in intramembranous particle distribution and membrane surface antigens and charge). This regional specialization, evidenced by biochemical and immunological parameters, reflects both the specific surface properties and the unique structure of this membrane. Moreover, membrane domains established during passage through the male reproductive tract are not fixed, but undergo reorganization during the capacitation process within the female reproductive tract (see Chap. 7). Thus, after the many modifications occurring in the testis, epididymis and oviduct, mammalian spermatozoa must be capable of fertilizing the oocyte (Thaler and Cardullo 1995; Curry and Watson 1995). The surface of boar sperm is in fact highly heterogeneous and has a molecular ordering that reflects the polar distribution of the main organelles (acrosome, nucleus, and mitochondria) and the cytoskeletal elements that lie under the surface (annulus, fibrous sheath and axoneme) (Phelps et al. 1990; Gadella et al. 1995). In this sense, different domains of the sperm surface (up to five) can be distinguished with separate functions in the fertilization process (Brewis and Gadella 2010). These various membrane domains differ in their binding affinity for lectins, thereby reflecting differences in the extent and composition of their glycocalyx (Fig. 1.17) (Fàbrega et al. 2011a, b).

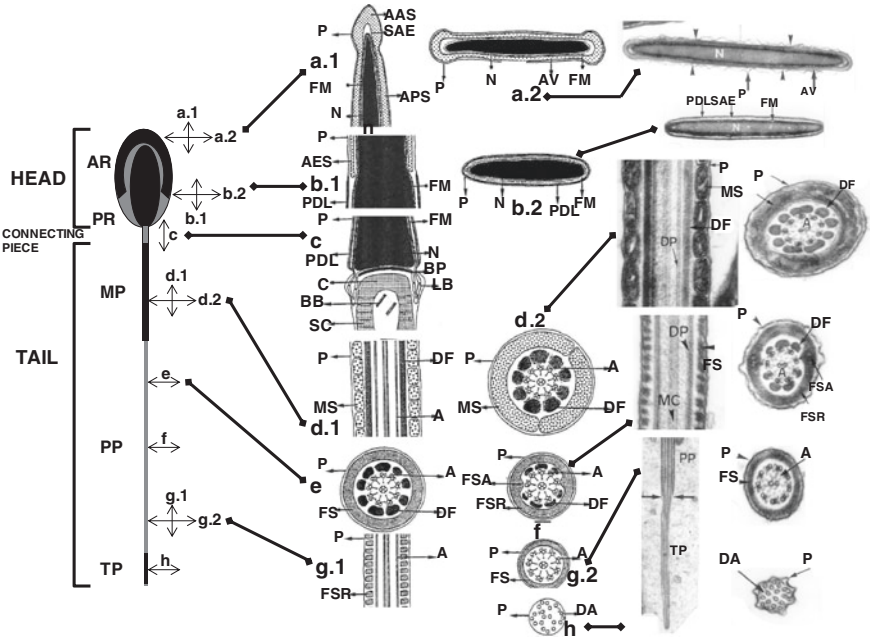


Fig. 1.4 Diagrammatic representation and micrographs of longitudinal and cross sections through the head (**a**–**b**), connecting piece (**c**) and tail (**d**–**h**) of the boar spermatozoon illustrating the main components. **a.1** Apical and principal segments of the acrosomal region (*AR*); **a.2** acrosomal region; **b.1** equatorial segment of the acrosomal and postacrosomal region (*PR*); **b.2** postacrosomal region; **c** postacrosomal region and connecting piece (or neck); **d.1** and **d.2** midpiece (*MP*); **e** proximal region of the principal piece (*PP*); **f** Intermediate region of the principal piece; **g.1** and **g.2** distal region of the principal piece; **h** terminal piece (*TP*). Axoneme (*A*); acrosomal vesicle or acrosome (*AV*); acrosome apical segment (*AAS*); acrosome equatorial segment (*AES*); acrosome principal segment (*APS*); axoneme (*A*); basal body (*BB*); basal plate (*BP*); capitulum (*C*); circular neckline (*CN*); disorganized axoneme (*DA*); dense fibers (*DF*); peripheral microtubule doublet (*DP*); perinuclear fibrous material (*FM*); fibrous sheath (*FS*); fibrous sheath axes (*FSA*); fibrous sheath ribs (*FSR*); laminar bodies (*LB*); mitochondria (*M*); central microtubule pair (*MC*); midpiece (*MP*); mitochondrial sheath (*MS*); nucleus (*N*); plasmalemma (*P*); postacrosomal dense lamina (*PDL*); peripheral granules (*PG*); subacrosomal space (*SAE*); segmented columns (*SC*)

1.2 The Ejaculated Spermatozoon

This section describes the general and detailed traits of mature boar spermatozoa present in normal ejaculates (Figs. 1.1–1.6).

1.2.1 General Structure and Function

The general structure of spermatozoa responds to their basic function, to reach and fertilize the oocyte, thereby becoming specialized for their reproductive role.

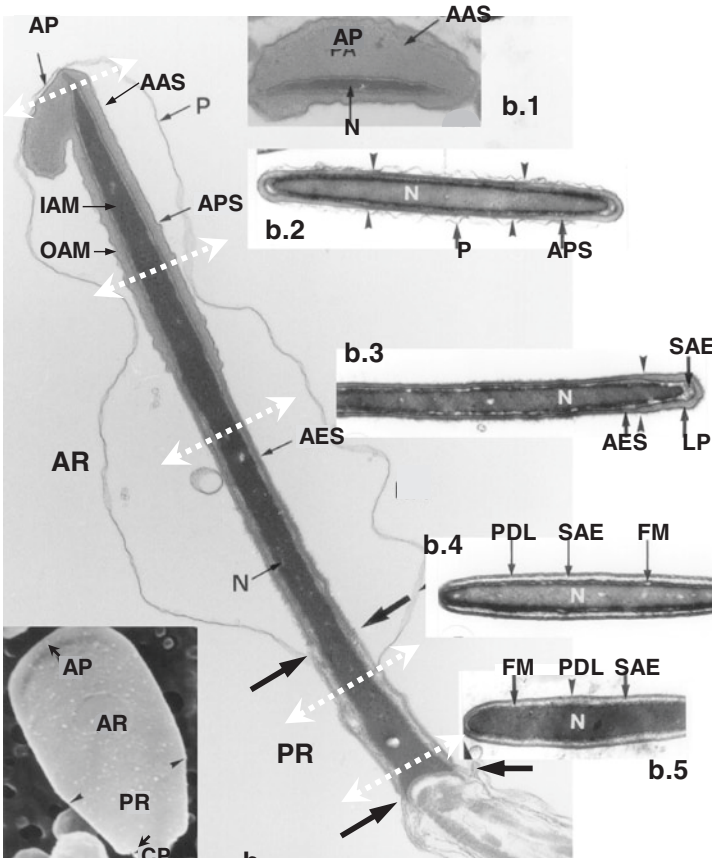
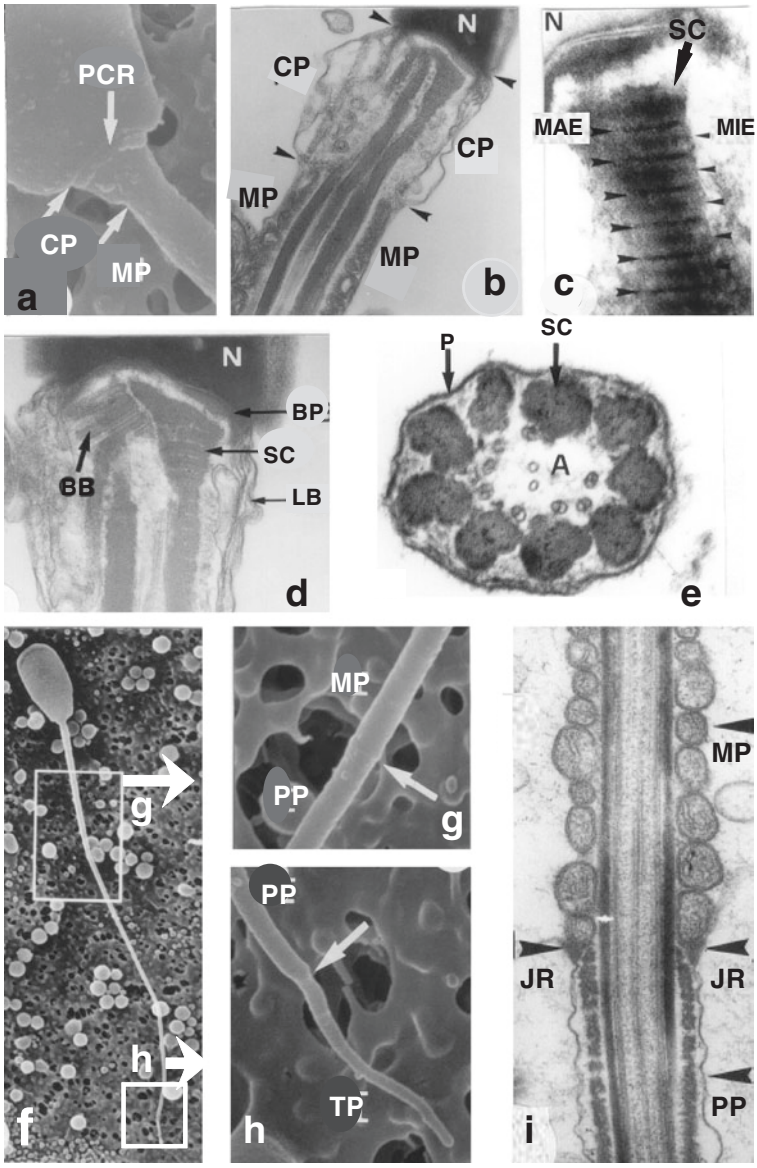


Fig. 1.5 Scanning (**a**) and transmission (**b**) electron micrographs showing the ultrastructure of the head of a mature boar spermatozoon. **a** Detail of a mature spermatozoon head; the *arrows* point to the boundaries between several spermatozoic regions: acrosomal protuberance (*AP*), acrosomal region (*AR*), postacrosomal region (*PR*) and connecting piece (*CP*). (×11,400). **b** Midsagittal section through the mature spermatozoon head; the *black thicker arrows* point to the limit between the connecting piece (*CP*), the postacrosomal region (*PR*) and the acrosomal region (*AR*). The plasmalemma (*P*) is detached from the acrosomal region and firmly adhered to the postacrosomal region (small changes in the osmolarity of the media used for the process of the seminal samples provoke this plasmolysis that, when slight, only affects the acrosomal cephalic region). Notice the following structures: acrosome apical segment (*AAS*); acrosome principal segment (*APS*); acrosome equatorial segment (*AES*); inner acrosomal membrane (*IAM*); outer acrosomal membrane (*OAM*) and nucleus (*N*) (×30,000). The *white arrows* indicate the orientation and the level of the sections depicted in figure **b.1–b.5**. **b.1** Cross section through the acrosome apical segment (*AAS*) at the level of the acrosomal protuberance (*AP*) (×31,500). **b.2** Cross section through the acrosome principal segment (*APS*) showing that the acrosome is slightly thinner in the central zones of the two faces of the head; the *arrowheads* indicate the limit of these differences in thickness of the acrosome. (×30,000). **b.3** Cross section of the acrosome equatorial segment (*AES*) showing that in this region the acrosome is thicker in the lateral zones of the head (lateral protuberance, *LP*); the *arrowheads* indicate the beginning of the lateral protuberance. Notice the subacrosomal space (*SAE*) underlying the lateral protuberance is highly developed (×30,000). **b.4** Cross section of the postacrosomal region closer to the acrosomal region and, **b.5** *closer to the connecting piece*. Notice the postacrosomal dense lamina (*PD*), the subacrosomal space (*SAE*) and the perinuclear fibrous material (*FM*) (×30,000)

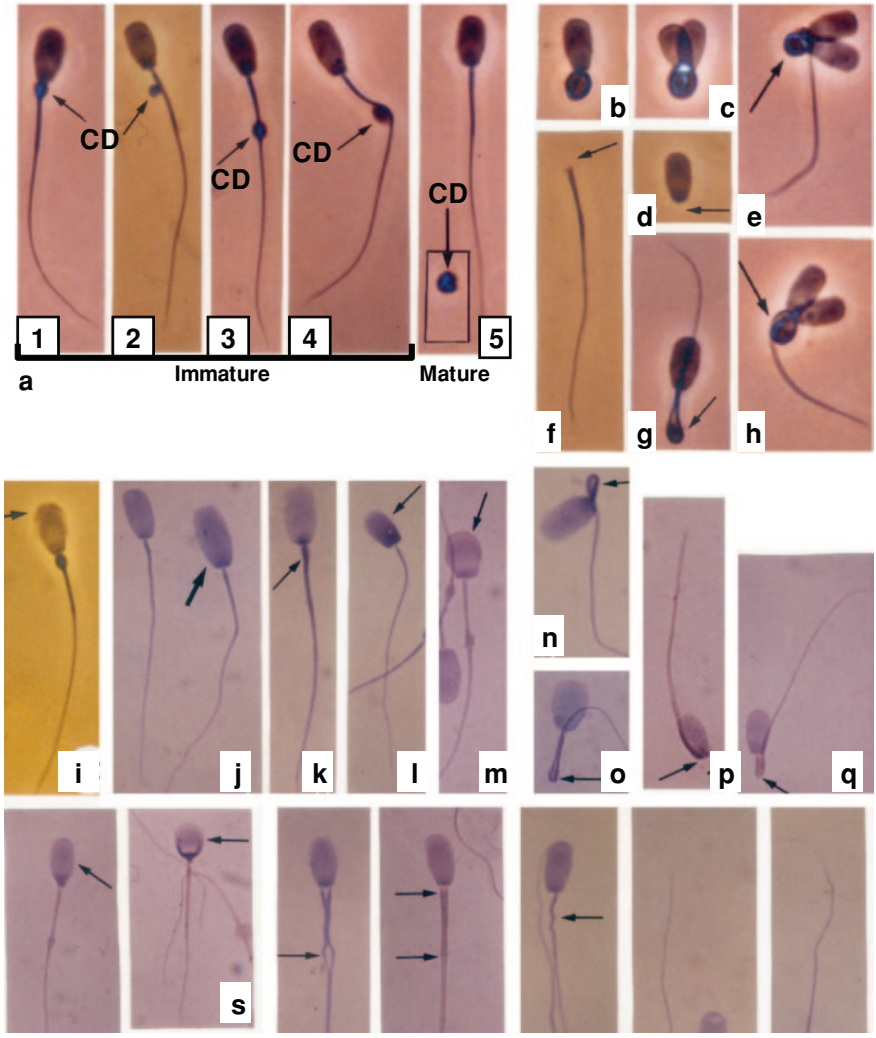


Spermatozoa are small in size and are highly mobile cells, containing the DNA that the male will contribute to the next generation and little more than the elements required to move toward the oocyte and achieve fertilization. Many details in the structure and function of spermatozoa are determined by their own genome (most of the sperm phenotype is controlled by the diploid genotype of the male), and possibly this is a component of the mechanism whereby speciation is controlled (Austin 1995). The spermatozoon has developed a highly specialized morphology

◀ **Fig. 1.6** Scanning (**a**, **f**–**h**) and transmission (**b**–**e**, **i**) electron micrographs showing the ultrastructure of the connecting piece and the tail of a mature boar spermatozoon. **a** Detail of the connecting piece (*CP*) of a mature spermatozoon; notice the postcephalic ring (*PCR*) (×10,000). **b** Longitudinal section of the connecting piece (*CP*); the *arrowheads* point to its limits (×40,000). **c** Longitudinal section through the segmented columns (*SC*); the *arrowheads* show the periodicity of the major (*MAE*) and minor striation (*MIE*) (×110,000). **d** Longitudinal section of the connecting piece; detail of the basal plate (*BP*), basal body (*BB*), segmented columns (*SC*) and laminar bodies (*LB*) (×65,000). **e** Cross section through the connecting piece at the mid-posterior region level (without laminar bodies); notice the microtubules of the axoneme (*A*) (×100,000). **f** General view of a mature spermatozoon; the framed areas are enlarged in figures **g** and **h**. **g** The arrow points to the junction of the midpiece (*MP*) and the principal piece (*PP*) of the spermatozoon tail (×14,000). **h** The *arrow points* to the limit between the tail principal (*PP*) and terminal (*TP*) pieces (×14,000). **i** Longitudinal section through the limit between the midpiece (*MP*) and the principal piece (*PP*) of the spermatozoon tail; notice the Jensen's ring (*JR*) prevents the mitochondria from migrating down the principal piece (×30,000). Plasmalemma (*P*); nucleus (*N*)

with its various structural components tailored to specific aspects of function. At first glance, as in many other mammal species, the boar ejaculated spermatozoon can be divided into two major parts: (1) the head containing the cell DNA (haploid nucleus) and the mechanisms for sperm-oocyte recognition and subsequent fusion (acrosome) and (2) the tail concerned with sperm motility being the site of energy production (it contains mitochondria that generate the energy necessary for movement) and the propulsive apparatus for the initiation and maintenance of cell motility (axoneme); both regions can be further subdivided into a number of cellular components, each with its own functional correlate (Figs. 1.3 and 1.4). This compartmentalization is a critically important feature of the sperm structure enabling this cell to perform the variety of tasks it must undertake. The structural and ultrastructural characteristics of the different organelles and cytoskeletal elements of the boar ejaculated spermatozoon will be described in the following sections.

The mature boar spermatozoon is an elongated cell of about 43–45 μm in length (Briz 1994; Holt et al. 2010) with two major distinguishable regions, the head and the tail, separated by a short linking segment called the connecting piece (or neck). The head is bilaterally flattened and oval shaped, with the following dimensions: 7 μm in length, 3.7 μm at its widest point and 0.4 μm in thickness. The two surfaces of the head are not exactly the same; while one is almost completely flat, the other has a half moon-shaped apical protuberance (or apical ridge), 0.4 μm in width and extending 1.2 μm along the edges of the head. The tail has a filamentous and cylindrical shape and can be subdivided into three major regions: the midpiece (or mitochondrial region), the principal piece and the terminal piece. The midpiece is 9 μm in length and 0.7 μm. in diameter; the principal piece is 26.2 μm in length and 0.4 μm in diameter; finally, the terminal piece is 2.2 μm in length and 0.2 μm in diameter. The connecting piece is 0.7 μm in length per 0.5 μm in thickness and has a trapezoidal shape with the wider base (1.3 μm) in contact with the head and the narrower base (0.6 μm) toward the midpiece; a small ring-shaped protuberance (or postcephalic ring) can be observed in the zone closer



to the head (Fig. 1.6a) (Briz 1994; Bonet et al. 2000). Each of these regions and pieces has a distinct anatomy directly related to its function (Figs. 1.3 and 1.4).

1.2.2 Ultrastructure

The ultrastructural study is helpful because the electron microscope can actually display the structural elements of small cells like spermatozoon that light microscopy cannot reveal, i.e., there are a large number of ultrastructural abnormalities of boar ejaculates that can only be seen directly with electron microscopy. The two

◀ **Fig. 1.7** Positive phase-contrast (**a–i**) and bright-field (**j–z**) light micrographs showing immature, mature, and aberrant boar spermatozoa. **a** Migration and release of the residual cytoplasmic droplet (*CD*) during the process of epididymal sperm maturation: (*1*) immature spermatozoon with proximal CD; (*2*) immature spermatozoon with intermediate CD; (*3*) immature spermatozoon with distal CD; (*4*) immature spermatozoon with distal CD bending the tail before shedding the droplet; (*5*) mature spermatozoon just after having shed the CD. **b** Spermatozoon with intensely coiled tail. **c** Bicephalic spermatozoon with intensely coiled tail. **d** Tailless spermatozoon. **e** Bicephalic spermatozoon with tail coiled at the midpiece displaying a moderate intensity coiling (→). **f** Acephalic spermatozoon. **g** Immature spermatozoon with distal cytoplasmic droplet and tail folded at the Jensen's ring level (→). **h** Bicephalic spermatozoon with tail coiled displaying high intensity coiling (→). **i** Immature spermatozoon with damaged acrosome (→). **j** Mature spermatozoon and macrocephalic spermatozoon (→). **k** (→). Macrocephalic spermatozoon with thickened tail (→). **l** Microcephalic spermatozoon (→) with slightly shortened tail. **m** Immature spermatozoon with distal cytoplasmic droplet and round head (→). **n** Macrocephalic spermatozoon with tail folded at the midpiece level (→). **o** Macrocephalic spermatozoon with roundish head and tail folded at the Jensen's ring level (→). **p** Spermatozoon with tail folded at the connecting piece level (→). **q** Spermatozoon with tail folded at the midpiece level (→). **r** Immature spermatozoon with distal cytoplasmic droplet and a slightly thin head (→). **s** Spermatozoon with small and round head (→). **t** Spermatozoa displaying different intensities of tail coiling. **u** Spermatozoon with two fused tails (→). **v** Spermatozoon with two completely fused tails; notice the limits of the midpiece (→). **x** Spermatozoon with corkscrew defect affecting the midpiece (→). **y** Spermatozoon with tail folded at the midpiece (→). **z** Immature spermatozoon with proximal droplet and tail folded at the connecting piece level (→). (Magnification = ×1,400)

major regions of the boar mature spermatozoon, the head and the tail, as well as the connecting piece in between, will be considered in turn.

1.2.2.1 Head

The small and compact boar sperm head contains a very limited number of components; the only other major organelle in the head region, apart from the cell nucleus, is the acrosome. Neighboring structures are the postacrosomal dense lamina, the subacrosomal space, and the perinuclear fibrous material (Figs. 1.2a–c; 1.4a.1, a.2, b.1, b.2 and 1.5).

The nuclear shape determined by the sperm genotype is highly species-specific and most species show a very high degree of uniformity (Curry and Watson 1995). This is the case with the flattened ovoid-shaped nucleus of the ejaculated boar spermatozoon whose dimensions are 6.6 μm in length and variable in thickness at the proximal and distal cephalic regions (Figs. 1.1d, e and 1.5a, b); in the proximal region, the nucleus is 220 nm in thickness and in the distal region it is approximately 320 nm (Fig. 1.5b.1–b.5). The nucleus constitutes the major part of the sperm head, and as in many other mammalian species, it consists of a very rigid structure attributable to its extremely condensed and electrodense chromatin fibers.

Accompanying the nucleus is the sac-like acrosome, a membrane-bound vesicle that forms a cap over the anterior part of the nucleus covering approximately 80 % of its length (Fig. 1.5b). Two broadly parallel acrosomal membranes are considered in

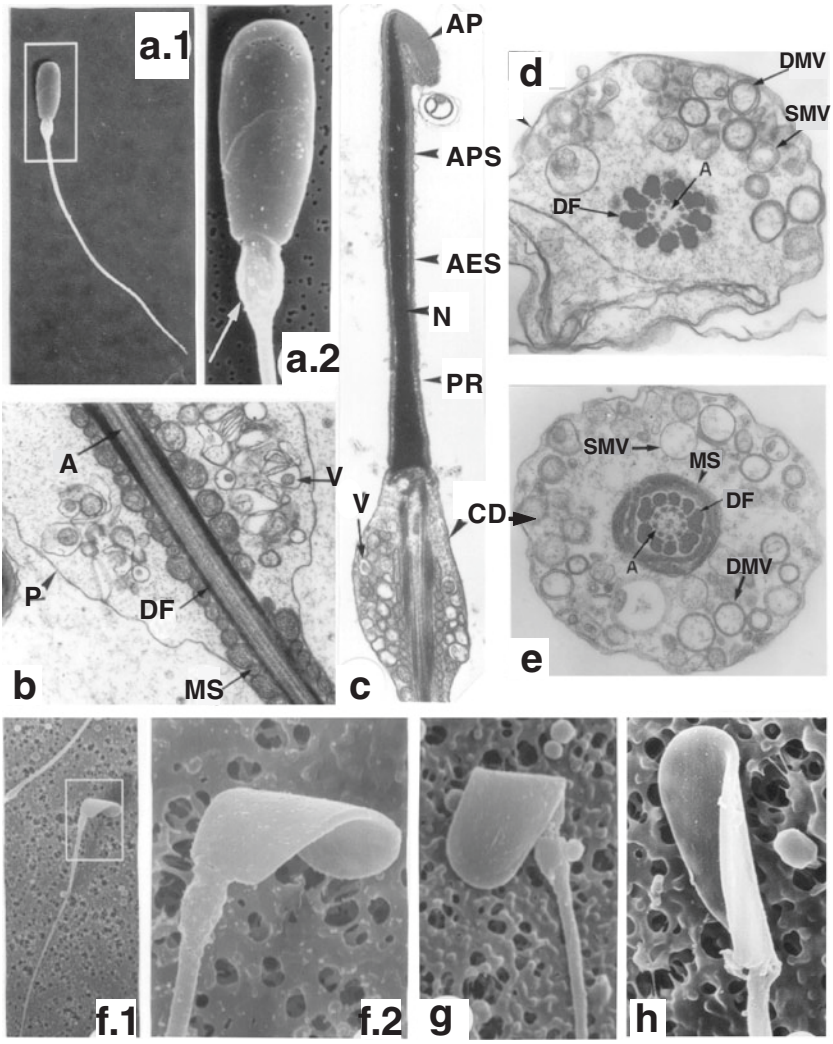


Fig. 1.8 Scanning (**a**, **f–h**) and transmission (**b–e**) electron micrographs showing the ultrastructure of the immature boar spermatozoa. **a.1** General view of an immature spermatozoon with proximal cytoplasmic droplet; the framed area is enlarged in figure **a.2** (×1,100). **a.2** Head and cytoplasmic droplet detail (→) (×4,400). **b** Longitudinal section through the cytoplasmic droplet; notice the abundant vesicles (*V*) (×24,000). **c** Longitudinal section of the head and the connecting piece; note the proximal cytoplasmic droplet (*CD*) (×13,000). **d** Cross section through the connecting piece showing double membrane vesicles (*DMV*) and simple membrane vesicles (*SMV*) inside the cytoplasmic droplet (×53,000). **e** Cross section through the cytoplasmic droplet; notice the midpiece occupies the droplet geometrical center (×60,000). Axoneme (*A*); acrosome equatorial segment (*AES*); acrosome principal segment (*APS*); acrosomal protuberance (*AP*); dense fibers (*DF*); mitochondrial sheath (*MS*); nucleus (*N*); plasmalemma (*P*); postacrosomal cephalic region (*PR*). **f.1** General view of an immature spermatozoon with proximal cytoplasmic droplet; the framed area is enlarged in figure **f.2** (×1,500). **f.2** Head transversely bent along its complete extension (×7,500). **g** Immature spermatozoon with proximal cytoplasmic droplet and the head transversely folded at the limit between the acrosomal and postacrosomal cephalic regions (×5,400). **h** Spermatozoon head rolled from its base (×6,500)

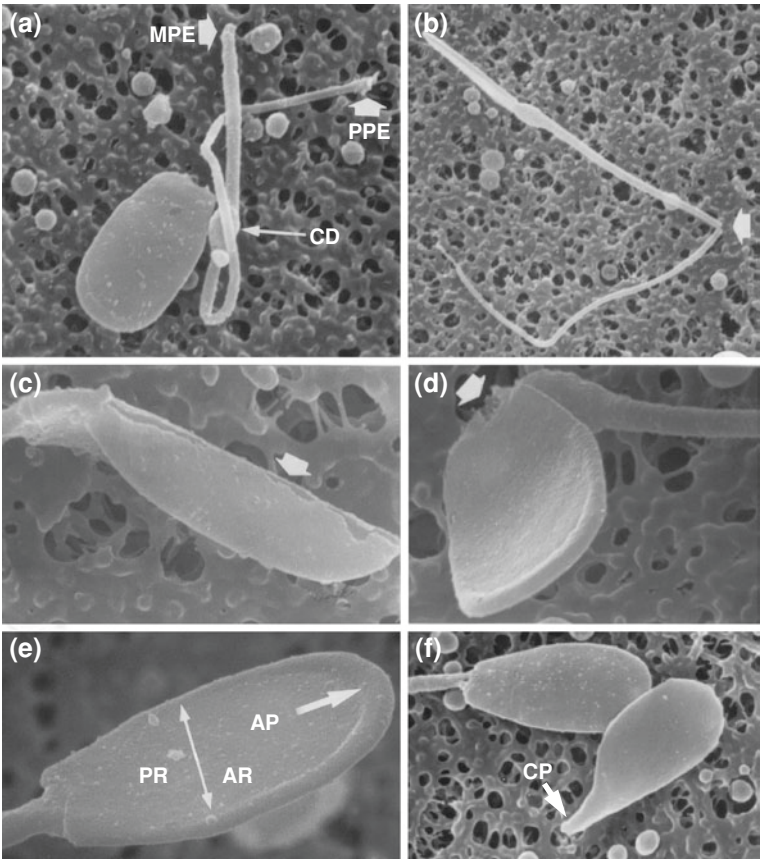


Fig. 1.9 Scanning electron micrographs showing characteristic malformations of the immature boar spermatozoa. **a** Separated head and tail of an immature spermatozoon with distal cytoplasmic droplet (*CD*); the tail's principal piece has been broken and has lost the terminal piece. midpiece proximal end (*MPE*); principal piece distal end (*PPE*) (×5,400). **b** Immature spermatozoon with proximal cytoplasmic droplet and tail broken at the point of the principal piece (→) (×3,600). **c** Immature spermatozoon with distal cytoplasmic droplet and head broken lengthwise (→) (×9,000). **d** Immature spermatozoon with proximal cytoplasmic droplet and head transversely broken (→) at its boundary with the cytoplasmic droplet (×9,000). **e** Detail of the head of an immature spermatozoon with distal cytoplasmic droplet; note the great development of the acrosomal protuberance (*AP*) which extends down to the boundary of the acrosomal region (→) (×9,500). Acrosomal region (*AR*); postacrosomal region (*PR*). **f** Spermatozoon with the tail broken at the beginning of the midpiece; note the severely pyriform head presents an intact connecting piece (*CP*) (×6,300)

this sac-like vesicle. An inner membrane overlays the nuclear envelope, which continues at the posterior margins of the acrosome forming an outer membrane lying directly beneath the plasmalemma. Enclosed inside these two acrosomal membranes is a narrow space, the acrosomal matrix, filled with amorphous material distributed

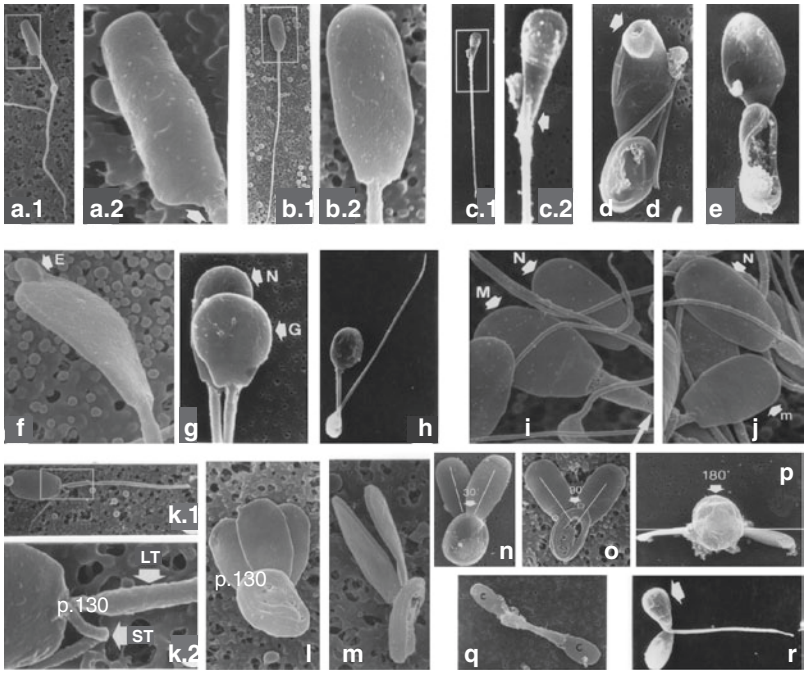


Fig. 1.10 Scanning electron micrographs showing different boar sperm malformations affecting the cephalic and the tail shape, size, and number. **a.1** General view of an immature spermatozoon with distal cytoplasmic droplet and abnormal head; the framed area is enlarged in figure **a.2** (×2,000). **a.2** Detail of the cylindrical or bacillary head displaying an abaxial tail attachment (→). (×8,000). **b.1** General view of a mature spermatozoon; the framed area is enlarged in figure **b.2** (×1,500). **b.2** Detail of the narrower and longer profile of the head (×6,000). **c.1** General view of a microcephalic mature spermatozoon; the framed area is enlarged in Figure. **c.2** (×1,500). **c.2** Detail of the elongated pear-shaped head; the *arrow* points to the head–tail junction (×6,000). **d** Coiled tail spermatozoon with a long flame-shaped head showing the craterlike appearance of the apical acrosomal protuberance (→) (×5,000). **e** Coiled tail spermatozoon with a short flame-shaped head (×5,400). **f** Spermatozoon with an apical acrosomal protuberance characterized by a well-developed knob (*E*) (knobbed acrosome defect) (×10,000). **g** Normal-headed spermatozoon (*N*) and spermatozoon with a globular head (*G*) (×5,000). **h** Folded tail spermatozoon with entirely roundish head (×2,600). **i** Macrocephalic spermatozoon (*M*) with two fused tails (→); note the normal spermatozoa (*N*) for size comparison (x5,400). **j** Microcephalic spermatozoon or with undersize head (**m**); note the normal-sized head (*N*) for comparison (×7,100). **k.1** General view of a macrocephalic spermatozoon with two unfused tails of different length and thickness; the framed area is enlarged in figure **k.2** (×1,500). **k.2** Detail of the cephalic region showing the attachment of the two tails, one of them short and thin (*ST*) and the other long and thick (*LT*) (×6,000). **l** Tricephalic spermatozoon with completely coiled tail (×4,400). **m** Side view of a bicephalic spermatozoon with a completely coiled tail; notice the two heads are overlapped on parallel planes (×5,600). **n** Spermatozoon with two heads on the same plane giving rise to about a 30º angle between them (×5,000). **o** Spermatozoon with two heads located on the same plane giving rise to about a 90º angle between them (×5,400). Notice in micrographs *n* and *o* that the completely coiled tail gives rise to a flattened structure which is on the same plane as the heads. **p** Spermatozoon with two heads on the same plane, but located in opposite directions giving rise to a 180º angle between them; note the coiled tail appears as a globular mass placed between the two heads (×3,000). **q** Spermatozoa with two uncoiled and antiparallel midpieces, and the principal and terminal pieces coiled over the midpieces; notice that one of the two heads is more voluminous than the other one (C, bigger head; c, smaller head) (×3,200). **r** Bicephalic spermatozoon with two uncoiled and fused tails; note the short tail and the pyriform shape of one of the two heads (→) (×2,500)

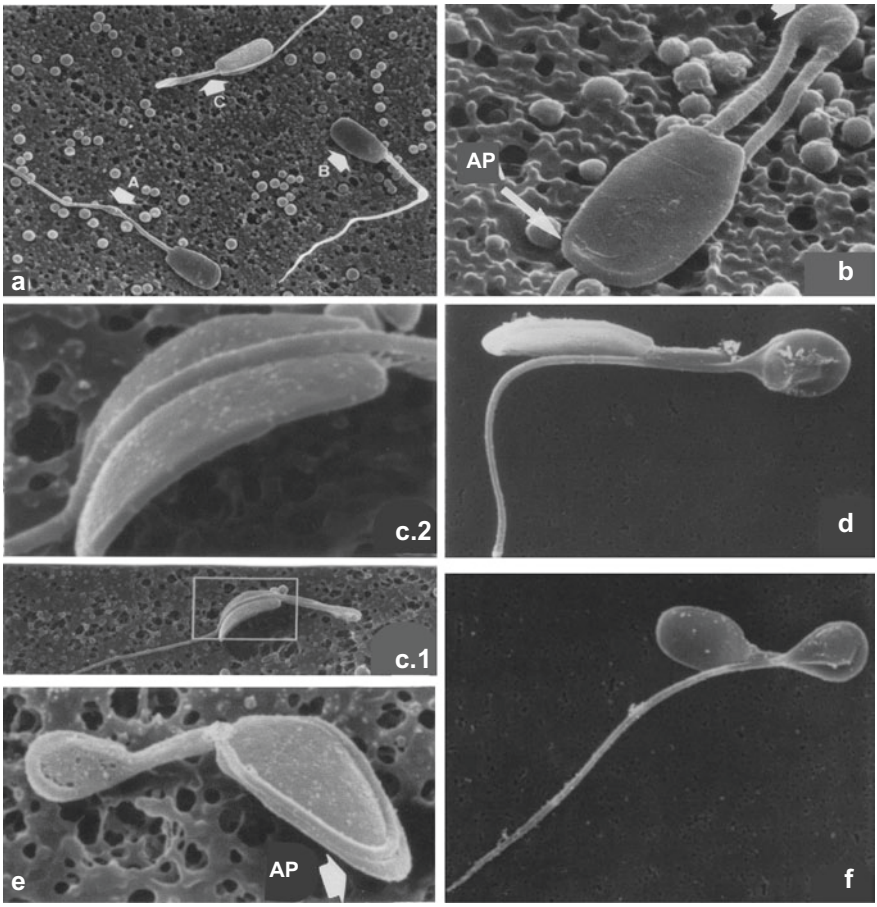


Fig. 1.11 Scanning electron micrographs showing different boar sperm malformations affecting the tail trajectory (*tail folding*). **a** Principal stages of tail folding at the location of Jensen's ring. *A*, immature spermatozoon with distal cytoplasmic droplet; *B*, immature spermatozoon with distal cytoplasmic droplet and its tail folded at Jensen's ring, displaying a 90º angle between the midpiece and the principal piece; *C*, immature spermatozoon with distal cytoplasmic droplet and a completely folded tail (×3,000). **b** Immature spermatozoon with distal cytoplasmic droplet (→); notice the 0º angle formed between the midpiece and the principal piece of the tail; in this phase of tail folding, these two pieces are not fused and the tail overruns the flat face of the head, leaving the cephalic face presenting the acrosomal protuberance (*AP*) uncovered (×5,000). **c.1** General view of a spermatozoon with its tail folded at Jensen's ring; the framed area is enlarged in figure **c.2** (×2,000). **c.2** Detail of the fusion of the principal piece along the midsagittal axis of the head; note the cephalic bending imprinted by the head and tail fusion (×8,000). **d** Spermatozoon with its tail folded at Jensen's ring; notice the cytoplasmic droplet consolidating the tail folding at Jensen's ring (×3,500). **e** Immature spermatozoon with a distal cytoplasmic droplet, a triangular-shaped head, its tail folded at the posterior third of the midpiece and coiled around the head, just below the acrosomal protuberance (*AP*) (×4,500). **f** Spermatozoon with its tail folded at the midpiece; notice that the tail is folded at the midpiece location because the zone affected by the bending shows a greater development than the kind which characterizes tail folding at Jensen's ring (×3,000)

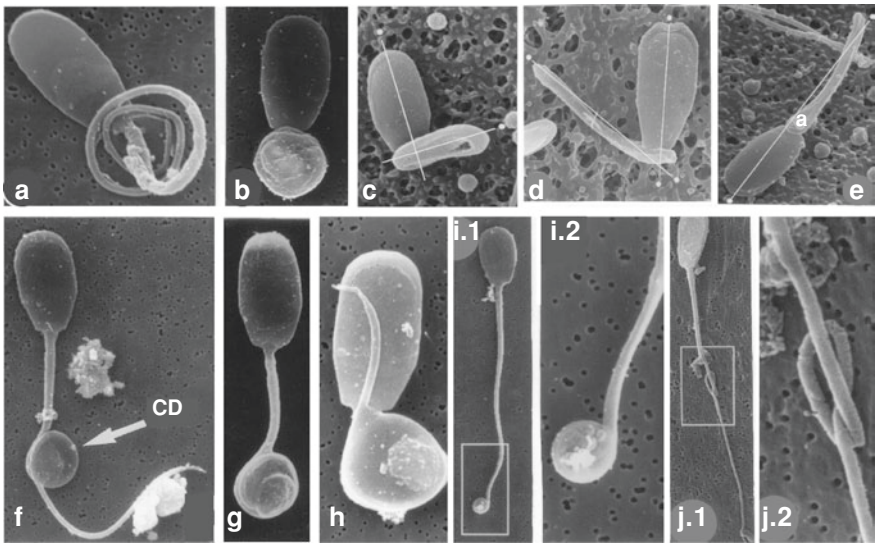


Fig. 1.12 Scanning electron micrographs showing different boar sperm malformations affecting the tail trajectory (*tail coiling*). **a** Spermatozoon with a coiled tail, but not fused; notice the tail turns in circles concentrically around itself (×6,800). **b** Spermatozoon with a coiled and fused tail (the plasmalemma covers the internal coiling of the tail); note the coiling displays a circular shape (×5,000). **c** Spermatozoon with a small elliptical head, along with an intensely coiled and fused tail; note the coiling displays an elliptical shape and is at 90º in regard to the midsagittal axis of the head (×5,200). **d** Spermatozoon with a coiled tail, giving rise to 45º angle in regard to the midsagittal axis of the head (×6,300). **e** Spermatozoon with its tail coiled along the entire midpiece; note the coiling begins by the tail folding at the Jensen's ring (×3,500). **f** Immature spermatozoon with its cytoplasmic droplet placed on the first segment of the principal piece; note that the principal piece displays a complete loop around the cytoplasmic droplet (*CD*) (×2,800). **g** Immature spermatozoon with its cytoplasmic droplet placed on the first segment of the principal piece; notice that the principal and terminal pieces turn completely around the cytoplasmic droplet (×3,000). **h** Spermatozoon with partially coiled tail; note the great development of tail coiling (×4,700). **i.1** Mature spermatozoon with its tail coiled at the distal end; the framed area is enlarged in figure **i.2** (×1,800). **i.2** Detail of the tail coiling which only affects the terminal piece and the distal segment of the principal piece (× 7,200). **j.1** General view of a spermatozoon with a false knot in the principal piece; the framed area is enlarged in figure **j.2** (×1,800). **j.2** Detail of the false knot of the principal piece (×7,200)

homogeneously and mostly corresponding to densely packed hydrolytic enzymes. The part of the head containing the acrosome is called the acrosomal region. Three acrosomal segments are clearly distinguishable: the apical segment, the principal segment and the equatorial segment; in this latter segment, the electrodensity of the acrosomal matrix is slightly higher than that of the first two segments. The acrosome apical segment is the most expanded zone of the vesicle, and this vesicular expansion does not have the same development in the two faces of the head; while in the non-flattened face of the head the apical segment stretches down to 0.72 μm in length per 270 nm at its widest point (Fig. 1.5b.1), in the flat face the apical segment reaches 0.50 μm in length per 100 nm at its widest point. The principal segment is 2.5 μm in

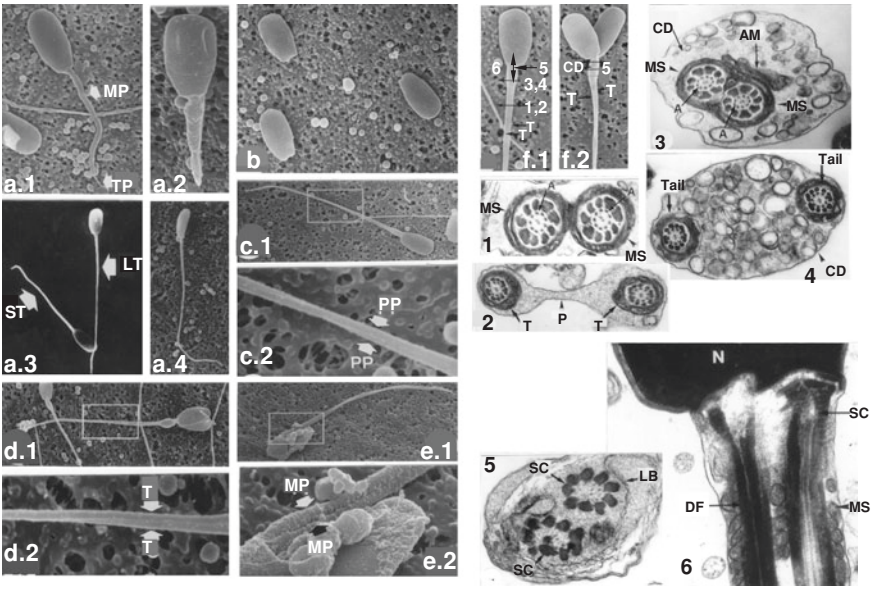


Fig. 1.13 Scanning and transmission electron micrographs showing different typologies of boar sperm malformations affecting the tail size and number. **a.1** Spermatozoon with short tail (14 m); note the tail presents the midpiece (*MP*)—that is longer than normal—and the terminal piece (*TP*), but not the principal piece (×3,600). **a.2** Spermatozoon with very short tail (7 m); notice the tail is thicker than normal and appears to be only formed by a short and thick midpiece (×4,500). **a.3** Spermatozoon with long tail (42 m) (*LT*) and spermatozoon with short tail (30 m) (*ST*) (×1,300). **a.4** Spermatozoon with long tail (43 m) (×1,400). **b** Tailless spermatozoa; note the tail breaking came about the base of the head (×2,700). **c.1** General view of an immature spermatozoon with proximal cytoplasmic droplet and two fused tails; the framed area is enlarged in figure **c.2** (×1,500). **c.2** Detail of the helix path of the two principal pieces (*PP*) (×6,100). **d.1** General view of an immature spermatozoon with proximal cytoplasmic droplet, with two heads and two fused tails; the framed area is enlarged in figure **d.2** (×1,500). **d.2** Detail of the principal piece region; note the two fused tails (*T*) (×6,000). **e.1** General view of a microcephalic spermatozoon with two fused tails; the framed area is enlarged in figure **e.2** (×1,500). **e.2** Detail of the initial region of the tail midpiece; notice the presence of two midpieces (*MP*) (×6,100). **f.1** General view of a spermatozoon with one head and two fused tails (*T*); the numbered arrows indicate the direction and the level of the sections depicted in Figs. 1.1–1.6 (×5,200). **f.2** General view of a spermatozoon with two heads and two fused tails (*T*); notice the residual cytoplasmic droplet (*CD*) (×5,200. (*1*) Cross section through the anterior region of the midpiece of a spermatozoon with two tails; note the mitochondrial sheaths (*MS*) coming in contact (×45,000). (*2*) Cross section through the mid-anterior region of the midpiece of a spermatozoon with two tails (*T*) (×36,000). (*3*) Cross section through the anterior region of the midpiece of a spermatozoon with two tails (*T*); notice the fused mitochondrial sheaths (*MS*) and the additional mitochondria (*AM*) (×42,000). (*4*) Cross section through the mid-anterior region of the midpiece of a spermatozoon with two tails (×36,000). (*5*) Cross section through the anterior part of the connecting piece of a spermatozoon with two tails (×45,000). (*6*) Frontal longitudinal section of a spermatozoon with one head and two fused tails (×33.000). Axoneme (*A*); cytoplasmic droplet (*CD*); nucleus (*N*); dense fibers (*DF*); laminar bodies (*LB*); mitochondrial sheath (*MS*); plasmalemma (*P*); segmented columns (*SC*)

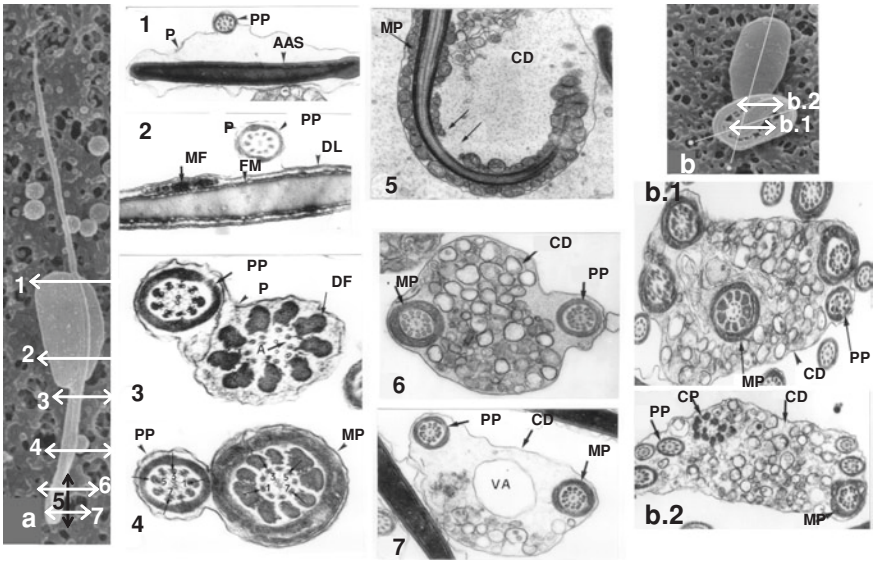


Fig. 1.14 Scanning and transmission electron micrographs showing different typologies of boar sperm malformations affecting the tail trajectory (*folded and coiled tails*). **a** General view of a spermatozoon with tail folded at the Jensen's ring; the *numbered arrows* indicate the direction and the level of the sections depicted in Figs. 1.1–1.7 (×6,400). (*1*) Cross section through the acrosome apical segment (*AAS*); note the plasmalemma (*P*) surrounds the acrosomal cephalic region and the tail principal piece (*PP*) (×25,000). (*2*) Cross section through the postacrosomal cephalic region; notice the myelin figures (*MF*) arranged between the postacrosomal dense lamina (*DL*) and the perinuclear fibrous material (*FM*) (×45,000). (*3*) Cross section through the posterior part of the connecting piece; note the proper organization of dense fibers (*DF*) and axoneme (*A*) (×85,000). (*4*) Cross section through the mid-anterior part of the midpiece (*MP*); notice the antiparallel disposition of the principal piece (*PP*) from the numbering of the axoneme microtubule doublets (×75,000). (*5*) Longitudinal section through the midpiece region (*MP*) where the tail folding occurs; note the disorganization of the mitochondrial sheath in this region (×30,000). (*6*) Cross section through the distal cytoplasmic droplet (*CD*) which consolidates tail folding (×30,000). (*7*) Cross section through the distal cytoplasmic droplet; notice the high vacuolation (*VA*) of the cytoplasmic droplet (×25,000). **b** General view of a spermatozoon with completely coiled tail; the *numbered arrows* indicate the direction and the level of the sections depicted in figures **b.1** and **b.2** (×8,100). **b.1** Section of a spermatozoon coiled tail; the midpiece (*MP*) appears in four sections and the principal piece (*PP*) in a single section (×25,000). **b.2** Section of a spermatozoon coiled tail at the connecting piece (*CP*) level (×22,000)

length in the flat face of the head and 2.8 μm in the other face; the thickness of this segment is about 80 nm. The equatorial segment is 2.3 μm in length, 40 nm in thickness in the two head faces, and 80 nm in thickness in the edges of the head.

The postacrosomal dense lamina consists of a homogeneous layer of fibrous and electrodense material, 1.4 μm in length and 25 nm in thickness, that lies parallel beneath the plasmalemma and takes up 20 % of the nuclear length, coinciding with the region of the nucleus not covered by the acrosomal vesicle (Figs. 1.4b.1, b.2 and 1.5b.4, b.5). The region of the head containing the postacrosomal dense

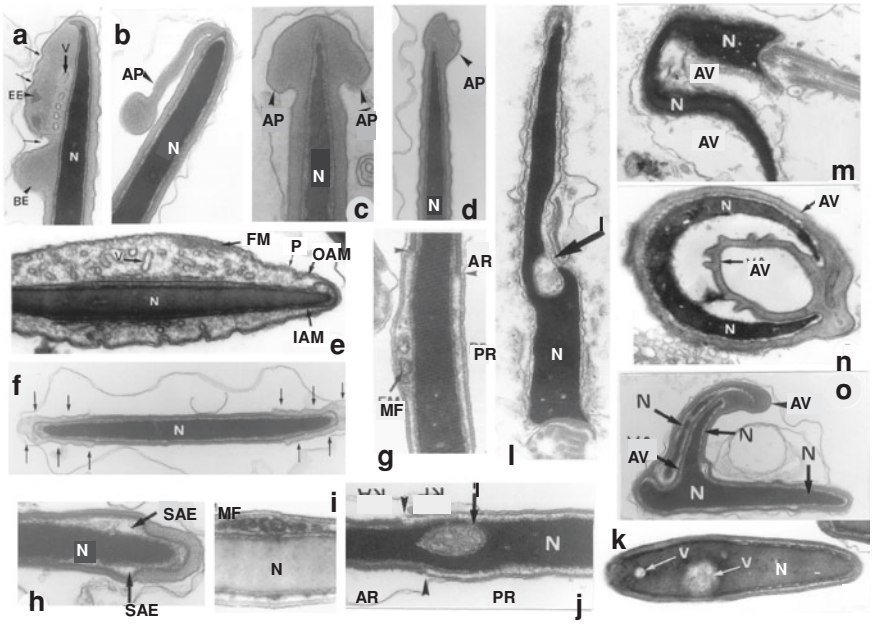
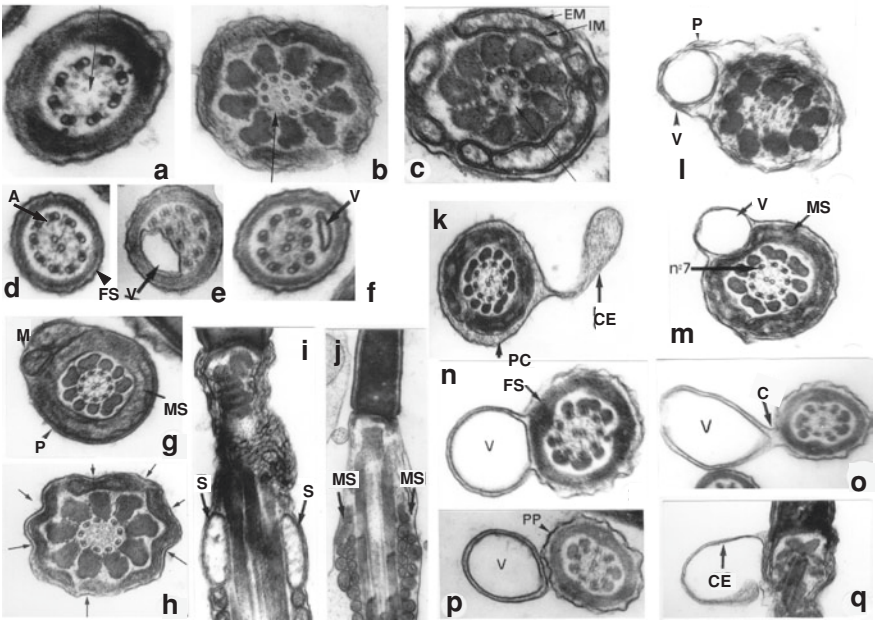


Fig. 1.15 Transmission electron micrographs showing different typologies of boar sperm cephalic malformations. **a** Longitudinal section of the acrosome apical segment; note the festooned perimeter of the acrosomal protuberance (→), the high inner vesiculation (*V*) and the matrix heterogeneity with highly electron dense zones (*EE*) and low electron dense zones (*BE*) (×50,000). **b** Longitudinal section of the acrosome apical segment; notice the drumstick-shape of the acrosomal protuberance (*AP*) (×40,000). **c** Longitudinal section of the acrosome apical segment; notice the symmetrical distribution of the acrosomal protuberance (*AP*) at both sides of the head (×63,000). **d** Longitudinal section of the acrosome apical segment; notice the scarce development of the drop-shaped acrosomal protuberance (*AP*) which is found in the cephalic apex (×25,000). **e** Cross section of the acrosome principal segment; notice the highly expanded acrosomal vesicle with a low matrix electron density and containing abundant vesicles (*V*) and electron dense fibrous material structures (*FM*). Inner acrosomal membrane (*IAM*); outer acrosomal membrane (*OAM*); plasmalemma (*P*) (× 45000). **f** Cross section of the acrosome equatorial segment; notice the festooned surface of the acrosomal vesicle at the end sides of the head (→) (×25,000). **g** Longitudinal section through the anterior postacrosomal region (*PR*) where myelin figures (*MF*) can be seen arranged only in the zone of the postacrosomal region closer to the acrosomal region (*AR*) (×46,000). **h** Cross section of the acrosome equatorial segment; note the high development of the subacrosomal space (*SAE*) (×30,000). **i** Cross section through the postacrosomal region where the myelin figures (*MF*) shows a linear arrangement which provokes a slightly protruding contour of the cephalic region (×80,000). **j** Longitudinal section through a spermatozoon head showing a false nuclear vacuole due to the refolding of the nuclear envelope within the invagination (*I*); note that this invagination is found in the anterior portion of the postacrosomal region (*PR*) closer to the acrosomal region (*AR*) (×37,000). **k** Cross section of the basal postacrosomal region; note the appearance of real non-membrane-bound nuclear vacuoles (*V*), thus being nuclear zones with low electron density due to the loosely compacted chromatin (×45,000). **l** Longitudinal section through a spermatozoon head showing a membrane-bound nuclear invagination giving rise to a false nuclear vacuole (*I*) (or nuclear pouche) (×32,000). **m** Longitudinal section of a spermatozoon with folded nucleus (*N*); notice the impaired appearance of the acrosomal vesicle (*AV*) (×24,000). **n** Cross section of a spermatozoon rolled head showing the curvature of the nucleus (*N*) and the appearance and placement of the acrosomal vesicle (*AV*) (×45,000). **o** Cross section of a spermatozoon with V-shaped nucleus (*N*) (or crested nucleus); note the appearance and placement of the acrosomal vesicle (*AV*) (×20,000)



lamina is called the postacrosomal region. The plasmalemma enclosing the sperm head region overlays is firmly adhered to the postacrosomal dense lamina, but detaches very easily from the outer acrosomal membrane (Fig. 1.5b).

The subacrosomal (or perinuclear) space is the limit separating the nucleus from the inner acrosomal membrane or the innermost face of the postacrosomal dense lamina (Figs. 1.4a.1 and 1.5b.3–b.5). This space consists of a scarce electrodense matrix and a perinuclear fibrous material of greater electrodensity. The subacrosomal space is especially developed in the region beneath the acrosome apical segment; in this supranuclear region this space has a conical shape, with a base of 80 nm in diameter and 120 nm in length (Figs. 1.2a and 1.5b, b.1). The subacrosomal space is reduced to 25 nm in thickness along the acrosome principal segment, and in the two faces of the head; in contrast, it increases up to 40 nm in thickness along the acrosome equatorial segment and also in the two faces of the head, and comes to its maximum development in the postacrosomal region (70 nm) (Fig. 1.2b). The subacrosomal space increases considerably in the borders of the head (70 nm) corresponding to the acrosome principal and equatorial segments (Fig. 1.5b, b.2, b.3); in contrast, in the margins of the head corresponding to the postacrosomal region it is practically non-existent and the perinuclear fibrous material becomes connected with the postacrosomal dense lamina (Fig. 1.5b, b.4, b.5).

Finally, the perinuclear fibrous material (or subacrosomal fibrous material) consists of an electrodense layer coating the nucleus with a separation of 15 nm between them. The maximum development of this layer is found in the postacrosomal region, reaching about 15 nm in thickness, whereas in the acrosomal region

◀ **Fig. 1.16** Transmission electron micrographs showing different typologies of boar sperm tail malformations. **a** Cross section through the mid-posterior region of the principal piece showing the absence of the central microtubule pair (→) of the axoneme (×140,000). **b** Cross section through the anterior region of the midpiece showing the absence of the peripheral microtubule doublet number 9 (→) (×100,000). **c** Cross section through the anterior region of the midpiece showing the absence of the peripheral microtubule doublet number 8 (→); note the extreme swelling of the mitochondrial sheath, and that the inner mitochondrial membrane (*IM*) has no type of folding, and is parallel to the outer mitochondrial membrane (*EM*) (×80,000). **d** Cross section through the mid-posterior region of a normal principal piece where a complete axoneme (*A*) can be observed inside the fibrous sheath (*FS*) (×140,000). **e** and **f** Cross sections through the mid-posterior part of the principal piece; note the presence of cytoplasmic vacuoles (*V*) which provoke an axonemal deformation affecting the peripheral microtubule doublets 4, 5 or 6 (figure **e**, ×61,000; figure f, ×70,000). **g** Cross section through the mid-region of the midpiece; note the presence of some additional mitochondria (*M*) located between the plasmalemma (*P*) and the mitochondrial sheath (*MS*) (×60,000). **h** Cross section through the anterior region of the midpiece; notice the festooned contour (→) attributable to the lesser thickness and greater fragility of the mitochondria which form the mitochondrial sheath (×70,000). **i** Longitudinal section of the connecting piece and the anterior region of the midpiece; note the great swelling (*S*) of the first mitochondria of the mitochondrial sheath (×45,000). **j** Longitudinal section of the connecting piece and the anterior region of the midpiece; note the arrangement of the double layer of mitochondria forming the mitochondrial sheath (*MS*) (×40,000). **k** Cross section through the posterior region of the midpiece; notice the highly developed peripheral cytoplasm (*PC*) and the formation of a great digitiform cytoplasmic evagination (*CE*) (×45,000). **l** Cross section of the connecting piece showing the presence of a voluminous vacuole (*V*) located below the plasmalemma (*P*) (×60,000). **m** Cross section through the mid-region of the midpiece; note the presence of a voluminous vacuole (*V*) located in front of the peripheral microtubule doublet number 7, which provokes a noticeable deformation on the mitochondrial sheath (*MS*) (×75,000). **n** Cross section through the anterior region of the principal piece; note the presence of a voluminous vacuole (*V*) and that the fibrous sheath (*FS*) provokes a slight deformation of the circular perimeter of the vacuole (×90,000). **o** Cross section through the anterior region of the principal piece; note the process of the shedding of the vacuole (*V*), which only maintains contact with the principal piece by a slender thread of cytoplasm (*C*) (×47,000). **p** Cross section through the anterior region of the principal piece showing the loss of contact between the vacuole (*V*) and the principal piece (*PP*) (×75,000). **q** Longitudinal section of the connecting piece; note the formation of a vacuole caused by the folding of a digitiform cytoplasmic evagination over itself (*CE*); this cytoplasmic evagination is found in the region of the boundary between the head and the connecting piece (×45,000)

this layer is thinner and poorly condensed showing less electrodensity (Figs. 1.2a–c; 1.4b.1, c and 1.5b, b.4, b.5).

1.2.2.2 Connecting Piece

The connecting piece (or neck) of the boar spermatozoon is a short linking segment between the base of the nucleus and the first mitochondrion of the tail midpiece (Figs. 1.3, 1.4c, 1.6a–e). It firmly attaches itself to the distal end of the head, being trapezoid in shape (Figs. 1.4c and 1.6a). The outline of the plasmalemma along the connecting piece and the postacrosomal cephalic region shows a slight circular neckline (or postcephalic ring) just in the head–tail junction (Figs. 1.2d

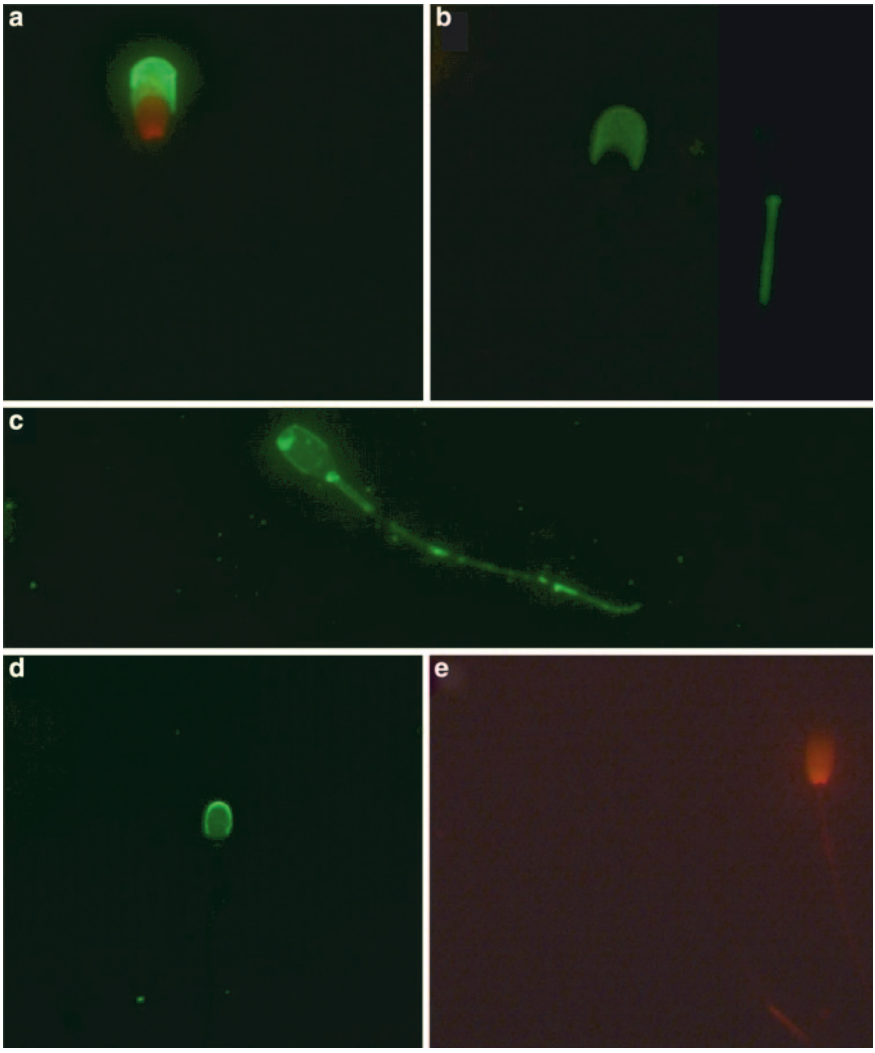


Fig. 1.17 Fluorescence micrographs of boar spermatozoa from epididymal cauda for galactose (**a**), glucose/mannose (**b**), N-acetyl-D-glucosamine (**c**), N-acetyl-D-galactosamine (**d**) and fucose moieties (**e**). **a** Galactose residues (FICT-PNA lectin) expressed over a membrane-damaged spermatozoa (PI labeled) with reacted acrosome (×600). **b** Glucose/mannose residues (FICT-PSA lectin) expressed over the acrosome and mitochondrial sheath (×600). **c** N-acetyl-D-glucosamine residues (FICT-WGA) expressed over the whole sperm surface (×600). **d** N-acetyl-D-galactosamine residues (FICT-SBA) expressed over the apical ridge (×400). **e** Fucose residues (TRITC-UEA-I) expressed over the acrosome and mitochondrial sheath (×400)

and 1.6a, b). Its major components are: the basal plate, the laminar bodies, the capitulum, the segmented columns, the basal body, and the axoneme (Fig. 1.4c).

The basal plate consists of an electrodense material very similar to that of the postacrosomal dense lamina lying at the caudal surface of the nucleus, and which

extends along about 0.6 μm (30 nm thick), adhered to the outer membrane of the nuclear envelope (Figs. 1.2d and 1.6d).

The laminar bodies come from pronounced folds of redundant nuclear envelope and enclose chromatin-free nuclear space. These folds have cylindrical disposition with walls of 150 nm in thickness, arise from the perimeter of the nuclear base and extend along 1.2 μm, terminating at the first mitochondrion of the tail midpiece (Figs. 1.2d, e, 1.6b, d).

The capitulum is a dome-shaped structure lying beneath the basal plate and separated from it by approximately 40 nm. The segmented columns arise from the capitulum ends and go down to the tail midpiece. The capitulum and also the segmented columns are electrodense structures of about 130 nm in thickness. While the laminar bodies are attached to the mitochondrial sheath of the tail midpiece, the segmented columns are attached to the outer dense fibers of the tail midpiece (Fig. 1.2d).

The nine segmented columns are individualized at the more distal end of the capitulum (0.3 μm of distance from it) (Fig. 1.6d and e). Along the first 0.3 μm of distance, the segmented columns fuse between them, and therefore, it is more proper to refer to them as capitulum extensions than as columns. The individualized segmented columns are cylinder-shaped and stretch along 0.8 μm down to the midpiece, enclosing the axoneme in a cylindrical space of 220 nm in diameter (Figs. 1.2e and 1.6e). These columns present two types of cross-striations with alternate periodicity; one type eminent and the other type less well defined. The distance between two consecutive eminent striations is 80 nm, and between an eminent striation and the consecutive less well defined striation, 40 nm (Fig. 1.6c).

The basal body is located at the feet of the convexity defined by the capitulum and the segmented columns, i.e., a space of 220 nm in diameter and 260 nm in height. It is positioned at an angle of 45º with respect to the longitudinal axis of the spermatozoon (Fig. 1.6d). The microtubular triplets of the basal body are arranged in small depressions of the capitulum which indicate the limits of the segmented columns drafts. This basal body marks the origin of the axoneme, which stretches progressively throughout the full length of the spermatozoon tail. The respective nine evenly spaced peripheral microtubule doublets of the axoneme are surrounded by the nine segmented columns, which in turn are surrounded by the laminar bodies (Fig. 1.2e).

1.2.2.3 Tail

Higher vertebrates have a noteworthy complexity in sperm tail organization showing the presence of a significant number of additional structures to the axoneme as compared to the invertebrate species (Curry and Watson 1995). Three regions are clearly distinguishable in the boar spermatozoon tail: the midpiece (or mitochondrial region), the principal piece and the terminal piece (Figs. 1.3, 1.4 and 1.6f–h).

The midpiece of the spermatozoon tail stretches from the distal end of the connecting piece to the annulus or Jensen's ring, an electrodense band marking the junction of the midpiece and the principal piece (Fig. 1.6b, g, i). The major structures of the midpiece are: the axoneme, the mitochondrial sheath, the outer dense fibers (or coarse fibers) and the peripheral granules (Figs. 1.2d, f and 1.4d.1, d.2). The axoneme occupies the central axis of the midpiece and has the classic 9 + 2 microtubular pattern, with the characteristic axoneme elements (dynein arms, nexin links, radial spokes, connecting bridge, and central sheath) (Figs. 1.2f and 1.4d.2). The nine peripheral microtubule doublets are numbered 1–9 in a clockwise direction, with number 1 being the only doublet situated on a plane perpendicular to that of the two central microtubules. As mentioned above, the axoneme stretches along the full length of the three pieces of the spermatozoon tail (midpiece, principal, and terminal pieces) (Figs. 1.2d–i and 1.4d–h). The mitochondrial sheath lies directly beneath the plasmalemma and consists of several elongated mitochondria (150 nm in diameter) lying end to end in a helical arrangement around the underlying axoneme. This sheath is approximately 80 nm thick. The mitochondrial matrix consists of very electrodense material and the crests lie parallel to the double mitochondrial membrane (Figs. 1.2f and 1.4d.1, d.2). The outer dense fibers (or coarse fibers) are filamentous cytoskeletal structures consisting of electrodense material lying between the mitochondrial sheath and each of the peripheral microtubule doublets of the axoneme. The dense fibers extend along the whole midpiece and the first third of the principal piece. Maximum thickness and diameter of the dense fibers are found in the distal end of the connecting piece and they decrease progressively along the two following pieces. Individual dense fibers, numbered by their association with the microtubular doublets of the axoneme, have a characteristic shape in cross section, all of them acquiring a truncated pyramid-like form with the narrower base toward the microtubular doublets and the wider base, round-edged, toward the mitochondrial sheath (Figs. 1.2f and 1.4d.2, e). The dense fibers are not all of equal thickness and diameter. In the proximal region of the midpiece, fibers number 1, 5, and 6 average 110 nm in thickness and 150 nm in diameter, and fibers number 2, 3, 4, 7, 8 and 9 average 80 nm in thickness and 110 nm in diameter. In the distal region of the midpiece, fibers number 1, 5 and 6 average 160 nm in thickness and 40 nm in diameter, and the remaining fibers average 70 nm in thickness and 60 nm in diameter. The peripheral granules distributed in the interstices between the outer dense fibers are only found in the proximal region of the midpiece. They consist of electrodense granules, of 15 nm maximum diameter, which disappear progressively as the dense fibers reach the distal region of the midpiece (Fig. 1.2f).

The principal piece is the longest segment of the spermatozoon tail, extending from the annulus or Jensen's ring to the proximal end of the terminal piece (Fig. 1.4e, f, g.1, g.2). The principal piece is characterized by the presence of: the fibrous sheath, the outer dense fibers, the axoneme, and the Jensen's ring. The fibrous sheath of the principal piece has a very high electrodensity, and replaces the mitochondrial sheath of the midpiece. It consists of two continuous longitudinal axes or columns (dorsal and ventral) coplanar with the central

microtubule pair; these two columns are joined by a series of circumferential ribs regularly distributed around the principal piece (Fig. 1.4e). The fibrous axes lie between the plasmalemma and the dense fibers 3 and 8 (Fig. 1.4f). The axes reach their maximum thickness (110 nm) at the first third of the principal piece and they decrease progressively along the following two thirds until being equal to the ribs' thickness (75 nm). Along the first third of the principal piece the great development of the axes determines their overlying and fusion with the dense fibers 3 and 8 beyond the termination of these fibers; thus, the axes continue attached to their underlying microtubule doublets (Fig. 1.4f). The remaining dense fibers, which stretch only along the first third of the principal piece, decrease progressively in thickness until they disappear. The two posterior thirds of the principal piece differ from the first third by their lack of dense fibers and because the thickness of the fibrous axes equals that of the fibrous ribs (75 nm) (Fig. 1.4g.1, g.2). The fibrous ribs are approximately 15 nm apart and about 40 nm in diameter along the whole principal piece. The thickness of the ribs, together with that of the axes, decreases progressively as the fibrous sheath draws nearer the terminal piece. The annulus or Jensen's ring consists of an electrodense ring-shaped structure underlying the plasmalemma, marking the junction of the midpiece and the principal piece. The plasmalemma is firmly attached to the Jensen's ring and this separates the mitochondrial sheath from the fibrous sheath. The Jensen's ring links up with the last mitochondrion of the mitochondrial sheath and is detached from the first ribs of the fibrous sheath by approximately 15 nm. This structure is about 130 nm thick and 160 nm in diameter (Fig. 1.6g, i).

The terminal piece is the last and shorter segment of the spermatozoon tail and has no accessory cytoskeletal structures, consisting only of the axoneme enclosed by the plasmalemma (Figs. 1.3, 1.4 and 1.6f, h). Whereas in other tail pieces the plasmalemma has a more or less smooth circular outline, in this last piece it adopts a festooned appearance. The axoneme becomes progressively disorganized as it stretches down the principal piece, with the B microtubule being the first element to disappear; finally, the microtubular disorganization makes it impossible to distinguish the typical axonemal 9 + 2 pattern (Fig. 1.4h).

1.3 Sperm Malformations

The careful study of structural and ultrastructural details in boar ejaculated spermatozoa has greatly assisted the development of an improved systematic classification for sperm malformations. Thus, an electron microscopic examination of sperm can be diagnostically helpful in case the source of a fertility alteration cannot be identified by means of other analyses.

Boar sperm malformations, understood as the whole range of immature and aberrant gamete forms, can have their origin in the testis (primary malformations) or in the epididymis (secondary malformations) (Bonet et al. 1992; Briz 1994; Briz et al. 1995, 1996).

1.3.1 Classification

The percentage of mature spermatozoa in a normal boar ejaculate is between 80 and 95 %; the percentage of immature spermatozoa ranges from 5 to 15 %; and the percentage of sperm malformations or aberrant spermatozoa is between 1 and 5 % (Martin 1982; Briz 1994; Bonet et al. 2000).

Among other features (described below), the immature spermatozoon mainly differs from the mature spermatozoon by the presence of a residual cytoplasmic droplet. This droplet consists of the remains of residual cytoplasm made redundant toward the end of spermiogenesis (see Sect. 3.2.3). It can be found at the connecting piece level or at a variable level along the midpiece, because the droplet forms at the connecting piece and moves down the midpiece, being released just at the Jensen's ring level of the spermatozoon (Fig. 1.7a) during the process of epididymal sperm maturation (see Sect. 3.3.4). Hence, the cytoplasmic droplet has two end positions: the proximal position (when placed at the connecting piece) (Fig. 1.7a.1, i, z) and the distal position (when placed at the Jensen's ring) (Fig. 1.7a.4, m, r). In domestic species, if the droplet is normally shed, then its retention on sperm in the ejaculate may be associated with infertility, and there is considerable evidence that this is the case; most reports relate the retention of proximal droplets (at the connecting piece) to be indicative of a failure of normal epididymal maturation (Cooper and Yeung 2003; Cooper 2005). All boar ejaculates show spermatozoa with residual cytoplasmic droplets but not all must be a priori considered abnormal; a distinction must be drawn between proximal and distal cytoplasmic droplets. Only, the immature spermatozoa with proximal cytoplasmic droplet are classified as sperm malformations in boar ejaculates, since this is a clear sign of a default in maturation that may cause impairment of the sperm fertilizing capacity under natural conditions. It has been demonstrated that ejaculates with more than 5 % of proximal cytoplasmic droplets produce a decrease in fertility; in contrast, there is no direct correlation between the percentage of distal cytoplasmic droplets and fertility (Gonzalez-Urdiales et al. 2006). However, Waberski et al. (1994) reported that infertility characterized by reduced pregnancy rate and litter size was also associated with retention of the distal droplet (at the Jensen's ring) in boars, although proximal droplets were also retained in these males. Immature sperm with proximal cytoplasmic droplet originate in the testis and this droplet normally moves distally, during passage through the epididymis, until it reaches the Jensen's ring and, once these spermatozoa with distal cytoplasmic droplet reach the epididymal cauda, they shed the cytoplasmic droplet and take on the appearance of mature spermatozoon (Fig. 1.7a.1–a.5). Therefore, the greater or lesser incidence of immature spermatozoa with proximal cytoplasmic droplet in the boar ejaculate enables us to determine the degree of sperm epididymal maturation. Inadequate or poor sperm epididymal maturation correlates with the inability to fertilize the oocyte due to shortcomings in sperm motility and/or interaction and fusion processes between the spermatozoon and the oocyte. Moreover, the presence of retained cytoplasmic droplets in ejaculated

spermatozoa may also be considered as a primary failure originated in the testes involving a membrane defect, and makes it difficult later on for the cytoplasmic droplet to migrate from the connecting piece to the end of the midpiece during epididymal maturation (Cooper and Yeung 2003; Cooper 2005). In conclusion, in boars, as in other mammalian species, the presence of a high percentage of abnormal spermatozoa with cytoplasmic droplets in their ejaculates has been related to poor fertility (Waberski et al. 1994; Cooper 2005; Gonzalez-Urdiales et al. 2006), lower binding capacity to oviductal explants (Petrunkina et al. 2001), and chromatin instability (Ardón et al. 2008).

The classification of sperm malformations may be carried out taking into account the external or internal morphology of the ejaculated spermatozoon, as well as their site of origin (Briz 1994; Briz et al. 1995; Bonet et al. 2000). Even though spermatozoa malformations are classified with regard to one aberrant sperm structure, often several abnormal structures converge in the same spermatozoon. Macrocephaly or microcephaly, additional heads or tails, longer or shorter tails, different shape malformations of the head, folding or coiling of the tail, etc. can be malformations found in the same aberrant spermatozoon (Fig. 1.7). Theoretically, there can be as many typologies of aberrant spermatozoa as paired combinations of the several possible malformations. Therefore, it is common to observe aberrant spermatozoa that, besides the highlighted malformation, present a second abnormality (e.g. aberrant spermatozoa with two or three heads and coiled tail, with macrocephaly and folded tail or two tails, with a flame-shaped head and coiled tail, etc.) (Figs. 1.7c, e, h, n, o and 1.10d, e, k–q). In order to establish a methodology for the description of the different morphological incidences it is useful to establish a classification criterion of cephalic malformations (affecting shape, size and number) and tail malformations (affecting shape or trajectory, size, and number), which frequently appear simultaneously. Moreover, the different aberrations present in the ejaculate may also be classified, in accordance with their place of origin, as primary or secondary: (1) primary malformations are those developed in the testis during spermatogenesis or spermiogenesis, and (2) secondary malformations are those developed during the sperm maturation process along the epididymis. In general, cephalic and tail size and number malformations tend to be of primary origin, but those affecting the tail trajectory are usually of secondary origin. Other malformations can be observed as a result of processing ejaculates in the laboratory (see Sect. 4.4.4).

Therefore, and according to the external and internal morphology of spermatozoa the following types of sperm malformations can be observed more or less frequently in boar ejaculates: immature spermatozoa with proximal cytoplasmic droplet (Figs. 1.7, 1.8 and 1.9); sperm malformations affecting the cephalic shape, size, and number (Figs. 1.7 and 1.10); and, sperm malformations affecting the tail trajectory, size, and number (Figs. 1.7, 1.11, 1.12 and 1.13). They are described one by one in the next section, some of them accompanied by ultrastructural details as seen by transmission electron microscopy (spermatozoa with two fused tails, with tail folded at the Jensen's ring, with coiled tail and with cephalic and tail malformations) (Figs. 1.7, 1.13, 1.14, 1.15 and 1.16).

1.3.2 Structure and Ultrastructure

The immature boar spermatozoon presents three distinctive traits compared to the mature spermatozoon: presence of the residual cytoplasmic droplet, greater development of the acrosome apical protuberance (or apical ridge) and greater flexibility of the head (Figs. 1.7a.1, i; 1.8 and 1.9).

The residual cytoplasmic droplet is approximately 1.5 μm in diameter and habitually encloses a small segment of the midpiece and contains an electrolucent cytoplasm rich in vesicles (Fig. 1.8a–c). The midpiece can be located at the center of the droplet or much more displaced from the center. The number and development of the vesicles increase as the cytoplasmic droplet moves distally from the connecting piece and along the midpiece (Fig. 1.8c–e). Two types of vesicles can be observed: double membrane vesicles and simple membrane vesicles. Double membrane vesicles have a clear matrix and a constant diameter of approximately 0.2 μm; they come from the cisternae of the smooth endoplasmic reticulum which close up over themselves enclosing hyaloplasmic material. Simple membrane vesicles also have an electrolucent matrix and arise from expansions of the endoplasmic reticulum. These last vesicles fuse together giving rise to greater vesicles exhibiting a large variety of diameters ranging from 0.1 to 0.9 μm (Fig. 1.8d, e).

The acrosome apical protuberance (or apical ridge) shows more development in immature than in mature spermatozoa. Its external width and length in the mature spermatozoon are 400 nm and 1.2 μm, respectively (Fig. 1.5a); and they increase until 600 nm and 4.5 μm, respectively, in the immature spermatozoon (Fig. 1.9e).

The head of the immature spermatozoa has greater flexibility than on reaching maturity. Different types of folds and deformations are often observed in the head of the immature spermatozoa. The more or less intense cephalic folds occur frequently through a cross plane (Fig. 1.8f, g), and more rarely through a frontal longitudinal plane (Fig. 1.8h). The most common cephalic malformation is a slight lateral deviation of the acrosomal region of the head with regard to the longitudinal axis of the cell. The immature spermatozoon is fragile, so it is not rare to observe transverse or longitudinal broken heads and tail ruptures (Fig. 1.9a–f).

Spermatozoa with malformations affecting the cephalic shape, such as elongation and flame, oval and round shapes, can be observed through light microscopy (Fig. 1.7m, r, s, u). The higher resolution of the scanning electron microscope, as compared to conventional light microscopy, allows for a more accurate detection of such anomalies (Fig. 1.10).

Two main types of spermatozoa with elongated heads can be found: spermatozoa with cylindrical heads and spermatozoa with narrow heads. Spermatozoa with elongated and cylindrical heads, about 8 μm in length and 2.7 μm in diameter, are characterized by an abaxial tail attachment (Fig. 1.10a). Spermatozoa with elongated and narrow heads, about 9–10 μm in length and 3.5–4.1 μm in diameter, are characterized by a normal tail attachment (Fig. 1.10b). Likewise, one can distinguish spermatozoa with elongated pear-shaped heads, detectable by the scarce development of the acrosomal cephalic region and the narrower postacrosomal region (Fig. 1.10c).

Spermatozoa with flame-shaped heads can also be classified regarding two major typologies: short flame-shaped heads and long flame-shaped heads (Fig. 1.10d, e). In this last typology, the apical ridge acquires a crater-like appearance (Fig. 1.10d).

Spermatozoa with oval heads usually present an apical acrosomal knob of variable development (Fig. 1.10f). Spermatozoa with round heads display many typologies ranging from globular to flat shapes and exhibiting different degrees of roundness (Fig. 1.10g, h).

With regard to the size of the head, two types of spermatozoa can be observed: macrocephalic and microcephalic spermatozoa (Figs. 1.7j–l, n, o, s and 1.10i, j). Within the macrocephalic or giant head spermatozoa, two typologies can be found: spermatozoa with normal head length (7 μm) and large width (4.7 μm), and spermatozoa with longer (7.7 μm) and wider (5.5 μm) heads. This last type of spermatozoa often has two tails (Fig. 1.10k). Within the microcephalic or small head spermatozoa, two typologies can also be noted: spermatozoa with normal head width and reduced length (5.2 μm), and spermatozoa with shorter (4.5 μm) and narrower (2.4 μm) heads.

Spermatozoa with more than one head frequently have as many fused tails as heads; these tails often exhibiting different degrees of coiling. Among spermatozoa with more than one head, those with three heads and those with two heads can be distinguished. Tricephalic spermatozoa tend to present completely coiled fused tails and three positioned coplanar heads giving rise to an angle not greater than 30º between them (Fig. 1.10l). Bicephalic spermatozoa tend to present completely or partially coiled fused tails and two heads arranged in coplanity in crossed planes (Figs. 1.7c, e, h and 1.10m, q). When the two heads are coplanar an angle ranging from 0º (overlapped heads) to 180º (opposite heads) can be formed between them (Fig. 1.10n–p). In this last case, heads are fused at their bases or they are separated by the tail coiling. When the two heads are positioned in crossed planes, these are orthogonal. In general, the two heads are often flat-shaped and attached to the connecting piece ending. However, a flat-shaped head and a globular or pyriform head can be found in some bicephalic spermatozoa (Fig. 1.10r). Head-to-head and tail-to-tail agglutinations are the possible origins of spermatozoa with more than one head.

Depending on the type of malformation affecting tail bending, one can distinguish two types of spermatozoa: spermatozoa with folded tails and spermatozoa with coiled tails.

Folding of tails can occur at the Jensen's ring level, at the midpiece level or at the connecting piece level. A tail folding is considered an anomaly when the tail bends completely in a way that the two parts of the folded tail run antiparallel (Figs. 1.11 and 1.14a). The most habitual folding occurs in immature spermatozoa with distal cytoplasmic droplet at the Jensen's ring level (Figs. 1.7g, o, 1.10h and 1.11a–d). The tail bends in the form that the principal piece contacts and fuses with the midpiece initially and with the flat face of the head later; finally, the tail can coil and fuse progressively over the flat face of the head (Figs. 1.11c–e and 1.14a). A more uncommon folding can also be observed; it concerns spermatozoa

whose tails bend at the midpiece level (Figs. 1.7n, q, y). This folding tends to be very apparent because of the midpiece thickness and the cytoplasmic droplet diameter and, in some cases, could be confused with a spermatozoon head under light or scanning electron microscopy due to its size and shape (Fig. 1.11d–f). The last category of spermatozoa with tail folded at the connecting piece level is even rarer and is scarcely found in boar ejaculates (Fig. 1.7p, z).

Spermatozoa with coiled tail exhibit complete or partial coiling (Fig. 1.7b, c, e, h, t). The most typical form is that of spermatozoa whose tails wrap around the first half of the midpiece (Fig. 1.12a). Within this modality, several typologies can be identified in regard to the placement of the coiling with respect to the head, and to the size and shape of this coiling. The coiling size may vary from approximately one half of the head size (Fig. 1.12b) to that of a normal-sized head (Fig. 1.12c, d). This variability relies on the degree of coiling and on the length of the coiled tail (depending on whether any tail piece is missing or is shorter). Moreover, the major axes of tail coiling and those of the head may be arranged in such a way as to give rise to angles of 180º, 90º, 45º or 30º (Fig. 1.10n–q). Tail coiling may adopt circular, spherical, oval or elliptical shapes. A second type of tail coiling is that of spermatozoa whose tails twist around the whole midpiece. In this case, the tail coiling adopts a very clear elliptical shape. The major axis of the coiling and the major axis of the head may be arranged so as to give rise to angles of 180º, 90º or 45º. Finally, more unusual tail coiling forms can be observed, for instance: coiling affecting solely the principal piece (Fig. 1.12f), coiling involving the principal and terminal pieces (Fig. 1.12g), coiling impacting the midpiece and partially the principal piece (Fig. 1.12h), coiling affecting solely the terminal piece or also partially the principal piece (Fig. 1.12i), false coiling (Fig. 1.12j), etc.

There are two modalities of sperm malformations affecting tail size: spermatozoa with short tails and spermatozoa with long tails (Fig. 1.13a). When the spermatozoon tail is shorter than normal it is generally due to the absence of some piece or to the lesser development of one of them; the affected piece is often the principal one (Fig. 1.13a.1, a.2). When the spermatozoon tail is longer than normal it is habitually due to a longer principal piece (Fig. 1.13a.3, a.4).

Two types of malformations affecting the tail number can be distinguished: tailless spermatozoa (Figs. 1.7d, 1.9f and 1.13b) and spermatozoa with two tails (Figs. 1.7u, v, 1.10k and 1.13c–e). The first typology is characterized by the presence of detached heads in the ejaculate; the majority of these forms correspond to artifactual breaking of heads and tails during handling that are often concentrated at the edges of the semen smears. Therefore, this malformation is really determined by the count of detached heads minus the number of detached tails. Spermatozoa with two tails may be classified according to whether the two tails have the same length or not, and whether they are fused or not (Figs. 1.10k and 1.13c–e). Moreover, it is also possible to find spermatozoa with two heads and with two completely fused tails (Fig. 1.13d).

Regarding the type of ultrastructural malformation, as observed on transmission electron micrographs, aberrant boar spermatozoa can also be classified into: (1) spermatozoa with two fused tails, (2) spermatozoa with tail folded at the

Jensen's ring, (3) spermatozoa with coiled tail, (4) spermatozoa with cephalic malformations, and (5) spermatozoa with tail malformations.

Spermatozoa with two fused tails have two complete axonemal axes (Figs. 1.7v and 1.13f). The two axonemal axes run parallel along their full path (Fig. 1.13f.5, f.6). Depending on the region examined one can find: (a) the two axonemal axes separated by a highly developed cytoplasmic mass (Fig. 1.13f.2, f.4), or (b) the two axonemal axes in contact with their respective mitochondrial and fibrous sheaths (Fig. 1.13f.1, f.3). Residual cytoplasm, actually abundant from the connecting piece to the intermediate region of the midpiece, declines considerably as it reaches the distal end of the midpiece.

Spermatozoa with tail folded at the Jensen's ring consolidate the folding by means of the distal cytoplasmic droplet (Figs. 1.7g, 1.10a–c and 1.14a.5–a.7). Beyond the distal cytoplasmic droplet domain, the principal piece and the midpiece overrun antiparallel (Fig. 1.14a). The fibrous and mitochondrial sheaths of both pieces come into contact, and the fibrous sheath provokes a slight deformation on the mitochondrial sheath in the proximal end of the midpiece. The principal piece overruns the midpiece following a helix path which becomes apparent by the evolution of the series of doublets faced between the midpiece and the principal piece (Fig. 1.14a.4). Along the postacrosomal cephalic region, the principal piece lies between the plasmalemma and the postacrosomal dense lamina (Fig. 1.14a.2). Along the acrosomal cephalic region, the principal piece is located between the plasmalemma and the acrosomal vesicle (Fig. 1.14a.1).

Spermatozoa with coiled tail have an axonemal axis that traces several loops inside a cytoplasmic mass rich in double membrane vesicular elements and the remainder of smooth endoplasmic reticulum (Fig. 1.14b). Such cytoplasm morphology is very similar to that of the cytoplasmic droplet of an immature spermatozoon. The axonemal axis is positioned at the periphery of the cytoplasmic mass (Fig. 1.14b.1). Degenerated cell figures can also be observed and characterized by their cytoplasmic plasmolysis and by the absence of some microtubular structures of the axonemal axes (Fig. 1.14b.1, b.2).

Spermatozoa with ultrastructural cephalic malformations consist of abnormalities affecting the different acrosomal segments, the postacrosomal cephalic region and the nucleus.

The acrosome apical segment may display several malformations: high or low development, anomalous shapes and distributions, internal vesicalization and heterogeneous acrosomal matrix (Fig. 1.15a–d). The acrosome principal segment can exhibit the following aberrations: acrosomal vesicle expansion, scarce matrix electrodensity, and vesicalization (Fig. 1.15e). The acrosome equatorial segment can also present different malformations: high development of the subacrosomal space, and expanded and festooned edges of the acrosomal vesicle (Fig. 1.15f, h).

In the postacrosomal region, a greater or lesser development of myelin fibers between the postacrosomal dense lamina and the perinuclear fibrous material can be found (Fig. 1.15g, i).

Finally, the most typical ultrastructural nuclear anomalies are membrane-bound nuclear invaginations and nuclear vacuoles (Fig. 1.15l, j, k). Moreover, other

nuclear malformations can also be observed (e.g. nucleus folded by a longitudinal or by a transverse axis) accompanied by acrosomal anomalies (generally, vesicalization or plasmolysis) (Fig. 1.15m–o).

Spermatozoa with ultrastructural tail malformations affect the axoneme, the mitochondrial sheath and the perimitochondrial cytoplasm (Fig. 1.16).

Several modalities of axonemal anomalies consisting of the absence of microtubular elements can be detected: deficiency of the peripheral microtubule doublets (6, 8, 9 and, 5, and 6) and absence of the central microtubule pair (Fig. 1.16a–c). Furthermore, we can also observe a general disorganization and deformation of the axonemal structure by the presence of more or less developed vesicles lying between the fibrous sheath and the axoneme (Fig. 1.16d–f).

The two main ultrastructural abnormalities affecting the mitochondrial sheath are mitochondrial swelling (Fig. 1.16c) and the irregular arrangement of the mitochondria in the sheath (Fig. 1.16g). Mitochondrial swelling affects generally the first mitochondria of the mitochondrial sheath (Fig. 1.16i). These mitochondria can be five times greater in diameter compared to the others in the sheath and be characterized by a very clear matrix and by the absence of mitochondrial cristae. The inner mitochondrial membrane does not form cristae and runs parallel to the outer membrane (Fig. 1.16c). The other alteration of the mitochondrial sheath consists of an anomalous distribution of mitochondria. In this case, there are additional mitochondria regularly distributed in a helix path external to the sheath which determine a slight deformation of the circular perimeter of the midpiece (Fig. 1.16g, j). Moreover, the mitochondrial sheath sometimes exhibits reduced thickness and a festooned contour (Fig. 1.16h).

Concerning the perimitochondrial cytoplasm, several ultrastructural malformations may be highlighted. Peripheral vesicalization can be found at any level of the first three tail pieces of the spermatozoon disturbing the circular perimeter of the tail, the development of the vesicles being greater from the connecting piece to the principal piece. At the connecting piece level, peripheral vesicles (about 0.30 μm in diameter) lie between the laminar bodies and the plasmalemma (Fig. 1.16l). At the midpiece level, vesicles (about 0.35 μm in diameter) lie between the mitochondrial sheath and the plasmalemma, provoking a noticeable deformation on some mitochondria (Fig. 1.16m). At the principal piece level, vesicles (about 0.40 μm in diameter) lie between the fibrous sheath and the plasmalemma and do not provoke noteworthy deformations on the fibrous sheath ribs (Fig. 1.16n). These vesicles are released along the proximal region of the principal piece by narrowing at their base and the posterior fusion of the plasmalemma (Fig. 1.16o, p). These vesicles may come from large vesicles within the cytoplasmic droplet of immature spermatozoa or from cytoplasmic expansions present in the connecting piece or midpiece of some spermatozoa. The residual cytoplasm appears under two forms at the connecting piece and midpiece level: (a) in the form of cytoplasmic expansions and (b) in the form of perimitochondrial cytoplasmic films. The cytoplasmic expansions lie at the base of the head, just in the junction of the connecting piece and the postacrosomal cephalic region (Fig. 1.16q). They are digitiform cytoplasmic evaginations, 1 μm in length per

0.1 μm in diameter, filled with a homogeneous and slightly electrodense cytoplasm. These evaginations may bend until fusing at their free end with the plasmalemma enclosing the connecting piece, and remain attached or are occasionally released. Digitiform or fungiform cytoplasmic evaginations can also be found at the more distal region of the midpiece. In these cases, the cytoplasmic expansion arises from a highly developed perimitochondrial cytoplasmic film (Fig. 1.16k).

In any case, most of the defects described above prevent proper sperm function, either impairing motility (tail malformations) or causing weaknesses in sperm-oocyte interaction (cephalic malformations).

1.4 Sperm Cell Surface

The mature spermatozoon is a highly polarized cell with a minimal amount of cytosol and organelles, which has lost its potential for gene expression and, consequently, for protein synthesis (there are no ribosomes and chromatin is extremely condensed and tightly packed) (Boerke et al. 2007). This seems to make mammalian spermatozoa proteomically simpler than somatic cells. However, the spermatozoon has a highly complex degree of plasma membrane specialization and more membrane proteins than many other cell types. In addition, spermatozoa undergo significant post-testicular maturation in the epididymis and reorganization during capacitation in the female reproductive tract in order to acquire their complete functionality to fertilize the oocyte (Brewis and Gadella 2010). Epididymal maturation and fertilizing ability acquisition convert the sperm plasma membrane into a very dynamic structure, with polarized domains of intramembranous particles, subjected to several modifications by the releasing, the redistribution or the adsorption of proteins and lipids that change the lipid/protein ratio and composition (Flesch and Gadella 2000).

As a result of these cell surface modifications, occurring from spermatogenesis to fertilization, the mature spermatozoon emerges as a highly polarized and differentiated cell which shares some cell surface similarities with other highly differentiated cell types such as epithelia, photoreceptors, and neurons (Thaler and Cardullo 1995). Like many cells involved in recognition, binding and signaling events, the mammalian spermatozoon exhibits a high degree of molecular mosaicism on its surface, which applies not only to the membrane proteins but also to the lipid environment as well (for a detailed review see Jones et al. 2007, 2008). According to the pattern of the membrane, the sperm surface may be considered to have five major membrane domains, each closely associated with an underlying cell compartment or cytoskeletal element and involved in different aspects of cell function. The head has three major domains covering the acrosomal region in its three distinguishable segments (the apical ridge, and the principal and equatorial segments), and the tail has distinct domains over the midpiece and the principal piece (Figs. 1.4 and 1.5).

This regional membrane specialization allows the underlying cellular components to interact independently with their external environment, thereby enabling

efficient performance of the various tasks necessary for successful fertilization (i.e. recognition, binding and fusion with the oocyte) (Curry and Watson 1995). In particular, the acrosome apical region of the sperm head specifically recognizes and binds to the oocyte zona pellucida; the acrosome principal region is involved in the acrosome reaction required for zona penetration; and the acrosome equatorial region specifically recognizes and fuses with the oocyte plasma membrane. Despite the fact that the surface of the midpiece and principal piece of the sperm tail are also hetereogeneous, the function of these plasma membrane specializations is not yet understood but it is likely that these domains are involved in the organization of optimal sperm motility characteristics (Brewis and Gadella 2010).

1.4.1 The Glycocalyx

Mammalian spermatozoa are covered by a carbohydrate-rich dense coating zone. Several hundred different glycoproteins comprise the sperm glycocalyx forming a 20–60 nm thick coat essential for the acquisition of full sperm fertilizing ability. The mature glycocalyx becomes an immunoprotective barrier for the spermatozoa in the female tract, allows intercellular gamete communication and has an important role in the early interaction steps during sperm-oocyte recognition (Kirchhoff and Hale 1996; Schroter et al. 1999; Flesch and Gadella 2000; Diekman 2003). Some of these sugar residues are synthesized within the testis, while others are produced in the efferent ducts, by the epididymis epithelium or by the accessory glands, and incorporated during the spermatozoa post-testicular maturation.

The carbohydrate residues are linked to proteins and lipids of the sperm membrane. Sugar residues can be intercalated or anchored within the lipid bilayer, or superficially associated with the membrane via polar groups or through hydrophobic interactions. They can be either integrated within the sperm membrane or loosely associated with it (Schroter et al. 1999).

Lectins are, commonly, proteins of plant origin and are able to recognize specific oligosaccharide structures. The conjugation of lectins with fluorochromes or biotin systems permits cell-surface sugars and the changes that these sugars undergo during cell growth, differentiation or malignancy, to be investigated (Lis and Sharon 1998). This association is also useful for the structural characterization of the carbohydrate moieties of glycoproteins, as lectins can discriminate different glycoconjugates in the spermatozoon surface. Several studies have been performed with lectins to analyze the carbohydrate composition of sperm plasma membrane in species such as rodents (Brown et al. 1983; Liu et al. 1991; Tulsiani et al. 1993; Calvo et al. 1995); poultry (Pelaez and Long 2007), rabbits (Nicolson et al. 1977), marsupials (Cooper et al. 2001), monkeys (Navaneetham et al. 1996; Srivastav 2000), rams (Hammerstedt et al. 1982; Magargee et al. 1988), bovine livestock (Taitzoglou et al. 2007), stallions (Desantis et al. 2010), porcine livestock (Harayama et al. 1998; Jiménez et al. 2002, 2003) or humans (Kallajoki et al. 1985; Bains et al. 1992).

The glycocalyx carbohydrate composition of the sperm surface is strongly modified throughout the epididymis and the net negative surface charge increases (López et al. 1989). Most lectins intensely label almost every part of the sperm surface, illustrating the importance of the glycocalyx around the spermatozoon. The principal piece of the boar spermatozoon is only coated by N-acetyl-D-glucosamine and N-acetyl-galactosamine residues (Fàbrega et al. 2011a). The global galactose, glucose/mannose, and N-acetyl-D-glucosamine content increases significantly over the surface throughout the epididymal duct, whereas N-acetyl-D-galactosamine and fucose residues are maintained in spermatozoa coming from the proximal regions of the epididymis and slightly decrease in those from the epididymal cauda (Fàbrega et al. 2011a). These changes are probably mediated by the secretion of specific sialoproteins into the epididymal corpus lumen of boars (Harayama et al. 1999) and their arrangement over the sperm surface (Calvo et al. 2000), which can mask sperm surface carbohydrate residues during examinations.

The specific location of the different carbohydrate residues throughout the epididymis has been also studied. Galactose residues are practically absent over the intact acrosome surface and they are localized mostly over the outer acrosomal membrane (Fazeli et al. 1997) and over the cytoplasmic droplet and midpiece of immature boar spermatozoa from the epididymal caput (Fig. 1.17a) (Fàbrega et al. 2011a). Galactose residues are also found to be abundant over the cytoplasmic droplet of bull and ram epididymal spermatozoa (Arya and Vanha-Perttula 1985; Magargee et al. 1988) and they could probably be involved in cytoplasmic droplet migration along the spermatozoon tail midpiece during the process of epididymal sperm maturation (Fàbrega et al. 2011a) (see Sect. 3.3.4). Fucose residues are rarely detected on the epididymal boar sperm surface and they are only significantly observed on spermatozoa from the epididymal corpus (Fig. 1.17e). This exclusive distribution may respond to the secretion of specific sialoproteins into the epididymal lumen (Harayama et al. 1999). In contrast, N-acetyl-glucosamine residues are highly spread over the sperm surface (Fig. 1.17c), especially over the flagellum and over the apical ridge in boar spermatozoa coming from distal epididymal regions and ejaculates (Töpfer-Petersen et al. 2008; Fàbrega et al. 2011a). Glucose/mannose residues are principally located over the sperm acrosomal surface and over the sperm tail midpiece throughout the epididymis (Fig. 1.17b). The N-acetyl-galactosamine residues intensely coat the acrosomal apical ridge (Fig. 1.17d) and the cytoplasmic droplet surface of boar epididymal sperm (Wagner et al. 2002; Fàbrega et al. 2011a). As can also be observed for several sperm surface proteins (Phelps et al. 1990; Petruszak et al. 1991), the migration of these residues toward the sperm head during maturation (Wagner et al. 2002; Fàbrega et al.2011a) is consistent with their involvement in the capacitation process of ejaculated boar sperm (Jiménez et al. 2003), in sperm-oocyte interaction (Nimtz et al. 1999; Töpfer-Petersen 1999), and in the prevention of head-to-head sperm agglutination (Calvo et al. 2000). Furthermore, oligomannose or mannosyl residues are specifically known to have an important role in the creation of the sperm reservoir in pig oviducts (Wagner et al. 2002).

A study performed by our group with different lectins has demonstrated changes in the composition of the carbohydrate moieties that coat the sperm surface glycoproteins during the epididymal transit for sperm maturation. Among the most visible, a 112–102 kDa glycoprotein, which exhibits galactose, glucose/mannose, N-acetyl-glucosamine, N-acetyl-galactosamine and fucose moieties, is present throughout the entire epididymal transit; a 151 kDa glycoprotein containing glucose/mannose, N-acetyl-glucosamine, and fucose moieties has been identified only in spermatozoa from the proximal caput; a 133 kDa glycoprotein with fucose moieties is present from the distal caput to the cauda; multiple glycoproteins from 91 to 73 kDa with N-acetyl-glucosamine, N-acetyl-galactosamine, and fucose moieties are only present on proximal and distal caput epididymal spermatozoa; three glycoproteins of 16, 19 and 23 kDa with glucose/mannose moieties appear in succession along the epididymal regions and could represent transient forms of a same protein for which glycans are modified during the epididymal transit (Fàbrega et al. 2011a). These complex modifications on the sperm surface during maturation are the consequence of several interactions with the epididymal milieu, in particular with glycosidases, known to be present at elevated concentrations in this medium (Syntin et al. 1996).

During boar sperm capacitation and acrosome reaction the glycocalyx composition and distribution over the sperm surface is also hardly modified. The presence of N-acetyl-glucosamine residues over the sperm head and flagellum in epididymal spermatozoa decreases in the ejaculated spermatozoa after capacitation and even further after the acrosomal reaction. Glucose/mannose residues mainly increase over the acrosomal region in capacitated spermatozoa and are concentrated over the apical ridge in the acrosome-reacted sperm. Furthermore, fucose residues are scarcely present over ejaculated, capacitated, and acrosome-reacted spermatozoa as previously described for the epididymal spermatozoa (Jiménez et al. 2003).

1.4.2 Features of Boar Sperm Membrane Proteins and Lipids

It has been suggested that boar sperm protein and lipid composition are found in similar total amounts, the phospholipid/protein weight ratio of an isolated plasma membrane being approximately 0.68 (Nikolopoulou et al. 1985). However, spermatozoa epididymal transit and capacitation change both ratio and composition (Flesch and Gadella 2000).

Sperm plasma membrane proteins play a key role in sperm–oocyte interaction. Several changes in the protein composition of the sperm surface membrane occur during post-testicular differentiation, maturation of spermatozoa throughout the epididymal transit, storage in the epididymal cauda and in the female tract. Also, several seminal plasma proteins, such as sperm surface protein DQH and several seminal plasma spermadhesins such as porcine seminal plasma glycoprotein (PSPII), spermadhesins with N-terminal amino acid sequence Ala–Gln–Asn

(AQN-1 and AQN-3) and with N-terminal amino acid sequence Ala–Trp–Asn (AWN) and their respective glycoslyated isoforms (Sanz et al. 1991; Jonáková et al. 2000) are known to bind the sperm surface during maturation or to participate at ejaculation in the sperm reservoir in the porcine oviduct (Manásková et al. 2007), in sperm capacitation and to assist primary sperm interactions with the zona pellucida (Petrunkina et al. 2000). Boar spermadhesins are synthesized by the epididymis and the accessory glands of the male genital tract, and some of them, such as AWN, also in the Fallopian tube of the female genital tract (Ekhlasi-Hundrieser et al. 2002). Calcium-binding proteins (CBPs) are other boar seminal plasma proteins that bind specifically during epididymal transit onto the boar sperm plasma membrane that overlies the principal segment and which are known to be removed during capacitation (Peterson et al. 1989).

Epididymal maturation results in the progressive disappearance of most of the testicular compounds on the boar sperm surface membrane, which are either renewed or masked by new permanent or transient low molecular weight polypeptides (Dacheux et al. 1989) and processed according to their specific function: (1) proteins located to specific domains, such beta-fertilin (ADAM2) or PH-20 are processed and relocated (Primakoff et al. 1985; Overstreet et al. 1995; Blobel 2000) in order to participate in oocyte–sperm interaction and fecundation (Jury et al. 1997; McLaughlin et al. 1997; Waters and White 1997); (2) some proteins secreted by epithelial epididymal cells are apparently added to the sperm surface (Gupta 2005), such as MEPs or SMA-4 in mice, E-3 in rats, HEP64 or P26 h in other rodents, and a 135 kDa protein in large domestic animals (Okamura et al. 1992); (3) finally, other proteins are released into the epididymal medium, like the angiotensin-convering enzyme (ACE) (Gatti et al. 1999). The study of boar epididymal plasma membrane proteins has resulted in the identification of 32 proteins by mass spectrometry (Belleannée et al. 2011), which have been suggested to control modifications of surface proteins and their correct folding during sperm maturation (TCP-1 complex, H1P1, and HSPs), to participate in sperm-oocyte membrane fusion (VCP with other HSPs) and to mediate the defence of male gametes against oxidative stress (especially from VCP, HSPA2, PRDX5 and GSTM5, which have been described to vary in concentration along the epididymal duct) (Belleannée et al. 2011). Further studies demonstrate that Hsp70 on boar sperm is relocalized and translocated from the inner to the outer leaflet of the sperm plasma membrane as sperm undergo capacitation and acrosome reaction, suggesting an important role of this protein during porcine gamete interaction (Spinaci et al. 2005). Some of the sperm surface proteins identified by Belleannée et al. (2011) have already been reported in the male gamete surface and luminal fluids, such as the angiotensine conversing enzyme (ACE) (Gatti et al. 1999), aldose reductase (AR) (Frenette et al. 2003; Pruneda et al. 2006), arylsulfatase A (ARSA) (Carmona et al. 2002), α-enolase (ENO) (Gitlits et al. 2000), glutathione S-transferase (GST) (Hemachand and Shaha 2003), huntingtin interacting protein 1 (HIP1) (Rao et al. 2001), heat shock 90 and 70 kDa proteins (HSP-90; HSP-70) (Spinaci et al. 2006), α-mannosidase (MAN) (Kuno et al. 2000), lactadherin (MFGE8) (Petrunkina et al. 2003), peroxiredoxin 5 (PRDX5) (Van Gestel et al. 2007), and T-complex protein 1 (TCP1) (Dun et al. 2011). Other identified proteins

have been found to belong to the acrosome (probably released from reacted spermatozoa), such as the acrosine (Puigmulé et al. 2011), LYP4 (Shetty et al. 2003), and sp38 (Mori et al. 1995), or to have cytosolic origins, such as β-tubulin (Pěknicová et al. 2001), valosin-containing protein (VCP) (Geussova et al. 2002), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ; 14-3-3) (Puri et al. 2008), which are known to interact with several membrane proteins.

The knowledge of protein processing during sperm maturation in the epididymal duct could address the question about their role on fertility and their potential use as fertility markers. The fertilin complex (ADAM-1 and ADAM-2) can be mentioned as an example. Fertilin complex expression was first described in guinea pigs (Blobel 2000) and mice (Kim et al. 2006), and a similar pattern was later suggested in bulls (Walker et al. 1996) and more recently in monkeys (Kim et al. 2010) and boars (Fàbrega et al. 2011b). In boars, fertilin maturation involves a regionalized proteolytic processing; most of the 70 kDa testicular ADAM-1 precursors are reduced to the mature 50–55 kDa form when spermatozoa get into the testis-efferent ducts; however, the 70 kDa precursor is not found until the distal corpus. For ADAM-2, a sequential proteolytic cleavage pattern of maturation restricted to proximal caput and corpus has been suggested: the testicular form is processed in the proximal caput into a precursor form of 90 kDa, and then to transient forms of 70–75, 65–70 and 50–55 kDa in the distal caput and corpus, which are further processed in the corpus leading to a final form of 40–43 kDa in the cauda (Fàbrega et al. 2011b). Both, ADAM-1 and ADAM-2 processing may have an essential role in the fertilin complex migration from the whole acrosomal domain to the acrosomal ridge that takes place in sperm at the distal epididymal corpus (resulting in an increase in the local concentration of fertilin that may be crucial for primary sperm-oocyte interactions) (Jones et al. 2007; Van Gestel et al. 2007; Fàbrega et al. 2011b).

Other important plasma membrane proteins present in mature spermatozoa are the GLUT family. This protein family allows the transport of glucose, fructose and related hexoses and pentose through the lipidic bilayer of the spermatozoon, necessary for the uptake of energy sources to maintain basic cell activity as well as specific functions such as motility and fertilization ability (Bucci et al. 2011). In boar, GLUT 1, 2, 3 and 5 have been described and localized (Bucci et al. 2010), and a clear decrease in GLUT 3 transporters has been observed in cryopreserved spermatozoa, suggesting that the cooling/freezing-associated alterations induce changes in the ability of boar sperm to manage their energy levels, thus altering the overall sperm function after thawing (Sancho et al. 2007; Casas et al. 2010).

Furthermore, 14 sperm-specific membrane proteins (SSMPs) ranging from 7.5 to 66 kDa have been described in boar plasma membrane. The identified SSMPs are immunodominant proteins of the sperm membrane; however, the SSMPs repertoire is probably larger and includes minor sperm membrane components that have not yet been identified. Most of them are glycoproteins with an acidic pI and at least one disulfide bond. They overlay all major regions of the spermatozoon and mediate key molecular events of the fertilization process (Haden et al. 2000).

Sperm plasma membrane contains much less cholesterol and glycolipids in boars than in humans (Mann and Lutwak-Mann 1982) due to the loss of cholesterol during the epididymal transit (Nikolopoulou et al. 1985). In contrast, the boar sperm plasma membrane contains relatively high amounts of plasmalogens and other ether-linked phospholipids and lipids with long, polyunsaturated aliphatic chains (Evans et al. 1980). The boar sperm cholesterol/phospholipids molar ratio is about 0.12; 70 % of sperm membrane lipids are phospholipids, choline being the most abundant followed by steroids (Nikolopoulou et al. 1985). Neutral lipids correspond to 25 % of the sperm plasma membrane lipid content and the remaining 5 % correspond to glycolipids (Mann and Lutwak-Mann 1982). This characteristic lipid composition leads to the formation of jellified lipid phases during cooling (Parks and Lynch 1992), which makes boar sperm highly sensitive to cold-shock (Simpson et al. 1987). There is abundant evidence that when boar spermatozoa are refrigerated or frozen both motility and metabolic activity are irreversibly depressed and the acrosome and plasma membranes disrupted. Cold-shock causes loss of cholesterol from sperm and extenders used during cryopreservation must partially supply it (White 1993) (see Sect. 11.2).

Furthermore, during sperm capacitation the organization of plasma membrane proteins and lipids also changes dramatically, allowing sperm to bind to the zona pellucida and thereafter to acrosome react (Gadella et al. 2008) (see also Chap. 7).

1.5 Conclusion

Bearing in mind the particular cell anatomy of boar spermatozoon reinforces the view that, in principle, the function determines the structure. However, for some features of the spermatozoon misunderstandings about structural–functional relationships still exist because certain functions of some structures have not yet been definitively elucidated. Moreover, what is still scarcely understood is the synergistic and concerted fusion in which a variety of identified sperm membrane proteins interact with one another, leading ultimately to the syngamy of sperm and oocyte. It is expected that recent and future advances in proteomics will aid greatly in determining the correct sequence of molecular events leading to sperm–oocyte adhesion (capacitation, induction of acrosome reaction, and sperm-oocyte plasma membrane fusion). Undoubtedly, the highly specialized spermatozoon represents a considerable intellectual challenge to biologists and researchers in the field of reproduction, and persistent studies of the structure, biochemistry, biophysics and functionality of this unique cell will certainly yield still more stimulating rewards in the future. Apart from their basic scientific interest, the thorough knowledge of the wide variety of morphological forms of spermatozoa occurring in boar ejaculate, and also the progress in determining the composition and modus operandi of the specific molecules present in sperm membrane, can be very helpful for improving the efficiency of animal production by means of the currently available biotechnology procedures.

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Chapter 2
Biological Aspects of the Mature Boar Spermatozoon

Joan E. Rodríguez-Gil

Abstract A practical consequence of the particular reproductive cycle of sows is that the functional features that distinguish boar spermatozoa cannot be extrapolated to other species, thus preventing an overall picture that explains mammalian sperm function from being assumed. Furthermore, the extraordinary complexity of the molecular mechanisms implied in the control and modulation of mature boar sperm functions makes it impossible to provide a complete description of these mechanisms in the limited space of this chapter. Taking this into account, this chapter centers on the description of three highly important specific aspects of boar sperm function. The first aspect is the mechanisms by which boar sperm cells manage their energy levels. The second aspect is the functional role of mitochondria as controllers of boar sperm function. The third aspect will address the existence of functional, separate subpopulations in boar ejaculates, and the hypothetical biological role of these subpopulations.

2.1 Introduction

The mammalian spermatozoon is a very specialized cell designed to accomplish an ultimate goal: the transmission of the paternal genome to the next generation. To achieve this goal, mammalian spermatozoa are finely designed, following the specific evolutionary reproductive strategy chosen by each species. This specificity of design implies that mature mammalian sperm are very complex cells, with several functional features that clearly distinguish them from other eukaryotic cells. Focusing on swine, boar spermatozoa are perfectly adapted to a female reproductive cycle based on oestrous phases of about 2–3 days, poly-ovulatory events, and a

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tendency of the sow toward monogamy. This differentiates boar sperm functionality from other species and thus, their sperm must accomplish particular steps in order to yield optimal “in vivo” fertility rates.

2.2 Mature Boar Sperm Energy Resource Management

Control mechanisms of boar sperm energy management play an essential role. This is due to the fact that practically all the reactions that maintain the functional status of the cell (control of tyrosine phosphorylation levels, maintenance of the glycosylation of membrane proteins, etc.), as well as those related to the maintenance and further control of molecular mechanisms controlling sperm resistance to environmental changes, need significant energy consumption. Thus, the optimal function of all these mechanisms will depend, to a great extent, on a correct functioning of control mechanisms modulating boar sperm energy management. Unfortunately, and despite vast amount of knowledge accumulated by many investigators in the past 20 years, there are several commonplaces regarding sperm energy metabolism, which, in fact, obstruct an optimal practical application of this knowledge. Thus, everybody knows that the mammalian spermatozoon is strictly a glycolytic cell. However, the adoption of this assertion can undoubtedly lead to the opinion that spermatozoa are almost exclusively glycolytic, therefore, they have practically no other modulator system to manage their energy levels (Mann 1975). On the other hand, if the spermatozoon is an exclusively glycolytic cell, what is the role of sperm mitochondria and the associated mitochondrial respiration? In this respect, it is noteworthy that many investigators indicate as an absolute fact that the energy obtained through the mitochondrial respiration is, under all conditions, absolutely necessary for the maintenance of sperm motility in all species (Nevo et al. 1970; Ford and Harrison 1985; Halangk et al. 1985; Folgero et al. 1993; Ruíz-Pesini et al. 1998), despite the fact that the same investigators maintain the absolute preeminence of glycolysis to obtain sperm energy, without realizing the contradiction in terms of energy that the simultaneous assumption of both principles implies. These contradictions highlight the complexity of the question, which has to be approached with an open mind. Only in this way can some valid and general conclusions with practical applicability on sperm conservation be attained.

Taking into account all these aspects, the study of boar sperm energy management can be structured around the following questions:

- First question: what energy sources can be utilized by mature boar sperm during their journey inside the female genital tract?
- Second question: what are the main metabolic pathways by which these energy sources are utilized?
- Third question: what are the main control mechanisms that coordinate energy management and changes in boar sperm function?

2.2.1 Energy Sources of Mature Boar Sperm

A general consensus exists among researchers that the main external energy sources of mature boar sperm are monosaccharides. In this way, it has been described that boar spermatozoa are able to metabolize a great variety of monosaccharides, from the ubiquitous glucose to other less common sugars, such as fructose, sorbitol, and mannose (Mann 1975; Jones and Connor 2000; Rigau et al. 2002; Marín et al. 2003; Medrano et al. 2006a). However, it is noteworthy that the fact that boar sperm is able to metabolize a specific sugar does not imply that this sugar is a usual energy substrate. In fact, the ability of boar sperm to utilize a specific monosaccharide is different depending on the utilized sugar. In this sense, it has been described that hexokinase activity, which phosphorylates monosaccharides thereby enabling their utilization through the cellular metabolic pathways, is more sensitive to glucose than to other monosaccharides, such as fructose, sorbitol, and mannose (Medrano et al. 2006a). This implies that glucose will be utilized faster than the other monosaccharides and, in this way, glucose will be a more suitable energy source than, for instance, fructose. However, there are other factors modulating the different availability of monosaccharides for boar sperm energy production. In fact, the first control step in monosaccharide utilization is not sugar phosphorylation, but monosaccharide uptake from the extracellular environment. This uptake is carried out through specific transmembrane transporters. To date, several hexose transporters have been described in boar spermatozoa. These are mainly of the GLUT family transporters. Boar spermatozoa present at least four separate GLUTs, namely GLUT-1, GLUT-2, GLUT-3, and GLUT-5 (Bucci et al. 2010). These transporters have separate affinities for specific monosaccharides, as well as presenting different hexose uptake rates. Thus, GLUT-3 is a highly specific glucose transporter, whereas GLUT-5 is completely fructose-dependent (Mueckler 1990). The presence of separate transporters with highly different affinity and uptake rate characteristics entails a complex and very sophisticated system to control hexose uptake. This is further complicated by the existence of a very specific distribution of each GLUT over the entire sperm surface. Thus, GLUT-3 is preferentially placed at the midpiece,whereas the preferential location of GLUT-5 is at the periacrosomal area, although researchers differ as to the utilized fixative technique (Medrano et al. 2006a; Sancho et al. 2007; Bucci et al. 2010). These specific locations indicate that a specific hexose, like glucose, can only be taken up by boar sperm through specific points of the cellular membrane, initiating thus its metabolism at strictly limited locations in the boar sperm cellular structure. We can only speculate about the biological significance of this strict hexose uptake location, although it seems to indicate the existence of directionality in hexose metabolism, by which a precise monosaccharide would be metabolized in a precise location inside boar sperm. This directionality, in turn, would indicate that hexose metabolization could follow specific and separate pathways depending on the precise uptake point. This would imply in turn that mammalian sperm regulate hexose utilization not only through the control of the enzymatic activities that modulate this

utilization, but also through the presence of specific metabolic pathways with a precise, spatial location inside the cell. These spatial mechanisms would allow for a better optimization of the utilization of energy sources, since they are metabolized in an almost instantaneous manner throughout the metabolic pathway, which could render maximal efficiency depending on the specific physiological status of the sperm.

Sugars are not the only extracellular substrate that boar sperm can utilize to obtain energy. Boar sperm can also utilize non-hexose compounds, such as lactate, pyruvate, glycerol ,and citrate (Jones et al. 1992; Jones 1997; Medrano et al. 2006b). The role of these substances as energy sources of mature boar sperm is, however, not well understood. These compounds do not seem to be present in significant amounts, at least, several of them like glycerol, are either in seminal plasma or inside the female genital tract. Moreover, whereas some metabolites, such as glycerol, can be metabolized by glycolysis, others like lactate, pyruvate, and citrate will be directly metabolized through the mitochondrial Krebs cycle. This poses a problem, since the energy yielding rate of boar sperm mitochondria is very low, about two magnitude orders below that determined for other highly energy-producing mitochondria, such as those from pig hepatocytes (Balis et al. 1999; Ramió-Lluch et al. 2011). This implies that these substrates are, in fact, inefficient at providing energy, especially when compared to monosaccharides. This low efficiency seems to point another role of these substrates other than merely obtaining energy. In this regard, it is noteworthy that citrate is directed by boar sperm to the Krebs cycle, but the obtained citrate-derived metabolites are subsequently processed through the pyruvate carboxylase step, finally synthesizing lactate. This lactate is subsequently secreted to the extracellular medium and then reentered into the Krebs cycle through the lactate dehydrogenase step. At a first glance, this very complicated utilization mechanism is not easy to understand. Nevertheless, this mechanism will yield important amounts of the reductive agent NAD^+, which is of paramount importance to stabilize sperm function (Baker and Aitken 2004). In this manner, these substrates would be primarily utilized to obtain reductive potential, energy production being thus a less important function.

There is a final point of interest regarding the sources of boar sperm energy substrates. What is the origin of these sources? It seems clear that mature boar sperm, after ejaculation, can only obtain their energy substrates in two ways. The first way will be from compounds present in the seminal plasma. The second way will be from substrates already present inside the female genital tract before sperm entry. Regarding substrates from seminal plasma, it is noteworthy that the great volume of boar ejaculate allows spermatozoa to maintain contact for a time with substances contained in the seminal plasma. This is different from other species, such as bovine or human, in which the sum of a low volume ejaculate with a fast semen deposition inside a genital tract or a relatively great volume implies that the contact of sperm from these species with compounds of seminal plasma will be brief. Moreover, boar seminal plasma

has been described to contain measurable amounts of glucose, although there are great discrepancies regarding the real concentration of the sugar (Baronos 1971; Mann 1975). This is important if we remember that glucose is the most efficient sugar to produce energy for boar sperm (Medrano et al. 2006a). Other sugars, like fructose and inositol, are also present (Baronos 1971; Mann 1975), thus the question arises of what the exact role of these minority compounds is in boar ejaculates. One possibility is that minority energy substrates could play another role than just being an energy source. We have already explained how boar sperm metabolizes citrate and the primary role of this compound as source of reduction potential (Medrano et al. 2006b). A similar role could be played by other substrates, like lactate. Another role could be that of a direct functional modulator by acting on, for instance, tyrosine phosphorylation levels of specific intracellular proteins. This effect has been demonstrated for both glucose and fructose in dog, but not in boar sperm (Fernández-Novell et al. 2011 and Fig. 2.1). However, this lack of effect does not necessarily imply that a sugar-specific action on some specific intracellular proteins is not present in boar sperm, and further investigations are needed to elucidate the role of minority substrates found in both seminal plasma and the female genital tract.

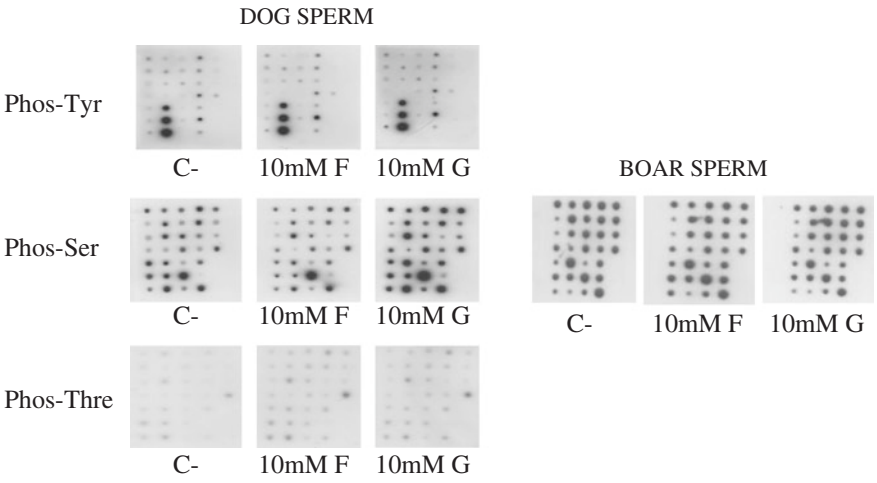

Fig. 2.1 Mini-array analysis of the tyrosine, serine, and threonine phosphorylation status of several proteins involved in the regulation of cell cycle and overall cell function in dog and boar spermatozoa after incubation with glucose or fructose. Dog and boar spermatozoa were incubated for 5 min in the absence (C-) or presence of either 10 mM fructose (10 mM F) or 10 mM glucose (10 mM G). The tyrosine- (Tyr-Phos), serine- (Ser-Phos), and threonine-phosphorylation (Thre-Phos) levels of each spot in the mini-arrays were then analysed. The figure shows a representative image for five separate experiments. Figure taken from Fernández-Novell et al. (2011)

2.2.2 Main Metabolic Pathways for Energy Production in Mature Boar Spermatozoa

As indicated above, the most important metabolic pathway by which mature boar sperm obtain energy is glycolysis. In a metabolomic study, it has been observed that approximately 95 % of the energy yielded by glucose in boar sperm originates through the glycolytic pathway, the mitochondrial respiration-produced energy being only about 5 % of the total glucose energy yield (Marín et al. 2003). At a first glance, this result can seem contradictory, since the glycolytic energy yield is much lower than that obtained through other metabolic pathways like mitochondrial respiration. Thus, glucose is not efficiently metabolized by boar sperm in these conditions. However, there is an aspect that could explain this apparent contradiction. As any biochemistry student knows, the mitochondrial respiration requires an aerobic environment to work in optimal conditions, whereas glycolysis is highly efficient in an anaerobic environment. The environment that mature boar sperm will find after ejaculation inside the female genital tract would be, generally speaking, mostly anaerobic. In these conditions, the mitochondrial respiration will not work at good rates, whereas glycolysis will. Taking into account this aspect, it is logical that the overall metabolic machinery of mature boar spermatozoa has been designed to maximize the generation of energy in anaerobic conditions, thus giving a prominent role to the glycolytic pathway. Obviously, all these results do not preclude the existence of a precise role for the energy obtained through the minority mitochondrial respiration route. Thus, it has been observed that the achievement of a feasible, progesterone-induced "in vitro" acrosome reaction is concomitant with a rapid and intense increase in the oxygen consumption rate, which indicates a rapid, transitory, and intense peak of the mitochondrial respiratory activity (Ramió-Lluch et al. 2011). This seems to indicate that this minority energy would be necessary for the achievement of acrosome reaction; meanwhile glycolysis-synthesized energy would be the main energy source for all other boar sperm necessities.

The assumption of a main glycolytic source for obtaining energy in boar sperm suggests that energy production is a very fast phenomenon, mainly controlled by the rate of exogenous substrates and subsequent phosphorylation. However, several data seem to indicate that this overview is more complex. Thus, it has been shown that boar sperm are able to accumulate energy resources in the form of glycogen in a limited way (Medrano et al. 2006a). This is similar to that observed in other species, such as dogs and horses (Ballester et al. 2000), although the role of an active glycogen metabolism seems to be different among species. Thus, glycogen metabolism in dog sperm is related to the maintenance of energy levels during "in vitro" capacitation through a complex mechanism involving glycogenesis and an indirect glycogen synthesis pathway (Albarracín et al. 2004). Boar sperm, instead, do not show signs for the existence of a functional gluconeogenic pathway from substrates like lactate or citrate (Marín et al. 2003). This implies that glycogen must be obtained directly from phosphorylated substrates that are

diverted at the start of the glycolytic pathway. We do not know what is the exact role of boar sperm glycogen . Taking into account its origin, this glycogen could be a supplementary source of energy in case of an urgent need for energy supply. Notwithstanding, more information is needed to elucidate this intriguing characteristic of energy metabolism in mature boar spermatozoa.

2.2.3 Control Mechanisms Involved in the Coordination of Energy Management of Boar Sperm

Information regarding the control mechanisms of energy management in boar spermatozoa is scarce. Recompilation of data from several authors indicate the existence of two main check points in the glycolytic pathway, which is the main energy source for boar sperm as indicated above. The first check point has been already described, since it is linked to the uptake and subsequent phosphorylation of exogenous substrates. Thus, the placement and activity of GLUT transporters and subsequent hexokinase activity will regulate this point. Taking into consideration the specific characteristics of both GLUTs and hexokinase activity, it seems evident that GLUTs perform the most relevant control role. This is due to the fact that overall boar sperm hexokinase activity is very fast and, moreover, reaches its maximal level at very low substrate concentrations and at micromolar orders (Fernández-Novell et al. 2004; Medrano et al. 2006a). This implies that practically all the substrates taken up by the cell are immediately phosphorylated and processed either toward glycolysis or glycogen synthesis (another possible pathway, the pentose phosphate cycle, was not detected in metabolomic studies, see Marín et al. 2003). In fact, the velocity of this step is such that boar sperm rarely achieve their theoretical stoichiometric ATP yield (Hammersted and Lardy 1983). In this way, a hexokinase-linked regulation of hexose metabolization is almost impossible. On the other hand, the presence of at least four separate GLUTs with different characteristics of uptake velocity and hexose specificity creates a more complex control system, in which the metabolization of a specific hexose will vary depending on the specific transporter that makes contact with this specific hexose. This is further complicated by the fact described above of the heterogeneous presence of each GLUT in specific sperm membrane positions. This will mean that, for instance, fructose will be taken up and subsequently metabolized at a much faster rate in the post-acrosomal region than in the acrosomal area, since the distribution of the GLUT-5, fructose-specific transporter is much more abundant in the post-acrosomal area (Sancho et al. 2007). This also seems to indicate the existence of metabolic zonation, in which separate areas of boar spermatozoa would have different rates of energy management, depending on the velocity and specificity of the relevant substrates in each separate sperm location.

Notwithstanding, there is another important check point to regulate glycolytic flux. This second check point involves modulation of the lactate dehydrogenase

activity (LDH), which controls the final fate of the obtained lactate, directing it either to the Krebs cycle or its secretion to the extracellular medium. It is noteworthy that mammalian spermatozoa present a specific LDH isozyme, which is mainly located at the principal piece of the tail (Jones 1997; Medrano et al. 2006b). The regulation of this LDH is very complex, involving the migration of the LDH protein from an insoluble cellular fraction, presumably linked to the axoneme of the principal piece of tail, to a soluble cellular one, loosely linked to the inner tail structures (Medrano et al. 2006b). Thus, boar sperm LDH regulates its activity by changes of its position inside a specific cellular sector. This system of regulation seems to imply the existence of a regulation mechanism based on the spatial contact among all the constituents of the entire glycolytic pathway. This pathway would work at different rates depending on the specific positions of some of the enzymes implied in glycolysis. In this way, changes in the location inside a precise zone of the sperm structure of proteins like GLUT-3, GLUT-5 of LDH would automatically lead to changes in the rate of the glycolytic flux. This would be a precise mechanism able to modulate rapid and intense changes in the energy formation rhythm, thus allowing for a very fine modulation mechanism of boar sperm intracellular energy levels.

2.3 Roles of Mitochondria in the Control of the Overall Mature Boar Sperm Function

As indicated in the previous section, the main energy source for mature boar sperm are ATPs obtained through the glycolytic pathway, with a minority role for mitochondria-originated energy through the mitochondrial respiration. As a result, an important question is raised: if boar sperm mitochondria do not seem to have a predominant role in obtaining energy, what is their main role? We can only speculate on this point, although several data can help us gain greater insight into this issue. The first data correspond to the observation of the boar sperm mitochondria ultrastructure (Fig. 2.2). Electron microscopic images of boar sperm mitochondria show an organelle with few and small inner membrane cristae. Instead, the inner mitochondrial space is mainly occupied by an amorphous and homogeneous matrix. This is very different to the classical image for mitochondria, which, like those from hepatocytes, show an inner structure crowded with prominent inner cristae (Fig. 2.2). Taking into account the most important steps of the electronic transport system and subsequent ATP synthesis are structurally linked to inner mitochondrial cristae (this information can be obtained from any of the excellent biochemistry books for students utilized worldwide), it is easy to assume that boar sperm mitochondria would not be very efficient as energy suppliers. In fact, as described above, the oxygen consumption rate of boar sperm, which is a direct measure of mitochondrial ability to generate energy, is about 2 magnitude orders lower than that measured in pig hepatocytes (Balis et al. 1999; Ramió-Lluch et al. 2011). This further reinforces

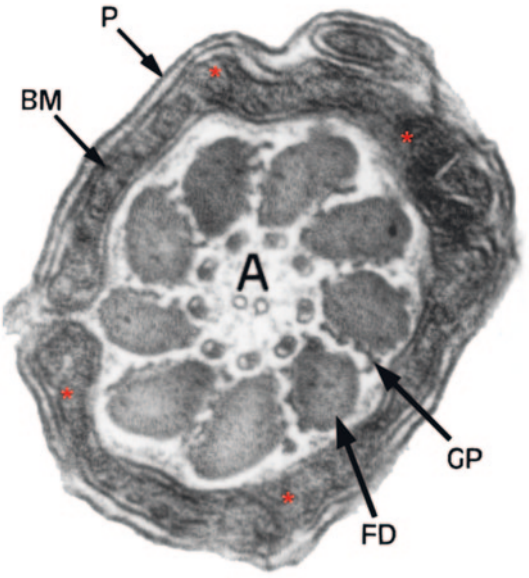

Fig. 2.2 Ultrastructural image of boar sperm mitochondria. The low development of inner cristae is noticeable (*asterisks*). *BM* inner mitochondrial membrane. *P* cell membrane. *A* axoneme. *FD* dense fibres. *GP* peripheral granules. From Bonet et al. (2000)

the low efficiency of boar sperm mitochondria as an energy synthesizer. However, this does not preclude that mitochondria-originated energy would not be important for boar sperm function. Thus, the achievement of a feasible, progesterone-induced "in vitro" acrosome reaction is concomitant with a sudden and intense peak of the O_2 consumption rate and also of intracellular ATP levels (Ramió-Lluch et al. 2011). This peak is not present in conditions in which progesterone-induced acrosome reaction is prevented (unpublished data from our laboratory), indicating thus a close relationship between mitochondria-generated energy and the achievement of the acrosome reaction, despite the low energy efficiency of these organelles.

However, the fact that mitochondrial respiration seems to be important only in specific moments of the boar sperm's lifespan does not necessarily indicate that boar sperm mitochondria are only important in this aspect. In fact, mitochondria have many more roles than mere energy-producing factories. It is well known that mitochondria play a key role in eukaryotic cells in the control of other highly important aspects of eukaryotic cell function, such as the modulation of apoptosis and the control of calcium metabolism. Thus, it is likely that the most important functions of boar sperm mitochondria would be linked to the control of other cellular functional aspects rather than to energy management. In this regard, unpublished results from our laboratory strongly indicate that the incubation of boar sperm in a capacitation medium in the presence of oligomycin A, a specific inhibitor of the electronic chain-to-chemiosmosis step (Chappell and Greville 1961), immobilizes boar sperm and prevents them from achieving "in vitro" capacitation, without modifying either the rhythm of O_2 production or the intracellular ATP

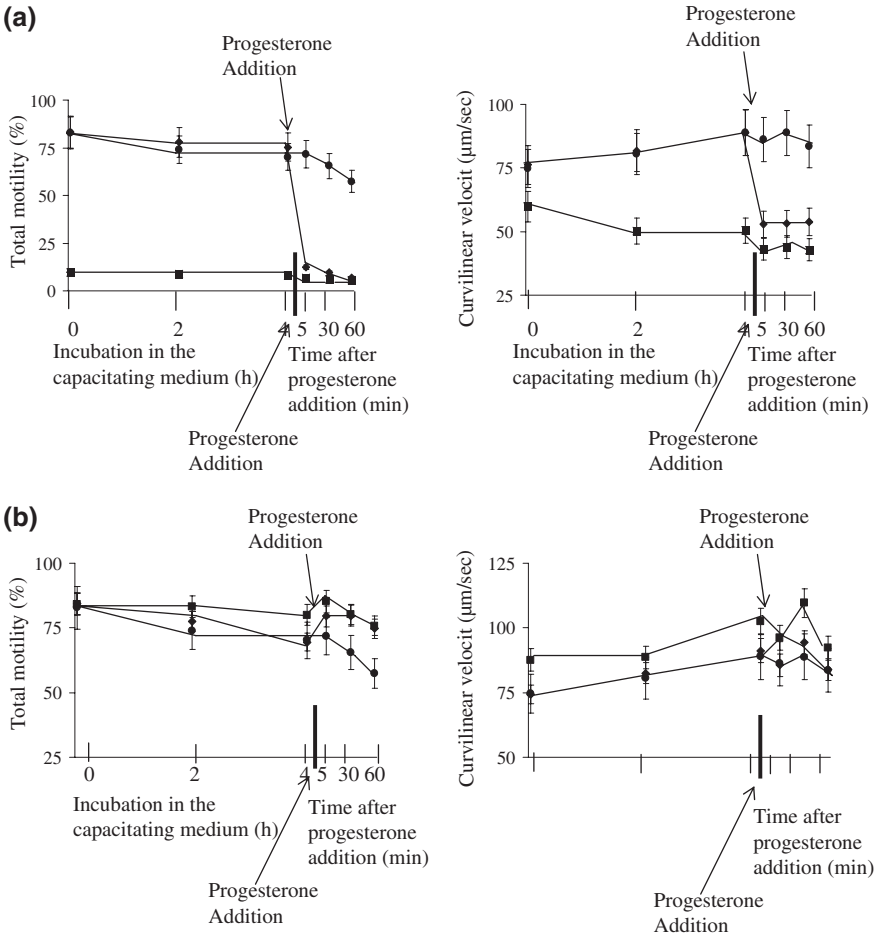

Fig. 2.3 Percentages of total motility and curvilinear velocity values of boar sperm subjected to "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosome reaction in a capacitation medium with or without the presence of either oligomycin A or $Ca2^{+}$ and EGTA. (**a**) spermatozoa incubated in a medium with or without oligomycin A. (**b**) spermatozoa incubated in a standard capacitation medium or a medium without Ca^{2+} and with 2 mM EGTA added. ● sperm incubated in a standard capacitation medium. ■ sperm incubated in a medium with oligomycin A or in a medium without Ca^{2+} and with 2 mM EGTA added. ♦ sperm incubated in a standard medium for 4 h and afterwards simultaneously with progesterone and either oligomycin A or 2 mM EGTA added. Results are mean ± S.E.M. for 7 different experiments. Unpublished results

levels (Figs. 2.3 and 2.4 and data not shown). On the contrary, the incubation of boar sperm in a capacitation medium without calcium induces an increase in the velocity parameters of these cells that was complementary to the already observed bicarbonate-induced, protein kinase A-modulated motility activation (Aparicio

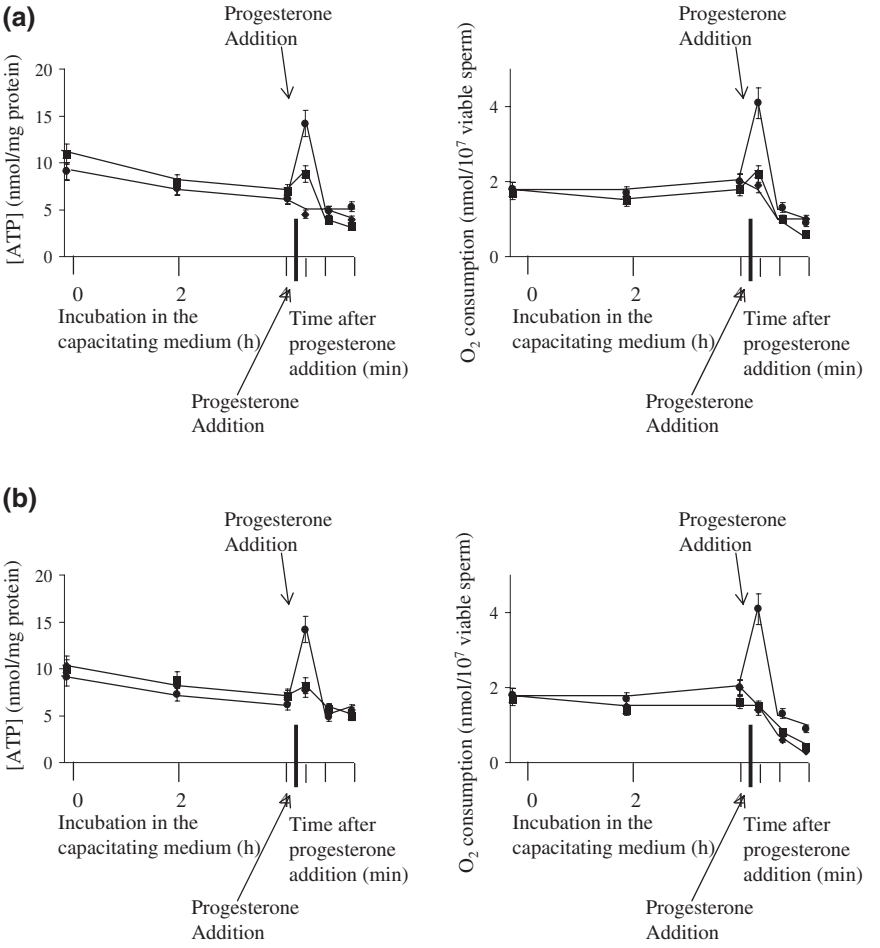

Fig. 2.4 Rates of O_2 production and intracellular ATP levels of boar sperm subjected to "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosome reaction in a capacitation medium with or without the presence of either oligomycin A or $Ca2^+$ and EGTA. (**a**) spermatozoon incubated in a medium with or without oligomycin A. (**b**) spermatozoa incubated in a standard capacitation medium or a medium without Ca^{2+} and with 2 mM EGTA added. ● sperm incubated in a standard capacitation medium. ■ sperm incubated in a medium with oligomycin A or in a medium without Ca^{2+} and with 2 mM EGTA added. ♦ sperm incubated in a standard medium for 4 h and afterwards simultaneously with progesterone and either oligomycin A or 2 mM EGTA added. Results are mean ± S.E.M. for 7 different experiments. Unpublished results

et al. 2007; Harayama and Nakamura 2008; Kaneto et al. 2008). Notwithstanding, the lack of extracellular calcium prevents the achievement of capacitation (Fig. 2.3 and data not shown). This effect linked to the lack of extracellular calcium, however, is carried out without changes in either the rate of O_2 production or the

intracellular ATP levels (Fig. 2.4 and data not shown). Thus, these results clearly indicate that mitochondria play an important regulatory role in the control of functional aspects such as motility patterns and the achievement of "in vitro" capacitation by ways that are not directly linked to energy production, but to the control of intracellular reductive potential and intracellular calcium storage. This opens a new perspective in the way in which investigators should approach the understanding of the role played by mitochondria in the control of sperm function. However, a great deal of more work is needed in order to achieve a thorough insight into this complex phenomenon.

2.4 Sperm Subpopulations: Do they have a Definite Biological Role?

In a seminal article published in 1996 within the framework of the Third International Conference on Boar Semen Preservation, Holt described the presence of a specific subpopulation structure in boar ejaculates when sperm motility of these ejaculates was analyzed by using a computerized, automatized system (CASA system; see Holt 1996). In the same article, Holt described how these subpopulations would change according to the presence of several stimuli like bicarbonate in the dilution medium, thus suggesting a functional role for this subpopulation structure in boar ejaculate (Holt 1996). Since then, an increasingly large number of papers have described the presence of a similar subpopulation structure, not only in boar (Holt 1996; Abaigar et al. 1999), but also in other mammalian species, such as gazelle (Abaigar et al. 1999), horse (Quintero-Moreno et al. 2003), donkey (Miró et al. 2005), dog (Dorado et al. 2011), rabbit (Quintero-Moreno et al. 2007), deer (Martinez-Pastor et al. 2005), bovine (Muiño et al. 2008), and ovine (Rodríguez-Gil et al. 2007). This wide spectrum seems to indicate that, as a common characteristic, mammalian ejaculates have a subpopulation structure of their sperm. Moreover, these subpopulations have been observed not only when analyzing motility, but also after the observation of other ejaculate characteristics like sperm morphology (Rubio-Guillén et al. 2007), and even midpiece mitochondria activity through JC-1 stain (Ramió-Lluch et al. 2011). These findings further reinforce the existence of a subpopulation structure inside mammalian ejaculates.

Taking into account the existence of sperm subpopulations, a highly important question is raised regarding this structure; what is the real, biological role of a subpopulation structure in mammalian ejaculates? This is currently an open question, and we can only speculate on this point. Notwithstanding, and centering on boar spermatozoa, we have enough information to establish some basic, preparative principles. In this regard, no definitive relationship has been established between subpopulations and "in vivo" fertility in a pig farm entourage (Quintero-Moreno et al. 2004). On the contrary, a subtle although significant relationship has been established between a specific subpopulation structure and the ability of boar spermatozoa to resist cryodamage after standard freezing-thawing procedure (Thurston

et al. 2001; Flores et al. 2009). This indicates that the capacity for resistance of boar ejaculates to freezing-thawing is related in some way with the specific subpopulation structure that this ejaculates presents before freezing. This feature could be related to specific functional aspects of boar sperm biology. In this respect, Satake et al. (2006) have observed that the interaction of boar sperm with oviductal proteins is related to the sensitivity of each spermatozoon to respond to bicarbonate stimulation. This result links the specific sensitivity of each boar sperm when undergoing capacitation to its ability to reach oocytes, thus opening the door to the presence of separate sperm subpopulations with different abilities to reach capacitation and subsequent sperm-oocyte interaction. These differences in sensitivity have also been observed by our laboratory after conducting "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosome reaction experiments. In these experiments, the achievement of feasible "in vitro" capacitation and subsequent "in vitro" acrosome reaction has been linked to specific changes in the boar sperm subpopulation structure, especially affecting those sperm that present higher velocity characteristics at the start of incubation in the capacitation medium (Ramió et al. 2008). This seems to indicate that those sperm that show higher velocity characteristics in an ejaculate are prone to achieve feasible "in vitro" capacitation and further acrosome reaction. Interestingly, analysis of mitochondrial activity through JC-1 stain has shown not only the presence of a subpopulation structure based on boar sperm mitochondria activity, but also specific changes in this subpopulation structure after "in vitro" capacitation and acrosome reaction, with higher changes in those sperm that show the lowest mitochondrial activity in freshly obtained ejaculates (Ramió-Lluch et al. 2011). These results indicate the existence of a close relationship between the specific motility characteristics of specific boar sperm and their mitochondrial activity. This close relationship between boar sperm motility and their mitochondrial activity status would then suggest a biological role in the subpopulation structure. This role would be related to the separate ability of sperm from each subpopulation to present a specific functional activity, resulting in separate mitochondria activity profiles. In turn, these specific functional changes would be related to the ability of each sperm to yield changes such as those related to the achievement of capacitation, thus linking the subpopulation structure with the fertilizing ability of an ejaculate. However, a great deal of work is necessary in order to elucidate this important question. In any case, it is obvious that boar sperm quality analysis would have to be modified in order to introduce the subpopulation concept if practitioners sought to obtain optimal information regarding the specific quality of the analyzed ejaculate.

2.5 Conclusion

In the past few years, an increasing amount of information has been gathered to alter our overall vision of boar sperm biology. Therefore, the old image of a simple, straightforward cellule must be abandoned to make way for a concept involving a highly complex cell, which is able to adapt its resources to the changes in the

environment through a myriad of complex mechanisms regulated with very fine and sensitive systems. Moreover, the concept of a complex, subpopulation structure of boar ejaculates must be considered as an important characteristic involved in the fertilizing ability of boar sperm, and semen quality analysis should be designed in order to incorporate this biological characteristic.

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Chapter 3
The Boar Reproductive System

Sergi Bonet, Estela Garcia and Lilian Sepúlveda

Abstract The boar reproductive system consists of six different structures (testes, epididymis, deferent ducts, urethra, accessory sex glands, and penis) and the main function is the production and the ejaculation of semen. The two testes are the male gonads and their functions are sperm production and secretion of hormones. The main functions of two epididymis are: sperm transport, sperm maturation, and sperm storage. The sperm maturation is a complex process after which sperm acquire progressive motility and fertilizing ability. Male accessory sex glands include: the seminal vesicles, the prostate, and the bulbourethral glands (Cowper's glands). These exocrine glands release their secretion into the urethra and their secretory activity is androgen-dependent. The noncellular fraction of the ejaculate (seminal plasma) is mainly composed of fluids from the accessory sex glands.

3.1 Introduction

3.1.1 General Anatomy and Functions

The boar reproductive system consists of two testes, two epididymis, two deferent ducts, the urethra and its accessory sex glands (two seminal vesicles, the prostate and two bulbourethral glands or Cowper's glands), and the penis (copulatory organ) (Hafez and Hafez 2000; Knobil and Neill 2006; Badia 2003) (Fig. 3.1).

The main function of the boar reproductive system is the production and the ejaculation of semen, composed of a cell fraction, the spermatozoa, and a noncellular fraction, the seminal plasma, which is constituted by a mix of secretions from the testes, the epididymis, and the accessory sex glands (Knobil and Neill 2006).

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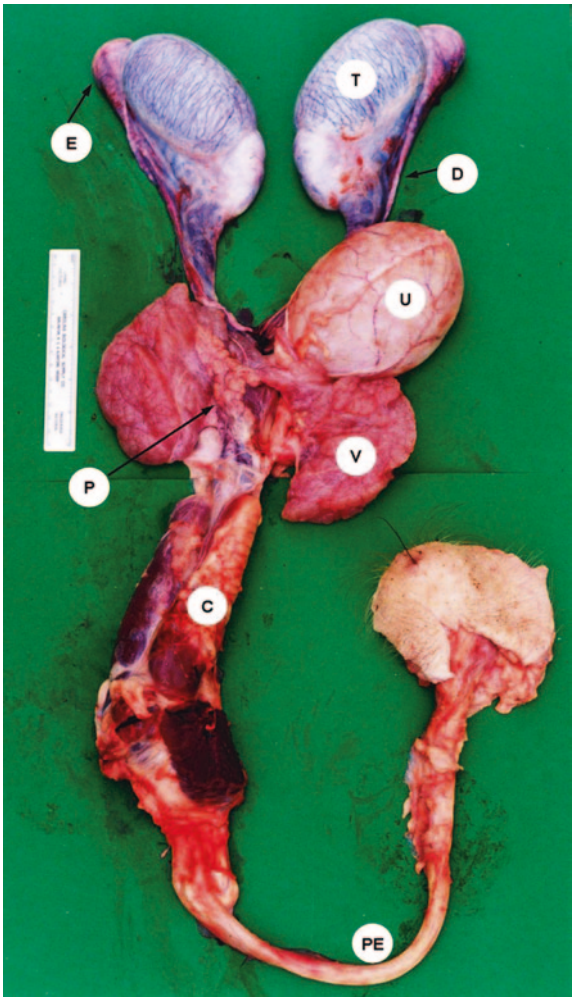

Fig. 3.1 General aspects of the boar reproductive system. *C* Cowper's glands; *D* deferent duct; *E* epididymis; *P* prostate; *PE* penis; *T* testicle; *U* urinary bladder; and *V* seminal vesicles

The testes are the male gonads and their main function is sperm production but they are also endocrine glands that contribute to sexual characters through the secretion of hormones. In the testicular stroma, numerous Leydig cells responsible for the synthesis and secretion of androgens (testosterone) are present, whereas in the parenchyma (seminiferous epithelium), a large number of Sertoli cells are responsible for the synthesis of Activin and Inhibin, among other factors (Garcia-Gil 2002).

Testicular sperm are carried from the seminiferous tubules through the rete testes and the efferent ducts to the epididymis, where they undergo a complex maturation process in order to acquire motility and fertilizing ability. Mature sperm are then stored in the latter portion of the epididymis (caudal epididymis) until its ejaculation, perhaps by directly binding to epididymal epithelial cells has been recently reported in in vitro studies (Yeste et al. 2012). Spermatozoa are

transported during this latter process through the deferent duct toward the urethra, where the secretions from the prostate, the seminal vesicles, and the Cowper's glands are collected. The urethra conducts semen to the urethral orifice, located at the outermost part of the penis, where it is expelled in the so-called process of ejaculation (Calvo et al. 2000; Sancho 2002; Bassols 2006; Yeste et al. 2010).

The ejaculate volume varies from 150 to 300 ml depending on many factors, such as breed, age, rhythm of collection, etc. (Pruneda et al. 2005a, b; Smital 2009; Yeste et al. 2010). In the ejaculate, secretions from the testes and the epididymis represent 2–5 % of the ejaculate volume, those from the seminal vesicles, 15–20 %, contribution from the bulbourethral glands is 10–25 %, and from the prostate, 55–75 % (Badia 2003). The ejaculate can be divided into three main fractions depending on its composition: prespermatic, spermatic, and postspermatic (Briz 1994; Sancho 2002; Yeste 2008).

3.1.2 Prenatal and Postnatal Development

Sexual differentiation is a sequential process that starts with chromosomal sex determination (presence or absence of the Y chromosome) at the time of fertilization; it continues with gonadal sex determination (testes or ovaries), and ends with phenotypic sex determination (boar or sow). Each step of the process depends on the previous one and, under normal conditions, chromosomal sex concurs with phenotypic sex (Pinart 1997).

Throughout the embryonic process, the expression and the presence of the Y chromosome is directly related to testicular differentiation. Nevertheless, the presence of the two X chromosomes is needed for the proper development of the ovary (Pinart 1997). Between days 20 and 40 of fetal age, the first signs of gonadal sex dimorphism start to become evident. Differentiation of primary gonads in the testes begins a few days before differentiation in the ovaries (Pinart 1997). Testicular development is associated with the progressive definition of the male reproductive tract and the virilization of the external genitalia. Testosterone secretion, produced by primary Leydig cells, masculinizes the external genitalia, maintains the Wolffian ducts (which will develop into the epididymis, the deferent duct, and the seminal vesicles), and determines the differentiation of the prostate and the Cowper's glands. The secretion of AMH (Anti-Müllerian hormone), produced by primary Sertoli cells, provokes the regression of the Müllerian ducts (which would have turned into a uterus and a Fallopian tube) (Pinart 1997; Badia 2003). So, the development of the male external genitalia occurs after the virilization of the Wolffian ducts has begun (Pinart 1997).

After 90 days of gestation and after the adenohypophyseal secretion of LH (luteinizing hormone), the testes shift from the abdominal cavity to the scrotum through two independent stages: the transabdominal descent, or sliding of the testes to the inguinal ring; and the inguinoscrotal descent, or descent of the testes from the inguinal ring to the scrotum (Pinart 1997). The first stage, which is not dependent

on androgens, is regulated by AMH; the absence of this hormone may lead to permanency of the testes within the abdominal cavity (cryptorchidism). However, the inguinoscrotal descent of the testes is regulated by androgens. The descent of the testes from the abdominal cavity into the scrotum takes place within a short period of time and it will only be satisfactorily accomplished if no inappropriate stimulus is produced by the hypothalamus-hypophysis- gonadal axis (Pinart 1997).

After birth, the proliferation of Sertoli cells is stimulated by follicle-stimulating hormone (FSH) (Pinart 1997). Differentiated Sertoli cells produce estrogens, which in conjunction with LH stimulate the proliferation and differentiation of Leydig cells, and paracrine factors, which encourage the movement of gonocytes (primary germ cells) to the basal lamina of the future seminiferous tubules (Pinart 1997). The evolution from gonocytes to spermatogonium occurs within the first weeks of postnatal development. At the age of 3 months, the processes of spermatogenesis and spermiogenesis begin, and after 4 months spermatozoa are found in the lumen of the seminiferous tubules. At around 5 months of age, puberty begins and the ejaculate contains its first sperm cells. Then, from the age of 6 months, the size of the testes, the ejaculate volume, and the concentration of ejaculated sperm will continue to increase until becoming stable from 18 months onwards.

3.1.3 Comparative Aspects with Other Species

As previously indicated, the male reproductive system presents three fundamental functions. The endocrine function is basically carried out by Leydig cells (testosterone synthesis and secretion) and Sertoli cells (inhibin synthesis and secretion). The production, maturation, and storage function of sperm with fertilizing ability is carried out by the testes and the epididymis (Hafez and Hafez 2000).

The reproductive physiology (estrous cycle, seasonal reproduction, anatomophysiological characteristics of the oviduct, mating patterns, etc.) determines the existence of anatomical and functional differences between the reproductive systems of different mammalian species with productive interest (porcine, equine, bovine, ovine, etc.). Some of the most noteworthy differences between swine and other species are listed in the following paragraph.

In relation to the ejaculate, notable differences in parameters such as volume, sperm concentration, and total sperm per ejaculate are found. Boars (porcine) present the largest ejaculate volume (between 150 and 300 ml), followed by horses (equine) with 60–70 ml, bulls (bovine) with 5–7 ml, rams (ovine) with 1–1.5 ml, goats (caprine) with 0.8–1.1 ml, and rabbits with 0.6–0.8 ml. The greatest sperm concentration ($\times 10^9$ sperm/ml) is for rams (3.0) followed by goats (2.4), bulls (1.1), rabbits (0.5), boars (0.2), and horses (0.15). Total sperm number per ejaculate ($\times 10^9$ sperm) is much greater in boars (10–100) than in the rest of species of productive interest (horse, 9.0; bull, 5.5; ram, 3.0; goat 2.0; and rabbit, 0.03). In regard to the number of ejaculates with good semen quality that a breeding male can produce per week, rams (ovine) and goats (caprine) stand out with up

to 20 ejaculates per week, followed by rabbits (6), bulls (4), boars, and horses (3). Regarding ejaculation time, important differences are also observed; while in ovine, caprine, bovine, rabbit, and equine species, it lasts a few seconds or fractions of a second, in porcine species it lasts 10–30 min. (Hafez and Hafez 2000; Knobil and Neill 2006).

The copulatory organ or penis presents important differences in size (length and diameter), shape, and internal anatomy. Whereas in bovine, porcine, caprine, and ovine livestock, the penis has a fibroelastic nature, in horses it is mainly musculocavernous; that is, the horse's penis presents a corpus cavernosum and an expandable glans that become engorged with blood during erection, thus increasing considerably in size. The horse usually has a penis with a highly differentiated glans, but without a retractor penis muscle, a fact that explains why the penis stays out for a while after copulation. In bovine, caprine, ovine, and porcine livestock, the penis presents a small glans covered by a thick and fibrous tunica albuginea (mainly composed of collagen fibers), which limits its enlargement during erection. In bovine livestock, the retractor penis muscle is highly developed but the glans, which is slightly flatter, is poorly differentiated. In ovine and caprine livestock, the glans forms a penile appendage with a sharp end that enables a specific structure located in the cervix of the female during erection to be lifted. In porcine livestock, the penis is highly developed and the glans presents an S-shaped structure. Finally, in rabbits the glans is sharp-edged and the retractor penis muscle is well developed.

The length of the epididymal duct also presents large differences among species. In equine species, it is 70–90 m long, in bovine species, around 50 m, and in porcine species, between 17 and 18 m. The accessory sex glands also have differences in size. For instance, seminal vesicles in horses measure 10–15 cm in length, in bulls 14 cm, and in boars from 7 to 12 cm (Hafez and Hafez 2000; Knobil and Neill 2006).

3.2 Testis

3.2.1 General Aspects: Anatomy and Functions

In healthy and sexually mature boars, the left testicle hangs down lower than the right one inside the scrotal sac. However, there are no differences in weight between the testes (Pinart et al. 1999a, 2001c). For instance, in boars aged 9–10 months and with a body weight of around 145 kg, each testicle can weigh from 310 to 360 g. The weight percentage of both testes in relation to body weight does not exceed 0.45 (Garcia-Gil 2002).

The testes are surrounded by the tunica albuginea, from which several radial septa extend inwards to divide up the testis. Between these septa, the seminiferous tubules and a connective tissue, which is innervated, vascularized, and rich in Leydig cells, are located (Garcia-Gil 2002). The set of seminiferous tubules is known as testicular parenchyma, and the connective tissue as testicular stroma.

Testicular parenchyma occupies 55–60 % of the left testicle and 60–65 % of the right one. In fact, the diameter of the seminiferous tubules in the right testicle (475–525 μm) is slightly higher than that in the left testicle (450–525 μm) (Pinart 1997). The seminiferous tubules anastomose in the mediastinum testis forming a complex network of tubules called rete testis, which gives rise to several efferent ducts that flow into the epididymis (Garcia-Gil 2002; Pinart 1997).

The two primary functions of the testis are carried out by the testicular parenchyma and the testicular stroma. The seminiferous tubules are responsible for sperm production, which include the processes of spermatogenesis, spermiogenesis, and spermiation. The abundant Leydig cells located in the testicular stroma are responsible for the androgen synthesis, mainly testosterone (Hart and Phillip 2007).

3.2.2 Structure and Ultrastructure

3.2.2.1 Testicular Capsule

Three tunicae can be distinguished in the testicular capsule: tunica albuginea, tunica vaginalis, and tunica vasculosa (Pinart 1997; Garcia-Gil 2002):

- Tunica vaginalis is the outermost layer covering the testis and is 30–50 μm thick. It consists of a mesothelium and a submesothelial connective tissue in which fibroblasts, abundant circular smooth muscle cells, and collagen fibers are distinguished (Fig. 3.2).

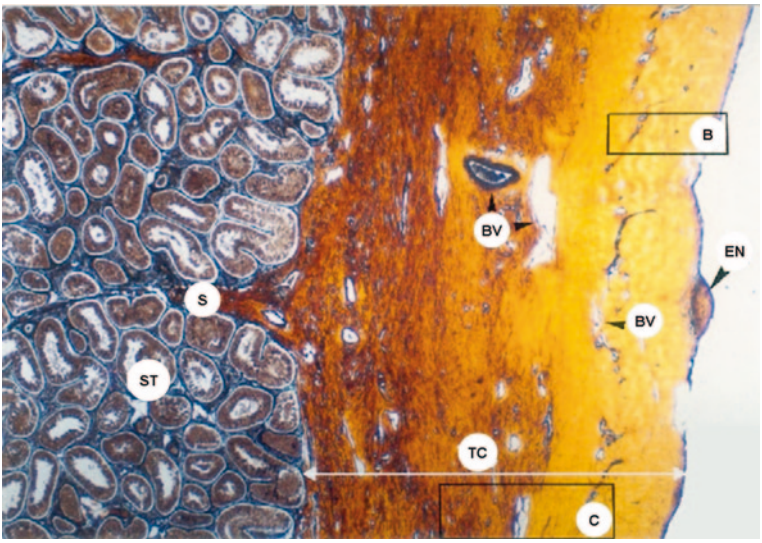

Fig. 3.2 Cross-section through the testis. Gomori's silver impregnation stain. *BV* blood vessels; *S* septa; *ST* seminiferous tubules; *TC* testicular capsule

- Tunica albuginea is the central layer covering the testis and is 800–950 μm thick. It consists of a dense connective tissue rich in collagen fibers and fibroblasts, but poor in elastic fibers (Fig. 3.2).
- Tunica vasculosa is located in the innermost part of the testicular capsule. It is 1,400–1,650 μm thick and consists of a connective tissue rich in collagen fibers and elastic fibers.

Tunica albuginea and tunica vasculosa are both well vascularized (Pinart 1997) and the irrigation pattern of the blood vessels can be appreciated observing the testicular surface.

The tunica vasculosa folds inwards in several *septula testis*. Between these septa, the testicular parenchyma (set of seminiferous tubules) and the testicular stroma (loose connective, highly vascularized tissue, rich in Leydig cells) are located.

3.2.2.2 Testicular Parenchyma

The testicular parenchyma comprises of a set of highly coiled up seminiferous tubules between the *septula testis*. With a mean diameter of 230 μm, each tubule consists of a lamina propria and a seminiferous epithelium (Garcia-Gil 2002) (Fig. 3.3).

The 4–5 μm thick lamina propria covers the external surface of the seminiferous epithelium and is composed of a basal lamina and peritubular cells (Garcia-Gil 2002; Pinart 1997).

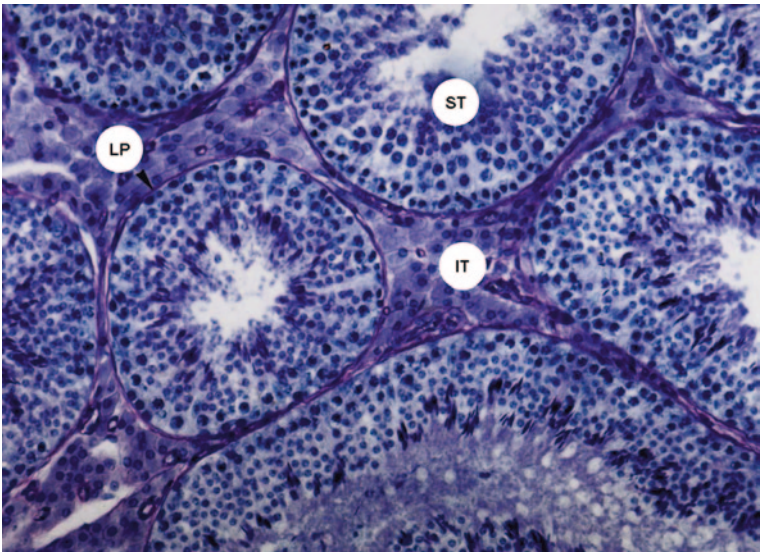

Fig. 3.3 General aspect of the testicular parenchyma. PAS-Groat's hematoxylin stain. *ST* seminiferous tubules; *LP* lamina propria; *IT* interstitial tissue or stroma

The 0.5–1 μm thick basal lamina is located in the innermost part of the lamina propria, in contact with the seminiferous epithelium, and is made up of a layer containing collagen fibers, reticular fibers, and neutral glycoconjugates. Peritubular cells are arranged in two concentric lines separated from each other by a matrix rich in collagen fibers, reticular fibers, and neutral glycoconjugates. The innermost line of peritubular cells is in close association with the basal lamina and is formed by myoid cells, which are elongated cells (about 10 μm length) with an irregular outline and rich in actin filaments (Garcia-Gil 2002; Pinart 1997). The outermost line of peritubular cells is in contact with the testicular stroma and is mainly composed of fibroblasts (Pinart 1997; Garcia-Gil 2002; Pinart et al. 2001a).

The seminiferous epithelium, about 55–75 μm in thickness, contains two cell types: germ cells and Sertoli cells (Pinart et al. 1999a; Garcia-Gil et al. 2002a, b). Sertoli cells are fixed to the basal lamina and they extend upwards to the free surface of the seminiferous tubule, the lumen (Garcia-Gil 2002). Germ cells are distributed in layers between Sertoli cells.

Basically, three categories of cells are involved in this process: spermatogonia, spermatocytes, and spermatids (Garcia-Gil et al. 2002a, b; Calvo et al. 2000; Pinart et al. 2000, 2001b). As germ cells divide and differentiate into different cell types, they move from the basal region to the apical region of the seminiferous epithelium (Garcia-Gil 2002).

The process in which testicular sperm is developed from spermatogonia is known as spermatogenesis (sensu lato) and, in pigs, it usually lasts 34–36 days. However, this process is divided into two phases: spermatogenesis (sensu stricto) and spermiogenesis (sensu stricto). Spermatogenesis (sensu stricto) is the phase that includes the transition, through mitotic divisions, from spermatogonia to primary spermatocytes, and the transition, through meiotic divisions, from primary spermatocytes to secondary spermatocytes, and then from these to early round spermatids (Pinart et al. 1999b, 2000). Spermiogenesis constitutes the complex process of cell differentiation of early round spermatids into mature spermatozoa, which are released in the lumen of the seminiferous tubules. Therefore, spermatogonia are divided up through mitosis several times until they become primary spermatocytes (spermatocytogenesis) (Garcia-Gil 2002). Then, primary spermatocytes, which are still diploids, undergo a meiotic process until they develop into spermatids (Sancho 2002). The first meiotic division, or reduction division, results in the formation of secondary spermatocytes (haploids) that go through the second meiotic division, or equatorial division, giving rise to early round spermatids (Pinart et al. 1998, 1999b). After these cell division processes, early round spermatids, which are closely attached to the free surface of Sertoli cells, undergo a complex cell differentiation process leading to the formation of spermatozoa that will be released by Sertoli cells into the lumen of the seminiferous tubules (Garcia-Gil 2002).

Different generations of germ cells coming from a single spermatogonium are interconnected by cytoplasmic bridges so that they remain bound to each other. These clusters of germ cells derived from the same spermatogonium are known as “isogenous groups”. All cells belonging to the same isogenous group are close to

each other and evolve synchronously. The plasma membrane of the cytoplasmic bridges displays a remarkable electrodense widening. The nuclear envelope near the cytoplasmic bridges has numerous nuclear pores (Garcia-Gil 2002). In the cytoplasm of these bridges the presence of annulate lamella is quite common, i.e., concentric and organized distributions of smooth endoplasmic reticulum cisternae (Fig. 3.4).

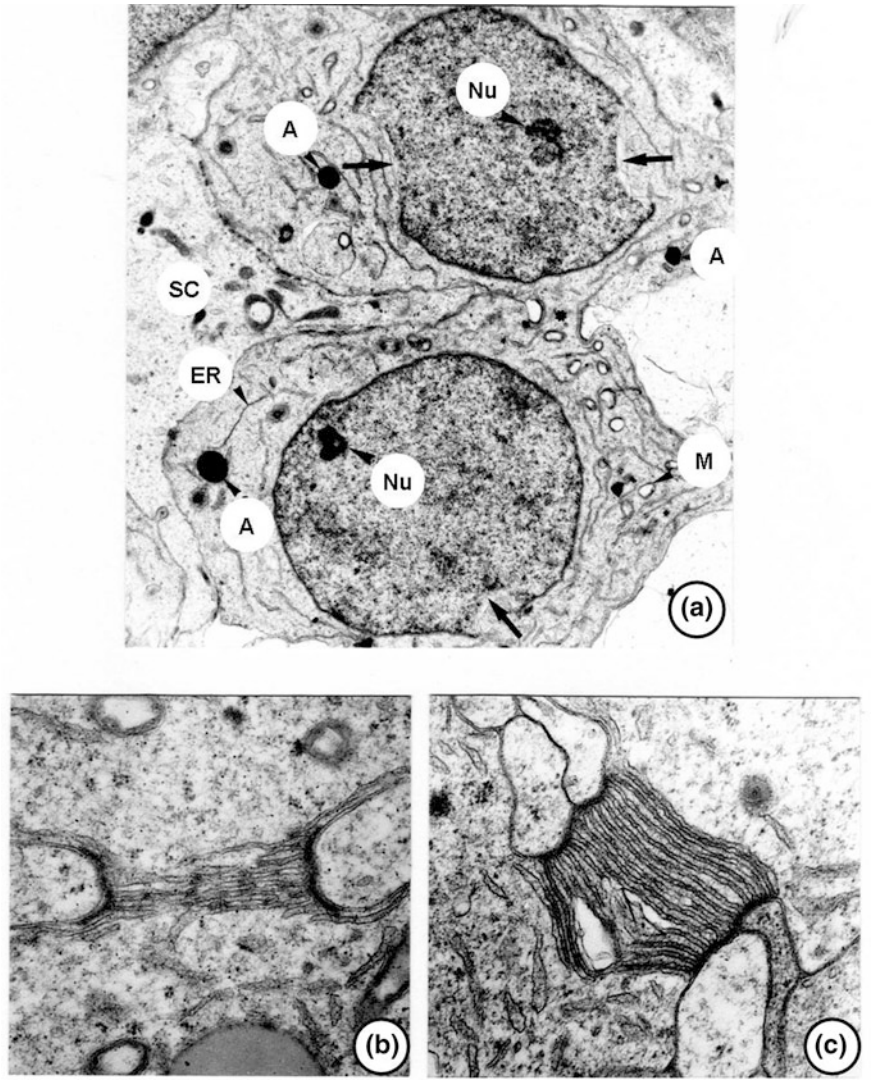

Fig. 3.4 **a** General aspect of two round spermatids interconnected by a cytoplasmic bridge. *A* electron dense aggregates; *ER* endoplasmic reticulum; *M* mitochondria; *Un* nucleolus; *SC* Sertoli cells (**b** and **c**) micrograph of two cytoplasmic bridges. The transversal disposition of endoplasmic reticulum cisternae (annulate lamella) can be appreciated

Sertoli cells are large pyramidal cells that extend upwards from the basal lamina to the lumen of the seminiferous epithelium (Garcia-Gil 2002; Pinart et al. 1999a, 2000). Structural changes of germ cells and their movement from the basal lamina to the free surface of the seminiferous epithelium during the process of spermatogenesis constantly promote Sertoli cells to undergo significant changes in shape and contour (Garcia-Gil 2002). They show a large rounded or oval nucleus of approximately 10–14 μm, with an irregular perimeter (Pinart et al. 2000). The nucleus is usually located in the basal cytoplasmic region, even though it can be situated in the medial cytoplasmic region in the postmeiotic stages of the seminiferous epithelium. The nucleus is composed of an evenly distributed euchromatin, a nuclear envelope with deep invaginations and a single prominent nucleolus or two little nucleoli surrounded by small associated heterochromatic masses (Garcia-Gil 2002; Pinart 1997).

The basal membrane of Sertoli cells is attached to the basal lamina through hemidesmosomes (Garcia-Gil 2002; Pinart 1997). Their lateral surfaces emit a complex system of protrusions that permit them to be in contact with the adjacent Sertoli cells. Between neighboring Sertoli cells, tight junctions and desmosome-like junctions are abundant in the basal region of the seminiferous epithelium (Garcia-Gil 2002; Pinart 1997; Pinart et al. 2000). Thus, two compartments are delimited in the seminiferous epithelium on both sides of the junction: the basal compartment and the adluminal compartment (Garcia-Gil 2002). The basal compartment extends from the basal lamina to the closure defined by the junctions between neighboring Sertoli cells, and contains all the diploid germ cells. The adluminal compartment extends from the closure to the lumen of the seminiferous tubules and is filled with the haploid germ cells. The aforesaid closure is known as blood–testis barrier and its main function is to protect haploid germ cells from autoimmune reactions. Any substance that has to reach the haploid germ cells must first go through Sertoli cells. Therefore, Sertoli cells, apart from acting as an autoimmune barrier, also act as the main source of metabolites for haploid germ cells (Garcia-Gil 2002). The frequency and type of junction between Sertoli cells and germ cells vary in the basoapical direction (Garcia-Gil 2002; Pinart 1997). Type A spermatogonia and intermediate spermatogonia are attached to Sertoli cells via desmosomes. The number of desmosomes decreases gradually as germ cells approach the blood–testis barrier. Thus, type B spermatogonia, preleptotene primary spermatocytes, and leptotene primary spermatocytes are poor in intercellular junctions. Once germ cells reach the adluminal compartment the number of intercellular junctions increases again. So, zygotene primary spermatocytes, pachytene primary spermatocytes, secondary spermatocytes, and round spermatids bind to Sertoli cells via desmosome-like junctions, tight junctions, and adherens junctions (Pinart et al. 2000).

The cytoplasm of Sertoli cells is rich in organelles, basically in the basal region, the smooth endoplasmic reticulum being well developed (Garcia-Gil 2002; Pinart 1997). It also contains several vesicles varying in size, mitochondria, primary and secondary lysosomes, phagolysosomes, lipid droplets, and a Golgi apparatus. In stages I and VII of the seminiferous epithelium cycle lysosomes fuse together with residual bodies emerging from mature spermatids, thereby forming

phagolysosomes that go toward the basal cytoplasm of Sertoli cells. Mitochondria of Sertoli cells are usually cup-shaped (Garcia-Gil 2002).

The cytoplasm also contains numerous intermediate filaments concentrated in the basal region, around the nucleus, or in the apical region in association with developing spermatids (Garcia-Gil 2002).

In stages VII and VIII of the seminiferous epithelium cycle, Sertoli cells are more electrodense and characterized by a more enlarged endoplasmic reticulum cisternae, longer or spherical mitochondria, and abundant ribosomes and polyribosomes (Garcia-Gil 2002).

The presence of germ cells, mainly mature spermatids, in the cytoplasm of Sertoli cells suggests that they play a role against degenerated germ cells by removing them (Garcia-Gil et al. 2002a, b). Primary modifications of Sertoli cells during the spermatogenic cycle can be observed in their apical pole and in their relationship with the development of spermatids (spermiogenesis).

3.2.2.3 Testicular Stroma

Testicular stroma or interstitial tissue is located among the seminiferous tubules and consists of a connective tissue rich in clusters of Leydig cells, poor in fibroblasts and mast cells, and with small blood and lymphatic vessels (Pinart et al. 1999a, 2001c, 2002).

Leydig cells are polyhedral cells of about 15 μm in length (Pinart et al. 1999a, 2001c), exhibiting a rounded nucleus with two well-developed nucleoli and a great number of cytoplasmic granules. Their nucleus contains euchromatin and small heterochromatic masses associated with a nuclear envelope, which displays an irregular contour and one or several deep invaginations (Garcia-Gil 2002; Pinart 1997). The cytoplasm is occupied by a Golgi apparatus and a well-developed smooth endoplasmic reticulum (Pinart 1997; Pinart et al. 2001c). The latter extends throughout the cytoplasm forming a far-flung complex system with large compact areas containing small lipid droplets. Endoplasmic reticulum aggregates often appear as electrondense granules or multivesicular bodies (Garcia-Gil 2002).

Spherical mitochondria, of variable size, are usually abundant all over the cytoplasm except at the perinuclear region (Pinart 1997). They commonly contain electrodense granules in their matrix and exhibit tubular cristae (Pinart et al. 2001c).

There is a close spatial relation between mitochondria and the endoplasmic reticulum, both organelles directly involved in the synthesis of testosterone (Garcia-Gil 2002).

Two types of Leydig cells can be distinguished: dark and pale cells. The content of cellular organelles in both modalities is similar, differing only in the content of ribosomes and glycogen granules (Pinart 1997). The pale type of Leydig cells exhibit a higher content in glycogen granulates while the dark Leydig cells display a greater amount of ribosomes. Another existing difference between the two cell types is the electrodensity of the nucleus and the volume of the nucleolus.

3.2.3 Spermatogenesis and Spermiogenesis

3.2.3.1 Phase I of Spermatogenesis: The Mitotic Phase

Phase I of spermatogenesis or spermatocytogenesis includes the set of germ cells that are divided through mitosis and are located in the basal compartment of the seminiferous epithelium, i.e., between the basal lamina and the blood–testis barrier, which is defined by the unions between Sertoli cells.

Spermatogonia are immature germ cells attached to the basal lamina via hemidesmosomes. These cells are classified in several types on the basis of their nuclear characteristics and their relationship with the basal lamina: reserve spermatogonia or type A0, type A1 spermatogonia, type A2 spermatogonia, type A3 spermatogonia, intermediate spermatogonia, type B1 spermatogonia, and type B2 spermatogonia (Garcia-Gil 2002; Pinart 1997).

Reserve spermatogonia or type A0 spermatogonia are elongated cells of 17 × 5 μm with their major axis oriented parallel to the basal lamina (Pinart et al. 2000). The surface in contact with the basal lamina is very large. Their nucleus is elongated and shows an irregular perimeter. The euchromatin is finely granular and homogeneously distributed, with small heterochromatic areas associated with the nuclear envelope (Garcia-Gil 2002; Pinart et al. 2000). The nucleolus is tiny and situated near the nuclear envelope. Cytoplasm is scarce, with mitochondria forming aggregates near the nucleus, and with abundant polyribosomes (Garcia-Gil 2002; Pinart 1997).

Type A1 spermatogonia are electrondense cells elongated in shape (17 × 7 μm) (Pinart 1997; Pinart et al. 2000) and their longitudinal axis is parallel to the basal lamina, thus creating a large contact surface between them. The nucleus, also elongated, shows a well-developed central nucleolus. The euchromatin is granular (Garcia-Gil 2002; Pinart et al. 2000) and with small heterochromatic masses scattered throughout the nucleoplasm. The cytoplasm presents a higher degree of development than type A0 spermatogonia and contains several endoplasmic reticulum cisternae and lysosomes; mitochondria are arranged in the vicinity of the nucleus forming small aggregates and ribosomes, which are present in large quantities, conferring greater electrodensity to the cytoplasm (Garcia-Gil 2002; Pinart 1997) (Fig. 3.5).

Type A2 spermatogonia are also placed on the basal lamina but this contact is lost in some areas. Their shape gradually changes from elongated to round (from 17 × 10 to 13 × 12 μm). The nuclear envelope shows few but very deep invaginations that can define nuclear lobes. The euchromatin is finely granular and homogeneously distributed, with small heterochromatic areas associated with the nuclear envelope. The nucleolus is well developed. The cytoplasm contains several mitochondria forming aggregates, an endoplasmic reticulum, some vesicles of low electrodensity, and abundant polyribosomes (Garcia-Gil 2002; Pinart 1997).

Type A3 spermatogonia are oval or conical cells (14 × 7 μm) still in contact with the basal lamina. The nucleus is rounded and possesses an underdeveloped

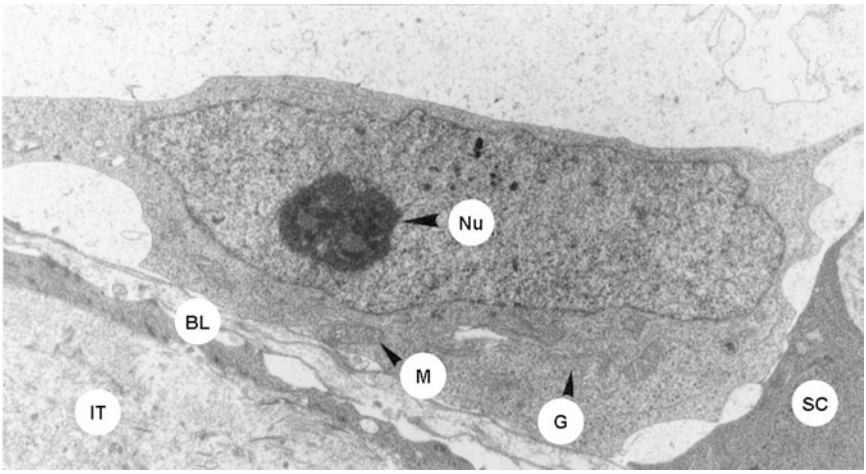

Fig. 3.5 Type A1 spermatogonium. *Nu* nucleolus; *BL* basal lamina; *G* Golgi apparatus; *IT* interstitial tissue; *SC* Sertoli cell; *M* mitochondria

nucleolus. The euchromatin is finely granular and the small heterochromatic masses are scattered throughout the nucleoplasm or associated with the nuclear envelope. The features of the cytoplasm are very similar to type A2 spermatogonia (Garcia-Gil 2002; Pinart 1997).

Intermediate spermatogonia or IN are rounded cells of about 7.5 μm in diameter. The nucleus exhibits granular euchromatin with a well-developed nucleolus. The cytoplasm contains abundant mitochondria, endoplasmic reticulum cisternae, and ribosomes (Garcia-Gil 2002; Pinart 1997).

Type B1 spermatogonia are small and elongated cells (10 × 7 μm) attached to the basal lamina. They show a strongly electrodense nucleus with large heterochromatic masses and one or two well-developed nucleoli (Garcia-Gil 2002; Pinart et al. 2000). The cytoplasm contains dilated endoplasmic reticulum cisternae, vesicles that vary in size and have abundant polyribosomes (Garcia-Gil 2002; Pinart 1997).

Type B2 spermatogonia are very similar to type B1 ones. However, they show a spherical nucleus with an underdeveloped nucleolus. These cells gradually lose contact with the basal lamina (Garcia-Gil 2002; Pinart et al. 2000) and when they become detached they enter meiosis (Garcia-Gil 2002; Pinart 1997).

3.2.3.2 Phase II of Spermatogenesis: The Meiotic Phase

Spermatocytes are cells involved in the process of meiosis, in which two phases can be distinguished: meiosis I or reductional division, and meiosis II or equatorial division. Primary spermatocytes are involved in meiosis I, while secondary spermatocytes are involved in meiosis II (Pinart 1997; Garcia-Gil 2002). One single

spermatocyte, after going through these two divisions, produces four haploid cells called spermatids (Garcia-Gil 2002).

During the period prior to the prophase of the first meiotic division, primary spermatocytes are known as preleptotene spermatocytes. These cells have completely lost their contact with the basal lamina (Pinart et al. 2000), but are still in the basal compartment of the seminiferous epithelium. They are oval in shape (12 × 7 μm) and contain a rounded nucleus with fine, granular euchromatin (Garcia-Gil 2002; Pinart et al. 2000). The cytoplasm displays a Golgi apparatus near the nucleus, a well-developed endoplasmic reticulum, vesicles that vary in size and electrodensity, and abundant polyribosomes (Garcia-Gil 2002; Pinart 1997).

As preleptotene spermatocytes differentiate the electrodensity of both the nucleus and the cytoplasm increases. The nucleus is characterized by the presence of several heterochromatic areas (Garcia-Gil 2002; Pinart 1997).

The prophase of the first division is subclassified into four successive stages: leptotene, zygotene, pachytene, and diplotene.

Leptotene spermatocytes are cells in transit toward the adluminal compartment of the seminiferous epithelium (Garcia-Gil 2002; Pinart et al. 2000). At an early stage, they show strongly electrondense nucleus and cytoplasm, and a well-conformed nucleolus. At a later stage, the cell volume increases (15 × 9 μm) and chromosomes appear as fibers attached to the nuclear envelope (Garcia-Gil 2002; Pinart 1997; Pinart et al. 2000). The endoplasmic reticulum is well developed (Garcia-Gil 2002; Pinart 1997).

Zygotene primary spermatocytes are rounded cells about 16 μm in diameter, located in the adluminal compartment of the seminiferous epithelium (Garcia-Gil 2002; Pinart 1997). They display a peripheral nucleolus and chromosome fibers attached to the nuclear envelope (Garcia-Gil 2002). The endoplasmic reticulum is still well developed (Garcia-Gil 2002; Pinart 1997).

Pachytene primary spermatocytes are the largest cells of the germ line (20 × 13 μm) (Garcia-Gil 2002; Pinart 1997). The nucleus is characterized by the presence of the synaptonemal complex (Garcia-Gil 2002; Pinart et al. 2000), and the cytoplasm by the presence of concentrically arranged endoplasmic reticulum cisternae (Garcia-Gil 2002).

In primary spermatocytes, the last stages of prophase I (diplotene), metaphase I, anaphase I, and telophase I take place very rapidly to form secondary spermatocytes (Garcia-Gil 2002). Secondary spermatocytes then enter meiosis II, or equatorial division (metaphase II, anaphase II, and telophase II), to form spermatids (Garcia-Gil 2002; Pinart 1997).

Secondary spermatocytes are rounded cells much smaller (15 μm in diameter) than pachytene primary spermatocytes. The nucleus contains granular euchromatin and small heterochromatic areas. The cytoplasm presents a well-developed Golgi apparatus near the nucleus, rough and smooth endoplasmic reticulum cisternae, some mitochondria, and a large number of vesicles that vary in size and electrodensity (Garcia-Gil et al. 2002a, b; Pinart et al. 2000).

Young spermatids, which are newly formed, are rounded cells (10–12 μm in diameter) and, compared to secondary spermatocytes, a reduction in the volume of

the nucleus and the cytoplasm is observed. The nucleus, which is spherical, occupies a central position; the content of the cytoplasm is very similar to that of secondary spermatocytes (Pinart 1997; Pinart et al. 2000; Garcia-Gil 2002).

3.2.3.3 Spermiogenesis

In swine, the differentiation process of a spermatid into a sperm cell and its subsequent spermiation, i.e., the releasing of spermatozoa from Sertoli cells to the lumen of the seminiferous tubules, lasts 14 days. Spermiogenesis begins at stage VI of the seminiferous epithelium cycle. During spermiogenesis, up to nine forms of spermatids can be observed according to their differentiation degree, and four phases can be distinguished: Golgi phase, cap phase, acrosome phase and maturation phase (Pinart 1997; Pinart et al. 2000).

In Golgi phase, type 1 and 2 spermatids are found; in cap phase, type 3 and 4 spermatids. In the aforesaid spermatids, the nucleus contains evenly distributed granular euchromatin, with few heterochromatic areas, and a small nucleolus close to the nuclear envelope. The first signs of chromatin condensation are observed at the end of the cap phase (type 4 spermatids) with the presence of a greater degree of heterochromatic areas. The Golgi apparatus is well developed and in association with the formation of the acrosomal vesicle and the chromatoid body. During these two phases, migration of the pair of centrioles from the cell periphery to the vicinity of the nucleus takes place. Near the proximal centriole, the capitulum and the segmented columns will be developed. Growth of the axoneme begins from the distal centriole. The formation of the fibrous sheath starts in type 2 spermatids and ends in type 8 spermatids. Nevertheless, only the formation and growth of the two longitudinal axes of the fibrous sheath take place in the Golgi and cap phases. The growth of these axes is initiated at the distal end of the axoneme, heading toward the distal centriole. The ribs of the fibrous sheath appear in the acrosome and maturation phases (type 5–8 spermatids). Mitochondria are spherical and mostly distributed in the periphery of the cytoplasm (Garcia-Gil et al. 2002a, b).

Type 1 spermatids display a spherical nucleus and an acrosomal system formed by a set of proacrosomal vesicles, which are derived from the Golgi apparatus. These vesicles fuse to form an acrosomal vesicle containing a small and incipient acrosome granule.

Type 2 spermatids contain a spherical nucleus and a more voluminous acrosomal vesicle, in contact with the nucleus, with a well-developed acrosome granule. These spermatids show a slight nuclear invagination, or implantation pit of the capitulum, in which electrodense material associated with the nuclear envelope will appear, thus forming the basal plate.

In type 3 spermatids, the nucleus maintains its spherical shape, the nucleolus disappears and the acrosomal vesicle extends as a cap until covering half of the nucleus.

Type 4 spermatids are introduced into the invaginations of the free surface of Sertoli cells, with the acrosomes located at the bottom of these crypts. Most of the

cytoplasm displaces to the opposite pole of the acrosome. The nuclei initiate their elongation despite still containing granular euchromatin. The chromatoid body (electrodense granular material) moves until placed near the distal centriole and the incipient axoneme.

The acrosome phase includes types 5, 6, and 7 spermatids. A feature of this phase is the emergence of the perinuclear manchette, a structure formed by microtubules arranged in spiral along the surface of the nucleus and closely related with its elongation. At this stage, the nucleus considerably lengthens and most of the cytoplasm occupies the opposite pole of the acrosome together with the tail in formation, so that polarization of spermatids occurs Chromatin condensation begins at the most apical part of the nucleus (i.e., close to the acrosome) and progresses distally (i.e., close to the centriole) from the periphery to the nuclear centre. As a result, chromatin condensation shortens the nuclear volume. The nuclear envelope reduces its nuclear pores in the areas of the nucleus where chromatin condenses. In some peripheral parts, in those areas in which the nuclear envelope is in excess, it folds up intensely constituting the so-called lamellar bodies. These areas of the nuclear envelope are rich in nuclear pores and allow the exchange of substances between the cytoplasm and the nucleoplasm. The spermatid portion containing the acrosome is found in contact with the bottom of the crypt, followed by the portion containing the nucleus; finally, a voluminous cytoplasmic portion (or cytoplasmic lobe), containing a large number of mitochondria, vesicles, endoplasmic reticulum, and two centrioles from one of which the axoneme elongates. The Golgi apparatus also moves toward the cytoplasmic portion. Mitochondria are arranged near the axoneme and around dense fibers describing a helical trajectory (Garcia-Gil 2002).

Type 5 spermatids already exhibit a cylindrical nucleus, though with a higher degree of chromatin condensation, and the acrosome develops a well-defined apical projection (Fig. 3.6).

In type 6 spermatids the nucleus takes the form of a spatula and flattens out, while the chromatin continues its condensation process. The apical projection of the acrosome is highly protruding. Dense fibers start to differentiate.

In type 7 spermatids the nucleus maintains its spatula shape and the apical projection of the acrosome takes the form of a hook. Dense fibers gradually increase in length and diameter.

Type 8 and 9 spermatids are found at maturation phase. Lamellar bodies experience an intense regression and they are only visible in testicular sperm in the distal part of the nucleus, surrounding the capitulum. The microtubular manchette disappears, and the annulus (Jensen's ring) will develop from the chromatoid body. Dense fibers reach their definitive length and diameter. Mitochondria elongate and their mitochondrial matrix is reduced and becomes more electrodense. Mitochondria not arranged around the dense fibers are gathered in the residual cytoplasm (Garcia-Gil 2002).

Type 8 spermatids display well-differentiated nucleus and acrosome, and the mitochondrial sheath begins to become organized, increasing the thickness of the dense fibers along the axoneme.

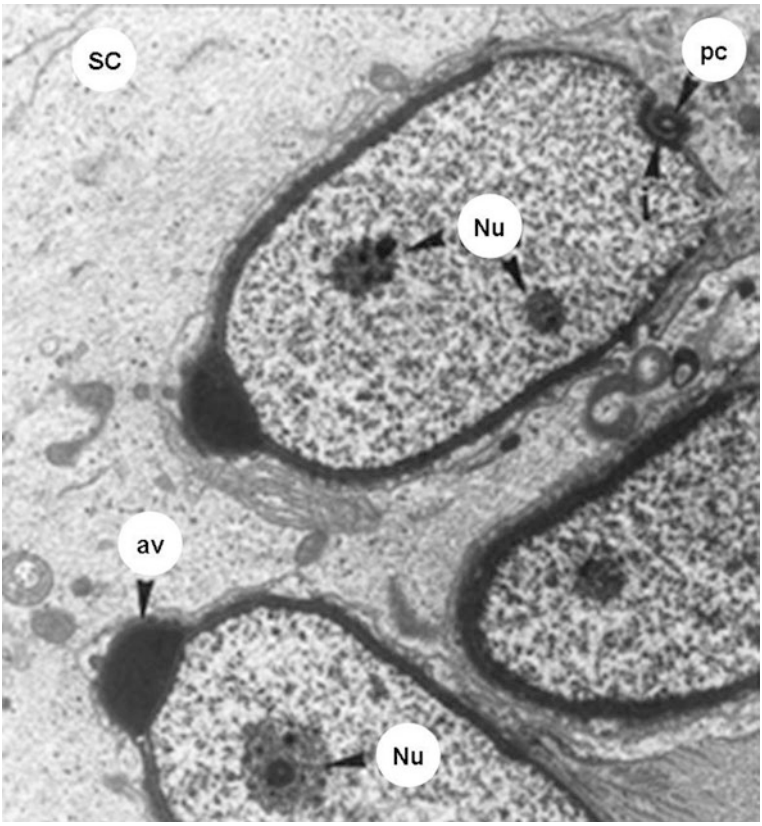

Fig. 3.6 General aspect of type 5 spermatids. *SC* Sertoli cell; *Nu* nucleolus; *pc* proximal centriole; *av* acrosomal vesicle

In type 9 spermatids, the mitochondrial sheath is complete and the tail is released from the crypts of the Sertoli cells toward the lumen of the seminiferous tubule. The distal centriole disappears and only the proximal centriole is observed at an angle of 45° relative to the axoneme. The residual cytoplasm is concentrated in a spherical mass located between the head (region containing the acrosome and nucleus) and the tail (region containing the axoneme) of the mature spermatid. Such residual cytoplasm mass will give rise to the proximal cytoplasmic droplet, characteristic of testicular sperm.

3.2.3.4 Sertoli Cell and Germ Cell Interactions

There is metabolite exchange among Sertoli cells, spermatogonia, and spermatocytes, due to the presence of desmosome junctions and tight junctions (Fig. 3.7). Sertoli cells play a nutritional role for germ cells and regulate spermatogenesis.

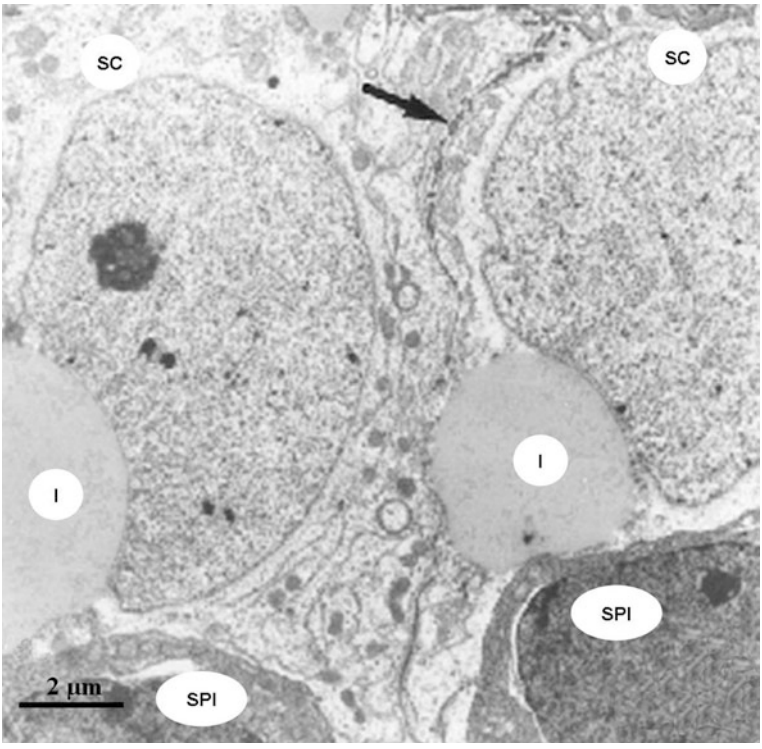

Fig. 3.7 Blood–testis barrier. The arrows indicate the tight junctions and desmosome junction between two sertoli cells (*SC*). *SPI*, primary spermatocytes; *I*, lipid droplets

Sertoli cells are also responsible for the blood-testis barrier that divides the seminiferous epithelium in two compartments: the basal compartment, in contact with the basal lamina of the epithelium; and the adluminal compartment, in contact with the lumen of the seminiferous tubule. Diploid germ cells (spermatogonia and primary spermatocytes) are located in the basal compartment, and haploid cells (secondary spermatocytes and spermatids) in the adluminal compartment. The blood-testis barrier is composed of desmosomes and tight junctions between cytoplasmic evaginations of neighboring Sertoli cells and prevents the autoimmune response toward haploid cells of the germ line (Fig. 3.7).

Sertoli cells exert a very important influence on spermiogenesis and spermiation of mature spermatids. They are directly involved in spermatogenesis beginning with type 5 spermatids. In type 8 spermatids, i.e., at the maturation phase, the excess of membrane after elongation folds back forming a lamellar structure known as the tubulobulbar complex. Some of these complexes are separated from the head of the spermatids and invaginated by Sertoli cells.

At the end of spermiogenesis, another type of interaction between Sertoli cells and spermatids is observed, which allows mature spermatids (or type 9 spermatids) to get rid of the excess of cytoplasm. Sertoli cells retain residual cytoplasm

from spermatids, which is accumulated in cytoplasmic lobes, while spermatids are displaced toward the free surface of the Sertoli cell. This residual cytoplasm, rich in mitochondria and lipid droplets and presenting some vesicles and ribosomes, detaches from the connecting piece of type 9 spermatids. Then, residual cytoplasm is phagocytized by Sertoli cells and subsequently digested after lysosome fusion. The small amount of cytoplasm that is still retained at the connecting piece of type 9 spermatids will constitute the well-known proximal cytoplasmic droplet of testicular sperm (Garcia-Gil 2002).

In the apical surface of Sertoli cells some invaginations called crypts are formed. Each crypt contains one spermatid in maturation phase. The union of spermatids to the crypt is maintained through specialized junctions, which are present in Sertoli cells and known as ESER complexes. The ESER complexes consist of endoplasmic reticulum cisternae (arranged around the spermatid) and an electrondense material located between the aforesaid cisternae and the plasma membrane of the Sertoli cell, surrounding the head of the spermatid (Garcia-Gil 2002; Pinart 1997).

In the seminiferous epithelium, numerous cells with signs of degeneration are observed. They tend to be spermatogonia and spermatids at initial stages of spermiogenesis and are removed after their phagocytosis by Sertoli cells.

3.2.4 Cycle and Wave of the Seminiferous Epithelium

The spermatogenic cycle at the seminiferous epithelium in swine is divided into eight stages. Each stage is distinguished by the shape and position of the spermatid nuclei, the presence of meiotic divisions, and the cellular composition of the epithelium. Likewise, these eight stages can be grouped into three categories (Garcia-Gil 2002):

1. premeiotic stages represent 31.9 % of the spermatogenic epithelium cycle and include stages I, II, and III
2. meiotic stages represent 16.4 % of the cycle and include stages IV and V
3. postmeiotic stages represent 50.6 % of the cycle and include stages VI, VII and VIII.

Following the cross-section of a seminiferous tubule, only one stage can be observed. A spermatogenic cycle lasts 9.6 days and its total length as measured in the seminiferous epithelium is about 4672.7 μm (Garcia-Gil 2002). In swine, around four cycles are needed to complete the process of spermatogenesis (Fig. 3.8).

The “cycle of the seminiferous epithelium” concept and the “wave of the seminiferous epithelium” concept are different. While the seminiferous epithelium cycle is a dynamic phenomenon that occurs at different times in a particular area of the seminiferous epithelium, the wave of the seminiferous epithelium refers to the more or less orderly distribution of the eight different stages along the seminiferous epithelium at a given point in time (Garcia-Gil et al. 2002a, b).

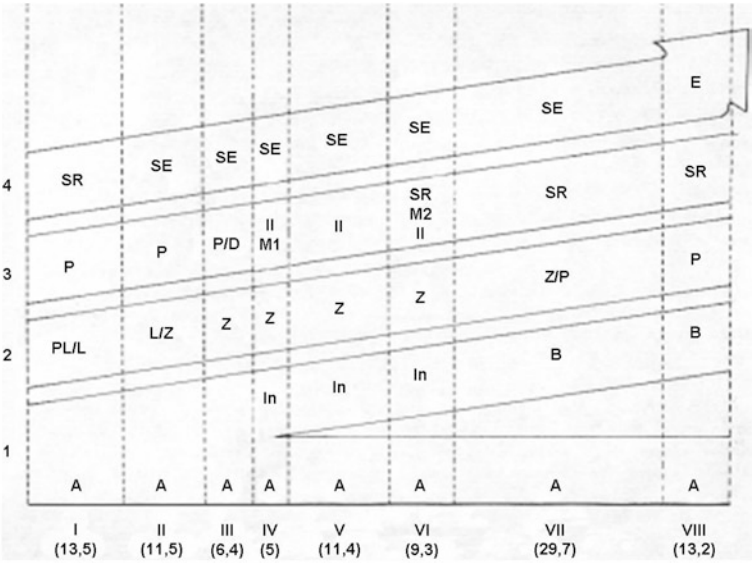

Fig. 3.8 Schematic diagram showing the process of spermatogenesis. The stages of the cycle are indicated by *Roman numbers*, and the cycles comprising the process of spermatogenesis in pigs are indicated by *Arabic numbers*. Relative frequencies of occurrence (%) of each stage are shown in *brackets*. The column width is proportional to the duration of each stage. The letters inside each column represent the types of germ cells characteristic of each stage. *A*, type A spermatogonia; *In*, intermediate spermatogonia; *B*, type B spermatogonia; *PL* preleptotene spermatocytes; *L* leptotene spermatocytes; *Z* zygotene spermatocytes; *P* pachytene spermatocytes; *D* diplotene spermatocytes; *M1*, primary spermatocytes at first meiotic division; *II*, secondary spermatocytes; *M2*, secondary spermatocytes at second meiotic division; *RS* round spermatids; *ES* spermatids at elongation phase; *S* spermatozoa (Garcia-Gil 2002)

3.3 Epididymis

3.3.1 General Aspects: Anatomy and Functions

The term epididymis is derived from the Greek words *epi* (=upon, over) and *didymoi* (=testicles) and refers to the organ that conducts spermatozoa from the efferent ducts outside the testis to the vas deferens (Cooper 1998). The boar epididymis contains an epididymal duct, 54 m long, tightly-coiled, and included in a matrix of vascularized and innervated connective tissue. The epididymis is covered by a fibrous capsule and can be anatomically divided into three main regions: *caput* (head or cephalic, proximal or initial region), *corpus* (body or intermediate region), and *cauda* (tail or caudal, distal or terminal region) (Cooper 1998). The cephalic region, which is the most flattened one, maintains the epididymal duct attached to the testicle through the efferent ducts. The body region, narrow and elongated, is located on a side of the testicle and joins the

cephalic region with the caudal region. The latter is the most prominent one and contains an epididymal duct that communicates directly with the vas deferens (Fig. 3.9) (Pruneda 2006).

Sperm leaving the efferent ducts and entering the epididymal duct take about 12–15 days to travel its entire length (3 days for the caput, 2 days for the corpus, and 7 to 10 days for the cauda). The distance travelled each day is, approximately, 5 m (Pruneda 2006).

The main functions of the epididymis, i.e., the epididymal duct, are (Cooper 1998):

1. Sperm transport from the efferent ducts to the vas deferens.
2. Sperm maturation (a complex process after which sperm acquire progressive motility and fertilizing ability) (Robaire and Hermo 1988; Hinton and Palladino 1995; Cooper 1998; Dacheux et al. 1998; Jones 1998; Bassols et al. 2004).
3. Sperm storage until ejaculation (Jones 1989; Jones and Clulow 1994).

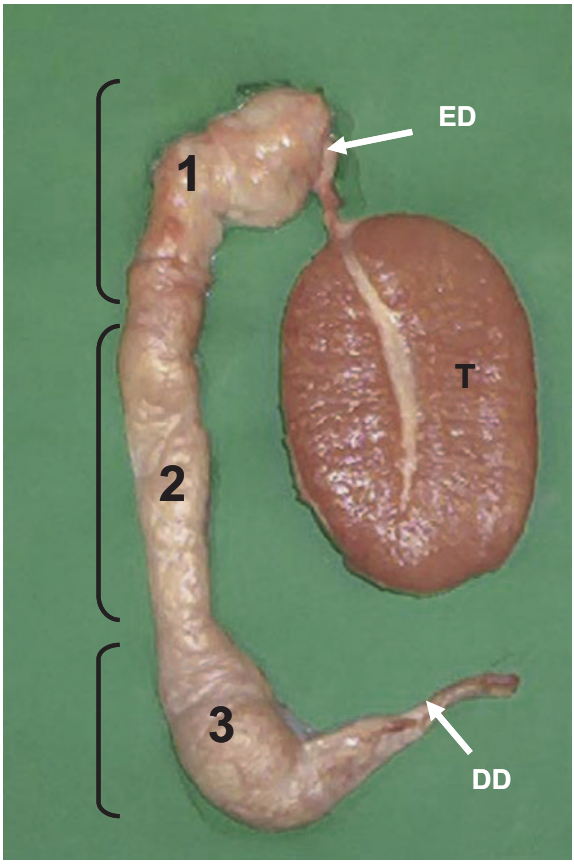

Fig. 3.9 Regions of the epididymis. *1* Caput; *2* Corpus; *3* Cauda. *ED* efferent ducts; *DD* deferent duct; *T* longitudinal section through the testis

The transport of sperm along the epididymal duct is facilitated by contractions of the smooth surrounding muscle (Robaire and Hermo 1988; Bassols 2006; Pruneda 2006). While the maturation process takes place in the cephalic and body regions, sperm storage occurs in the caudal region (Holtz and Smidt 1976). In this last part of the epididymal duct luminal concentrations of sodium and bicarbonate ions are low and those of potassium are high; osmolarity and sperm concentration are high and oxygen pressure and energy substrates are low; secretion of viscous mucoproteins; and motility inhibiting factors are characteristic (Cosentino and Cockett 1986). Such physicochemical conditions maintain the spermatozoa in a quiescent state (Bassols 2006; Fàbrega et al. 2011a, b). During ejaculation, the number of sperm stored in the tail of the epididymis is reduced by around 60 % is emptied (Hughes and Varley 1984; Strzezek et al. 1995; Pruneda 2006).

3.3.2 Structure and Ultrastructure

Regardless of the region the epididymal duct has a common and basic histological structure: a pseudostratified epithelium with stereocilia surrounded by a musculo-connective sheath. However, it has specific features for each anatomical region (Table 3.1) (Briz et al. 1993).

The maximum density of stereocilia is observed in the body region. The musculo-connective sheath is more developed in the cauda but better vascularized in the caput and corpus (Briz et al. 1993). The lumen of the epididymal duct is filled with fluid, spermatozoa, and basophilic mononuclear somatic cells, which are more frequent in the caudal region.

Five cell types can be distinguished in the epithelium of the epididymal duct: principal cells, basal cells, clear cells, apical cells, and halo cells (Fig. 3.10) (Bassols 2006).

Principal cells outnumber all other cell types (Bassols et al. 2006a, b), representing 65–80 % of the total population of epithelial cells. These are columnar cells with abundant stereocilia (Hamilton 1975; Briz et al. 1993; Stoffel and Friess 1994; Pruneda 2006) and their cytoplasm exhibits a well-developed, rough, endoplasmic reticulum at its basal region, a Golgi apparatus at a supranuclear position, smooth endoplasmic reticulum cisternae, and several vacuoles, pinocytosis vesicles, secretory granules, endosomes, multivesicular bodies, lysosomes, and

Table 3.1 Histological features of the three regions of the epididymal duct (Briz et al. 1993)

Epididymal region (μm)	Caput	Corpus	Cauda
Internal diameter of the duct	216 ± 22	205 ± 19	175 ± 14
Height of the epithelium without stereocilia	43 ± 2	46 ± 4	37 ± 3
Length of stereocilia	12 ± 0.5	7 ± 1.0	9 ± 2.0

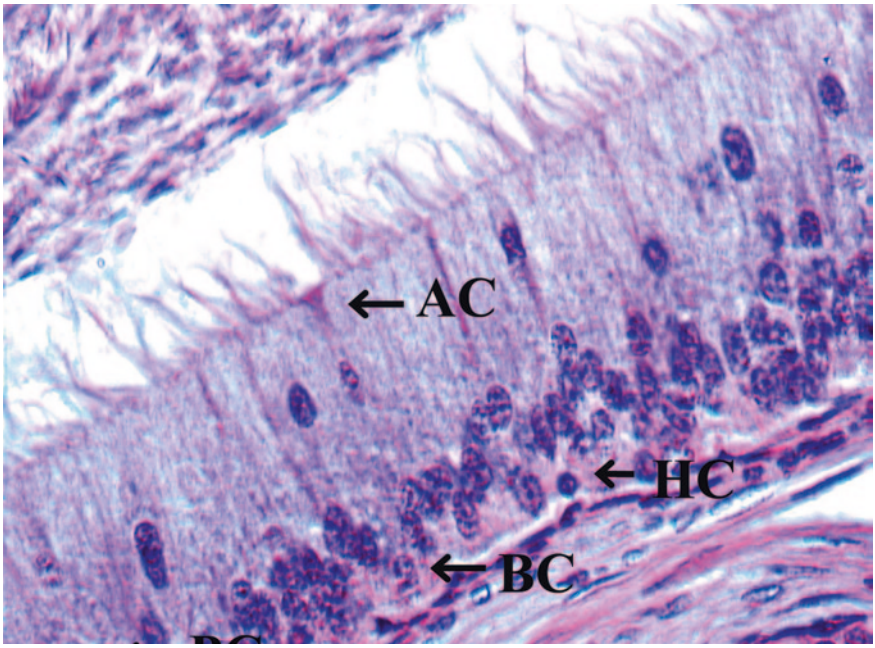

Fig. 3.10 General aspect of the epididymal duct epithelium. *PC* principal cells; *BC* basal cells; *AC* apical cells; *HC* halo cells

mitochondria (Briz 1994; Stoffel and Friess 1994; Bassols 2006). Principal cells establish tight junctions among them, thus constituting the well-known blood-epididymis barrier. Therefore, all metabolites needing to pass through the epididymal epithelium must be actively transported through the principal cells. These cells have secretory and absorptive functions, synthesizing a large number of proteins that are secreted or retained within them. Moreover, they are involved in the uptake of ions, water, and organic solutes from the luminal fluid and in the transport of low molecular weight osmolytes.

Basal cells and clear cells are pyramidal and isodiametric cells, respectively, in touch with the basal lamina of the epithelium but they do not reach its free surface, i.e., the lumen of the duct (Briz et al. 1993). The cytoplasm of basal cells does not present strong electrodensity and it contains scarce mitochondria and endoplasmic reticulum cisternae (Robaire and Hermo 1988; Briz 1994; Stoffel and Friess 1994). Basal cells express antigens inherent to macrophages (Yeung et al. 1994; Seiler et al. 1999), as well as different enzymes such as glutathione-S-transferase (GTS) (Veri et al. 1993) and SOD (superoxide dismutase) (Nonogaki et al. 1992). Their role is controversial but they are mostly related to phagocytic activities and to the immune and oxidative protection of sperm (Bassols 2006). Clear cells are considered as T lymphocytes or macrofhages.

Apical cells are very narrow and have no stereocilia. Their cytoplasm is rich in mitochondria and vesicles (Sun and Flickinger 1980; Briz 1994; Stoffel and Friess 1994; Adamali and Hermo 1996), contains several proteolytic enzymes (Adamali and Hermo 1996) and carbonic anhydrases (Ekstedt et al. 1991), and also abundant proton pumps in their plasmalemma (Brown et al. 1992; Hermo et al. 2000). Basically, they are located at the epididymal caput. Therefore, their function has been associated with the acidification and alkalinization of the epididymal fluid (Au and Wong 1980; Rodriguez-Martinez et al. 1990; Calvo et al. 2000) and, consequently, with the quiescent state of sperm.

Finally, halo cells are cuboidal basal cells that are basically considered as T lymphocytes or intraepithelial macrophages (Goyal and Williams 1991).

The epididymal duct epithelium in the region of the caput is composed of a layer of circularly oriented smooth muscle cells. The thickness of this layer progressively increases and, when it reaches the caudal region, two layers can be observed: an inner and well-developed one, in which the smooth muscle cells are longitudinally arranged, and an outer and less developed one with the smooth muscle cells arranged circularly. The connective tissue surrounding the muscle sheath is more fibrous and dense in the caudal region (Bassols 2006).

Blood vessels and nerves are present between the connective tissue of the muscle sheath. The epididymis has two irrigation routes, one coming from the internal spermatic artery, which, after crossing the testicle, supplies blood to the efferent ducts and epididymal caput and corpus, and another coming from the deferential artery, which irrigates the epididymal cauda.

3.3.3 Epididymal Fluid

In the testis, Sertoli cells secrete the testicular fluid that transports sperm toward the efferent duct and epididymal caput, where much of the testicular fluid is reabsorbed (Setchell 1967 and 1969; Tuck et al. 1970; Cheung et al. 1977; Wong et al. 1979; Cooper 1998). Different processes (water and electrolyte transport across the epididymal epithelium, secretion and reabsorption of substances, and sperm metabolic activity) throughout the different regions of the epididymis, modify the epididymal fluid composition on an ongoing basis (reviewed by Bassols 2006). The most influential phenomenon on epididymal fluid composition is the secretory and reabsorptive activity of the epididymal duct epithelium. This fluid exchange varies along the epididymal duct so that the epididymal fluid composition is both qualitatively and quantitatively different depending on the region of the epididymis. Reabsorption mainly takes place in the efferent ducts and epididymal caput, progressively decreasing toward the epididymal corpus and cauda. Secretion mainly occurs in the epididymal caput and, as for reabsorption, it progressively decreases toward the epididymal corpus and cauda. So, in the epididymal cauda, reabsorption, and secretion reach their minimum values and are balanced.

In swine, six regions can be distinguished according to the quantitative and qualitative composition of the epididymal fluid: proximal caput, distal caput, proximal corpus, distal corpus, proximal cauda, and distal cauda. Obviously, these sequential microenvironments are critical to the proper development of the complex process of sperm maturation that spermatozoa experience along the epididymal duct. Any factor affecting the secretory and absorptive activity of the epididymal epithelium (stress, diet, temperature, collection frequency, etc.) leads to an incomplete sperm maturation process (Briz et al. 1996) and, consequently, to the loss of progressive sperm motility and fertilizing ability. For example, a high extraction rate of semen entails a change in the reabsorption/secretion pattern of the epididymal fluid as a result of a decrease in the fluid reabsorption rate in the caput and of a net reabsorption balance in the corpus. In boars submitted to a high extraction rate during 4 days, alterations in sperm motility, in migration of the cytoplasmic droplet, in sperm head size and shape, and in the stability of the connecting piece, have been observed (Pruneda et al. 2005a, b; Pruneda 2006).

Epididymal fluid composition differs from the composition of blood plasma. The former is hypertonic with regard to the latter (Cooper 1998) and the blood-epididymis barrier, which is formed by tight junctions among epididymal principal cells, prevents the resemblance of fluids from both compartments.

Water transport in the epididymis is very important, affecting both the concentration of different metabolites and sperm concentration. In the epididymal caput, sperm concentration significantly increases as a result of the large amount of reabsorbed water (Wong 1986). High sperm concentration facilitates the interaction between spermatozoa, luminal metabolites, and the surface of the epididymal epithelial cells.

In the epididymal fluid, ions (Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, PO_4^{3-}), low molecular weight molecules (glycerophosphocholine, sialic acid, carnitine, glycerol, inositol, etc.), lipids, proteins (clusterin, immobiline, angiotensin, etc.), and enzymes (alkaline phosphatase, acid phosphatase, alpha-glucosidase, N-acetylglucosaminidase, etc.) have been described (Robaire and Hermo 1988; Syntin et al. 1996). Some low molecular weight molecules, such as water, can cross the blood-testis barrier; however, high molecular weight molecules, such as glucose, must be transported through the epididymal epithelium (Hinton and Howards 1982; Turner and Howards 1985). The transport of some molecules, such as glucose, occurs by facilitated diffusion, (Brooks et al. 1974; Hinton and Howards 1982) while for other molecules, such as L-carnitine, transport is active, and mediated by androgens (James et al. 1981).

L-carnitine concentration of the epididymal fluid increases along the epididymal duct, (Casillas 1973; Hinton et al. 1979; Jeulin et al. 1994) and it is in the distal corpus and proximal cauda where it reaches its highest values. However, L-carnitine content of sperm does not vary throughout the epididymal duct (Pruneda et al. 2007). Carnitine has been related to the progressive movement of sperm (Jeulin and Lewin 1996). Myo-inositol concentration of the epididymal fluid decreases along the epididymal duct; myo-inositol content of sperm

decreases from the distal caput. Glutamate concentration of epididymal fluid increases along the epididymal duct, mainly in the distal corpus and proximal cauda. The glutamate content of sperm decreases in the epididymal caput and increases in the epididymal corpus and cauda (Pruneda et al. 2006 and 2007). Other energy substrates, such as glucose or fructose, also present a distinct concentration pattern throughout the epididymal duct. Glucose in the epididymal fluid increases from the distal caput to the proximal cauda, where it reaches its highest values, while it is virtually undetectable in the distal cauda (Jones and Montague 1991; Pruneda et al. 2006). Fructose is undetectable in the epididymal fluid of proximal caput but it gradually increases from the distal caput to the proximal cauda; at the distal cauda, fructose concentration drops again (Jones and Montague 1991; Pruneda et al. 2006). On the other hand, the sorbitol concentration of the epididymal fluid is 10-fold higher than glucose and fructose over all the regions. Sorbitol, as well as being an energy substrate, is a determining osmolyte in sperm volume regulation. In swine, the polyol pathway has been observed at the epididymal duct. In this pathway, glucose entering the lumen of the duct and coming from the testes or blood is reduced to sorbitol by the aldose reductase enzyme. The resulting sorbitol is accumulated in the epididymal fluid and subsequently transformed to fructose by the sorbitol dehydrogenase (Kinoshita and Nishimura 1988; Pruneda et al. 2006). Both aldose reductase and sorbitol dehydrogenase are present in the epididymal fluid throughout the epididymis although the distribution of aldose reductase in the epithelium varies slightly along the duct. It is only present in the proximal caput at the apical region and stereocilia of principal cells, while in the distal caput it is distributed all over the cytoplasm of principal cells. In the corpus, aldose reductase is present in the cytoplasm of principal and basal cells, and in the cauda it is only present in the cytoplasm of some basal cells. Sorbitol dehydrogenase is found in the stereocilia of principal cells of all the epididymal regions but it is also present in the cytoplasm of principal cells at the caput (Pruneda et al. 2006).

Over 125 different proteins and enzymes have been described in porcine epididymal fluid (Syntin et al. 1996). Total protein concentration is approximately 35 mg/mL in the caput and around 20 mg/mL in the corpus and cauda. Proteins synthesized and secreted in a given region of the epididymis are reabsorbed, metabolized, or modified in the same region or in later regions. Thus, as examples, most of the synthesis and secretion of glutathione peroxidase (GPX) is produced in the proximal caput as well as the synthesis of the retinoic acid binding protein (E-RABP); in the distal caput an intense secretion of clusterin and lactoferrin takes place; the epididymal fluid at the distal corpus characteristically presents two unidentified proteins; finally, in the epididymal cauda both the quantity and the typology of its particular proteins are very low (Syntin et al. 1999).

Many substances synthesized and secreted by epididymal epithelial cells are subsequently expressed on the sperm surface. These substances are transferred from the fluid to the sperm plasma membrane where they bind through covalent bonds thanks to the action of enzymes such as transferases or by direct exchange mediated by lipids such as glycosylphosphatidylinositol.

Since the epididymal function is androgen-dependent, testosterone from blood or directly from the testicular fluid is incorporated into the epididymis, mostly at the epididymal caput, where it is converted into dihydrotestosterone (DHT) (Aafjes and Vreeburg 1972). The ABP protein (androgen binding protein) is synthesized by Sertoli cells; most of it (80 %) is secreted in the testicular fluid (French and Ritzen 1973) and a small portion (20 %) is secreted in blood (Weddington et al. 1975). Within the principal cells of the epididymal duct epithelium, testosterone is separated from ABP and transformed into dihydrotestosterone (DHT) by the action of 5alpha-reductase, an enzyme much more frequent in the epididymal caput than in the other regions. Afterwards, DHT targets an androgen receptor (AR) present in the nucleus of principal cells, finally activating their DNA and the transcription of various proteins (Fabrega et al. 2012a, b; Dacheux et al. 2009).

3.3.4 Sperm Maturation

The sperm maturation process includes a complex set of modifications that testicular sperm experience to acquire progressive motility and fertilizing ability, i.e., the ability to recognize and bind to the oocyte (Holtz and Smidt 1976; Moore 1990; Briz et al. 1993). Among the major modifications experienced by spermatozoa along the epididymal duct, the following ones are highlighted:

1. Cytoplasmic droplet displacement from the connecting piece (proximal droplet) to the Jensen's ring (distal droplet) of the axoneme and its detachment (Table 3.2) (Bedford 1975).

The cytoplasmic droplet is composed of residual cytoplasm retained by sperm after spermiation, i.e., after leaving the Sertoli cells crypts. This residual cytoplasm is initially located at the sperm connecting piece and, as it progresses through the epididymal duct, it migrates to the boundary between the principal and mid pieces of the axoneme, i.e., the Jensen's ring (Bloom and Nicander 1961; Bedford 1975; Bassols et al. 2005a, b; Bassols 2006); at this point, the cytoplasmic droplet becomes detached. At the moment of detachment, the sperm tail bends

Table 3.2 Percentage of sperm with proximal droplet, distal droplet, and without cytoplasmic droplet over the three epididymal regions in boars subjected to a collection rate of twice a week

Epididymal region (%)	Caput	Corpus	Cauda
Spermatozoa with proximal cytoplasmic droplet	45–50	0–1	0–1
Spermatozoa with distal cytoplasmic droplet	0–1	45–50	10–15
Spermatozoa without cytoplasmic droplet	45–50	45–50	80–95

slightly at the Jensen's ring and, once released, the tail returns to its linear trajectory (Fig. 3.11).

Sperm with tails bent by the Jensen's ring are abnormal sperm (malformations) produced after membrane fusion of mid and principal pieces when the cytoplasmic droplet is about to detach. The percentage of sperm with tails folded at this region is at a maximum at the epididymal cauda (0.04 % in caput and corpus, and 0.45 % in cauda) (Bonet et al. 2012).

The cytoplasmic droplet shows a diameter of around 1.5 μm and is rich in membranous vesicles that increase as the droplet displaces through the midpiece. There are two types of vesicles: double-membrane vesicles and single-membrane vesicles. Double-membrane vesicles exhibit an electrolucent matrix, have a diameter of around 0.2 μm and are derived from smooth endoplasmic reticulum cisternae that close upon themselves. Single-membrane vesicles are derived from smooth endoplasmic reticulum dilations which fuse together giving rise to vesicles highly variable in diameter (0.1–0.9 μm).

The presence of the cytoplasmic droplet on ejaculated sperm can be used as an indicator of sperm maturation (Gomez et al. 1996; Keating et al. 1997; Amann et al. 2000; Thundathil et al. 2001; Bassols 2006). The more similar the percentages of sperm with distal or proximal droplet are to the values in corpus or caput, the more immature the ejaculated sperm is (Table 3.3).

2. Acrosomal protuberance reduction (Bassols 2006).

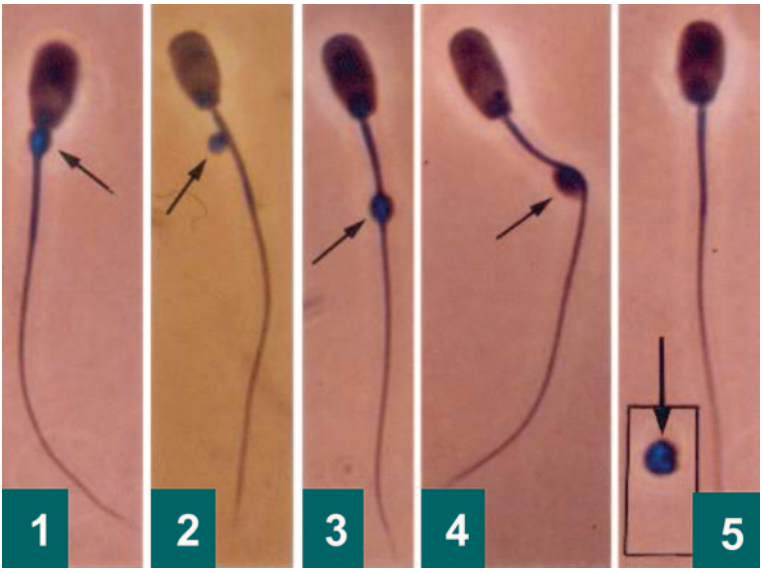

Fig. 3.11 *1* Sperm with proximal cytoplasmic droplet. *2* Displacement of the droplet through the mid-piece. *3* Sperm with distal cytoplasmic droplet. *4* Sperm with distal cytoplasmic droplet bending the tail before its detachment. *5* Sperm with a detached cytoplasmic droplet. Positive phase-contrast light microscopy. Glutaraldehyde-osmium tetroxide fixation

Table 3.3 Percentage of sperm with and without cytoplasmic droplets in boars subjected to a semen collection rate of once a day and in boars subjected to a semen extraction rate of twice a week

Collection rate (%)	Once daily	Twice a week
Sperm with cytoplasmic droplet	60–65	10–15
Sperm without cytoplasmic droplet	35–40	85–90

Within the epididymal caput, spermatozoa show a highly protuberant acrosomal vesicle in its apical and lateral limits (Bonet et al. 2012). As sperm pass through the epididymis, the acrosomal protuberance is gradually reduced in volume until it is only apparent at the apical region of ejaculated sperm. Thus, the development and extension of the acrosomal protuberance in ejaculated sperm can also be used as an indicator of sperm maturation.

3. Nuclear chromatin condensation through the formation of disulfide bonds between protamine cysteines (Calvin and Bedford 1971; Bassols 2006; Pruneda 2006).

The formation of disulfide bonds provides greater stiffness and thus minor flexibility to the sperm head. Boar sperm protamine molecules contain 10 cysteine groups each, which is higher than in other species. The chromatin crosslinking in boar sperm is therefore much more than in other mammalian species and DNA is more stable. The presence of sperm with bent heads in an ejaculate is another proof of failed sperm maturation. The plane by which the head is folded is related to the degree of sperm immaturity and to the presence of a cytoplasmic droplet; sperm with heads transversely folded usually exhibit a proximal cytoplasmic droplet; sperm with heads laterally folded usually exhibit a distal cytoplasmic droplet.

4. Stabilization of dense fibers through disulfide bonds between the protein cysteines constituting these structures (Calvin and Bedford 1971; Bassols 2006; Pruneda 2006).

Like the sperm head, the formation of disulfide bonds between cysteines provides greater stiffness and resistance to bending and coiling of the sperm tail (Briz et al. 1996). The percentage of sperm with tails folded at the midpiece region decreases as sperm move through the different epididymal regions (0.65 % in caput, 0.40 % in corpus, and 0.30 % in cauda), and screening for their presence in the ejaculate constitutes yet again a good parameter for the degree of sperm maturation.

5. Changes in the nature and distribution of membrane glycoproteins and formation of membrane domains rich in transport proteins and specific membrane receptors (Jones 1998; Bassols 2006).

The sperm plasma membrane undergoes significant changes in the nature and distribution of glycoproteins and lipids. The plasma membrane is enriched in

proteins secreted by the epididymal epithelium, and the enzymes present in the epididymal fluid modify membrane carbohydrate residues, proteins, and lipids. Recent studies using specific lectins against certain carbohydrate residues have demonstrated that many of them show a different and specific distribution pattern in the three epididymal regions (Fàbrega et al. 2011a, b); among them: galactose, glucose, manose, N-acetylglucosamine, N-acetyl-D-galactosamine, and fucose. Simultaneously, a redistribution of membrane glycoproteins and lipids occurs, leading to new membrane domains. Identity and distribution of glycoproteins and membrane lipids are also good indicators of sperm maturation.

The new antigenic identity and the new membrane domains acquired by mature sperm allow for its recognition with oviductal cells and with the zona pellucida and the plasma membrane (oolemma) of the oocyte. In addition, these modifications provide sperm with greater resistance to challenge osmotic shocks; after application of the ORT test (osmotic resistance test, see Sect. 9.3.5.3) 45–50 % of sperm resists an osmotic shock of 150 mOsm, while the percentage rises to 95–100 % in sperm coming from the epididymal corpus and cauda.

6. Redistribution of acrosomal enzymes and cytosolic proteins

The nature and distribution of acrosomal enzymes along the epididymal duct are also modified. Acrosin distribution in the sperm acrosomal vesicle is an example; in sperm extracted from epididymal caput, acrosin is located at its apical region and, when sperm reach the epididymal cauda, acrosin has been dispersed throughout the acrosomal vesicle. The correct distribution of acrosin is highly important for the digestion of the oocyte envelopes and its distribution in the acrosomal vesicle of ejaculated sperm is a good indicator of sperm maturation (Puigmule et al. 2011).

The nature and distribution of some cytosolic proteins are also modified. Thus, for example, the chaperone HSP90AA1, present in the cytosol of the sperm tail, is found in the proximal cytoplasmic droplet too. The HSP90AA1 is involved in the activation of the signals responsible for tyrosine phosphorylation of flagellar proteins. When the sperm maturation process has been carried out correctly the presence of HSP90AA1 in the distal cytoplasmic droplet occurs only in sperm from epididymal caput and corpus. In the epididymal cauda, the presence of sperm with significant amounts of HSP90AA1 in the distal cytoplasmic droplet is very rare. Thus, absence of HSP90AA1 in the distal cytoplasmic droplet of ejaculated sperm is a molecular marker of correct sperm maturation (Bonet et al. 2012).

7. Changes in the sperm movement pattern.

Sperm cells modify their movement pattern along the epididymal duct and acquire progressive motility. In the caput region, sperm acquire an irregular and vibrant movement; in the corpus, acquire a circular and nonprogressive movement; in the cauda, it acquires progressive linear motility (Briz 1994; Bassols 2006; Pruneda 2006). Forward motility protein (FMP) induces sperm motility. Carnitine

and acetylcarnitine (Dacheux and Paquignon 1980; Hinton et al. 1981; Goyal and Williams 1991; Yeung et al. 1993), in the epididymal fluid, and the intracellular cAMP, responsible for the protein tyrosine residues phosphorylation (Tash and Means 1983; Hoskins and Vijayaraghavan 1990), are involved in the changing of the motility pattern (Dacheux et al. 1990; Bassols 2006).

To sum up, the sperm maturation process is essential for the acquisition of progressive linear motility and sperm fertilizing ability, i.e., the recognition of the oocyte and the digestion of its envelopes. As mentioned before, many ambient factors to which boars are exposed affect the secretory and reabsorptive activity of the epididymal duct epithelium and, thus, alter the sequential modifications to sperm during their transit through the epididymis. The most common indicator to determine the degree of maturation of ejaculated sperm is the presence or absence of cytoplasmic droplets. If significant percentages of sperm with proximal cytoplasmic droplet are detected in an ejaculate, it can be confirmed that its quality is low, displaying nonprogressive sperm motility and no fertilizing ability. However, if an ejaculate shows significant percentages of sperm with distal cytoplasmic droplet then the sperm motility and fertilizing ability may be normal. To determine whether the sperm maturation process has been successfully completed, different morphological indicators or molecular markers should be examined. Presence or absence of proximal or distal cytoplasmic droplets, acrosomes with more or less development of the apical protuberance, folded heads, or tails folded at the midpiece, may be used as morphological indicators of sperm maturity while the presence or absence of fertilin, acrosin, and HSP90AA1 may be used as molecular markers of this very same characteristic (Fàbrega et al. 2011a, b).

3.4 Accessory Sex Glands

3.4.1 General Aspects

The noncellular fraction of the ejaculate, called seminal plasma, is composed of fluids from the testis and epididymis, and, mainly, of secretions from the accessory sex glands. Male accessory sex glands include: the seminal vesicles, the prostate and the bulbourethral glands (or Cowper's glands) (Badia 2003). They are all exocrine glands that release their secretion into the lumen of the urethra and their secretory activity is androgen-dependent.

In swine, 55–75 % of the ejaculate volume comes from the secretions of the prostate and urethral glands, 10–25 % from the secretions of the Cowper's glands, and 15–20 % from the seminal vesicles. Only 2–5 % of the ejaculate volume comes from the caudal region of the epididymis, consisting of sperm and epididymal/testicular fluid (Dyce et al. 1999; Badia 2003; Badia et al. 2005, 2006).

The seminal plasma presents a pH of around 7.3–7.9 and a water content of 94–98 %. Accessory sex glands synthesize and secrete several organic and inorganic substances, among which proteins are particularly important.

3.4.2 Seminal Vesicles

The seminal vesicles, also known as seminal or vesicular glands, are a pair of glands that are pinkish in color and spongy in appearance, located on both sides of the urinary bladder. In swine, they are highly developed, extending over the pelvic and abdominal cavities [see Badia (2003) for review]. Vesicular glands can weigh 100–130 g and have a length and width of 11–15 and 8–9 cm, respectively (Badia et al. 2006b). These vesicles secrete their content, via an excretory duct, into the urethra, close to where the deferent ducts coming from epididymal cauda also lead (Badia 2003).

Histologically, these vesicles are tubular glands limited by a peritoneal covering (or *tunica serosa*) and a connective capsule, and formed by glandular parenchyma and stroma of a connective nature. The capsule is very thin and composed of two layers. The outermost layer, 60–90 μm thick, is formed by a dense connective tissue rich in collagen fibers. The innermost layer, 80–150 μm thick, consists of a loose connective tissue rich in fibroblasts, smooth muscle fibers, mast cells, collagen fibers, elastic fibers, and reticular fibers. This layer is highly irrigated and innervated and is the origin of the radial septa that divide up the inside of the gland, thus forming the glandular lobes.

The glandular parenchyma consists of secretory tubules surrounded by a simple epithelium mainly composed of columnar secretory cells and, to a lesser extent, of basal and mast cells (Badia et al. 2006b), which are recognizable by the presence of metachromatic granules.

Principal cells are extended upwards from the basal lamina to the free surface of the epithelium and their nucleus occupies a mid-basal position. Their cytoplasm is slightly basophilic and the most apical region is characterized by the presence of periodic acid-schiff staining (PAS) positive vesicular or digitiform blebs (Badia et al. 2006b) (Fig. 3.12).

Lectin histochemical studies show that columnar cells synthesize and secrete N- and O- glycosylated glycoproteins.

Basal cells are scarce pyramidal or flattened cells disposed between columnar cells. Their cytoplasm is scant and no secretory granules are present (Badia 2003; Badia et al. 2006b).

The accessory sex glands provide seminal plasma with most of its ion content, energy substrates, and proteins. Although these products are not essential to ensure the breeding pig male fertility they exert an important nutritional and antioxidant function (Strzezek al. 2000; Badia 2003). Regarding the ion content secreted by the accessory sex glands, sodium, potassium, calcium, magnesium, zinc, manganese, and iron ions can be highlighted (Shivaji et al. 1990; Aumüller and Riva 1992; Gatti et al. 1993; Lavon and Boursnell 1971; Dubei et al. 1980; Saiz-Cidoncha et al. 1991; Strzezek et al. 1995). Sperm motility depends on sodium and potassium levels, sperm capacitation depends on calcium and magnesium levels, and the structure of several proteins in seminal plasma depends on the levels of zinc, manganese, and iron (Saiz-Cidoncha et al. 1991; Badia 2003).

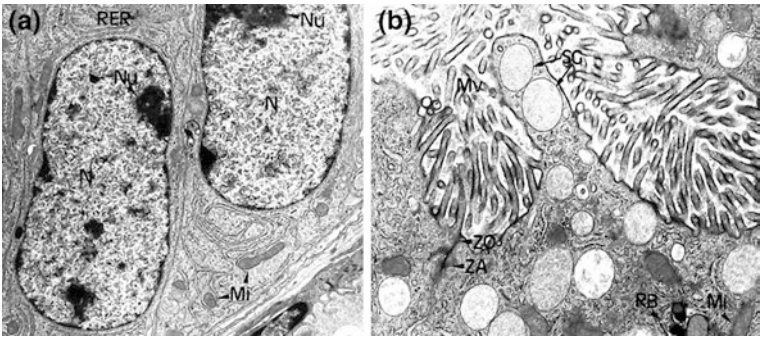

Fig. 3.12 Columnar cells. Transmission electron microscopy. **a** Sub-basal cytoplasm. **b** Sub-apical cytoplasm. *N* nucleus; *Nu* nucleolus; *M* mitochondria; *RER* rough endoplasmic reticulum; *RB* residual body; *SG* secretory granule; *MV* microvilli; *Zo* zonula occludens; *Za* zonula adherens

The energy substrates secreted by the seminal vesicles and used by boar sperm are: sorbitol, glycerophosphocholine and fructose (Setchell et al. 1994; Jones and Bubb 2000; Sancho 2002; Badia 2003).

Proteins secreted by the seminal vesicles represent 80–90 % of total seminal plasma protein content (Lavon and Boursnell 1971). One of the most important class of proteins is the spermadhesins, which are glycoproteins weighing about 20 kDa (Dostalova et al. 1994; Töpfer-Petersen et al. 1995; Calvete et al. 1996) (PSP-I, PSP-II, AWN, AQN, etc.). Distinct functions have been attributed to them, such as promoting sperm-oocyte interaction and sperm capacitation (Dostalova et al. 1994; Töpfer-Petersen and Calvete 1995), or protecting sperm from head-to-head agglutination (Harayama et al. 1994, 1999, 2000) and from peroxidation (Kordan et al. 1998).

Another interesting protein secreted by seminal vesicles is the acid phosphatase enzyme, which is involved in sperm metabolism and maintains its plasmalemma integrity (Wysocki and Strzezek 2000, 2003). Its activity depends on the interaction with ergothioneine, glutathione, and ascorbic acid. These nonenzymatic substances, also secreted by the seminal vesicles, show an additional antioxidant function by protecting the membrane phospholipids (Strzezek et al. 1999, 2000; Wysocki and Strzezek 2000; Badia 2003).

3.4.3 Prostate

The prostate is a single gland divided in two parts: the prostatic body and the disseminate prostate (Luke and Coffey 1994; Setchell et al. 1994). The prostatic body is located below the urinary bladder while the disseminate prostate, which is more developed, is extended along the pelvic urethra (Setchell et al. 1994; Dyce et al. 1999; Badia 2003).

The prostatic body presents a whitish, spongy appearance, is irregularly shaped (5–6 cm long and 0.7–0.8 cm thick) and weighs approximately 10 g. Its rough surface unveils a highly developed vascular network (Badia 2003).

The prostatic body is crossed by numerous excretory ducts that collect the prostatic secretion and culminate in the pelvic urethra, very close to where the excretory ducts of the vesicular glands also lead (Badia 2003).

The prostate is a tubular gland limited by a fibrous capsule from which major septa depart toward its interior, thus dividing the gland into glandular lobes. Furthermore, major septa divide into smaller ones that cross the glandular lobes, thereby constituting the so-called prostatic lobules. The capsule and major and minor septa are composed of highly innervated and vascularized dense fibrous tissue, rich in collagen fibers, elastic fibers, and smooth muscle cells (Badia 2003).

The glandular parenchyma consists of secretory tubules formed by a simple epithelium of columnar cells and scarce basal cells. Columnar cells are acidophilic, containing neutral glycoproteins N or O glycosylated. Their apical free surface exhibits vesicular cytoplasmic blebs of about 6 μm in diameter. The cytoplasm contains several osmiophilic secretory granules of around 0.8 μm in diameter, and a highly developed rough endoplasmic reticulum (Badia 2003).

The interstitial tissue is underdeveloped and vascularized, and contains fibroblasts, abundant smooth muscle cells, and perivascular mast cells (Badia 2003).

The disseminate prostate is located along the submucosa of the caudal region of the pelvic urethra, where its secretion leads through small excretory ducts. It is a compact glandular mass with a spongy, whitish appearance, and limited by a fibrous capsule, of a connective nature, from which septa come out to divide the glandular parenchyma (Badia 2003). Its histological nature is very similar to that of the prostatic body.

The secretory tubules are composed of three cell types: columnar cells, mucosal cells, and basal cells.

Columnar cells (Fig. 3.13) exhibit a slightly acidophilic and PAS negative cytoplasm, but in some cells a PAS positive reaction can be observed in the most apical cytoplasmic portion. The secretory granule content is not as elevated as in its prostatic body counterparts. Two types of columnar cells can be distinguished in the secretory tubules under an electron microscope: type I and type II. Type II columnar cells are very similar to columnar cells belonging to the prostatic body. Their secretion mechanism is apocrine and regulated by hormones. Type I columnar cells are the most abundant and their mechanism of secretion is merocrine. Their free surface is filled with microvilli, and their cytoplasm with a large number of mitochondria and electrodense membranous bodies. These cells secrete mucin, rich in highly hydrated O-glycoproteins resistant to proteolysis, to the lumen of the pelvic urethra. This mucin constitutes a viscous gel that lubricates and protects the urethral epithelium against pathogenic organisms (Jentoft 1990; Devine and McKenzie 1992; Strous and Dekker 1992; Lamblin and Roussel 1993; Logow et al. 1999; Badia 2003). Their secretion is constitutive and not regulated by hormones.

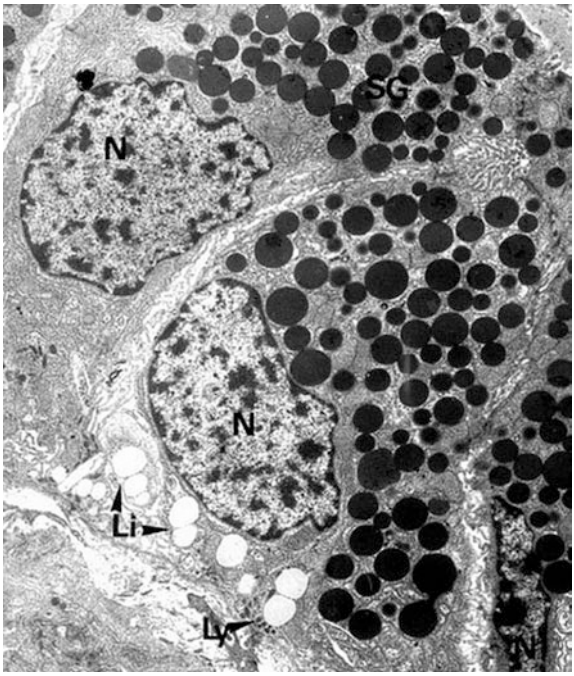

Fig. 3.13 Columnar cells of the prostatic body. Transmission electron microscopy. *SG* secretory granules; *N* nucleus; *Li* lipid droplets; *Ly* lysosomes

Mucosal cells are very scarce and scattered among columnar cells. These cells are distinguished by their content in secretory granules rich in neutral and carboxylated acid mucosubstances (Badia 2003).

Basal cells, as is characteristic of secretory epithelia, are pyramidal cells. They are located on the basal lamina of the epithelium, but they do not reach the lumen of the secretory tubule (Badia 2003). Multiple functions are attributed to them, such as being the stem cells of columnar cells, phagocytic cells or even cells that regulate the epithelial secretion.

The prostate also synthesizes immunomodulatory spermadhesins, such as PSI and PSII, which prevent the uterus from an immune response and protect sperm from agglutination, and acid phosphatases that provide stability to the plasma membrane and are involved in cellular metabolism by promoting sperm motility (Ekhlasi-Hundrieser et al. 2002; Manaskova et al. 2002; Garcia et al. 2006, 2008; Manaskova and Jonakova 2008).

3.4.4 Cowper's Glands

Cowper's glands, also called bulbourethral glands, are two compact glands, pinkish in color, located dorsoventrally to the pelvic urethra. Each gland weighs about 90–110 g, has a cylindrical shape 12–15 cm long and a diameter that increases from 3 to 4 cm at the apical region to 4–6 cm at the caudal region. They produce

a whitish, highly viscous secretion that is discharged, through an excretory duct, into the urethral region near the beginning of the penile urethra (Badia 2003; Badia et al. 2006).

The bulbourethral gland is a tubular gland covered by a fibrous capsule from which a few septa depart radially, thus sectioning the glandular parenchyma (Setchell et al. 1994). Its outer surface is coated, dorsolaterally, by striated musculature (Badia 2003).

Secretory tubules are limited by a simple epithelium of pyramidal cells rich in secretory granules containing neutral and carboxylated acid mucosubstances and O-glycosilated sulphated acid mucosubstances (Badia et al. 2005; Badia et al. 2006). They are mostly sialoproteins (with a content in sialic acid of over 25 %) mainly released at the end of the ejaculation and organized to form a gel plug that prevents backflow of semen (Boursnell et al. 1970; Boursnell and Butler 1973; Setchell et al. 1994; Dyce et al. 1999; Badia 2003; Badia et al. 2005, 2006).

Secretory ducts of Cowper's glands are covered by a glandular monostratified epithelium of cubic-prismatic cells that have a voluminous nucleus and a cytoplasm rich in mitochondria, a well-developed Golgi apparatus, and rough and smooth endoplasmic reticulum cisternae. The apical region of the cytoplasm is filled with small and highly electrodense secretory granules (Fig. 3.14), which are O-glycoproteins that protect and lubricate the free surface of excretory ducts.

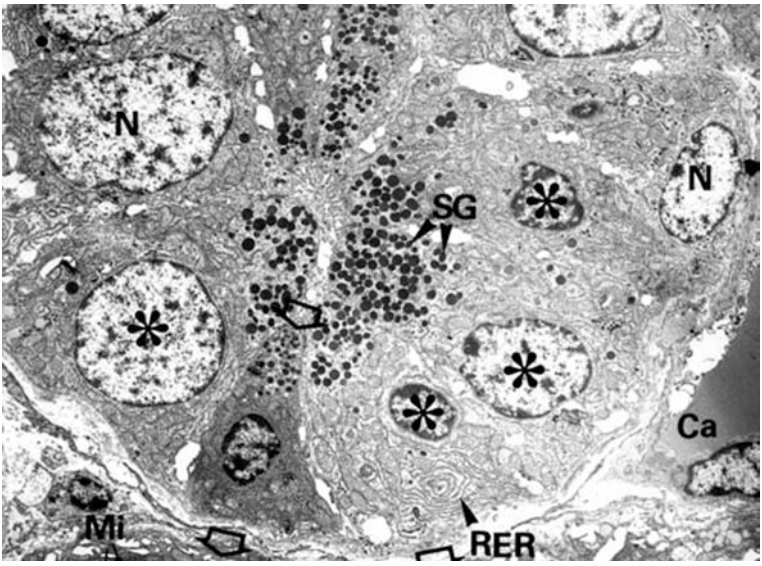

Fig. 3.14 Epithelial cells from an excretory duct of Cowper's glands. Transmission electron microscopy. *Asterisks* indicate nuclei (N). *Mi* mitochondria; *SG* secretory granules; *RER* rough endoplasmic reticulum; *Ca* capillary

3.5 Conclusion

The male reproductive system has three distinct functions. The endocrine function is performed by testicular Leydig cells and Sertoli cells and regulates sperm production. Sperm production (spermiogenesis and spermatogenesis) takes place in the seminiferous tubules of the testes. Sperm maturation (the acquisition of progressive sperm motility and fertilizing aptitude) takes place in the epididymis. In its most caudal region, matured sperm are stored until ejaculation. At ejaculation, sperm from the caudal region of the epididymis, is mixed with secretions from the accessory sex glands (seminal vesicles, prostate, and Cowper's glands). These secretions, chemically complex, interact with the ejaculated sperm, provide energy substrate, are involved in sperm capacitation and act as immunomodulators.

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Chapter 4
Factors Affecting Boar Reproduction, Testis Function, and Sperm Quality

Elisabeth Pinart and Marta Puigmulé

Abstract Sperm quality of boars depends on both intrinsic (genetic) factors and extrinsic (environmental/husbandry) factors. In relation to intrinsic factors, an increased reproductive efficiency of crossbred boars as compared with purebreds manifests the importance of heterosis in this context. Studies on semen traits have demonstrated that some parameters have greater heritability than others, such as semen volume. At the same time there is a poor relationship between seminal parameters and fertility that limits the sensitivity and specificity of cut-off values based on these traits to select boars. Recent studies have pointed out the importance of selecting high-fertility boars according to their testis size at pre-pubertal age. Genetic defects in testicular size and structure, such as in cases of cryptorchidism, result in partial or total arrest of spermatogenesis at post-pubertal age. In relation to extrinsic factors, the ambient temperature, photoperiod, and rhythm of semen collection are negatively correlated with the reproductive performance of boars, whereas food supplementation, social contact with other pigs and the accuracy of semen processing protocols are positively correlated with artificial insemination (AI) outcomes.Certain divergences in the effects of these factors on individuals could be mainly attributed, although not exclusively, to the nature of the breed.

4.1 Introduction

The reproductive performance of boars is usually monitored by analyzing semen quality. In assisted reproduction, threshold values for sperm concentration, sperm motility, and sperm morphology have been established in order to obtain high fertility and prolificacy rates (Bonet et al. 2000; Casas et al. 2009, 2010). Semen quality is not only a proxy measure of boar fertility, but it also has sire effects on pig production in terms or reproductive performance of progeny (Smital 2009; Huang et al. 2010).

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Monitoring and analyzing the quality of boar semen has great economic importance for pig breeders; the economic return of an artificial insemination (AI) center primarily depends on the boar's ability to produce spermatozoa (Robinson and Buhr 2005). Boars consistently producing high-quality ejaculates are crucial in AI programs due to the limited number of insemination doses that can be obtained from one ejaculate (Ciereszko et al. 2000; Estienne and Harper 2004). During the practice of AI the characteristics of collected semen are usually evaluated to ensure its quality before service (Petrunkina et al. 2007). Unfortunately, sperm production of boars can fluctuate up and down as much as 25–30 % throughout the year, forcing AI centers to keep additional boars to compensate for these shifts (Ciereszko et al. 2000; Sancho et al. 2004).

The reproductive efficiency of AI boars can be evaluated by different indicators such as semen quality (Smital 2009; Dyck et al. 2011; Yeste et al. 2010, 2011), fertility (Flowers 2002; Gadea 2005) and sexual behavior (Levis and Reciks 2005; Hemsworth and Tilbrook 2007; Frydrychová et al. 2011). A number of tests have also been developed to assess sperm functionality and in vitro fertility (Popwell and Flowers 2004; Bussalleu et al. 2005; Lovercamp et al. 2007; Foxcroft et al. 2008). Nevertheless, the limited repeatability, technical complexity, and high cost of these tests have impeded their widespread application in field conditions (Dyck et al. 2011).

Semen quality is routinely assessed by determining semen volume, sperm concentration, total number of spermatozoa per ejaculate, sperm viability, sperm motility, and sperm morphology (Pruneda et al. 2005; Casas et al. 2009, 2010). All these traits are only partial semen characteristics and it is impossible to determine semen quality on the basis of only one of them (Smital et al. 2004; Bussalleu et al. 2005). In vivo fertility of boars is currently evaluated from the farrowing rate and the litter size, the latter being expressed as the total number of piglets born or the number of piglets born alive (Gadea 2005). Sexual behavior is assessed by recording the duration of time between the boar entering the collection pen and mounting the dummy, the number of mounts before ejaculation starts, and the duration of ejaculation (Levis and Reicks 2005).

Reproductive performance of boars depends on genetic or intrinsic factors such as breed (Smital 2009; Wolf 2009), age (Huang et al. 2010) and testicular size (Clark et al. 2003; Pinart et al. 1999a), on environmental extrinsic factors like temperature (Ciereszko et al. 2000; Yeste et al. 2010) and photoperiod (Sancho et al. 2004, 2006; Yeste et al. 2010), and on husbandry extrinsic factors such as semen collection rhythm (Pruneda et al. 2005), nutrition (Yeste et al. 2010, 2011) and, social environment (Kunavongkrit et al. 2005).

4.2 Genetic or Intrinsic Factors

Little data exist about the heritability of reproductive traits. Despite the fact that heterosis (also known as hybrid vigor or outbreeding enhancement) might have an important impact on the pig industry (Smital et al. 2004), only few reports exist on the evaluation of crossbreeding effects on reproductive traits of boars and most of them are based on small datasets and/or a small number of males.

Numerous studies involving multiple genetic lines have reported large individual variations in the seminal quality of boars, whereas others have demonstrated that crossbred boars usually produce the highest seminal quality, followed by purebred terminal lines and lastly, purebred maternal lines (Sonderman and Luebbe 2008). The differences between purebreds and crossbreds also include sensitivity to seasonal infertility, ejaculate volume per age interactions, optimum collection frequency, rate of ejaculates discarded due to poor quality, age of puberty, libido, and trainability, and sperm lifespan (Sonderman and Luebbe 2008).

4.2.1 Breed

4.2.1.1 Differences Among Breeds in Reproductive Parameters

Breed Differences in Semen Quality

There are few comparative studies focusing on the seminal characteristics of boar breeds. Among these, five comprehensive trials should be mentioned since they present a good overview of the importance of the breed on seminal features (Park and Yi 2002; Smital et al. 2004; Smital 2009; Wolf 2009; Wolf and Smital 2009). Briefly, many ejaculates from boars of different purebred populations were collected and assessed over a long period of time. Data obtained from these extensive studies indicate that Large White boars appear to have the highest seminal volume, followed by other breeds in this sequential order: Yorkshire, Czech Landrace and Czech Large White, Pietrain, Czech Meat Pig, Prestice Black-Pied Hampshire, and Duroc boars. The coefficient of variation among breeds for semen volume is estimated at 30–40 % (Smital et al. 2004) (Table 4.1).

Table 4.1 Breed ranking for seminal volume

Breed	Seminal volume (ml)	References
Large white	350	Smital et al. (2004)
	270	Wolf (2009); Wolf and Smital (2009)
Yorkshire	280	Park and Yi (2002)
Czech landrace	270	Smital et al. (2004); Wolf (2009)
Czech large white	270	Smital et al. (2004); Wolf (2009)
Pietrain	270	Wolf 2009; Wolf and Smital 2009
	240	Smital et al. (2004)
Czech meat pig	250	Wolf (2009)
	215	Smital et al. (2004)
Prestice black-pied	235	Smital et al. (2004)
Hampshire	220	Smital et al. (2004)
Duroc	200	Wolf 2009; Wolf and Smital (2009)
	160	Park and Yi (2002); Smital et al. (2004)

Smital et al. (2004) reported that the Large White breed also has the highest total number of spermatozoa (120 × 10^9 spermatozoa/ejaculate), whereas in the remaining breeds the total number of spermatozoa ranges between 100 × 10^9 and 80 × 10^9 spermatozoa/ejaculate. Ranking breeds from the highest to the lowest in sperm number resulted in the following sequence: Czech Landrace, Czech Large White, Hampshire, Prestice Black-Pied, Pietrain, Czech Meat Pig, and Duroc. According to these results it was concluded that the coefficient of variation among breeds for total number of spermatozoa is of 30–40 %, the same as for semen volume. In contrast, Wolf and Smital (2009) reported that the Pietrain breed produces the highest total number of spermatozoa (118 × 10^9 spermatozoa/ejaculate) followed by Large White (101 × 10^9 spermatozoa/ejaculate) and Duroc breeds (94 × 10^9 spermatozoa/ejaculate). Such variability in records reflects a differential behavior among individuals and stresses the difficulty of validating single values for a given breed when dealing with reproductive parameters.

Differences also exist among breeds in terms of sperm concentration within the ejaculate, so that higher values have been recorded in Duroc breed and lower values in Large White breed (Wolf 2009; Wolf and Smital 2009). As for seminal volume and total number of spermatozoa per ejaculate, the coefficient of variation among breeds for sperm concentration ranges between 30 and 40 % (Smital et al. 2004) (Table 4.2).

Little data has been published about the differences among breeds in sperm membrane integrity, sperm motility, and sperm morphology. The total number of live (intact membrane) spermatozoa is about 86 × 10^9 spermatozoa/ejaculate in Large White boars, 72 × 10^9 spermatozoa/ejaculate in Czech Landrace and Prestice Black-Pied boars, 71 × 10^9 spermatozoa/ejaculate in Czech Large White boars, 68 × 10^9 spermatozoa/ejaculate in Hampshire boars, 66 × 10^9 spermatozoa/ejaculate in Czech Meat Pig, 64 × 10^9 spermatozoa/ejaculate in Pietrain boars, and 59 × 10^9 spermatozoa/ejaculate in Duroc boars (Smital et al. 2004). The coefficient of variation among breeds in sperm membrane integrity is also of 30–40 % (Smital et al. 2004). Total sperm motility (expressed as percentage of motile spermatozoa) has an average of 74–77 %, with low differences among breeds (Wolf 2009; Wolf and Smital 2009). According to this, Smital et al. (2004)

Table 4.2 Breed ranking for sperm concentration

Breed	Sperm concentration (spermatozoa/mm^3)	References
Duroc	490 × 10^3	Wolf (2009); Wolf and Smital (2009)
Czech meat pig	480 × 10^3	Wolf (2009)
Pietrain	450 × 10^3	Wolf (2009); Wolf and Smital (2009)
Czech large white	424 × 10^3	Wolf (2009)
Czech landrace	417 × 10^3	Wolf (2009)
Large white	400 × 10^3	Wolf (2009); Wolf and Smital (2009)

found a coefficient of variation for sperm motility of less than 10 % among breeds (Smital et al. 2004). On the other hand, divergences exist between studies with regard to sperm morphology. Wolf (2009) and Wolf and Smital (2009) found little differences among breeds in the percentage of aberrant spermatozoa, calculating ranges between 8.5 and 11.5 %, whereas Smital et al. (2004) found that the highest coefficient of variation among breeds for a seminal parameter was manifested in the proportion of aberrant spermatozoa, with a variation of 80 %.

In the study by Smital et al. (2004), Large White boars exceeded by far all other purebreds in all semen parameters analyzed, whereas Duroc boars had the poorest sperm output. Despite these results, most authors agree that no breed excels in all semen characteristics and that breeds differ basically in semen volume and total number of spermatozoa per ejaculate (Kennedy and Wilkins 1984; Ciereszko et al. 2000; Park and Yi 2002; Flowers 2008; Sonderman and Luebbe 2008; Smital 2009; Wolf 2009). In a more recent study, Smital (2009) also concluded that differences among breeds in semen traits correlate with sperm production, but they do not with sperm quality. Considering the total number of spermatozoa, Smital (2009) classified Pietrain, Czech Meat Pig, and Czech Large White as above-average breeds, Landrace, Hampshire, and Large White as average breeds, and Duroc as a below-average breed. In another extensive study examining the sperm production of purebred boars, Kennedy and Wilkins (1984) found that Yorkshire boars routinely produce 10–12 $\times$ 10^9 more spermatozoa than Hampshire, with Landrace and Duroc producing intermediate amounts. It is likely that these differences among studies in the sperm production of boar breeds are related to different environmental, social, and husbandry factors.

Boar breeds also differ in the refrigerability of seminal doses. Sonderman and Luebbe (2008) found that ejaculates from Hampshire boars are very sensitive to dilution and refrigeration at 15–17 °C, resulting in a significant decrease in sperm quality and fertility of refrigerated doses 24 h after semen collection. In contrast, ejaculates from both Yorkshire and Duroc are resistant to refrigeration conditions since their sperm quality and fertility are stable during the first 3 days of refrigeration, whereas Landrace boar ejaculates exhibit average refrigerability (Sonderman and Luebbe 2008). Maternal line breeds are reported to be more sensitive to storage conditions than terminal line breeds (Sonderman and Luebbe 2008).

Discrepancies exist about the freezability of semen doses from different breeds. According to Waterhouse et al. (2006), ejaculates from Duroc boars exhibit better freezability than those of Landrace boars, as confirmed by the higher percentage of spermatozoa with intact plasma membrane and intact acrosome in post-thawed samples from Duroc males. Moreover, a positive correlation exists between the proportion of viable post-thaw spermatozoa and the ratio of polyunsaturated fatty acids (PUFA), which is of 0.64 and 0.67 for Landrace and Duroc boars, respectively (Waterhouse et al. 2006). In contrast with these results, Roca et al. (2006) found that Landrace and Pietrain boars have higher post-thaw sperm motility, membrane integrity, mitochondrial membrane potential, and acrosomal integrity than Large White, Duroc, and Yorkshire boars. Nevertheless, according to this last reference the variance in post-thaw semen quality measurements is not explained

by breed due to the considerable variability among boars within the same breed. Other studies found farrowing rates 20 % greater in post-thawed spermatozoa from Large White boars as compared with Landrace boars, whereas farrowing rates of fresh semen from the same boars were higher for Landrace than for Large White males (Johnson et al. 1981, 1982).

Effect of Heterosis on Semen Quality

Heterosis is manifested in an increased reproductive efficiency of crossbred animals as a result of a faster development in comparison to purebreds, a lower age at puberty, a greater weight at a constant age, a greater testicular weight and a higher seminal quality (Smital et al. 2004; Flowers 2008; Smital 2009). Hybrid boars below 8 months of age have a stronger libido, greater semen volume, higher sperm motility, lower frequency of abnormal spermatozoa and higher pregnancy rates than purebred boars; nevertheless, these differences diminish at the age of maturity (Smital et al. 2004).

For each semen trait, heterotic effects are estimated from the average value of semen characteristics of the hybrid progeny and the mid values of purebred parents (Wolf and Smital 2009). The heterotic effect of crossing on semen traits is calculated from:

$$h_{A\times B} = \text{crossing}_{A\times B} - {}^{1}/_{2}\,(\text{breed}_A + \text{breed}_B)$$

where, $\text{breed}_{A\times B}$ equal the effect for the crossbred combination A × B, and breed_A and breed_B equal the effects of breeds A and B. Heterosis can range from 0 (lack of heterotic effect) to 1 (total heterosis), the results being expressed in absolute terms or in percentage.

Although heterosis for semen traits is high, not all crossings result in increased semen quality (Smital et al. 2004; Smital 2009; Wolf and Smital 2009). The effects of heterosis on semen traits are extensively studied in two studies using large numbers of purebred and crossbred boars over a long time period. In the first study (Smital et al. 2004), a significant heterotic effect on sperm quality was found; manifestation of heterosis was less impressive in the second study (Smital 2009). The differences between both studies could probably have been related again to external ambient factors, husbandry factors, age of boars, and/or semen collection rhythm. Despite these differences, results from both studies indicate that heterosis results in increased semen volume and total number of spermatozoa in nearly all crossings, which usually ranges from 3 to 10 %. A favorable heterotic effect for sperm concentration, sperm membrane integrity, and sperm motility is only described in a few crossings. The heterotic effect on the percentage of abnormal spermatozoa in the Duroc × Large White crossing is manifested only at 5 % (Smital 2009). In the crossings of Duroc × Large White, Duroc × Pietrain, and Large White × Pietrain, the decrease in the frequency of abnormal spermatozoa ranges from 10 to 23 % as compared with their purebreds (Wolf and Smital 2009) (Table 4.3).

Table 4.3 Heterotic effect of different crossings on semen traits

Crossing	Heterotic effect		References
Duroc × Large white	Semen volume	3 %	Smital et al. (2004)
	Total number of spermatozoa/ejaculate	No heterosis	Smital (2009)
	Sperm concentration	4 %	Smital (2009)
		No heterosis	Wolf and Smital (2009)
	Sperm motility	No heterosis	Smital (2009)
Large white × Pietrain	Semen volume	4 %	Smital (2009)
		6–7 %	Wolf and Smital (2009)
	Total number of spermatozoa/ejaculate	3 %	Smital (2009)
	Sperm concentration	No heterosis	Wolf and Smital (2009)
	Sperm motility	No heterosis	Smital (2009)
Czech landrace × Duroc	Semen volume	10 %	Smital et al. (2004)
	Total number of spermatozoa/ejaculate	13 %	Smital et al. (2004)
	Sperm viability	10 %	Smital et al. (2004)
Czech meat pig × Pietrain	Semen volume	17 %	Smital et al. (2004)
		No heterosis	Smital (2009)
	Total number of spermatozoa/ejaculate	10 %	Smital et al. (2004)
	Sperm concentration	17 %	Smital (2009)
	Sperm viability	5 %	Smital et al. (2004)
	Sperm motility	No heterosis	Smital (2009)
Duroc × Hampshire	Semen volume	23 %	Smital et al. (2004)
		12 %	Smital (2009)
	Total number of spermatozoa/ejaculate	8 %	Smital et al. (2004); Smital (2009)
	Sperm viability	No heterosis	Smital et al. (2004)
	Sperm motility	2 %	Smital (2009)
Hampshire × Pietrain	Semen volume	32 %	Smital et al. (2004)
		12 %	Smital (2009)
	Total number of spermatozoa/ejaculate	18 %	Smital et al. (2004)
		6 %	Smital (2009)
	Sperm viability	10 %	Smital et al. (2004
	Sperm motility	2 %	Smital (2009)
Duroc × Pietrain	Semen volume	6–7 %	Wolf and Smital (2009)
		No heterosis	Smital (2009)
	Total number of spermatozoa/ejaculate	No heterosis	Smital et al. (2004); Smital (2009)
	Sperm concentration	No heterosis	Wolf and Smital (2009)
	Sperm viability	No heterosis	Smital et al. (2004)
	Sperm motility	No heterosis	Smital (2009)

Breed Differences in Fertility

Boars, as independent individuals, are a significant source of variation with regards to the success of both in vivo (Long et al. 1999) and in vitro (Flowers 1997) fertilization. However, owing to several technical difficulties only a few studies have attempted to investigate whether there is a genetic component for these differences. The low number of sows that can be inseminated from a single ejaculate makes it difficult to evaluate boar effects independently from those of the sow (Flowers 2008). Moreover, in commercial production, pooling ejaculates from several boars has gained widespread acceptance, especially in the USA, because it has been reported to increase the efficiency of insemination doses (Flowers 2008). Consequently, most commercial boar studs do not routinely collect data that can be used to assess individual male fertility and this practice prevents litter size and farrowing rates being used as descriptors of the fertility of individual males (Flowers 2008).

An extensive study conducted in a 200-boar stud provides some insight into the phenotypic variation of fertility among AI boars (Flowers 2002). Individuals vary in terms of the insemination dose required to reach a plateau, and the number of piglets born alive at the plateau. For some boars, there is a robust response in litter size as the sperm number in the insemination dose increases, whereas for others the change is less pronounced. Moreover, there are no clear differences among breeds in fertility patterns; and the proportion of boars that exhibit asymptotic and linear responses to increases in sperm numbers and the overall number of piglets born alive is similar among breeds (Flowers 2002, 2008). It is widely accepted that crossbred boars are more likely to produce higher litter sizes than purebred boars (Flowers 2008). On the other hand, regardless of the breed or the crossing, some boars used in AI programs are capable of producing exceptional fertility results with low numbers of spermatozoa (Flowers 2008). Therefore, development of prospective fertility tests for semen is of paramount importance for the identification of boars with extreme phenotypes.

The farrowing rate differs considerably among breeds. Sonderman and Luebbe (2008) reported that Meishan boars have the highest farrowing rates (92.2 %) followed by Duroc boars (78.1 %), whereas Yorkshire (73.8 %) and Landrace (71.8 %) boars have the lowest farrowing rates. Some studies have suggested that the differences among breeds, and even among individual boars in fertilizing ability, are related to differences in sperm head dimensions (Peña et al. 2006; Saravia et al. 2007), high-fertility boars producing spermatozoa with smaller and shorter heads than low-fertility boars (Hirai et al. 2001).

Breed Differences in Reproductive Performance

Sexual behavior in boars has not been studied to the same extent as other reproductive traits. In a trial based on the exposition of boars to gilts in estrus, the authors concluded that little differences exist within and among breeds in the

sexual behavior of males, despite crossbred boars exhibiting greater libido than their purebred counterparts (Sonderman and Luebbe 2008). Purebred Duroc boars are very docile and need a more hands-on approach in training (Sonderman and Luebbe 2008); Hampshire influence in crossbred boars makes them more aggressive, whereas boars with Duroc in their pedigrees are more reluctant to mate than crossed boars with Yorkshire (Neely and Robison 1983). These results suggest that the sexual behavior of the boar may have a heritable basis (Hemsworth and Tilbrook 2007).

Differences also exist among genetic lines in their libido and the age at which they reach puberty; maternal lines appear to be approximately 2 months delayed in contrast with terminal lines (Sonderman and Luebbe 2008). Nearly all terminal line boars reach puberty and are receptive to training to mount a dummy sow between 6 and 7 months of age, whereas the age of maternal lines is 8/9 months. However, in maternal lines a correlation exists between size and training ability; therefore, maternal boars of low size seem to lag behind in training until they attain both the minimum age and weight (Sonderman and Luebbe 2008). The trainability of boars also favors terminal lines over maternal lines. The ability to train different terminal lines is nearly the same although differences exist in how to handle them. Maternal lines need more training time, effort, and visual and competitive stimulation in order to get them properly trained; moreover, there is a greater percentage of untrainable boars within maternal lines due to lack of libido (Sonderman and Luebbe 2008).

Nevertheless, AI boars are, on rare occasions, allowed contact with females after they reach sexual maturity (Flowers 2008). Thus, the small genetic differences observed in natural mating might be magnified in AI boars whose sexual behavior is typically evaluated on the basis of their interest in a dummy sow (Flowers 2008). In swine, the receptive female assumes the dominant role in finding a sexual partner and it is reasonable to speculate that females in estrus provide more stimulation than the collection dummy (Flowers 2008). In most commercial studs, the only measure of mating behavior that is routinely monitored is the proportion of boars that could be trained for semen collection (Flowers 2008). Divergences exist among boars in their capacity to mount a dummy. While some boars quickly mount the dummy sow after entering the collection pen, others are very cautious (Levis and Reicks 2005). These divergences in mounting behavior are not dependent on the genetic line (Flowers 2008). Moreover, the phenotypic variation associated with the mounting behavior of terminal boar lines is small, so that the possibility of enhancing these traits through their identification and posterior recruitment appears to be low (Flowers 2008). Improvements in training programs seem to be the best way to enhance the sexual behavior in boars at the present time.

References about the correlation between sexual behavior traits and semen traits are scarce. In crossbred boars, the total number of spermatozoa per ejaculate does not correlate with either time for first mounting ($r = 0.03$) or number of mounts per ejaculation ($r = 0.18$), but it is positively correlated with the duration of ejaculation ($r = 0.38$) (Thiengthan 1992). Other studies have also reported a significant correlation between duration of ejaculation and semen

volume ($r = 0.58$), total number of spermatozoa ($r = 0.58$) and sperm motility ($r = 0.29$), as well as between duration of mounting time with collection frequency ($r = 0.29$), semen volume ($r = 0.29$), sperm concentration ($r = 0.30$), and sperm abnormalities ($r = 0.44$) (Kuciel et al. 1983).

Testosterone is the hormone most closely associated with male sexual behavior, so it is reasonable to expect that boars with high testosterone levels also have high libido (Flowers 2008). In the Yorkshire breed, serum testosterone levels are significantly higher than in the Duroc breed (Park and Yi 2002), whereas no differences have been found among Meishan, Fengjing, Min zhu and Duroc breeds (Borg et al. 1993).

4.2.1.2 Criteria for Boar Selection According to Reproductive Parameters

Genetic Evaluation of Boars

Selection practices for AI boars are similar throughout the global swine industry and are universally based on genetic evaluation for economically important traits. For maternal line boars selection criteria focus on the growth rate or age at a specific weight, fatness, and productivity of their daughters, whereas terminal sire boars are selected for their genetic potential to produce offspring that grow quickly and efficiently, and have commercially desirable meat qualities, with minimal emphasis placed on semen quality and fertility (Robinson and Buhr 2005; Safranski 2008; Wolf 2010).

It is appropriate to review a historical perspective of boar selection criteria. Visual selection was the practice of selecting a boar based on his appearance at a certain point in time; this method resulted in a slow rate of genetic progress for the most economically important traits (Safranski 2008). In the mid twentieth century individual boar performance for growth rate and backfat thickness was measured and used to provide objective comparisons (Safranski 2008). This approach was more effective than pure visual appraisal, but comparisons among boars were difficult unless males were in the same contemporary group; however, the development of standard guidelines for measures and selection improved this system (Miller 2000). In the 1980s, computing power led to the next step in genetic improvement by recording both phenotypic performance and pedigree information, which are used to generate genetic evaluations expressed as estimated breed values (EBV) for a number of economically important traits, expected progeny difference (EPD), and best linear unbiased predictors (BLUP) (Robinson and Buhr 2005; Safranski 2008). Using individual performance data, adjusted for environmental factors, and data from relatives of selection candidates, EBV and EPD allow objective calculation of the genetic merit of individual boars (Safranski 2008). An advantage of this procedure is the fact that it allows calculation of the genetic merit of an animal based on pedigree alone (Safranski 2008).

The use of EBVs can double the accuracy of selection for lowly heritable reproductive traits of sows, such as litter size, and it also improves the accuracy of selection for growth rate and backfat thickness (Schinckel 1991). Moreover, EBVs are combined into two different overall indices to rank individual boars and gilts on their suitability for producing fast efficient growth in their offspring (sire line index) and for producing reasonable and efficient growth along with prolificacy (dam line index) (Robinson and Buhr 2005). These indices are computed by combining EBV with economic values appropriate for the swine industry to rank the animals (Robinson and Buhr 2005). Breeding companies follow the same approach by measuring phenotypes and computing EBV, but frequently they must also compute specialized indexes that reflect the market niche in which their genetics fits (Robinson and Buhr 2005).

By utilizing objective measures and computing power, it also becomes possible to simultaneously select for multiple traits using these selection index approaches. These practices require knowledge of genetic and phenotypic correlations among traits of interest and also lead to estimate the economic value of change for each; these data are used to derive a single numeric value for selection candidates (Safranski 2008). This method is currently in use and will continue to be critical for long-term genetic improvement (Safranski 2008).

In addition to the quantitative trait selection, specific genetic markers are used in selection programs (Dekkers 2003). The marker-assisted selection (MAS) approach requires associating genetic markers with variations in phenotype (Safranski 2008). The RYR1 (PSS) gene and the RN gene are two well-known examples of genetic tests for deleterious alleles affecting the meat quality of pigs carrying one or two copies of the "bad" allele (Robinson and Buhr 2005). Nevertheless, for other genetic markers the relationship between the marker and the phenotype is not consistent in all swine populations (Safranski 2008). One of the challenges of incorporating molecular genetic markers in the selection process is deciding upon the optimal combination of selection based on MAS and conventional selection on EBV. The goal is to seek a balance between emphasizing the marker information and the EBV information (Robinson and Buhr 2005).

The next step beyond MAS is the identification of causative mutations that lead to different phenotypes. The first example in swine genetics was the identification of a mutation that changed a nucleotide in ryanodine receptor, resulting in leaky calcium channels and the condition known as Porcine Stress Syndrome (Fuji et al. 1991). The PCR tests lead to fast identification of pigs with the mutant allele and the eradication of the mutation in commercial circuits (Safranski 2008). Like MAS, a big advantage of this approach is the ability to test the animals at birth, so that it allows an assessment for traits that are only expressed late in life or that are sex-linked. Nevertheless, a major limitation of this approach is that most economically important traits are under the control of several genes (Safranski 2008). Molecular technologies are useful for traits that are measured late in life (reproduction, longevity), traits that are only expressed in one sex (litter size), or traits that are difficult to measure (disease resistance, meat quality) (Safranski 2008).

The EBVs for growth and carcass traits are generally high; however, the litter size has a low heritability (about 10 %) and is not measured on the boar himself (Robinson and Buhr 2005). The EBV of the litter size of a boar has a low accuracy compared with growth traits as it is necessary to accumulate data on the litter size produced by a number of his daughters (Robinson and Buhr 2005). Selection is therefore more effective on growth and fatness traits than for litter size (Robinson and Buhr 2005).

Boar selection has become increasingly important in parallel with AI (Robinson and Buhr 2005; Safranski 2008; Dyck et al. 2011). Nevertheless, and as stated above, this selection is based on genetic evaluation of economically important traits and minimal emphasis is placed on semen quality and fertility (Robinson and Buhr 2005; Safranski 2008; Wolf 2010); this practice results in the culling of valuable selected boars because of poor semen quality (Robinson and Buhr 2005). This fact highlights the importance of including reproductive traits in boar selection, even though the incorporation of additional traits into a selection index will reduce the establishment of other traits (Safranski 2008). There are few reports on genetic correlations among male reproductive traits and growth or carcass traits, which show a low correlation with growth and fatness or even a negative correlation with muscle depth (Oh et al. 2006). This is clearly an area where more data are needed to allow effective inclusion in selection decisions (Safranski 2008).

Heritability of Semen Traits

As for heterosis, values of heritability of any specific trait range from 0 (lack of heritability) to 1 (total heritability) and results can be expressed either in absolute terms or in percentage. Little data exist about the heritability of reproductive traits, despite preliminary results suggesting that selection based upon EBVs is possible (Safranski 2008; Wolf 2010). Semen volume and sperm concentration (Robinson and Buhr 2005), together with the proportion of morphologically normal spermatozoa (Roca et al. 2006), are heritable traits, but estimates of heritabilities and repeatabilities for these parameters vary widely. Large variations in semen traits are probably related to the number of ejaculates and/or boars used; recent data suggest that large numbers of ejaculates (>26,000) and boars (>600) have to be analyzed in order to obtain robust results about the heritabilities of semen traits (Wolf and Smital 2009; Wolf 2010). Moreover, the heritability of sperm quality parameters is difficult to evaluate since they are affected by several extrinsic factors (Robinson and Buhr 2005; Wolf 2009, 2010).

Heritabilities for semen traits are reported to be similar among breeds. Comparative studies show that semen volume has the greatest heritability, followed by sperm concentration and total number of spermatozoa. Moreover, some studies state that the heritability estimates for total sperm cells tend to increase according to boar age (Huisman et al. 2002; Oh et al. 2006). The heritability of sperm motility, sperm viability and sperm morphology is reported to be low (Wolf

Table 4.4 Heritabilities of semen traits in different breeds

Semen trait	Breed	Heritability	References
Semen volume	Pietrain	0.28	Wolf and Smital (2009)
	Large white	0.28	Wolf and Smital (2009)
	Duroc	0.28	Wolf and Smital (2009)
	Czech landrace	0.25–0.24	Wolf 2009, (2010)
	Czech large white	0.20	Wolf (2010)
		0.14	Wolf (2009)
Sperm concentration	Pietrain	0.20	Wolf and Smital (2009)
	Large white	0.20	Wolf and Smital (2009)
	Duroc	0.20	Wolf and Smital (2009)
	Czech landrace	0.20–018	Wolf (2009), (2010)
	Czech large white	0.18	Wolf (2010)
		0.13	Wolf (2009)
Total number of spermatozoa/ejaculate	Pietrain	0.18–0.17	Wolf and Smital (2009)
	Large white	0.18–0.17	Wolf and Smital (2009)
	Duroc	0.18–0.17	Wolf and Smital (2009)
	Czech landrace	0.10	Wolf (2010)
	Czech large white	0.10	Wolf (2010)
Sperm motility	Czech landrace	0.16	Wolf (2009)
		0.10	Wolf (2010)
	Czech large white	0.10	Wolf (2010)
		0.06	Wolf (2009)
	Pietrain	0.05	Wolf and Smital (2009)
	Large white	0.05	Wolf and Smital (2009)
	Duroc	0.05	Wolf and Smital (2009)
Frequency of aberrant spermatozoa	Pietrain	0.16	Wolf and Smital (2009)
	Large white	0.16	Wolf and Smital (2009)
	Duroc	0.16	Wolf and Smital (2009)
	Czech landrace	0.12	Wolf (2009)
		0.10	Wolf (2010)
	Czech large white	0.10	Wolf (2010)
		0.04	Wolf (2009)

2009, 2010). This low heritability may explain the treatment of these traits as variable parameters in several studies (Table 4.4).

In boars, a negative genetic correlation exists between semen volume and sperm concentration of about −0.68 and −0.69 (Smital et al. 2005; Wolf 2009); in cattle (Basso et al. 2005) and sheep (David et al. 2006) a negative relationship but of lower magnitude has also been reported. Both sperm motility and proportion of aberrant spermatozoa are considered genetically independent from both semen volume and sperm concentration (Smital et al. 2005; Wolf 2009). In contrast, a high negative correlation exists between sperm motility and frequency of aberrant spermatozoa (Wolf 2009); this fact agrees with previous studies that noticed significant differences in sperm movement in relation to head shape (Thurston et al. 2001), and negative correlations between head width and flagellum length (Kondracki et al. 2005). Sperm length is positively correlated with sperm velocity

(Gomedio and Roldan 1991), and negatively correlated with total number of spermatozoa per ejaculate (Wysokinska et al. 2009).

Taken together, the results obtained by different researchers show that heritability values for semen traits, especially those for semen volume, sperm concentration and sperm morphology, allow their effective selection using genetic evaluations. Nevertheless, the negative genetic correlation between semen volume and sperm concentration is unfavorable for selection for total number of sperm (Smital et al. 2005; Oh et al. 2006; Wolf and Smital 2009; Wolf 2009, 2010). On the other hand, the negative correlation between sperm motility and the percentage of aberrant spermatozoa is favorable for selection to improve male reproduction (Smital et al. 2005; Wolf and Smital 2009; Wolf 2009, 2010).

Despite the fact that several investigations have been focused on detecting reproductive boars producing high quality ejaculates (Lovercamp et al. 2007), others studies have demonstrated the low predictive value of seminal parameters in terms of fertility outcomes (farrowing rate and litter size) (Gadea et al. 2004; Popwell and Flowers 2004; Gadea 2005; Sancho et al. 2006). In commercial AI, threshold values for seminal parameters have been established for boar selection in order to ensure high fertility rates (Briz et al. 1996; Bonet et al. 2000). However, the poor relationship between seminal parameters and fertility limits the sensitivity and specificity of such a cut-off (Gadea 2005).

Negative correlations have been reported between semen volume and number of piglets born (Gadea et al. 2004; Smital et al. 2005), and between total number of spermatozoa and litter size (Gadea et al. 2004; Smital et al. 2005; Wolf 2010). Correlations for the litter size are near zero in relation with sperm concentration (Gadea et al. 2004; Wolf 2010), and either positive (from 0.12 to 0.35) (Gadea et al. 2004; Smital et al. 2005), near zero or negative (Wolf 2010) with sperm motility. In contrast, sperm motility has a low positive but significant correlation with the farrowing rate ($r = 0.05$) (Gadea et al. 2004). Contradictory results regarding the relation between sperm motility and litter size may be caused by differences in the experimental procedures to measure sperm motility. Sperm morphology has usually been related to fertility; in standard semen analysis this parameter provides information on the status of spermatogenesis and it can facilitate the selection of boars for AI programs (Gadea 2005). An inverse relationship exists between the percentage of sperm abnormalities and fertility; Xu et al. (1998) reported that sperm morphology explains 59 % of variation in litter size in a commercial setting, whereas Hirai et al. (2001) established a significant relationship between sperm head dimensions and fertility. Lack of correlation between sperm viability and fertility is supported by several studies (Gadea et al. 2004; Roca et al. 2006; Yeste et al. 2010). Sperm viability is currently measured by assessing the integrity of the sperm plasma membrane, which is not related to fertility (Gadea et al. 2004). Intactness of the sperm plasma membrane is a prerequisite for correct sperm metabolism and function (Harrison 1997); therefore, functional assays on sperm plasma membrane have to be performed in order to correlate sperm viability with fertility.

In conclusion, sperm traits are poor predictors of male fertility outcome in AI (Gadea 2005); they are good indicators only in cases of altered testicular and/or

epididymal function, which is manifested in low quality ejaculates and poor fertility rates (Xu et al. 1998; Rodríguez-Martínez 2003; Gadea 2005; Pruneda et al. 2005). In contrast, this is not an accurate way to distinguish samples with excellent fertility from those with medium fertility (Gadea 2005). This could be related to the highly limited variation in these parameters in mature fertile boars (Xu et al. 1998) or to the fact that these tests do not properly evaluate the functionality of spermatozoa (Bussalleu et al. 2005). According to Flowers and Turner (1997) the common estimates of semen quality are good qualitative, but poor quantitative, indicators. To solve this problem, new procedures have been developed which include multiple functional tests (Bussalleu et al. 2005) or in vitro fertilization tests (Selles et al. 2003), but they are expensive and time-consuming, and cannot be applied under commercial field conditions.

Selection of Artificial Insemination (AI) Boars for Testis Size

According to Flowers (2008) the best way to improve semen quality of AI boars is to select them according to testis size. In boars the existence of a gene or group of genes on the X chromosome that affect testicular size has been confirmed (Rohrer et al. 2000; Ford et al. 2001).

Testis size correlates not only with daily sperm production but also with testosterone levels (Ford et al. 2001; Hemsworth and Tilbrook 2007; Flowers 2008). Therefore, boars with a large testicular size show high sperm concentration and testosterone levels, as well as high libido (Flowers 2008) and large epididymis (Walker et al. 2004). Greater epididymal weight may result in a larger capacity for sperm storage, which can improve overall fertility (Walker et al. 2004). A threshold concentration of testosterone is required to elicit sexual behavior; above this threshold, testosterone concentration is ineffectual at increasing copulatory behavior (Hemsworth and Tilbrook 2007). In addition to maintaining sexual behavior, androgens stimulate the latter stages of spermatogenesis and prolong the lifespan of epididymal spermatozoa (Hemsworth and Tilbrook 2007).

Testis size also affects the age of the onset of puberty; therefore, young boars with large testicular size begin puberty at an early age and also reach sexual maturity 2–3 months earlier, according to the breed; besides, these boars exhibit a higher daily sperm production (Johnson et al. 1994; Harder et al. 1995; Rathje et al. 1995; Huang and Johnson 1996), superior mating efficiency (Schinckel et al. 1983), and a longer reproductive longevity (Flowers 2008). Moreover, a significant positive response occurs in ovulation rate from selection for testis weight not only in swine (Schinckel et al. 1983, 1984), but also in sheep (Hanrahan and Quirke 1977) and mice (Islam et al. 1976). In swine, the genetic correlation between testis size and ovulation rate varies from 0.10 (Johnson et al. 1994) to 0.65 (Schinckel et al. 1983), whereas the genetic correlations between testis size and both female traits (Schinckel et al. 1983) and age of gilt puberty (Johnson et al. 1994) are negligible. Nevertheless, these findings suggest that it might be

possible to base genetic selection for increased fertility in the female on the reproductive characteristics of the male (Johnson et al. 1994).

Recent reports also highlight the relevance of male selection for increased testosterone levels as it results in increased estrogen levels of daughters (Walker et al. 2004). The heritability of testosterone production is reported to be similar to other male reproductive traits (Lubritz et al. 1991). However, at the present time, there are technical limitations associated with collecting and using testosterone concentrations in selection programs (Flowers 2008). As boars selected for increased testis size also have elevated testosterone concentrations, selection for testis size might be the best way to enhance sexual behavior in boars (Flowers 2008). Consequently, there is an economic value for AI studs in producing boars with larger testes (Ford and Wise 2011).

Weight of testes at a constant age may be a useful indicator trait to select for increasing reproductive efficiency of boars (Harder et al. 1995; Rathje et al. 1995). Determination of testicular diameter from three to 5 months of age would provide a method to rank boars of a specific genetic line (Ford and Wise 2011). Little data exist about the heritability of testis size, nor other characteristics related with boar reproductive capacity; estimated heritability ranges from 0.6 to 0.3 (Schinckel et al. 1983; Young et al. 1986; Johnson et al. 1994). Flowers (2008) stated that the heritability of testis size could be similar to other corporal traits. Estimates of genetic correlations between testis size and body weight range between 0.4 and 0.5, between testis size and backfat thickness are of 0.25, and between testicular size and age of puberty of 0.16 (Johnson et al. 1994). Daily sperm production of adult boars is highly correlated with testicular weight (Ford et al. 2001); correlations between 0.50 and 0.65 (Schinckel et al. 1983; Young et al. 1986) and even 0.90 (Rathje et al. 1995) have been reported between testis size and daily sperm production.

Boars selected for large testis size produce 6×10^9 more spermatozoa per ejaculate than control boars (Huang and Jonhson 1996); this represents almost a 10 % increase in sperm production. Daily sperm production shifts more rapidly and reaches its plateau at younger ages in the lines selected for testis size as compared with controls (Rathje et al. 1995; Ford and Wise 2011). Replacement rates in terminal sire lines are high, so that swine production companies can take advantage of superior genetics. Consequently, boars that can produce large quantities of semen at young ages and maintain them over their productive life are of premium value (Flowers 2008). Selection for testis size appears to be a valid approach for enhancing spermatogenesis and can be applied to any type of genetics (Flowers 2008; Ford and Wise 2011).

Very little is known about the impact of the boar on the farrowing rate (Robinson and Buhr 2005). In bulls, increasing pregnancy rates after AI were reported positive for a fertility-associated antigen (FAA) in semen samples, a 31 kDa protein present on the sperm surface (Sprott et al. 2000). In contrast, recent data have demonstrated that the identification of FAA-positive and FAA-negative status is not a successful procedure to select AI bulls (Dalton et al. 2012). In mice the activin receptor gene II and estrogen receptor beta (ESR) have been directly related to fertility. Knock-out males lacking the activin receptor gene

are fertile, although they reach puberty later and with smaller testes than healthy males; in contrast, knock-out females are completely infertile (Matzuk and Lamb 2002). Male mice lacking ESR are phenotypically normal and completely fertile, although their prostates do enlarge in later life, whereas females lacking this receptor are subfertile (Krege et al. 1998). These results indicate that an apparently normal fertile male can transmit a genetic cause of subfertility to his female offspring (Robinson and Buhr 2005). All these studies provide great evidence of male genetic influence on pregnancy rate, which warrants further investigations on pigs.

In contrast, evidence exists in pigs for genetic impact on litter size (Robinson and Buhr 2005). Certainly, sows can be selected for improved litter size (Bolet et al. 2001), but males also contribute (Robinson and Buhr 2005). Reciprocal translocation occurs when different chromosomes exchange pieces, and subsequent segregation during meiosis produces gametes that can be balanced or unbalanced with respect to the chromosomes carrying the translocations; the unbalanced gametes carrying a chromatid that is either too long or too short (Robinson and Buhr 2005). If such a spermatozoon penetrates an egg, the unbalanced spermatid cannot pair properly with the female partner chromatid, resulting in early embryonic death and therefore smaller litter size (Robinson and Buhr 2005). A number of such translocations has been identified in boars; in Finnish York boars these translocations are reported to produce a reduction of two pigs per litter as compared with the breed average (Makinen et al. 1999). Furthermore, although the offspring that receives the unbalanced chromosome dies, half of the living offspring carries the balanced translocation; thus, a spontaneous translocation can be perpetuated in subsequent generations (Robinson and Buhr 2005). AI stations have been strongly recommended to perform cytogenetic assays to detect and exclude boars with genetic abnormalities (Makinen et al. 1999; Robinson and Buhr 2005).

Recent studies also highlight the importance of boar selection for semen freezability (Safranski 2008; Casas et al. 2010). Although it has been demonstrated that genetics is responsible for freezability in boars (Thurston et al. 2002a, b; Roca et al. 2006) no data exist about the heritability of this semen trait. Whereas ejaculates collected from the same boar tend to maintain freezability (Roca et al. 2006), heritability could explain the large differences among breeds and among boars in this parameter (Waterhouse et al. 2006; Safranski et al. 2011). In the Pietrain breed, 75–79 % of freezability features of ejaculates can be predicted by using different linear combinations of sperm quality parameters in the first steps of the cryopreservation process (17º and 5º C) (Casas et al. 2009). This range of percentages emphasizes the weight of individual boar genetics on sperm freezability (Casas et al. 2009).

Today, boar selection criteria for sperm freezability are only based on pre-freeze and post-thaw sperm quality (Casas et al. 2009, 2010). Nevertheless, it seems unlikely that conventional measures of sperm quality result in accurate prediction of freezability, and therefore the identification of genetic markers for freezability might be the most efficient approach (Safranski 2008). In this sense, Thurston et al. (2002a) have identified 16 candidate gene markers presumably linked to genes associated with sperm freezability. This again suggests the potential of genetic improvement in sperm chilling resistance (Safranski 2008).

In conclusion, identifying and enhancing the genetics of sperm production and quality will benefit the reproductive performance of AI boars (Safranski 2008).

4.2.2 Cryptorchidism and Testicular Activity

4.2.2.1 Testicular Activity

Testicular activity is frequently assessed by measuring the daily sperm production per gram of testis or the total number of spermatozoa per ejaculate, both parameters being correlated with the age of boars (Kondracki et al. 2005; Huang et al. 2010), testicular weight (Ford et al. 2001; Lunstra et al. 2003; Almeida et al. 2006; Ford and Wise 2011) and number of Sertoli cells (Lunstra et al. 2003; Caires et al. 2008; Ford and Wise 2009). The ratio spermatid-to-Sertoli cell is a measure of the Sertoli cell efficiency based on the assumption that relative changes in daily sperm production would alter the number of spermatids supported by a stable Sertoli cell population (Okwun et al. 1996). Despite its accuracy such a ratio is not routinely used due to technical limitations in field conditions, so available data are scarce. Another ratio, the germ cell-to-Sertoli cell ratio, is reported to be significantly higher in the Meishan breed than in Whitecross and West African breeds (Okwn et al. 1996); these results indicate that Meishan boars are more efficient in supporting spermatogenesis than Whitecross and West African boars, due both to a lower degeneration index of germ cells and to the accommodation of more germ cells by an equal population size of Sertoli cells (Okwun et al. 1996).

Despite significant differences in total number of spermatozoa per ejaculate and testosterone levels, daily sperm production per gram of testis in adult boars is fairly constant within a breed and also among breeds, being approximately 21.7×10^6 in West African (Okwun et al. 1996), 23.6×10^6 in Yorkshire (Swierstra 1970), 24.1×10^6 in Lacombe (Swierstra 1970), 24.5×10^6 in Meishan (Okwun et al. 1996), 24.8×10^6 in Whitecross (Okwun et al. 1996), 25.9×10^6 in Landrace (Egbunike et al. 1975), and 27.3×10^6 in Piau breeds (França 1992). This indicates that although spermatogenesis requires testosterone, there may be a threshold effect whereby further increases do not result in higher sperm production once a certain level of testosterone is achieved (Walker et al. 2004). This could explain the low correlation ($r = 0.12$) between sperm production and testosterone levels in the ejaculate of boars (Peter et al. 1980). As the duration of spermatogenesis (Almeida et al. 2006) and the rate of germ cell apoptosis during both spermatogonia and spermatocyte stages (De Rooij and Rusell 2000) do not differ substantially among breeds and genetic lines, variations among boars in daily sperm production are related with testicular size, as well as the number of A spermatogonia and Sertoli cells per testis (Orth et al. 1988; Huang and Jonhson 1996; Okwun et al. 1996; Lunstra et al. 2003).

The number of A spermatogonia per testis is highly positively correlated with the number of Sertoli cells per testis ($r = 0.95$) and with the parenchymal mass

($r = 0.88$), and it also accounts for 77 % of variability in daily sperm production among breeds (Okwun et al. 1996). The number of A spermatogonia which initiate the spermatogenic cycle depend on: (1) the number of stem cells per testis, (2) the process of stem cell renewal, (3) the number of cell divisions from stem cells to primary spermatocytes, and (4) the degeneration index of spermatogonial subtypes (Okwun et al. 1996). Given that (2), (3) and (4) are similar among breeds, differences in daily sperm production are partly related with the number of A spermatogonia (Okwun et al. 1996).

It has been suggested that Sertoli cells establish a ceiling in the rate of sperm production (Johnson 1986; Berndtson et al. 1987). Sertoli cells can place this limit in spermatogenic potential by controlling testis size (Kluin et al. 1984), modulating type A spermatogonial populations (Hochereau-de Reviers 1981; Johnson 1986), and/or limiting the number of germ cells supported (Almeida et al. 2006). In boars the number of Sertoli cells correlates with parenchymal mass ($r = 0.87$), number of A spermatogonia ($r = 0.95$) and daily sperm production ($r = 0.87$) (Okwun et al. 1996). Therefore, the number of Sertoli cells is responsible for 76 % of variability in daily sperm production among breeds. Breed differences in the total number of Sertoli cells and A spermatogonia per testis are manifested in divergences in sperm production (Okwun et al. 1996; Almeida et al. 2006).

Although the testicular activity of boars is measured after the onset of puberty, the daily sperm production is dependent on changes occurring during the prepubertal period (Caires et al. 2008).

In boars a significant increase in both testis weight and Sertoli cell number occurs during the first 3–4 weeks of neonatal life (McCoard et al. 2003; Caires et al. 2008; Huang et al. 2011). Throughout this period, Sertoli cells maintain an immature appearance and secrete high levels of anti-Mullerian hormone (AMH) (Rey et al. 2003; Caires et al. 2008); the germ cell population consists of mitotically quiescent prespermatogonia, located centrally in the seminiferous tubules, among immature Sertoli cells (Caires et al. 2008). Prespermatogonia start to migrate towards the basal lamina after 14 days of age and begin to express progesterone receptors (Kohler et al. 2007; Caires et al. 2008); the expression of progesterone receptors is a key process for further maturation of prespermatogonia into spermatogonia and later mitosis (Kohler et al. 2007). In 60-day-old boars, the seminiferous tubules are constituted by immature Sertoli cells and prespermatogonia (Rey et al. 2003); 85 % of prespermatogonia that have reached the basal lamina express progesterone receptors, whereas only 18 % of preespermatogonia remaining in the center of the seminiferous epithelium express this receptor (Kohler et al. 2007). The signals inducing the expression of progesterone receptors in the prespermatogonia in the way to or upon reaching the basal lamina remain obscure, but it might result from a paracrine stimulation of Leydig cells mediated by estrogens (Kohler et al. 2007). In sexually mature boars expression of progesterone receptors is maintained in 78 % of A and B spermatogonia, regardless of age and stage of spermatogenesis; this expression disappears when spermatogonia enter in the developmental stage of primary spermatocytes (Kohler et al. 2007). Therefore, progesterone is not just an intermediary in the synthesis of testicular androgens

and estrogens by Leydig cells, but it also acts as a paracrine factor involved in the control of spermatocytogenesis (Kohler et al. 2007).

It is well known that Sertoli cell numbers established before puberty determine adult testis size, spermatogenic activity, and lifetime in boar fertility (Caires et al. 2008). In the porcine testis, maximum Sertoli cell proliferation occurs during the first 2 weeks of life under follicular stimulating hormone (FSH) stimulus, resulting in a four-fold increase in Sertoli cell numbers during this period (McCoard et al. 2001, 2003); nevertheless, porcine Sertoli cells still express markers of proliferating cells around 4 months of age (Klobucar et al. 2003). In vivo studies have suggested the potential to manipulate the establishment of Sertoli cell populations in rodents (Orth et al. 1988), bulls (Madjdic et al. 1998) and boars (Lunstra et al. 2003). In order to obtain a notable impact on boar lifetime fertility, manipulations must be performed during the first 2 weeks of neonatal life (Caires et al. 2008).

In response to testosterone, Sertoli cells cease proliferation and initiate maturation (Buzzard et al. 2003). The maturation of Sertoli cells starts at about 60 and 90 days of age, depending on the breed (Ford and Wise 2009; Huang et al. 2011), and is characterized by the arrest of proliferation (Ahmed et al. 2009), the progressive decrease in AMH expression (Rey et al. 2003; Ford and Wise 2009), the increase in the diameter of the seminiferous tubules (Rey et al. 2003; França et al. 2005; Ford and Wise 2009), the formation of the blood-testis barrier (França et al. 2005; Ford and Wise 2009), the secretion of tubular fluids (Ford and Wise 2009, 2011), and the onset of spermatogenesis in some seminiferous areas (Kohler et al. 2007; Ford and Wise 2009). Increased androgen secretion by early pubertal testes marks the onset of sexual maturity (Ford and Wise 2009). The development of Leydig cells in boar testes follows a different pattern than that of Sertoli cells, and it can be divided into three stages (Huang et al. 2011): the first stage occurs before the first 35 days of gestation; the second stage begins 3 weeks before parturition and continues for approximately 3 weeks postnatally; and the third stage starts at approximately 90 days of age throughout puberty. Intratesticular androgens act as negative regulators of AMH secretion (Rey et al. 2003). In humans, the lack of androgen receptors on Sertoli cells during neonatal development (Chemes et al. 2008) offers a means whereby Sertoli cells avoid the negative influence of androgen on AMH expression (Ford and Wise 2009). Then, at the onset of puberty, acquisition of androgen receptors within Sertoli cells provides a link for suppressing AMH production (Ford and Wise 2009). In neonatal boars, published reports disagree regarding the presence of androgen receptors in Sertoli cells (Ramesh et al. 2007; Caires et al. 2008), leaving unresolved the role of androgens in the regulation of AMH production during early postnatal development.

Differences exist among breeds in the rate of decrease of AMH expression in Sertoli cells; in this sense, Ford and Wise (2009) found that AMH expression increases from seven to 28 days of age in Meishan and crossbred boars; after 28 days AMH declines in both genetic lines but at a more rapid rate in Meishan than in crossbred boars (Ford and Wise 2009). In Meishan boars, AMH expression is nearly absent at 70 days of age, whereas in crossbreds it is absent at 112 days of age (Ford and Wise 2009). These findings correlate with an earlier onset of pubertal

development in Meishan boars as compared with crossbred boars (Kanematsu et al. 2006). Longer expression of AMH in crossbred boars reflects a less mature status of their Sertoli cells, and a prolonged potential to proliferate (Ford and Wise 2009).

In boars, Sertoli cell maturation begins next to the mediastinum and progresses outwardly towards the interior of the testis (Ford and Wise 2009, 2011). This outwardly progressing pattern supports the hypothesis of a paracrine regulation of Sertoli cell maturation within a given tubule (Ford and Wise 2009). Adjacent sections of the same seminiferous tubules have a defined sequence of change, with AMH production decreasing before much expansion of seminiferous tubules, followed by a change in gene expression in Sertoli cells before the formation of a distinct lumen and the onset of spermatogenesis (Ford and Wise 2009).

A progressive transformation of spermatogonia into spermatocytes begins at approximately 100–115 days of age, which is followed by the transformation of spermatocytes into immature spermatids and the differentiation of immature spermatids into mature spermatids with advancing age (Kohler et al. 2007). A low concentration of spermatozoa can be found in the epididymal cauda at 125 days of age (Andersson et al. 1998a, b). The germ cell differentiation is followed by an extremely rapid increase in the number of germ cells together with the organization of the seminiferous epithelium, which acquires a sexually mature appearance at approximately 180 days of age (Andersson et al. 1998a, b). In domestic boars, puberty starts at 150 days of age with the completion of the first spermatogenic wave (Caires et al. 2008; Ford and Wise 2009), and it involves rapid expansion of seminiferous tubules that manifests itself in a marked increase in testicular size (França et al. 2005). Thereafter, the quantity and quality of spermatozoa improve over time (Malgrem et al. 1996) and reach their maximum at approximately 24 months of age (Kennedy and Wilkins 1984).

Testicular weight increases significantly from 3 to 10 months of age, the monthly progression being of 25 g (3 months), 100 g (4 months), 205 g (5 months), 300 g (6 months), 350 g (7 months) and 375 g (10 months) (Ford and Wise 2011). The percentage of testicular parenchyma occupied by seminiferous tubules increases at 3 months (38 %) and reaches its maximum by 5 months of age (68 %); at 10 months of age it decreases slightly to 65 % (Ford and Wise 2011). The mean diameter of seminiferous tubules is also greater at 7 months than at 4 or 5 months of age (Ford and Wise 2011). A high coefficient of correlation exists between testis weight and volume, although it decreases progressively with age, from 0.95 at 4 months to 0.78 at 7 months of age (Ford and Wise 2011). The correlation coefficients of the mean diameter of seminiferous tubules with testicular diameter and weight decrease with advancing age and become non-significant at 6 months for testicular diameter, and at 7 months for testicular weight; the values of both coefficients were 0.71 and 0.63 at 4 months, 0.75 and 0.66 at 5 months, 0.15 and 0.35 at 6 months, and 0.14 and 0.10 at 7 months (Ford and Wise 2011). The coefficient of variation of the mean seminiferous tubule diameter decreases from 21.6 % at 3 months and 20.6 % at 4 months to 11.2 % at 5 months, 10.9 % at 6 months and 6.6 % at 7 months of age, thus indicating less variation in testicular diameter as the boar progresses throughout puberty (Ford and Wise 2011).

Similarly, the coefficient of variation for testicular diameter decreases from 16.1 % at 4 months to 12.6 % at 5 months, 8.1 % at 6 months, and 5.6 % at 7 months of age, thus reflecting the significant correlation between seminiferous tubule diameter and testicular diameter in pubertal boars (Ford and Wise 2011).

The stage of pubertal development is determined by the mean diameter of seminiferous tubules (Ford and Wise 2011). Due to the high correlation between mean diameter of seminiferous tubules and testicular weight and diameter (Ford and Wise 2011), it is possible to obtain an accurate estimation of testicular development of pubertal males by evaluating the testis size (Clark et al. 2003; Rawlings et al. 2008). Ford and Wise (2011) proposed determining the testis size as an estimator of tubular diameter following a two-phase protocol: (1) ranking boars by testicular size at 4 months of age, the period of greatest variation in diameter of seminiferous tubules, and (2) ranking boars by testicular size at 8 months of age. At both evaluations, testicular traits would be adjusted for age and weight of the boar within a defined genetic line.

The progressive maturation of the seminiferous epithelium during the pubertal period leads to the onset of spermatogenesis between around 5 and 8 months of age, with a gradual increase in daily sperm production and also in semen volume until boars reach 18 months of age (Kondracki et al. 2005). In a tropical environment, the progressive maturation of testicular function in Duroc boars occurs from nine to 33 months (Suriyasomboon et al. 2005). In general terms, at 18 months of age, the total number of spermatozoa per ejaculate ranges between 20 and 80 $\times$ 10^9 and the seminal volume between 200 and 400 ml depending on the breed (Park and Yi 2002); this level of production is maintained until a gradual decline starts at the age of 60 months (Park and Yi 2002; Wolf and Smital 2009; Huang et al. 2010). Testosterone levels also increase during puberty, reflecting the high activity of steroid synthesis by Leydig cells (Andersson et al. 1998b) and remain fairly constant at >2 mg/ml during adult life (Tan and Raeside 1980). There are significant breed differences in sperm production and heavy breeds such as Yorkshire and Large White generally tend to produce greater semen volume and total number of spermatozoa over a period, although it is not clear how weight of boars at maturity affects sperm concentration (Park and Yi 2002). The maximum quality of boar semen may last for a certain period but the duration has not yet been established (Huang et al. 2010). In temperate environments the highest total number of spermatozoa per ejaculate has been set at the 32nd month of age (Swierstra 1973), whereas the maximum semen volume, sperm concentration and number of seminal doses is obtained in 24–28-month-old boars (Kennedy and Wilkins 1984). Nonetheless, beyond a certain optimal age semen quality inevitably declines at an unknown rate to a level that is no longer acceptable for service. Little data exist about the longevity of a boar's reproductive performance in terms of acceptable semen quality for AI. According to Huang et al. (2010) it ranges between 60 and 70 months of age.

The decrease in semen quality with boar age is due to a reduction in the number and the impairment of Leydig cell function, and to the narrowing and sclerosing of seminiferous tubules. All these alterations result in decreased spermatogenic activity and increased degeneration of germ cells (Johnson 1986), as well as in increased frequency of spermatozoa with altered chromatin condensation, gene

mutations and aneuploidies (Wyrobek et al. 2006). In Duroc boars, semen volume declines at a slower rate than sperm parameters (Huang et al. 2010), thus indicating that the spermatogenic function is more vulnerable to aging effects than accessory glands and chiefly seminal vesicles, since they produce the bulk of semen volume (Badia et al. 2006). A different declining rate of testes and accessory gland function as a result of aging has also been reported in other mammals (Huang et al. 2010).

4.2.2.2 Types of Cryptorchidism: Unilateral or Bilateral and Abdominal or Inguinal

Cryptorchidism is the most frequent male sexual disorder in mammals, arising from a failure in the descent of one testis towards the scrotum (unilateral cryptorchidism) or of both testes (bilateral cryptorchidism). The alteration of the testicular descent can be either total, the ectopic testes remaining in the abdominal cavity (abdominal cryptorchidism), or partial, with the ectopic testes lodged at different levels of the inguinal canal (inguinal cryptorchidism) (Mieusset et al. 1995, 1997; Pinart et al. 2000). The etiology of cryptorchidism is not clear; it can develop as a result of genetic defects, disrupted endocrine regulation, or anatomical lesions (Demircan et al. 2006; Matuszczak et al. 2011).

Several authors have reported anomalies in the seminiferous tubules, lamina propria and interstitial tissue of ectopic testes that become apparent at pre-puberty (Paninagua et al. 1990; Regadera et al. 1991; Lee 1993; Antich et al. 1995; Sasagawa and Yanagimachi 1997; Matuszczak et al. 2011). In humans, the purpose of early treatment of cryptorchidism is to recover testicular activity but it does not always restore complete function, leading to decreased semen quality at adulthood (Mieusset et al. 1995; Rozanski and Bloom 1995; Foresta et al. 1996; Lee and Coughlin 2001; Kvist et al. 2006; Hutson et al. 2010; Thorup et al. 2010; Marchetti et al. 2012). It has been established that up to 30 and 54 % of patients treated during childhood of unilateral (Thorup et al. 2010) and bilateral (Lee and Coughlin 2001) cryptorchidism, respectively, are subfertile in their adulthood. Subfertile patients with a history of pre-pubertal cryptorchidism usually show hyperthermia on the cryptorchid side, so in these patients treatment of cryptorchidism does not result in normalization of testicular temperature (Mieusset et al. 1995; Hutson et al. 2010). In other cases, treatment does not warrant the maintenance of the testes in a scrotal position; these patients exhibit a retractile testis (Mieusset et al. 1995, 1997) or an ascended testis (Gracia et al. 1997) on the cryptorchid side. A significant relationship exists between spontaneous ascent and high localization of the ectopic testis before treatment (Mieusset et al. 1995, 1997). Therefore, subfertile cryptorchid men treated at pre-puberty show similar anatomopathological alterations to those of untreated cryptorchid men (Gracia et al. 1997).

Cryptorchid testes also show an increased risk of cancer at adulthood; in humans, early successful correction of cryptorchidism does not appear to reduce the incidence of malignant transformation of the testes (Mieusset et al. 1995, 1997; Hutson et al. 2010; Ma et al. 2011). The majority of testicular tumors, usually originated by malignant transformation of germ cells (Ma et al. 2011), occur during adolescence and after puberty (James et al. 2009; Hutson et al. 2010).

Malignant degree risk is proportional to the higher position of the testis; in unilateral cryptorchidism, the scrotal testis also shows increased probabilities of developing cancer (Ma et al. 2011).

Great controversy exists about the severity of testicular lesions in cryptorchid testes, not only in humans but also in other mammals (Pinart et al. 1999a). Similar abnormalities have been reported in the scrotal testes of unilateral cryptorchid males by some authors (Mieusset et al. 1995), but not by others (Nistal et al. 1990). Divergences are due to the different conditions in which the study of cryptorchidism is performed (Pinart et al. 1999a). This pathology has been studied in humans (Mieusset et al. 1995, 1997; Demircan et al. 2006; Matuszczak et al. 2011) and in rodents under experimentally-induced conditions (Antich et al. 1995; Sasagawa and Yanaguimachi 1997). Spontaneous cryptorchidism brings about a hormonal imbalance that is not found in the same way or in the same intensity in experimental cryptorchidism; this makes both types of cryptorchidism not comparable (Heyns and Hutson 1995; McMahon et al. 1995; Pinart et al. 1998).

Most studies focused on the alterations induced by spontaneous cryptorchidism in mammals do not discriminate between inguinal or abdominal, and unilateral or bilateral, although the effects are different (Paniagua et al. 1990; Lee 1993; Mieusset et al. 1995; Pinart et al. 1998, 2000, 2001d). Additionally, in unilateral cryptorchidism, significant differences have been described between right- and left-sided cryptorchidism (Mieusset et al. 1995). Moreover, the effects of this pathology in post-pubertal males are different depending on age; thus, differences exist between young adults and elderly males in the alterations of the ectopic testes (Lee 1993). Likewise, data in humans are not comparable between men with cryptorchidism and men with a history of pre-pubertal cryptorchidism (Lee 1993). Besides, in men with a history of cryptorchidism the abnormalities correlate to the age of the orchidopexy (Lee 1993; Mieusset et al. 1995).

These discrepancies lead us to focus on the study of cryptorchidism using the boar as an animal model (Pinart et al. 1998). The main advantages of using this species are: (1) spontaneous cryptorchidism in boars has an incidence of 6–10 % (McMahon et al. 1995), so there is availability of individuals; (2) cryptorchid conditions both in boars and humans involve similar hormonal disorders and morpho-functional abnormalities of the ectopic testes (Heyns and Hutson 1995; McMahon et al. 1995); (3) parallelism in the time of testicular descent between humans and boars, as testicular descent influences semen quality (Heyns and Hutson 1995; McMahon et al. 1995); (4) the structural analogy between human and boar spermatozoa (Guraya 1987); and (5) the correspondence in the mechanisms of testicular thermoregulation between humans and boars (Dadoune and Demoulin 1993).

An extensive study was carried out using three groups of post-pubertal boars of the same age; one group was composed of three boars with spontaneous unilateral abdominal cryptorchidism on the right side, another group was formed by three boars with spontaneous bilateral abdominal cryptorchidism, and the third group was formed by healthy boars. For each male group we performed an analysis of the alterations in the testicular structure (Pinart et al. 1999a, b, 2001d), ultrastructure (Pinart et al. 2000, 2001a), lectin histochemistry (Pinart et al. 2001b, c,

2002), and of the alterations in semen quality (Bonet et al. 1998; Pinart et al. 1998, 1999c). It resulted in a thorough description of the effects on spermatogenesis and steroidogenesis of unilateral abdominal cryptorchidism on the right side and bilateral abdominal cryptorchidism at post-pubertal age.

4.2.2.3 Structural and Ultrastructural Alterations of Cryptorchid Testes

Macroscopic Characteristics

Despite the fact that few differences exist in size and weight between the right and the left testis in post-pubertal healthy boars, in unilateral boar cryptorchidism on the right side, the left (scrotal) testis is two-fold more voluminous and 3.35 times heavier than the right (abdominal) testis (Pinart et al. 1999a). Moreover, it is 1.29 times longer, 1.47 times wider, and 1.24 times heavier than the left testis of healthy boars; the right testis of unilateral cryptorchid boars is smaller (×0.73 in length and ×0.68 in width) and lighter (×0.38 in weight) than the right testis of healthy boars (Pinart et al. 1999a). In bilateral abdominal cryptorchid boars the left testis is slightly more voluminous (×1.70 in length and ×1.12 in width) and heavier (×1.13 in weight) than the right (abdominal) testis; moreover, the right testis has a degenerative appearance. Left and right testes of bilateral abdominal cryptorchid boars are smaller and lighter than the left (×0.29 in length, ×0.52 in width, and ×0.29 in weight) and right (×0.38 in length, ×0.52 in width, and ×0.26 in weight) testes of healthy boars. Differences also exist between unilateral and bilateral cryptorchidism in testicular appearance (Pinart et al. 1999a). The left testis of the bilateral abdominal cryptorchid boars is significantly smaller (×0.49 in length and ×0.40 in width) and lighter (×0.23 in weight) than the left (scrotal) testis of unilateral cryptorchid boars. The right testis of bilateral cryptorchid boars is also smaller (×0.52 in length and 0.77 in width) and lighter (×0.68 in weight) than the right (abdominal) testis of unilateral cryptorchid boars. These findings are congruent with those found in dogs, in which paired testicular weight is also significantly lower in bilateral than in unilateral cryptorchidism (Pathirana et al. 2011).

Therefore, at post-pubertal age unilateral and bilateral cryptorchidism induces hypotrophia of the abdominal testes, whereas unilateral cryptorchidism results in hypertrophia of the scrotal testes of boars (Pinart et al. 1999a, 2001d). Abnormalities in the volume and weight have also been reported in the abdominal testes of unilateral and bilateral cryptorchid men (Paniagua et al. 1990; Regadera et al. 1991) and in the testes of men with a history of unilateral and bilateral cryptorchidism (Mieusset et al. 1995). Supporting the above-mentioned findings some authors reported that alterations are more severe in the bilateral disease (Mieusset et al. 1995), whereas others do not find differences between unilateral and bilateral cryptorchidism in testicular appearance (Paniagua et al. 1990). Both reduced size of abdominal testes and increased size of scrotal testes are early phenomena that develop during the pre-pubertal period and become progressively more severe with age (Antich et al. 1995; Mieusset et al. 1995; Matuszczak et al. 2011). In the

abdominal testes of unilateral and bilateral cryptorchid boars the low testicular weight results from the defective development of the seminiferous tubules, whereas in the scrotal testis of unilateral cryptorchid boars the increased weight correlates with a compensatory hypertrophy (Pinart et al. 1999a). This latter malformation has not been described in the scrotal testis of unilateral cryptorchid men. Some authors have reported a significant decrease in the volume of the left testis in the case of unilateral cryptorchidism on the right side, whereas unilateral cryptorchidism on the left side is not believed to alter the volume of the right testis (Mieusset et al. 1995).

Testicular Structure and Histochemistry

Left (scrotal) and right (abdominal) testes of unilateral cryptorchid boars are surrounded by the tunica albuginea, which extends from the large fibrous septa to the testicular parenchyma in the right testis. The seminiferous tubules occupy 48 % of the testicular parenchyma in the left testis and the 44 % in the right testis, the amount of interstitial tissue being, respectively, of 52 and 56 % (Pinart et al. 1999a). These differences between both testes in the proportion of seminiferous tubules and interstitial tissue are not significant; nevertheless, in comparison to healthy boars, the left and right testes show a significant reduction in the amount of seminiferous tubules in unilateral cryptorchid boars of 10 and 20 %, respectively (Pinart et al. 1999a).

In the left (scrotal) testis of unilateral cryptorchid boars the tubular diameter (averaging 515 μm) and the height of the seminiferous epithelium are similar to that of healthy boars, whereas the tubular lumen is greater (Pinart et al. 1999a); the lamina propria shows the same structural (Pinart et al. 1999a) and ultrastructural (Pinart et al. 2001c) pattern as in healthy boars. The seminiferous epithelium, despite showing a normal structural appearance (Pinart et al. 1999a), is characterized by a low density of germ cells (Pinart et al. 1999b) and altered sugar composition in the apical compartment of the seminiferous epithelium (Pinart et al. 2001b). As compared with healthy boars, the apical cytoplasm of Sertoli cells displays a decreased content of fucosyl and galactosyl residues; spermatogonia have similar sugar residues as in healthy boars, but spermatocytes exhibit higher amounts of α-D-glucose. Spermatids present an altered nature of both glucosyl and galactosyl residues (Pinart et al. 2001b). On the other hand, although all four stages of the seminiferous epithelium cycle are found in the scrotal testis of unilateral cryptorchid boars, stages I and II occur most frequently (Pinart et al. 1999a). Increased frequency of stages I and II results in impaired maturation of spermatids (Pinart et al. 1999a) and corresponds to the incidence of spermatozoa with primary abnormalities in the ejaculate (Pinart et al. 1998). The analysis of the meiosis indicates that in the scrotal testis of unilateral cryptorchid boars transformation from primary to secondary spermatocytes (meiosis I) is low, whereas transformation from secondary spermatocytes to round spermatids (meiosis II) shows similar values to healthy boars (Fig. 4.1a–h) (Pinart et al. 1999b). Moreover, pathological germ cells under such conditions correspond to giant-cell forms of primary

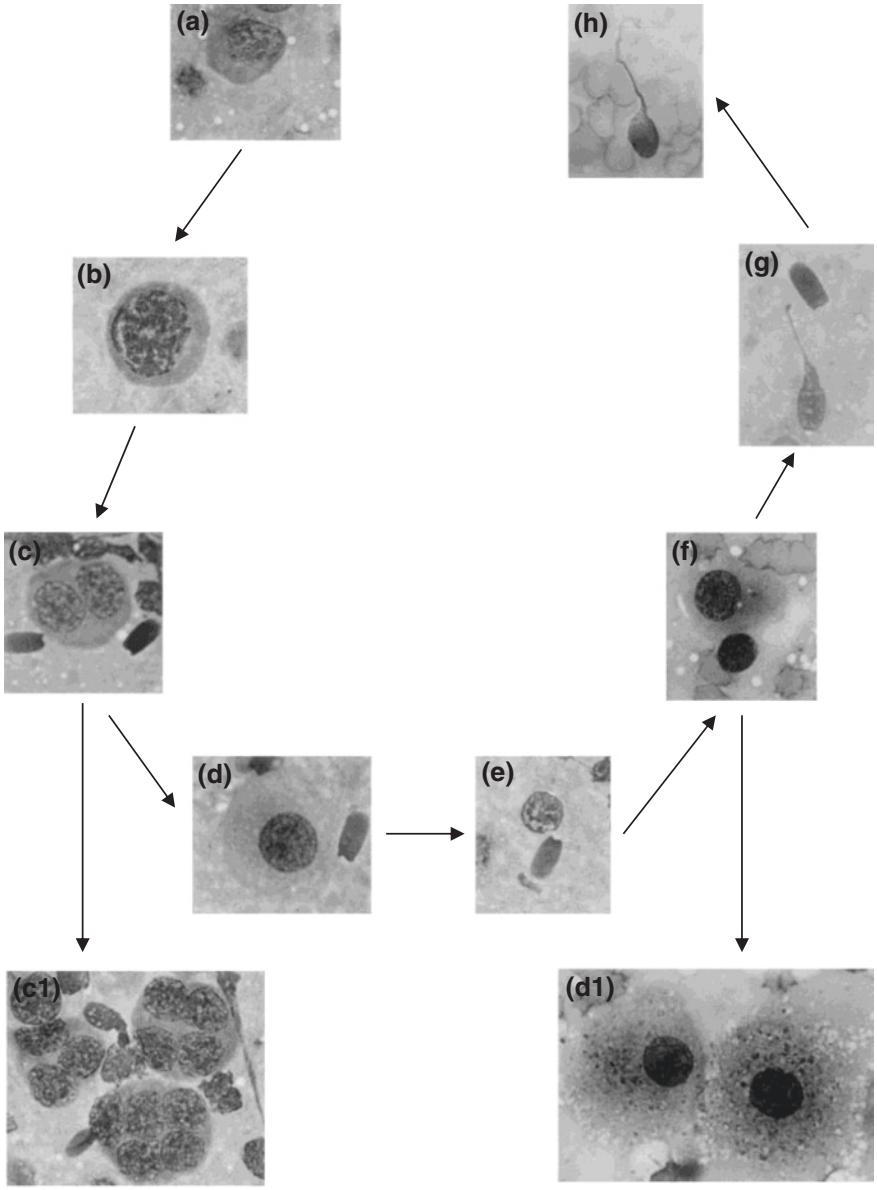


Fig. 4.1 Appearance of germ cells in the scrotal testis of post-pubertal boars with spontaneous unilateral abdominal cryptorchidism on the *right side* by light microscopy Quick Panoptic Method (*QCA*). **a** Ap type spermatogonium. **b** Pachytene prophase stage of a primary spermatocyte. **c** Advanced telophase of a primary spermatocytes. $\mathbf{c_1}$ Pathological giant-cell forms of primary spermatocytes showing a tetranucleated cytoplasm. **d** Secondary spermatocyte. **e** Early spermatid. **f** Intermediate spermatid (Golgi phase). $\mathbf{f_1}$ Pathological giant-cell forms of intermediate spermatids, showing a granular cytoplasm. **g** Elongating spermatid. **h** Elongated spermatid. ×500. Reproduced from Pinart et al. (1999b) with permission

spermatocytes with a tetranuclear cytoplasm (Fig. 4.1c$_1$), and to aberrant intermediate spermatids with a granular cytoplasm (Fig. 4.1d$_1$). Impaired meiosis I leads to a low production of round spermatids in the scrotal testis of unilateral cryptorchid boars, which is 50 % lower than in the left testis and 30 % lower than in the right testis of healthy boars (Pinart et al. 1999b).

Taken together, boar unilateral abdominal cryptorchidism on the right side affects sperm production of the scrotal testis by inducing a partial arrest of spermatogenesis at both the primary spermatocyte stage (Pinart et al. 1999b) and the spermatid stage (Pinart et al. 1999a). Mieusset et al. (1995) state that unilateral abdominal cryptorchidism affects the spermatogenic potential of the scrotal testis more severely in the case of right-sided than of left-sided cryptorchidism.

Partial arrest of spermatogenesis at the primary spermatocyte stage has been related to several testicular disorders associated with oligospermia and azoospermia (Gargiulo et al. 1991; Foresta et al. 1996; Jannes et al. 1998). Defective transformation of primary spermatocytes could appear as a result of disturbed mitosis in spermatogonia, which leads to the formation of spermatocytes with an abnormal DNA content (Setchell et al. 1998), or to anomalies in the metabolic activity and in the organization of the cytoskeleton of primary spermatocytes (Kojima 1991; Jannes et al. 1998). Moreover, defective meiosis can lead to the formation of aberrant spermatids that are not capable of achieving the process of spermiogenesis, resulting in arrest at the spermatid stage (Pinart et al. 1998, 1999a), as well as in the presence of degenerative spermatids in the scrotal testis of unilateral cryptorchid boars (Pinart et al. 1999b). The degeneration of intermediate spermatids correlates with an abnormal activity of both endoplasmic reticulum and Golgi complex that is manifested in an increased granule amount in the cytoplasm (Kojima 1991). It has been suggested that these granules have a defective content of glycoproteins implicated in the formation of the acrosome vesicle (Kojima 1991). Multinucleated spermatocytes have also been detected in men with severe oligospermia (Hofmann et al. 1992; Miething 1995). These cells are not capable of further development, so it has been suggested that they are forms of spermatogenic arrest at the spermatocyte and spermatid levels (Miething 1995). According to Miething (1995), multinuclearity comes from the confluence of cell membranes of neighboring spermatocytes, whereas Hofmann et al. (1992) suggest that it appears from disturbed mitosis.

In adult males, FSH and testosterone are required for the maintenance of quantitatively and qualitatively normal spermatogenesis (Chemes et al. 2008; Pathirana et al. 2011). Receptors for these hormones are present in Sertoli cells but not in germ cells (Kohler et al. 2007; Caires et al. 2008); therefore, the effects of both hormones are indirect and mediated by Sertoli cells, which secrete factors that regulate germ-cell differentiation in a paracrine manner (Ford and Wise 2009). In the scrotal testis of unilateral cryptorchid boars defective spermatogenesis correlates with abnormalities in the activity of Sertoli cells (Kojima 1991; Miething 1995; Pinart et al. 1999a); altered Sertoli cells produce abnormal paracrine signals that damage germ-cell differentiation, leading to spermatogenic arrest at different stages (Antich et al. 1995; Jannes et al. 1998). Anomalies in Sertoli-cell function

could be related to a genetic origin of unilateral cryptorchidism (Lee 1993; Foresta et al. 1996) or an underlying endocrinopathy generated by the cryptorchid testis (Antich et al. 1995; Rozanski and Bloom 1995).

Altered lectin affinity of the seminiferous epithelium is also an indicator of both impaired Sertoli cell function and spermatogenic arrest in scrotal testes of unilateral cryptorchid boars (Pinart et al. 2001b). Therefore, the increased content of glucosyl conjugates in spermatocytes and spermatids suggests that an abnormal ion transport could be implicated in the arrest of spermatogenesis at spermatocyte and spermatid steps; according to Santi et al. (1998), adequate ion transport is crucial for normal spermatogenesis. The decrease in galactose residues in spermatids and Sertoli cells is indicative of anomalies in cell-to-cell adhesion; defective attachment of germ cells to Sertoli cells has also been reported in subfertile rats (Courtens and Ploen 1999). The low galactosyl content of spermatids in case of partial arrest agrees with the notion that these sugars are important for spermiogenesis (Pinart et al. 2001b). In healthy boars, the residues of α-fucose at the apical cytoplasm of Sertoli cells are markers of lysosomal enzymes implicated in the degradation of residual bodies formed during spermiation (Ueno et al. 1991; Pinart et al. 2001b); in the scrotal testis of unilateral cryptorchid boars, the low amount of α-fucose residues in these cells correlates with the partial spermatogenic arrest, which results in low numbers of mature spermatids and residual bodies, and therefore in low content of lysosomes (Pinart et al. 2001b).

As in healthy boars, the tail region of testicular spermatozoa in the left (scrotal) testis of unilateral cryptorchid boars shows a poor lectin affinity. However, differences exist in the affinity of the acrosome, with a decreased content of galactosyl residues and increased content of glucosyl residues (Pinart et al. 2001b). This abnormal content of sugar residues may interfere with sperm maturation during epididymal transit and also with sperm fertilizing ability (Töpfer-Petersen 1999). Both abnormal testicular differentiation and defective epididymal maturation of the acrosome correlate with the increased frequency of spermatozoa with acrosomal anomalies in the ejaculate of post-pubertal boars affected by unilateral abdominal cryptorchidism (Pinart et al. 1998, 1999a).

The interstitial tissue of the left (scrotal) testis of unilateral cryptorchid boars has a normal appearance, with small blood and lymph vessels (Pinart et al. 1999a) and a similar Leydig cell, fibroblast and mast cell density as in healthy boars (Pinart et al. 2001d). Structure and ultrastructure of both Leydig cells and fibroblasts are also similar to scrotal testis of healthy boars (Pinart et al. 1999a, 2001d). Controversies exist about the effects of unilateral cryptorchidism on the Leydig cell population of the scrotal testis. Some authors describe anomalies either in Leydig cell number or in steroid production (Ezeasor 1985; Sirvent et al. 1989), whereas others link an increase in both Leydig cell number and steroidogenic activity to a compensatory mechanism for the low testosterone production of the abdominal testis (Jansz and Pomerantz 1986). Besides, some authors describe similar, but less severe anomalies than in the ectopic testis, in the scrotal testis of unilateral cryptorchid adults (Zakaria et al. 1998). These divergences are probably correlated with age; according to Kawakami et al. (1993) the testosterone

production of the scrotal testis of unilateral cryptorchid males peaks in the postpubertal period, and then decreases progressively in adulthood.

Although structural (Pinart et al. 1999a) and ultrastructural (Pinart et al. 2001d) evaluations do not show abnormalities in Leydig cells of scrotal testes of unilateral cryptorchid boars, the histochemical approach reveals anomalies in the sugar nature of O-linked glycans (Pinart et al. 2002). Therefore, as compared with healthy boars, Leydig cells of scrotal testes of unilateral cryptorchid boars have an altered content of glucosyl and galactosyl residues, which are indicative of disturbances in the transport of fluid and ions and in membrane permeability, and also of defective steroidogenesis (Arenas et al. 1998; Pinart et al. 2002). Alterations in substrate transport and in membrane permeability of Leydig cells are described in ectopic (either abdominal or inguinal) testes but not in the scrotal testes of unilateral cryptorchid males (Tanigawa et al. 1990). On the other hand, divergent views exist about the effects of unilateral cryptorchidism on the steroidogenic activity of the scrotal testis; thus, whereas some authors describe unaffected androgen synthesis (Ezeasor 1985; Sirvent et al. 1989; Toppari et al. 2006), others report increased (Jansz and Pomerantz 1986) or even decreased (Pinart et al. 2001d, 2002) testosterone production. This variability in testosterone production probably reflects the different aetiology of cryptorchidism (Kvist et al. 2006). In normal and pathological conditions, Leydig cell function depends on the presence of specific cell populations in the seminiferous tubules (Wu and Murono 1996). Pachytene spermatocytes and spermatids modulate the secretion of specific paracrine factors by Sertoli cells, which are involved in the regulation of Leydig cell activities (Jegou and Sharpe 1993; Wu and Murono 1996). In several disturbances, including varicocele, X-irradiation, vitamin A deficiency, efferent duct ligation or heat stress, damage of spermatogenesis results in impaired testosterone production (Jegou and Sharpe 1993). In the scrotal testes of unilateral abdominal cryptorchid boars, alterations in Leydig cells correlate with partial spermatogenic arrest at the spermatocyte and spermatid stages (Pinart et al. 1999a, b, 2000).

In the right (abdominal) testis of unilateral cryptorchid boars a significant reduction of the diameter of seminiferous tubules has been found (average 129 μm) (Pinart et al. 1999a). Moreover, spermatogenesis is totally inhibited in all seminiferous tubules, as evidenced by the structural appearance of the seminiferous epithelium (Pinart et al. 1999a); therefore, in most tubules, the seminiferous epithelium is pseudostratified and constituted by Sertoli cells and few spermatogonia (Fig. 4.2a), whereas in others a complete absence of germ cells is shown (Fig. 4.2b). The nucleus of Sertoli cells is located in the basal cytoplasm and the apical cytoplasm fills the lumen of seminiferous tubules (Pinart et al. 2000). The lamina propria displays a variable degree of thickening (from 4.5 to 10 μm) and collagenization; this thickness usually being greater in the seminiferous tubules exhibiting only Sertoli cells (Pinart et al. 1999a, 2001c). Peritubular cells of inner and outer layers of the lamina propria appear as fibroblasts of immature appearance, whereas myoid cells are not observed (Pinart et al. 2001c). The interstitial tissue shows a heterogeneous pattern, with three different areas irregularly distributed: areas with abundant Leydig cells, areas crossed by large fibrous septa, and areas with abundant blood vessels and erythrocytes (Pinart et al. 1999a, 2001d).

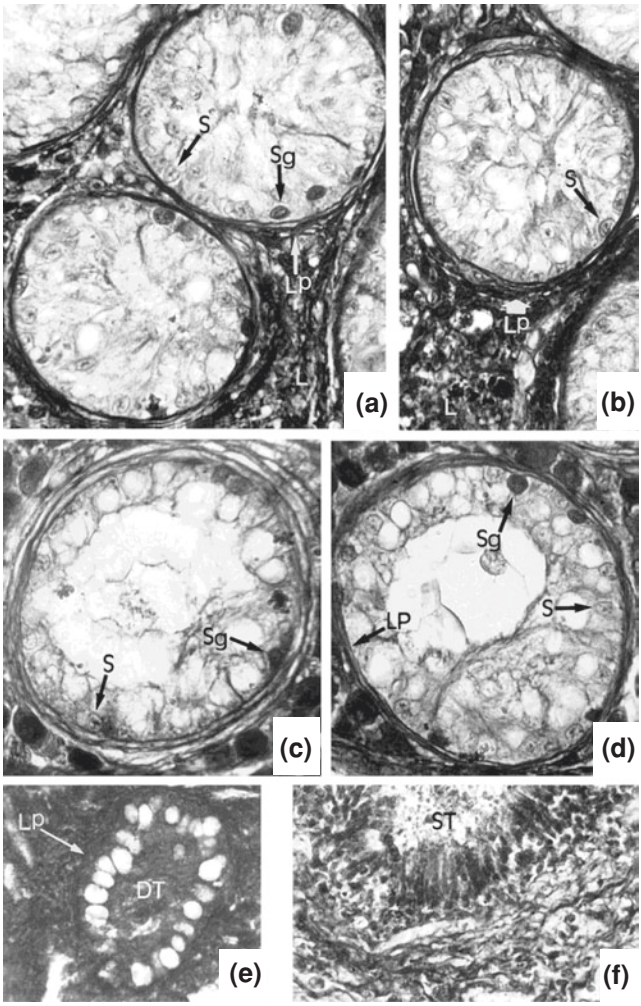


Fig. 4.2 Structure of the seminiferous tubules in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral cryptorchidism. Mallory's trichrome stain. **a** Seminiferous tubule with Sertoli cells (*S*) and spermatogonia (*Sg*) of the *right* (abdominal) testis of unilateral cryptorchid boars. **b** Seminiferous tubule with only Sertoli cells (*S*) of the *right* (abdominal) testis of unilateral cryptorchid boars. **c, d** Seminiferous tubule with Sertoli cells (*S*) and spermatogonia (*Sg*) of the *left* (abdominal) testis of bilateral cryptorchid boars; note the height of Sertoli cells and the considerable thickening of the lamina propria (*LP*). **e** Degenerative seminiferous tubule (*DT*) of the *right* (abdominal) testis of bilateral cryptorchid boars. **f** Sclerotic seminiferous tubule (*ST*) of the *right* (abdominal) testis of bilateral cryptorchid boars. ×170. L, Leydig cells. Reproduced from Pinart et al. (1999a) with permission

Therefore, unilateral abdominal cryptorchidism provokes an important regression of the Leydig cell population, which appears in an unpatterned distribution, and a great vascularization of the abdominal testis. Under these circumstances, Leydig cells can have either a mature but degenerative appearance or an immature

appearance (Pinart et al. 2001d). The fibroblast population, constituted by immature cells, displays a higher density than in healthy boars.

The left and right testes of bilateral abdominal cryptorchid boars are covered by a thickened tunica albuginia; the testes show marked abnormalities in the structural organization of the seminiferous tubules, lamina propria and interstitial tissue. In the left testis, seminiferous tubules and interstitial tissue occupy 38 and 62 % of the testicular parenchyma, respectively. In the right testis, scattered seminiferous tubules occupy about 6 % of the testicular parenchyma; thus, the amount of interstitial tissue is of 94 % (Pinart et al. 1999a). Compared to healthy boars, both testes of bilateral cryptorchid boars show a significant reduction in the amount of seminiferous tubules that is of 20 % in the left testis and practically complete in the right one. In both abdominal testes of bilateral cryptorchid boars, spermatogenesis is inhibited. In the left testis, the diameter of seminiferous tubules is 181 μm, significantly greater than in the abdominal testis of unilateral cryptorchid boars (Pinart et al. 1999a). The seminiferous epithelium, about 50 μm high, is simple cuboidal and contains Sertoli cells and few spermatogonia (Fig. 4.2c–d) or only Sertoli cells; in this last case the tubules usually display a degenerative pattern. The lamina propria shows a great thickening, which can double or even triple the thickness of the lamina propria of scrotal testes from either healthy or unilateral cryptorchid boars. Inner and outer peritubular cell layers of the lamina propria are composed of fibroblasts of immature appearance; myoid cells are lacking (Pinart et al. 2001c). The right testis contains few seminiferous tubules without germ cells, which can exhibit either a degenerative (Fig. 4.2e) or sclerotic (Fig. 4.2f) appearance; the diameter of these tubules is 182 μm. The lamina propria is composed of a thickened basal lamina (2–3 μm thick) with a high density of collagen fibers and low glycoconjugate content; peritubular cells are lacking (Pinart et al. 2001c).

Regadera et al. (1991) have also reported anomalies in the seminiferous tubules of ectopic testes from unilaterally cryptorchid men. However, their results are quite divergent:

(a) 10 % of cryptorchid testes contain seminiferous tubules with germ cells, mainly spermatogonia, but also some spermatocytes and occasionally spermatids,
(b) 57 % of cryptorchid testes show seminiferous tubules with only Sertoli cells, and
(c) 33 % of cryptorchid testes have sclerotic tubules (Regadera et al. 1991).

These divergent results may be attributed to the different positions (abdominal or inguinal) in which the ectopic testes can be found and also to the age of the patients at the time of the experiment, which ranged from 16 to 63 years. Probably the most severe abnormalities are related to a higher position of ectopic testes and/or advanced age (Pinart et al. 1999a). The structural alterations of the Sertoli cells in cryptorchid testes can be due to either a primary defect or acquired damage provoked by high temperatures (Paniagua et al. 1990; Lee 1993; Antich et al. 1995). In any case, abnormalities in Sertoli cells produce the absence of spermatogenesis in abdominal testes (Nistal et al. 1990; Paniagua et al. 1990; Pinart et al. 1999a).

Lectin histochemistry indicates that few differences exist in sugar content among non-degenerating, degenerating and degenerated immature Sertoli cells, as well as between the basal and apical cytoplasm of immature Sertoli cells, and between immature Sertoli cells and spermatogonia. Compared to healthy boars, the seminiferous epithelium of abdominal testes displays a decreased content of fucosyl residues and an increased content of glucosyl and galactosyl residues. A similar sugar composition has been found in the degenerating testes of rodents (Vanha-Perttula and Arya 1985). These results indicate that degeneration of the seminiferous epithelium may induce increased ion transport and high membrane permeability (Pinart et al. 2001b). Some studies report that in abdominal testes the degeneration of spermatogonia occurs by apoptosis whereas the degeneration of Sertoli cells is necrotic (De Rooij and Russell 2000); contrarily, more recent data suggest that abdominal cryptorchidism, both unilateral and bilateral, does not result in an increased apoptotic degeneration of spermatogonia as compared with the scrotal testes of healthy boars (Bernal-Mañas et al. 2005).

Increased thickness of the lamina propria has also been described in men with unilateral and bilateral cryptorchidism (Paniagua et al. 1990; Santamaria et al. 1990), in men with a history of cryptorchidism (Gotoh et al. 1987), in bulls with testicular hypoplasia (Veeramachaneni et al. 1987), and in bulls with azoospermia (Gargiulo et al. 1991). The thickening of the lamina propria is due to abnormalities in the cooperation between damaged Sertoli cells and peritubular cells that lead to both increased synthesis and secretion of extracellular components by peritubular cells and decreased turnover of glycoconjugates (Gargiulo et al. 1991; Richardson et al. 1995). Besides, the thick lamina propria impairs interactions between tubular and interstitial compartments, further interfering with testicular functions (Gargiulo et al. 1991; Pinart et al. 2001c).

The interstitial tissue of the right (abdominal) testis of unilateral cryptorchid boars is composed of mature but degenerative Leydig cells, immature Leydig cells and fibroblasts, and few mast cells. In the left testis of bilateral abdominal cryptorchid boars the interstitial cell population is composed of mature and immature Leydig cells, immature fibroblasts and mast cells, but also of lymphocytes and erythrocytes and few plasma cells (Pinart et al. 2001d). The numerical density of Leydig cells does not differ from the right (abdominal) testis of unilateral cryptorchid boars, whereas fibroblast and mast cell density is higher than in unilateral cryptorchid boars. The interstitial tissue of the right testis of bilateral abdominal cryptorchid boars exhibits large fibrous connective tissue areas with immature fibroblasts and mast cells, and also adipose areas. The Leydig cell population shows notable regression with scarce immature cells in advanced pignosis. Both fibroblasts and mast cell density are higher than in healthy boars (Pinart et al. 2001d).

Regression of the Leydig cell population and steroid production is also observed in ectopic testes of unilateral and bilateral cryptorchid men (Regadera et al. 1991; Sheth et al. 1996) and dogs (Kawakami et al. 1993), in unilateral abdominal cryptorchid boars and stallions (Raeside et al. 1988), and in men with a history of pre-pubertal cryptorchidism (Mieusset et al. 1995, 1997). Instead,

divergences exist regarding the degree of alteration in Leydig cell numbers; in cryptorchid testes of post-pubertal men some authors describe Leydig cell hyperplasia (Sirvent et al. 1989; Regadera et al. 1991), whereas others report a reduction in the number of Leydig cells that begins in the first months of life and becomes progressively more severe with age (Sasagawa et al. 1994). These divergences reflect different etiologies of cryptorchidism (Pinart et al. 1999a, 2000). Leydig cells from adult cryptorchid testes have impaired testosterone production (Regadera et al. 1991). The anomalies in Leydig cells are due to defective stimulation during the pre-pubertal period (Rozanski and Bloom 1995) as a result of abnormal paracrine influence from altered Sertoli cells (Antich et al. 1995). The abnormal differentiation of Leydig cells may be the cause of the marked fibrosis in the abdominal testes of unilateral and bilateral cryptorchid boars (Pinart et al.1999a).

The presence of adipocytes in the interstitial tissue has also been observed by Ezeasor (1985) in abdominal testes of adult goats. Testicular adipocytes develop from an abnormal differentiation of mesenquimatic precursors of Leydig cells during the pre-pubertal period (Huhtaniemi and Pelliniemi 1992) and/or from immature fibroblasts (Hill et al. 1999). Both the anomalies in Sertoli cell population (Antich et al. 1995) and impaired blood irrigation (Setchell et al. 1995) result in a defective differentiation of interstitial cells.

Therefore, in post-pubertal boars the abnormalities in the seminiferous tubules and the interstitial tissue of the abdominal testes are more severe in bilateral cryptorchidism than in unilateral cryptorchidism, and in the case of bilateral cryptorchidism, alterations are more marked in the right than in the left testis (Pinart et al. 1999a). In young adult men, Paniagua et al. (1990) do not find differences in testicular alterations between the unilateral and the bilateral disease.

Ultrastructure of the Seminiferous Epithelium

Sertoli cells of the right (abdominal) testis of unilateral cryptorchid boars show an oval or roundish nucleus, smaller than in healthy boars (averaging 10×8 μm), placed in the basal or medio-apical cytoplasm and forming few small infoldings on the nuclear envelope; the long axis lies parallel to the basal lamina (Pinart et al. 2000). Sertoli cell nuclei contain homogeneously distributed euchromatin with small heterochromatic areas and one or two prominent centrally placed nucleoli (Fig. 4.3a). Sertoli cells adhere to the basal lamina by numerous but poorly developed hemidesmosomes. Neighboring Sertoli cells form abundant small desmosomes and a few gap and occludens junctions located at different levels of the seminiferous epithelium. The defective establishment of junctional complexes leads to an incomplete development of the blood-testis barrier (Pinart et al. 2000). Few junctions also exist between Sertoli cells and germ cells, and they are usually small desmosomes. The cytoplasm has low organelle content, with perinuclear mitochondria, flat cisternae of rough endoplasmic reticulum, and polysomes. Regularly outlined nuclei and apical cytoplasm filling the tubular lumen are considered markers of Sertoli cell immaturity

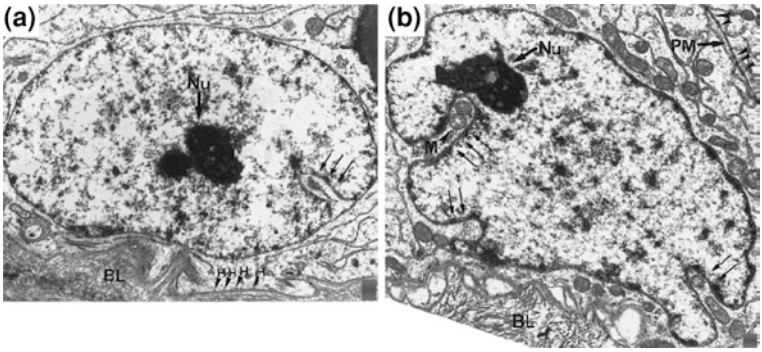


Fig. 4.3 Ultrastructural appearance of Sertoli cells in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral cryptorchidism. **a** Ultrastructure of Sertoli cells in the *right* (abdominal) testis of unilateral cryptorchid boars. The oval nucleus contains one prominent and centrally located nucleolus (*Nu*) and one small infolding on the nuclear surface (*arrows*). **b** Ultrastructure of Sertoli cells in abdominal testes of bilateral cryptorchid boars. The nuclear envelope forms deep infoldings (*arrows*) giving rise to nuclear crypts; neighboring Sertoli cells attach by poorly developed desmosomes (*arrowheads*). ×10,080. *BL* basal lamina, *ER* endoplasmic reticulum, *M* mitochondria, *PM* plasma membrane. Reproduced from Pinart et al. (2000) with permission

(Paniagua et al. 1990; Bruning et al. 1993). These ultrastructural features have also been observed in young men with unilateral and bilateral cryptorchidism (Paniagua et al. 1990) and in other testicular diseases such as hypogonadotropic hypogonadism (Nistal et al. 1990), tubular hypoplasia (Bruning et al. 1993), Klinefelter's syndrome (Regadera et al. 1991), and ovotestis (Bruning et al. 1993). Thus, different etiological factors may induce the persistence of immature Sertoli cells in adulthood.

In bilateral cryptorchid boars, Sertoli cells are cuboidal and have an elongated basal nucleus, smaller than in healthy boars (averaging 12 × 6 μm); it encloses granular euchromatin and one large nucleolus (Fig. 4.3b). The nuclear envelope forms abundant deep infoldings that produce nuclear crypts (Pinart et al. 2000). The basal cell membrane adheres to the basal lamina by abundant but poorly developed hemidesmosomes. Neighboring Sertoli cells form abundant small desmosomes, and gap and occludens junctions are scarce; as in unilateral cryptorchid boars the development of the blood-testis barrier is defective (Pinart et al. 2000). The apical cytoplasm forms lateral processes that are superimposed and attached by small desmosomes with lateral processes of adjacent Sertoli cells. The cytoplasm contains flat cisternae of endoplasmic reticulum, scattered vesicles and polysomes, and abundant mitochondria usually forming perinuclear aggregates lying inside the nuclear crypts. Elongated nuclei with deep membrane indentations and lateral cytoplasmic processes have been identified in the degenerative Sertoli cells of pre-pubertal cryptorchid testes of goats (Singh and Ezeasor 1989) and humans (Rune et al. 1992), and of post-pubertal cryptorchid testes of humans (Nistal et al. 1990). In cryptorchid testes, degeneration of immature Sertoli cells involves, in initial stages, the formation of deep infoldings of the nuclear

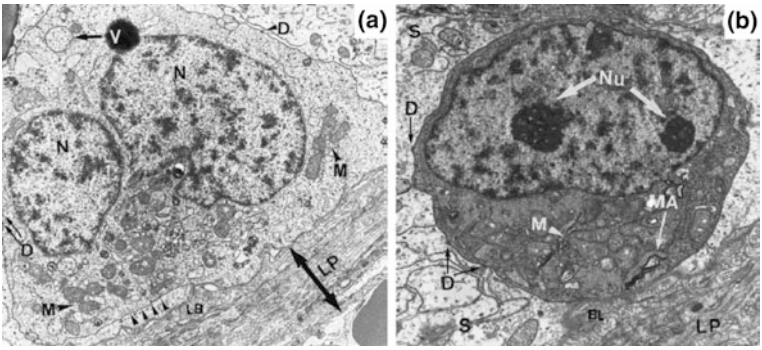


Fig. 4.4 Ultrastructural appearance of spermatogonia in abdominal testes of post-pubertal boars with spontaneous unilateral cryptorchidism. **a** Pyramidal spermatogonium; the cell is partially attached (*arrowheads*) to the basal laminal (BL). ×8,000. Ultrastructure of Sertoli cells in the *right* (abdominal) testis of unilateral cryptorchid boars. **b** Rounded spermatogonium. ×12,800. *D* desmosomes, *LP* lamina propria, *M* mitochondria, *MA* membrane aggregates, *N* nucleus, *Nu* nucleolus, *S* Sertoli cell, *V* vesicles. Reproduced from Pinart et al. (2000) with permission

membrane and retraction and convolution of the apical cytoplasm, thus forming lateral processes. More advanced degeneration includes dilation of endoplasmic reticulum cisternae, increased lipid content, and atrophy of Golgi complex and mitochondria (Singh and Ezeasor 1989; Rune et al. 1992).

Spermatocytes and spermatids are not observed in the abdominal testes of unilateral and bilateral cryptorchid boars (Pinart et al. 1999a, 2000). Seminiferous tubules contain only scattered spermatogonia with an abnormal appearance. Reduction of germ cell number is more severe in bilateral cryptorchidism than in unilateral cryptorchidism. In unilateral cryptorchid boars, two different types of spermatogonia have been distinguished (Pinart et al. 2000). Some appear as large cells (averaging 16 × 15 μm) with pyramidal profiles and partially attached to the basal lamina (Fig. 4.4a). The large nuclei (averaging 12 × 8 μm) usually show irregular profiles, with one or two deep infoldings dividing them into separate lobes. Nuclei are euchromatic and the cytoplasm contains abundant mitochondria aggregated basally, poorly developed endoplasmic reticulum, electrolucent vesicles and polysomes. Other spermatogonia appear as highly electrodense cells, with roundish profiles (averaging 10 × 8 μm) that have practically lost their contact with the basal lamina (Fig. 4.4b). The apical oval nucleus (averaging 7 × 5 μm) is strongly electrodense and contains granular euchromatin with scattered heterochromatic areas and one or two prominent nucleoli. The cytoplasm has abundant mitochondria, scattered cisternae of endoplasmic reticulum and membrane aggregates. In some cases, these spermatogonia have completely lost their attachment to the basal lamina and can be found at different levels of the seminiferous epithelium; some detached cells show degenerative patterns.

Spermatogonia with round profiles are also observed in bilateral cryptorchid boars; however, the cells are slightly larger (averaging 11 × 9.5 μm) than in

unilateral cryptorchid boars. The nucleus contains granular euchromatin with abundant heterochromatic areas and one large peripherically placed nucleolus, and the cytoplasm encloses few cellular organelles and displays a degenerative appearance (Pinart et al. 2000).

Therefore, abdominal testes of both unilateral and bilateral cryptorchid boars at post-pubertal age are likewise constituted by immature Sertoli cells, showing degenerative signs in bilateral cryptorchid boars (Pinart et al. 2000). The alterations of Sertoli cells of post-pubertal abdominal testes are attributed to an abnormal differentiation during the pre-pubertal period (Antich et al. 1995). In normal testes, proliferation and differentiation of Sertoli cells occur post-natally under FSH and testosterone stimulus, respectively (McCoard et al. 2001, 2003; Ford and Wise 2009). Differentiated Sertoli cells produce inhibin, which is involved in feedback control of FSH levels; as FSH levels decrease, Sertoli cell replication declines (McCoard et al. 2001, 2003). Testosterone produced by perinatal Leydig cells under LH stimulus also prevents enhancement of Sertoli cell proliferation and promotes their differentiation (Buzzard et al. 2003). In abdominal testes the hormonal cascade that stimulates Sertoli cell differentiation is blunted (Antich et al. 1995; Rozanski and Bloom 1995), leading to the persistence of immature Sertoli cells. Divergences between unilateral and bilateral cryptorchidism could be due to different alterations in endocrine and paracrine factors implicated in the regulation of these cells (Mieusset et al. 1995; Pinart et al. 1999a; Pinart et al. 2000). It has been reported that endocrine and paracrine disfunctions are more severe in the bilateral disease (Mieusset et al. 1995; Foresta et al. 1996; Matuszczak et al. 2011).

The degeneration of the seminiferous epithelium in abdominal testes has been extensively described in humans, boars, and rams (Rune et al. 1992; Courtens and Ploen 1999; Pinart et al. 1999a, 2000). At an initial degeneration stage, the tubules exhibit immature Sertoli cells with regularly outlined nuclei and apical cytoplasm filling the tubular lumen, and few degenerating spermatogonia placed at different levels of the seminiferous epithelium (Pinart et al. 1999a, 2000). More advanced degeneration involves nuclear elongation, formation of deep infoldings in the nuclear envelope, and retraction and convolution of the apical cytoplasm in immature Sertoli cells, as well as the disappearance of degenerated spermatogonia, the tubules being composed of a cuboidal epithelium (Rune et al. 1992; Courtens and Ploen 1999; Pinart et al. 1999a, 2000). Total degeneration includes disappearance of the nucleus, atrophy of organelles, and increased lipid content in Sertoli cells forming a disorganized nodule (Rune et al. 1992; Pinart et al. 1999a). Therefore, the right (abdominal) testis of unilateral cryptorchid boars, and the left and right testes of bilateral abdominal cryptorchid boars are representative of these three different stages of seminiferous epithelium degeneration (Pinart et al. 2001b).

In cryptorchidism, abnormal development of the blood-testis barrier has also been described by several authors (Cinti et al. 1993; Maekawa et al. 1995; Rozanski and Bloom 1995). Paniagua et al. (1990) and Nistal et al. (1990) reported that the lack of Sertoli cell maturation in abdominal testes of post-pubertal men does not hinder the development of junctional specializations, which

contradicts with explanations above. Some studies suggest that the establishment of the blood-testis barrier requires the complete maturation of Sertoli cells, the onset of spermatogenesis, and the development of the tubular lumen (França et al. 2005; Ford and Wise 2009, 2011). In unilateral and bilateral cryptorchid boars, the basal membrane of immature Sertoli cells forms small hemidesmosomes. Anomalies in hemidesmosomes have been correlated with the increased thickness of the basal lamina in cases of cryptorchidism (Paniagua et al. 1990; Maekawa et al. 1995; Rozanski and Bloom 1995). Such alterations in basal and lateral junctions of Sertoli cells are attributed to an abnormal development of actin and intermediate filaments (Maekawa et al. 1995).

The seminiferous epithelium of abdominal testes from unilateral and bilateral cryptorchid boars contains few spermatogonia; the decrease in germ cell number is greater in the case of bilateral cryptorchidism (Pinart et al. 2000). Differences also exist between the unilateral and bilateral disease in the ultrastructural appearance of spermatogonia. In abdominal testes, abnormally differentiated Sertoli cells may produce abnormal paracrine signals that damage germ cell differentiation (Antich et al. 1995). Alterations in germ cell number are attributed to delayed and defective maturation of prespermatogonia into spermatogonia, decreased proliferation of spermatogonia, and complete failure of the transformation of spermatogonia into primary spermatocytes during the perinatal period (Kohler et al. 2007; Caires et al. 2008). Untransformed gonocytes and abnormal spermatogonia degenerate progressively throughout the pre-pubertal period (Codesal et al. 1993; Rozanski and Bloom 1995), resulting in decreased numbers of spermatogonia in the abdominal testes of post-pubertal males with unilateral and bilateral cryptorchidism.

The presence of roundish spermatogonia with electrodense nuclei and large nucleoli has also been observed in cryptorchid testes of children and men (Codesal et al. 1993). It has been suggested that these cells have abnormal DNA content as a result of disturbances in DNA replication at above-scrotal temperatures of 35 °C (Codesal et al. 1993; Jannes et al. 1998). Giant pyramidal spermatogonia have been described in cryptorchid and retractile testes of children (Cinti et al. 1993; Codesal et al. 1993), and in testes of sub-fertile men (Hofmann et al. 1992). Some studies state that these cells are multinucleate and develop as a result of complete DNA replication but abnormal cell division (Hofmann et al. 1992; Cinti et al. 1993; Codesal et al. 1993).

In conclusion, the seminiferous epithelium of abdominal testes from unilateral and bilateral cryptorchid boars is constituted by immature Sertoli cells and few spermatogonia. Differences in the ultrastructural appearance of immature Sertoli cells indicate that testicular degeneration is initiated earlier in bilateral cryptorchidism than in unilateral cryptorchidism. Sertoli cell degeneration may be the cause of the degenerative appearance of germ cells and also of their lower number in the abdominal testes of bilateral cryptorchid boars (Pinart et al. 2000). These results differ from those reported by Singh and Ezeasor (1989) and Rune et al. (1992), who found Sertoli cell degeneration of cryptorchid testes at pre-pubertal age. These divergences reflect different etiologies of cryptorchidism. Tubular degeneration at pre-pubertal age could be due to an intrinsic defect of Sertoli cells,

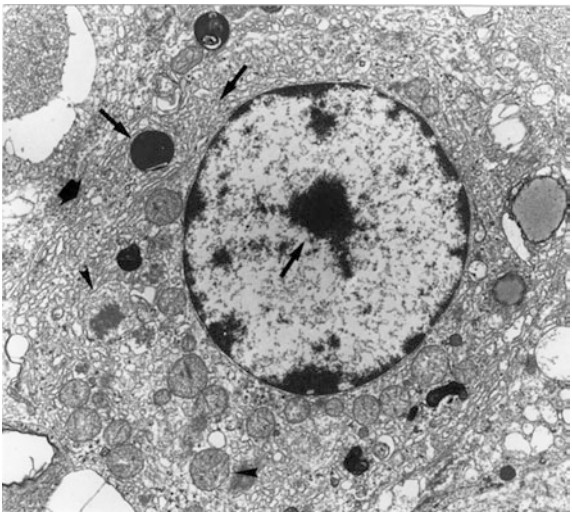


Fig. 4.5 Ultrastructure of mature Leydig cells in initial degeneration stage in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral abdominal cryptorchidism. ×10,080. *ER* endoplasmic reticulum, *Ly* lysosomes, *M* mitochondria, *Nu* nucleus, *PM* plasma membrane, *V* vesicles. Reproduced from Pinart et al. (2001b) with permission

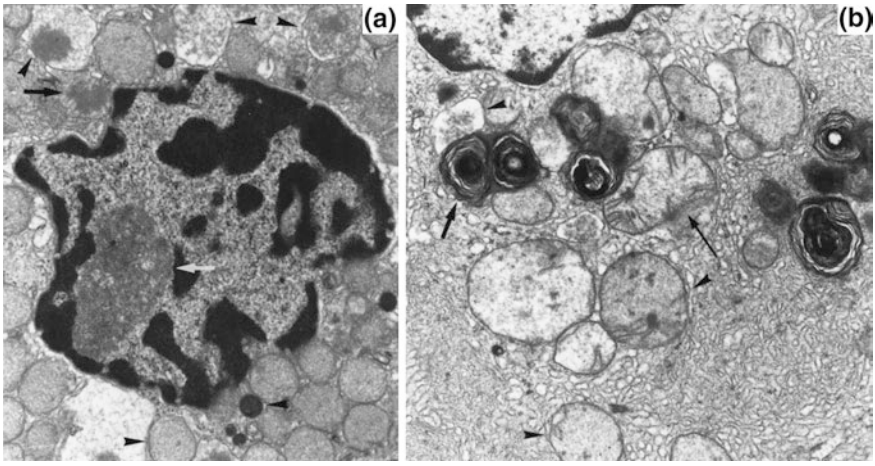


Fig. 4.6 Ultrastructure of mature Leydig cells in mid degeneration stage in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral abdominal cryptorchidism. ×10,080. **a** Appearance of the nucleus (×12,800). **b** Appearance of the cytoplasm (×20,000). *ER* endoplasmic reticulum, *He* heterochromatin, *Ly* lysosomes, *M* mitochondria, *MF* myelin figures, *N* nucleus, *Nu* nucleus, V vesicles. Reproduced from Pinart et al. (2001b) with permission

whereas tubular degeneration at post-pubertal age may be due to the adverse effect generated by high temperature (Pinart et al. 2000).

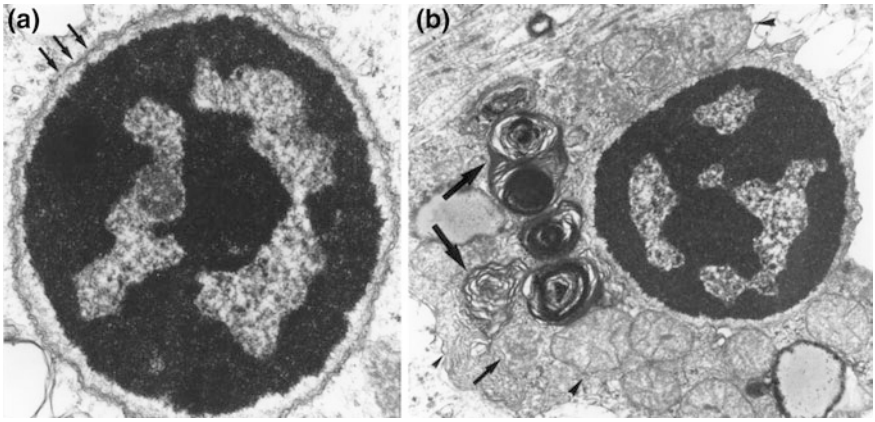


Fig. 4.7 Ultrastructure of mature Leydig cells in advanced degeneration stage in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral abdominal cryptorchidism. ×10,080. **a** Appearance of the nucleus (×20,000). **b** Appearance of the cytoplasm (×25,000). *En* nuclear envelope, *He* heterochromatin, *M* mitochondria, *MF* myelin figures, *PM* plasma membrane, *V* vesicles. Reproduced from Pinart et al. (2001b) with permission

Ultrastructure of the Interstitial Tissue

In abdominal testes of unilateral and bilateral cryptorchid boars, mature Leydig cells are located in association with blood capillaries and show degenerative signs (Figs. 4.5, 4.6, 4.7) (Pinart et al. 2001d). In an initial degeneration stage, these cells are characterized by a decreased cellular size (averaging 10 × 12 μm) as compared to mature Leydig cells of scrotal testes, and the presence of a round nucleus (averaging 5.5–6.5 μm in diameter) enclosing coarse euchromatin, heterochromatic masses and a prominent nucleolus (Fig. 4.5). The cytoplasm contains poorly-developed Golgi complex and smooth endoplasmic reticulum, myelin figures, few lysosomes and spherical mitochondria (averaging 0.6–0.8 μm in diameter) with tubular cristae. Mid degeneration involves a reduction in nuclear size (averaging 4–4.5 μm in diameter) and a greater development of heterochromatic areas (Fig. 4.6a); the nucleus can exhibit either a regular or an irregular profile (Pinart et al. 2001d). The decrease in cytoplasmic size (averaging 8 × 10.5 μm) results in a disorganization of smooth endoplasmic reticulum and increased numbers of myelin figures. Mitochondria maintain their size and profile but show lower numbers of cristae (Fig. 4.6b). Advanced degeneration leads to a notable decrease in cellular diameter (averaging 6 μm) and nuclear diameter (averaging 3.5 μm), total heterochromatinization of the nucleus, and swelling of the envelope (Fig. 4.7a). In the cytoplasm, myelin figures show great development whereas mitochondria (averaging 0.6 μm in diameter) display swelling, scarce cristae and low electrodensity (Fig. 4.7b).

Non-degenerating immature Leydig cells of abdominal testes from unilateral and bilateral cryptorchid boars lie in contact with seminiferous tubules. They

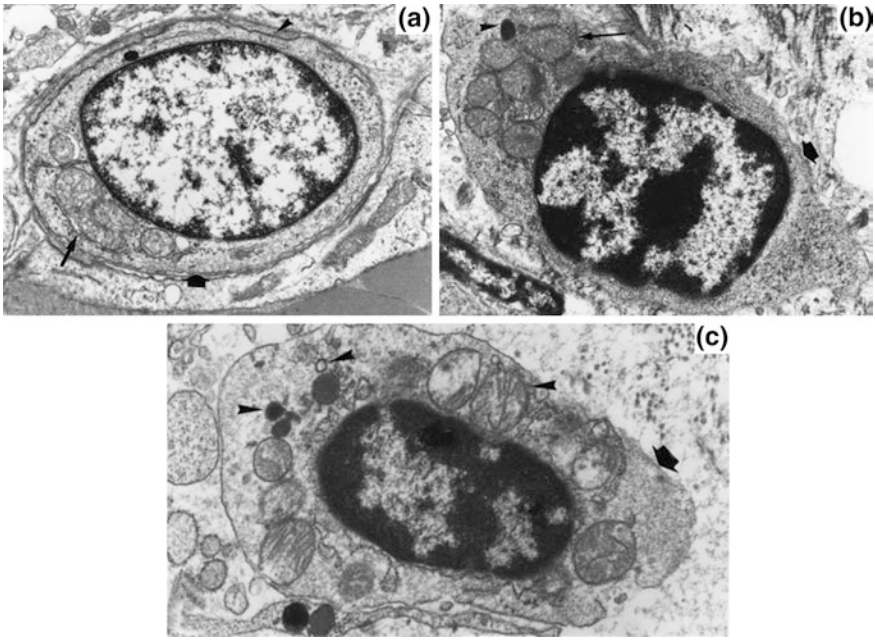


Fig. 4.8 Ultrastructure of immature Leydig cells in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral abdominal cryptorchidism. **a** Appearance of non-degenerating immature leydig cells in the *right* (×8,000. **b** Appearance of immature leydig cells in initial degeneration (×16,000). **c** Appearance of immature Leydig cells in advanced degeneration the nucleus (×20,000). *ER* endoplasmic reticulum, *He* heterochromatin, *Ly* lysosomes, *M* mitochondria, *N* nucleus, *PM* plasma membrane, *V* vesicles. Reproduced from Pinart et al. (2001b) with permission

show an oval profile (averaging 7.5 × 12 μm) and a rounded nucleus (averaging 3.5–4.5 μm in diameter) constituted by fine euchromatin and one small nucleolus (Pinart et al. 2001d). The cytoplasm is poorly electrodense and encloses a few large, oval mitochondria (averaging 0.8 × 1.2 μm), usually arranged in aggregates and exhibiting lamellar cristae, poorly developed rough endoplasmic reticulum, and some vesicles (Fig. 4.8a). In bilateral cryptorchid boars, picnosis of immature Leydig cells is initially manifested in a decrease in cellular size (averaging 4.5 × 8.5 μm) and a greater development of heterochromatic areas (Fig. 4.8b) (Pinart et al. 2001d). The cytoplasm contains abundant polysomes and round mitochondria (averaging 0.5 × 0.6 μm). Advanced degeneration results in decreased size (averaging 2 × 2.5 μm), heterochromatinization and swelling of the nucleus, with a cytoplasm characterized by polysomes, few lysosomes, and swollen mitochondria (averaging 0.7 × 0.8 μm) (Fig. 4.8c).

Differences in the maturation degree between intersticial and intertubular Leydig cells are also found in men with unilateral and bilateral cryptorchidism (Sirvent et al. 1989; Regadera et al. 1991), in goats with unilateral abdominal cryptorchidism (Ezeasor 1985), and in patients with primary testicular disorders

(Regadera et al. 1991). It has been reported that in both immature and mature testes of healthy males, interstitial, and intertubular Leydig cells differ in number, type, and activity of LH and hCG receptors, so that they respond differently to the same hormonal stimulus (Sasagawa et al. 1994; Antich et al. 1995; Setchell et al. 1995); besides, the morphology and function of intertubular Leydig cells are under the control of neighboring Sertoli cells by means of paracrine factors (Antich et al. 1995). In cryptorchid testes, the presence of immature Leydig cells in peritubular localization correlates mainly with the immaturity of Sertoli cells (Pinart et al. 1999a, 2000), which results in an abnormal paracrine stimulation of neighboring Leydig cells (Antich et al. 1995).

Immature Leydig cells are characterized by lack of smooth endoplasmic reticulum and Golgi complex, and by low mitochondrial content; degenerating mature Leydig cells display regressive changes in smooth endoplasmic reticulum, Golgi complex, and mitochondria (Pinart et al. 2001d). In Leydig cells, the degree of development of these organelles is used as a marker of their capacity to synthesize steroids (Haider and Servos 1998). Therefore, the ultrastructural study indicates that both unilateral and bilateral abdominal cryptorchidism inhibit the steroidogenesis in the abdominal testes at post-pubertal age (Pinart et al. 2001d). Lack of steroidogenesis in abdominal testes results in low testosterone levels in bilateral cryptorchid males (Kawakami et al. 1995; Pathirana et al. 2011). In contrast, no differences arise in plasma testosterone concentrations between healthy and unilateral cryptorchid dogs (Pathirana et al. 2011).

The degeneration of Leydig cells of abdominal testes from post-pubertal boars is more severe in bilateral than in unilateral cryptorchidism. Similar differences are found between unilateral and bilateral cryptorchidism in the picnotic degree of immature Sertoli cells of post-pubertal abdominal testes (Pinart et al. 1999a, 2000). These results indicate that the degeneration of abdominal testes is initiated earlier in bilateral cryptorchidism than in unilateral cryptorchidism (Pinart et al. 2000) due to more severe endocrine, paracrine, and vascular alterations in the former disease (Mieusset et al. 1995). As has been reported in bilateral cryptorchid men (Sheth et al. 1996), the anomalies of bilateral cryptorchid boars are more severe in the right testis than in the left testis (Pinart et al. 2001d); nevertheless, the causes of these differences between the right and left side are still unknown.

In the interstitial tissue of abdominal testes of unilateral and bilateral cryptorchid boars, fibroblasts appear as large immature cells (averaging 4.5 $\times$ 15 μm) containing a fused nucleus (averaging 3.5 $\times$ 13 μm) of either regular or irregular profile (Pinart et al. 2001d). The nucleoplasm has granular euchromatin, small heterochromatic areas, and one prominent nucleolus. The organelle content is composed of aggregates of rough endoplasmic reticulum cisternae, mitochondria, vesicles and lysosomes.

In scrotal testes of healthy adult males, fibroblasts are characterized by a low rate of synthesis and turnover of extracellular matrix components, their main function being the regulation of Sertoli cell activities (Schteingart et al. 1999). Immature fibroblasts with well-developed rough endoplasmic reticulum and prominent nucleolus have high secretory activity, which correlates with the

collagenization and impaired substrate diffusion of the interstitial tissue in abdominal testes of post-pubertal boars (Pinart et al. 2001d, 2002). In healthy adults, mature Sertoli cells secrete paracrine factors implicated in the maintenance of the cytologic features of the fibroblast population (Schteingart et al. 1999). In post-pubertal cryptorchid testes, the alterations in fibroblast number and metabolism correlate with Sertoli cell immaturity (Antich et al. 1995; Pinart et al. 2000), mast cell proliferation (Jezek et al. 1996; Pinart et al. 2001d), and defective testicular perfusion (Setchell et al. 1995; Pinart et al. 2001a).

Unilateral cryptorchidism does not induce abnormalities in the mast cell population of either scrotal or abdominal testes (Pinart et al. 2001d). Instead, the interstitial tissue of the left testis of bilateral abdominal cryptorchid boars has been reported to increase the number of mast cells, as well as that of lymphocytes and, to a lesser degree, the number of plasma cells; the right testis also exhibits frequent mast cells (Pinart et al. 2001d). Abundance of lymphocytes has been found in rats with bilateral abdominal cryptorchidism (Kort et al. 1991); this anomaly is usually associated with inflammatory and autoimmune diseases (Itoh et al. 1998). In this species, defective Leydig cell differentiation and Leydig cell depletion result in mast cell proliferation (Meinhardt et al. 1998). In men, idiopathic infertility (Yamamoto et al. 1994), seminiferous tubule atrophy (Jezek et al. 1996), malignant transformation of germ cells (Arber et al. 1998), and cryptorchidism (Montella and Pirino 1990) lead to testicular mastocytosis. In severe disturbances, mast cells are implicated in testicular fibrosis by enhancing proliferation and collagen production of fibroblasts, and in chronic inflammation by promoting capillary proliferation and lymphocyte infiltration; the presence of abundant mast cells and plasma cells is also considered as a sign of testicular regression (Antón et al. 1998; Ohtsuka 2000). In abdominal testes of bilateral cryptorchid boars, the mastocytosis correlates with the degeneration of Sertoli cells (Pinart et al. 2000) and of Leydig cells (Pinart et al. 2001d).

Despite the great fibrosis of the interstitial tissue (Pinart et al. 1999a, 2001d), abdominal testes from unilateral and bilateral cryptorchid boars also exhibit increased number of blood capillaries interspersed among interstitial cells (Pinart et al. 2001a); at ultrastructural level, interstitial capillaries can display either a mature or an angiogenic appearance. Both capillary angiogenesis and capillary degeneration are greater in bilateral cryptorchidism than in unilateral cryptorchidism. Increased number and altered permeability of capillaries, resulting in a reduction of blood flow, are also reported in ectopic testes of children and men with unilateral and bilateral cryptorchidism (Setchell et al. 1995). Besides, early correction of cryptorchidism at pre-pubertal age does not always improve the testicular blood flow (Mieusset and Bujan 1995). Abnormal vascular supply results in impaired oxygen and nutrient exchange that is manifested in increased fibrosis of the interstitial tissue (Setchell et al. 1995). The increased content of erythrocytes in the right (abdominal) testis of unilateral cryptorchid boars, and of erythrocytes and lymphocytes in the left testis of bilateral abdominal cryptorchid boars is also indicative of abnormalities in vascular permeability (Collin and Bergh 1996; Haider and Servos 1998).

As compared with capillaries of scrotal testes, angiogenic capillaries show an increased number but a decreased size of endothelial cells, lack of pericytes, and decreased thickness and collagen content of the basal lamina (Pinart et al. 2001a). These capillaries are usually associated with small migrating cells (Fig. 4.9a_1–a_2). The morphological events in angiogenesis were first described by Korpelainen et al. (1998) and Pettersson et al. (2000). New blood capillaries develop from preexisting ones by means of degradation of the basal lamina components by endothelial cells and detachment of pericytes; the pericyte loss results in proliferation of endothelial cells, which can further migrate to the surrounding tissue, and finally, associate to form new capillaries (Pettersson et al. 2000). At postpuberty, the proliferation of testicular capillaries can be due to the malignancy of endothelial cells, but also to abnormal stimulation from neighboring tissue as a

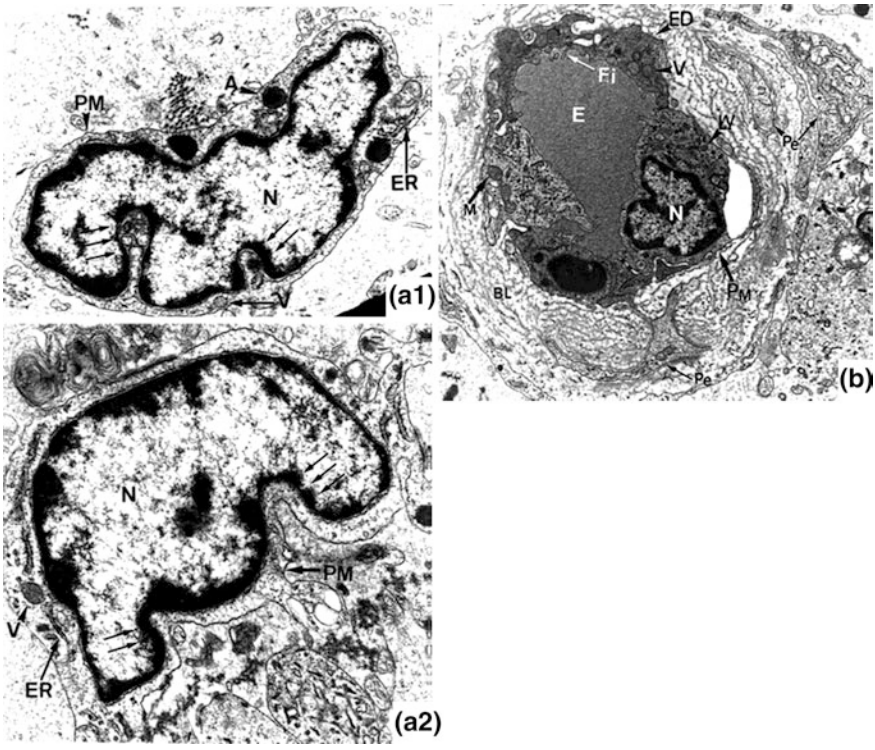


Fig. 4.9 Ultrastructure of blood capillaries in abdominal testes of post-pubertal boars with spontaneous unilateral and bilateral abdominal cryptorchidism. **a** Appearance of a migrating endothelial cell in the right testis of unilateral cryptorchid boars (**a_1**) and in the *left* testis of bilateral cryptorchid boars (**a_2**) (×15,000). Note the presence of deep invaginations (*arrows*) on the nucleus (*N*). **b** Cross section of a degenerative capillary of the left testis of bilateral cryptorchid boars (×8,960). *A* electrodense aggregates, *BL* basal lamina, *E* erythrocyte, *ED* endothelial cell, *ER* endoplasmic reticulum, *Fi* finger-like processes, *Ly* lysosomes, *M* mitochondria, *Pe* pericytes, *PM* plasma membrane, *V* vesicles. Reproduced from Pinart et al. (2001a) with permission

consequence of tumor development (Korpelainen et al. 1998; Feng et al. 2000), severe Leydig cell dysfunctions (Collin and Bergh 1996; Ergün et al. 1998), and stress conditions generated by hypoxia (Kerbel 2000), oxidative damage (Markey et al. 1994), or high temperature (Korpelainen et al. 1998). Tumoral cells (Feng et al. 2000; Kerbel 2000) or altered Leydig cells (Collin and Bergh 1996) secrete high amounts of vascular endothelial growth factor (VEGF), which stimulate several stages of blood vessel formation including endothelial cell proliferation and migration, basal lamina degeneration, and tube formation (Korpelainen et al. 1998). VEGF also acts as a potent pro-survival factor of endothelial cells in newly formed vessels (Kerbel 2000; Otani et al. 2000).

Degenerative blood capillaries of abdominal testes exhibit decreased diameter, narrowing of the lumen, pyknotic transformation of the endothelium, thickening and collagenization of the basal lamina and a great increase in pericytes (Fig. 4.9b) (Pinart et al. 2001a). Similar alterations are also reported in degenerative capillaries of men, rams, bulls and mice with testicular involution caused by age, ischemia, varicocele, cryptorchidism and idiopathic infertility (Markey et al. 1994; Jezek et al. 1996). The degeneration of intertubular capillaries is activated by the thickening and collagenization of the lamina propria of the seminiferous tubules (Jezek et al. 1996; Pinart et al. 1999a, 2001c). In advanced testicular involution, capillary pyknosis extends towards the interstitial vessels (Setchell et al. 1995).

4.2.2.4 Main Semen Abnormalities of Cryptorchid Boars

Fertility is impaired in adult males with unilateral cryptorchidism, although the degree of alteration of semen parameters remains controversial (Mieusset et al. 1995). Divergent results are due to the different conditions in which the studies of cryptorchidism are performed. Thus, the effect of unilateral cryptorchidism in semen quality is different depending on whether it is spontaneous or artificially induced, abdominal or inguinal, and right- or left-sided (Mieusset et al. 1995; Pinart et al. 1999c). Moreover, the sperm quality of unilaterally cryptorchid males is different depending on age since significant differences have been reported between young adults and elderly males (Lee 1993). The effects of bilateral cryptorchidism is different when it is inguinal than when it is abdominal; post-pubertal boars with bilateral abdominal cryptorchidism are sterile due to lack of semen production (Pinart et al. 1998, 1999a, c, 2000).

An extensive study of seminal quality has been performed with post-pubertal boars characterized by unilateral abdominal cryptorchidism on the right side (Pinart et al. 1999c). The ejaculate volume of unilateral abdominal cryptorchid boars is reported to be between 30 and 60 % lower than in healthy boars, whereas the volume of the cell-rich fraction does not differ between both groups of males (Pinart et al. 1999c). In humans, Puri and O'Donnell (1990) state that 50 % of subfertile men with a history of cryptorchidism have decreased semen volume; in these patients, the ejaculate volume can be up to 80 % lower than in healthy men.

The decrease in semen volume in unilateral cryptorchid males is due to an abnormal production of seminal fluid by the accessory glands (Pinart et al. 1999c).

Unilateral abdominal cryptorchidism significantly affects sperm concentration and sperm production per gram of testicular parenchyma, which are 80 and 75 % lower than in healthy boars, respectively (Pinart et al. 1999c). In pubertal dogs with unilateral cryptorchidism, sperm concentration is 50 % lower than in normal dogs (Kawakami et al. 1995). Oligospermia has also been observed in all subfertile men with a history of unilateral cryptorchidism, with a decrease in sperm concentration ranging from 65 to 85 % (Puri and O'Donnell 1990; Mieusset et al. 1995; Rozanski and Bloom 1995; Foresta et al. 1996). In post-pubertal boars with unilateral abdominal cryptorchidism on the right side, the decrease in sperm concentration is due to the lack of sperm production of the abdominal testis (Pinart et al. 1999a, 2000), and the impaired sperm production of the scrotal testis as a result of the partial arrest of spermatogenesis at the meiosis stage (Pinart et al. 1999b) and at the spermatid stage (Pinart et al. 1998, 1999a).

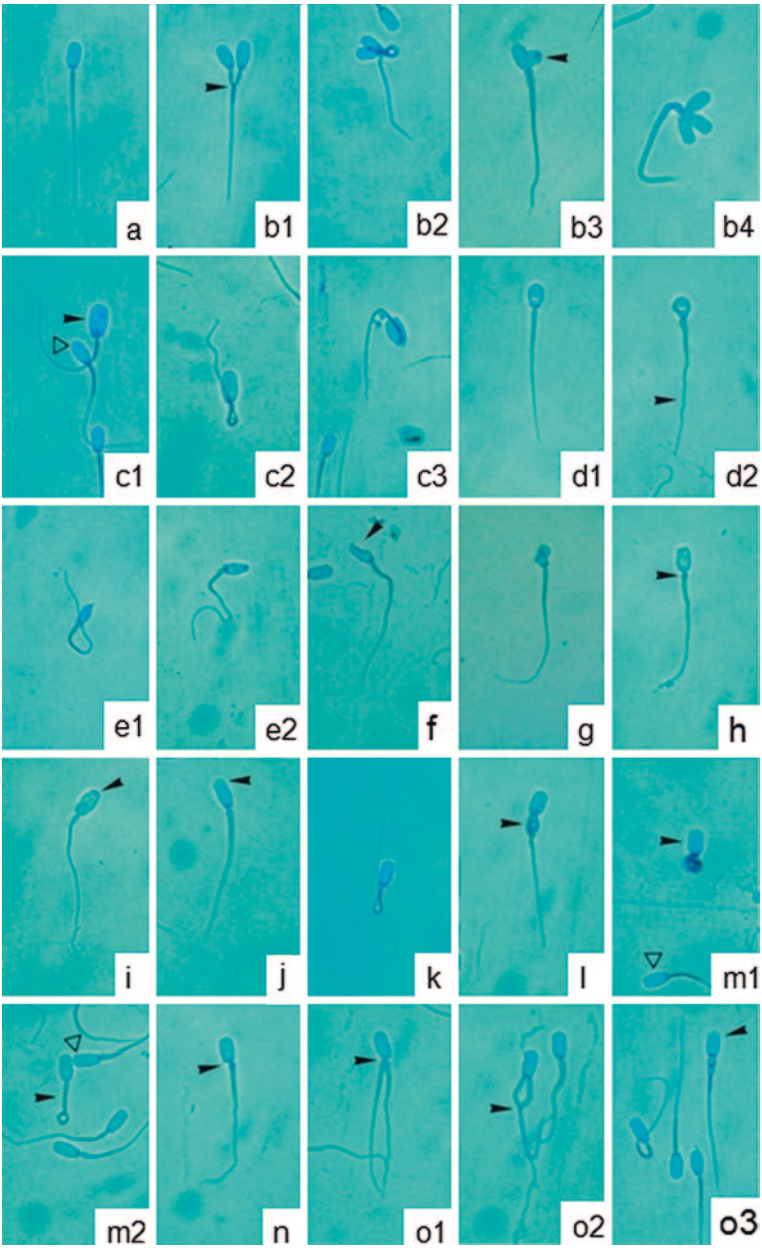
Few data exist about the effect of cryptorchidism on DNA integrity of spermatozoa and the studies performed are quite dated. In a study using the acridine orange test (AO test), it was concluded that this parameter is not significantly affected in post-pubertal boars with unilateral abdominal cryptorchidism (Pinart et al. 1999c). The AO test is based on determining the denaturalization degree of DNA (Evenson and Wixon 2006; Lamb 2010). DNA denaturalization can be originated by an abnormal protaminization during spermatogenesis and/or by anomalies in the epididymal maturation which result in an impaired formation of S–S cross-links between the cysteine-rich protamines (O'Brien and Zini 2005; Evenson and Wixon 2006). In a recent review, Lamb (2010) stated that in both fertile and subfertile patients DNA damage increases progressively during epididymal maturation, although the molecular basis of this damage is completely unknown. According to all these findings, results of Pinart et al. (1999c) seem to indicate that unilateral abdominal cryptorchidism on the right side does not result in an impairment of DNA integrity in boar spermatozoa, either at the testicular level or at the epididymal level. Nevertheless, more studies are required using more robust tests of chromatin integrity and DNA fragmentation in order to better establish the effect of cryptorchidism on the nuclear damage of spermatozoa.

The sperm motility of post-pubertal boars with unilateral abdominal cryptorchidism can be up to 22 % lower than in healthy boars and have non-progressive trajectories (Pinart et al. 1999c). Alterations in the progressive movement of spermatozoa have been reported in unilateral cryptorchid men (Urry et al. 1994) and boars (Bonet et al. 1998), as well as in subfertile men with a history of unilateral cryptorchidism (Puri and O'Donnell 1990; Urry et al. 1994; Mieusset et al. 1995). The most frequent causes of decreased sperm motility are: (1) abnormal spermiogenesis that leads to the formation of spermatozoa with intrinsic defects in the tail ultrastructure (Gopalkrishnan et al. 1995); (2) anomalies in the activity of the epididymal epithelium, which result in imbalance in the ionic and proteic components of epididymal fluid that interferes with the normal development of sperm motility along the duct (Pruneda et al. 2005); or (3) impaired activity of the

accessory glands, which does not only produce alterations in the ejaculate volume but also changes in the osmolality of semen, with further effects on sperm motility (Smital et al. 2005). Impaired activity of the epididymides and accessory glands is attributed to an underlying endocrinopathy generated by the cryptorchid testis (Pinart et al. 1999c).

Sperm morphology, expressed as the percentage of mature, immature and aberrant spermatozoa, and the cephalic stability of spermatozoa, expressed as the frequency of detached heads, do not differ between unilateral abdominal cryptorchid boars and healthy boars (Pinart et al. 1999c). In contrast, oligospermic men with a history of unilateral cryptorchidism usually show a significant decrease in the frequency of mature spermatozoa (Urry et al. 1994; Mieusset et al. 1995). Aberrant spermatozoa of the ejaculate may be of primary origin if they develop in the testis as a result of abnormalities in spermatogenesis and spermiogenesis (i.e. testicular origin), or of secondary origin if they develop along the epididymal duct as a result of an abnormal maturation of spermatozoa (i.e. epididymal origin) (Briz et al. 1995, 1996). The detailed study of sperm abnormalities in unilateral abdominal cryptorchid boars shows that although the frequency of aberrant spermatozoa is normal, a significant increase in the frequency of primary abnormalities occurs, whereas the frequency of secondary abnormalities maintains low values; moreover, the types of primary and secondary abnormalities differ significantly between unilateral abdominal cryptorchid boars and healthy boars (Fig. 4.10a–o) (Pinart et al. 1998).

Among the primary abnormalities, unilateral abdominal cryptorchid boars have a lower percentage of spermatozoa with anomalies in head size (Fig. 4.10c_1–c_3) and in head shape than healthy boars (Fig. 4.10d_1–d_2, e_1–e_2), but a higher proportion of spermatozoa with folded tail (Fig. 4.10 c_2–c_3, k) and with tail length and/or thickness anomalies (Pinart et al. 1998). The percentage of spermatozoa with one head and multiple tails is similar to healthy boars (Fig. 4.10o_1–o_3). Moreover, some primary sperm abnormalities are specific to unilateral abdominal cryptorchid boars: (1) spermatozoa with a nuclear crest, which appears as a transversal ridge on the surface of the spermatozoon head; (2) spermatozoa with crater defect, which is commonly manifested at the apex of the sperm head (Fig. 4.10h), (3) spermatozoa with knobbed acrosome defect, characterized by the flattening of the apex of the acrosome (Fig. 4.10i), (4) spermatozoa with abaxial tail, in which the tail attaches eccentrically to the base of the spermatozoon head (Fig. 4.10l, n), and (5) spermatozoa with mitochondrial swelling (Fig. 4.10l) (Pinart et al. 1998). Spermatozoa with deformed (Fig. 4.10f) or folded (Fig. 4.10g) heads as well as spermatozoa with thin tails have only been observed in unilateral cryptorchid boars (Pinart et al. 1998). The percentage of spermatozoa with primary abnormalities by tail folding is significantly higher in unilateral cryptorchid boars. Moreover, healthy boars and cryptorchid boars also differ in the localization of tail folding; unilateral cryptorchid boars have a significantly higher frequency of spermatozoa with folded tail at the connecting piece (Fig. 4.10c_3) and at the midpiece (Fig. 4.10c_2), and a significantly lower frequency of spermatozoa with folded tail at the principal piece (Pinart et al. 1998).



Among the secondary abnormalities, unilateral abdominal cryptorchid boars have a lower percentage of spermatozoa with folded tails at the Jensen's ring (Fig. 4.10k), whereas the proportion of spermatozoa with multiple heads and spermatozoa with coiled tails does not differ from healthy boars (Pinart et al. 1998). In

◀ **Fig. 4.10** Sperm malformations of the ejaculate of post-pubertal boars with spontaneous unilateral abdominal cryptorchidism on the right side (Quick Panoptic Method of QCA). **a** Mature spermatozoon. **b_1** Bicephalic spermatozoon with two tails fused from the distal end of the midpiece (*arrow*). **b_2** Spermatozoon with two elongated heads, and two tails totally fused and folded at the midpiece. **b_3** Spermatozoon with two heads, one of them aberrant (*arrow*), and two totally fused tails. **b_4** Tricephalic spermatozoon with three totally fused tails. **c_1** Macrocephalic spermatozoon (*arrow*); note the presence of an elongated head spermatozoon (*triangle*). **c_2** Macrocephalic spermatozoon with folded tail at the midpiece. **c_3** Macrocephalic spermatozoon with folded tail at the connecting piece. **d_1** Roundish head spermatozoon. **d_2** Round head spermatozoon; note the corksrew defect affecting the principal piece (*arrow*). **e_1**, **e_2** Pointed head spermatozoon. **f** Deformed head spermatozoon due to a cephalic break (*arrow*). **g** Spermatozoon with a transversely folded head. **h** Spermatozoon with crater defect and proximal droplet (*arrow*). **i** Macrocephalic spermatozoon with knobbed acrosome (*arrow*). **j** Spermatozoon with acrosomal vacuolization (arrow). **k** Spermatozoon with folded tail at the Jensen's ring. **l** Macrocephalic spermatozoon with mitochondrial swelling (*arrow*). **m_1** Macrocephalic spermatozoon with folded tail; note the presence of a spermatozoon with crater defect and abaxial tail (*triangle*). **m_2** Spermatozoon with coiled tail at the terminal and principal pieces (*arrow*); note the presence of a spermatozoon with acrosomal vacuolization (*triangle*). **n** Immature spermatozoon with proximal droplet and abaxial tail (*arrow*). **o_1** Spermatozoon with two unfused tails abaxially attached (*arrow*). **o_2** Spermatozoon with two tails fused from the principal piece (*arrow*). **o_3** Macrocephalic spermatozoon with vacuolated acrosome (*arrow*) and two fused tails. ×500. Reproduced from Pinart et al. (1998) with permission

contrast, spermatozoa with vacuolated acrosome (Fig. 4.10j), which show swelling of this vesicle, disruption of the plasma and acrosomal membranes in the apical region and loss of the acrosomal content, and spermatozoa with tail-to-tail agglutination (Figs. 4.10b_1–b_4) have only been observed in cryptorchid boars (Pinart et al. 1998). Cryptorchid and healthy males do not differ in the percentage of spermatozoa with coiled tails, but they differ in the proportion of spermatozoa with totally coiled or partially coiled tails (Fig. 4.10m_1–m_2). Unilateral cryptorchid boars have a significantly lower percentage of spermatozoa with totally coiled tails, and a significantly higher proportion of spermatozoa with partially coiled tails at the midpiece, at the principal piece and at the terminal piece (Pinart et al. 1998).

At ultrastructural level, sperm abnormalities of the ejaculate of unilateral abdominal cryptorchid boars can be classified as (Pinart et al. 1998): (1) nuclear inclusions, mainly located in the postacrosomal region and manifested as large areas without chromatin or with non-condensed chromatin (nuclear vacuoles), and with membrane aggregates inside them (see Fig. 1.15j, k); (2) myelin figures, which appear between the postacrosomal dense lamina and the perinuclear fibrous material and cause a slight deformation of the cephalic surface (see Fig.1.15g, i); (3) membrane folding, which appears in the equatorial region between the perinuclear fibrous material and the acrosomal vesicle and causes a slight deformation of the cephalic surface; (4) nuclear crests, originated by the presence of three unequal nuclear arms inclined to each other, with membranous material placed between these arms (see Fig.1.15o); (5) acrosomal inclusions, characterized by the presence of large numbers of vesicles and granules of different sizes immersed in a low electrodense acrosomal matrix, leading to an alteration of the acrosome shape (see Fig.1.15e); (6) knobbed acrosome defect, the prominence being originated in some cases by apical and lateral

protrusions (see Fig.1.15c–d), which manifest themselves in association with other defects like the presence of small vesicles and inclusions in the protrusions (see Fig.1.15a) and the lower electrodensity of the acrosome (see Fig.1.15b); in other cases, the acrosomal prominence is due to a big vacuole, with variable electrodensity, that results in a large deformation of the organelle and the non-uniform thickness of the spermatozoon head; (7) festooned acrosome, with an irregular outline, lateral, and apical protrusions and a slightly electrodense matrix (see Fig.1.15f); (8) tail folding, which can be located at the connecting piece, the midpiece, the Jensen's ring (see Fig. 1.14a) or the principal piece; in tail foldings at the principal piece the absence of peripheral microtubular doublets has also been observed; (9) tail coiling, exhibiting axonemal disarrangements (see Fig. 1.14b); (10) two fused tails, with two axonemal structures correctly formed, (11) additional mitochondria, with extra mitochondria placed at the proximal region of the midpiece between the mitochondrial sheath and the plasma membrane and causing a local thickening of the midpiece; and (12) mitochondrial swelling, characterized by the presence of mitochondria without cristae and an electrolucent matrix on the mitochondrial sheath.

The types of sperm malformations observed at both structural and ultrastructural level indicate that unilateral abdominal cryptorchidism affects the spermiogenesis of the scrotal testes by inducing disturbances in the late stages of nuclear and acrosomal differentiation, as well as in the late stages of flagellum remodeling (Pinart et al. 1998). The low percentage of immature spermatozoa suggests that unilateral abdominal cryptorchidism does not alter the migration of the cytoplasmic droplet along the epididymal duct (Briz et al. 1995) and its detachment during ejaculation (Pruneda et al. 2005).

4.3 Extrinsic Environmental Factors

Sperm quality characteristics show clear seasonal changes. The causes of seasonal fluctuations in semen quality are not fully understood but may be mediated by hormonal mechanisms controlled by the photoperiod (Ciereszko et al. 2000). In temperate climates wild pigs are seasonal breeders and do not mate during the summer and fall months (Almeida et al. 2006). Similarly, the lowest reproductive performance of commercial swine is observed in summer. Ancient seasonal mechanisms still influence semen characteristics of boars, especially the semen volume and the number of spermatozoa per ejaculate, the levels of both parameters being the highest during fall and winter (Ciereszko et al. 2000; Huang et al. 2010). On the other hand, many factors may modify semen characteristics of the boar, including high ambient temperature leading to heat stress, frequency of semen collection, age, social environment, and nutrition.

4.3.1 Temperature

Ambient temperature is considered the most important environmental parameter affecting semen quality. Low ambient temperature does not seem to influence

semen composition whereas excessive warm (up to 29 °C) does not only decrease testicular function, leading to a fall of up to 35 % in sperm concentration, but also induces disturbances in the spermatogenic process (Sonderman and Luebbe 2008; Wolf and Smital 2009) that manifests itself in an impairment of sperm motility along with a rise in the frequency of abnormal spermatozoa in the ejaculate (Joseph et al. 2005). The decrease in steroidogenesis results in low testosterone levels and, therefore, in reduced libido (Hemsworth and Tilbrook 2007; Huang et al. 2010); nevertheless, the adverse effect of high temperatures on sexual behavior is generally only temporary (Hemsworth and Tilbrook 2007). On the other hand, pregnancy rates and embryo survival are dramatically reduced in gilts inseminated with semen from heat-stressed boars (Trudeau and Sanford 1986). To ensure constant and high semen quality, boars must be kept at a temperature below 22 ° C (Huang et al. 2010).

Despite the importance of this factor few reliable data correlating high temperature values with specific alterations on semen parameters are available. Most studies on the effects of temperature are focused on the comparative analysis of semen quality among seasons. Semen quality of reproductive boars differs throughout the year but significant breed-by-season interactions have been reported, especially in sperm production. For instance, North European breeds have lower tolerance to heat stress than breeds developed along the Equator (Sonderman and Luebbe 2008).

Comparisons among breeds of semen quality throughout the year show that while the total number of spermatozoa in Meishan boars is the highest in winter, in Duroc boars it is higher during autumn and spring than in the other seasons; in contrast, in Fengjing and Min zhu breeds the total number of spermatozoa is not affected by the season (Borg et al. 1993). In temperate climates, decreased sperm production during summer months occurs concomitantly with a decrease in testicular size (Trudeau and Sanford 1986). Differences also exist among genetic lines in their sensitivity to ambient temperatures; thus some lines experience a modest decrease in sperm production during summer months whereas others recover more quickly, thus indicating that a genetic component may exist governing the homeostatic mechanism of heat stress adaptation (Flowers 2008). In some genetic lines, sperm production is not impacted significantly after exposure to elevated ambient temperatures; exploitation of these genetic lines could conceivably lead to the development of “heat tolerant” AI boars (Flowers 2008). In cattle, genetics definitely affects heat tolerance (Hammond et al. 1996), so it is reasonable to assume the same mechanism in swine (Flowers 2008).

Seasonality has no effect on sperm concentration of Yorkshire and Duroc breeds (Park and Yi 2002); in contrast, in Large White and Pietrain breeds sperm concentration peaks in spring and maintains consistently lower values during autumn and winter (Ciereszko et al. 2000).

Differences in sperm morphology throughout the year have been reported by some authors but not by others. Thus, in temperate climates minimum and maximum percentages of aberrant spermatozoa have been noted in September and March, respectively, and correlate with changes in the rate of spermatozoa with folded tails (Trudeau and Sanford 1986). In contrast, Wolf and Smital (2009) have

reported that seasonal differences in the percentage of abnormal spermatozoa are less than 1 %, and Park and Yi (2002) state that the effects of seasons on sperm morphological traits cannot be considered biologically important. Despite the lack of significant differences in sperm morphology throughout the year, some authors have reported changes in sperm functionality due to disturbances in acrosin activity (Ciereszko et al. 2000) and in membrane integrity (Trudeau and Sanford 1986). Acrosin activity peaks in summer and falls in autumn, probably as a result of disturbances in spermatogenesis caused by high temperatures during summer, which could provoke either or both decreased acrosin concentration and/or poor quality of acrosomal membranes (Ciereszko et al. 2000). According to the former Trudeau and Sanford (1986) have found that membrane integrity of boar spermatozoa is affected by seasonality, with a significant decrease in summer and autumn. Other reports describe an increased frequency of spermatozoa with nuclear vacuoles (Malmgrem and Larsson 1984) and of spermatozoa with knobbed acrosome defect (Sánchez et al. 1989) as a result of heat stress.

The effect of seasonality on sperm membrane integrity has scarcely been studied; in Yorkshire and Duroc breeds the maximum percentages of membrane intact spermatozoa have been recorded in fall (Park and Yi 2002). The effects of season on sperm motility are controversial. Sperm motility is not affected by season in Duroc (Borg et al. 1993; Park and Yi 2002), Min zhu (Borg et al. 1993), and Yorkshire (Park and Yi 2002) breeds, whereas Meishan and Fengjing breeds have lower sperm motility in the autumn than in the subsequent seasons (Borg et al. 1993). Other authors have noticed that despite this apparent lack of seasonality effect on sperm motility the type of sperm movement varies considerably throughout the year, with a greater frequency of progressive motile spermatozoa from October to March (Trudeau and Sanford 1986). This variability throughout the year in the type of sperm movement has been attributed to qualitative changes in sperm metabolism and/or in flagellum activity (Trudeau and Sandford 1986).

Semen volume is not affected by season in Meishan (Borg et al. 1993), Duroc (Borg et al. 1993; Park and Yi 2002), and Yorkshire breeds (Park and Yi 2002). In contrast, in Large White and Pietrain breeds, as well as in Pietrain × Duroc crossbreds, ejaculate volume peaks in autumn and falls in spring (Ciereszko et al. 2000); in Fengjing and Min zhu breeds, the ejaculate volume is the lowest in autumn (Borg et al. 1993). Semen pH varies with the season, the values tending to be lower in winter and higher in spring and early summer (Trudeau and Sanford 1986). Seasonal changes in pH correlate not only with temperature but also with daylight (Trudeau and Sandford 1986). Protein content (Murase et al. 2007, 2010) and acid citric content (Trudeau and Sanford 1986) in seminal plasma exhibit similar seasonal variations, being lower in summer months; conversely, alkaline phosphatase activity does not change appreciably throughout the year (Trudeau and Sanford 1986). These results reflect variations in the behavior of vesicular glands according to the season (Trudeau and Sanford 1986). Since in boars the vesicular gland function is supported by the synergistic action of testosterone and estrogens, seasonal changes in seminal plasma components probably

reflect alterations in testicular steroid levels (Trudeau and Sanford 1986) that ultimately result in sperm DNA instability (Strzezek et al. 1995).

In Duroc and Yorkshire boars, testosterone concentration in serum is significantly higher in spring (3.06 and 5.11 ng/ml, respectively) (Park and Yi 2002), whereas in Meishan, Fengjing, and Min zhu boars, testosterone levels are markedly elevated in autumn (2.8, 5.0 and 4.0 ng/ml, respectively) (Borg et al. 1993). In Yorkshire boars, testosterone levels maintain constant throughout summer, autumn, and winter (2.57 ng/ml) (Park and Yi 2002), as well as in Fengjing (1.0–1.2 ng/ml) (Borg et al. 1993), Meishan (1.2–1.4 ng/ml) (Borg et al. 1993) and Min zhu (0.8–1.6 ng/ml) (Borg et al. 1993), whereas in Duroc boars a dramatic fall in testosterone levels occurs in summer (0.73 ng/ml), followed by a moderate increase in autumn and winter (1.31–1.36 ng/ml) (Park and Yi 2002).

Ambient temperature also affects refrigerability (Murase et al. 2010) and freezability (Park and Yi 2002) of seminal doses, which are significantly lower in summer. During summer, the significantly reduced farrowing rate of refrigerated doses is associated to a higher rate of acrosome reactions as compared to other periods (Murase et al. 2007, 2010). This fast induction of the acrosome reaction in refrigerated doses correlates with the low protein content of seminal plasma during summer (Murase et al. 2007, 2010), as well as with alterations in sperm and acrosomal membrane integrity (Trudeau and Sanford 1986; Ciereszko et al. 2000). In frozen-thawed doses of Yorkshire and Duroc boars, Park and Yi (2002) detected a higher number of motile and aberrant spermatozoa in the spring season than in summer, autumn and winter. Nevertheless, more studies are needed in order to better correlate semen freezability and seasonality.

Results obtained by different authors regarding seasonal effects on sperm quality are somewhat discrepant, and the divergences cannot only be attributed to the breed but also to latitude, age of the animals, and frequency of semen collection. Moreover, most studies are performed without taking into account that the seasons do not only differ in temperature but also in photoperiod; besides, these studies do not provide detailed information about changes in temperature during the experimental period. All the above-mentioned trials have been carried out including few individuals, which leads to unreliable results, and/or throughout only 1 year, so it is not possible to conclude whether the changes in semen quality are specific of a particular year or are general across years. Therefore, to fully characterize the repeatability of seasonal changes in semen quality traits, further trials should include a large number of samples and should be carried out for multiple years.

In order to correlate the behavior of sperm parameters with the ambient temperature, a comprehensive study has been performed on the effect of mean temperature on semen quality in Pietrain, Large-White and Duroc breeds over a 20-week period comprised between February and July (Yeste et al. 2010). Throughout this period, mean temperature ranged from 8 to 15° C during the first 10 weeks, but it increased suddenly to 18 °C degrees at week 11 and progressively to 21 °C from week 12 to 20 (Yeste et al. 2010).

Data obtained by Yeste et al. (2010) show that semen volume is affected by mean temperature, being inversely correlated in all three breeds. From this study,

Pietrain boars, but not Large-White and Duroc boars, suffer a progressive reduction of sperm concentration from May (week 12) to July (week 20) as a result of the increased temperature. Mean temperature affects sperm viability, measured as the percentage of spermatozoa with intact plasma membrane, intact acrosome and intact mitochondrial sheath, in Pietrain and Large-White breeds, but not in the Duroc breed (91 %); in Pietrain boars sperm viability decreases from 92 % in February to 86 % in July, whereas in Large-White boars the decrease is from 90 to 85 %. Sperm motility is also affected by ambient temperature in Pietrain (88 % at week 0 and 82 % at week 20) and Large-White boars (87 % at week 0 and 81 % at week 20), but not in Duroc boars (89 %). The study of kinematic parameters of sperm movement shows that the percentage of fast spermatozoa is inversely correlated with temperature in Pietrain and Duroc breeds. Therefore, in Pietrain boars the frequency of fast spermatozoa falls from 59 % at week 0 to 53 % at week 20, and in Duroc boars from 64 to 59 %. In Large-White boars the frequency of fast spermatozoa (53 %) is not affected by mean temperature, although a significant decrease in the rate of progressive motile spermatozoa has been observed from week 0 (58 %) to week 20 (52 %). Increased temperature also results in a decrease in the percentage of mature spermatozoa in Large-White boars and in Pietrain boars from week 12 (May), but not in Duroc boars; in Pietrain boars the decrease in the proportion of mature spermatozoa is manifested in association with an increase in the percentage of immature spermatozoa, whereas in Large-White boars it is linked to an increase in the frequency of aberrant spermatozoa. In Pietrain, Large-White and Duroc breeds the osmotic resistance of spermatozoa does not correlate with mean temperature (Yeste et al. 2010).

In a comprehensive 3-year study, Sonderman and Luebbe (2008) compared the percentage of discarded (trashed) ejaculates among three purebred maternal lines and two crossbred terminal lines. They concluded that during cold months trash rates are higher in purebreds Yorshire (14 %), Landrace (12 %) and Duroc (10 %) than in both crossbreds Duroc × Hampshire (F1) and ¾ Duroc × ¼ Hampshire (5 %). Moreover, the three maternal purebred lines appear to be more sensitive to seasonal infertility than the crossbred terminal lines, which results in an 18 % increase in trash rate during warm months in Yorkshire and Landrace breeds, and a 16 % increase in Duroc breeds; in crossbreds the frequency of discarded ejaculates averages 7–9 % (Sonderman and Luebbe 2008).

There is an interaction of season with age that has been reported to involve changes in the thermoregulatory ability of boars during aging (Huang et al. 2010). Age influences the susceptibility of pigs to heat stress through either physical or physiological functions and, in consequence, affects semen quality (Huang et al. 2010). A better ability in thermoregulation of boars aged 10 months than those of full maturity (36 months) correlates with higher body surface area (cm^2) and lesser subcutaneous fat content, which facilitate heat loss (Huang et al. 2010). A comparative study of the semen quality of purebred Duroc boars from 10 to 80 months of age during cold and hot seasons shows that the decrease in semen quality during the hot season is less significant in young boars (from 10 to 30 months) than

in adult boars (>35 months) (Huang et al. 2010). The average semen volume in 10 month old boars is similar during hot and cold seasons (about 185 ml). From this starting point, a progressive increase in semen volume is found with age; in the cold season semen volume reaches a maximal point of 242 ml at 46.2 months of age, while in the hot season the maximum semen volume is of 218 ml at 36.0 months of age. Afterwards, semen volume declines in both hot and cold seasons but at different rates, thus resulting in different reproductive longevities; therefore, in the cold season the semen volume drops to 180 ml at 81.0 months, whereas in the hot season it occurs at 62.8 months of age. The correlation between semen volume and age is $r = 0.45$ in the cold season and $r = 0.49$ in the hot season (Huang et al. 2010).

A similar pattern of variation has also been found for the total number of spermatozoa per ejaculate and for the number of motile spermatozoa per ejaculate. Despite the lack of differences in the total number of spermatozoa per ejaculate in 10 month old boars between hot and cold seasons, the highest total number of spermatozoa per ejaculate (84.3×10^9) during the cold season is reached at 38.7 months of age, whereas in the hot season the highest value (70.0×10^9 spermatozoa per ejaculate) is reached at 34.5 months (Huang et al. 2010). Again, a progressive decline in the total number of spermatozoa per ejaculate occurs from this maximum; in this study, the lower threshold for this parameter has been established in 56.0×10^9 spermatozoa per ejaculate and it has been reached at 69.6 months in the cold season and at 60.5 months of age in the hot season (Huang et al. 2010). A significant correlation exists between total number of spermatozoa per ejaculate and age in the cold season ($r = 0.66$) and in the hot season ($r = 0.76$) (Huang et al. 2010).

Similarly, the total number of motile spermatozoa per ejaculate reaches its maximum at 37.8 months in the cold season and at 32.1 months of age in the hot season, the values being 66.0×10^9 and 54.8×10^9 spermatozoa, respectively (Huang et al. 2010). From this highest point, the number of motile spermatozoa per ejaculate starts to decrease; the lowest threshold for this parameter has been established in 44.9×10^9 spermatozoa and it has been recorded at 72.3 months of age in the cold season and at 55.7 months in the hot season. Correlation between the number of motile spermatozoa and age is 0.60 in the cold season and 0.77 in the hot season (Huang et al. 2010).

Taken together, these results provide compelling evidence that the hot season does not only decrease the sperm quality of ejaculates but also accelerates aging in reproductive biology (Huang et al. 2010). Therefore, Duroc boars submitted to heat stress have reproductive longevity extended until 51 months of age, but if summer heat stress is avoided it may extend to 70 months (Huang et al. 2010).

Decrease of sperm production associated with high temperatures is an universal problem for the swine industry (Flowers 2008). Development of genetic lines of "heat tolerant" boars based on the phenotypic variation that seems to be present in modern terminal sire lines would substantially enhance reproductive efficiency (Flowers 2008).

4.3.2 Photoperiod

Wild boars are short-day breeders whose entire breeding activity takes place in winter and early spring (Almeida et al. 2006). In contrast, the domestic boar has traditionally been considered a non-seasonal breeder even though sperm quality and libido are not constant throughout the year (Andersson et al. 1998a). Controversies exist on the effects of photoperiod on the sperm quality of boars. Autumn photoperiod with short or decreasing day length stimulates the reproductive capacity of boars, which manifests itself in an increase of seminal volume and sperm concentration. It is not clear if photoperiods with long or increasing day length alter the reproductive capacity of boars; the decrease in sperm quality during summer is due to temperature increase and not to hours of light (Huang et al. 2010).

The effects of photoperiod in semen quality and fertility of selected AI boars have been extensively studied in different field experiences. Landrace boars housed under controlled temperature and humidity have been tested for different natural (Sancho et al. 2004) or artificial (Sancho et al. 2006) regime light conditions during 2.5 and 3 months, respectively. The effects of increasing daylight and of long daylight natural photoperiods have been assayed as well on Pietrain, Large-White and Duroc boars (Yeste et al. 2010).

The seminal volume of Landrace boars does not differ between increasing and decreasing natural photoperiods (Sancho et al. 2004). In Pietrain, Large-White and Duroc boars semen volume is neither affected by natural increasing of daylength, but it decreases significantly during a long-day natural photoperiod (Yeste et al. 2010). The sperm concentration is significantly higher in Landrace boars exposed to increasing day length as compared with those exposed to decreasing daylength (Sancho et al. 2004). In Large-White and Duroc, sperm concentration does not differ between increasing day length and long-day natural photoperiods; nevertheless, in Pietrain boars a progressive decrease in sperm concentration has been reported throughout the long-day photoperiod (Yeste et al. 2010). These observations indicate that the effect of photoperiod on sperm concentration is dependent on the breed. In a previous experiment, Claus and Weiler (1985) showed that penning of boars in an artificial photoperiod of long days results in increased sperm production. This increased sperm production has been attributed to a rise in the blood levels of testosterone (Minton et al. 1985) and follicle-stimulating hormone (FSH) (Lee et al. 1987) when boars receive supplemented light.

Increasing and decreasing natural photoperiods have no effect on sperm motility, sperm viability (estimated as the frequency of membrane intact spermatozoa), and sperm morphology of the Landrace breed (Sancho et al. 2004) or those of the crossbreds Large White × Pietrain and Duroc × Landrace × Large White (Rivera et al. 2005). The detailed analysis of sperm morphology indicates that the frequency of immature spermatozoa with proximal droplet is higher in Landrace boars exposed to decreasing photoperiods, and that the frequency of immature spermatozoa with distal droplet is higher in boars exposed to increasing photoperiods (Sancho et al. 2004). These results lead to conclude that in Landrace boars

decreasing photoperiod promotes epididymal deficiencies that alter the migration process of the cytoplasmic droplet and that the increasing photoperiod results in anomalies in the shedding process of the distal droplet during ejaculation (Sancho et al. 2004). In the Duroc breed, sperm motility, sperm viability (estimated as the frequency of spermatozoa with intact plasma membrane, intact acrosome and mitochondrial sheath integrity), and sperm morphology do not differ between increasing photoperiod length and long-day photoperiod (Yeste et al. 2010). In contrast, in Pietrain and Large White breeds, sperm motility, viability and morphology are significantly lower in long-day than in increasing day length (Yeste et al. 2010). The osmotic resistance of spermatozoa is affected by the natural photoperiod in Pietrain, Large White and Duroc boars, being significantly lower in long-day than in increasing day-length (Yeste et al. 2010).

In crossbred boars (Large White × Pietrain and Duroc × Landrace × Large White), no differences have been found between natural increasing and decreasing photoperiods on the sperm quality of both fresh semen and refrigerated doses for 72 h (Rivera et al. 2005). Taken together, these results indicate that significant differences exist among breeds in their response to natural light regimes.

Trudeau and Sanford (1986) reported a negative correlation between testis length and width and daylength ($r = -0.62$). Seasonal variations in testicular size may be mediated in part by photoperiod-induced endocrine mechanisms since testosterone concentrations are also negatively correlated with daylength (Trudeau and Sanford 1986; Andersson et al. 1998b). Thus, testosterone is necessary to maintain spermatogenesis, especially by regulating meiosis, and although FSH is necessary to initiate meiosis, further development of spermatocytes is only reached through maintenance of appropriate testosterone signaling (Zirkin 1998). Likewise, testosterone seems to be an important survival factor that protects germ cells from apoptosis during spermatogenesis; in this sense, a positive correlation between the number of spermatozoa in the testes and testosterone levels of the seminiferous tubule fluid has been reported (Zirkin 1998). Furthermore, testosterone plays an important role in sperm maturation throughout the epididymal duct (Lan et al. 1998). Therefore, it seems that light-induced fluctuations in testosterone levels may promote changes in both the spermatogenic process and the epididymal maturation (Andersson et al. 1998b). Drastic alterations in photoperiod can induce an unparallel synthesis of androgens and estrogens in Leydig cells of both peripubertal (Claus and Weiler 1985) and adult (Andersson et al. 1998a) boars.

Extreme artificial photoperiods affect the semen quality of healthy boars selected for AI, the response being more severe in the boar group kept in absolute darkness (0 h of artificial light per day) than in continuous light (24 h of artificial light per day) (Sancho et al. 2006). As compared with boars exposed to 12 h of artificial light per day, boars kept in continuous light exhibit lower sperm concentration, whereas sperm membrane integrity and sperm motility maintain normal values. Boars kept in absolute darkness show reduced sperm motility and membrane integrity as well as decreased percentages of spermatozoa with intact acrosomes, but they do not differ in sperm concentration from boars exposed to 12 h of artificial light per day (Sancho et al. 2006).

Continuing with the previous approach, semen volume has been observed to decrease in boars housed in continuous light conditions (24 h light), whereas it is not affected in boars housed in absolute darkness (0 h light). Seminal plasma composition determined from glucose, fructose, and sorbitol contents, as well as from total protein levels and their pattern, does not differ among the three artificial light regimens, although glucose contents are lower in both 24 and 0 h artificial light groups (Sancho et al. 2006). The lack of effect of darkness on semen volume is supported by observations by Strzezek et al. (1999). Brandt and Diekman (1985) hold that photophase length does not affect the total semen volume of the ejaculate; in the study by Sancho et al. (2006) artificial daylength is described as affecting seminal plasma in a quantitative, more than in a qualitative way. Lack of qualitative alterations is an interesting finding since the change in the protein composition of seminal plasma is frequently associated with reduced sperm viability and fertilizing ability due to anomalies in the capacitating process (Pérez-Pe et al. 2001). Seminal plasma sugars are the main energy source of ejaculated spermatozoa and necessary for sperm movement (Marin et al. 2003). In both extreme artificial light conditions sperm motility maintains high values, thus indicating that despite the decrease in glucose levels the overall sugar content is not dramatically affected by photoperiod (Sancho et al. 2006).

Results described by Sancho et al. (2006) are in disagreement with a previous study by Trudeau and Sanford (1986), who reported that protein and citric acid content of seminal plasma are highly correlated ($r = 0.98$) and inversely correlated with changes in daylength ($r = -0.80$). Protein and citric acid content are both indicators of the seminal gland function, which is under control of testosterone and estrogens; therefore, photoperiodic changes in glandular activity reflect changes in circulating testicular steroid levels (Trudeau and Sanford 1986). Total protein content in seminal plasma may influence transmembrane cation movement, whereas variations in citric acid levels could indicate changes in the osmotic pressure of seminal plasma (Trudeau and Sanford 1986). Seasonal changes in pH are also correlated with changes in daylength ($r = 0.64$) and total protein content of seminal plasma ($r = 0.64$) (Trudeau and Sanford 1986).

Acrosome integrity of spermatozoa diminishes in boars exposed to extreme artificial photoperiods, especially in those of absolute darkness (Sancho et al. 2006). Trudeau and Sandford (1986) reinforce the previous finding by reporting that the functional status and composition of sperm membranes vary with photoperiod. Indeed, the stability of plasma and acrosome membranes of spermatozoa mainly depends on the rate at which proteins and lipids become incorporated during spermiogenesis and epididymal maturation, as well as on the adsorption of proteins secreted by seminal vesicles during ejaculation (Ciereszko et al. 2000). Since no changes are detected in the total protein content of seminal plasma, anomalies in the acrosome membrane probably develop during spermiogenesis and/or epididymal maturation (Sancho et al. 2006).

An artificial light regime also affects reproductive performance. In boars exposed to continuous light (24 h light) or to absolute darkness (0 h light) both farrowing rate and libido scores are reduced by 30 %; however, the total number of piglets born is

unaffected (Sancho et al. 2006). In boars exposed to 12 h of artificial light, the farrowing rate also decreases at the end of the treatment, whereas the number of piglets born and the boar's libido remain constant (Sancho et al. 2006). To our knowledge, this is the only report correlating artificial photoperiod with boar libido, fertility, and prolificacy. Lack of clear correlation between semen traits and male fertility is in agreement with other reports stating that indicators of semen quality are not useful for predicting boar fertility (Gadea 2005; Sancho et al. 2006; Wolf 2010; Yeste et al. 2011). Extreme artificial photoperiods of constant light and absolute darkness may induce subtle defects on spermatozoa that cannot be detected using classical sperm parameters, but that result in decreased fertility and prolificacy rates.

In boars, increased blood and seminal plasma steroid levels are reported during artificial photoperiods of short daylength (Claus and Weiler 1985), whereas FSH blood levels increase when boars have supplemental lighting (Lee et al. 1987). Other studies have suggested that artificial photoperiods can influence prolactin levels in domestic pigs (Andersson et al. 1998a). Nevertheless, more studies are needed in order to better understand the effect of photoperiod on the hormone levels and reproductive performance of boars.

Results obtained in the three trials mentioned above (Sancho et al. 2004, 2006; Yeste et al. 2010) also suggest that the effect of photoperiod differ when boars are exposed to natural or artificial light, or to constant or variable daylength. Nevertheless, further research is needed to better determine the consequences of light exposition regimes on the reproductive performance of boars.

4.4 Extrinsic Husbandry Factors

Management of AI boars plays an important role in efficient semen production (Colenbrander et al. 1993; Flowers 1997). Several husbandry factors, such as nutrition, herd health, routine animal care, semen collection rhythm, and physical environment (in terms of housing, ventilation systems and collection pens) are reported to be essential for the maintenance of high semen quality and high field fertility (Kunavongkrit et al. 2005; Flowers 2008). A goal of large swine companies is to standardize management conditions across farms so that production is uniform throughout the entire system (Flowers 2008).

4.4.1 Frequency of Semen Collection

Frequency of semen collection is considered as one of the main husbandry factors significantly affecting both semen quality and fertility of boars (Pruneda et al. 2005; Smital 2009). Studies on the effect of collection frequency on boar semen quality have yielded variable results. While few authors have determined that boars submitted to a high collection rhythm produce semen with similar

progressive motility and sperm morphology (Audet et al. 2009), other authors have reported that boars under a high semen collection frequency have poor semen quality as a result of the forced passage of sperm cells from caput to cauda of the epididymis (Bonet et al. 1991; Strzezek et al. 1995; Pruneda et al. 2005). These divergent results indicate that consequences of the semen collection rhythm on sperm quality depend not only on the intensity of collection, but also on breed and age, as well as on ambient factors (Pruneda et al. 2005).

Regarding the intensity of semen collection, after a single extraction 60 % of the sperm cells stored in the epididymal cauda is emptied, and when three to four ejaculates have been collected at intervals of 12 h the epididymal cauda is almost completely depleted (Pruneda et al. 2005). Moreover, this high semen collection rhythm forces the passage of sperm cells through the epididymis and leads to defective sperm maturation (Briz et al. 1995, 1996) that results in a decreased percentage of motile spermatozoa and a rise in immature spermatozoa with proximal droplet (Bonet et al. 1991; Pruneda et al. 2005). Different semen parameters such as semen volume and sperm concentration are also altered (Bonet et al. 1991; Pruneda et al. 2005).

Other results demonstrate that the breed also modulates the effects of the collection rhythm. When submitting one-year-old boars characterized by high seminal quality to a rhythm of three collections per week during 2 months, sperm concentration in the Landrace breed is reduced by 43 % and sperm motility drops to only 35 % (Bonet et al. 1991), whereas in Landrace-Large White boars, sperm motility remains at 72 % (Huang and Johnson 1996). The analysis of sperm morphology of stressed Landrace boars also shows a significant decrease in the percentage of mature spermatozoa as a result of a significant increase in the proportion of immature spermatozoa with proximal droplet, which reaches 61 %, and in the percentage of aberrant spermatozoa, of about 6.5 % (Bonet et al. 1991). Aberrant spermatozoa are characterized by disturbances in the acrosome and/or the mitochondrial sheath (Bonet et al. 1991). In Duroc boars submitted to a collection of three ejaculates per week, Audet et al. (2009) found a significant decrease in sperm concentration and total sperm production, of 24 and 30 %, respectively, and a decrease of 11 % in semen volume; however, the percentages of total motile spermatozoa, progressive motile spermatozoa and normal spermatozoa were not affected.

Another approach was used on Pietrain boars submitted to two daily semen collections during 2 days (Pruneda et al. 2005). In these animals, the decrease in semen volume and sperm concentration was of 50 and 95 %, respectively, as compared with control males; the total number of spermatozoa per ejaculate was calculated to be $33.30 \pm 1.16 \times 10^9$ in control boars, whereas in stressed boars it was only $1.40 \pm 0.35 \times 10^9$ spermatozoa per ejaculate (Pruneda et al. 2005). Although sperm membrane integrity was not affected (85 % of spermatozoa with intact membrane) sperm motility dropped to 20 %. Other studies, including boars submitted to daily ejaculations during 10 days, corroborate that sperm membrane integrity decreases (Strzezek et al. 1995). The analysis of sperm morphology in the study by Pruneda et al. (2005) reveals increased percentages of immature

spermatozoa with proximal droplet (around 50 %) and of aberrant spermatozoa (around 6.5 %). Stressed boars, as compared with control boars, show an increased percentage of spermatozoa with abnormal head and tail shape and of spermatozoa with head and tail number abnormalities. In a similar trial performed on Duroc boars submitted to daily collections during 14 days, Audet et al. (2009) found a decrease of 19 % in semen volume, of 50 % in sperm concentration, and of 55 % in sperm production; nevertheless, these authors do not report alterations in sperm motility and sperm morphology.

The study performed by Pruneda et al. (2005) was not restricted to the analysis of semen quality on stressed boars, but it also focused on the effects of semen collection rhythm in the pattern of absorption and secretion of the epididymal epithelium, and in the maturation process of spermatozoa throughout the epididymal duct. To our knowledge this is the only study assessing the effects of increased semen collection on epididymal cell function and sperm maturation. Results obtained highlight that intensive sexual exploitation of Pietrain boars has a significant effect on the pattern of absorption and secretion of the epididymal fluid, decreasing the resorption in the proximal caput and altering the dynamics of resorption and secretion from the distal caput to the distal cauda (Pruneda et al. 2005). This change in the pattern of epididymal fluid resorption and secretion also results in significant alterations in intraluminal sperm concentration throughout the epididymal duct. An increase in sperm concentration associated to epididymal fluid resorption is observed from the proximal to distal caput of stressed boars, as in control boars; nevertheless, both intraluminal sperm concentration and percentage of fluid resorbed are significantly lower than in control boars. In stressed boars, intraluminal sperm concentration and percentage of fluid resorbed in the caput are 99 and 80 % lower respectively than in control boars (Pruneda et al. 2005). In control males a significant decrease in luminal sperm concentration is found throughout the proximal corpus due to intense fluid secretion; in stressed boars, intraluminal sperm concentration is maintained due to lack of fluid secretion (Pruneda et al. 2005). Intraluminal sperm concentration increases progressively from distal corpus to distal cauda of control boars as a result of fluid resorption; the distal corpus of stressed boars also exhibits a clear resorption activity, but throughout the proximal and distal cauda there is a decrease in intraluminal sperm concentration as a result of fluid secretion (Pruneda et al. 2005).

Therefore, high semen collection frequency brings about an altered resorption and secretion pattern of the epididymal fluid, which results in defective sperm maturation and abnormal development of sperm motility (Pruneda et al. 2005). Corroborating these outcomes, Strzezek et al. (1995) demonstrated that increased semen collection frequency induces changes in the biochemical properties of the sperm cells.

The comparative analysis of sperm quality throughout the epididymal duct between stressed and control boars also reveals significant differences. Sperm membrane integrity, used as an estimation of sperm viability, has been set at about 85 % along the entire epididymal duct in control boars; in stressed boars sperm membrane integrity has been of 65 % in the proximal caput, and of 85 % from

distal caput to distal cauda (Pruneda et al. 2005). This decrease in sperm membrane integrity only in the proximal caput has been correlated with the short duration of the stress period, of only 4 days; bearing in mind that stress caused by the semen collection rhythm results in an altered activity of epididymal cells, it is reasonable to suppose that longer stress situations may result in a progressive decrease in sperm membrane integrity throughout the epididymal duct. In control boars, the percentage of total motile spermatozoa increases progressively from 12 % in the proximal caput to 82 % in the distal cauda, achieving maximum values in the ejaculate (90 %); in contrast, in stressed boars the percentage of total motile spermatozoa increases steadily along the epididymis, and reaches only 20 % in the ejaculate. These results indicate that the decrease in sperm motility observed in boars submitted to stress by semen collection frequency is due to the shortened time that spermatozoa spend in the epididymal duct, in addition to the alteration in secretion-absorption flows at the epididymal epithelium (Pruneda et al. 2005).

Moreover, it has been concluded that the incidence of spermatozoa with proximal, intermediate or distal cytoplasmic droplet in control boars varies along the length of the epididymis. Most of the spermatozoa found in the proximal caput retain a proximal droplet (90 %) and only 0.5–1 % have intermediate droplets; spermatozoa with distal droplets have not been found. The percentage of spermatozoa with proximal droplet decreases to 18 % in the distal caput, whereas for spermatozoa with intermediate and distal droplet the percentages increase to 50 and 20 %, respectively. In the proximal corpus of control boars there is no presence of spermatozoa with proximal and intermediate droplets, while the frequency of spermatozoa with distal droplets reaches 75 %. The rate of spermatozoa with distal droplets does not change throughout the distal corpus and the proximal and distal cauda, but it decreases to 5 % in the ejaculate. In stressed boars, the pattern of migration of the cytoplasmic droplet throughout the epididymal duct is similar to that of control boars; nevertheless, the percentage of spermatozoa with proximal droplets is significantly higher than in control boars (Pruneda et al. 2005). Therefore, it can be concluded that a high semen collection rhythm results in anomalies in the migration of proximal droplets. As stated above for sperm motility, anomalies in cytoplasmic droplet migration correlate to both the shortened time spermatozoa spend in the epididymal duct and to the altered absorption-secretion pattern of the epididymal fluid (Pruneda et al. 2005). These latter changes probably cause alterations in the balance of proteins and ions of the epididymal fluid that interfere with the sperm maturation process (Briz et al. 1996).

The detailed study of sperm abnormalities shows in healthy boars that the percentages of spermatozoa with head and tail shape anomalies remain constant at around 10 % along the epididymal duct, while the proportion of spermatozoa with abnormalities in the head size, tail shape, and head and tail number are close to 0 % (Pruneda et al. 2005). In all the epididymal regions of stressed boars, the percentage of aberrant spermatozoa with abnormal head and tail shape is greater than in control boars; moreover, in the stressed boars the frequency of both anomalies

is significantly higher in the caput than in the cauda of epididymis. The frequency of spermatozoa with abnormalities in head size does not differ significantly between healthy and stressed boars throughout the caput and corpus, but it is significantly higher in the epididymal cauda of stressed boars (Pruneda et al. 2005). As stressed boars in this study were submitted to high semen collection frequency for only 4 days, and taking into account that spermatogenesis lasts 30 days, all the anomalies observed in the epididymis and ejaculate of these stressed boars are of epididymal origin. Throughout epididymal maturation, abnormalities in the shape and the size of the sperm head develop as a result of anomalies in the maturation of the nucleus and acrosome, whereas spermatozoa with abnormal tail shape are the result of tail coiling or bending along the epididymal duct (Briz et al. 1996). Anomalies in the number of heads and tails are the result of sperm agglutination throughout the epididymis (Briz et al. 1996).

The interval between semen collections has also a great effect on sperm concentration. Therefore, a progressive boost in sperm concentration occurs when dilating the interval between collections, reaching a plateau at 10 days (Wolf and Smital 2009). A slight gain in semen volume is observed when the elapsed time between collection intervals goes from two to 7 days; for longer intervals, the values do not change markedly (Wolf and Smital 2009). Motility tends to slow down, and the percentage of aberrant spermatozoa tends to increase when lengthening the interval between collections, but these changes are not significant (Wolf and Smital 2009). The optimum collection interval to magnify the number of sperm cells per ejaculate and the total sperm output ranges from 2 to 5 days (Smital 2009).

4.4.2 Nutrition

A positive correlation also exists between nutrition and semen quality. Supplemented diets with vitamins, antioxidants, or PUFA have been developed as a potential strategy for enhancing boar fertility (Audet et al. 2004; Strzezek et al. 2004; Castellano et al. 2010a, b; Yeste et al. 2010, 2011). Despite the large number of studies performed, several controversies still arise about the benefits generated by a specific substance.

To illustrate this controversy we will analyze the effects of the addition of PUFA to the diet of reproductive boars of different breeds on sperm quality. PUFA have more than one double bond within their molecular structure; depending on the position of the first double bond regarding the methyl end of the molecule we obtain omega-3 (ω-3), omega-6 (ω-6) or omega-9 (ω-9) PUFA (Howe et al. 2002). Two long chain ω-3 PUFA, eicosapentaenoic (EPA; 20:5, ω-3) and docosahexaenoic (DHA; 22:6, ω-3) acids, are especially abundant in natural sources as fish oils (Howe et al. 2002). PUFA influence the physical nature of cell membranes and are involved in membrane protein-mediated responses, lipid-mediator generation, cell signaling, and gene expression in many different cell types (Calder and Yaqoob

2009). High content in ω-3 PUFA is involved in plasma membrane fluidity and integrity, both being crucial for sperm fertility (Conquer et al. 2000). DHA has been detected as the main PUFA in the spermatozoa of human and other mammalian species (Tavilani et al. 2006) and it binds to phospholipids, which play a major role in the fluidity of the sperm plasma membrane (Ollero et al. 2000).

Supplementing the boar diet with PUFA has been used up to now as a strategy to improve the quality of seminal doses (Strzezek et al. 2004), and could also be used to improve the freezability of sperm samples (Safranski 2008). The effects of feeding boars with ω-3 PUFA differ depending on the breed; in Yorkshire × Landrace boars, supplementing the feed with ω-3 PUFA results in an increased number of sperm cells in the ejaculate but has an impact on their sexual behavior (Estienne et al. 2008); it improves sperm motility in Pietrain boars (Mitre et al. 2004). Different effects have been reported on sperm in the ejaculate of Large-White boars: increased percentage of progressive motility (Rooke et al. 2001; Mitre et al. 2004), increased percentage of intact plasma membranes and osmotic resistance (Strzezek et al. 2004), and decreased frequency of aberrant spermatozoa (Rooke et al. 2001). In contrast, Castellano et al. (2010a) reported that in Duroc boars, although supplementing their diet with ω-3 PUFA modifies the fatty acid composition of the sperm plasma membrane, it does not affect sperm production and quality. Divergences are probably related to the low number of animals used in most of these different approaches.

In order to obtain robust and reliable data about the effects of dietary supplementing with ω-3 PUFA on boar sperm quality, our group performed a comparative study among three different breeds including large numbers of animals: 48 Duroc boars, 48 Large-White boars, and 48 Pietrain boars (Yeste et al. 2011). The evaluation period lasted 26 weeks; at the beginning of the experiment, the age of the boars ranged between 12 and 14 months. Within each breed, boars were randomly separated into two groups; one group was fed with a diet supplemented with ω-3, and the other group was fed with a control diet. Throughout the experiment, the boars were housed in climate-controlled conditions and submitted to a semen collection rhythm of once per week (Yeste et al. 2011).

The results obtained indicate that supplementing boars' diet with ω-3 PUFA does not affect semen volume, sperm concentration, sperm functionality (estimated from the analysis of the integrity of the plasma membrane, the acrosome and the mitochondrial sheath), and sperm motility of Duroc, Large White, and Pietrain boars. Nevertheless, a significant increase in the percentage of mature spermatozoa is observed in Large-White and Pietrain breeds, but not in Duroc breeds, from week 10 until the end of the treatment (Yeste et al. 2011). In Large-White boars the increase in the percentage of mature spermatozoa manifests itself in association with a decrease in the frequency of immature spermatozoa from week nine until the end of the experimental period; in Pietrain boars, the increased proportion of mature spermatozoa is associated with a decrease in both the percentage of immature spermatozoa from week nine and of aberrant spermatozoa from week 12 (Yeste et al. 2011). The osmotic resistance of spermatozoa is not affected by

ω-3 PUFA supplementing in Large-White and Duroc boars, but it is significantly increased in Pietrain boars from week 11 to the end of the trial. Improved sperm morphology has also been reported in Large-White boars fed with a ω-3 PUFA supplemented diet (Rooke et al. 2001), but not in Yorkshire × Landrace boars (Estienne et al. 2008). In agreement with the results obtained by Yeste et al. (2011) concerning the osmotic resistance of spermatozoa, Rooke et al. (2001) reported an increase in the percentage of spermatozoa with intact acrosomes. Despite lack of abnormalities in osmotic resistance, Wathes et al. (2007) notice that ω-3 PUFA make spermatozoa vulnerable to reactive oxygen species.

The effects of ω-3 PUFA supplementing on sperm morphology and osmotic resistance of spermatozoa in both Pietrain and Large-White boars became statistically significant by weeks 9–12 (Yeste et al. 2011); in other studies, the effects of ω-3 PUFA (Estienne et al. 2008), as well as other diet supplements based on herbal preparations (Frydrychová et al. 2011), were also manifested 8 weeks after the experiment began. This may be related to spermatogenesis and sperm transit within the epididymis, which lasts 49–52 days (França et al. 2005). Differences among breeds regarding the effects of ω-3 PUFA supplementing could be related to the different composition of sperm plasma membrane, and to different influences of these fatty acids on spermatogenesis (Yeste et al. 2011). Further research is needed to determine how plasma membrane structure influences sperm morphology parameters.

Feeding ω-3 PUFA has also been considered a potential strategy to improve the storage of semen both in refrigeration and in cryopreservation (Castellano et al. 2010b), but no success has been achieved in cryopreservation and inconsistent responses have been obtained after long-term liquid storage. The results of PUFA supplementing on boar fertility in terms of farrowing rate and litter size have not yet been investigated.

Such disparity in outcomes indicates that the addition of a dietary supplement does not have a direct effect on sperm quality in itself, and that the effects depend on other physiological, genetic, and ambiental factors. Another study was set-up in order to show this interrelation among factors. It consisted of the evaluation of L-carnitine as a dietary supplement in boars of different breeds housed in variable ambient conditions (Yeste et al. 2010). L-carnitine (β-hydroxy-γ-trimethyl ammonium butyrate) is a vitamin-like compound synthesized in the liver, kidney, and brain through the conversion of two essential amino acids, lysine, and methionine (Krajcovicova-Kudlackova et al. 2000; Hoppel 2003). It plays an essential role in the cellular energetic metabolism because it functions as a carrier of fatty acids across the inner mitochondrial membrane, thereby facilitating β-oxidation and enhancing cell energy production (Hoppel 2003). L-carnitine is also known to fulfill important roles in mammalian sperm maturation and metabolism throughout the epididymis (Yakushiji et al. 2006), although its exact mechanism of action is unknown. An antiapoptotic effect of L-carnitine on different cells, including neurons, myocytes, hepatocytes, and lymphocytes has also been reported (Ng et al. 2004).

The first contact of spermatozoa with L-carnitine occurs within the lumen of the epididymis. In boars, the levels of L-carnitine in epididymal plasma vary from 24.0 ± 5.7 nmol/mg of protein in the proximal caput to 442.9 ± 39.1 nmol/mg of protein in the distal cauda (Pruneda et al. 2007). In humans, L-carnitine supplementation results in increased sperm concentration (Vitali et al. 1995). In boars, the addition of 500 mg/day of L-carnitine in the diet increases the semen volume and sperm concentration, thereby augmenting the number of AI doses produced per ejaculate (Baumgartner 1998). Nevertheless, Kozink et al. (2004) pointed out that the addition of 500 mg/day L-carnitine is only worthwhile when boars are submitted to an intensive collection period.

In the study of L-carnitine, Yeste et al. (2010) evaluated a total of 120 boars of Pietrain, Duroc, and Large-White breeds, with 40 boars equally sampled per breed. Boars in each breed were separated into two experimental groups, one group being fed with a control diet and the other group with a supplemented diet (625 mg/day L-carnitine) over a 20-week period to account for fluctuations in temperature and photoperiod (from February to July). All boars were aged between 10 and 15-months old at the beginning of the experiment.

Results obtained indicate no effect of L-carnitine supplementation on semen volume, sperm concentration, sperm viability, sperm motility, and osmotic resistance of spermatozoa in Pietrain, Duroc, and Large White boars, but a significant effect on sperm morphology in Pietrain boars (Yeste et al. 2010). Lack of effect of L-carnitine supplementation on semen volume and sperm concentration supports the hypothesis that L-carnitine has a key role in the epididymis by influencing sperm survivability, but it does not act directly upon spermatogenesis and accessory glands (Kozink et al. 2004; Jacyno et al. 2007). In asthenozoospermic patients, treatment with L-carnitine results in increased sperm motility (Ng et al. 2004); these results lead to speculate that high concentration of L-carnitine in the epididymal fluid, or even within the spermatozoa, produces energy for sperm motility (Yeste et al. 2010). Such an effect has not been observed, although this sperm parameter is negatively affected by high temperature and long-day photoperiod in Pietrain and Large White breeds (Yeste et al. 2010).

The main effects of feeding L-carnitine are observed in the sperm morphology of Pietrain boars after 13 weeks of treatment, whereas no remarkable effects on Duroc and Large-White breeds in terms of sperm morphology have been found (Yeste et al. 2010). In Pietrain boars, sperm morphology is negatively affected by high temperature and long-day photoperiods, resulting in a decrease in the percentage of mature spermatozoa associated to an increase in the frequency of immature spermatozoa with proximal and medial cytoplasmic droplet (Yeste et al. 2010). However, feeding L-carnitine may have a compensatory effect in terms of percentage of mature spermatozoa during summer weeks, because it prevents the increase in the frequency of immature spermatozoa (Yeste et al. 2010). These results are in agreement with the potential role of L-carnitine in sperm maturation. Jacyno et al. (2007) also describe a positive effect of L-carnitine on the sperm morphology of Pietrain boars after 1 week of administration.

It has been reported that epididymal spermatozoa accumulate both free L-carnitine and acetylated L-carnitine; L-carnitine acts as a transporter of fatty acids to the mitochondria, which are the main energy source for epididymal spermatozoa (Jeulin and Lewin 1996). Moreover, L-carnitine possesses antioxidant properties, plays an important role in cellular detoxification, and protects cell membranes against oxidative damage (Kalaiselvi and Panneerselvam 1998). L-carnitine enters spermatozoa by passive diffusion in the proximal cauda (Jeulin and Lewin 1996). According to this fact, Yeste et al. (2010) hypothesize that in Pietrain boars the levels of L-carnitine in the epididymal fluid are reduced during long-day photoperiods and high temperatures, and that feeding them with a dietary L-carnitine supplement may compensate for this reduction.

The effects of other supplements on the diet are poorly studied and the results are somewhat controversial. Prolonged selenium (Se) deficiency in boars manifests itself in low sperm concentration and sperm motility, and increased frequency of immature spermatozoa (Liu et al. 1982); however, the addition of inorganic Se to the diet of non-deficient boars does not improve sperm quality (Henson et al. 1983) or their reproductive performance (Marin-Guzman et al. 1997). Dietary Se in growing boars results in increased numbers of Sertoli cells and germ cells in the immature testes, thus leading to increased sperm production in adulthood (Marin-Guzman et al. 2000).

Dietary supplementation with fat-soluble and water-soluble vitamins also leads to increased semen production (Yousef et al. 2003) even in boars submitted to intensive semen collection (Audet et al. 2004). In Duroc boars, fat-soluble and water-soluble vitamin supplementation does not influence sperm production or sperm quality regardless of collection frequency (Audet et al. 2009). Vitamin E deficiencies cause testicular degeneration which results in a decreased number of germ cells and, therefore, in low sperm production (Cooper et al. 1987). Marin-Guzman et al. (1997) found a positive effect of vitamin E supplementation on sperm motility and the percentage of mature spermatozoa, which manifests itself in an increased fertility rate. Nevertheless, their results overlapped with environmental temperature and semen collection rhythm, so they could not infer a clear effect of vitamin E supplementation on boar reproductive performance. In a previous report, Brzezinska-Slebodzinska et al. (1995) found increased sperm concentration in the semen of boars supplemented with vitamin E.

The combined results indicate that the effects of supplemented diets are variable depending on the breed but also on ambient factors (Yeste et al. 2010, 2011). Others studies have also highlighted the importance of taking into account physiological factors such age or illness, genetic factors such as breed, and husbandry factors such as semen collection rhythm, when using dietary supplements (Marin-Guzman et al. 1997; Audet et al. 2004, 2009). It is also important to note that inadequate dietary supplementing could result in a lack of effect (Marin-Guzman et al. 1997) or even in an adverse effect (Frydrychová et al. 2011) on the reproductive performance of boars.

4.4.3 Social Factors

Social factors on the sexual behavior of boars can be profound since they can exert a long-term, and perhaps even permanent, effect. Nevertheless, few studies have been performed to determine the effects of social contact during rearing. Boars reared from three to 30 weeks of age without visual or physical contact with pigs achieve fewer copulations and display less courting behavior than boars reared in either all-male or mixed sex groups (Hemsworth and Tilbrook 2007). Since mating dexterity of socially restricted boars appears satisfactory, it is likely that sexual motivation is affected by social restriction during rearing. Subsequent research indicates that the lack of physical contact *per se* is predominantly responsible for depression in sexual behavior caused by rearing in social restriction (Hemsworth and Tilbrook 2007). Furthermore, the age at which social restriction is imposed affects the extent of depression in sexual behavior: boars reared in social restriction from 3 weeks of age have lower testicular size (Trudeau and Sanford 1986) and lower levels of sexual activity (Hemsworth and Tilbrook 2007) than boars reared in social restriction from 12 weeks. Moreover, boars reared in groups display a fully coordinated mating response at an earlier age than boars reared individually (Thomas et al. 1979).

The social environment around puberty and after puberty may also affect the sexual behavior of boars. Isolation of mature boars from sows depresses their sexual behavior; however, this effect is not permanent since rehousing isolated boars near females restores their sexual behavior within 4 weeks (Hemsworth and Tilbrook 2007). Housing in isolation of females does not affect testosterone concentrations. Interestingly, the oestrus status does not influence the effectiveness of females in maintaining the sexual behavior of mature boars (Hemsworth and Tilbrook 2007). Isolation from sows of young post-pubertal boars from 6 to 9 months of age reduces their subsequent sexual behavior at 26–40 months of age, even though they are housed near females when tested; in adult boars, short-term isolation from females does not affect semen quality, but it is associated with a reversible depression in copulatory performance (Hemsworth and Tilbrook 2007). The effects on sexual behavior of young boars of housing near mature boars or near a semen collection area, in which olfactory, visual, and auditory stimulations are provided, are unknown (Hemsworth and Tilbrook 2007). However, the interest in using exogenous hormones such as $PGF_{2\alpha}$ to expedite the training of young, sexually inexperienced boars for semen collection suggests the need to improve understanding in this area (Kozink et al. 2002). The benefits of social contact have clear implications for boars used in natural mating but might also have implications for boars used for semen collection, although sexual motivation may be less critical because of the moderate collection frequency generally required (Hemsworth and Tilbrook 2007).

Sexual stimulation, like observation of other animals mating, can increase short-term levels of sexual behavior in males but the presence of dominant males, even when physically separated, is proven to inhibit sexual behavior in rams and

bulls (Price 1987). Allowing boars to observe other pigs mating immediately prior to semen collection has been shown to increase the number of spermatozoa in the ejaculate (Thiengtham 1992). This form of sexual stimulation has been demonstrated in a number of studies to produce acute increases in plasma cortisol and testosterone concentration in boars (Jongman 1993). Other attempts, such as sexually stimulating boars by briefly delaying collection after mounting, have been unsuccessful in increasing sperm output (Arkins et al. 1988). Kuciel et al. (1983) reported a significant correlation between duration of ejaculation and volume of the ejaculate ($r = 0.58$), number of spermatozoa per ejaculation ($r = 0.30$) and sperm motility ($r = 0.29$).

In a study performed in Landrace boars from a temperate climate, Trudeau and Sanford (1986) demonstrated that semen volume for socially non-restricted boars (boars penned adjacent to each other and allowed minimal physical contact with gilts) is maximal during autumn and winter, declines in spring, and gradually increases again during late spring and summer. In contrast, socially-restricted boars have consistently lower volumes than socially non-restricted boars, and the seasonal variation is less pronounced (Trudeau and Sanford 1986). Differences in semen quality between socially non-restricted and restricted boars are more obvious in autumn and winter, although the increase in semen volume during spring and summer is also noted in socially-restricted boars (Trudeau and Sanford 1986). The social environment does not appear to have a major effect on seminal pH, sperm concentration, total number of spermatozoa per ejaculate, sperm motility or sperm morphology; the protein content per ejaculate is significantly lower in socially-restricted than in non-restricted boars (Trudeau and Sanford 1986). The reasons for the dramatic differences in semen volume between socially-restricted and non-restricted boars are unclear, but they could be related to differences in ejaculatory capability (Hemsworth and Tilbrook 2007) and in gonadal steroid production (Joshi and Raeside 1973).

Flowers (2006) performed a study in which boars were fostered at birth to be reared in litters of six or more than eight individuals. When trained for semen collection and mated with sows under controlled conditions, boars reared in litters of six were found to mount the dummy earlier, have higher sperm output between 39 and 54 weeks of age, and sire more piglets in inseminations with pooled semen from different males than boars in litters of more than eight. Therefore, adequate handling of individuals at early stages in life can have a profound influence on their productivity, although more research is needed in order to better establish the effects of social environment on boar fertility.

4.4.4 Sperm Handling

During semen processing (see Parts 3 and 4), spermatozoa are exposed to light and oxygen and subjected to several diluents, temperatures, pH gradients, and mechanical forces that can potentially alter the structure and function of the plasma

membrane (Leahy and Gadella 2011a). Prolonged exposure of cells to light and oxygen can create an oxidative environment that will trigger detrimental peroxidative processes (Browners et al. 2005).

Sperm handling procedures such as centrifugation and resuspension (Matás et al. 2011), density gradients (Berger and Parker 1989; Matás et al. 2003; Suzuki and Nagai 2003; Morrell et al. 2009), filtration (Bussalleu et al. 2009; Ramió-Lluch et al. 2009), refrigeration (Waterhouse et al. 2004; Gogol et al. 2009) and cryopreservation (Leahy and Gadella 2011a, b) induce plasma membrane (Bailey et al. 2008; Kim et al. 2011) and DNA alterations (Love et al. 2002; Fraser and Strzezek 2004), increase the susceptibility to oxidative damage (Neild et al. 2005; Leahy et al. 2010a), and/or activate capacitation-like processes and acrosome reaction (Leahy and Gadella 2011b). Maternal line breeds are reported to be more sensitive to sub-optimal semen handling than terminal line breeds (Sonderman and Luebbe 2008).

The majority of procedures which involve washing and pelleting cause a partial stripping of loosely associated extracellular coating material in ejaculated spermatozoa (Caballero et al. 2009), and also remove decapacitation factors and other proteins from seminal plasma (Leahy and Gadella 2011a). Density gradient washings, as well as filtration procedures (see Chap. 10), lead to the separation of immotile spermatozoa and debris from progressively motile spermatozoa, thus providing a sub-population of spermatozoa with signs of superior maturation, higher chromatin condensation and removal of cytoplasmic droplets (Bussalleu et al. 2009; Leahy and Gadella 2011a). These selection mechanisms are an analogy of sperm surface decoating during transport in the female tract and of competitive selection of mature spermatozoa mediated by the cervix and the oviductal isthmus (Holt and Fazeli 2010), and are also common practice for in vitro fertilization (Mortimer 2000). Selected spermatozoa are very sensitive to additives in the extenders, especially to the presence of capacitative agents such as bicarbonate, bovine serum albumin (BSA), and calcium ions (Leahy and Gadella 2011a, b; Puigmulé et al. 2011).

On the other hand, most handling procedures are performed below the physiological temperature of 37 °C. It must be taken into account that boar spermatozoa are very susceptible to cooling below 15 °C, the sperm plasma membrane being the primary site of cold-induced damage (Watson 2000; Bailey et al. 2008). It is likely that this damage is related to lipidic phase changes and the altered functional status of the plasma membrane (Watson 2000). The major phase change occurs during cooling from 15 to 5 °C (Drobnis et al. 1993), this being the most sensitive temperature range for temperature-dependent injury (Kim et al. 2011). Therefore, as temperature declines there is an inevitable reduction in the proportion of spermatozoa that maintains membrane integrity, ultrastructure and biochemical components, which is manifested in decreased sperm viability and sperm motility (Johnson et al. 2000; Casas et al. 2010), and increased acrosome alterations (Flores et al. 2008).

Capacitation-like processes have been extensively studied throughout the cryopreservation procedure (see Chap. 11). Boar spermatozoa undergo “cryo

capacitation" or capacitation-like changes when submitted to frozen-thawed protocols (Watson 2000; Bravo et al. 2005). In physiological or real capacitation, increased Ca^{2+} uptake due to cholesterol removal in the oviducts promotes a cascade of phosphorylations, which lead to changes in enzymatic activity and to hyperactivation of boar sperm motility (Guthrie and Welch 2005; Fàbrega et al. 2011; Puigmulé et al. 2011). Similar modifications have also been observed during cryo capacitation, which result in diminished lifespan of spermatozoa (Green and Watson 2001) and activation of apoptosis (Yoshimoto et al. 2008). Thus, cryo capacitation could be described as a premature capacitation in which most of the features of the physiological process are only partially accomplished (Green and Watson 2001).

Synthetic freezing diluents commonly contain tris(hydroxymethyl)aminomethane (TRIS) or citrate to provide an isotonic environment and pH buffering, glucose or fructose as an energy source, and non-penetrating, sand penetrating factors to protect spermatozoa from freezing-thawing damage, such as egg yolk and glycerol. It is accepted that egg yolk minimizes cold shock damage but its mechanism of action is still debated (Leahy and Gadella 2011b). Direct associations between lipids of egg yolk and lipids of the sperm membrane have been reported, minimizing lateral phase separations involved in cold shock deterioration (Ricker et al. 2006). Glycerol and other penetrating cryoprotectants are used to protect the cell content and prevent intracellular ice formation; however, inclusion of such agents must be carefully managed to reduce potentially detrimental osmotic effects (Kim et al. 2011; Leahy and Gadella 2011b).

In spite of considerable improvement in the last decades, cryopreservation remains highly damaging, resulting in significant lethal and sub-lethal effects on boar spermatozoa (Sancho et al. 2007; Casas et al. 2009, 2010; Kim et al. 2011). Regarding sperm surface, cryopreservation has three major effects: (1) decoating of extracellular matrix components and concomitant coating of proteins and lipids from the cryoprotective diluents (milk, albumin or egg yolk) (Ricker et al. 2006), (2) lateral phase separation of lipids and thus a lateral reordering of membrane components (Drobnis et al. 1993) and (3) the permeability of the sperm surface to water, ions, and cryoprotectants is altered (Leahy and Gadella 2011a). The extent of these changes determines whether or not spermatozoa from certain ejaculates (good freezers vs. poor freezers) can be used for cryopreservation (Hagiwara et al. 2009; Casas et al. 2009, 2010; Oldenhof et al. 2010). Compared to bovine and human spermatozoa, cryopreservation of boar spermatozoa is more critical due to the relatively low levels of cholesterol (Rath et al. 2009) and to the high content of PUFA (Cerolini et al. 2000) in the plasma membrane.

Temperature fluctuations and cell dehydration during cooling and freezing procedures induce lateral phase separation of lipids and, therefore, reordering of membrane components (Drobnis et al. 1993) and loss of PUFA and cholesterol (Chakrabarty et al. 2007). This alters the permeability of the sperm surface to water, ions and cryoprotectants (Oldenhof et al. 2010), and leads to cell weakening, reducing its ability to withstand future stresses (Leahy and Gadella 2011b). Proteins and lipids of cryoprotective diluents provide a partial protection from

these deleterious effects of freezing (Ricker et al. 2006). Rewarming of cryopreserved spermatozoa evidences the capacitating-like changes occurred during freezing, which follow a different pathway than true capacitation (Green and Watson 2001). These capacitating-like changes are activated by both temperature changes and osmotic stress, and they correlate with the poor fertility of frozen-thawed boar spermatozoa following AI (Guthrie and Welch 2005; Leahy and Gadella 2011b). Lateral phase separation of lipids in frozen-thawed spermatozoa is not completely restored upon thawing, inhabilitating the specific lateral rearrangements of the sperm surface lipids and proteins that spermatozoa undergo during true capacitation (Leahy and Gadella 2011a), which result in alterations to semen quality and fertility (Flores et al. 2008; Casas et al. 2009, 2010).

Due to the decapacitation effects of seminal plasma outlined above and its partial or complete removal during freezing, several researchers have investigated the protective effect of the addition of seminal plasma during sperm cryopreservation. Incubation of frozen-thawed boar spermatozoa with 50 % (v/v) crude seminal plasma improved motility and viability (Garcia et al. 2010), although the effect of seminal plasma varies according to the boar (Hernández et al. 2007), the ejaculate portion (Garcia et al. 2009) and the seasonality (Leahy et al. 2010b), as well as the previous processing of seminal plasma (i.e. fractionation) (Ollero et al. 1997) or sperm samples (i.e. washing) (Pérez-Pe et al. 2001). Finally, protocol variations such as time of addition (i.e. pre-freeze vs. post-freeze) and protein concentration also affect the response of spermatozoa to seminal plasma (Leahy et al. 2010b). These variants contribute to the controversial effects of seminal plasma on field fertility (Leahy and Gadella 2011b).

Besides stabilization of the sperm plasma membrane, inclusion of the entire seminal plasma during the freezing process holds the sperm sample in a non-capacitated state (Leahy and Gadella 2011b). Other studies report that supplementation with 10 % of crude seminal plasma to a capacitation medium prevents induced capacitation-like changes during liquid storage, but it is unable to prevent them during freezing (Vadnais and Roberts 2010). On the other hand, supplementation of 10 % seminal plasma to frozen-thawed spermatozoa is reported to have no effect on the farrowing rate (Abad et al. 2007a) but in other cases it is observed to improve it (Okazaki et al. 2009). Likewise, thawing boar spermatozoa in 50 % of seminal plasma is reported to increase litter size (Garcia et al. 2010), but post-thaw supplementation with 10 % of seminal plasma has no effect on this parameter (Abad et al. 2007a) or on the establishment of an oviductal sperm reservoir (Abad et al. 2007b).

Despite being poorly studied, freeze–thawing procedures also induce an impairment of DNA integrity, which could explain the low fertilizing ability of thawed samples despite showing reasonably good results in other sperm quality parameters, such as sperm viability, sperm motility, and acrosome integrity (Flores et al. 2008). Both the cooling phase and thawing process do not seem to result in DNA fragmentation in spermatozoa, but they do induce significant alterations in protamine-1-DNA complexes (Flores et al. 2008) and disrupt the disulfide bonds between cysteine residues in sperm head proteins (Flores et al. 2011). Alterations

in these complexes lead to the loss of DNA compactness, which manifests itself in a slight increase in the nuclear volume of spermatozoa. Moreover, boar ejaculates with poor survival after freeze–thawing show less homogeneous sperm chromatin than those with good freezing characteristics (Flores et al. 2008). Both the alteration in protamine-1-DNA structure and the disruption of disulfide bonds between cysteines could be induced by the oxidative damage of spermatozoa during the cooling process and by osmotic and mechanical stress during the thawing process (Flores et al. 2008).

Significant differences among animals have been reported in the ability of their spermatozoa to withstand freeze-thawing damage (Roca et al. 2006; Casas et al. 2009, 2010). In swine, this is the main factor affecting cryosurvival (Roca et al. 2006). The reasons for this significant male-to-male variation are not well understood but they could be related to differences among boars in the regulation of sperm volume (Chaveiro et al. 2006), to genetic factors (Thurston et al. 2002a, b), to the expression of housekeeping proteins (Casas et al. 2009, 2010), and to the content of seminal plasma proteins (Jobim et al. 2004).

In refrigerated AI seminal doses, oxidative damage of spermatozoa has been widely reported (Guthrie and Welch 2006; Awda et al. 2009). An extense range of commercial diluents have been developed for optimal preservation of boar semen at 15–17 °C during three or more days post-collection (Gadea et al. 2004); according to their conservative capacities, commercial extenders are classified as short-term (3–4 days), medium-term (5–6 days), long-term (7–8 days), and extra-long term (9 or more days) extenders (see Chap. 10). Not only do long term extenders enhance the longevity of sperm cells but they also preserve sperm motility and the structural integrity of the plasma membrane and the acrosome throughout the claimed period (Waterhouse et al. 2004; Estienne et al. 2007). The exact composition of commercial extenders is unknown but they contain an energy source (usually glucose) that allows the spermatozoa to maintain basal function, protecting substrates against thermal shock (usually BSA), buffer salts to avoid the negative effects of pH fluctuations [sodium bicarbonate, sodium citrate, TRIS, or N-2-hydroxyethylpiperzaine-N'-2-ethanesulphone acid (HEPES)], basic salts to ensure a proper osmotic balance (NaCl, KCl, and/or sodium citrate), and antibiotics to inhibit bacterial growth (Gadea et al. 2004; Gogol et al. 2009).

Semen dilution with an extender reduces the concentration of proteins present in seminal plasma, thus increasing the risk of functional and structural damage during storage, even for a short period (Waterhouse et al. 2004; Estienne et al. 2007; Gogol et al. 2009; Pérez-Llano et al. 2009). The plasma membrane is a dynamic structure that plays a crucial role in maintaining sperm viability by keeping the intracellular ambient intact, but also in the control of sperm function prior and during the fertilization process (Eddy and O'Brian 1994; Flesch and Gadella 2000). Therefore, the preservation of the ultrastructural characteristics of the sperm membrane is important for maintaining the fertilizing ability of spermatozoa during prolonged storage (Waterhouse et al. 2004). Semen dilution results in decreased fluidity of sperm membranes when using short-term extenders, but in increased fluidity when using long-term extenders (Dubé et al. 2004; Waterhouse et al. 2004; Gogol et al.

2009). This different effect correlates with the specific composition of short- and long-term extenders; therefore, several long-term extenders contain BSA, which favours the efflux of membrane cholesterol (Dubé et al. 2004; Matás et al. 2010), and HEPES, a zwitterionic organic buffer that is known to capture heavy metals and to control the pH (Johnson et al. 2000; Dubé et al. 2004). Increased membrane permeability leads to a rise in the intracellular calcium content that induces a capacitation-like destabilization in the membrane during storage, which results in a premature acrosome reaction and, subsequently, in the loss of the fertilizing ability of refrigerated doses (Waterhosue et al. 2004; Oh et al. 2010).

A subtle decrease in membrane fluidity in stored spermatozoa with short-term extenders is due to peroxidative damage; peroxidative damage occurs as a result of aging in stored spermatozoa with long-term extenders (Awda et al. 2009; Leahy et al. 2010a; Pérez-Llano et al. 2010). Peroxidative damage develops as a result of the reduced activity of antioxidant enzymes in spermatozoa (Neild et al. 2005; Leahy et al. 2010a; Pérez-Llano et al. 2010), and because of metabolic changes, which lead to an enhanced production of free oxygen radicals during storage (Hammerstedt 1993). PUFA of the plasma membrane are the preferred substrates of free radicals, resulting in the generation of reactive oxygen species (ROS) (Cerolini et al. 2000; Browners et al. 2005) and the irreversible reduction of the fluidity of the plasma membrane (Gogol et al. 2009). Decreased membrane permeability leads to anomalies in the activity of several proteins and enzymes, such as membrane ATPases, which finally results in altered levels of intracellular ions (Gogol et al. 2009). One intracellular source of ROS may be the leakage of electrons from the mitochondrial transport chain and the impairment of enzymes such as the NADPH-oxidase, synthesizing the superoxide anion (Baker and Aitken 2005; Guthrie and Welch 2006). The presence of ROS also leads to the formation of substances having cytotoxic properties (Aitken et al. 1998), which inhibit numerous cellular enzymes and metabolic processes including anaerobic glycolysis, further impairing ATP generation (Gogol et al. 2009) and protein phosphorylation (De Lamirande and Gagnon 1992). Altered content of ATP and intracellular ions (Gogol et al. 2009) and decreased phosphorylation of axonemal proteins (De Lamirande and Gagnon 1992) manifest themselves in reduced sperm motility.

Sperm motility has been used as an indicator of active metabolism and membrane integrity, and of the fertilizing capacity of spermatozoa diluted in commercial extenders (Estienne et al. 2007; Oh et al. 2010). Some authors have reported a significant boar effect with regard to the preservation of sperm motility (Gogol et al. 2009) and sperm viability (Pérez-Llano et al. 2009) during the conservation period, whereas others have pointed out the importance of identifying the most successful extenders in maintaining sperm quality during storage (Estienne et al. 2007). The addition of bicarbonate (Holt and Harrison (2002) and caffeine (Yeste et al. 2008) in short-term extenders has been correlated with increased sperm motility of semen doses, but also with an increased percentage of capacitated spermatozoa and acrosome-reacted spermatozoa.

Regardless of the type of extender, prolonged liquid storage results in the aging of spermatozoa due to increased DNA instability, mainly as a result of DNA

fragmentation (Love et al. 2002; Fraser and Strzezek 2004; Pérez-Llano et al. 2009, 2010; Kim et al. 2011). The increase in DNA fragmentation has been reported to be coincident with the progressive decrease in sperm motility during liquid storage (Pérez-Llano et al. 2010). Significant differences exist among boars in the percentage of spermatozoa with altered DNA during liquid storage (Fraser and Strzezek 2004), each boar showing a different critical time for DNA fragmentation (Pérez-Llano et al. 2010). Individual variation in the rate of DNA damage could be related to the inherent chromatin packaging of the spermatozoa (Love et al. 2002), and the time of storage before insemination is also a key factor to reduce the incidence of this alteration (Pérez-Llano et al. 2010). Storage temperature has a significant effect on the rate of DNA fragmentation, being significantly higher and earlier as temperature increases from 15 to 37 °C (Pérez-Llano et al. 2010). Therefore, DNA fragmentation begins at day two or three during sperm storage at 37 °C for 10 days, depending on the boar; it is detected in over 80 % of spermatozoa at day seven and nearly in 100 % of the sperm population at day 10; in contrast, during sperm storage at 15° for 15 days, DNA fragmentation begins at day seven or eight, and at day 15 it ranges between 5 and 43 % depending on the male (Pérez-Llano et al. 2010). Not only the temperature but also the extender type affects DNA status (Love et al. 2002); thus, extenders containing lipoprotein fractions result in lower levels of DNA damage, indicating that these fractions help to slow down sperm aging changes associated with genome alterations during storage (Fraser and Strzezek 2004).

DNA damage as a result of the aging of refrigerated spermatozoa is associated with oxidative damage (Aitken et al. 1998; Zini and Libman 2006; Kim et al. 2011), which produces single-stranded DNA breaks (Sundaram and Panneerselvam 2006); ROS are particularly efficient at producing 8-oxyguanosine residues ubiquitously distributed along single DNA strands (Oger et al. 2003). DNA damage during liquid storage can also be related to the increased activity of acrosomal enzymes released to the extracellular medium from non-viable spermatozoa; these enzymes could trigger a cascade of detrimental effects on viable spermatozoa, as a consequence of side-by-side cell death (Pérez-Llano et al. 2010). This hypothesis agrees with the decline in sperm quality parameters before observing any defects on DNA.

Antioxidant supplementation has been shown to increase the oxidative resistance of liquid-stored spermatozoa (Johnson et al. 2000; Aurich 2008) and of frozen-thawed spermatozoa (Kalthur et al. 2011). Such optimization techniques have resulted in increased fertility rates, particularly in liquid-stored semen (Leahy and Gadella 2011b).

4.5 Conclusions

Purebreds differ in sperm production, but not in sperm quality. The coefficient of variation among breeds is estimated at 30–40 % for semen volume, total number of spermatozoa and sperm concentration, whereas the coefficient of variation for sperm motility, sperm viability and sperm morphology is below 10 %. The

heritability of semen traits ranges depending on the breed from 0.14 to 0.28 for semen volume, from 0.13 to 0.20 for sperm concentration, and from 0.10 to 0.18 for total number of spermatozoa. Sperm motility, sperm viability, and sperm morphology have a low heritability, usually below 0.10; for this reason, they are frequently considered as variable parameters, not heritable seminal traits.

The selection of boars consistently producing high quality ejaculates which are resistant to refrigeration and cryopreservation procedures are key events in AI. In order to ensure high fertility rates, threshold values for seminal parameters have been established. However, the poor relationship between seminal parameters and fertility limits the sensitivity and specificity of such a cut-off. In order to improve the efficiency of AI procedures in terms of fertility and prolificacy, boar selection must be performed according to testis size at pre-pubertal age. In boars, testis size is positively correlated with daily sperm production, testosterone levels, libido, and reproductive longevity. Genetic defects in testicular size and structure, such as in cases of cryptorchidism, result in partial or total arrest of spermatogenesis at post-pubertal age.

The reproductive performance of AI boars in terms of sperm quality, fertility, and sexual behavior show clear seasonal changes. High ambient temperatures do not only decrease testicular function, but also induce transient disturbances in spermatogenesis and a decrease in steroidogenesis. Differences exist among breeds in their tolerance to heat stress, breeds from northern Europe being less tolerant than those from along the Equator. Divergences exist regarding the effect of photoperiod in the reproductive efficiency of AI boars. While some authors state that autumn photoperiods with decreasing day length stimulate the reproductive capacity of boars as compared with photoperiods of increasing daylength, others report significant differences among breeds in their response to light regimes.

Management of AI boars plays an important role in efficient semen production. One of the main husbandry factors affecting sperm quality and fertility of males is the frequency of semen collection. The optimal collection interval ranges between 2 and 5 days, and the reduction in this interval manifests itself in a decrease in semen volume, sperm concentration, and sperm motility, and in an increase in the frequency of immature spermatozoa with proximal droplet. Such disturbances in sperm quality are the result of both the forced passage of spermatozoa throughout the epididymis and the altered pattern of absorption-secretion of epididymal cells. The alteration degree of sperm quality is dependent on the breed, semen collection rhythm, and extent of stress.

A positive correlation exists between nutrition and semen quality. In order to enhance the reproductive performance of AI boars, different supplemented diets with vitamins, antioxidants, or PUFA have been tested. Nevertheless, controversies still arise about the benefits generated by a specific substrate, due to the fact that effects of supplementation are variable depending on the breed and the physiological status of males, as well as on environmental and husbandry factors. Moreover, inadequate dietary supplementing could result in lack of effect or even in an adverse effect on the reproductive performance of boars.

Social factors can exert profound and long-term effects on the reproductive efficiency of AI boars, and an adequate handling of boars during early stages of life can have great influence on their productivity. In this sense, social restriction results in reduced levels of sexual activity, the extent of depression inversely correlated with the age at which social restriction is imposed. Despite these results, further research is needed in order to better establish the effects of social environment on boar reproductive efficiency.

In AI practice, semen processing results in the exposition of spermatozoa to light and oxygen, and to several diluents, temperatures, pH gradients, and mechanical forces, which alter the structure and function of the plasma membrane and trigger detrimental peroxidative processes. Procedures which involve washing and pelleting cause the stripping of loosely associated extracellular coating material in ejaculated spermatozoa, and also remove decapacitating factors. Therefore, these washed and pelleted spermatozoa become very sensitive to certain additives in the extenders, especially to capacitative agents. Changes in temperature alter both membrane integrity and composition, which is manifested in decreased sperm viability and motility. Freeze-thawing protocols also induce both the "cryo capacitation" or capacitation-like changes in spermatozoa, as a result of lateral phase separation of membrane lipids, and the impairment of DNA integrity due to the loss of DNA compactness. Nevertheless, significant differences exist among boars in the ability of their spermatozoa to withstand freeze-thawing damage. Refrigeration of seminal doses results in the oxidative damage of spermatozoa and altered membrane permeability; prolonged liquid storage leads to the aging of spermatozoa which manifests itself in increased DNA fragmentation. Again differences exist among boars in the rate of DNA damage during liquid storage.

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Part II
Boar Spermatozoa Within the Female Genital Tract

Chapter 5
Boar Spermatozoa Within the Uterine Environment

Marc Yeste and Miriam Castillo-Martín

Abstract The present chapter overviews some aspects of the anatomy, histology and physiology of the female genital tract, focusing especially on the uterus, through which sperm are transported. Moreover, some aspects of sperm interaction with uterinic epithelial cells are referred to in line with recent studies. This chapter also deals with other relevant aspects, such as the functional role of ejaculate volume on sperm transport, and the effects of placing sperm at different parts of the sows' tract in fertility and prolificacy rates. Finally, it ends with the role of reproductive immunology in response to spermatozoa, seminal plasma and short- and long-term extenders within the intrauterine environment.

5.1 Introduction: The Female Reproductive Tract

5.1.1 A General Overview of the Swine Genital Tract

The sow's reproductive tract is formed by the following organs, listed here in the reverse direction of the pathway followed by spermatozoa: ovaries, oviducts, uterus, cervix, vagina and external genitalia (Fig. 5.1). All these organs, except the ovaries, form the tubular genitalia.

Each of the two ovaries has a length of approximately 5 cm and presents an irregular shape as a result of numerous follicles and corpora lutea protruding from the surface (Edström 2009). Their main function is to produce follicles, oestrogens (mainly oestradiol), and progesterone (Michael and Schofield 1969).

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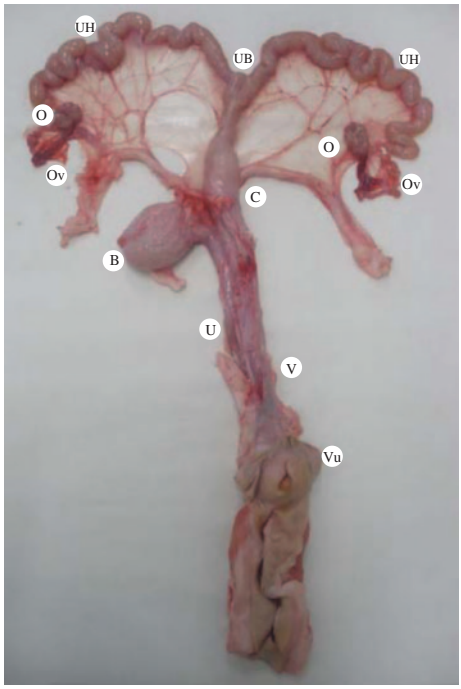

Fig. 5.1 General view of the sow reproductive tract. Abbreviations mean *O* ovaries, *Ov* oviducts, *UH* uterine horns, *C* cervix, *B* urinary bladder, *V* vagina, *Vu* vulva, *U* urethra and *UB* uterine body

The oviducts are tubular conduits connecting the ovaries with the uterus. Each oviduct has a length of about 20 cm and can be divided into three parts starting from the ovarian side: infundibulum, ampulla and isthmus. The ampullary-isthmic junction is the site of fertilisation (Hafez 1993).

The uterus is formed by two long uterine horns and a short body. In non-pregnant sows, the length of each uterine horn is about 60–90 cm. Spermatozoa pass through both horns before reaching the oviducts, and both horns are the sites of implantation and foetal development. The other part of the uterus, the uterine body, which is small if compared to other domestic species, is located at the junction of the two uterine horns (Hafez 1993; Thibault et al. 1993).

The cervix is a muscular conduit connecting the vagina and the uterus and the site of semen deposition during natural mating and artificial insemination (AI). Its length is about 25 cm and it has internal interdigitating mucosal prominences. Cervical morphology depends on the stage of the oestrus cycle, since it is dilated when the sow is in heat and constricted during pregnancy and the remaining period of the oestrus cycle (Hafez 1993).

The vagina reaches from the cervix to the urethral orifice. Since the urethra connects the bladder to the vagina, the vagina serves at the same time as a passageway for urine and for the piglets at birth.

Finally, there are the external genitalia, consisting of the vestibulum and the vulva, which is in turn formed by the labia, the clitoris and the vestibular glands. The vestibulum extends from the urethral orifice to the vulva, and the vulva is the

external portion of the genital tract of the sow. Its aspect also changes depending on the stage of the oestrus cycle and on sow parity, so that it becomes red and swollen just prior to the oestrus and more swollen in gilts than in sows (Edström 2009).

Although the books covering the female genital tract usually start with the description of the ovaries and end with that of the external genitalia, this chapter will follow the opposite direction starting with the external genitalia. The main purpose is to focus on the transit of a spermatozoon within the female reproductive tract, i.e. from the deposition site towards the ampullary-isthmic junction.

5.1.2 Histology of the Swine Reproductive Tract: General Pattern

The tubular genitalia follow a pattern that is commonly observed in most tissue sections (Edström 2009).

First, we can distinguish the mucosa, which surrounds the lumen and consists of an epithelium that fulfils different functions depending on the organ (e.g. uterus vs. oviduct). Underlying the epithelium, there is a layer of connective tissue with varying thickness and structure among the reproductive organs. In this connective tissue, we can find other cell types such as the immune cells, which play a relevant role as will be explained below (Finn and Porter 1975; Hafez 1993; Edström 2009; Yáñiz et al. 2006).

Surrounding the mucosa, there is the muscularis, made up of two layers of smooth muscle cells, a circular inner and a longitudinal outer layer.

Finally, there is an outer layer of connective tissue that surrounds the organs. While in the peritoneal cavity the organs are covered by the peritoneum, a serosa consisting of a simple squamous epithelium, in the pelvic cavity there is a tunica adventitia of loose connective tissue. The reproductive organs are contained within the pelvic cavity, except for the most cranial part of the vagina (which has a serosa), and are therefore surrounded by loose connective tissue (Edström 2009).

As observed from histological samples, ovaries, oviducts and the uterus are mainly innervated by autonomous nerves, while the pudendum nerve is the one that innervates the vagina, the vulva and the clitoris by sensorial and parasympathetic fibres (Hafez 1993).

5.2 Concepts Relating to Swine Reproductive Physiology

5.2.1 Puberty and Sexual Maturation

In pigs, females reach puberty at the age of 6–7 months. However, this age can be influenced by breed, season, management and/or nutritional factors (see Chap. 4).

In fact, the ovaries are controlled by the hypothalamus and the pituitary gland and their activity determines both the onset of puberty and other subsequent events.

Pregnancy lasts approximately 113 days and during lactation, sows are still in anoestrus. The interval between weaning and oestrus is about 4–6 days (Knobil and Neill 1994).

5.2.2 The Oestrous Cycle

The domestic pig (*Sus domesticus*) is a poly-oestral species, which means that the female has regular oestrus cycles throughout the year, the cycle being interrupted when the females are pregnant or lactating (the anoestrus period).

In swine, the oestrus cycle averages 21 days, ranging between 18 and 24 days, and is defined as the period of time from the onset of one oestrus to the onset of the next. This cycle can be divided into three different stages: proestrus (1–3 days), oestrus (1–3 days), metoestrus (2–3 days) and dioestrus (13–18 days), and the first day of standing oestrus is generally considered to be the first day of the cycle.

The oestrus corresponds to the ovarian follicular phase, as follicles are the predominant structures in the ovaries. About 3 days after the onset of standing heat, follicular growth is accelerated by the follicle-stimulating hormone (FSH) (Fig. 5.2). Around 20 follicles, between 6 and 10 in each ovary, are ovulated in each oestrous cycle leading to an increase in the corresponding number of corpora lutea (Hafez 1993; Knobil and Neill 1994).

There are three different organs involved in the control of the oestrous cycle: the hypothalamus, the hypophysis and the ovaries. The hypothalamus secretes the gonadotropin-releasing hormone (GnRH), which stimulates the hypophysis to secrete FSH and luteinising (LH) hormones (Blödow et al. 1990). FSH stimulates follicles to produce oestradiol (Fig. 5.2); each follicle contains a maturing oocyte and the granulosa cells responsible for its secretion. This oestradiol stimulates, in turn, the follicular growth and acts on the external genitalia, on the cervix and on the uterus, thereby preparing the female both for mating and for implanting the fertilised egg in the endometrium (Eiler and Nalbandov 1977). Oestradiol produces the typical signs of the oestrus, i.e. swollen and hyperaemic vulva, restlessness and riding behaviour, increase in secretory activity and hypertrophy and oedema of the genital tract (Mburu et al. 1998). Finally, the increase in oestradiol levels stimulates the hypophysis to release LH, a peak of this hormone leading to ovulation (Fig. 5.2) (Eiler and Nalbandov 1977). Ovulation occurs at the beginning of the last third of the oestrus stage, i.e. approximately 40 h after the onset of oestrus.

As far as the different stages of the oestrous cycle are concerned, pro-oestrus lasts, as mentioned, between 1 and 3 days and is characterised by follicular growth and regression of the corpus luteum of the previous cycle (Espey and Lipner 1994). Granulose cells inside the developing follicles produce oestrogen, responsible for the typical outer signs of an approaching oestrus.

During oestrus (1–3 days), the sow or gilt is sexually receptive and thus accepts mating by showing a standing reflex (stiffness) when the loin is firmly pressed in

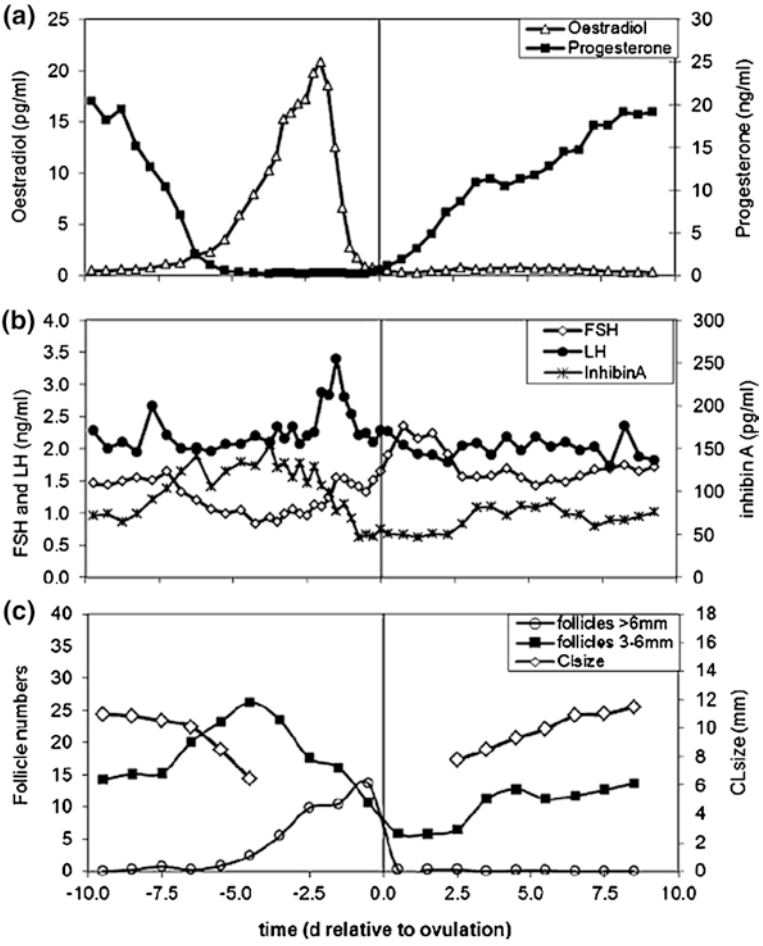

Fig. 5.2 Oestrous cycle in pigs. **a** Plasma concentrations of 17β-oestradiol and progesterone, **b** Plasma concentrations of FSH, LH and Inhibin-A, and **c** development of follicular and corpora lutea (*CL*). The *vertical line* in the figures represents time of ovulation (Soede et al. 2011, Reproduced with permission).

the presence of a boar. The increase in oestradiol levels provokes oedema of the oviducts, endometrium, cervix and vulva, this effect being more pronounced in gilts than in sows, an increase in the production of vaginal mucus, and, finally, ovulation itself. There are secondary signs that the female exhibits during the oestrus: increased nervous activity, desire to seek the boar, loss of appetite, changes in vocalisation and a male-like sexual behaviour (pursuing, nosing and mounting other females) (Eiler and Nalbandov 1977; Espey and Lipner 1994).

Metoestrus (days 2–3) and dioestrus (days 13–18) are collectively called the luteal phase, with the corpora lutea being the main functional ovarian structures during this phase. After ovulation, ruptured follicles evolve into luteinised cells,

which form the corpora lutea and produce progesterone, about 1–2 days after mating (Shille et al. 1979). Then, there is a decrease in the oestradiol levels and an increase in progesterone (Noguchi et al. 2010; Soede et al. 2011).

Finally, at dioestrus the corpora lutea continue producing progesterone, which results in stimulation of secreting activity in the uterine glands. During this stage, the uterus is prepared for foetal membrane attachment and placentation. If the sow becomes pregnant, the corpora lutea continue to release progesterone together with relaxin until parturition is approaching, so that progesterone is responsible for maintaining pregnancy. On the other hand, the high levels of progesterone block the onset of a new oestrus cycle because they inhibit GnRH secretion in the hypothalamus, which in turn impedes complete maturation and ovulation of new follicles (Hafez 1993; Mburu et al. 1998; Mwanza et al. 2000; Razdan et al. 2001).

If there is no conception, luteolysis occurs when the end of dioestrus is approaching in the presence of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a hormone secreted by the non-pregnant uterus that diffuses from the uterine vein and the ovarian artery to the ovaries (Shille et al. 1979). Luteolysis leads to a decrease in progesterone levels by day 17 of the cycle, which also involves return of hypothalamic stimuli. Release of GnRH is restored in this way, and a new cycle starts with the development of new follicles in the ovary (Knobil and Neill 1994; Mirando et al. 1995).

Lactation is also capable of inhibiting the oestrous cycle, which will be restored 4–7 days after piglet weaning; this period of time depending on several factors, such as the length of lactation, parity, nutrition or season (Knobil and Neill 1994). In fact, shortening lactation and performing weaning as soon as possible is very important in economic terms for AI centres and pig breeders.

The length of oestrus is variable and may last from only 12 h in gilts to up to 60 h or more in sows. Since the real time of its onset is rarely known, it is recommended that a female receives at least two matings or two inseminations in oestrus. This practice ensures that spermatozoa will be present in the oviduct when fertilisation occurs, with the corresponding optimisation of farrowing rates and litter size (Thibault et al. 1993). Here, the role of the sperm reservoir is very important, as will be discussed in the Chap. 6. Other aspects of reproductive physiology relating to AI and mating will be taken up in the (Chap. 12) of this book.

5.3 External Genitalia

The external genitalia comprise the vestibulum, the major and minor labia, the clitoris and the vestibular glands (Hafez 1993).

5.3.1 Vestibulum

The union between vestibulum and the vagina is characterised by the presence of the urethral orifice, and sometimes by the vestigial hymen. During the development of sexual organs, two ducts, the Müllerian and the Wolffian, coexist until the

sex of the embryo is defined. In females, the Wolffian duct regresses to a remnant called Gartner's duct that leads to the vestibulum. Particularly, the vestibulum has subepithelial lymphatic nodules in the connective tissue stroma. Bartholin ducts are the conduit between vestibulum and Bartholin glands, which are located on the external lateral wall of the vaginal vestibulum (Knobil and Neil 1994). These glands secrete a viscous liquid into the vestibulum that is more viscous during oestrus, and presents a tubo-alveolar structure similar to that of the boar bulbourethral glands (see Chap. 3).

5.3.2 Major and Minor Labia

The major labia contain fat deposits, elastic tissue and a thin muscular layer, and the external surface presents the same structure as the epidermis. The integument of major labia contains numerous sebaceous and tubular glands.

Minor labia have a nucleus made of spongeous connective tissue, their surface containing numerous, large sebaceous glands (Hafez 1993).

5.3.3 The Clitoris

The ventral commissure of the vestibulum hides the clitoris, which has the same embryonic origin as the boar penis. It is formed by erectile tissue covered by a squamous and stratified epithelium and presents numerous sensorial nerve terminations. It is long and sinuous and ends in a small cone or tip (Hafez 1993).

5.4 The Vagina

5.4.1 Anatomy and Histology

The vagina extends from the cervix to the urethral orifice and is the receptacle for the boar penis during copulation (Fig. 5.1). The height of its epithelium also depends on the stage of the oestrus cycle, so that the maximum thickness is observed in the late proestrus (Knobil and Neill 1994).

The vaginal wall consists of a superficial epithelium, a muscularis and a serosa.

The muscularis is not as developed as in the external parts of the uterus, and consists of two thinner smooth muscle layers. The inner is circular and the outer is longitudinal and continuous with the uterus. The muscularis contains a large number of blood vessels, nervous bundles and connective tissue both dense and lax (Hafez 1993).

The superficial epithelium is formed by squamous stratified cells and does not contain secretory glands. The surface of epithelial vaginal cells contains microborders ordered longitudinally or in circles. In this pluristratified epithelium, cells form a firm and consistent structure because the microborders of one cell interact with those of another.

The reproductive cycle influences the morphology and disposition of microborders, so that they exhibit a regular pattern during pregnancy but present inner pores throughout the oestral cycle (Hafez 1993).

5.4.2 Physiological Responses: Contractions and Vaginal Fluid

Vaginal contractility plays a main function in psychosexual responses and seems to play an indirect role in sperm transport (Langendijk et al. 2005; Levin 2011; Suarez and Pacey 2006). It is stimulated by the vaginal fluid during stimulation previous to coitus.

This vaginal fluid consists of transuded secretions through the vaginal wall and also contains vulvar secretions coming from sebaceous and sudoriparous glands. Moreover, this fluid contains traces of endometrial fluid and cervical mucus, as well as exfoliated cells from the vaginal epithelium (Hafez 1993).

5.4.3 Functions of the Vagina

The vagina is the copulatory organ in the female. After coitus and ejaculation, the seminal plasma is not transported into the uterus but it is either expelled or absorbed through the vaginal wall. When absorbed, the components of seminal plasma trigger physiological responses in other parts of the female reproductive tract, such as the endometrium and the ovary, that affect sow reproductive physiology and improve the chances of conception and pregnancy success (Robertson 2007; O'Leary et al. 2011). Accordingly, within these physiological responses triggered by seminal plasma components, we can find inflammatory responses, including altered patterns of cytokine secretion that facilitate embryo development and implantation (Robertson 2007), and regulation of ovulation timing, corpus luteum development and steroid production in the ovary (O'Leary et al. 2002). Specifically, the cytokines and prostaglandins that seminal plasma contains bind to receptors on target cells in the cervix and uterus, thereby activating changes in gene expression that lead to modifications in structure and function of the female tissues (Kaczmarek et al. 2010) (see also Sect. 5.8). In the case of endometrium, seminal plasma induces pro-inflammatory cytokines and cyclooxygenase-2 and causes recruitment of macrophages and dendritic cells. Apart from seminal plasma components, spermatozoa also contribute in male–female signalling

because interaction with seminal plasma factors modulates neutrophil influx into the uterine luminal cavity. Recently, transforming growth factor-β (TGF-β), a potent immune-modulating cytokine present in the seminal plasma of boars, mice and humans, has been suggested to be involved in the immune changes of female reproductive tract elicited by seminal fluid (O'Leary et al. 2011).

On the other hand, vaginal secretions present a low pH which is unfavourable for spermatozoa. A complex interaction between cervical mucus, vaginal secretion and seminal plasma form a buffer system that protects sperm until it is transported towards the micelles of cervical mucus.

Finally, we must mention that the vagina acts as an excretory duct for secretions of the uterine body, endometrium and oviducts, and it also functions as parturition canal. Different physiological features are involved in all these functions: contraction, extension, involution, secretion and absorption (Hafez 1993).

5.5 The Cervix

5.5.1 Anatomy and Histology

The cervix is located in the pelvic cavity, where semen is deposited after mating or after conventional AI (Fig. 5.1). As stated, its length is about 25 cm and has internal interdigitating mucosal prominences (*pulvini cervicales*). It can be divided into two different regions: the shorter is the uterine region, with a length of 6–7 cm, while the larger is the vaginal region, which is about 12–14 cm long. The cervix has two different ends, the so-called utero-cervical end being more pronounced than the cervico-vaginal end (Edström 2009).

In terms of histological structure, the cervical epithelium undergoes cyclic variations and is formed by stratified squamous cells and columnar cells that can cover more than 90 % of the mucosa. The mucosa presents folds and the underlying stroma is made up of connective tissue containing capillaries and small blood vessels.

The muscularis is formed by two layers, the inner circular layer and the outer longitudinal one, that are arranged in bundles and do not extend into the mucosal prominences. The muscularis is surrounded by a serosa of loose connective tissue towards the abdominal cavity (Hafez 1993).

5.5.2 Cervical Changes During the Oestrous Cycle

The cervix becomes increasingly firm and projects horizontally into the abdominal cavity when the oestrus is coming, and it progressively softens to hang limply over the pubic border by 7 of the oestrous cycle (Rigby 1967; Meredith 1977; Edström 2009).

The literature has reported inconsistent results regarding the histological properties of the cervix during oestrus. Thus, while Steinbach and Smidt (1970) did not report any significant cyclic variation in the height of the epithelium at the uterine part of the cervix, Smith and Nalbandov (1958) observed that the cervix was mostly constricted during oestrus. Indeed, and according to these authors:

1. The constriction/firmness reaches its maximum by days 1 and 2 of the oestrous cycle.
2. After this, the cervix progressively relaxes and softens up to day 9 of the cycle.
3. From days 13–14, the cervix gradually increases its constriction/firmness (Edström 2009).

Although the oestrous cycle does not affect the size of cervical lumen at the uterine portion, the changes in cervical consistency seem to be caused by fluctuations in the rigidity of the tissue rather than by modulations of the muscular contractility. In fact, cervical consistency depends on oestrogen levels. Thus, the increase in firmness of the cervix is related to the rise in oestradiol levels that precedes the oestrus (Kunavongkrit et al. 1983; Edström 2009), while softening of the cervix during the post-oestrus phase occurs as a consequence of the absence of oestradiol in plasma. In this respect, there are contradictory reports on the effects of the presence/absence of sexual hormones during post-oestrus (Smith and Nalbandov 1958; Kunavongkrit et al. 1983), but it seems that it is the absence of oestrogen rather than the presence of progesterone which is responsible for cervical softening (Edström 2009).

5.5.3 Cervical Changes Related to Pregnancy and Parturition

The cervix also changes during pregnancy and parturition and its physiological status differs from that described during the oestrous cycle.

When sows are pregnant, the main function of the cervix is to protect the uterus (Eldridge-White et al. 1989). This explains why the extensibility and the lumen diameter of the uterine part is lower than those of the vaginal portion during the first 80 days and suggests that, at least during this period of time, the uterine part is more involved in the protection of the uterus than the vaginal region. By day 80 of pregnancy, the uterine cervix increases its softness and extensibility, with no differences when compared to the consistency of the vaginal portion. Here, it is important to keep in mind that the cervix has to be extensible during parturition to allow passage of the foetuses. The increase in firmness is related to progesterone levels, which are high throughout the entire pregnancy, while the softness of the uterine portion appears to be related to relaxin (O'Day et al. 1989) and oestradiol levels, increasing from day 80 until the end of pregnancy (Eldridge-White et al. 1989). It must be also taken into account that progesterone and relaxin are produced by the corpora lutea, and oestrogen is produced by the placenta (Edström 2009).

Finally, it is noteworthy that relaxin influences the cervical connective tissue, reducing the collagen concentration and increasing water content, dry weight and the glycosaminoglycan to collagen ratio (O'Day-Bowman et al. 1991). During late pregnancy, relaxin appears to cause histological changes in the cervix, thereby reducing collagen density and influencing the organisation of muscle fibres and collagen fibre bundles (Winn et al. 1993).

5.6 The Uterus

5.6.1 Anatomy and Histology

In the domestic pig, the uterus is formed by a short (3–4 cm) body (*corpus*) and two long uterine horns (*cornuae uteri*) (Fig. 5.1). The length of each uterine horn depends on age and whether the female is pregnant or not. Thus, it measures about 60 cm in gilts, 100 cm in non-pregnant sows and may reach 200 cm in the pregnant sow. Notwithstanding, the uterus acquires its full morphological traits by the second oestrus after puberty (Schnurrbusch and Erices 1979; Norrby 2010).

Like the oviducts and ovaries, the uterus is located in the abdominal cavity and is suspended from the abdominal wall by the broad ligaments, including the mesometrium and continuing as mesosalpinx and mesovarium (Finn and Porter 1975).

In the uterine wall, we can distinguish three different layers: the endometrium, surrounding the uterine lumen, the myometrium, and the perimetrium, which is the most outer layer in the vicinity of the abdominal cavity (Edström 2009; Norrby 2010).

The endometrium is the uterine mucosa and consists of a simple to pseudostratified, columnar epithelium (Eslaminejad et al. 2007), depending on the phase of the oestrous cycle, and a loose connective tissue stroma with capillaries and small blood vessels with ciliated and secretory cells, which produce and secrete hormones and nutrients for embryo development (Walter and Bavdek 1997). The subepithelial layer is highly vascularised and infiltrated by several types of cells of the immune system (Romek and Karasinksi 2011).

The myometrium is the tunica muscularis of the uterus and it is made up of three layers of smooth musculature. The inner layer is thicker and forms two circular patterns that come from the oviducts and extend to the cervix. The middle layer is constituted by fibres randomly organised, which run lengthwise, widthwise and diagonally, and supports the large blood vessels that nourish the myometrium. Finally, the outer layer is thinner and longitudinal (Finn and Porter 1975). On the other hand, the myometrium thickens during pregnancy to allow embryo post-implantation development, and its contractile activity depends on the status of the (pregnant or non-pregnant) female and the stage of foetal development (Michael and Schofield 1969), so that it increases at the moment of parturition.

Finally, there is the perimetrium, which is the outer layer of the uterus and is formed by a layer of connective tissue covered by a serosa, in a similar fashion to

the oviducts (Edström 2009). It has the typical composition of loose connective tissue, but it contains a large number of lymphatic vessels.

5.6.2 Uterine Changes During the Oestrus Cycle

During the oestrous cycle, the morphological properties of the endometrium vary (Walter and Bavdek 1997). Thus, and in accordance with what we stated previously, the nature of the uterine epithelium changes depending on the phase of the oestrous cycle, as follows:

1. At oestrus and early dioestrus, the epithelium is high columnar and pseudostratified (Stroband et al. 1986; Kaeoket et al. 2001).
2. During dioestrus, it is low columnar.
3. At late dioestrus and proestrus it is simple cuboidal or low columnar (Kaeoket et al. 2001).

The mitotic activity in the epithelium is maximal at proestrus and oestrus (Stroband et al. 1986), whereas the secretory activity in the uterine glands is the highest at dioestrus. During late dioestrus, proestrus and oestrus, there is uterine oedema in the subepithelial connective tissue (Walter and Bavdek 1997).

On the other hand, mast cells, macrophages, lymphocytes, plasma cells and eosinophils have also been found in the endometrium (Kaeoket et al. 2001), and the phase of the oestrous cycle also influences the number of lymphocytes, neutrophils, eosinophils and plasma cells. Thus, and regarding the surface epithelium, lymphocytes are mainly found during oestrus and early dioestrus, while macrophages are mainly present at proestrus and oestrus (Kaeoket et al. 2001). As far as the submucosa is concerned, lymphocytes are the dominating cell type during all the stages of the oestrous cycle especially at oestrus and early dioestrus, when they are more numerous. Moreover, there is a massive infiltration of neutrophils in the submucosa during proestrus and oestrus (Fig. 5.3), but these immune cells are not observed during the other stages of the oestrous cycle (Stroband et al. 1986; Kaeoket et al. 2001). More information relating to reproductive immunology will be given in Sect. 5.8.

5.6.3 Interaction of Spermatozoa with Cells of the Uterine Epithelium

Although billions of spermatozoa arrive at the uterus after copulation or AI, only a few thousand of them ever reach the oviducts (Matthijs et al. 2003) so that the uterine passage involves a large number of sperm cells being lost. This radical decrease may be explained by two different phenomena (Taylor et al. 2008):

1. The backflow within 4 h after AI (Viring and Einarsson 1980, 1981; Steverink et al. 1998), and

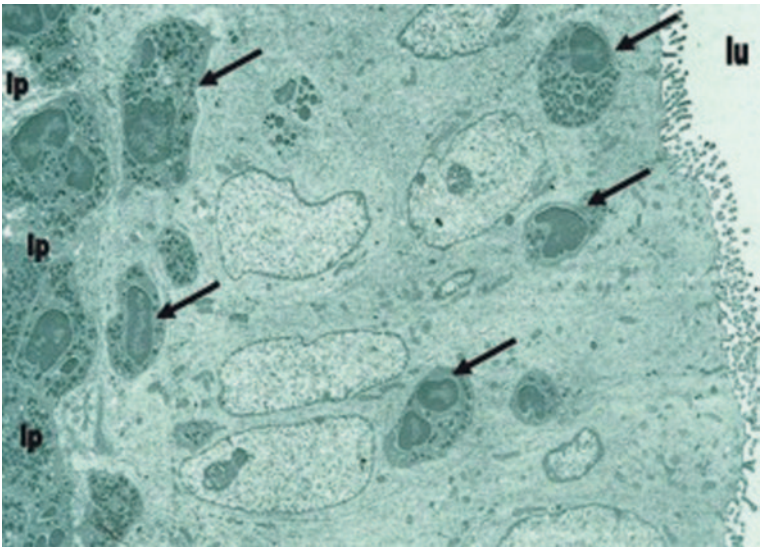

Fig. 5.3 Transmission electron micrograph showing the entry of polymorphonuclear leucocytes (*arrows*) from the lamina propria (*lp*) into the luminal epithelium and towards the lumen (*lu*) of the uterus in an oestrous sow. (Rodríguez-Martínez et al. 2010, Reproduced with permission)

2. The possible selection of the spermatozoa before entering the oviduct (Matthijs et al. 2003).

Upon arrival, sperm cells have to face two different cell types: uterine epithelial cells (UEC) and neutrophilic granulocytes [polymorphonuclear neutrophils; see Nathan (2006) for a general review], and both seem to play an active part in a selective-like process by becoming attached to viable spermatozoa (Taylor et al. 2008, 2009a). Indeed, spermatozoa can be attacked and phagocytosed by neutrophilic granulocytes, which migrate in great number after insemination/mating into the uterus lumen (Pursel et al. 1978; Rozeboom et al. 1999; Matthijs et al. 2003). However, the influx of neutrophilic granulocytes takes place within the first 3–4 h post-insemination, and due to the backflow, they are confronted by a reduced number of viable spermatozoa (Taylor et al. 2008).

Although the exact molecular mechanisms by which spermatozoa bind to UEC and neutrophilic granulocytes is unknown, it seems quite clear that only viable spermatozoa are able to bind these two cell types (Taylor et al. 2008). Indeed, using ex vivo and in vitro experiments in pigs, Taylor et al. (2008) observed that many spermatozoa were retained within the uterus strongly enough to resist vigorous flushing. These authors also showed that the number of non-viable spermatozoa that were flushed out was similar to the number of non-viable spermatozoa in the insemination dose. In contrast, the number of viable spermatozoa recovered after flushing was lower than that of the dose. These inseminations

were performed with spermatozoa diluted in a long-term extender (Androhep™, Minitüb) or in seminal plasma, the number of viable spermatozoa retained in the uterine segments being higher in the former than in the latter.

As far as the mechanism by which viable sperm cells adhere to UEC is concerned, it seems that specific surface molecules (carbohydrates) could mediate the binding between the two cell types. This possibility is reinforced by observations by Taylor et al. (2008) when comparing the acrosome integrity of spermatozoa diluted in a commercial extender with spermatozoa diluted in seminal plasma. The assessment of the acrosome integrity in this study was performed using the lectin peanut agglutinin (PNA; see also Sect. 9.3.10 for more information about lectins and acrosome integrity), which binds to damaged acrosomes, so that PNA-stained spermatozoa could not be found, in principle, attached to UEC. The PNA modulated binding was more obvious with sperm cells in the commercial extender, whereas the seminal plasma inhibited the binding of PNA. Although PNA-specific sugars may not be the dominant structures involved in the interaction between viable spermatozoa and UEC, this could be a valid hypothesis of why the number of retained spermatozoa decreases when they are suspended in their seminal plasma. Apart from this evidence, and bearing in mind that in vitro cultured UEC retain functional characteristics (Cox and Leese 1997), it is also well-known that viable spermatozoa preferentially attach to oviductal epithelial cells (Töpfer-Petersen et al. 2002; Yeste et al. 2009) and to epididymal epithelial cells (Yeste et al. 2012), this fact being explained by the function of the sperm reservoir in the former case. In addition, sperm binding to oviductal epithelial cells (OEC) is also mediated by carbohydrate interactions (DeMott et al. 1995; Green et al. 2001; Töpfer-Petersen et al. 2002; Wagner et al. 2002), and the ability of sperm cells to adhere to OEC depends on their viability and their functional status. Therefore, as will be seen in the Chap. 6, only viable, morphologically normal and uncapacitated spermatozoa are able to bind oviductal (Fazeli et al. 1999; Green et al. 2001; Töpfer-Petersen et al. 2002; Yeste et al. 2009) and epididymal epithelial cells (Yeste et al. 2012). Furthermore, this preferential binding has not only been seen in reproductive but also in non-reproductive epithelial cells (Yeste et al. 2009, 2012), although it occurs to a lower extent in the latter case. All these data back the hypothesis put forward by Taylor et al. (2008) by which only viable spermatozoa are able to bind UEC, and emphasises the functional role of sperm binding to epithelial cells in different parts of male and female reproductive tracts (epididymal, uterine and oviductal).

Finding an explanation for the reduction in the number of spermatozoa within the uterus is complex, but the most reasonable one is that only viable spermatozoa are able to bind UEC. The other possible explanation for the reduction in viable spermatozoa in the experiment of Taylor et al. (2008) could be that they were damaged during ex vivo incubation and subsequently retained within the female tract. However, this possibility is quite unlikely because if damaged sperm cells increase their binding ability to UEC, the number of non-viable spermatozoa would also decrease after flushing, and Taylor et al. (2008) did not report changes in the number of non-viable spermatozoa.

Regarding the role of sperm binding to UEC, Taylor et al. (2008) have hypothesised that the retention of sperm cells in the uterus could protect the viable spermatozoa from being removed with the backflow, or that it plays some role in sperm

maturation. As such, this phenomenon should be considered as a positive selection process. On the other hand, a negative-selection process might also exist that would consist of a certain part of the viable sperm population being actively prevented from reaching the oviduct. Thus, given that the preference of neutrophils to interact with viable spermatozoa has been reported (Taylor et al. 2008), a negative-selection process involving subsets of viable sperm cells unable to attach UEC has been proposed. Although this hypothesis suggests that these spermatozoa would not be considered suitable for fertilisation, we must also say that finding a possible biological meaning for this process is quite difficult and speculative. Another possible explanation for the role of sperm binding to UEC is that spermatozoa might induce signals favouring subsequent inflammatory responses. Intriguingly, Rozeboom et al. (1999), comparing the influx of neutrophils after insemination between extenders with and without spermatozoa, observed an increase in neutrophils in the former but not in the latter.

Finally, seminal plasma significantly inhibits sperm binding ability to UEC and to neutrophilic granulocytes. This indicates its important protective role and also shows its relevance, which has to be considered for AI, especially when using low dosages of spermatozoa.

5.6.4 Communication from Uterus to Ovarium

Uterus and ovary are in communication (Michael and Schofield 1969), but the exact mechanism is unknown. A possible signal transfer from the uterus to the ovary could involve cells present in the uterus (epithelial and others, including spermatozoa) that would release or lead to release cytokines such as the granulocyte–macrophage colony-stimulating factor (GM-CSF) and the TNF-α (Schuberth et al. 2008). Local mediators would then reach the ovarian stroma and pre-ovulatory follicles and would bind to receptors expressed on the surface of ovarian cells. The physiological route for these cytokines would be the lymphatic ducts and the transfer from the uterine vein to the utero-ovarian artery (Schuberth et al. 2008).

This hypothesis is supported by previous observations. Thus, for example, boar seminal plasma stimulates uterine epithelial cells to secrete GM-CSF (O'Leary et al. 2004), and can induce the advancement of ovulation when contacting with the epithelium at the utero-tubal junction (Waberski et al. 1995, 1996, 1999, 2006) (Fig. 5.4).

5.6.5 Distribution of Spermatozoa Within the Intrauterine Environment, UTJ, and Oviduct After Artificial Insemination: CAI, IU and DIU

Tummaruk et al. (2007) and Tummaruk and Tienthai (2010) have investigated the number of spermatozoa in the crypts of the utero-tubal junction (UTJ) (Fig. 5.5) and the oviduct of sows, approximately 24 h after intrauterine insemination (IU; Belstra

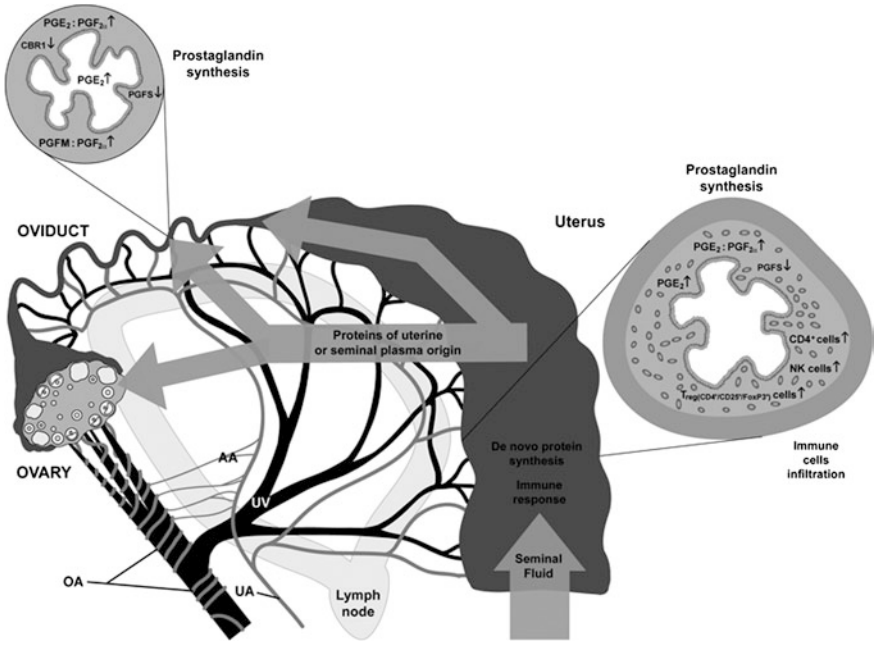

Fig. 5.4 Communication from uterus to ovary, where seminal plasma may contribute to the advancement of ovulation when contacting with the epithelium at the utero-tubal junction. In addition, seminal plasma also initiates the immune cell infiltration ($CD4^+$, T_{reg} cells and a small increase in NK cells) and de novo protein synthesis in the endometrium that may prepare the uterine environment for embryonic development. The biologically active molecules from uterine or seminal plasma origin can reach the ovarian and oviduct tissues directly or via the ovarian and uterine arteries. Abbreviations mean: *OA* Ovarian artery, *UA* Uterine artery, *UV* Uterine vein, T_{reg} T regulatory cells, *NK* natural killer cells and *AA* arterio-arterial anastomoses connecting uterine and ovarian arteries (Kaczmarek et al. 2010, Reproduced with permission)

2002) and deep intrauterine insemination (DIU; Martínez et al. 2001a, b; Vázquez et al. 2005), and compared them with that of (conventional) cervical artificial insemination (CAI). These studies have been the first to show, by using the histological examination technique, the distribution of spermatozoa in the UTJ, caudal isthmus, cranial isthmus and ampulla of the oviduct in sows after low-dose IU and DIU, compared with conventional AI. Accordingly, these authors have observed that the number of spermatozoa in the UTJ and caudal isthmus depend on the insemination technique used (2296 in conventional, 729 in post-cervical/IU, and 22 in DIU). Notwithstanding, they have observed that most of the viable spermatozoa are located in groups in the epithelial crypts within the oviduct in pre- and peri-ovulatory periods during standing oestrus (Mburu et al. 1996 ; Sumransap et al. 2007). These spermatozoa will remain uncapacitated until ovulation takes place (Rodríguez-Martínez et al. 2005).

When using conventional and post-cervical AI, the spermatozoa are found on both sides of the UTJ and caudal isthmus (Tummaruk and Tienthai 2010).

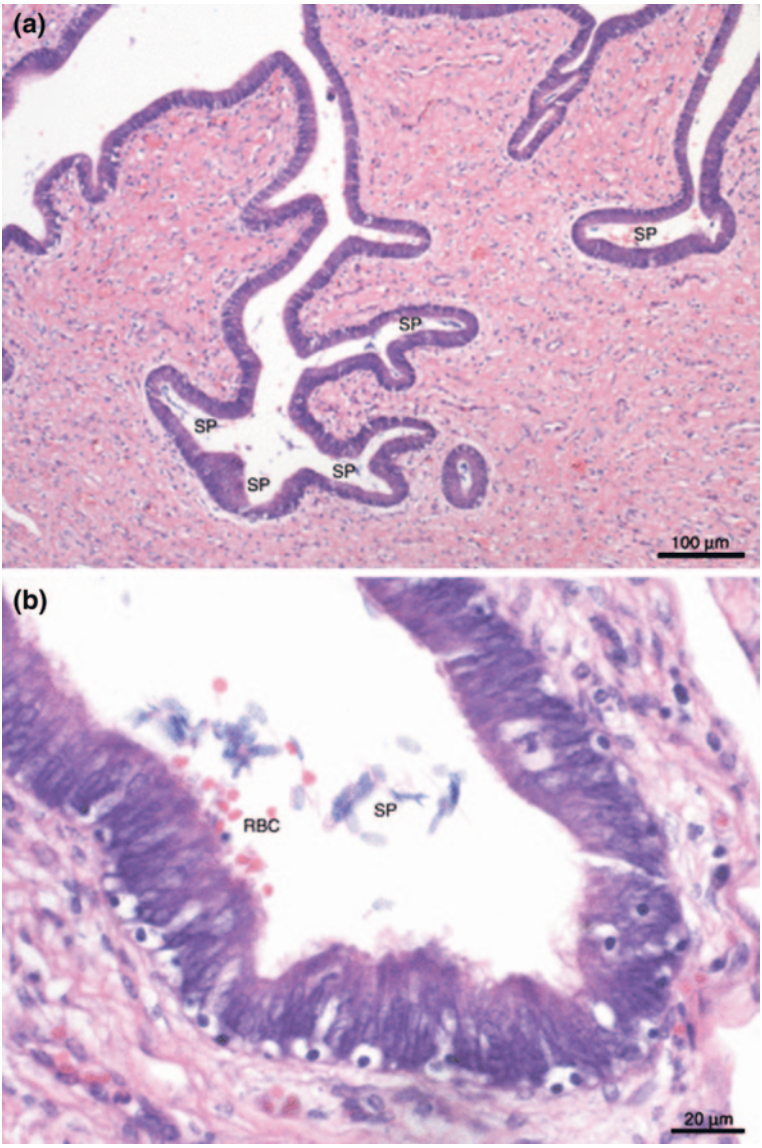

Fig. 5.5 Distribution of spermatozoa in epithelial crypts of the utero-tubal junction (UTJ) of a sow approximately 24 h after insemination at **a** 100× magnification, and **b** 400× magnification. Abbreviations mean: *SP* spermatozoa, *RBC* red blood cell, *E* epithelium and *S* subepithelium. (Tummaruk and Tienthai 2010, Reproduced with permission)

In contrast, DIU results in a significantly diminished number of spermatozoa in the sperm reservoir (UTJ and caudal isthmus) after 24 h of insemination compared with AI and IU. This lower number of sperm cells in the sperm reservoir is

associated with decreased litter size compared to conventional AI (Martínez et al. 2001a, b, 2006; Vázquez et al. 2005). Moreover, partial and unilateral fertilisation in sows is higher when using DIU than when using CAI (Martínez et al. 2006). According to Tummaruk and Tienthai (2010), this finding may be related to the formation of the sperm reservoir on only one side.

Finally, and still regarding with sperm distribution throughout female reproductive tract, it is worth noting that the formation of the sperm reservoir relies on both spermatozoa and seminal plasma components (Rodríguez-Martínez et al. 2005) as a certain number of them are passively transported throughout the uterine lumen and escape from phagocytosis. In addition, Rodríguez-Martínez et al. (2005) have also shown that within the boar ejaculate, the first 10 ml of the sperm-rich fraction contain a sperm subpopulation that is more effective in terms of sperm reservoir colonising than the rest of the fraction.

5.7 Sperm Transport Throughout the Uterus

5.7.1 Introduction

Semen is deposited in the female reproductive tract, the location depending on the species (Suarez and Pacey 2006) and whether natural mating or AI is used. For example, in cattle, semen is deposited in the cranial segment of the vagina in natural mating (First et al. 1968), while spermatozoa are left in the uterine body in AI. In porcine species, semen is deposited in the narrow cervical canal in natural mating and CAI (Rodríguez-Martínez 2007). Alternative techniques of artificial insemination involve other places of semen deposition; thus, intrauterine insemination leaves semen in the uterine body, and deep intrauterine insemination leaves it in the vicinity of the utero-tubal junction (UTJ) (see also Chap. 12).

Three phases can be distinguished in semen transport through the sow tract:

1. A passively transuterine transport immediately after semen deposition.
2. The colonisation of the lower oviduct, forming the semen reservoir.
3. A slow release from the reservoir towards the venue of fertilisation, i.e. the ampullary-isthmic junction, which is a peri-ovulatory event (Barratt and Cooke 1991).

Thus, semen is first deposited in the cervix of the sow after natural mating or CAI (First et al. 1968; Langendijk et al. 2005). From this point, it is flushed into the lumen of the uterine body and the spermatozoa are then transported through the uterine horns up to the oviducts, where fertilisation takes place.

Although the exact mechanism by which male gametes are transported through the mammalian uterus is not completely known (Rousseau and Ménézo 1993), it seems that it consists of a passive process relying on the flow of sperm-containing fluids in the uterine lumen. This process appears to be driven by gravitational force and uterine contractility rather than by the intrinsic motility of spermatozoa (Langendijk et al.

2002a, 2005). Thus, because the contractile activity of the uterus in sperm transport plays such a key role (Suarez and Pacey 2006), the next subsections are focused on myometrial activity and on the factors that modulate this activity.

Another important aspect to be considered in sperm transport throughout the uterus is that this organ provides a hostile environment for spermatozoa due to phagocytosis. However, phagocytosis inhibitors such as caffeine and calcium increase the number of viable spermatozoa when added to insemination doses (Woelders et al. 2000; Woelders and Matthijs 2001). Only when spermatozoa arrive at the end of the uterine horns, i.e. when the sperm cells reach the UTJ and the first part of the oviduct, are they safe, because they can form the sperm reservoir by directly contacting the oviductal epithelial cells (Töpfer-Petersen et al. 2002).

Finally, we must mention that it is not completely known how spermatozoa survive in the uterus despite sperm-UEC interaction, or which role myometrial contractions play in sperm transport throughout the uterine horns. It also remains unclear what happens when the sperm cells stay in the uterine horns for a long time and whether this fact may affect their ability to enter the oviduct and to fertilise.

5.7.2 Contractile Activity of the Uterus

One important aspect in swine reproductive physiology is uterine activity around oestrus. This activity has been studied using invasive (Brüssow et al. 1988; Claus et al. 1989) and non-invasive methods (Von Döcke and Worch 1963; Langendijk et al. 2002b), both of which have advantages and disadvantages, as Langendijk et al. (2005) have successfully reviewed.

Specifically, by using non-invasive methods for monitoring the intraluminal pressure of the uterus, Von Döcke and Worch (1963) inserted a fluid-filled balloon at the cervical end of the uterus, while Langendijk et al. (2002b) adapted a catheter developed by Hazeleger and Kemp (1994) for non-surgical embryo transfer in sows. From these and other studies, several authors have observed that uterine activity varies during the oestrus cycle, distinguishing the following four different stages:

1. From 2 to 4 days before oestrus, the myometrial activity, in terms of frequency and amplitude of contractions, is low. In a study by Langendijk et al. (2002b), about 50 % of females did not show contractility within this period, whereas the others presented a lower frequency and amplitude of contractions when compared with uterine activity during oestrus.
2. When oestrus is coming, both the number of sows showing myometrial activity and the frequency and the amplitude of myometrial contractions increase.
3. During oestrus, the number of sows showing myometrial activity and the frequency and amplitude of contractions are at their highest. At this time, the amplitude and duration of electrical bursts are more pronounced and the myometrium is more sensitive to electrical inputs (Claus et al. 1989). This leads to an increase of sperm transport.
4. After oestrus, myometrial activity decreases again.

5.7.3 Uterine Contractions: Cervico-Tubal and Tubo-Cervical

As stated, sperm transport is a passive process. When the spermatozoa are deposited in the cervix, their passage into the uterine body depends on gravity and on the relaxation of the myometrium. The luminal pressure of the uterus consists of baseline pressure in the relaxed state, with periodical increases owing to electrical bursts.

Stimulating uterine contractions can extend the time needed for the uptake of semen during insemination (Langendijk et al. 2002c) and can also increase semen backflow so that this may delay the influx of semen from the AI catheter into the uterus. In this case, it is very important to keep in mind that a high ejaculate backflow may reduce farrowing rates (Steverink et al. 1998; Langendijk et al. 2002c), especially in some unfavourable situations such as using low sperm concentration or low ejaculate volume, or when there is a long period of time between insemination and ovulation.

Uterine contractions are involved in the transport of spermatozoa from the cervix to the UTJ through the uterine horns, and they also take part in the transfer of sperm cells into the oviducts (Baker and Degen 1972). The main role of the uterine contractions is evident when myometrial contractility is reduced as a result of administering a β-adrenergic agonist before insemination. In this case, spermatozoa spend more time in their transport through the uterine horns. This fact provokes, in turn, a decrease in both the number of spermatozoa at the oviduct and fertilisation rates (Langendijk et al. 2002c).

Contraction waves originate at the cervical and tubal ends of the uterine horns, where Taverne (1982) has observed the most pacemaker activity during parturition. Depending on the direction, three types of uterine contractions can be distinguished: tubo-cervical, cervico-tubal and undirected contractions (Brüssow et al. 1988); all three simultaneously observed during oestrus. In all cases, the direction of contractility seems to be a coordinated process, which depends on the phase of oestrus and can be affected by stimuli involved in mating. In the propagation of these contractions, communication between myometrial cells plays a key role. This cell-to-cell communication depends on the number of gap junctions, which increases during the oestrus when oestradiol levels are high (Verhoeff et al. 1986).

The tubo-cervical directed contractions are important to eject the seminal plasma after mating and are also important in the distribution of spermatozoa in the two uterine horns (Woelders and Matthijs 2001), while the cervico-tubal contractions play a key role in sperm transport towards the oviduct (Langendijk et al. 2005).

5.7.4 Female Factors Influencing Myometrial Activity

Myometrial activity is affected by several factors that are intrinsic to the female. In this respect, it is noteworthy to mention the modulating role of female hormones (oestrogens, progesterone and LH), parity and the individual variation within and among animals (First et al. 1968).

5.7.4.1 Influence of Female Hormones

Langendijk et al. (2005) have mentioned the modulating role of oestradiol and progesterone on the tissue and on plasma levels in sows. Thus, oestrogens have been reported to increase uterine activity, while progesterone exerts a decreasing effect. In other mammalian species like human, oestrogens increase both the myosin content of myometrial cells (Michael and Schofield 1969) and the pacemaker activity of myometrial cells (Finn and Porter 1975), while in sheep they increase gap junctions among myometrial cells, as mentioned above (Verhoeff et al. 1986).

In swine, oestradiol and oxytocin affect the levels of $PGF_{2\alpha}$ released by the uterus (Edgerton et al. 2000) so that uterine activity is modulated upstream by oestradiol and oxytocin. However, the exact role of both oxytocin and prostaglandins is not exactly known, since, whereas some authors have reported a diurnal fluctuation in the levels of these hormones (Edgerton et al. 2000), others have observed that this variation is quite low (Schille et al. 1979).

Apart from oestradiol, progesterone and oxytocin, LH also seems to be involved in the regulation of uterine activity (First et al. 1968; Ziecik et al. 1992) since LH/human chorionic gonadotropin (hCG)-receptors are present in the myometrium during oestrus, and (Flowers et al. 1991) have demonstrated that hCG suppresses the myometrial activity of the swine uterus. However, it is still unclear whether the high levels of LH during the pre-ovulatory period exert some effects on the regulation of uterine activity.

Finally, the activity of the myometrium not only depends on the levels of circulating hormones but also on the sensitivity of their receptors (Smith and Toft 1993; Weigel 1996). In this regard, Thilander et al. (1990) and Wathes et al. (1996) have reported that numbers of receptors interacting with oestradiol, progesterone and oxytocin depend on the circulating levels of oestradiol and progesterone.

5.7.4.2 Influence of Parity: Primiparous Versus Multiparous

Apart from the hormonal modulating role, myometrial activity is also affected by parity. Indeed, the frequency of contractions during oestrus has been reported to be higher in primiparous sows than in multiparous sows, while the amplitude of uterine contractions is lower in the former (59 mm Hg vs. 45–51 mm Hg) (Langendijk et al. 2005).

5.7.4.3 Inter- and Intra-Individual Variations in Female Uterine Activity

On the other hand, there is high inter-individual variation in uterine activity in swine (Langendijk et al. 2002a, b). This variation is detected in the frequency and amplitude of contractions throughout the entire oestrous cycle, i.e. not only during the standing oestrus but also within the period around oestrus. Thus, sows

presenting a relatively high level of uterine activity during the days before oestrus have also been reported to display a relatively high level of uterine activity during oestrus. Furthermore, when sows present a longer heat (from 2 to 3 days), they also maintain the high level of uterine activity during oestrus for a longer period of time. In these sows, the decline of oestradiol levels in peripheral blood occurs later.

Apart from the mentioned inter-individual variation, intra-individual differences also exist when comparing oestrous cycles within the same sow (Langendijk et al. 2002a, b). Thus, and according to the above-mentioned, the inter- and intra-individual variations reasonably depend on individual factors such as circulating hormone levels, sensitivity of their receptors and sow parity.

5.7.5 Boar Factors Influencing Myometrial Activity of Females

Apart from female factors influencing uterine activity, there are male factors that are related to sexual stimuli before, during and after copulation. Langendijk et al. (2005), reviewing the state-of-the-art of this issue, have distinguished between two boar-dependent stimuli: sensory and seminal plasma-related stimuli.

5.7.5.1 Sensory Stimuli

Within sensory stimuli, at least four different factors can be distinguished: visual (boar presence), olfactory, tactile and auditory.

First, one of these sensory stimuli is the visual presence of a boar, which increases the levels of oxytocin in the sow and her myometrial activity (Claus and Schams 1990). However, these boar-mediated effects are only observed in those sows that have a below average frequency of uterine contractions (Langendijk et al. 2002a). On the other hand, the increase in myometrial activity due to the presence of a boar is not only related to the magnitude of oxytocin release, although the same effect is observed when sows are treated with oxytocin injected intramuscularly (Langendijk et al. 2002c).

As for the effect of olfactory stimulation on uterine activity, a review of the literature provides inconsistent results. Thus, whereas some papers report that olfactory stimulation with 5-α-androstenone increases the release of oxytocin and uterine activity in a similar fashion to what occurs during mating (Maffeo et al. 1993; Mattioli et al. 1986), others (Langendijk et al. 2002c) observe no effect of olfactory stimulation on oxytocin release and myometrial activity.

As far as tactile stimulation is concerned, touching the back and the flanks of the female in the absence of a boar triggers receptive behaviour but has no effect either on oxytocin levels or on uterine activity (Langendijk et al. 2002c). Moreover, although tactile stimulation of the female cervix, either by using normal and transcervical catheters (Claus and Schams 1990), or by massaging the

vulva and the clitoris, does not alter oxytocin release, stimulating the cervix does enhance uterine activity. Furthermore, flushing a significant volume of semen extender or saline solution also stimulates myometrial activity (Claus et al. 1989) but this increase seems to be due to the effect of catheter insertion rather than to the infused volume (Langendijk et al. 2005).

Since this cervical stimulation effect is independent on oxytocin release, modulation of myometrium contractility appears to involve adrenergic and/or cholinergic pathways (Langendijk et al. 2005). Accordingly, the swine myometrium presents adrenergic and cholinergic receptors (Claus and Schams 1990), adrenergic receptors being mainly present in the longitudinal muscle layer and cholinergic receptors being mainly located in the circular muscle layer (Taneike et al. 1990).

The effect of the adrenergic and cholinergic receptors on myometrial contractility depends on the type of receptor. Therefore, contractility is initiated when cholinergic and α-adrenergic receptors are stimulated, while β-adrenergic receptors suppress myometrial contractility (Langendijk et al. 2005).

5.7.5.2 Seminal Plasma-Related Stimuli

The effect of seminal plasma on uterine activity is clearer than that of sensory stimulation (Langendijk et al. 2005). Seminal plasma stimulates myometrial contraction in vitro, owing to the composition of seminal plasma, which contains oestradiol (Claus 1990; Langendijk et al. 2002c).

Therefore, after copulation and AI, the oestrogens present in the boar ejaculate stimulate the endometrium, inducing an immediate release of $PGF_{2\alpha}$. Moreover, the effect of oestrogens on LH and follicular $PGF_{2\alpha}$ is likely to contribute to the timing of ovulation in response to mating (Claus 1990; Waberski et al. 2006), and this stimulation of myometrium contractility mediated by the oestrogens that boar ejaculate contains is maintained for a few hours. Although the intrauterine infusion of oestrogens at the same level as the boar ejaculate causes similar effects on uterine contractility, the effect mediated by mating with a boar has a higher extent (Claus 1990; Langendijk et al. 2005).

In short, oestrogens in seminal plasma have a clear effect on endometrium and uterine activity by stimulating $PGF_{2\alpha}$-release during the standing oestrus, while, from a review of the literature, the effects of sensory stimulation are, in contrast, less clear and sometimes controversial. In fact, although it is quite evident that both cervical stimulation and the presence of a boar increase uterine activity, it is less clear that tactile and olfactory stimuli have any effect (Langendijk et al. 2005).

5.7.6 Relevance of Ejaculate Volume in Sperm Transport in the Female Reproductive Tract

Another relevant issue concerns the putative functional role of the ejaculate volume. In AI, the infused ejaculate volume depends on the catheter used, thereby

distinguishing among conventional or cervical (CAI) and intrauterine insemination (IU), this latter being either post-cervical (post-CAI) or deep intrauterine (DIU) (see Chap. 12).

While CAI requires high semen volume (≥80 mL), less volume is needed when using IU (Casas et al. 2010; Martínez et al. 2001a, b). These data underline the importance of the functional role of the ejaculate volume on fertility and prolificacy rates, because when CAI is performed using low semen volume, fertility rates decrease. This fact indicates that large semen volume is needed in CAI, because it probably plays a role in flushing the spermatozoa from the cervix into the uterine body (Langendijk et al. 2005). This would prevent the retention of spermatozoa in the cervical folds. In contrast, the sperm volume would not play such a key role in the transport of spermatozoa throughout the uterine horns and the oviducts, since good farrowing rates are obtained when much lower volumes of semen (~30 mL in IUI, ≤15 mL in DUI) are deposited in the uterus.

On the other hand, a large amount of semen in the sow's genital tract may be required to accelerate the transport of male gametes from the uterine body to the tubal end of the uterine horns (Langendijk et al. 2005). Given that phagocytosis of spermatozoa occurs in the uterine horns and this reduces the number of available sperm cells, large semen volume may be needed, therefore, when insemination takes place before ovulation (Woelders and Matthijs 2001).

5.7.7 Effects of Stimulating Myometrial Contractility on Sperm Transport and Farrowing Rates

Stimulating myometrial contractility can also positively affect farrowing rates, i.e. fertility and prolificacy, and these effects can be assessed through two different approaches. The first consists of infusing seminal plasma before insemination, which stimulates uterine activity, as mentioned above. By using this methodology, Viring and Einarsson (1980) observed that sperm transport increased at the oviduct from 1 to 6 h after insemination, while Waberski (1996) reported an increase in the number of accessory spermatozoa in the zona pellucida (ZP) of 3- to 4-day-old embryos without noting any effect on fertilisation rates.

The other approach for stimulating uterine activity consists of combining low semen volume with an intravenous injection of a high dose of oxytocin after insemination. This approach seems to improve the percentage of fertilised oocytes (from 58 to 72 %) (Stratman et al. 1959).

Despite the positive effects mentioned of stimulating myometrial contractility on sperm transport and fertilisation, this also entails disadvantages such as the increase in backflow. Thus, although Peña et al. (1998, 2000) have demonstrated that the hormonal stimulation of myometrial contractility at the time of insemination increases the farrowing rates during the low-fertility season, stimulating uterine contractility can also decrease sperm transport to the oviducts and fertilisation. Furthermore, Hazeleger and Kemp (1994), in another study, infused a high dose of cloprostenol before insemination, and negative rather than positive effects were observed. Thus, the degree of stimulation is key here and a critical concept.

In short, uterine contractility under physiological conditions plays an important role for rapid transport of sperm cells throughout the uterus and up to the oviducts, because the spermatozoa are safer in the oviducts than in the uterine horns (Langendijk et al. 2005). However, it is important to find the right balance when stimulating myometrial contractility, because it can increase sperm transport and fertilisation rates when used at a suitable level, but it can reduce the uptake of semen by the uterus and increase the risk of backflow at a higher level.

5.7.8 *Effects of Prostaglandins on Reproductive Performance: Myometrial Contractility, Sperm Transport and Quality*

Prostaglandins (PGs) are eicosanoids that are widely distributed in vertebrate tissues and play multiple roles in a wide array of physiological processes (Kingsley et al. 2005; Flower 2006). These hormones are produced by the *bis*-dioxygenation of arachidonic acid (20:4) to form hydroperoxy endoperoxide (PGG_2), followed by the reduction of the PGG_2 to hydroxyl endoperoxide (PGH_2), in a process catalysed by cyclooxygenases. Hydroxyl endoperoxide is then transformed by different enzymes to PGs and thromboxane A_2 (Kingsley et al. 2005). Such cyclooxygenases are present in the apical region of the head, the post-acrosomal region and the midpiece of the tail of ejaculated and epididymal bovine spermatozoa, as immunohistochemical studies have shown (Shalev et al. 1994).

Prostaglandins are related to several reproductive processes, being present in seminal fluid (Templeton et al. 1978; Kaczmarek et al. 2010) and in cervical mucus (Charbonnel et al. 1982). Human spermatozoa are even able to synthesise prostaglandins (Roy and Ratnam 1992) and, in bovine, spermatozoa have even been reported to induce prostaglandin synthesis and secretion in oviductal epithelial cells (Kodithuwakku et al. 2007). In vitro, PGs produce different effects on tubal smooth muscle because prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) increases tubal muscle contractility (Pérez-Martínez et al. 1998), whereas prostaglandin E_2 (PGE_2) inhibits the contraction of circular muscles (Lindblom et al. 1978). However, both are needed since the transport of the embryo and the communication between the embryo and the oviduct involves prostaglandin action through PGE_2 and $PGF_{2\alpha}$ receptors (Mwanza et al. 2002a; Wanggren et al. 2006; Kaczmarek et al. 2010) (Fig. 5.4).

On the other hand, some prostaglandins affect sperm function (PGE_1, PGE_2, 19-OH-PGE, 19-OH-PGF and $PGF_{1\alpha}$), while others do not, or just to a lower extent ($PGF_{2\alpha}$) (Gottlieb et al. 1988; Maes et al. 2003; Yeste et al. 2008). Indeed, prostaglandins E_1 and E_2 (PGE_1 and PGE_2) increase the velocity and the penetrating ability of human spermatozoa (Aitken and Kelly 1985), thereby changing their functional competence. Moreover, seminal plasma and follicular fluid contain PGE_1 and PGE_2 that promote a Ca^{2+}-influx in human spermatozoa (Blackmore et al. 1990; Baldi et al. 1991; Joyce et al. 1987; Margalioth et al. 1988; Thomas and Meizel 1988; Shimizu et al. 1998), and in the case of PGE_1 acts as well as an in vitro capacitating factor for mouse spermatozoa (Herrero et al. 1997).

Prostaglandin $F_{1\alpha}$ reduces sperm motility, while 19-OH-PGE increases sperm motility and penetration ability, and 19-OH-PGF diminishes ATP concentration in human spermatozoa (Bendvold et al. 1984).

As far as the hormone $PGF_{2\alpha}$ is concerned, it has been used in swine operations for the synchronisation and induction of farrowing and to increase the libido of boars (Hawk 1983; Estienne and Harper 2004; Mwanza et al. 2002b; Szurop et al. 1986). This hormone contributes like other components of seminal plasma (Waberski et al. 1996, 1999, 2006; Kaczmarek et al. 2010) to the timing of ovulation in response to mating in sows (Claus 1990) and, as stated before, it is an important smooth muscle contractile agent that exerts a significant uterotonic effect via the specific $PGF_{2\alpha}$-receptor that has been identified in the myometrium of humans, swine, sheep and rats (Friel et al. 2005). Prostaglandin $F_{2\alpha}$ binds to the $PGF_{2\alpha}$-receptor and a signal transduction pathway, which leads to the mobilisation of intracellular Ca^{2+}, is then activated (Olson et al. 2003). The role of this mechanism is so important that failure in parturition occurs when the $PGF_{2\alpha}$-receptor gene is knocked out (Sugimoto et al. 1997). Furthermore, significant changes in the plasma concentration of $PGF_{2\alpha}$ are observed within 15–21 min after starting stimulation and AI, reaching a plateau after 30 min (Madej et al. 2005). The effects of oxytocin are partially mediated by $PGF_{2\alpha}$, which also augments the expression of an oxytocin receptor (Mirando et al. 1995).

All this background finds its practical application in AI procedures. Indeed, one strategy for increasing fertility outcomes consists of adding different substances to cooled or frozen seminal doses in order to improve their storage, maintain their function and survival and/or increase farrowing rates (Yeste 2008). Accordingly, the addition of $PGF_{2\alpha}$ to extended semen used in AI increases farrowing rates (Gustaffson et al. 1975; Gamcik et al. 1980; Hawk 1983; Kos and Bilkei 2004) because it enhances myometrial contractility (Gil et al. 1998; Cheng et al. 2001; Kos and Bilkei 2004; Friel et al. 2005). However, some concentrations of $PGF_{2\alpha}$ can be cytotoxic (Maes et al. 2003; Yeste et al. 2008) and this is for boar spermatozoa when added to extended seminal doses at concentrations higher than 12.5 mg·100 mL^{-1}. Sperm viability drops dramatically above this threshold and the reduction in general sperm motility, in specific kinematic parameters (VSL, VCL and VAP) and in the osmotic resistance of spermatozoa is very significant. In contrast, $PGF_{2\alpha}$ concentrations of 2.5, 5 and 10 mg·100 mL^{-1} are not harmful to spermatozoa and the addition of 5 mg of $PGF_{2\alpha}$·100 mL^{-1} has still been reported to have a positive effect on maintaining sperm viability after 6 and 10 days of storage in a short-term extender at 15 °C (Yeste et al. 2008).

5.8 Reproductive Immunology in the Female Tract

5.8.1 Introduction

Spermatozoa within the uterus are not only able to attach to UEC but they can also interact and be phagocytosed by resident leucocytes. This may explain the reduction in the number of spermatozoa in the sperm population flushed out from the sow. However, the number of these leucocytes appears to be too low to explain

solely the loss of so many spermatozoa in such a short time period. Schuberth et al. (2008) have reviewed the state-of-the-art of reproductive immunology in sows and gilts. In this regard, some crucial aspects have to be kept in mind.

First, insemination is followed in many species by attraction and activation of leucocytes, with subsequent biological consequences (Robertson 2007). Nonetheless, relevant advances have been made in this field in recent years, but more research is still required to unveil the exact molecular mechanisms that regulate the post-mating inflammatory reaction.

Second, the immune response to copulation depends on the species, amount and composition of seminal plasma, semen extenders and number of spermatozoa (Schuberth et al. 2008). Thus, there are differences among species in the volume of ejaculate reaching the uterine lumen directly or loosely after passage through the cervix. As an example, the immune response induced by spermatozoa triggers a neutrophil influx similar to the one induced by bacteria in equine species (Gorgens et al. 2005a).

The interaction of spermatozoa with neutrophilic granulocytes has been described in several species, including pigs (Matthijs et al. 2000, 2003; Rozeboom et al. 2001), horses (Troedsson et al. 2005), ruminants (Strzemienski 1989) and humans (Blanco et al. 1992). However, in this direct neutrophil-spermatozoa interaction, neutrophilic granulocytes preferentially target aged, non-viable or capacitated spermatozoa, as described for porcine and other mammalian species like humans (Vogelpoel and Verhoef 1985; Eisenbach 2003; Matthijs et al. 2003).

On the other hand, as previously stated, some aspects of this interaction are known, whilst others remain unclear. Despite complementary factors, natural anti–sperm antibodies or carbohydrate–protein interactions have been suggested in this regard (Matthijs et al. 2000; Rozeboom et al. 2001; Troedsson et al. 2005). It remains unknown whether the interaction of spermatozoa with neutrophilic granulocytes is due to random attachment or involves sperm-specific molecules that are recognised by the leucocytes. From these three possible interactions, neither complementary factors (Matthijs et al. 2000; Rozeboom et al. 2001) nor natural anti-sperm antibodies (Kalaydjiev et al. 2002; Troedsson et al. 2005) seem to be involved in pigs, even though more research is needed on this point. The other speculated possibility would involve carbohydrate-mediated interactions of spermatozoa with neutrophilic granulocytes (Ofek and Sharon 1988), since the former exhibit lectins on their surface, which mediate, in turn, interaction with other cells like OEC (Green et al. 2001; Ekhlasi-Hundrieser et al. 2005; Töpfer-Petersen et al. 2002, 2008; Wagner et al. 2002). However, more recently Taylor et al. (2008) have shown that lectins do not seem to mediate binding between neutrophilic granulocytes and spermatozoa. In short, viable spermatozoa can bind neutrophilic granulocytes, while non-viable male gametes cannot, so that this interaction seems to be specific rather than random and involves membrane surface molecules, although their exact nature has remained hitherto unveiled. This topic will be taken up again in a specific subsection of the present chapter (Sect. 5.8.4).

Insemination is the starting point of communication with the female organism, which allows optimal pregnancy success. Immune reactions in response to mating/AI have an influence on the ovulation process, sperm selection, induction and maintenance of immunological tolerance regarding paternally derived

antigens, restructuration of endometrial tissue for implantation and placentation and immunological support of foetal tissues during pregnancy (Robertson 2005; 2007) (Fig. 5.6). As an example of this phenomenon, boar semen may specifically accelerate ovulation in sows, as Waberski et al. (1995, 1997) showed by using a surgical model that consisted of gilts with a clamped uterine horn. In this experiment, these authors observed that semen accelerated ovulation only on the infused uterine side, while it did not affect ovulation timing on the other one.

Specifically, semen constituents induce a series of immunological reactions when contacting with cervical and uterine tissues (Figs. 5.4 and 5.6). This response seems to be logical, since semen is a foreign material for the female reproductive tract organism and the aim of the sow's immune system is to eliminate it (Schuberth et al. 2008). In fact, the mucosal immune system in the female reproductive tract has to maintain a balance between the presence of commensal bacteria, sexually transmitted bacterial and viral pathogens, allogeneic spermatozoa and an immunologically distinct foetus (Wira et al. 2005; Ochiel et al. 2008). In this regard, it is worth noting that seminal plasma induces changes in cell populations of the uterine mucosa by increasing the amount of MHC class II-positive cells, which means an immediate and local cellular response against seminal plasma, especially at the UTJ (Waberski et al. 2006).

Epithelial cells that line the cervix, the uterus and the oviducts provide a first line of defence that confers continuous protection by providing a physical barrier as well as secretions that contain bactericidal and virucidal agents. These epithelial cells of the female reproductive tract are also able to respond to pathogens, in part through Toll-like receptors. Toll-like receptors (TLRs) are a broad family of innate immunity receptors that play critical roles in detecting and responding to invading pathogens. Thus, epithelial cells, macrophages, natural killer cells and neutrophils in the oviducts, uterus and cervix act via TLRs, which confer protection through the production of chemokines and cytokines. Chemokines and cytokines recruit and activate immune cells, as well as bactericidal and virucidal agents, which provide protection at times when adaptive immunity is down-regulated by steroid hormones to meet the constraints of procreation. Thus, in the female reproductive tract, TLRs enhance innate immune protection and, when necessary, contribute to the initiation of an adaptive immune response (Ochiel et al. 2008).

Finally, we must mention that some members of the TLR family (Tlr1–Tlr9) and some TLR-adapter proteins, such as TLR adaptor molecule 1 and NFKBIA, have also been indentified in some organs of the rat male reproductive tract (testis, epididymis and vas deferens) (Palladino et al. 2007). These TLRs have also been detected on epididymal rat spermatozoa (Palladino et al. 2008), while TLR2 and TLR4 have also been found in the membranes of human and mouse ejaculated spermatozoa (Fujita et al. 2011). In addition, bacterial endotoxins have been reported to negatively affect sperm function and survival by activating TLR-dependent pathways that lead to cell death. Therefore, TLRs appear to play important roles in innate immunity not only in female but also in male reproductive tract (Wira et al. 2005; Palladino et al. 2007, 2008; Fujita et al. 2011).

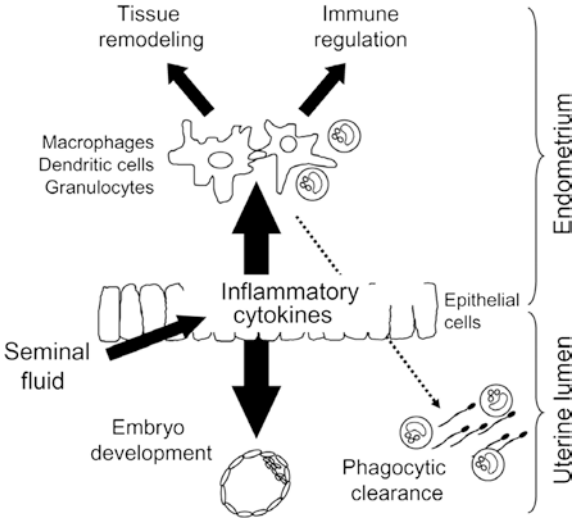

Fig. 5.6 Actions of seminal plasma in the female reproductive tract. Active moieties in seminal plasma and associated with spermatozoa interact with cervical and uterinic epithelial cells at mating to induce synthesis of pro-inflammatory cytokines. These cytokines cause the recruitment and activation of inflammatory cells in the uterine endometrium, including macrophages, dendritic cells and granulocytes. The macrophages and dendritic cells have roles in remodelling of the endometrial tissue and in activating maternal immune tolerance of pregnancy. Neutrophils traversing the endometrial epithelium into the lumen act to clear debris and maintain uterine sterility. Epithelial cytokines activated by seminal plasma are also secreted into the luminal fluid, where they exert trophic actions on the developing pre-implantation embryo (Robertson 2005, Reproduced with permission)

5.8.2 Variation of Local Resident Leucocytes During the Oestrous Cycle

The uterus has features of a mucosa-associated lymphoid tissue similar to other tracts, like the digestive tract. However, as mentioned above, the uterus undergoes cyclic changes, which affect not only the endometrium but also the leucocyte populations within the endometrium and the uterine lumen (Bischof et al. 1994; Kaeoket et al. 2001).

Regarding the surface epithelium of endometrium, lymphocytes are mainly found during oestrus and early dioestrus, while macrophages are mainly found at proestrus and oestrus (Kaeoket et al. 2001). As far as the submucosa is concerned, lymphocytes are the dominating cell type during all stages of the oestral cycle, especially at oestrus and early dioestrus, when they are more numerous. Moreover, there is a massive infiltration of neutrophils in the submucosa during proestrus and oestrus, but these immune cells are not observed during the other stages of the oestrus cycle (Stroband et al. 1986; Rodríguez-Martínez et al. 1990;

Bischof et al. 1994; Kaeoket et al. 2001) (Fig. 5.3). These neutrophilic granulocytes form a resident population in the uterine lumen just before ovulation (Matthijs et al. 2003; Rozeboom et al. 1998, 1999).

Furthermore, a considerable variation in terms of this cell population has been observed among individuals. Thus, Schuberth et al. (2008) have reported that a leucocyte population within uteri of gilts varies before ovulation from 0 to 2.7×10^9 leucocytes, with variable fractions of monocytes and granulocytes. In fact, it still remains unclear whether these luminal leucocytes are significantly involved in the insemination-induced signalling cascade, even though this seems a reasonable assumption.

5.8.3 The Immunological Response After Mating/ Insemination Within the Intrauterine Environment

5.8.3.1 The Influx of Neutrophilic Granulocytes

As stated, an inflammatory response in the female reproductive tract of several mammalian species including porcine (Lovell and Getty 1968; Matthijs et al. 2003; Rozeboom et al. 1999), mice (Robertson et al. 1996; Robertson 2007) and equine (Gorgens et al. 2005a, b) occurs after copulation/insemination. This response is characterised by an influx of neutrophils in the uterine lumen, which is usually the highest from 1 to 12 h after mating or AI. The duration of this peak also depends on the species, as Katila (1995) reported an elevated number of uterine leucocytes in mares up to 48 h after insemination. In fact, this potent neutrophil influx in the equine species may be related to the mentioned intensity of immune response, which is similar to the one observed in response to bacteria (Gorgens et al. 2005a).

Spermatozoa are chemotactic as previous studies conducted in mice, pigs and humans have shown (Yoshida and Yoshida 2011; Zuccarello et al. 2011; Li et al. 2012). Indeed, chemotaxis of spermatozoa towards ovum is a widespread phenomenon that occurs in most forms of life from plants to mammals and plays important roles in ensuring fertilisation (Yoshida and Yoshida 2011). In fact, the fundamental mechanisms underlying sperm chemotaxis seem to be common among all mammalian species and the intracellular calcium concentration is an important factor for the regulation of chemotactic behaviour in spermatozoa (Yoshida and Yoshida 2011). Related to this, ion channels, such as CatSper (a pH-regulated calcium-selective ion channel), KSper (Slo3), voltage-gated proton channel Hv1 or P2X2, have been found in human and/or mouse sperm tails and are also involved in sperm chemotaxis and sperm hyperactivation (Lishko et al. 2012). Recently, Zuccarello et al. (2011) have investigated chemotaxis in human spermatozoa and they have observed that SDF-1 (stromal cell-derived factor-1), a chemokine expressed in the oocytes, endometrium and follicular fluid, is involved in sperm chemotaxis, since its specific receptor CXCR-4 (chemokine CXC motif receptor 4) is present on the sperm head. When SDF-1 interacts with CXCR-4,

sperm hyperactivation is induced and there is an increase of the intracellular calcium levels, without inducing the acrosome exocytosis (Zuccarello et al. 2011).

Schuberth et al. (2008) have proposed that spermatozoa may induce chemotaxis of neutrophils by binding to endometrial cells and/or present leucocytes. As mentioned, the interaction between spermatozoa and neutrophilic granulocytes has been reported in porcine, human and other mammalian species such as ruminants and horses (Strzemienski 1989; Blanco et al. 1992; Matthijs et al. 2000, 2003; Troedsson et al. 2005) (Fig. 5.6). However, it still remains unknown whether the interaction between both cell types occurs randomly or a specific binding exists. In this regard, Taylor et al. (2008) have reported that those sperm cells that interact with neutrophilic granulocytes have their membrane intact. Although membrane integrity does not seem the only functional parameter to be taken into account when assessing sperm-neutrophil interaction, these authors have observed that membrane-damaged spermatozoa do not bind polymorphonuclear neutrophils. In this context, sperm motility also seems to be involved in this interaction.

Finally, the interaction between spermatozoa and neutrophils also seems to be modulated by some seminal plasma components. In horses (Alghamdi et al. 2004) and in boars (Taylor et al. 2008; Li et al. 2012), seminal plasma reduces the chemotactic and phagocytotic activities of polymorphonuclear neutrophils when co-incubated with spermatozoa, while sperm diluted in semen extender does not. Here, it is also interesting to highlight the modulating role of seminal plasma constituents in sperm interactions since, as mentioned, they also inhibit the interaction of spermatozoa with uterine epithelial cells (see Sect. 5.6.3). All these aspects are discussed below.

5.8.3.2 Immune Mediators in Response to Spermatozoa and Seminal Plasma

After insemination, uterine and cervical cells synthesise and release cytokines, chemokines and other local mediators that are involved in the subsequent cellular response (Schuberth et al. 2008). These local mediators include cytokines, as granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6), and chemokines (Robertson 2007; Schuberth et al. 2008). Accordingly, Pandya and Cohen (1985) in human, and Robertson et al. (2000) in mice have reported that pro-inflammatory factors stimulate the infiltration of uterine and cervical tissues by macrophages, dendritic cells and granulocytes. In humans, Jeremias et al. (1998, 1999) found that semen induced the expression of IL-10 (anti-inflammatory TH2-related cytokine) and HSP1A1 (HSP70) in peripheral blood mononuclear cells from women in co-culture, but did inhibit the expression of interferon-gamma (IFN-γ) when phytohaemagglutinin, a potent IFN-γ-inducing T cell mitogen, was added to co-culture conditions. Hence, these authors suggested that human semen is both an inducer of an anti-inflammatory (TH2) immune response and an inhibitor of pro-inflammatory (TH1) cell-mediated immunity. In pigs, O'Leary et al. (2004) also observed a response to mating/insemination, since

seminal plasma induces the uterine expression of GM-CSF, IL-6 and monocyte chemotactic protein-1 (MCP-1), leading to monocyte and dendritic cell recruitment in the endometrial stroma.

Cytokines expressed by endometrial cells are considered as a part of the inflammatory response to insemination and are involved in the recruitment of leucocytes (Fig. 5.6). In swine, Rozeboom et al. (1998, 1999) and Matthijs et al. (2003) have proposed that cytokines are involved in the neutrophilic response to insemination. This hypothesis is in agreement with the findings obtained by Taylor et al. (2009b) who, in a study assessing the levels of mRNA of the five following transcripts: TNF-α, TGF-β, IL-10, CXCL8 and COX-2, demonstrated that only the concentrations of CXCL8-encoding mRNA in the presence of a commercial extender were correlated with the recruitment of neutrophilic granulocytes, while the other cytokines did not appear to be involved. As previously mentioned, seminal components elicit inflammatory responses in the female reproductive tract, including altered patterns of cytokine secretion, which have consequences for early embryo development and implantation (O'Leary et al. 2002). Indeed, boar seminal plasma appears to synergise in activating an inflammatory response and downstream changes in the female tract after insemination. In this regard, it is worth remembering that seminal plasma elicits endometrial changes, with induction of pro-inflammatory cytokines and cyclooxygenase-2, causing recruitment of macrophages and dendritic cells. Spermatozoa contribute by interacting with seminal plasma factors to modulate neutrophil influx into the luminal cavity. The cascade of changes in local leucocyte populations and cytokine synthesis persists throughout the preimplantation period (Robertson 2007).

As we have stated, the humoral response can be triggered by seminal plasma, spermatozoa, or by both. In humans and mice, it seems that it is the seminal plasma rather than sperm that is mainly responsible for triggering the immunological response (Robertson et al. 1996; Robertson 2007). Moreover, the components of the extenders (both short- and long-term) infused at the time of insemination may affect the immunological events, i.e. cytokine and chemokine release as well as cellular infiltration, and their nature and amount can vary, thereby triggering a different humoral response. Indeed, comparing the differential effects of seminal plasma, semen extender and sperm preparations on the endometrial expression of cytokines, Schuberth et al. (2008) observed that spermatozoa modulated the expression of interleukin-8, GM-CSF and TGF-β after 3 h post-insemination. However, the modulation of this cytokine expression did not appear to be exactly the same when spermatozoa were infused with seminal plasma or with semen extender. Related to this observation, Taylor et al. (2009b) found that the presence or absence of seminal plasma or extender also influenced the immune response in sows slaughtered after insemination. Accordingly, spermatozoa in semen extender (Taylor et al. 2009c) appear to stimulate the neutrophil influx in the uterine lumen, while seminal plasma suppresses it (Rozeboom et al. 1999; Taylor et al. 2009c). Specifically, seminal plasma components rather than sperm cells are thought to decrease the female immune response after insemination, but the down-regulated cytokines include pro- as well as anti-inflammatory mediators. This raises reasonable doubts about the real meaning of decreasing

cytokine expression in response to insemination, since both pro- and anti-inflammatory mediators are down-regulated in response to insemination.

Seminal plasma attenuates neutrophil immigration and function both in vivo (Rozeboom et al. 1998, 1999) and in vitro (Bischof et al. 1994; Gorgens et al. 2005a). This effect has also been reported in humans (Binks and Pockley 1999), rats (Galdiero et al. 1989) and cattle (Gilbert and Fales 1996). In vivo, the effects of seminal plasma (with or without spermatozoa) are partially reflected by a lower but still significant up-regulation of interleukin-8 expression compared to semen extender alone (Taylor et al. 2008). Schuberth et al. (2008) have reported that seminal plasma effectively blocks the interleukin-8-induced neutrophil chemotaxis. However, in this study it appeared that the anti-chemotactic effect of seminal plasma was more related to the strongly induced agglutination of neutrophils, which hinders in vitro migration.

In their study, Taylor et al. (2009b) found an inhibition effect on cytokine expression due to spermatozoa, since the presence of sperm cells abrogated the extender- or seminal plasma-induced up-regulation of interleukin-10 (IL-10), transforming growth factor-β (TGF-β), TNF-α, CXCL-8 (IL-8), COX-2 and ALOX-5 (Fig. 5.7). Moreover, Jiwakanon and colleagues (2011) have recently compared how inseminations with or without spermatozoa in extended seminal plasma affected the expression of pro-inflammatory (interleukin-1β, interleukin-6 and GM-CSF) and suppressive (interleukin-10 and TGF- β) cytokines (Fig. 5.8). Both Jiwakanon et al. (2011) and Taylor et al. (2009b) report no differences in the use of seminal plasma with or without spermatozoa on the expression of the three pro-inflammatory (IL-1β, IL-6 and GM-CSF) and one suppressive (TGF-β) cytokines. In the case of Taylor et al. (2009b) the study was performed 3 h after AI, while in that of Jiwakanon et al. (2011), it was carried out between 5 and 6 h after AI. Notwithstanding, the literature has reported the presence of interleukin-6, interleukin-10 and TGF-β in the oviductal endosalpinx epithelial cell layer (Jiwakanon et al. 2010) and the presence of TGF-β in the endometrial cells (Moussad et al. 2002).

Within the seminal plasma molecules involved in the immune response, the cytokine TGF-β seems to be the main factor (O'Leary et al. 2011). This cytokine is present as an inactive form, which is activated in the female reproductive tract by plasmin and other enzymes after insemination (Robertson 2005, 2007). Then, TGF-β acts indirectly by inducing cytokine and chemokine expression in the female genital tract as has been shown in the murine uterus (Tremellen et al. 1998). In addition, mammalian seminal plasma contains eicosanoids, such as prostaglandin E_2 (PGE_2) and interleukin-8, which are the strongest chemotactic agents for neutrophils and cooperatively interact with TGF-β (Palter et al. 2001; Robertson 2005). Indeed, the effects of TGF-β depend on the endometrial induction of appropriate cytokines. Thus, the presence of interleukin-6 is required for TGF-β to induce the generation of interleukin-17 and the production of pro-inflammatory Th-17 cells, which in turn favours the induction of interleukin-8 (Rubtsov and Rudensky 2007) that plays a neutrophil-chemotactic function.

Comparing seminal plasma vs. semen extender without spermatozoa, the former has been reported to induce the expression of interleukin-10, while the latter up-regulates the expression of CXCL-8, TNF-α and COX-2 (Fig. 5.7).

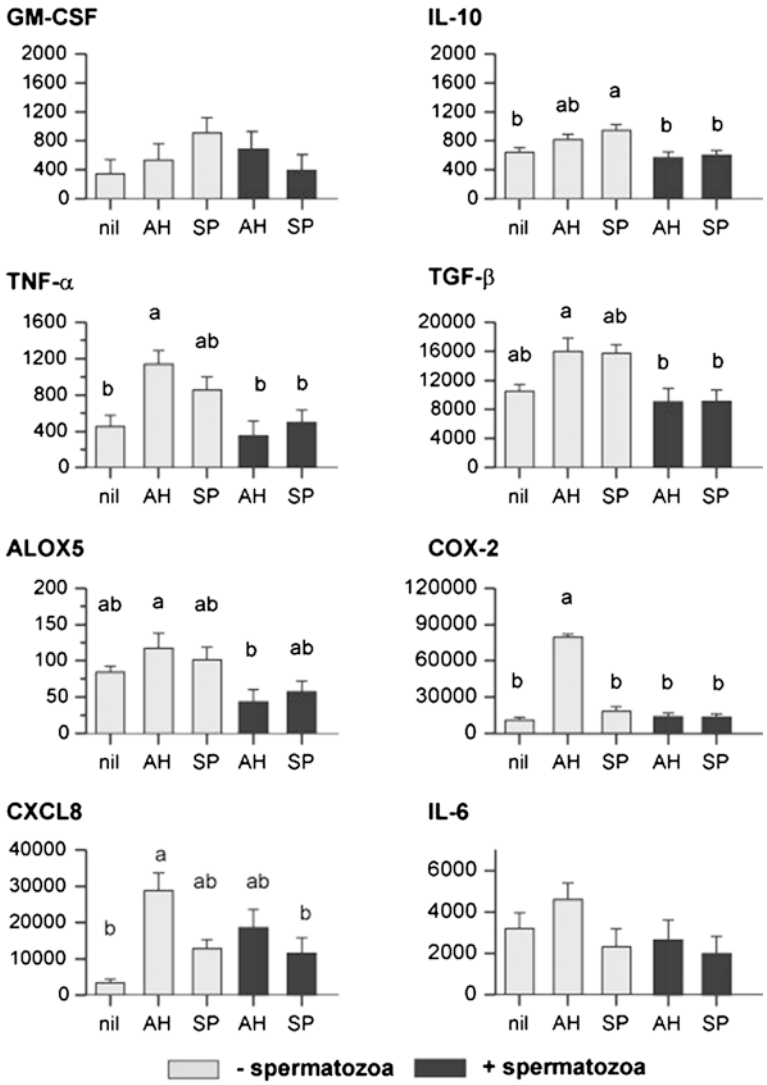

Fig. 5.7 Expression of cytokines, the chemokine CXCL8 (interleukin-8), ALOX-5 and COX-2 in the endometrial tissue 3 h after artificial insemination in the absence (*grey bars*) or presence (*black bars*) of spermatozoa. Abbreviations means *nil* not inseminated, *AH* inseminated with 98 % (v/v) Androhep™ and 2 % (v/v) seminal plasma, *SP* inseminated with 98 % (v/v) seminal plasma and 2 % (v/v) Androhep™ (Taylor et al. 2009b, Reproduced with permission)

This suggests, as previously mentioned, that the media used for extending semen also affects cytokine expression in the sow tract after insemination. In the case of the suppressive cytokine interleukin-10, Taylor et al. (2009b) observed a higher mRNA expression in the presence of seminal plasma without spermatozoa than in inseminations with both seminal plasma and spermatozoa. Moreover, Taylor et al.

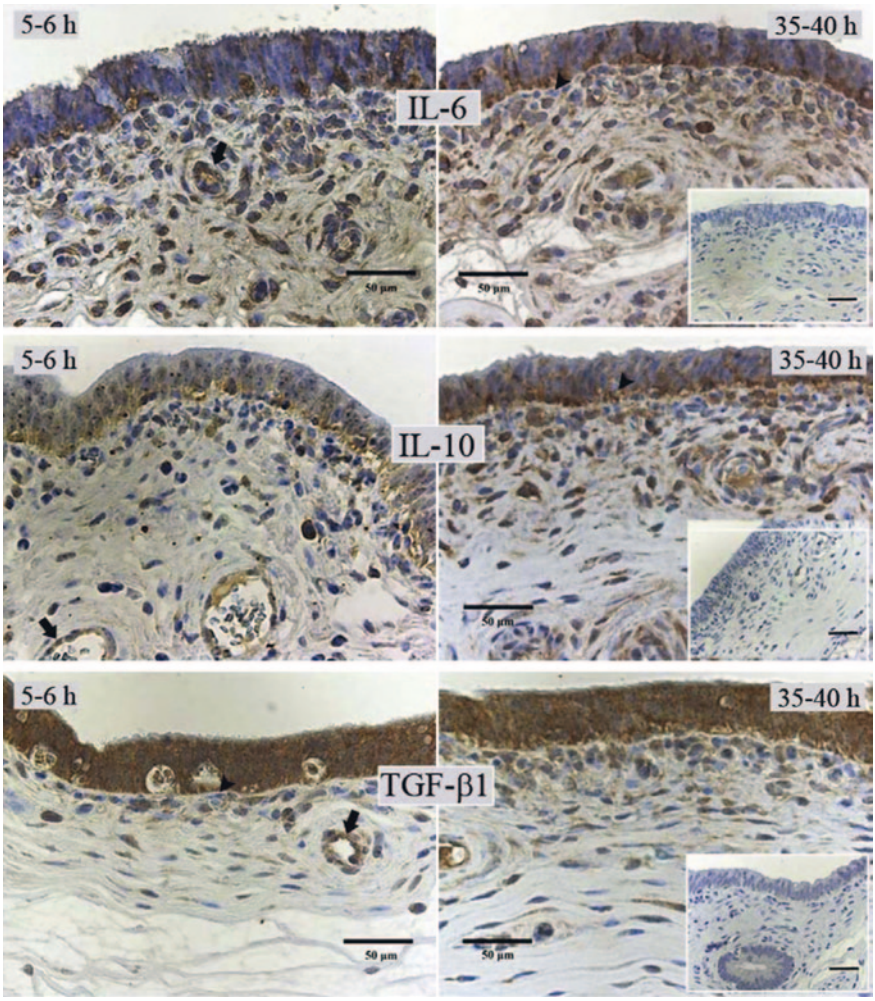

Fig. 5.8 Immunohistochemical labelling of *IL-6*, *IL-10* and *TGF-β1* in the surface epithelium and sub-epithelial connective tissue of the porcine endometrium collected 5–6 h and 35–40 h after treatment. *Arrows* (➡) indicate endothelial cells and *arrow heads* (⮝) indicate neutrophils (Jiwakanon et al. 2011, Reproduced with permission)

(2009b) also reported that the mRNA levels encoding TGF-β were higher when AI with a long-term commercial extender without spermatozoa took place than when insemination was performed with the commercial extender containing sperm cells.

5.8.3.3 Immune Response to the Short- and Long-Term Extenders

Inseminating with extended sperm doses induces a higher influx of neutrophils than insemination of spermatozoa in seminal plasma, or seminal plasma or extender alone (Matthijs et al. 2003). The composition of the various commercial extenders

differs among them (Yeste 2008; see also Sect. 10.2.1). Such heterogeneity entails a variety of immunological responses to insemination. In equine species, skim milk extender with egg yolk is strongly chemotactic for equine neutrophils in vitro (Gorgens et al. 2005a, b) and results in significant neutrophil migration into the uterus in vivo (Kotilainen et al. 1994). In pigs, Schuberth et al. (2008) have mentioned that a long-term extender (Androhep™, manufactured by Minitüb) can inhibit the migration of porcine neutrophils in vitro, but, in contrast, causes a massive and significantly higher influx of neutrophils when compared to seminal plasma, which also appears to be independent of the presence or absence of spermatozoa. According to Taylor et al. (2009b), the commercial extender (Androhep™) seems to induce leucocyte migration into the uterus owing to nonspecific irritation of epithelial cells or resident leucocyte populations rather than direct chemotaxis.

Both Taylor et al. (2009b), working with a long-term extender (Androhep™), and Jiwakanon et al. (2011), working with a short-term extender (BTS), have observed that commercial extenders with or without spermatozoa induce higher neutrophilic granulocyte migration into the uterine lumen than seminal plasma (Fig. 5.9). Indeed, in a study using a short-term extender (BTS) that involved suppressive cytokines (Jiwakanon et al. 2011), spermatozoa in BTS appeared to stimulate immune reactivity at about 35 to 40 h after insemination because of down-regulation in the expression of the suppressive cytokine TGF-β and interleukin-10 in the endometrium. These authors also observed that cervical stimulation alone also affects neutrophil infiltration because the presence of neutrophils in the endometrial subepithelial connective tissue, when stimulating the cervix after insemination, was higher than when inseminating with seminal plasma without spermatozoa. The response to this cervical stimulation is similar to the one derived from inseminating with seminal plasma and spermatozoa. This would be in agreement with Woelders and Matthijs (2001), who suggested that the intromission of a volume of liquid after insemination rather than the composition itself is responsible for recruiting neutrophils in the uterine lumen.

Both Taylor et al. (2009b) and Jiwakanon et al. (2011) have shown that insemination and/or inseminated components modulate cytokine expression in the swine endometrium too. Jiwakanon et al. (2011) observed that inseminations with seminal plasma in the absence of sperm cells decreased neutrophilic granulocyte infiltration in the gilt endometrium. In short, active seminal constituents, damaged and viable spermatozoa and semen extender, act in concert to modulate early and mid-term immune responses in the female genital tract, although Jiwakanon et al. (2011) did not find a clear relation between the cytokines studied and the presence of polymorphonuclear neutrophils. Moreover, all the mentioned data also suggest that gene regulation after insemination and, thus, the inflammatory response, also relies on the inseminate composition so that artificial extenders contain immune-stimulating agents, while seminal plasma appears to contain immune-suppressors. Thus, and when comparing the endometrial cytokine expression in presence and absence of sperm cells, the extender up-regulates the expression of four of the eight cytokines while the presence of spermatozoa leads to general inhibition (Taylor et al. 2009b) (Fig. 5.7).

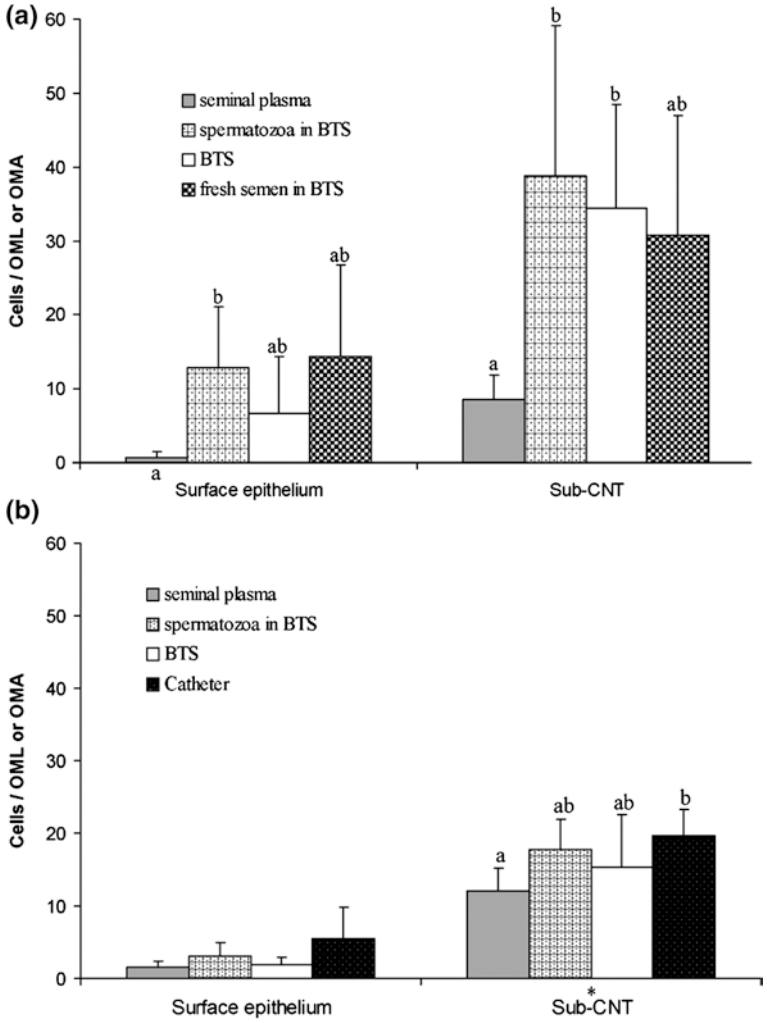

Fig. 5.9 Distribution of neutrophils in the surface epithelium and sub-epithelial connective tissue (*Sub-CNT*) of the sow endometrium collected at: **a** 5–6 h (insemination with seminal plasma, spermatozoa in BTS, fresh semen in BTS or BTS) or **b** 35–40 h after treatment (insemination with seminal plasma, spermatozoa in BTS, BTS or catheter only used). Abbreviations mean *BTS* Beltsville thawing solution, *OML* one ocular micrometre length and *OMA* one ocular micrometre area (Jiwakanon et al. 2011, Reproduced with permission)

5.8.3.4 Mechanisms and Modulation of Cytokine Expression

Seminal plasma components and spermatozoa modulate the cytokine expression in the endometrial cells (Fig. 5.6). As aforementioned, this modulating effect can be triggered by a direct binding sperm-UEC, by secretion of humoral factors, or by both. We proceed now to discuss these three speculated possibilities.

Direct contact between cells is one of the means by which sperm and epithelial cells interact, as Taylor et al. (2008) have observed. Their data indicate that an inhibition of cytokine expression is possible via direct contact between spermatozoa and endometrial epithelial cells, since up-regulation of such expression is absent when spermatozoa are infused into the uterus.

The other possible means consists of the secretion of humoral factors, which are involved in the communication between leucocytes. In this regard, Huleihel et al. (1999, 2000) have shown that seminal plasma contains cytokines (IL-1 and IL-6).

The degree and intensity of the immune response to insemination/mating is another question that merits discussion. After insemination, Taylor et al. (2009b) observed a significant but small increase in the expression of an array of cytokines, while the neutrophilic response was very strong when compared to baseline values. In agreement with these results, O'Leary et al. (2004) in swine and Gutsche et al. (2003) in humans also observed a quite low induction of cytokine expression in response to mating/exposure to seminal plasma. These findings have led to suggest that the endometrial cytokine response to insemination is moderate.

On the other hand, the mRNA expression of TGF-β in the oviduct mucosa (endosalpinx) is down-regulated when sows are inseminated in the absence of spermatozoa, while the presence of sperm cells up-regulates it (Jiwakanon et al. 2010). From these observations, it has also been concluded the different immunological response of endometrium and endosalpinx to spermatozoa obeys alternative pathways in different parts of the sow's reproductive tract. Therefore, up-regulation of the suppressive TGF-β in the oviductal mucosa would be consistent with its function, which consists of protecting early embryos in the oviduct at the pre-implantation stages. In contrast, down-regulation of TGF-β observed in the endometrium would take place to clean the uterus before the embryos enter the uterine lumen (Jiwakanon et al. 2010, 2011).

Apart from the role of semen on the swine's immunological response, the physiological status of the female is another factor to be considered in the immune response of endometrial tissues in insemination/copulation (Taylor et al. 2008). Taking into account various biological meanings of immune cells and immune mediators, the range of cellular responses to different inseminate preparations indicate specific immune responses and warrant a closer look at the level of regulating factors (Schuberth et al. 2008). In sows, an inflammatory reaction in the endometrium takes place after insemination. This process consists of the infiltration of leucocytes in the epithelium and in the sub-epithelial connective tissue (Kaeoket et al. 2003a) and of a massive influx of polymorphonuclear neutrophilic granulocytes into the uterine lumen within a few hours after insemination (Rozeboom et al. 1998; Kaeoket et al. 2003a). Upon arrival of the embryos at the uterus, i.e. between 2 and 3 days after ovulation, the neutrophils are eliminated (Kaeoket et al. 2003a). The immune response to insemination/mating also depends on the moment of insemination with respect to ovulation, i.e. whether insemination takes place before or after ovulation. This is related to the plasma levels of oestrogen and progesterone and to which of these is predominant (Kaeoket et al. 2002, 2003a, b), and may explain why the phagocytic role of neutrophils in the uterine lumen is not dominant in the early phases after insemination.

5.8.4 The Physiological Role of Neutrophils

5.8.4.1 Phagocytosis

Phagocytosis cleans the uterine environment after insemination (Robertson 2005). As previously stated, aged, non-viable or capacitated spermatozoa are preferentially targeted by neutrophilic granulocytes (Matthijs et al. 2003; Eisenbach 2003). This hypothesis is in agreement with the suggestion made by Tomlinson et al. (1992) that neutrophils take part in sperm cell selection in humans, removing superfluous, non-motile or damaged spermatozoa. Furthermore, in human (Vogelpoel and Verhoef 1985) and equine (Gorgens et al. 2005a) species, it has been reported that membrane-damaged sperm cells favour in vitro neutrophil migration.

It is not known exactly whether sperm cell phagocytosis is a selective or a random process, the former being more reliable than the latter. Indeed, under in vitro conditions boar spermatozoa are not phagocytosed by neutrophilic granulocytes when the antibodies and complements are absent (Schuberth et al. 2008), pointing to a selective process. Moreover, as stated above, neutrophils preferentially target sperm cells with intact mitochondrial membrane potential, which reflects a preference for certain sperm cell subpopulations.

5.8.4.2 Modulating the Decision of Immune Cells

Neutrophils play a key role in the recruitment, activation and programming of antigen-presenting cells (macrophages, dendritic cells) (Robertson 2007). These leucocytes secrete chemotactic signals that attract monocytes and dendritic cells, and influence macrophage differentiation to a pro- or anti-inflammatory state (Bennouna et al. 2003).

Although neutrophils release fewer molecules of cytokine than lymphocytes or macrophages, the number of neutrophilic cells during the inflammatory process is higher than that of mononuclear leucocytes and sources of cytokines.

Seminal plasma components, sperm cells and semen extender contribute to the regulation of neutrophil influx. Moreover, seminal plasma also takes the lead in the role that neutrophils play in the immunological response to insemination, so that it appears to shape the decision of neutrophils towards the activation or the suppression of other immune mechanisms (Schuberth et al. 2008).

5.8.4.3 The Biological Meaning of the Interactions Between Spermatozoa and Neutrophils

The biological meaning of the interactions between intact spermatozoa and neutrophils is still rather speculative. One of the most obvious reasons for spermatozoa–neutrophil interactions would be the initiation of sperm cell phagocytosis, even though this does not explain why neutrophils preferentially target viable spermatozoa. In this regard, it has been proposed that neutrophils play a negative-selection

role removing those spermatozoa that are not able to bind epithelial cells and, therefore, are not considered fit for fertilisation (Schuberth et al. 2008). Another possible biological meaning is that the attachment of spermatozoa to epithelial cells and/or to neutrophils induces signals, triggering the subsequent inflammatory responses. This hypothesis is supported by previous reports showing that inseminating with extended spermatozoa induces an influx of neutrophils but inseminating with extender alone does not (Rozeboom et al. 1999).

Under natural conditions, when a high number of sperm cells reach the uterus a neutrophil-mediated selective process may not be so relevant. However, new insemination techniques (e.g. with sex-sorted spermatozoa) require considerably reduced sperm dosages and conventional AI does not yield a satisfactory reproductive performance under this condition. In contrast, fertility rates improve significantly when spermatozoa are deposited in the vicinity of the site of fertilisation (intrauterine and deep intrauterine insemination) (Vázquez et al. 2005). Intriguingly, the failure caused by using conventional insemination with low sperm dosages suggests the relevance of sperm interactions with the intrauterine environment and possible selection processes.

5.8.4.4 Maternal Immune Tolerance and Remodelling of the Uterine Tissue

In the swine tract, as in the female genital tract of other mammalian species (Moffett and Loke 2006; Robertson et al. 2009), the establishment of pregnancy needs a proper balance between the immune reaction against foreign pathogens and the tolerance to allo-antigens like those of the embryo (Taylor et al. 2009b). Thus, given that implantation and development of foetal trophoblastic cells and the pregnancy take place within the female reproductive tract, a control of potentially harmful maternal immune mechanisms against paternally derived antigenic epitopes is needed. In this regard, the term immunotolerance has been adopted to refer to different complex mechanisms, such as the induction of a variety of regulatory T-cells (Treg).

Within the uterine tissues, the concept of maternal immune tolerance is very important to explain the turn from a pro-inflammatory to an anti-inflammatory reaction. In this process, seminal plasma contains and/or induces the release of TGF-β and $PGE_{2,}$ which participate, along with an increase in progesterone levels, in the development of maternal immune tolerance (Schuberth et al. 2008; O'Leary et al. 2011). In fact, this mechanism is quite complex. Immediately after insemination, TGF-β favours an initial pro-inflammatory reaction (Schuberth et al. 2008) while it induces the generation of antigen-specific regulatory T-cells in regional draining lymph nodes at later stages (Rubtsov and Rudensky 2007). These antigen-specific regulatory T-cells seem to control the activation and proliferation of putative harmful effector-T-cells by producing interleukin-10 in the periphery (Zou 2006). Then, interleukin-10 inhibits the generation of pro-inflammatory T-helper 1 cells and enhances the generation of T-cells that produce

anti-inflammatory cytokines (Schuberth et al. 2008). Generating anti-inflammatory cytokines inhibits the production of cytotoxic T-cells and complement-fixing antibodies. In this anti-inflammatory mechanism, PGE_2, progesterone and progesterone-induced blocking factor are also involved (Zhang et al. 2007), stimulating the antigen-presenting cells to secrete interleukin-10 (Liu and Kelly 2008) and the activated T-cells to inhibit interleukin-12 release (Par et al. 2003).

Reorganisation of the endometrial tissue needs growth factors, necessary for angiogenesis (e.g. VEGF, vascular endothelial growth factor), and matrix metalloproteinases for the rearrangement and restructuring of the endometrial stroma (Aplin 2002; Curry and Osteen 2003; Das et al. 1997; Sunderkotter et al. 1994). Both seminal plasma and spermatozoa indirectly contribute to endometrial tissue remodelling, since this reorganisation depends on leucocytes (including semen-induced attracted neutrophils) and on local epithelial cells (Schuberth et al. 2008). Moreover, seminal plasma plays a dual role in controlling the sow's immune response to mating/insemination, as it recruits macrophages and dendritic cells in the endometrial stromal tissue (McMaster et al. 1992; Robertson 2007; Robertson et al. 1996, 2009). First, macrophages and dendritic cells recruit foreign material, which then produce paternal antigen-specific T-cells that are inflammation-favouring T-cells. Later, macrophages and dendritic cells induce antigen-specific and regulatory T-cells (Robertson 2005, 2007). The seminal plasma-induced effects (i.e. cytokine expression, eicosanoid production) persist throughout early pregnancy (Robertson et al. 2006, 2009), are likely to be mediated by expansion of the Treg-cell pool and, thus, semen-induced attracted leucocytes modulate the course of inflammation and the mechanisms of tissue repair (Schuberth et al. 2008; O'Leary et al. 2011).

5.9 Conclusions

After mating or insemination, ejaculated spermatozoa are deposited in the female genital tract, being immediately and passively transported towards the oviduct throughout the uterus/uterine horns depending on the species. During this transit through the uterus, most of the male gametes are lost owing to immunological response, so that only a small number of them arrive at the distal portion of the isthmus, where they bind the oviductal epithelial cells. On the other hand, uterine epithelial cells are also able to bind viable spermatozoa, thereby suggesting that the uterus could protect viable spermatozoa from being removed with the backflow.

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Chapter 6
Boar Spermatozoa Within the Oviductal Environment (I): Sperm Reservoir

Marc Yeste

Abstract This chapter is the first part of three chapters dealing with the passage of boar spermatozoa within the oviduct. First, this chapter starts by outlining some aspects of the anatomy, histology and functions of the oviduct, together with the composition and functions of oviductal fluid. Most of the chapter deals with the formation of the sperm reservoir, including the molecular mechanisms and the role of sperm-surface adhering proteins. The interaction between spermatozoa and oviductal epithelium also focuses on the dialogue between oviductal epithelial cells (OEC) and spermatozoa, which comprises both the influence of OEC on sperm function and survival parameters, and the influence of spermatozoa on the oviductal proteome and secretome. The suitability of in vitro and in vivo studies is also discussed.

6.1 Introduction: The Relevance of the Oviduct in Reproductive Physiology

A great deal of research into the reproduction of pig and other mammalian species deals with the female reproductive tract, in relation to the physiology of the oviduct (Hunter 2005) and the uterus (Tabizadeh and Broome 1999), and IVF procedures (Romar et al. 2001, 2003). Specifically, the oviduct (also known as Fallopian tube in humans) has a significant role in the events leading to fertilisation, by providing an appropriate microenvironment for gamete support and transport leading to the ampulla, the region of the oviduct where fertilisation occurs, and for early embryonic development (Abe 1996; Romar et al. 2001, 2003; Hunter 2005; Rodríguez-Martínez et al. 2005).

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In general, three different steps characterise boar sperm passage within the oviduct (Hunter and Rodríguez-Martínez 2004; Tienthai et al. 2004; Rodríguez-Martínez 2007; Hunter 2008):

1. Upon arrival at the oviduct, spermatozoa bind the oviductal cells forming the sperm reservoir (also known as the oviductal reservoir or even as sperm oviductal reservoir) during the pre-ovulatory period. Spermatozoa are then stored until ovulation.
2. At the periovulatory stage, spermatozoa are gradually released from the reservoir.
3. Finally, sperm capacitation (see Chap. 7) occurs in response to post-ovulatory signals and spermatozoa move towards the ampullary-isthmic junction (AIJ), where fertilisation takes place (see Chap. 8).

The crucial events that take place in the oviduct, i.e. sperm storage, sperm capacitation and fertilisation, are related to regional/anatomical differences of this organ (Rodríguez-Martínez et al. 2001). Indeed, the oviduct can be anatomically divided into different parts regarding the reproductive-events (Fig. 6.1).

In the first part of the oviduct, a restricted tubal segment (utero-tubal junction (UTJ) and isthmus) forms the sperm reservoir, which entails the adhesion of spermatozoa to oviductal epithelial cells (OEC) and provides a safe environment for spermatozoa. Here, a portion of the inseminated spermatozoa undergoes a period of storage from hours to days and remains in a quiescent state (Rodríguez-Martínez 2007).

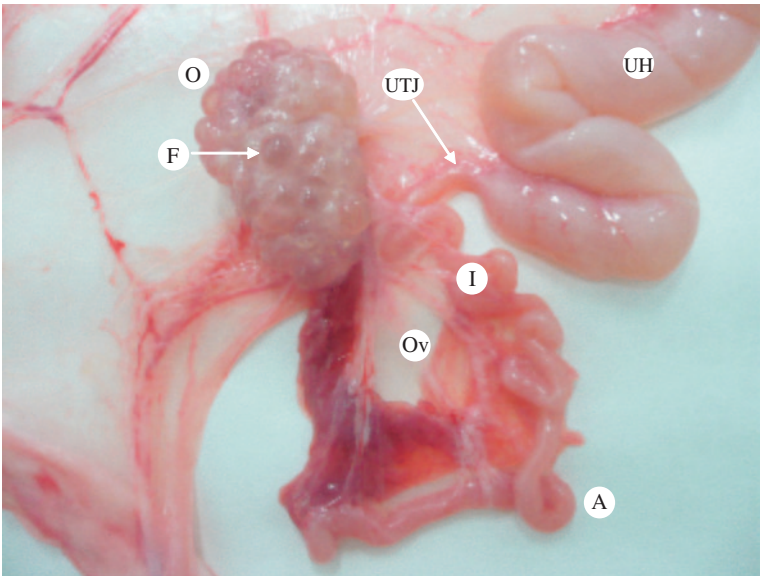

Fig. 6.1 General view of the porcine oviduct. Abbreviations mean *O* ovaries, *Ov* oviduct, *I* Isthmus, *A* ampulla, *F* follicle, *UTJ* utero-tubal junction, *UH* uterine horn

During this storage period, spermatozoa maintain their survival and fertilising ability, awaiting the arrival of the egg to the site of fertilisation (Rodríguez-Martínez 2007). This point is very important since spermatozoa are fully differentiated cells that, in principle, cannot be repaired if any damage is incurred.

The second part is constituted by the AIJ and ampulla. In this part, spermatozoa become capacitated and fertilisation takes place. Specifically, the events occurring in this second part will be taken up again in the next two Chaps. 7 and 8).

6.2 Anatomy and Parts of the Oviduct

The anatomic relationship between the oviduct and the ovary is highly important (Fig. 6.1). In domestic animals, such as pigs, the ovary is located in an open ovarian bursa, while in other mammal species, such as rats or mice, it is located in a closed bag (bursa). This bursa consists of a thin peritoneal fold of mesosalpinx, joined to a hanging handle in the superior region of the oviduct (Hafez 1993). In porcine species the ovarian bursa is well developed and, despite being open, contains most of the ovary.

Oviducts hang in the mesosalpinx, a peritoneal fold derived from the wide ovarian ligament, and bordered in front by the round ligament and behind by the ovarian ligament and mesovarium. It is attached medially to the uterine fundus and laterally to the infundibular pelvic ligament and to the ovary by means of an elongated mucosal fold, called the fimbria-ovarica.

The length of the oviduct and its degree of coiling are two features that vary among mammalian species. In sows, for example, the length ranges between 15 and 30 cm, while in ewes it is from 15 to 19 cm, and in cows it is about 25 cm long (Hafez 1993).

The oviduct can be divided into four functional segments: fimbriae, infundibulum, ampulla and isthmus, counted from the ovarian to the uterine ends. There are also three connecting areas: the UTJ, the AIJ, and the ostium, which is a terminal section connected to the ovarian fimbriae and bursa in the abdominal opening (Yáñiz et al. 2006).

Fimbriae are small finger-like extensions of the infundibulum that constantly beat, creating a vortex that sweeps the released egg into the oviduct. Since the ovary is not directly attached to the fallopian tube, the current made by the fimbriae is necessary in order to make sure the egg enters the ampulla, where fertilisation will occur if spermatozoa are present in the tube (Hafez 1993).

The infundibulum is an abdominal opening, shaped like a funnel, and is located near the ovary. Its size varies depending on the species and the age of the animal.

After the infundibulum, is the ampulla, which is more dilated and distal, and covers half the length of the oviduct. Between the ampulla and the isthmus, is the AIJ, which does not exhibit any special feature and whose existence is defined by its function as a temporary barrier to ova transport rather than by some tissue particularity. Fertilisation takes place in this oviductal region.

The isthmus, which is the proximal and narrowest section of the oviduct and forms the sperm reservoir (Suarez et al. 1991), connects directly with the uterus at the UTJ, which varies according to species and is surrounded by a fold of digitiform mucosa in sows (Töpfer-Petersen et al. 2002). This UTJ and the posterior part of the isthmus regulate the transport of spermatozoa in pigs, as early studies using scanning electron microscopy around ovulation showed (Fléchon and Hunter 1981).

There are also muscles that cover the area from these epithelial layers to the connective tissue of mucosa folds. The thickness of this musculature increases from the ovarian to the uterine ends of the oviduct (Hafez 1993).

6.3 Histology of the Oviduct

The histoarchitecture of the oviduct is very simple and formed by a non-glandular mucosa (endosalpinx), a double-layered smooth muscle (myosalpinx) and a covering serosa (mesosalpinx) continuous with the peritoneal covering. This histoarchitecture defines the presence of tubal compartments, each one with a specific function: maintenance and regulation of gametes, fertilisation, and the first steps of zygote development (Rodríguez-Martínez et al. 2001).

6.3.1 Oviduct Mucosa (Endosalpinx)

Oviduct mucosa consists of primary, secondary and tertiary foldings.

Ampullary mucosa is arranged in tall folds that become shorter towards the isthmus. The complex configuration of these ampullary mucosa folds largely fills the fluid-conducting lumen.

The tubal mucosa consists of a simple cylindrical epithelium lying on a richly vascular conjunctive stroma. The folds are of variable height and complexity, depending on the segment, the species and the stage of the oestrous cycle (Beck and Boots 1974). They are tall and complex in the infundibulum and the ampulla, becoming shorter and simpler in the isthmus. In sows, the mucosa fold at the UTJ extends into the uterine cavity forming a valve-like structure that can limit passage of the uterine contents into the oviduct (Fléchon and Hunter 1981).

The mucosa consists of a layer of cylindrical epithelial cells, and the adjoining mucosa, or submucosa, is formed by smooth musculature fibres and connective tissue and is highly vascularised. This epithelium consists of secretory and ciliated cells, which will be described separately.

6.3.1.1 Ciliated Cells

Ciliated cells of the oviductal mucosa present thin and motile cilia that become extended to the oviductal lumen. The beat frequency of these cilia depends on the concentration of ovarian hormones, and their activity is greatest at the moment of ovulation, when cilia movement in the fimbriated region of the oviduct is highly synchronised and leads in the direction of the infundibulum aperture.

It seems that ciliary beating enables the ovum to leave the follicular surface and go to the infundibulum. The relationship between ciliary and secretory cells along the oviduct varies, and is highly relevant (Hafez 1993; Abe 1996). Whereas ciliary cells are more abundant in the fimbriae and infundibulum, where the egg is harvested, secretory cells are more plentiful in the regions of the oviduct where oviductal fluid plays a relevant role in sperm-oocyte interaction, such as the ampulla.

Cilia beat their way to the uterus. This activity, jointly with oviduct contractions, is basic because fertilisation takes place in the oviduct (see Chap. 8) but the implantation of fertilised oocyte occurs in the uterus. However, oviducts vary in those mammal species that, unlike pigs, are subjected to an oestrous cycle. As a result, they atrophy and lose their cilia in the anoestrous stage, hypertrophy, recover their cilia in the proestrus and oestrus stages, and atrophy again, losing their cilia in gestation (Hafez 1993).

6.3.1.2 Secretory Cells

The secretory cells of the oviduct are not ciliated and characteristically contain secretory granules. The number and the size of these granules depend on both the species and the stages of the oestrous cycle. There are a large number of microvilli on the apical surface. Secretory granules accumulated during the follicular phase are released to the fluid- conducting lumen after ovulation, so that the height of the epithelium is reduced.

Secretory cells produce and release several macromolecules (Abe 1996) into the oviduct lumen, such as oviductal secretory glycoproteins that are present in many species of mammals (Buhi et al. 2000; Killian 2004). Such secreted proteins, observed both in vivo and in vitro conditions, provide a suitable environment for the events that occur in the oviductal lumen, such as fertilisation and embryo development, and maintain sperm function in vitro (Lippes and Wagh 1989; Abe et al. 1995; Abe 1996; Quintero et al. 2005; Georgiou et al. 2007). All these aspects will be taken up again in a specificion about Oviductal Fluid (Sect. 6.5).

Different studies have reported regional variations within the mammalian oviduct in both the morphological and ultrastructural features of these secretory cells in some species (Abe 1996). However, in in vitro conditions, Eyestone et al. (1991) found no effect of the cycle stage in cultured OEC on early bovine embryo development, and Fazeli et al. (2003) did not observe differences when comparing apical plasma membrane preparations (APM) coming from the isthmus and ampulla regions after co-incubation with spermatozoa in pigs.

6.3.2 Changes in the Oviductal Epithelium During the Oestrous Cycle

The oviductal epithelium is subjected to the cyclic changes of the oestrous cycle. In this regard, the epithelium in the ampulla and infundibulum is high columnar and pseudostratified with a high degree of mitotic activity and secretory granules during prooestrus and oestrus (Jiwakanon et al. 2005). In contrast, this epithelium

is low columnar with some degree of pseudostratification, fewer secretory granules and less mitotic activity during dioestrus. Degree of submucosal oedema is also highest at oestrus, when levels of oestrogen in blood are high (Jiwakanon et al. 2005).

As far as the differences between the oviductal regions are concerned, it must be noted that the isthmus presents a lower degree of morphological changes than the ampulla and infundibulum during all stages of the oestrous cycle (Abe and Oikawa 1992; Jiwakanon et al. 2005).

Finally, lymphocytes are the dominating immune cell in the oviductal epithelium, while the lymphocytes and plasma cells (also known as plasmocytes) are the most common immune cell types in the submucosa, even though such submucosa also contains low numbers of neutrophils, macrophages, mast cells and eosinophils (Jiwakanon et al. 2005). In this regard, it is worth noting that the number of intraepithelial lymphocytes and macrophages do not differ between oviductal regions and/or oestrous cycle stages, whereas the number of lymphocytes, plasma cells and neutrophils in the submucosa changes significantly throughout the different parts of the oviduct, but not between the different stages of the oestrus cycle.

6.3.3 Vascularisation and Innervation

Uterine and ovarian arteries supply the oviduct (Fig. 5.4). The increase in the number of blood vessels is largely regulated by ovarian oestrogens, and is related to an increase in the secretory activity of the oviduct (Hafez 1993).

On the other hand, short adrenergic neurons partially innervate the oviduct, similar to what happens in other segments of the female reproductive tract. In fact, the oviducts are mainly innervated by prevertebral and paravertebral ganglia (long adrenergic neurons), and by adrenergic ganglia formations in the uterovaginal region (short adrenergic neurons).

The degree of innervation varies, depending on the muscular layers and oviductal regions. Adrenergic innervation is particularly abundant in the circular musculature of the isthmus and at the isthmic-ampullary junction, where adrenergic terminations connect with smooth musculature. Dense adrenergic innervation allows the isthmus to act as a physiological sphincter that is important for the transport of eggs (Hafez 1993).

6.3.4 Musculature of the Oviduct and Related Ligaments

Throughout the length of the oviduct, there is a circular muscular layer under the mucosa. This layer is thicker in the isthmus, increasing from the AIJ to the UTJ. It is lined with an external layer of longitudinal bands (or peritoneal muscle), separated at the ampullary level but confluent around the isthmus to form a complete

layer. An internal longitudinal layer, terminating in the myometrium, is located in the proximal isthmus (Rousseau and Ménézo 1993).

Oviductal contractions homogenise the secretions, make fertilisation easier, and in part regulate ova transport. Oviductal peristalsis tends to retain the egg, instead of contributing to its movement, unlike what occurs in intestinal peristalsis (Hafez 1993).

6.3.4.1 Pattern of Oviduct Contractions

Oviduct musculature presents different kinds of complex contractions: located peristaltic contractions, previously originating from isolated segments or handles that only cover a short distance; segment contractions, and lumbricoid movements of the overall oviduct. Abovarian contractions are more common than adovarians, and the ampulla is generally less active than the isthmus in terms of muscular activity (Hafez 1993).

Since longitudinal muscular fibres (which shorten) and circular muscular fibres (which produce annular constrictions) are constantly activated, the pattern of oviductal contractions is complex. Other factors contributing to the complexity of this pattern are the contractile activity of mesosalpinx, myometrium and supporting ligaments, as well as ciliary movement.

The pattern and amplitude of contractions vary among oviductal regions. In the isthmus, peristaltic and antiperistaltic contractions are segmentary and continuous. In the ampulla, intense peristaltic segmentary waves advance to the middle of the organ. The variability of contractile patterns is associated with cyclical changes in the glycogen content of oviduct musculature, which is more abundant in circular (internal) musculature than in the longitudinal (external) layer.

6.3.4.2 Prostaglandins and Oviduct Contractions

The effect of prostaglandins on oviductal contractility depends on the chemical nature of these molecules. Thus, prostaglandin E1 (PGE_1) and prostaglandin E2 (PGE_2) fulfil a characteristic effect on oviduct longitudinal musculature, by contracting the proximal part while relaxing the rest of the organ. Conversely, prostaglandin E3 (PGE_3) induces oviduct relaxation completely, and prostaglandins F1 (PGF_1) and $PGF_{2\alpha}$ act as contractility stimulants in humans (Lindblom et al. 1978) and in rats (Pérez-Martínez et al. 1998), without changes being observed during the menstrual cycle.

Oviductal contractility has been reported to be mediated by the plasma levels of oestrogens and progesterone (Spilman et al. 1978; Mwanza et al. 2000a, b). In this sense, 17β-estradiol and $PGF_{2\alpha}$ are closely related, since it has been demonstrated that 17β-estradiol stimulates prostaglandin synthesis (Saksena and Harper 1975).

More recently, Kodithuwakku et al. (2007) have shown that motile but not dead spermatozoa increase the biosynthesis and secretion of PGE_2 and $PGF_{2\alpha}$

in cultured OEC. This study has been performed using real-time RT-PCR in homologous bovine co-culture and has demonstrated for the first time that live spermatozoa may accelerate their own transport towards the fertilisation site by upregulating the expression of PGE_2 and $PGF_{2\alpha}$ (both enhance the contractions of the oviduct, as has already been noted).

6.3.4.3 Musculature of Uterovarian Ligaments

Uterovarian ligaments contain smooth muscle fibres mainly arranged in longitudinal bundles. These muscle fibres are also present in the myometrium and inside ovarian stroma. The smooth musculature, consisting of mesovaries and different mesentery ligaments attached to the ovaries and fimbriae, intermittently contracts. These rhythmic muscular contractions ensure that the fimbriae remain in a constant position regarding the ovarian surface (Hafez 1993).

6.4 Oviduct Functions

Oviduct functions depend on the oviductal epithelium and fluid (i.e. ciliated and secretory cells) as well as on the oviductal region. The oviduct functions are mainly the transport of gametes, sperm storage and capacitation, fertilisation and early embryo development. In the present section, these different functions will be analysed separately.

6.4.1 Gamete Transport

The oviduct plays a fundamental role in transporting gametes, in maintaining their function, and in embryo development (Abe 1996; Hunter 2005). With regard to gametes, oocyte and spermatozoa advance simultaneously in opposite directions along the oviduct. Although this is a single function, the oviductal structure is well adapted to their roles. Fimbriae allow the transport of released ova from the ovarian surface to the infundibulum. Later, eggs are transported through the ampulla up to the uterotubal junction, where fertilisation takes place. The most proximal region of the oviduct, the UTJ, controls the transport of sperm cells from the uterus to the isthmus, and is a kind of mechanical valve that severely limits the number of spermatozoa penetrating the oviduct (Fléchon and Hunter 1981).

6.4.2 Sperm Reservoir and Capacitation

The oviduct is a dynamic organ that modulates gamete physiology. In this regard, it is worth noting that the oviduct mainly plays a dual function because (a) it stores

male gametes in the sperm reservoir until ovulation occurs (keeping the sperm at a 'quiescent state'), and (b) it provides a suitable environment for spermatozoa to undergo capacitation, thereby preparing them for interaction with oocytes and fertilisation (Fig. 6.2).

Therefore, on the one hand, spermatozoa transiently adhere to the oviductal epithelium forming the sperm storage reservoir at the UTJ and isthmus (Fig. 6.3). This allows the selection of spermatozoa with certain qualities in eutherian mammals, and maintains sperm function. In fact, direct contact between spermatozoa and the oviductal epithelium has been described as a crucial final phase for the fertilising ability of the sperm before encountering the oocyte (Hunter 1984). At the time of ovulation, only a few spermatozoa can be released from the reservoir (Suarez et al. 1991; Töpfer-Petersen et al. 2002). Thus, the reservoir basically serves two functions: to ensure that sperm are available in the oviduct at the time of ovulation, and to prevent polyspermy (Hunter 1990).

On the other hand, after spermatozoa are released from the reservoir, they progress towards the AIJ and another crucial physiological process takes place in the oviduct: sperm capacitation (Tienthai et al. 2004; see also Chap. 7).

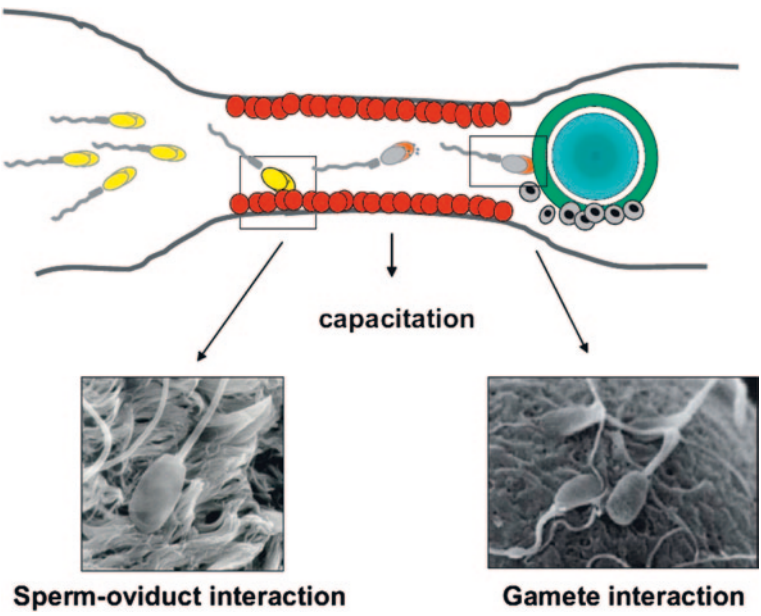

Fig. 6.2 Oviduct functions: sperm reservoir, capacitation and fertilisation. Firstly, spermatozoa enter the oviduct, are trapped in the isthmic region through carbohydrate-mediated binding mechanisms and are stored under protective conditions (sperm reservoir). Close to the time of ovulation the capacitation process is initiated and sperm dissociate from the epithelium and freely swim to the site of fertilisation thereby completing capacitation. Finally, capacitated spermatozoa meet and recognise the ovulated egg (Töpfer-Petersen et al. 2008, Reproduced with permission)

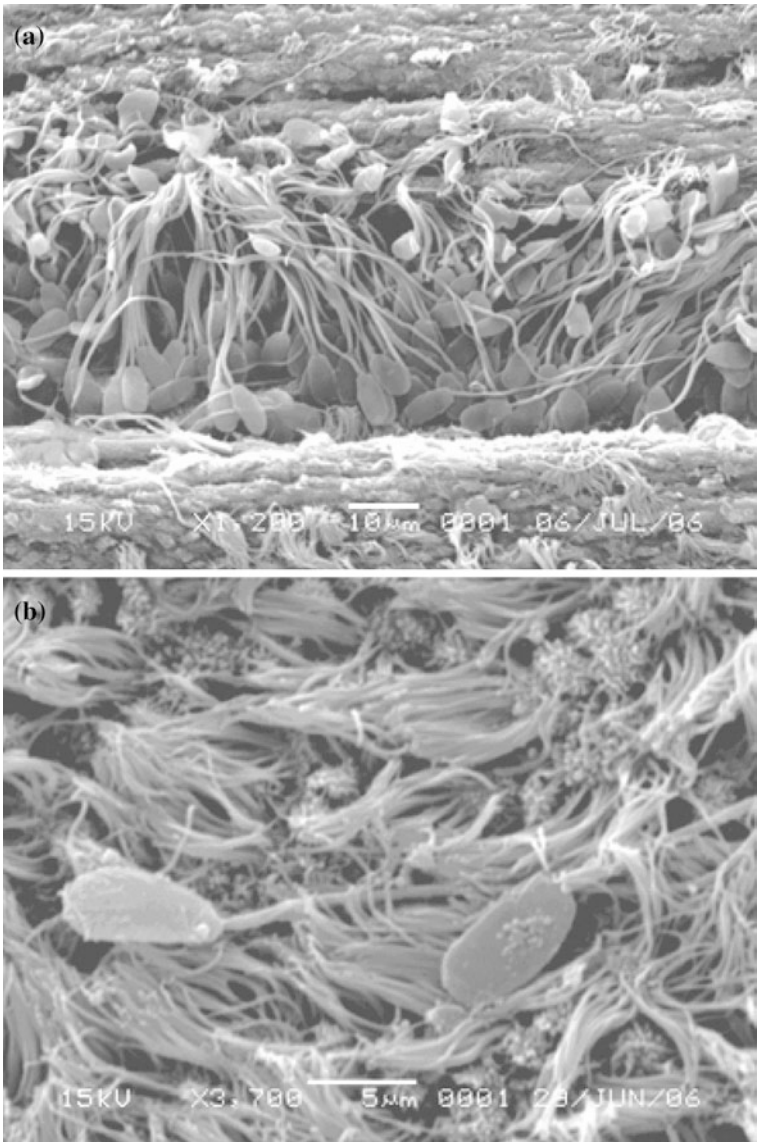

Fig. 6.3 Scanning electron micrographs of boar spermatozoa bound to the epithelia of the (**a**) utero-tubal junction and (**b**) oviductal isthmus, four hours after artificial insemination (Holt et al. 2010, Reproduced with permission)

In both cases, i.e. sperm reservoir and sperm capacitation, the coordination under strict oviductal control together with the presence of gametes is very important since sperm capacitation is an irreversible process, so that destabilisation of the sperm membrane when an oocyte is absent unavoidably leads to sperm death (Rodríguez-Martínez 2007).

6.4.3 Fertilisation and Early Embryo Development

The oviduct is involved in fertilisation, as the AIJ is the venue of sperm-oocyte interaction and fusion (Fig. 6.2; see also Chap. 8). In addition, the oviduct also plays a relevant role in early embryonic development, because embryos remain in this organ about three days before being transported to the uterus. In this regard, two aspects of oviduct physiology are important for embryo development: the oviductal environment and the active components. The oviduct environment includes temperature, pH, osmotic pressure, nutrients and oxygen tension, amongst others. The active components of the oviductal fluid, namely stimulatory and regulatory molecules and growth factors, regulate the fertilisation process and the first differentiative steps (Gandolfi 1995), and seem to also respond to prior gamete presence (Georgiou et al. 2007).

6.4.4 Control of Oviductal Function

Finally, the functions of the oviduct depend on the regulation of smooth muscle contractility, epithelial ciliated cells and oviductal secretions. Several studies have shown that ovarian hormones, nitric oxide, cytokines and endothelial growth factors relax or contract oviductal smooth muscle and modulate the oviductal ciliated cells (Pérez-Martínez et al. 2006). As will be discussed in the section about sperm-oviduct interaction (Sect. 6.12), gametes, both spermatozoa and oocytes, also control oviductal function (Georgiou et al. 2007).

6.5 Oviductal Fluid

6.5.1 Introduction

As stated, the oviduct provides the optimum medium for gamete union, for accomplishing fertilisation and early embryonic development, and for inducing sperm capacitation and hyperactivation, which occurs quickly in pigs, unlike in other species (Yanagamachi 1994a, b; Fazeli et al. 1999). For these reasons, this environment is both nutritive for and protective of spermatozoa, oocytes and embryos (Gandolfi 1995; Coy et al. 2010). In this regard, it is important to note the role of oviductal fluid, which is composed of oviductal secretions and mixed by ciliary beating and myosalpinx contractions (Rodríguez-Martínez 2007).

Since the isthmus completely or partially blocks the progress of fluids to the uterus, the direction of oviductal fluid is towards the ovary. Different physiological factors can contribute to create currents or cross-currents of the fluid, such as:

- Quantitative and qualitative changes of oviductal secretions during the oestrous cycle.
- Variations in the size and the morphology of ciliary beats in the oviduct compartments.
- Constant changes in the diameter of oviductal lumen in different regions as a result of mucosal fold reorientation and muscular contraction.

6.5.2 Cyclic Changes and Regional Differences Affecting Oviductal Fluid

Using different catheterisation techniques to obtain a continuous supply of oviductal fluid, it has been demonstrated that the accumulation and the volume of this liquid via the oviduct depends on the species and the oestrous cycle, under hormonal influence (Leese 1988). Thus, volume is low during the luteal phase, then increases at the beginning of oestrus, reaching a plateau during ovulation and for the following three days, and finally diminishes down to characteristic levels of atretic ovarian follicles (Hafez 1993; Rodríguez-Martínez et al. 2001; Killian 2004). On the other hand, the volume of liquid secreted by the isthmus is much lower than that secreted by the ampulla and the infundibulum, due to the presence of fewer epithelial secretory cells.

The oviductal cells have a specific secretory activity, because different studies have shown some proteins are present in the oviductal fluid but not in the serum. Moreover, oviductal fluid also differs from blood plasma in terms of ionic composition, osmolarity and pH (Leese 1988), so that the pH of the upper segments of the oviduct is slightly alkaline (Hugentobler et al. 2004), and bicarbonate levels (35–90 mM) are higher than in venous blood (Zhou et al. 2005) (see also Sect. 6.5.4). Regional differences also exist in the composition of oviductal fluid and this seems to be related with the process of gamete preparation for fertilisation (Leese et al. 2001). This issue is described in the next subsection.

6.5.3 Composition of Oviductal Fluid

As indicated by biochemical analyses, oviductal fluid is a complex mixture of components originating from plasma (mostly at the ampullar segment) obtained via selective transudation from the blood through the lamina propria, and specific products derived from tissue metabolism (Leese et al. 2001). Accordingly, oviductal fluid is formed by serum and molecules coming from the granules produced by the secretory cells of the oviductal epithelium (Oliphant et al. 1984), which are regulated by oestrogens (Xia et al. 1996) and gametes (Georgiou et al. 2007).

The most relevant components of this fluid are (a) sulphated and non-sulphated glycosaminoglycans (Tienthai et al. 2001; Buhi 2002; Bergqvist et al. 2005a), and (b) proteins, some of them known as oviduct-specific glycoproteins or ‘oviductins’ (Killian 2004; see also Sect. 8.6.4). These different components, together with variations through the oestrous cycle and oviductal segments, will be analysed separately below.

6.5.3.1 Sulphated Glycosaminoglycans

Concentrations of total sulphated glycosaminoglycans (GAGs) (chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin) in the oviductal fluid depend on the species, oviductal region and the moment of oestrous cycle.

On the one hand, levels of total sulphated GAGs depend on the species studied, as pigs (Tienthai et al. 2001) are endowed with a lower number of sulphated GAGs than bovine species (Bergqvist and Rodríguez-Martínez 2006). This could be related to the role of these sulphated GAGs in each species since, for example, heparin and heparin-like GAGs have been reported to be powerful inducers of sperm capacitation in bull (Parrish et al. 1988, 1989; Galantino-Homer et al. 1997; Marquez and Suarez 2004) but not in boar spermatozoa (Ekhlasi-Hundrieser et al. 2005).

With regard to oviductal segments, concentration of sulphated GAGs in the isthmus is higher than in the ampulla, probably because the latter presents a larger secretory capacity than the latter (Tienthai et al. 2001).

Finally, the levels of sulphated GAGs also rely on the moment of the oestrous cycle, so that they significantly increase in the isthmus during pre-ovulatory oestrus and they then decrease towards metoestrus. These changes are observed in the oviducts, mainly because of bilateral ovarian activity in swine.

6.5.3.2 Non-Sulphated Glycosaminoglycans: Hyaluronan

The levels of non-sulphated GAGs (such as hyaluronan (HA)) in oviductal fluid also depend on the species, sows presenting a higher concentration of this non-sulphated glycosamino-glycan than cows, and on the moment of the cycle, increasing at standing oestrus and highest around ovulation. In contrast, levels of non-sulphated GAGs do not rely on the oviductal region (Rodríguez-Martínez 2007).

OEC, specifically at the level of sperm reservoir, contain HA synthases, HA-binding proteins and specific membrane receptors in porcine (Tienthai et al. 2001, 2003a, b) and bovine (Bergqvist et al. 2005b) species. Intriguingly, there is an accumulation of mucus at the pre-ovulation stage at this level (Johansson et al. 2000; Rodríguez-Martínez 2007). For all these reasons, HA has been found to be involved in sperm survival, capacitation and the binding to and release from the sperm reservoir within the oviduct (Rodríguez-Martínez et al. 2005; Liberda et al. 2006). The specific role of HA in sperm release will be discussed in Sect. 6.10.3.

6.5.3.3 Proteins

Oviductal fluid has been described as a protein-rich fluid, containing a wide array of oviduct-specific proteins of different molecular masses (50, 80, 130 and 200 kDa) (Buhi et al. 1997; Buhi 2002; Buhi and Alvarez 2003). These proteins have partially been characterised, identifying oviductin (also named

oviduct specific glycoprotein, OSP or OVGP1) and the soluble forms of ORP150, LAMP-1 and aminopeptidase N (McCauley et al. 2003; Töpfer-Petersen et al. 2008).

Oviductin is the major secretory protein of the mammalian oviduct and in the case of porcine species, it contains a highly O-glycosylated mucin-like domain and three N-glycosylation sites, which are occupied preferentially by complex N-glycans (Töpfer-Petersen et al. 2008). The concentrations of oviductin are oestrous- and region-dependent, so that it is predominant in the ampulla on days 1–3 after ovulation (6.8–10.3 % of total oviductal protein) (Wollenhaupt and Brüssow 1995). Oviductin appears to be involved in relevant functions during fertilisation and embryo development (Buhi 2002) and is up-regulated by the presence of spermatozoa (Georgiou et al. 2007; see also Sect. 6.12.3). In this regard, this protein is attached to the in vivo embryos but not to intrafollicular oocytes or to the in vitro produced (IVP) embryos (Brüssow et al. 1998). Moreover, its addition to in vitro fertilisation (IVF) and maturation (IVM) media exerts a positive effect on the *de novo* protein synthesis in in vivo matured embryos (Wollenhaupt et al. 1997), reduces polyspermic penetration (Kouba et al. 2000), and increases cleavage and blastocyst rates (McCauley et al. 2003). Recently, Coy et al (2008) have suggested that oviductin reduces polyspermy in swine and cattle because it accounts for ZP hardening within the oviduct after ovulation and before fertilisation (see also Sect. 8.6.4).

On the other hand, aminopeptidase N is a multifunctional enzyme, which has also been identified in oviductal (Sostaric et al. 2006; see Sect. 6.12) and other non-reproductive cells. This protein can be found both in the plasma membrane of OEC and as a soluble form, and seems to play a relevant role in cell proliferation and antigen presentation (Luan and Xu 2007). In human oviducts, aminopeptidase-N has been reported to participate in the interleukin 8 system (Palter et al. 2001) (see also Sect. 5.8).

6.5.4 Role of Oviductal Fluid on Sperm Capacitation, Fertilisation and Early Embryo Development

When encountering the egg, the spermatozoon has to be prepared for fertilisation. With this aim, two aspects must be taken into account at this moment:

1. 'sperm quiescence', i.e. a mechanism for ensuring that sperm maintains its survival and fertilising ability and is available at the moment of fertilisation, and
2. sperm capacitation, which is a destabilisation process that is required for sperm to gain the ability to bind the zona pellucida (ZP) and to penetrate the oocyte.

These two aspects are influenced by oviductal fluid, environmental pH and the amounts of GAGs, which play a significant role in this regard. Thus, and in

the case of sperm quiescence, the sperm reservoir (isthmus) is surrounded by pH and bicarbonate levels lower than those found in AIJ and ampulla ($pH_{isthmus}$: 7.5 ± 0.2 vs. $pH_{ampulla}$: 8.8 ± 0.6; $[HCO_3^-]_{isthmus}$: 10.0 ± 1.2 vs. $[HCO_3^-]_{ampulla}$: 33.1 ± 1.5) (Rodríguez-Martínez 2007). These environmental features seem to allow the maintenance of sperm quiescence and the prevention of capacitation in the oviductal reservoir up to shortly before ovulation (Mburu et al. 1996). On the other hand, the main role of oviductal fluid on sperm capacitation has been reported by Tienthai et al. (2004), who found that porcine spermatozoa retrieved from the pre-ovulatory sperm reservoir only become capacitated when exposed to homologous post-ovulatory oviductal fluid, but not when exposed to pre- or peri-ovulatory oviductal fluid. Still according to Tienthai et al. (2004), bicarbonate is, not only in porcine species, but also in other species, such as bovine (Bergqvist et al. 2006), the main effector for triggering sperm capacitation (see also Sect. 7.5.2). This was concluded because boar spermatozoa retrieved from the sperm reservoir were only seen to capacitate when exposed to bicarbonate levels similar to those present in vivo at the ampullary isthmic junction (i.e. 33–35 mM) (Tienthai et al. 2004). From these observations, Rodríguez-Martínez et al. (2005) have suggested that the constant release of individual spermatozoa out of the sperm reservoir is enough to induce their capacitation when adequate levels of bicarbonate are encountered outside the sperm reservoir area, sperm capacitation being in vivo a periovulatory event (Hunter and Rodríguez-Martínez 2004). The role of bicarbonate as the main effector of sperm capacitation will be taken up again in Sect. 7.5.2.

Several studies have also reported that binding of oviductal fluid proteins to the sperm membrane facilitates fertilisation and has a beneficial effect on sperm function, by capacitating and hyperactivating the spermatozoa and by avoiding premature acrosome reaction in the absence of the female gametes. These studies have been carried out in humans (Wagh and Lippes 1989; Lippes and Wagh 1989; Quintero et al. 2005) and in other mammalian species, such as bovine (Parrish et al. 1989; McNutt and Killian 1991; King and Killian 1994; McNutt et al. 1994; Abe et al. 1995; Lapointe and Sirard 1996), porcine (Coy et al. 2010) and equine (Ellington et al. 1993a).

In addition, oviductal fluid also appears to be involved for the maturation of porcine ovulated oocytes inside the oviduct to acquire full competence (Rodríguez-Martínez 2007). Accordingly, the oviductal fluid may be involved in the final maturation of the ZP, thereby playing a supporting role for fertilisation and early embryo development. This hypothesis is supported by previous observations showing that IVP porcine embryos do not present a normal development and have higher percentages of polyspermic fertilisations than in vivo fertilised oocytes. This may be explained by a deficient formation of cortical granules and a subsequent constrained reaction of ZP after first sperm penetration. As previously mentioned, oviductin (OSP or OVGP1), which is more expressed 1–3 days after ovulation than before, is the major protein of oviductal fluid that could explain this effect (Coy et al. 2008), since its addition to the IVF and IVM media exerts a positive effect on de novo protein synthesis in in vivo matured embryos (Wollenhaupt et al. 1997) (see Sect. 8.6.4).

Finally, there are other factors influencing the composition of oviductal fluid and subsequently early embryo development, such as the alteration of the secretory proteomic profile mediated by male and female gametes. This issue will be taken up again in a specific section about sperm-OEC interaction (Sect. 6.12).

6.6 Sperm Reservoir (I): General Concepts

6.6.1 The Sperm Reservoir in the Animal Kingdom

One of the successful reproductive strategies in the animal kingdom is sperm storage in specialised regions of the female reproductive tract (Ekhlasi-Hundrieser et al. 2005; Talevi and Gualtieri 2010). In this storage mechanism, which has been observed in insects, fish, amphibians, reptiles, birds and mammals, specialised organs trap and retain the spermatozoa before fertilisation, thereby maintaining their function and viability for a given period of time that depends on the species (Neubaum and Wolfner 1999).

According to Holt and Lloyd (2010), the existence of the sperm reservoir in females in animal species seems to be related to asynchrony between mating and fertilisation, which can last from a few hours in most eutherian mammals, up to days, months, and even years in some reptiles. In lower vertebrates (fish, amphibians and reptiles), male gametes can be stored in the female genital tracts, even allowing fertilisation at successive ovulations without additional mating (Neubaum and Wolfner 1999). This highlights the relevance of having a fertile sperm population available upon arrival of a mature oocyte at some time after mating.

The capacity of the female reproductive tract to maintain sperm function and survival relies on the traits of the species. For instance, sperm storage is advantageous for species whose male and female do not live together or for others whose insemination may occur a long time before ovulation but still result in fertilisation (Holt and Lloyd 2010). Other explanations link the existence of sperm reservoir to cryptic female choice (Birkhead 1998; Hosken and Stockley 2003); this mechanism offers genetic and reproductive advantages that maximise offspring number and genetic quality. In this regard, Pearse et al. (1999) reported that turtle females are able to select which cohort of spermatozoa from different males that have copulated with them before should be used for fertilisation.

Although the sperm reservoir is widespread through animal species, not all the physiological mechanisms for maintaining sperm fertilising ability are equal, nor do they seem to have followed the same evolution despite appearing to be analogous through different taxonomic groups. Nonetheless, it must be highlighted that the result, i.e. preserving sperm fertilising ability within the female reproductive tract, is similar despite existing differences in storage sites and the time storage lasts (Holt and Lloyd 2010).

Spermatozoa can be stored in different locations within the female reproductive tract. Fish present different reproductive strategies, including both external and internal fertilisation. Some fish with internal fertilisation have been reported to store spermatozoa within ovarian follicles, the venue of fertilisation in these species (Vila et al. 2007). Gummy sharks (*Mustelus antarcticus*) store spermatozoa throughout the uterine sphincter, body of the uterus, isthmus, and the oviduct of maturing and mature animals and in the uterine sphincter of immature animals one year before the first ovulation (Storrie et al. 2008). In amphibians, all the females belonging to the suborder Salamandroidea store spermatozoa in cloacal glands known as spermathecae (Sever and Brizzi 1998; Sever 2002). In birds and some reptiles, male gametes are stored in blind-ended sperm tubules during variable periods prior to fertilisation, the sperm later emerging from these tubules and proceeding towards the venue of fertilisation. In the case of eutherian mammals, the cervix, uterus, and oviducts are the venues of the female tract where spermatozoa are stored, as observed in dogs (Doak et al. 1967), pigs (Hunter 1981; Suarez et al. 1991), sheep (Hunter and Nichol 1983), mice (Suarez 1987), hamsters (Smith and Yanagimachi 1991), cattle (Hunter et al. 1991), and humans (Baillie et al. 1997; Suarez 1998; Suarez and Pacey 2006). The specific case of oviducts in pigs will be specifically discussed in Sect. 6.6.2.

Differences in sperm reservoirs throughout the animal kingdom also entail the mechanism that allows sperm fertilising ability to be maintained. In some cases, as in most mammalian species, spermatozoa directly bind epithelial cells from the female reproductive tract, but in others they are simply positioned near to the epithelial cells without establishing a direct contact with them. In those fish species presenting internal fertilisation, spermatozoa are stored within the ovary, develop intimate interactions with the epithelium of the ovarian duct (Potter and Kramer 2000), and even enter directly into the ovarian duct and interact with the follicular epithelium (Koya et al. 2002). Vila et al. (2007), working with the ovary in blue mouth rockfish (*Helicolenus dactylopterus dacrylopterus*), observed that spermatozoa became embedded within the epithelial crypts and appeared to be enclosed within cytoplasmic storage bags, surviving for approximately 10 months. In some shark species, sperm storage is of especially long duration and occurs within the oviductal glands without needing direct contact with oviductal epithelium (Hamlet et al. 2002). In higher mammals, the sperm reservoir uses another method for storing spermatozoa, which consists of sperm-oviduct adhesion and controlled trapping/release, instead of the presence of specialised organs or crypts, as in lower mammalian species (Bedford 1999).

In general, and in contrast with other animal species and with the exception of bats, mammals have failed to evolve such long-term sperm storage capabilities. In these species, the existence of sperm reservoir has also been demonstrated but its ability to store spermatozoa is much lower (from hours to a few days) than lower vertebrates (fish, amphibians and reptiles). In this regard, Holt and Lloyd (2010) hypothesised that mammals have invested in the alternative tactics of delayed implantation and embryonic diapause for optimising the match between the respective timings for mating, birth, and seasonality.

Within the mammals class, there are also differences in terms of sperm storage ability, since five species of marsupials are able to store sperm between 1 and 14 days and thirteen species from other eutherian orders store spermatozoa from 0.6 to 5 days. In contrast, eleven species from the order *Chiroptera* (bats) are able exceptionally to store sperm from 16 days to 6 months (Birkhead and Møller 1993).

6.6.2 A General Overview of Sperm Reservoir in Pigs

As discussed in Chap. 5, among millions of ejaculated spermatozoa that are deposited in the cervix after insemination/mating, only a few are able to enter the oviduct. Spermatozoa traverse the uterus up to the tubal end, and quickly colonise the UTJ and the lower segment of the oviductal isthmus forming the sperm reservoir (Petrunkina et al. 2001a). This process is mediated by the interaction between oviductal glycoproteins that expose their high-mannose glycans ($Man_{5–9}GlcNAc_2$) to the extracellular surface and sperm-surface adhering proteins, the major secretory proteins within boar seminal plasma (Töpfer-Petersen et al. 1998).

The mechanisms of sperm binding to oviductal epithelia have been studied using oviductal explants and OEC monolayers, and it has been reported that biochemical and mechanical factors are involved in its formation (Rodríguez-Martínez et al. 2001, 2005). In vitro studies have also shown sperm adhesion to the oviductal epithelium and cultured OEC prevents sperm capacitation and depresses motility (Fazeli et al. 1999; Hunter 2008; Petrunkina et al. 2001a; Yeste et al. 2009a). Notwithstanding, such studies have reported that only viable and uncapacitated spermatozoa are able to bind the oviductal cells, thereby showing the selective capacity of sperm reservoir in hamsters (Smith and Yanagimachi 1990; DeMott et al. 1995), bovine (Lefebvre and Suarez 1996), equine (Dobrinski et al. 1996, 1997) and porcine species (Fazeli et al. 1999; Petrunkina et al. 2001b; Yeste et al. 2009a, 2012).

Overall, several functions have been attributed to the sperm reservoir not only in porcine, but also in other mammalian species, including the maintenance of sperm lifespan and the regulation of sperm transport (Hunter 1996, 1998, 2003, 2005; Hunter et al. 1998, 1999; Petrunkina et al. 2001a; Töpfer-Petersen et al. 2002; Gualtieri and Talevi 2003). In addition, the microenvironment of the reservoir allows the selection of competent spermatozoa, modulates their capacitation and ensures their release in limited numbers, diminishing the risk of polyspermy (Hunter 1995; Talevi and Gualtieri 2010). In this regard, different studies have reported that the sperm reservoir suppresses capacitation-related events, such as the influx of calcium ions into the sperm cell and tyrosine phosphorylation of sperm proteins (Murray and Smith 1997; Smith and Nothnick 1997; Fazeli et al. 2003; Petrunkina et al. 2001b, 2004). Therefore, the most important feature of the sperm reservoir is that it allows male gametes to maintain their fertilising ability for longer periods (up to 30 h from the onset of oestrus), awaiting the arrival of the eggs at the site of fertilisation (Rodríguez-Martínez 2007). This ensures that a suitable number of viable, potentially fertile spermatozoa are available for fertilisation

(Wilmut and Hunter 1984; Hunter 1988, Smith and Yanagimachi 1990). This point is highly important since spermatozoa are fully differentiated cells that, in principle, cannot be repaired if any damage is caused (Rodríguez-Martínez 2007).

Around ovulation, spermatozoa are released from the oviductal reservoir in a process that does not only depend on capacitation and hyperactivation (Yanagimachi 1994a), but is also affected by local and systemic ovarian control mechanisms (Hunter and Rodríguez-Martínez 2004). As previously mentioned, the mammalian oviduct is formed by secretory and ciliated cells (Hafez 1993). During their stay in the sperm reservoir, sperm cells are in contact with isthmic secretions that contain oviduct specific proteins and enzymes (Rodríguez-Martínez et al. 2005; Suarez 2007). In this regard, both capacitation and acrosome reaction in a fertilising spermatozoon appear to be induced by specific fluids/effectors, such as bicarbonate at the AIJ (Hunter 2002).

As will be described subsequently, the release of spermatozoa from the sperm reservoir appears to be a peri-ovulatory event and a very well-coordinated process. In addition, it must be stated that not all the spermatozoa stored in the sperm reservoir participate in fertilisation, but only a restricted number (about 5 %) that are released and undergo sperm capacitation (Hunter 1981; Rodríguez-Martínez 2007).

6.6.3 Effect of Stress Simulation on the Establishment of Sperm Reservoir

Management procedures in swine include a number of situations that can be stressful for the animals (Einarsson et al. 2008). In this regard, stress may affect reproductive function and performance, and its effects on reproduction depend on the critical timing of stress in relation to the stage of the oestrous cycle, the genetic predisposition for stress, and the type of stress (Madej et al. 2005; Einarsson et al. 2008).

Different reports have studied the factors affecting the establishment of the sperm reservoir. Thus, the question of whether ‘stressful’ events provoke unfavourable effects on the establishment of the sperm reservoir, on the release of spermatozoa from the reservoir to the site of fertilisation, and on fertilisation rates have been investigated. With this aim, previous investigators stressed sows by administering adrenocorticotropic hormone (ACTH) through intramuscular injection for up to 48 h from the beginning of standing oestrus to ovulation (Brandt et al. 2006a, b; Eirnarsson et al. 2008). This hormone acts by releasing other hormones, such as progesterone, from the adrenal glands (Brandt et al. 2006a). Following this approach, administering high levels of ACTH from days 16–18 to the end of oestrus appears to delay the beginning of the heat and the development of cystic follicles.

In addition, this hormone has also been reported to alter the distribution of spermatozoa along the UTJ and oviductal isthmus. Thus, sows treated with ACTH present a larger number of spermatozoa in the UTJ and in the oviduct than non-treated sows, so that this hormone, which we must bear in mind, stresses

sows, appears to act favouring the establishment of sperm reservoir. Finally, ACTH also increases amounts of mucus in the intraluminal environment of the sperm reservoir (Madej et al. 2005; Brandt et al. 2006a; Einarsson et al. 2008).

6.7 Sperm Reservoir (II): Sperm-Oviduct Interaction

6.7.1 Introduction

The interaction between spermatozoa and OEC is mediated by carbohydrate-recognition mechanisms, so that sperm and oviductal surfaces expose molecules that participate in sperm-OEC binding (Biermann et al. 1997; Ekhlasi-Hundrieser et al. 2005; Töpfer-Petersen et al. 1998, 2008; Walter and Bavdek 1997). In fact, OEC and spermatozoa surfaces contain a variety of oligosaccharides (Walter and Bavdek 1997; Kirchhoff and Schroter 2001) and carbohydrate-binding molecules (Töpfer-Petersen 1999) that appear to be involved in sperm-oviduct recognition and binding (Fig. 6.4).

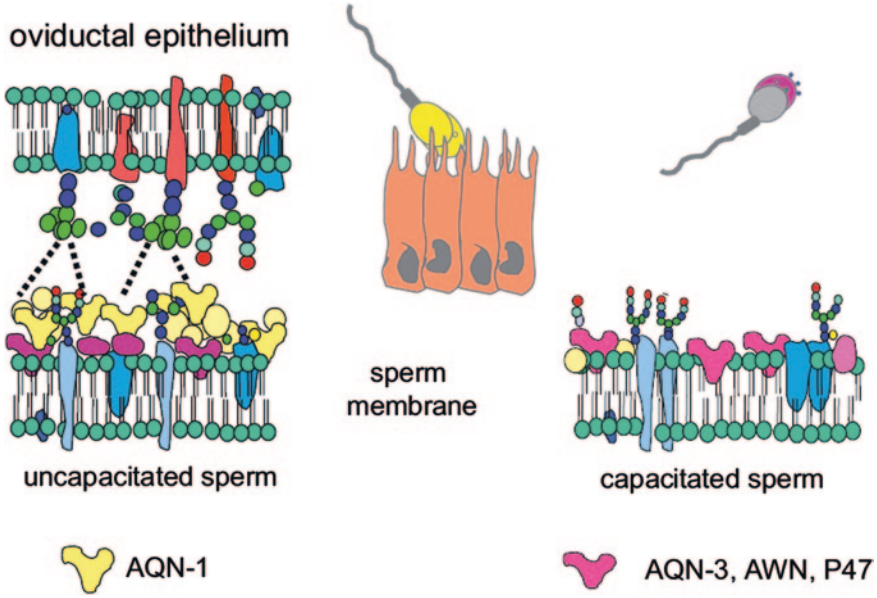

Fig. 6.4 Interaction between spermatozoa and the oviductal epithelium (sperm reservoir). Spermatozoa are associated with the oviductal cells through AQN-1 (a sperm-surface attached protein) that binds the exposed high-mannose type N-glycans of oviductal membrane glycoproteins such as LAMP-1/2 and others. When capacitation is initiated, AQN-1 dissociates from the sperm surface unmasking other sperm-surface attached proteins that are involved in ZP-binding (as AWN, AQN-3 and P47) (Töpfer-Petersen et al. 2008, Reproduced with permission)

As far as the sperm side is concerned, the mannosyl-oligosaccharide chains exposed by oviductal cells are recognised by sperm surface-binding proteins, which are produced by the boar reproductive tract and become associated with the sperm surface at the moment of ejaculation (Suarez 2001, 2002; Töpfer-Petersen et al. 2002). Taking this event into account, OEC-sperm interaction occurs when sperm surface-binding proteins attached to sperm cells, recognise flexible oviductal oligosaccharides (Revah et al. 2000; Ignotz et al. 2001; Ekhlasi-Hundrieser et al. 2005). As partially occurs in the case of sperm-ZP interaction in some mammalian species (see Chap. 8), the carbohydrate signals involved in OEC-sperm recognition depend on the species. These are terminal sialic acid in rodents (hamsters and rats) (DeMott et al. 1995; Cortés et al. 2004), fucose in cattle (Lefebvre et al. 1997; Suarez et al. 1998; Ignotz et al. 2001), galactose in equine species, and mannose in high-mannose or hybrid-type N-glycans in pigs (Green et al. 2001; Suarez 2001, 2002; Wagner et al. 2002).

6.7.2 Oviductal Surface

Different studies have investigated the distribution of carbohydrate moieties in the oviductal molecules involved in sperm-OEC interaction, since it is well known that cytoplasm, secretory granules, Golgi regions and the apical surface of the OEC express a variety of glycan structures containing α-mannosyl residues (Walter and Bavdek 1997) and oligomannosyl sequences (Ekhlasi-Hundrieser et al. 2005). Accordingly, Shibuya et al. (1988) used the lectin-affinity chromatography with *Galanthus nivalis* agglutinin (GNA) to specifically recognise mannose-residues in α(1–3)– and α(1–6)– linkages in the APM of oviductal cells and in oviductal fluid. These authors observed that both apical membrane of oviductal cells and oviductal fluid contained several proteins of a wide molecular mass range. Within the global amount of these proteins, they observed that glycoproteins carrying mannose-rich N-glycans were retained by the GNA-column, and presented a high molecular mass.

On the other hand, although molecules involved in sperm-OEC can vary between species, the mechanisms of sperm reservoir formation and activation successfully work in heterologous systems in in vitro conditions (Petrunkina et al. 2003, 2004). This suggests that oviductal cells expose carbohydrate chains that are flexible enough to trap heterologous spermatozoa, at least in in vitro conditions (Töpfer-Petersen et al. 2008).

The present section is divided into two parts. The first one addresses oviductal surface proteins that may be involved in the formation of the sperm reservoir (Sect. 6.7.2.1). The second one (Sect. 6.7.2.2) focuses on the oligosaccharide chains that oviductal cells expose to their extracellular surface, allowing them to recognise and trap spermatozoa.

6.7.2.1 Oviductal Surface Proteins

Two hundred seventy different proteins have been identified up to now in the oviductal surface (Sostaric et al. 2006). Within these surface proteins, we can find different lectin receptors, including glycoconjugates with terminal N-acetylgalactosamine, N-acetylglucosamine, galactose, and fucose residues (Walter and Bavdek 1997; Ekhlasi-Hundrieser et al. 2005) (Fig. 6.5) and various members of the heat shock protein (HSP) family that may be involved in sperm-oviduct interaction. These proteins are ORP150, LAMP-1 and LAMP-2, oviductal sperm binding protein (SBG), annexins, HSPA5 and HSPA8. The putative role of these oviductal surface proteins in the formation of the sperm reservoir is discussed below.

Glycoproteins Attaching the GNA-column: ORP150, LAMP-1 and LAMP-2

Different studies have been conducted to identify the various oviductal surface proteins and their functions. One approach involved isolating oviductal glycoproteins through GNA-affinity chromatography. Following this method, three major oviductal surface glycoproteins together with other minor oviductal components, which correspond to complex N-glycans with and without terminal α(1–3)-linked galactose residues, have been found to bind the GNA-affinity column

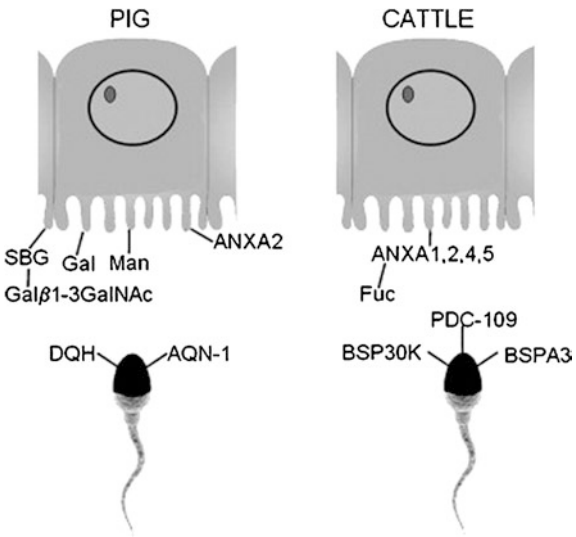

Fig. 6.5 Molecules/carbohydrate residues involved in sperm-oviduct binding in pig and cattle. Abbreviations mean *SBG* sperm binding glycoprotein, *Galβ1-3GalNAc* galactose-beta 1-3 *N*-acetylgalactosamine, *Gal* galactose, *Man* mannose, *ANXA2* annexin 2, *ANXA 1,2,4,5* annexins 1, 2, 4, 5, *Fuc* fucose, *BSP30K* bovine seminal plasma protein 30 KDa, *BSPA3* bovine seminal plasma protein A3 (Talevi and Gualtieri 2010, Reproduced with permission)

(Shibuya et al. 1988; Töpfer-Petersen et al. 2008). In all cases, the sequences of these oviductal glycoproteins binding the GNA-column contain several putative N-glycosylation sites or are already N-glycosylated at the extracellular domain. These N-glycosylation sites contain covalently linked N-glycans that allow oviductal cells to interact with spermatozoa through carbohydrate ligands.

As far as major glycoproteins are concerned, three different proteins, as aforementioned, have been hitherto identified: the porcine homolog of the human oxygen-regulated glycoprotein (ORP150) of 150 kDa, and two proteins of 100 kDa each; the porcine lysosome-associated membrane protein 1 (LAMP-1) and the porcine homolog to the human lysosome-associated membrane protein 2 (LAMP-2). In addition, these three glycoproteins have also been found in the oviductal fluid. The major neutral N-glycans species of these three glycoproteins is mannose N-glycans with 5–9 mannosyl residues.

Oxygen-regulated glycoprotein (ORP150, also known as HYOU1) is a molecular chaperone, expressed in response to cellular stress (Ikeda et al. 1997) that takes part in mechanisms of cell protection by facilitating protein transport and maturation (Sidrauski et al. 1998; Bando et al. 2000). In the case of the human variant, ORP150 presents nine N-glycosylation sites, of which at least seven asparagine residues have been shown to be glycosylated (Zhang et al. 2003). ORP150 has been reported to suppress hypoxia-induced apoptosis in human embryonic kidney cells (Ozawa et al. 1999) and to facilitate protein transport/maturation in an environment with low ATP levels (Bando et al. 2000). Furthermore, ORP150 is also present on the surface of mammalian spermatozoa, being identified as one of the five calcium-regulated proteins associated to the human sperm plasma membrane, together with other relevant proteins like HSPA2 (HSP70-1) and HSPA5 (also known GRP78; see Epigraph 6.12) (Naaby-Hansen et al. 2010). Taking into account all the above-mentioned, Naaby-Hansen et al. (2010) have suggested that ORP150 may participate in the protection of sperm membrane protein homeostasis and function during hypoxia-induced cellular perturbations, which are likely to occur as the gametes pass from the testis to the oviduct.

The other two major glycoproteins are LAMP-1 and LAMP-2. Although these two proteins are the main glycoprotein components of the lysosomal membranes (Fukuda 1991), both have also been identified at the extracellular surface of various cells, including oviductals, and LAMP-1 has even been found as a soluble form (Chang et al. 2004; Krishnan et al. 2005). In humans, both LAMP-1 and LAMP-2 are highly O- and N-glycosylated with up to eighteen N-glycosylation sites partially occupied with acidic poly-lactosaminylated complex N-glycans (Carlsson and Fukuda 1990). In the case of pigs, Töpfer-Petersen et al. (2008) have also predicted several N-glycosylation sites for the LAMP-1 homolog.

Oviductal Sperm Binding Glycoprotein

Apart from the GNA-chromatography binding proteins, another interesting protein that seems to mediate sperm-oviduct interaction in pigs is the Oviductal Sperm

Binding glycoprotein (SBG) (Marini and Cabada 2003; Teijeiro et al. 2009). This protein is located on the apical surface of the oviductal epithelium (Pérez et al. 2006) surrounding the oviductal lumen, and has the ability to attach to those sperm periacrosomal membranes that are not covered by spermadhesins (Marini and Cabada 2003).

Despite this, it remains to be seen what the actual function of SBG is, as the literature has been controversial in this regard. Thus, some authors have suggested that it plays a relevant role in the formation of the sperm reservoir (Pérez et al. 2006), while others have claimed a negative selection role (Teijeiro et al. 2008). On the one hand, Pérez et al. (2006) highlighted the interaction between SBG and the AQN1, since SBG would expose O-linked Galβ(1–3)GalNAc disaccharide (Marini and Cabada 2003) and the spermadhesin AQN1 would recognise galactose residues of this disaccharide. This led Pérez et al. (2006) to suggest that interaction SBG-AQN1 is one of the molecular systems responsible for the establishment of the sperm reservoir in pigs.

In contrast, some other reports offered a more suitable explanation, suggesting that this protein plays a negative-selection role since it might select those spermatozoa that have already started a capacitation process when reaching the oviduct (Teijeiro et al. 2008, 2009). In fact, SBG only binds early capacitated/capacitating spermatozoa, suppressing sperm motility as well as altering protein tyrosine phosphorylation patterns and acrosome integrity. Still backing this hypothesis, SBG has been found in the oviductal lumen where different authors have reported the presence of non-viable/altered sperm cells (Mburu et al. 1997; Tienthai et al. 2004). This suggests that SBG might attach to spermatozoa with altered plasma membranes that are unable to bind OEC, as previous co-culture studies have shown (Fazeli et al. 1999; Yeste et al. 2009a).

Annexins

Other protein-candidates from the oviductal surface that may be involved in the establishment of sperm reservoir are annexins. In cattle, these proteins interact with bull spermatozoa via sperm surface adhered proteins that belong to the Fibronectin-2 type protein family (such as the porcine DQH) and mediate sperm-oviduct interaction (Ignotz et al. 2007).

In the case of porcine species, Teijeiro et al. (2009) isolated the oviductal proteins interacting with sperm adhering proteins, and that might participate in the establishment of the sperm reservoir from the oviductal side. These authors identified different components in this extract, such as annexins A1, A2 and A5.

Annexin A2 is a polypeptide of about 38 kDa that has been suggested to be the main annexin involved in sperm-oviduct binding in pigs, working as a sperm receptor of oviductal cells. This annexin, which has also been identified in endothelial cells (Kim and Hajjar 2002), is located at the basal and apical surfaces of OEC and has been identified not only in the oviductal epithelium of pigs (Teijeiro et al. 2009), but also of rabbits (Chailley and Pradel 1992) and cattle (Ignotz et al. 2007) (Fig. 6.5). Annexin A2 might mediate the formation of

the sperm reservoir by interacting with spermadhesin AQN1, which is attached to the periacrosomal membrane of spermatozoa (Teijeiro et al. 2009; Talevi and Guatieri 2010). Moreover, given that it has been identified in different species, it has been suggested that Annexin A2 takes part in a generalised mechanism that mediates the formation of the sperm reservoir in eutherian mammals (Fig. 6.5). Additionally, Teijeiro et al. (2009) have speculated that the role of Annexin A2 would be the opposite to that of the SBG (see Sect. 6.7.2.1) (Teijeiro et al. 2009).

Apart from the specific role of Annexin A2 in the formation of the sperm reservoir, the other two identified annexins (A1 and A5) seem to play other physiological roles. However, the participation of these two annexins in the formation of sperm reservoir in pigs should not be excluded, especially because both seem to be involved in the establishment of sperm reservoir in bovine species (Ignotz et al. 2007).

Annexin A1 is present in the cytoplasm, basal membrane and apical surface of OEC in porcine (Teijeiro et al. 2009) and bovine species (Ignotz et al. 2007) (Fig. 6.5). It has also been identified in human serum and seminal plasma (Christmas et al. 1991), and it has been reported to inhibit the transendothelial migration of leukocytes (Walther et al. 2000), having an anti-inflammatory effect. For these reasons, Teijeiro et al. (2009) have suggested that Annexin A1 exerts an anti-inflammatory role within the oviductal environment.

In addition, Annexin A5 is able to bind phosphatidylserine residues of boar sperm surface upon bicarbonate induction (Gadella and Harrison 2002). This protein is also present in the cytoplasm and apical and basal membranes of OEC, which is why it has been suggested that it could be involved in sperm-oviduct binding in some way (Teijeiro et al. 2009).

Other Oviductal Proteins with a Putative Role in Sperm-Oviduct Interaction

Fazeli et al. (2003) isolated apical membrane plasma fractions from the pig oviduct and observed that these fractions bound to the apical region of the sperm head and maintained sperm viability in vitro conditions. Within these isolated oviductal proteins, one of the most relevant is Heat Shock 70 KDa Protein 8 (HSPA8; previously known as Heat Shock Cognate 70, HSC70) (Elliott et al. 2009). This protein appears to bind sulphated glycolipids of the rostral sperm region (Gadella et al. 1995) and may represent a common and evolutionary conserved mechanism of maintaining sperm lifespan in the oviduct. The beneficial effects of this protein on sperm survival have been observed not only in boar, but also in bull (Elliott et al. 2009) and ram (Lloyd et al. 2009, 2012) spermatozoa. The role of this oviductal protein on sperm function and survival will be taken up again in Sect. 6.12.2.

Finally, Lachance et al. (2007) found two other oviductal heat shock proteins, HSP 60 KDa (HSPD1) and glucose-regulated protein 78 kDa (HSPA5) that bind to human spermatozoa and increase the levels of intracellular calcium in spermatozoa. These two proteins may also play a relevant role in porcine sperm-oviduct interaction.

6.7.2.2 Glycans

As mentioned above, the apical surface of oviductal cells contain several glycoproteins that expose their glycan chains to the extracellular surface and are recognised by spermatozoa. The present section specifically focuses on these carbohydrate structures that can be sialylated or desialylated complex N-glycans, the former being more numerous than the latter (Töpfer-Petersen et al. 2008).

Within sialylated N-glycans, two components, namely major (acidic) and minor, have been distinguished. Major components are acidic N-glycans and consist of biantennary complex mono- and di-sialylated N-glycans containing N-acetylneuraminic and N-glycolyl-neuraminic acids. When these molecules are desialylated on-target, polylactosaminylated structures appear ($Hex_{5,6}HexNAc_{5,6}Fuc_1 + Man_3GlcNAc_2$), thereby indicating the presence of highly sialylated polylactosaminoglycans in the APM of oviductal cells.

As far as minor sialylated components are concerned, they are neutral N-glycans containing oligomannosyl ligands that consist of high-mannose type glycans with 5–9 mannosyl residues ($Man_{5–9}GlcNAc_2$) with a predominance of sialylated forms and one or two terminal α1–3-linked galactose residues.

Within desialylated glycans, the major components are biantennary complex N-glycans with two terminal hexose residues ($Hex_2HexNAc_2Fuc_1 + Man_3GlcNAc_2$) and with one additional hexose residue ($Hex_3HexNAc_2Fuc_1 + Man_3GlcNAc_2$), while the minor components are tri- and tetra-antennary glycans with and without additional hexose residues.

Finally, it is worth noting that a preferential occurrence of oligomannosyl glycans ($Man_{5,6}HexNAc_2$) and, to a minor degree, of hybrid N-glycans, ($Man_{3–6}GlcNAc_{3–4}$) has been observed in experiments isolating glycopeptides by Concavalin A-affinity chromatography and N-glycosidase F digestion (Wagner et al. 2002). Concavalin A binds molecules that contain α-D-mannose, α-D-glucose, and related residues with available C-3, C-4, or C-5 hydroxyl groups. These data suggest that in pigs sperm-oviduct interaction entails the cooperative interaction of oligomannose high affinity sites, and possibly low affinity sites, such as galactose in glycan chains (Töpfer-Petersen et al. 2008).

6.7.3 Sperm Surface

6.7.3.1 Introduction: Seminal Plasma and Sperm-Adhering Proteins

It is widely known that semen is constituted from a cellular fraction (sperm) and a liquid fraction (seminal plasma). Seminal plasma is a mixture of secretory products of organic and inorganic nature that come from the releasing activity of male reproductive organs (testis, epididymis, and male accessory sex glands, such as seminal vesicles, prostate, and bulbourethral glands) (Yanagimachi 1994b; Jonáková et al. 2006) (see also Sect. 9.2.1).

One of the main components in seminal plasma are sperm surface-adhering proteins. These proteins vary among mammalian species, and bind the surface of the

sperm head predominantly over the acrosomal region during ejaculation forming protein-coating layers (Suarez 2001, 2002; Töpfer-Petersen et al. 1998, 2002; Song et al. 2010) (see also Sect. 1.4). Sperm surface-adhering proteins maintain sperm topological organisation and participate in events that occur both in the male and the female reproductive tracts, such as sperm transport, protecting spermatozoa from premature capacitation and preventing an immune attack from the female tract (Evans and Kopf 1998; Jansen et al. 2001; Suarez 2001; Wassarman et al. 2001) (see also Chap. 5). Indeed, these proteins play a crucial role in the establishment of the sperm reservoir (sperm-oviduct binding), in sperm-oocyte recognition/binding and fertilisation (Gwathmey et al. 2003, 2006; Jonáková et al. 2007; Teijeiro et al. 2009) and in embryo development (Juyena and Stelletta 2012) (see also Chap. 8). The involvement of sperm surface-adhering proteins in these roles has been confirmed by their ability to bind glycoproteins from oviductal cells and from ZP in a cation-independent manner and via non-covalent interactions (Töpfer-Petersen 1999; Töpfer-Petersen et al. 2008).

In boars, spermadhesins are the main sperm surface-adhering proteins (Töpfer-Petersen et al. 1998; Song et al. 2010), even though the presence and relevance of other heparin-binding proteins that do not belong to the spermadhesin family (like DQH) has also been reported (Maňásková et al. 2007). Sperm surface-adhering proteins are lectins (i.e. carbohydrate-binding proteins, which must not be confused with glycoproteins that are proteins containing carbohydrate residues) that have a wide range of ligand-binding abilities, including saccharides, heparin and other sulphated GAGs, phospholipids, ZP glycoproteins, and proteinase inhibitors. However, not all sperm surface-adhering proteins interact with the same substrates. This may be explained by their different roles in the events leading to formation of the oviductal sperm reservoir, sperm capacitation and gamete recognition (Töpfer-Petersen et al. 1998; Ekhlasi-Hundrieser et al. 2005; Jonáková et al. 2007).

As far as their role in the sperm reservoir is specifically concerned, Liberda et al. (2006) found that heparin-binding proteins (spermadhesins AQN-1, AQN-3 and AWN spermadhesins, and DQH) and their aggregated forms interacted more strongly with both OEC and fluid than non-heparin-binding proteins (PSP-I and PSP-II spermadhesins) (Fig. 6.5). In addition, these authors also observed that the presence of mannan, hyaluronic acid and sialylated O-glycoproteins in the media inhibited the ability of heparin-binding proteins to adhere oviductal epithelium (Liberda et al. 2006), while the presence of sulphated polysaccharides (such as heparin and chondroitin sulphate) and simple monosaccharides did not affect the attachment ability of heparin-binding proteins to oviductal epithelium. These observations help us to understand how the establishment of the sperm reservoir works. Furthermore, the binding-inhibition effect between sperm surface proteins and oviductal epithelium mediated by hyaluronic acid suggests this non-sulphated glycosaminoglycan plays a role in sperm release from the oviductal reservoir (Liberda et al. 2006). This issue will be taken up again in Sect. 6.10 on sperm release from the oviductal reservoir.

In the present section, spermadhesins will be treated in a specific subsection (Sect. 6.7.3.3), even though not all of them are involved in the establishment of the sperm reservoir. The other subsection (Sect. 6.7.3.4) will deal with DQH, another heparin-binding protein involved in the formation of sperm reservoir that presents a different molecular structure.

6.7.3.2 Molecular Structure of Porcine Sperm Adhering Proteins: The CUB Domain

Boar sperm surface-adhering proteins (the spermadhesins AQNs, AWN and PSPs, and DQH protein) contain a characteristic CUB domain, an acronym that reflects where the protein was first found. Thus, C means Complement proteins C1r/C1s, U means UEGF (a sea Urchin epidermal growth factor with EGF-like domains), and B means bone morphogenetic protein 1 (BMP1). This domain is a structural motif, spanning approximately 110 residues, that has been evolutionary conserved and seems to be involved in protein–protein and glycosaminoglycan-protein interactions.

This CUB domain has also been found in a number of developmentally-regulated proteins, which are either extracellular or associated with the plasma membrane, including spermadhesins, TSG-6, the neuronal recognition molecule A5, and a variety of other proteins (DQH, PDGF-C, SCUBE1, SCUBE2, SCUBE3) (Bork and Beckmann 1993). The conformation of a CUB domain is made up of two β-sheets, each one consisting of four anti-parallel and one parallel strands (Romero et al. 1997; Varela et al. 1997), and two disulphide bonds between the nearest neighbour cysteine residues to stabilise its structure.

6.7.3.3 Spermadhesins

Spermadhesins and the Ungulate Order

Spermadhesins have been identified in seminal plasma and/or peripherally associated to spermatozoa from a mammalian order, the ungulates, which include porcine, bovine, ovine, equine and caprine species (Dostàlovà et al. 1995a; Töpfer-Petersen 1999). In contrast, seminal plasma and ejaculated spermatozoa from non-ungulates, such as humans, dogs and rodents, do not contain, in principle, spermadhesins (Töpfer-Petersen et al. 2008). Interestingly, genomes of humans, chimpanzees and dogs contain copies of spermadhesin genes. However, these copies have been inactivated by mutations that disrupt the coding sequence, in a putative evolutionary mechanism. In the case of rodents (mice and rats), their genomes do not contain the entire region of the hypothetical ancestral spermadhesin gene, so that possible deletion has been suggested (Leeb 2007).

Within the ungulates, the porcine species presents the highest diversity and expression levels of spermadhesins, representing >90 % of total boar seminal plasma proteins (Dostàlovà et al. 1994a). In bovine species, two spermadhesins have been identified (SPADH1 also known as aSFP and SPADH2 also known as Z13) (Wempe et al. 1992; Tedeschi et al. 2000), while in ovine, equine and caprine species, only one spermadhesin has been found. In the case of rams, RSP appears to be the homolog of the boar spermadhesin AQN-1 (Bergeron et al. 2005), while in horses and bucks, Horse Seminal protein 7 (HSP-7) (Reinert et al. 1996) and BSFP (Melo et al. 2008) are respectively the homologs of boar spermadhesin

AWN. In addition, spermadhesin ability to bind carbohydrates also differs between species. Thus, for example, while porcine spermadhesins recognise a wide spectrum of oligosaccharides, the array of glycans recognised by bovine spermadhesin SPADH1 (aSFP) is more restricted (Töpfer-Petersen et al. 1998).

Diversity of Spermadhesins in Porcine Species and Localisation in the Male and Female Tracts

In pigs, spermadhesins are able to bind carbohydrate chains of glycoproteins containing Gal-GalNAc sequences in O-glycans and carbohydrate chains containing Gal-GlcNAc sequences in N-glycans (Ekhlasi-Hundrieser et al. 2005).

Five different spermadhesins have been identified up to now in boar seminal plasma: AQN-1, AQN-3, AWN, DQH, PSP-I and PSP-II, the two latter being the most abundant (Sanz et al. 1991, 1992a, b, c; Kwok et al. 1993; Romero et al. 1997; Mánáskova et al. 2007; Töpfer-Petersen et al. 2008). Genes encoding these spermadhesins contain diverged and conserved regions and all of them have been reported to be closely related (Sanz et al. 1991, 1992d; Kwok et al. 1993) and have been located on the q arm of pig chromosome 14 (SSC 14q28–q29), in a region syntenic to human chromosome 10 [HAS 10q26 (Haase et al. 2005)].

On the other hand, the literature remains controversial about the actual localisation of spermadhesins through the male and female reproductive tracts (García et al. 2008; Maňásková and Janóková 2008; Veselský et al. 1999; Ekhlasi-Hundrieser et al. 2002; Song et al. 2010). Accordingly, Ekhlasi-Hundrieser et al. (2002) found that spermadhesins are synthesised within the boar reproductive tract (except AWN, which was also found to be expressed in the oviduct). These authors also found that spermadhesins AQN-1, AQN-3, PSP-I, and PSP-II were secreted by the seminal vesicles, while AWN was synthesised in the epididymis, was present on the epididymal spermatozoa, and was also expressed in the oviduct (Ekhlasi-Hundrieser et al. 2002). In contrast, in a more recent study performed by Song et al. (2010), which assessed transcripts of the five spermadhesins in both male and female genital tracts, AWN, PSP-I and PSP-II were found in seminal vesicles, prostate, bulbourethral glands, and caput, corpus and cauda epididymidymides. Spermadhesins AQN-1 and AQN-3 were found to be expressed in accessory glands and in the epididymal caput and cauda, but not in the corpus. Only AWN was reported to be expressed in the testis. Thus, Song et al. (2010) observed transcripts of AWN in all male tissues, while Ekhlasi-Hundrierser et al. (2002) found it only in the seminal vesicle, cauda epididymidis, and prostate. However, the observations made by Song et al. (2010) concerning the expression of PSP-I and PSP-II genes agreed with those of García et al. (2008).

Ekhlasi-Hundrieser et al. (2002) only found one spermadhesin (AWN) in the female reproductive tract, specifically in the uterus, uterotubal junction, isthmus and ampulla. However, Song et al. (2010) did not only observe AWN in the female reproductive tract, they also found PSP-I, PSP-II and AQN-3 in uterine horns, and AWN, PSP-I and PSP-II in the ovaries. Since Yang et al. (1998) reported that

PSP-I/PSP-II enhance in vitro immune activities of porcine peripheral lymphocytes, Song et al. (2010) hypothesised that the presence of mRNA transcripts of PSP-I and PSP-II could modulate immune responses within the female reproductive tract.

Overall, Song et al. (2010) observed that mRNA transcripts of spermadhesins were widely distributed along the male and female reproductive tracts. However, spermadhesin transcripts were higher in seminal vesicles than in prostate and caput epididymidis.

Mechanisms of Binding to Sperm Membrane and Interaction with Other Molecules

As stated above, spermadhesins can interact with a wide array of other molecules, such as carbohydrates, heparin, phospholipids and proteinase inhibitors, this feature being related to different biological activities (Jonáková and Tichá 2004; Töpfer-Petersen et al. 2008). In this regard, the specific roles played by spermadhesins are expressed in three relevant contexts: primary structure (sequence of aminoacidic residues), glycosylation levels and aggregation state (Töpfer-Petersen et al. 1998, 2008; Calvete and Sanz 2007).

Calvete et al. (1997) suggested that AWN, AQN-1 and AQN-3 attach the spermatozoa via direct or indirect phospholipid-binding, in a similar fashion to the case of cattle. Indeed, the first interaction of spermadhesins to sperm membranes appears to be mediated by non-aggregated AWN and AQN-3, since they are able to directly bind the lipids of the sperm membrane. In fact, this hypothesis was proposed by Dostàlovà et al. (1995b) who observed that these two spermadhesins were the only ones able to bind phosphorylethanolamine molecules (Dostàlovà et al. 1995b), the major phospholipid constituents of the boar sperm membrane (Watson 1981). Aggregated spermadhesins will then become coated on top of this first layer, serving as stabilising factors that protect the acrosome membrane from a premature/degenerative acrosome reaction (Töpfer-Petersen et al. 2008). Such spermadhesins are thought to stabilise the plasma membrane over the acrosomal vesicle and are mainly released from the spermatozoal surface during capacitation (Sanz et al. 1993; Dostàlovà et al. 1994b; Calvete et al. 1997).

Therefore, spermadhesins AWN and AQN-3 interact directly with membrane phospholipids (Dostàlovà et al. 1995b; Ensslin et al. 1995), whereas AQN-1 interacts with another seminal plasma protein DQH (also known as pAIF-1 or pB1) at the moment of ejaculation, forming a heterodimer (Calvete et al. 1997). This complex AQN-1/DQH interacts with the plasmalemma of the acrosomal region, since DQH contains two fibronectin type-II domains that interact with sperm membrane through the phosphorylcholine-binding sites (Calvete et al. 1997; Ekhlasi-Hundrieser et al. 2007). The crucial role of AQN-1 in the formation of the oviductal sperm reservoir by interacting with glycoconjugates of the oviductal epithelium (Wagner et al. 2002; Ekhlasi-Hundrieser et al. 2005) will be discussed in the next section) (Fig. 6.4).

AWN presents a discontinuous phosphorylethanolamine–binding region (Ensslin et al. 1995) and a heparin-binding domain in the opposite location to the

carbohydrate-binding region that partly matches the phosphorylethanolamine–binding region (Calvete et al. 1996). This protein remains bound to the periacrosomal sperm membrane after transport through the female genital tract and in vivo capacitation and participates in oocyte recognition (Rodríguez-Martínez et al. 1998). Proteins AQN-3 and AWN appear to stabilise the plasma membrane over the acrosomal vesicle and are released during capacitation (Töpfer-Petersen et al. 1998; Dostàlovà et al. 1995b; Calvete et al. 1997).

PSP-I and PSP-II interact with glycosylated spermadhesins to form a non-heparin binding heterodimer (Nimtz et al. 1999) and are immunostimulatory for lymphocyte activity in vitro. That is why it has been suggested that they prevent possible infections in the sow's genital tract and provide a uterine environment free from foreign cells for early embryos (Yang et al. 1998; Assreuy et al. 2003; Song et al. 2010). Spermadhesins PSP-I and PSP-II are present in boar seminal plasma as a heterodimer complex (PSP-I/PSP-II) and contribute to maintaining sperm with high viability, motility, and mitochondrial activity at physiological temperatures (Centurión et al. 2003).

In cattle, only two spermadhesins (SPADH1 and SPADH2) have been identified, in contrast to swine where five spermadhesins have been detected. Spermadhesin SPADH1, which also interacts indirectly with the surface of ejaculated spermatozoa, is released during in vitro capacitation, so that it does not take part in sperm-oocyte recognition (Dostàlovà et al. 1994b; Töpfer-Petersen et al. 2008), and like PSP-I and PSP-II, also stimulates the division of lymphocytes. This SPADH1 also favours the secretion of progesterone by bovine endometrium and granulosa cells (Einspanier et al. 1991), which suggests a stimulating effect on ovulation upon insemination (Töpfer-Petersen et al. 2008).

A probable carbohydrate recognition of 11 amino acids has been identified, which is highly conserved throughout the members of the spermadhesin family; these sugar-combining amino acids are located around the asparigine residue (Asn_{50}) (Ekhlasi-Hundrieser et al. 2008). Although 11 consecutive amino acids are also conserved in the respective cattle sequences, replacements within the potential key sequence may be responsible for the missing property of SPADH1 to interact with glycoproteins. The comparison to human species also emphasises the functional importance of this region, as nine amino acids are conserved when comparing the porcine spermadhesins AQN-1 and PSP-II with the human hypothetical spermadhesin SPADH2. It remains open to further studies whether human and pig spermadhesins also share their biochemical properties (Haase et al. 2005).

Finally, both binding site and amino acids sequences appear to be specific to spermadhesins, since they have not hitherto been observed in any other molecules recognising carbohydrates (Kaltner and Gabius 2001).

Role of Spermadhesins in the Formation of Sperm Reservoir: AQN-1

The main role of spermadhesins in the formation of sperm reservoir is confirmed when related-events of sperm release from the reservoir are analysed. Indeed, sperm

release from the oviductal reservoir seems to occur at an early stage of capacitation, when there is a reorganisation of sperm membrane that entails detachment of spermadhesins from the sperm surface. This leads to spermatozoa losing their contact with oviductal cells and allowing them to swim freely towards the site of fertilisation (Töpfer-Petersen et al. 2008). Related to this, in vitro studies conducted in bovine and porcine species showed that ejaculated spermatozoa presented a higher ability to bind oviductal explants and OEC than epididymal spermatozoa (Petrunkina et al. 2001a; Gwathmey et al. 2003; Yeste et al. 2012). This fact could be explained by the presence of spermadhesins secreted by seminal vesicles into seminal plasma, so that only when these proteins are present, as in the case of ejaculated spermatozoa, but not epididymal, can they mediate the interaction between spermatozoa and oviductal cells (Ekhlasi-Hundrieser et al. 2005).

Different studies have investigated which porcine spermadhesins are involved in the formation of sperm reservoir. One of these reports was the one carried out by Ekhlasi-Hundrieser et al. (2005), who investigated the role of AQN-1, AQN-3 and AWN in sperm-oviduct binding. These authors observed that AQN-1 recognised a broad spectrum of carbohydrates, including mannose and galactose structures, whereas AQN-3 and AWN presented a high specificity for galactose but not for mannose residues. Thus, AWN only interacted with α- and β-linked galactose residues, while AQN-1 also recognised Man α1-3(Manα1–6)Man structures (Ekhlasi-Hundrieser et al. 2005). In addition, AWN and AQN-3 present a similar binding affinity for oviductal- and ZP-glycoproteins that contain Galβ(1–3)GlcNAc and Galβ(1–4)GlcNAc sequences either in N- or O-linked oligosaccharides, but they differ regarding the recognition of tri-/tetra-antennary N-glycans (Dostàlovà et al. 1995a; Töpfer-Petersen et al. 2008) (Fig. 6.4).

Ekhlasi-Hundrieser et al. (2005), conducting in vitro experiments with oviductal explants, also found that AQN-1, but not AWN, inhibited the sperm binding ability to these explants in a concentration-dependent manner. Related to this, it is interesting to bear in mind that the apical surface of oviductal cells expresses mannose components that can be recognised by AQN-1, which exposes mannose-binding sites. Finally, the crucial role of AQN-1 and mannose matches capacitation-related events, since it has been reported that, at an early stage of capacitation, spermatozoa lose AQN-1 from their surface and decrease their binding ability to OEC (Fig. 6.4). In fact, the loss of mannose binding in capacitated spermatozoa is not due to ongoing acrosome reaction, but it is already observed at an early stage of capacitation when AQN-1 detaches from the sperm surface.

In contrast, despite small amounts of AWN being detected in viable spermatozoa (Petrunkina et al. 2000; Ekhlasi-Hundrieser et al. 2005), this spermadhesin, which remains attached to the spermatozoa until the male gametes reach the ovulated oocyte, has also been detected in sperm bound to the ZP (Rodríguez-Martínez et al. 1998). This is the opposite to what occurs in the case of AQN-1, which, as stated, detaches from the sperm surface after capacitation (Fig. 6.4). This detachment leads to a reduction in sperm binding ability to oviductal explants and to a decrease in the number of mannose-binding sites on the sperm surface.

All these findings led Ekhlasi-Hundrieser et al. (2005) to suggest that AQN-1 attaches the spermatozoa to OEC via its mannose-binding site, using a mannan-sensitive system, as Liberda et al. (2006) later confirmed. These authors also concluded that spermadhesin AQN-1, but not spermadhesins AQN-3 or AWN, plays a crucial role in sperm-OEC binding. Indeed, spermadhesin AQN-1 adheres mannosyl-components of apical glycocalyx of the ampulla, as well as of the isthmic and uterine tubal junction regions (Liberda et al. 2006). This confirms the participation of AQN-1 in the establishment of oviductal sperm reservoir.

Interestingly, AQN-1 seems to be the homolog of the bovine protein PDC-109, because even though PDC-109 (Wah et al. 2002) and AQN1 (Romero et al. 1997) do not belong to the same protein classes, both are endowed with a similar structure characterised by a β-domain structure and carbohydrate-binding ability. However, in the case of pigs, the role corresponding to PDC-109 appears to be mediated by AQN-1/DQH heterodimer (Fig. 6.5). This issue is discussed in the next section.

6.7.3.4 Role of DQH in the Establishment of Sperm Reservoir

Another seminal plasma protein that has been reported to take part in the formation of the sperm reservoir in pigs is DQH (pB1), which differs from the structure of spermadhesins but presents a CUB domain that exhibits very similar binding activities (Maňásková et al. 2007).

DQH has been described as a heparin-binding protein that presents low molecular mass (13 KDa) and basic pI (8.6) (Jonáková et al. 1998), and belongs to a large family of cell and matrix adhesion proteins (Fn-2 type proteins), which includes seminal plasma proteins, collagenases, fibronectins, large cell surface receptors and others (Bezouška et al. 1999). It seems to be originated in seminal vesicles, is present in seminal fluid (Maňásková et al. 2007), forms a heterodimer with AQN-1 and adheres to the acrosomal surface of ejaculated boar spermatozoa, interacting with mannose exposed components of the apical surface of oviductal cells (Maňásková et al. 2007; Talevi and Gualtieri 2010). A great deal of knowledge about DQH has come from its homolog in cattle: PDC-109. PDC-109, also known as BSP-A3, is a fucose-binding protein that belongs to the family of bovine seminal plasma proteins, a new emerging superfamily of proteins found in mammals (Seidah et al. 1987; Desnoyers and Manjunath 1992; Manjunath et al. 1994; Manjunath et al. 2007). These proteins are secreted by seminal vesicles, specifically bind the phosphorylcholine residues of sperm membrane and are important for initiating motility (Maňásková et al. 2000; Liberda et al. 2002; Juyena and Stelletta 2012). Such proteins have also been identified in horses and are known as horse seminal proteins 1 and 2 (HSP-1 and HSP-2) (Calvete et al. 1995).

In the case of cattle, PDC-109, the homolog of porcine DQH, is known to increase sperm motility and the pumping efficiency of Ca^{2+}-ATPase of plasma membrane in an irreversible-cooperative manner (Sánchez-Luengo et al. 2004), and to remodel the architecture of bull sperm membrane mediating the effects of

capacitation factors (Müller et al. 2002). Such fucose-binding protein is involved in sperm-oviduct recognition and subsequent formation of sperm reservoir (Ignotz et al. 2001), since it specifically binds the sperm head plasmalemma via the phospholipid-binding domain, intercalating into the sperm membrane (Müller et al. 1998), and also attaches the carbohydrate-ligands exposed by the OEC (Hadjisavas et al. 1994; Jonáková et al. 1998) (Fig. 6.5).

The complete DQH covalent structure, including its post-translational modifications, has been determined by Edman degradation and post-source decay MALDI-MS. Accordingly, DQH protein is composed of a 105 amino acid polypeptide with four disulphide bonds that consist of N-terminal O-glycosylated peptide on Threonine 10 (Thr_{10}) followed by two fibronectin-type II repeats. Plucienniczak et al. (1999) confirmed DQH protein sequence from cDNA constructed from seminal vesicle mRNA transcripts.

According to Maňásková et al. (2007), DQH mediates sperm-oviduct interaction but also participates in the primary sperm–ZP binding. In fact, DQH interacts strongly not only with sulphated polysaccharides (such as heparin, chondroitin sulphate, dextran sulphate and fucoidan) but also with hyaluronic acid (Tichá et al. 1998; Jonáková et al. 2000) and exhibits affinity to porcine ZP (Jonáková et al. 1998; Maňásková et al. 2000).

6.7.4 Conclusions

In conclusion, OEC trap spermatozoa through carbohydrate recognition mechanisms, the flexible high-mannose type glycans exposed by oviductal glycoproteins bound by the spermadhesin AQN-1. In turn, AQN-1 indirectly interacts with the sperm plasma membrane, because it needs the participation of another molecule (DQH) to bind spermatozoa.

Therefore, although there are still some aspects about spermadhesins-mediation that need to be addressed, it seems quite clear that the two sperm surface-binding proteins that take part in the establishment of the sperm reservoir are AQN1 (Ekhlasi-Hundrieser et al. 2005) and DQH (Maňásková et al. 2007) (Fig 6.5).

6.8 Sperm Reservoir (III): Selection of Spermatozoa Through Sperm Reservoir

Spermatozoa are subjected to two selection processes during their stay in the oviduct; the first involves sperm ability to attach to the oviductal cells (Petrunkina et al. 2001a; Yeste et al. 2009a, 2012), while the other occurs when spermatozoa are released from the sperm reservoir in a mechanism that has been suggested takes place together with sperm capacitation (Talevi et al. 2010). In both cases, the aim of this selection is to ensure successful fertilisation and embryo development.

The sperm reservoir in mammalian species has been described in pigs (Hunter 1981) and other mammalian species such as sheep (Hunter and Nichol 1983), mice (Suarez 1987), hamsters (Smith and Yanagimachi 1991; Kan and Esperanzate 2006), cattle (Hunter et al. 1991; Kan and Esperanzate 2006) and humans (Baillie et al. 1997). Therefore, spermatozoa arrive at the uterotubal junction and the oviductal isthmus coming from the uterine horns and are sequestered by the OEC, through a mechanism that involves carbohydrate-binding domains of oviductal cells and of spermatozoa as well as the participation of sperm surface-adhering proteins. As mentioned above, adhesion of spermatozoa to the oviductal cells from the isthmus and the uterotubal junction maintains sperm function and survival (Hunter 1981, 2002).

However, not all spermatozoa are able to bind OEC and thus take part in the sperm reservoir. In in vitro experiments conducted with oviductal explants and OEC monolayers, it has been shown that there is selectivity of the sperm that are able to attach to OEC (Töpfer-Petersen et al. 2002; Suarez and Pacey 2006). In the case of pigs, the population of spermatozoa that bind the OEC have to be viable (Fazeli et al. 1999; Yeste et al. 2009a, 2012), mature (Petrunkina et al. 2001a) and morphologically normal (Green et al. 2001; Waberski et al. 2006; Yeste et al. 2012), and uncapacitated (Fazeli et al. 1999; Petrunkina et al. 2001b; Yeste et al. 2009a). Related to this, sperm intracellular calcium levels and phosphorylation levels of some sperm membrane proteins have to be low (Petrunkina et al. 2001a), both features of non-capacitated spermatozoa. We must bear in mind, as will be described in Chap. 7, that sperm capacitation consists of a signal transduction mechanism that involves increases in intracellular calcium levels and tyrosine-phosphorylation of some sperm proteins like p32. In addition, boar sperm attached to OEC have been reported to be endowed with stable and non-fragmented chromatin structure (Ardón et al. 2008) and to have good osmoregulatory abilities (Petrunkina et al. 2007).

Such selection/selectivity of the sperm reservoir is not exclusive of boar spermatozoa but it has also been observed in other mammalian species. Thus, for example, only uncapacitated spermatozoa (Lefebvre and Suarez 1996) and with intact acrosomes in bulls (Gualtieri and Talevi 2000), spermatozoa without DNA fragmentation in men (Ellington et al. 1999), and uncapacitated and morphologically normal spermatozoa in stallions (Thomas et al. 1994) are able to bind the oviductal epithelium. All these data evidence that there are some common features of the sperm reservoir in eutherian mammals and that a preference for morphologically normal, viable and uncapacitated spermatozoa exists through different species. In addition, all these findings have led Talevi and Gualtieri (2004) to hypothesise that sperm-oviduct interactions not only form a sperm reservoir, but also enable the selection of a higher quality sperm subpopulation with high fertilising ability. Following this, the function of sperm reservoir would be to store and to prolong lifespan of the highest quality spermatozoa in order to ensure their availability for fertilisation upon arrival of the oocyte, which would finally lead to successful fertilisation (Tienthai et al. 2004). It can also be speculated that there is a sort of sperm competition within the female reproductive tract in porcine and other mammalian species, as suggested by other authors (Gualtieri and Talevi 2003; Satake et al. 2006).

On the other hand, Talevi and Guatieri (2004) have also suggested that not all the spermatozoa within a given ejaculate are able to bind the OEC, but only those exposing particular surface proteins, which confer upon them the capacity to adhere to oviductal epithelium. In fact, epididymal spermatozoa have a lower ability to bind the OEC than ejaculated spermatozoa in in vitro co-culture (Yeste et al. 2012). This also seems to be related to sperm adhering proteins AQN-1 (Ekhlasi-Hundrieser et al. 2005) and DQH (Maňásková et al. 2007), which are attached to the surface of ejaculated spermatozoa and are removed from sperm membrane during capacitation. The absence of these sperm-adhering proteins in sperm plasmalemma might explain why epididymal and capacitated spermatozoa are unable to bind the oviductal epithelium.

6.9 Sperm Reservoir (IV): A Putative Role for the Endocannabinoid System

6.9.1 Reproductive Physiology and Endocannabinoids

Cannabinoids are a group of terpenophenolic compounds present in Cannabis (*Cannabis sativa*) that are structurally related to tetrahydrocannabinol and bind to cannabinoid receptors. Endocannabinoids are cannabinoids produced by the organism itself and are present in biological fluids, involved in different physiological roles including reproduction. The main endocannabinoids are arachidonylethanolamide, also known as anandamide (AEA), and 2-arachidonoyl glycerol (Devane et al. 1992; Felder and Glass 1998).

The mechanism of action of AEA consists of 'on-demand' release from membrane phospholipid in a process mediated by a phospholipase enzyme (N-acylphosphatidyl-ethanolamine selective phospholipase D, NAPEPLD) and stimulated by cell depolarisation or mobilisation of intracellular calcium stores. The half-time life of AEA is low, since membrane-bound fatty acid amide hydrolase (FAAH) quickly degrades it, once the former is released (Habayeb et al. 2002). Arachidonylethanolamide plays different physiological roles in different tissues, through a signal-transduction pathway that involves its interaction with two specific G protein-coupled receptors (GPCRs): cannabinoid receptors 1 and 2 (CB1 and CB2).

Distribution and function of CB1 and CB2 receptors have been described in different animal models (McPartland et al. 2007). Both have been found together with NAPEPLD and FAAH, two enzymes that mediate AEA-release in the reproductive organs of mammals and in germ cells of vertebrates and invertebrates, and in non-neuronal somatic cells of peripheral organs (Habayeb et al. 2002; Talevi et al. 2010).

Schuel et al. (2002a, b) suggested a putative role for AEA in reproductive physiology because high levels of this endocannabinoid are found in reproductive

fluids. As previously mentioned, spermatogonia, spermatocytes and spermatids of vertebrates and invertebrates express CB1 (Cobellis et al. 2006). Furthermore, AEA has been reported to regulate spermatogenesis, since high levels of this endocannabinoid reduce germ cell proliferation and testis weight (Cacciola et al. 2008; Lewis and Maccarrone 2009). Matching this, a CB2-dependent mechanism regulates Sertoli cells (see also Chap. 3), which act supporting male germ cells enter into apoptosis (Maccarrone et al. 2003).

The function of AEA is not restricted to male germ cells and organs, but it is also relevant to the mature sperm cell and for the female reproductive tract (Taylor et al. 2007). On the one hand, the endocannabinoid system has been found in human (Rossato et al. 2005; Francavilla et al. 2009), boar (Maccarrone et al. 2005), mouse (Ricci et al. 2007; Sun et al. 2009), and bull (Gervasi et al. 2009) spermatozoa. Notwithstanding, Rossato et al. (2005) found AEA depressed motility and capacitation of human spermatozoa, while Talevi et al. (2010) observed that the plasma membrane of bovine spermatozoa also presented endocannabinoid receptors CB1 and CB2, and that AEA also depressed sperm capacitation. Related to this, Maccarrone et al. (2005) observed that activation of CB1 and CB2 receptorsin boar spermatozoa at different time-points is required for fertilisation. In addition, these authors also found that boar spermatozoa have a complete and efficient endocannabinoid system, containing the biochemical machinery to synthesise, bind, and degrade AEA.

On the other hand, as far as the role of AEA in the female tract is concerned, endocannabinoid signalling via CB1 appears to be involved during mouse embryo cleavage and transport in the oviduct (Wang et al. 2004). In fact, from fertilisation to the blastocyst stage, NAPEPLD and FAAH are up-regulated and this seems to reduce AEA levels at potential implantation sites, thereby facilitating such implantation (Schuel and Burkman 2005; Wang et al. 2004, 2006).

6.9.2 Endocannabinoids, Sperm-Oviduct Interaction, Release and Capacitation

The concentration of AEA throughout the oviduct varies, being higher in the isthmus than in the ampulla. According to Wang et al. (2006), these regional differences are related to the critical balance between AEA synthesis and degradation within the oviduct. Indeed, a suitable microenvironment containing the most appropriate AEA concentration is needed as this has been correlated with normal embryo development, oviductal transport, implantation, and pregnancy.

Despite the evident role of AEA in the oviduct after fertilisation being widely known, it is not clear where this endocannabinoid participates in sperm-oviduct interaction. To the best of our knowledge, although the AEA gradient within the mammalian oviduct (Wang et al. 2006) led to speculate on the possibility that endocannabinoids regulated sperm storage, capacitation, and/or selection within

the oviduct, this gradient does not seem to participate in the establishment of sperm reservoir and the selection of spermatozoa, or in sperm transport. However, AEA seems to modulate sperm physiology since it has been reported to affect sperm capacitation of human, bovine and porcine spermatozoa (Maccarrone et al. 2005; Rossato et al. 2005; Rossato 2008; Talevi et al. 2010).

In order to clarify the role of the endocannabinoid system in mammalian spermatozoa within the oviductal environment, Talevi et al. (2010) recently studied the localisation of endocannabinoid receptors CB1 and 2 in bull spermatozoa that bound to and were released from the oviductal epithelium in vitro. These authors also evaluated the effects of AEA on sperm viability and motility, and ZP-binding ability, and the expression of NAPEPLD in bovine OEC, reaching the following conclusions:

1. CB1 and CB2 are present in bull spermatozoa.
2. AEA does not influence sperm viability, but induces a rapid decrease in sperm motility and kinetic parameters.
3. AEA does not affect sperm-oviduct interaction.
4. Spermatozoa released from the oviduct through heparin, a powerful capacitating agent (Parrish et al. 1988), showed a decreased detection in CB1.
5. AEA reduces sperm–ZP binding, but failed to do so when spermatozoa were capacitated with heparin.
6. OEC express NAPEPLD, the main enzyme involved in AEA synthesis.

Taken together, these findings suggest that endocannabinoids may modulate the motility and capacitation of spermatozoa within the oviduct, suggesting that endocannabinoids via CB1 play a key role in the oviductal microenvironment modulating the physiology of spermatozoa. On the other hand, AEA does not seem to affect either sperm-oviduct binding or sperm release from the reservoir. This finding is in agreement with previous studies showing that sperm capacitation promotes sperm release from the reservoir (Lefebvre and Suarez 1996; Gualtieri et al. 2005; Talevi et al. 2007; Talevi and Gualtieri 2010). In contrast, AEA depresses sperm capacitation not only in bull (Talevi et al. 2010) but also in human and boar spermatozoa (Maccarrone et al. 2005; Rossato et al. 2005; Rossato 2008).

Regarding the localisation of CB1 receptors in bovine spermatozoa, Talevi et al. (2010) compared sperm responses to heparin and penicillamine in in vitro conditions. Despite being known as powerful inducers for sperm release from the oviductal reservoir of cattle, which trigger capacitation-related changes (Talevi and Gualtieri 2001; Gualtieri et al. 2005; Talevi et al. 2007), heparin and penicillamine do not act in the same way because only the sperm capacitated by the latter are able to revert this process (Gualtieri et al. 2009). Intriguingly, these two different mechanisms of action of heparin and penicillamine are related to the presence/localisation of CB1 after induced-capacitation. Thus, Talevi et al. (2010) found that CB1 receptor was not detectable in heparin-released spermatozoa, whereas the localisation of CB1 receptor in the penicillamine-released spermatozoa was the same as that of non-capacitated spermatozoa. These authors also observed that

heparin-released spermatozoa did not present CB1 receptor due to sperm surface remodelling induced by capacitation. Related to this, heparin-capacitated spermatozoa failed to respond to AEA in the sperm–ZP binding experiments. This reinforces the hypothesis that the CB1 receptor loses its functionality in heparin-capacitated spermatozoa as result of capacitation-related events.

In conclusion, although no studies in porcine species have been published to date, Talevi et al. (2010) have demonstrated that the endocannabinoid system plays an active role within the oviductal environment in cattle. Specifically, OEC secrete AEA, thereby contributing to the function of the sperm reservoir, namely prolongation of sperm fertile lifespan through depression of motility and capacitation.

6.10 Sperm Reservoir (V): Release Mechanisms

6.10.1 Introduction

Before ovulation, viable spermatozoa are sequestered by the oviductal epithelium at the level of the isthmus and uterotubal junction and remain transiently adhered to OEC (Hunter 2008). While spermatozoa remain attached to the OEC, their function and survival is maintained in a 'quiescent' state (see Sects. 6.4.2 and 6.11).

Around ovulation, the oviductal conditions change and the inhibitory influence of the oviduct on sperm function is reversed. The new oviductal conditions induce the continuation of sperm capacitation (see Sect. 7.6). Thus, at an early stage of capacitation, the sperm plasmalemma is reorganised and this allows spermadhesin AQN-1 to be detached from its surface (see Sect. 7.6.3). This leads spermatozoa to lose their contact with OEC, so that they can then be released from the reservoir. In addition, attached/attaching spermatozoa also undergo rapid metabolic changes at the time of release, such as increases in intracellular calcium levels, protein tyrosine phosphorylation, and in flagellar beat frequency (Gualtieri et al. 2005; Talevi et al. 2010). These changes allow spermatozoa to swim freely towards the site of fertilisation, the ampullary/isthmic junction. (Hunter and Rodríguez-Martínez 2004; Tienthai et al. 2004; Töpfer-Petersen et al. 2002, 2008).

Studies conducted more recently have changed the classical view about sperm capacitation as a continuous process that once triggered leads to hyperactivated motility, acquisition of ZP binding, and fertilisation competence (Yanagimachi 1994b). In contrast to this classical view, recent studies have suggested that different mechanisms promoting or suppressing capacitation may coexist, and act at different times and in different regions during the reproductive cycle (Hunter and Rodríguez-Martínez 2004). Thus, it has been hypothesised that sperm ascent through the oviduct may involve intermittent phases of adhesion and release in a gradual way (Smith and Yanagimachi 1990; DeMott and Suarez 1992; Nakanishi et al. 2004) (see also Sect. 7.4.2).

6.10.2 Sperm Release from the Reservoir as a Well-Coordinated Process

The mechanisms governing sperm inhibition (release) and activation (capacitation) within the oviductal environment remain largely unknown (Hunter 2002; Hunter and Rodríguez-Martínez 2004; Töpfer-Petersen et al. 2002; Tienthai et al. 2004). Different reports have observed the ovary is able to signal ovulation to the oviduct (Hunter 2002, 2003; Hunter and Rodríguez-Martínez 2004), but at the same time the sperm's presence has also been reported to modulate gene expression of oviductal cells (Georgiou et al. 2005, 2007).

Since sperm capacitation is a controlled destabilisation process (Harrison 1996; see also Chap. 7), which reduces sperm lifespan, the maintenance of sperm viability within a certain time window and the control of capacitation are closely interrelated events (Töpfer-Petersen 1999; Hunter and Rodríguez-Martínez 2004). For this reason, the interaction between spermatozoa and oviduct seems to be a well-regulated event, which synchronises sperm fertilising ability with the arrival of a mature oocyte (Mburu et al. 1996, 1997). This phenomenon, which ensures successful fertilisation, needs direct binding between the male gametes and the oviduct and then the release of sperm in a gradual way, which is why Hunter (2008) stated that sperm release starts before, and lasts during and after ovulation.

6.10.3 Signals Leading to Sperm Release

Spermatozoa are released from the reservoir around ovulation, following downstream events that are still poorly known. Despite this, it seems that in porcine, like in other mammalian species, a set of signals present in the oviductal environment leads to sperm release. In fact, the question about whether any effector exists that plays a key role in sperm release from the reservoir has been widely studied, but the results provided by the literature so far have been inconsistent. According to Talevi and Gualtieri (2010) these signals could function as alternative or synergic events, such as:

1. Preovulatory phase endocrine milieu,
2. Effect of cumulus-oocyte complexes (COCs) on sperm release, and
3. Presence of gametes, which may change the secretory activity of oviduct.

6.10.3.1 Influence of the Oviduct

Carrasco et al. (2008) observed that the activity of oviductal enzymes, such as β-D-galactosidase, α-D-mannosidase, and β-N-acetyl-galactosaminidase depends on the phase of the ovarian cycle, increasing their activities at the early follicular phase

and decreasing them after ovulation. Given that mannose- and galactose-exposed residues of the apical surface of oviductal cells are involved in the formation of sperm reservoir, these authors suggested that oviductal enzymes could reasonably play a role in remodelling the oviduct surface, so that their action leads to sperm release from the reservoir.

On the other hand, heparin and heparin-like GAGs induce sperm capacitation (Parrish et al. 1988; Galantino-Homer et al. 1997; Marquez and Suarez 2004) and detach the spermatozoa from oviductal explants and oviductal cell monolayers in bovine species (Gualtieri and Talevi 2003; Gualtieri et al. 2005). However, this effect is not observed in pigs, so it does not seem the major capacitating factor in the oviduct (Ekhlasi-Hundrieser et al. 2005).

Finally, the oviductal secretory proteome is specifically modulated by the presence of gametes (Georgiou et al. 2007), having a direct influence on oviductal redox pathways as it up-regulates thioredoxin and down-regulates superoxide dismutase and phospholipid-hydroperoxide glutathione peroxidase (Georgiou et al. 2005). In cattle, these proteomic alterations seem to influence sperm release directly since bull sperm release is a redox-regulated event (Talevi et al. 2007; Gualtieri et al. 2009). In the case of pigs, the possible involvement of these oviductal proteomic alterations on sperm release remains open and warrants further investigation, even though a similar mechanism can be speculated. Proteome/secretome alterations of the oviduct in response to the presence of gametes will be specifically dealt with in a separate section.

6.10.3.2 Involvement of Oocytes and Their Cumulus Cells in Sperm Release from the Reservoir: The Role of Hyaluronan

Sperm release from the reservoir can also be influenced by the oocytes and cumulus cells, by attracting them to the ova. To confirm this hypothesis, Brüssow et al. (2006) used a model in which ova from donor gilts were transferred at the periovulatory period into only one oviduct (the other served as a control) of bilaterally ovectomised (aspiration of oocytes from the follicle) recipient gilts that had previously undergone endoscopic intrauterine insemination with low sperm-doses in both uterine horns. These authors observed the presence of COCs in the oviduct significantly increased the percentages of spermatozoa in the ampullar and isthmic regions, compared to control oviducts, indicating that the presence of ova affected sperm release from the sperm reservoir.

Having found that the components of oocytes modulate sperm release from the reservoir, the next step involved determining which components were involved in this release. In this regard, Brüssow et al. (2006) suggested that these components might be GAGs, especially HA. HA is a non-sulphated GAG and is a main component of the extracellular matrix of the porcine cumulus and ZP (Yokoo et al. 2002; Tienthai et al. 2004; Liberda et al. 2006), which is in turn synthesised by COCs during cumulus expansion and increases around ovulation (Kimura et al. 2002; Yokoo et al. 2007).

Tienthai et al. (2000a) have suggested that additional HA would reach the oviduct together with the COCs, as it has been observed that the level of HA in the porcine ampullary fluid increases around ovulation. This possibility was studied by Brüssow et al. (2003, 2006), demonstrating that the numbers of accessory spermatozoa were the highest in those COCs transferred together with HA (COC + HA). Moreover, exogenous HA together with cumulus-free oocytes increase fertilisation rates and the number of accessory spermatozoa compared to oocytes or COCs (Brüssow et al. 2003). All these data launch the hypothesis that the HA participates in the process of sperm release from the oviductal reservoir (Rodríguez-Martínez et al. 2001, 2005; Liberda et al. 2006).

Other studies have suggested that HA plays a role in sperm release because it is a strong inhibitor of sperm-oviduct interactions and induces capacitation in vitro without acrosome exocytosis (Tienthai et al. 2000b). Furthermore, Liberda et al. (2006) reported that oviductal fluid is the strongest inhibitor of boar seminal plasma protein binding to the oviductal epithelium. According to these authors, oviductal fluid contains glycoproteins and GAGs that might induce the release of sperm from the sperm reservoir or act as inhibitors of sperm attachment to the oviductal epithelium. Still backing this hypothesis, seminal plasma acts as a decapacitating factor, but HA and freshly conditioned medium (CM) present capacitation action in boar spermatozoa in vitro (Suzuki et al. 2002).

Therefore, all the mentioned evidence suggests that HA inhibits the interaction of sperm surface proteins to the oviduct, thereby playing a relevant role in sperm release from the oviductal reservoir and in the capacitation process (Tienthai et al. 2000b; Bergqvist and Rodríguez-Martínez 2006; Bergqvist et al. 2006, 2007; Liberda et al. 2006; Brüssow et al. 2008).

6.10.3.3 Ovarian Influence: Progesterone

Finally, Hunter (2008) has demonstrated that the ovary also influences sperm release from the oviductal reservoir via progesterone, as this hormone would act as an indirect rather than direct signal for sperm release. However, the actual influence of this hormone is controversial, according to results from other authors (Brüssow et al. 2008).

In this regard, two observations have demonstrated that progesterone is the ovarian hormone that modulates the sperm oviductal reservoir. Accordingly, progesterone concentrations are from 100 to 1,000 times higher in the follicular fluid than in peripheral blood (Eiler and Nalbandov 1977; Blödow et al. 1990). Moreover, when progesterone or follicular fluid containing high levels of progesterone are injected into the oviduct, spermatozoa are released from the sperm reservoir (Hunter et al. 1999). In addition, progesterone induces acrosome reaction in in vitro conditions.

Furthermore, other studies have indicated that when follicular fluid does not enter the oviduct because, either it has been aspirated from pre-ovulatory follicles or the oviduct has been ligated, there is a reduction in the percentage of

spermatozoa at the ampulla and AIJ. This suggests that sperm release from the reservoir has been prevented (i.e. in this case, sperm remains adhered to the isthmus forming the sperm reservoir). We must bear in mind here that follicular fluid contains high concentrations of progesterone.

Despite all the above mentioned, the potential role of progesterone on sperm release has been contradicted by other findings that reported that follicular fluid does not seem to reach the oviduct (Brüssow et al. 2003, 2008). This excludes its direct effect on sperm reservoir in vivo (Brüssow et al. 1999, 2008). Thus, even though Hunter et al. (1983) and Hunter (1996) had previously suggested that follicular fluid components were involved in sperm migration and fertilisation by a local counter-current transfer into the blood and lymph circulation, this hypothesis remains to be proven (Brüssow et al. 2008).

6.11 Modulation of Sperm Function and Survival in the Oviduct: Lessons from In Vitro Studies

6.11.1 Experimental Studies in In Vitro Conditions

Most studies about the passage of spermatozoa within the mammalian oviduct and the related events that take place in this organ (i.e. sperm reservoir, sperm capacitation and fertilisation) have been conducted in in vitro conditions. The present section reviews the state-of-the-art on such in vitro studies.

6.11.1.1 Establishing OEC Monolayers

Since OEC play a key role in mammal reproduction, the latest research includes several reports on their primary cultures in order to understand their structure and ultrastructure (Eslaminejad et al. 2007), to set IVF systems, to assess sperm parameters after homologous or heterologous co-culturing and to determine the effects of cell-CM on sperm function.

Primary culture of OEC has been developed both to study OEC and develop IVF protocols in porcine (Romar et al. 2001; Sostaric et al. 2006; Yeste et al. 2009a, 2012) and other mammalian species, such as human (Vlad et al. 1996; Saridogan et al. 1997), equine (Thomas et al. 1995a), rabbit (Dickens et al. 1996), caprine (Rodríguez-Dorta et al. 2007), bovine (Walter and Miller 1996; Xia et al. 1996; Cox and Leese 1997; Reischl et al. 1999; Rottmayer et al. 2006), monkey (Rajagopal et al. 2006), mouse (Tan et al. 2007) and canine (Vannucchi et al. 2006) species.

Different studies performed on cattle (Eyestone and First 1989; Eyestone et al. 1991; Xu et al. 1992) and sheep (Gandolfi and Moor 1987) showed early on that OEC co-culture and the CM produced by OEC in culture (OEC-CM) promote

embryo development in vitro. In pigs, OEC in culture secrete products that provide suitable conditions for reducing polyspermy and increasing IVF fertilisation rates (White et al. 1989; Nagai and Moor 1990; Kano et al. 1994; Vatzias and Hagen 1999; Bureau et al. 2000), but has a lesser extent in influencing sperm survival (Yeste et al. 2009a, 2012).

6.11.1.2 Co-culture of OEC with Spermatozoa

Sperm-OEC co-culture systems have also been performed on several species, such as pigs (Suarez et al. 1991; Fazeli et al. 1999; Petrunkina et al. 2001a; Yeste et al. 2007, 2008, 2009a, b, 2012), humans (Bongso et al. 1993; Kervancioglu et al. 1994; Morales et al. 1996; Ellington et al. 1998; Yao et al. 1999), cattle (Lefebvre and Suarez 1996; Gualtieri and Talevi 2000, 2003), dogs (Ellington et al. 1995; Kawakami et al. 2001), horses (Ellington et al. 1993a, Thomas et al. 1994, 1995b; Dobrinski et al. 1996, 1999), rats (Cortés et al. 2004), sheep (Gutiérrez et al. 1993) and wallabies (Sidhu et al. 1998).

In most of these studies, it has been demonstrated that OEC specifically influence sperm by prolonging their viability, by stabilising the acrosome and by inducing capacitation, amongst other physiological processes (Kervancioglu et al. 1994; Yeung et al. 1994; Morales et al. 1996; Ellington et al. 1998). Later, these findings will be addressed again, and discussed in relation to what has been observed in the case of pigs.

In principle, the effects of OEC on sperm function in in vitro co-culture seem to depend on the ovarian state of the oviduct used to perform the primary culture, but this has been reported to be controversial through species and studies. Thus, whereas in pigs no difference has been found in sperm-OEC binding when comparing follicular and luteal states (Petrunkina et al. 2001a; Yeste et al. 2009a, b), in dogs, Kawakami et al. (2001) have observed that the percentages of both hyperactivated and acrosome-reacted spermatozoa were significantly higher in follicular than luteal OEC. This difference was attributed to GAGs and/or glycoproteins secreted by the follicular-OEC, but more research is warranted.

6.11.1.3 Incubation of Spermatozoa with OEC-Conditioned Media and APM Fractions

Reports of the effects of OEC on sperm function have not been limited to co-culture. Indeed, the effects on sperm function and survival of OEC-CM in vitro and the incubation of APM fractions coming from the oviduct have also been studied.

Concerning the former, OEC-CM prolongs both motility and viability in fresh (Ijaz et al. 1994; Abe et al. 1995; Yeste et al. 2009a) and cryopreserved (Zhu et al. 2001) spermatozoa, and induces sperm capacitation (Chian et al. 1995). Furthermore, King et al. (1994) showed that treatment with 17β-estradiol enhances the ability of OEC-CM for prolonging sperm motility. However,

although CM obtained from in vitro oviduct culture and oviduct fluid obtained via catheterisation contain oviduct secretions, their compositions are not identical. Oviduct fluid collected via a catheter contains some molecules that are not present in the CM produced by cultured oviduct tissue. Nevertheless, both oviduct fluid collection (Oliveira et al. 1999) and oviduct culturing have been used to study the oviduct, the composition of its fluid and the effect of oviduct secretions on gamete function and embryo development (Bureau et al. 2000; Killian 2004).

With regard to APM, Smith and Nothnick (1997) reported that the viability of rabbit spermatozoa incubated in the presence of APM vesicles is enhanced as a result of direct contact between spermatozoa and plasma membranes of oviductal cells. In pigs, Fazeli et al. (2003) observed that sperm survival was extended after incubation with APM, demonstrating that the factors involved in this prolongation were not integral OEC membrane proteins but peripheral membrane proteins. Several protein components of the solubilised APM fraction (sAPM) that binds to boar spermatozoa have been identified (Elliott et al. 2009); related to this, it has been suggested that knowledge about which APM proteins extend the life of sperm may be useful for developing long-term semen diluents. The role of some of these identified sAPM proteins will be taken up again when speaking about the modulation of oviductal gene expression in response to the presence of sperm (see Sect. 6.12).

6.11.1.4 IVF Studies Involving OEC Monolayers

With regard to the use of oviductal monolayers in IVF procedures, many studies have been carried out regarding porcine (Romar et al. 2001) and other mammalian species, such as human (Vlad et al. 1996; Saridogan et al. 1997; Tse et al. 2008), equine (Thomas et al. 1995a; Mugnier et al. 2009), rabbit (Dickens et al. 1996), caprine (Rodríguez-Dorta et al. 2007), bovine (Walter and Miller 1996; Xia et al. 1996; Cox and Leese 1997; Reischl et al. 1999; Rottmayer et al. 2006), monkey (Rajagopal et al. 2006), mouse (Lee and Yeung 2006; Tan et al. 2007) and canine species (Vannucchi et al. 2006).

In most of these studies, performing IVF in the presence of OEC monolayers increased the percentages of division at day 2 and the percentages of blastocyst at day 7, and decreased polyspermy.

6.11.2 Modulation of Sperm Function and Survival by OEC in In Vitro Co-culture

6.11.2.1 Introduction

In most mammalian species, including pigs, the contact between spermatozoa and OEC in vivo appears to be one of the final phases of maturation that gives spermatozoa the ability to penetrate oocytes (Hunter 1984). In fact, reproductive

physiology involves a significant coordination of different processes, one of them being the interactions between spermatozoa and OEC (Hunter and Rodríguez-Martínez 2004).

In vivo, when spermatozoa are within the oviduct, their lifespan is prolonged and their fertilising ability is maintained when they directly attach to oviductal epithelium. However, such epithelium is constituted by secretory and ciliated cells, so that the oviductal fluid composed of these secretions also contributes to the oviductal function. Therefore, secretory cells produce and release into the oviduct lumen several macromolecules, such as oviductin (OSP/OVGP1), which is conserved throughout many mammalian species (Buhi et al. 2000; Killian 2004). Secreted proteins in oviductal fluid support embryo development and also seem to be involved in the modulation of sperm function within the oviduct (Abe 1996; Pérez-Martínez et al. 2006; Rodríguez-Martínez et al. 2001; Tienthai et al. 2004).

Since performing in vivo studies is quite difficult, the in vitro studies dealing with co-culture of sperm with OEC and epididymal epithelial cells (EEC) enable us to understand what the role of these epithelial cells on the function of male gametes is (Yeste et al. 2007, 2009a, 2012). In the case of oviductal cells, it is very important to highlight the role of sperm-epithelial cells binding on the formation of the sperm reservoir during the preovulatory period. In vivo, this cell-to-cell contact takes place in the UTJ and isthmus, the regions of the oviduct where sperm is stored (Töpfer-Petersen et al. 2002). The molecular mechanisms of sperm-OEC binding, thereby forming the sperm reservoir, have been considered in a preceding specific section (see Sect. 6.7).

As previously mentioned, state-of-the-art research into the oviduct documents that OEC affect sperm cell parameters both by binding to sperm (Fazeli et al. 1999; Yeste et al. 2009a) and by releasing secretory products (McCauley et al. 2003; Quintero et al. 2005; Zhang et al. 2006; Kumaresan et al. 2006; Lachance et al. 2007). Accordingly, several reports have demonstrated that in vitro OEC affect sperm viability, capacitation and motility in human (Ellington et al. 1998) and other mammalian species such as stallions (Thomas et al. 1995b; Dobrinski et al. 1999), dogs (Kawakami et al. 2001), cattle (Kodithuwakku et al. 2007), and rats (Cortés et al. 2004). Taking into account all the above mentioned, it is interesting to investigate how boar sperm function and survival is modulated when they are directly co-cultured with homologous OEC, with other reproductive epithelial cells like epididymal ones (EEC), or with non-reproductive cells such as pig kidney epithelial cells (LLC-PK1). In addition, another relevant aspect when evaluating how OEC monolayers affect sperm function consists of unveiling whether this influence depends on the direct contact sperm-OEC, or whether OEC-CM resulting from the activity of cultured epithelial cells (oviductal, epididymal and LLC-PK1) also modulates such sperm parameters. Finally, it is also interesting to study the differences between the two sperm populations that can be distinguished in in vitro co-cultures: unbound and bound to epithelial cells (Yeste et al. 2007, 2009a, 2012).

Therefore, the effects of in vitro homologous OEC co-culture and CM on viability, motility, capacitation status and acrosome and mitochondrial sheath integrity of boar spermatozoa are discussed in the following pages of this section.

6.11.2.2 Influence of OEC on Sperm Viability and Acrosome Integrity

Maintaining sperm viability in co-culture systems with OEC and EEC has been observed in pigs (Fazeli et al. 1999; Yeste et al. 2007, 2009a, 2012), humans (Kervancioglu et al. 1994; Yeung et al. 1994; Akhondi et al. 1997; Yao et al. 1999), cattle (Ellington et al. 1991; Pollard et al. 1991), sheep (Gutiérrez et al. 1993) and horses (Ellington et al. 1991; Thomas et al. 1994). However, it must be noted that sperm viability is better maintained in spermatozoa bound to reproductive monolayers (OEC and EEC) than in spermatozoa that are not attached to these cells. Indeed, the viability of ejaculated normal spermatozoa bound to OEC has been reported to decrease by only about 15 % after 24 h of co-culturing, while the viability of spermatozoa that remained unbound from the OEC decreased by about 50 % (Yeste et al. 2009a) (Fig. 6.6).

On the other hand, reproductive cells (OEC and EEC) are more able to maintain sperm survival in vitro in both sperm populations (namely, bound to and unbound from epithelial cells) than in non-reproductive monolayers like LLC-PK1. This finding suggests that both secretions from and binding to OEC and EEC specifically influence sperm survival and also emphasises the relevance of reproductive nature of epithelial cells when co-culturing with ejaculated (Yeste et al. 2009a) and epididymal spermatozoa (Yeste et al. 2012).

As far as the effects of cell-CM on sperm survival are concerned, it must be mentioned that sperm viability is maintained better in co-incubation with CM

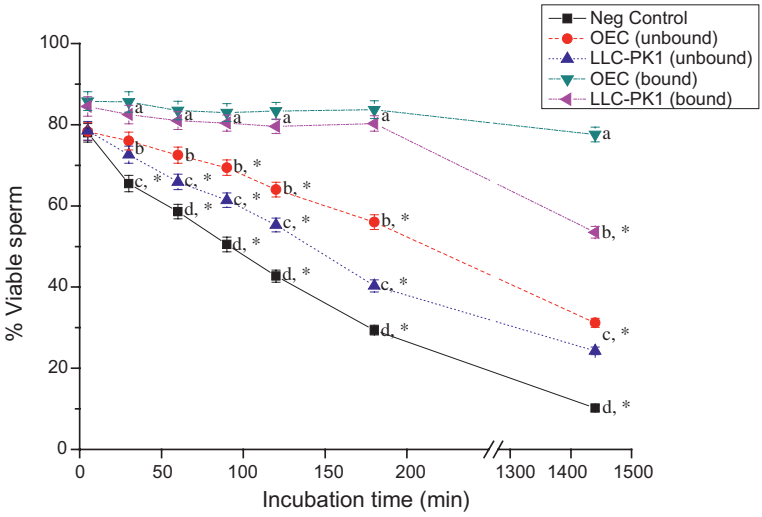

Fig. 6.6 Comparisons between viabilities of bound and unbound spermatozoa in co-culture with OEC or LLC-PK1 cells. Different superscripts (*a*, *b*, *c* and *d*) mean significant differences ($P < 0.05$) among treatments at the same time point. Superscript (*) means significant differences compared to the same treatment at 5 min (Yeste et al. 2009a, Reproduced with permission)

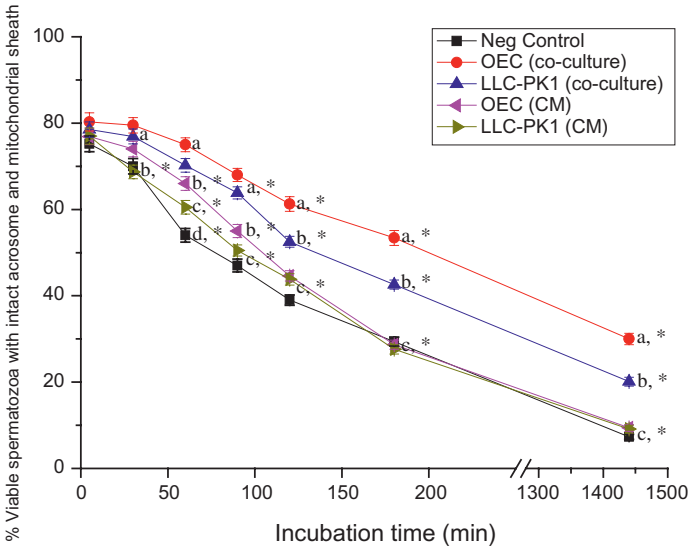

Fig. 6.7 Percentages of spermatozoa with intact plasma membrane, acrosome and mitochondrial sheath (mean ± SEM). Graphs show data for both unbound spermatozoa co-cultured with OEC or LLC-PK1, and for spermatozoa incubated with cell-CM. Different superscripts (*a*, *b*, *c* and *d*) mean significant differences ($P < 0.05$) within-times between treatments. Superscript (*) means significant differences ($P < 0.05$) compared to the same treatments at 5 min (Yeste et al. 2009a, Reproduced with permission)

from cultured reproductive cells (OEC and EEC) than in co-incubation with CM from non-reproductive cells (LLC-PK1 cells), and, in the latter, better than in unconditioned medium (Yeste et al. 2009a, 2012) (Fig. 6.7). This finding has also been observed in human and bovine species, where OEC-CM has also been reported to prolong sperm survival when compared to unconditioned medium (Ijaz et al. 1994; King et al. 1994; Abe et al. 1995; Zhu et al. 2001).

Sperm that retains the ability to bind to epithelial cells, especially to OEC and EEC, must be viable and thus present an intact plasma membrane, which will ensure that stored spermatozoa in the reservoir (i.e. attached to oviductal cells) will be able to fertilise the oocyte (see Sect. 6.8). Related to this, Khalil et al. (Khalil et al. 2006) working with frozen–thawed bull spermatozoa showed that sperm-oviductal epithelium binding capacity depends on plasma membrane integrity, since the ability of spermatozoa to modify their swelling in response to hypo-osmotic stress (volume regulatory ability) is related to their binding and further fertilising abilities. According to these authors, spermatozoa from sub-fertile bulls present a damaged plasma membrane, which leads to lower binding indices to oviductal epithelium and deficiencies in volume recovery after hypotonic challenges.

In porcine (Fazeli et al. 1999; Yeste et al. 2007, 2009a, 2012), as in other mammalian species like bovine (Gualtieri and Talevi 2000), only acrosome-intact sperm are able to bind to OEC and the acrosomes of bound spermatozoa

are preserved intact over time (Fig. 6.7). This also confirms that, in vivo, there is a sort of selection mechanism in the sperm reservoir (see Sect. 6.8) and binding to OEC is essential for preserving sperm fertilisation ability during the interval between the start of oestrus and ovulation (Hunter and Rodríguez-Martínez 2004). Related to this, the integrity of the acrosome is very important at the moment of fertilisation, because previous studies in bovine and humans have clearly shown that acrosome-intact spermatozoa are significantly more able to bind to the ZP than acrosome-reacted spermatozoa (Nishikimi et al. 1997; Liu et al. 2006).

In addition, the positive effect of OEC on acrosome integrity is also observed in unbound sperm populations and CM, since the percentage of spermatozoa with intact acrosomes is higher in OEC and EEC co-cultures and in OEC-CM and EEC-CM, than in LLC-PK1 co-cultures, LLC-PK1-CM and medium without cells. This finding reveals that OEC and EEC stabilise the acrosomal membranes of spermatozoa in pigs, as has also been observed in humans (Yao et al. 1999), thereby preserving sperm integrity and decreasing the percentage of false or degenerative acrosome reactions.

6.11.2.3 Influence of OEC on Sperm Motility

In vitro co-culture of spermatozoa with OEC also plays a beneficial role in some sperm kinetic parameters (such as straight line, curvilinear and average-path velocities and the percentage of linearity) in pigs (Yeste et al. 2009a), humans (Bongso et al. 1993; Yao et al. 2000) and sheep (Gutiérrez et al. 1993). These benefits are observed in both sperm populations (i.e. bound to and unbound from epithelial cells) when co-culturing with OEC is compared with LLC-PK1 and with medium without cells. In this case, OEC co-culturing maintains better progressive motility of unbound spermatozoa from epithelial cells than LLC-PK1 monolayers and medium without cells.

In addition, the percentage of progressive motile spermatozoa is also higher after incubation with OEC-CM than with LLC-PK1-CM, or with unconditioned medium (Yeste et al. 2009a), in agreement with other similar reports in humans and other species, where it has been documented that CM also prolongs sperm motility (Ijaz et al. 1994; King et al. 1994; Abe et al. 1995; Zhu et al. 2001). However, a differential effect is observed in sperm motility when kinetic parameters of unbound sperm population in co-culture are compared with sperm incubated with CM. Co-culturing reveals a greater ability to maintain this sperm parameter than incubating with CM. In fact, CM is not identical to the medium of co-culture experiment containing spermatozoa in suspension.

Influence of oviductal epithelium on sperm motility parameters has also been reported in other in vitro studies conducted with APM-oviduct fractions. Satake et al. (2006) observed that from the different sperm subpopulations existing within a given ejaculate (see also Sect. 2.4), oviductal-APM proteins acted on the fast-linear subpopulation by suppressing their motility.

6.11.2.4 Influence of OEC on Sperm Capacitation

In vitro, direct contact between OEC and spermatozoa also delays sperm capacitation. This is quite clear when the capacitation status of bound and unbound spermatozoa from OEC is compared in co-culture experiments. Indeed, the percentage of uncapacitated spermatozoa in bound sperm population is significantly higher than in unbound (Fazeli et al. 1999; Yeste et al. 2009a). This result confirms sperm-OEC attachment is selective because uncapacitated rather than capacitated spermatozoa bind preferentially to OEC and also demonstrates that it delays sperm capacitation (Fazeli et al. 1999; Petrunkina et al. 2001a; 2003; Töpfer-Petersen et al. 2002; see also Sect. 6.8). This finding also matches other reports in dogs (Smith and Nothnick 1997) where the tyrosine-phosphorylation of heads (a capacitation sign) was observed in unbound sperm populations, but not in bound suspensions. Thus, only spermatozoa with non-phosphorylated heads preferentially bind to epithelial cells, and after this attachment tyrosine phosphorylation of sperm head proteins and capacitation are delayed. Finally, Petrunkina et al. (2004) have reported that binding of uncapacitated sperm to OEC may be a species-independent phenomenon, at least in vitro conditions, since dog spermatozoa bind in a similar way to homologous and heterologous explants.

The induction of sperm capacitation is specifically observed when comparing the unbound sperm population co-cultured with OEC, or co-cultured with non-reproductive cells, or when spermatozoa are incubated in medium without cells (Kervancioglu et al. 1994; Fazeli et al. 1999; Yeste et al. 2009a) (Fig. 6.8). Moreover, the effects of CM on sperm capacitation are also dependent on reproductive cells, since the percentage of capacitated spermatozoa is higher after incubation with OEC than with LLC-PK1 cells or unconditioned medium (Fig. 6.9). Thus, in porcine as in bovine species (Abe et al. 1995), OEC-CM components are not identical to the LLC-PK1-CM and the unconditioned medium, or to the medium to which the unbound sperm population is exposed during OEC co-culturing. These observations would be explained by considering that sperm capacitation, with the specific involvement of OEC rather than non-reproductive cells, is a physiological process that takes place in vivo in the isthmus (Rodríguez-Martínez 2007).

Since sperm capacitation is a controlled destabilisation process (Harrison 1996) that reduces sperm lifespan, the control of capacitation and the maintenance of sperm viability during a certain period of time are associated-events (Töpfer-Petersen 1999; Hunter and Rodríguez-Martínez 2004) (see Chap. 7). In the case of in vitro co-culture, the percentages of uncapacitated sperm decrease and those of capacitated sperm increase in both systems (i.e. freely swimming spermatozoa in co-culture and sperm in CM), so that it seems that the specific secretory activity of cultured OEC influences sperm capacitation. This is in accordance with the finding that in pigs (Rodríguez-Martínez et al. 2001; Tienthai et al. 2004) and in cattle (Parrish et al. 1989; McNutt and Killian 1991; McNutt et al. 1994; Abe et al. 1995) oviductal fluid plays a basic role in vivo in sperm capacitation. In fact, it is likely that oviductal secretions are involved in sperm capacitation, although this effect is clearer in unbound sperm in co-culture than in CM experiments (Yeste et al. 2009a)

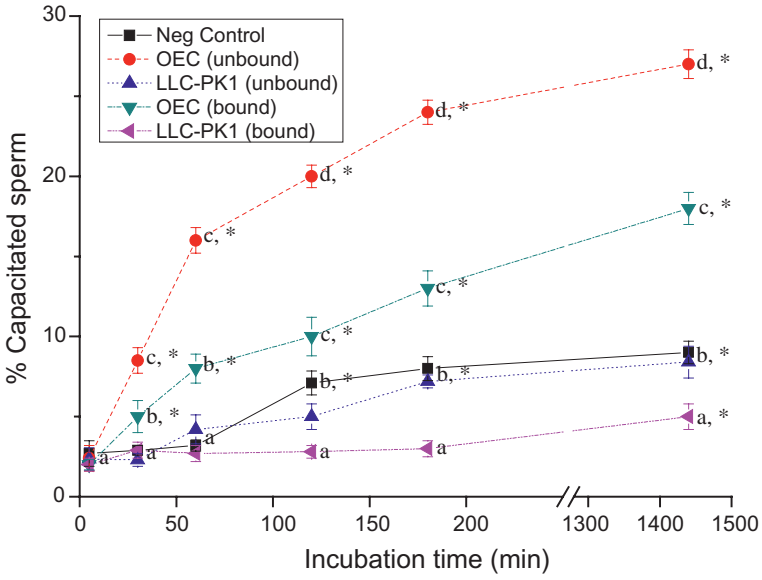

Fig. 6.8 Percentages of capacitated spermatozoa of unbound and bound sperm populations (mean ± SEM) after co-culture with OEC or LLC-PK1 cells. Different superscripts (*a*, *b*, *c* and *d*) mean significant differences ($P < 0.05$) within-times between treatments. Superscript (*) means significant differences ($P < 0.05$) compared to the same treatments at 5 min (Yeste et al. 2009a, Reproduced with permission)

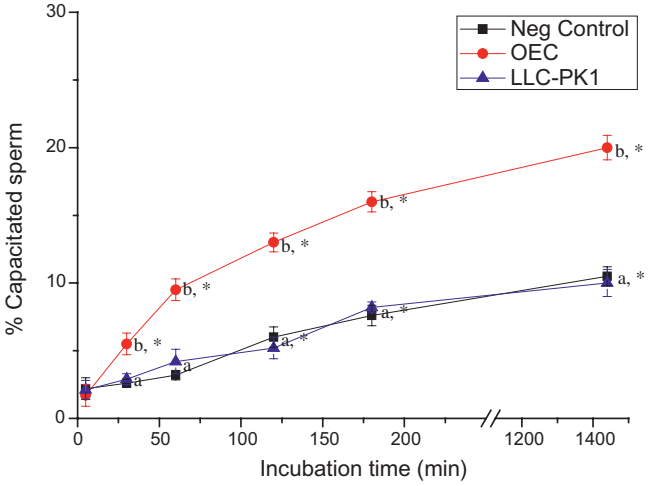

Fig. 6.9 Percentages of capacitated spermatozoa (mean ± SEM) after incubation with cell-CM. Different superscripts (*a* and *b*) mean significant differences ($P < 0.05$) within-times between treatments. Superscript (*) means significant differences ($P < 0.05$) compared to the same treatments at 5 min (Yeste et al. 2009a, Reproduced with permission)

(Figs. 6.8, and 6.9). However, in this case, it is difficult that in vitro conditions mimic what happens in vivo. Indeed, as aforementioned, in vivo capacitation stands for an active and specific coordination process within succeeding regions of the female tract and its completion is synchronised with the events of ovulation for those sperm cells involved in fertilisation. This issue will be taken up again in Chap. 7.

6.11.2.5 Overall View of OEC Effects on Sperm Function and Survival In Vitro

Analysing together sperm viability, motility and capacitation in co-culture with porcine OEC, it can be concluded that the in vitro co-culture system maintains boar sperm viability, especially when bound to OEC, affects sperm velocity parameters, and induces capacitation (Fazeli et al. 1999; Yeste et al. 2009a). This is consistent with previous reports in humans (Kervancioglu et al. 1994; Yeung et al. 1994; Morales et al. 1996; Yao et al. 1999), dogs (Petrunkina et al. 2003), cattle (Ellington et al. 1991; Pollard et al. 1991; Gualtieri and Talevi 2000), sheep (Gutiérrez et al. 1993) and horses (Ellington et al. 1991; Thomas et al. 1995b), where it has been observed that OEC monolayers promote sperm survival, enhance sperm motility, stabilise the acrosome, modify the frequency of tail beat, stabilise sperm chromatin structure, and induce sperm capacitation and the hyperactivation that follows, so that the maintenance of sperm function would appear to be a common characteristic of co-culture systems with OEC. The beneficial effects of OEC have even been observed in the heterologous co-culturing of human sperm with bovine OEC (Ellington et al. 1998).

OEC co-culture and cell-CM affect spermatozoa by maintaining their viability, inducing capacitation and changing motility parameters (Yeste et al. 2009a). Similarly, co-culture and cell-CM from EEC also play a positive part in maintaining sperm motility and survival in porcine (Yeste et al. 2012) and other mammalian species, such as humans (Akhondi et al. 1997). Even co-culture and CM from non-reproductive pig kidney epithelial (LLC-PK1) cells also extend sperm viability when compared to a medium without epithelial cells, although not as significantly as OEC and EEC (Yeste et al. 2009a, 2012). This observation, which is in accordance with previous observations in co-culture with OEC and Vero cells (non-reproductive green monkey kidney epithelium cells) (Kervancioglu et al. 1994), demonstrates that epithelium of the reproductive tract is the most advantageous for spermatozoa.

When separately unbound and bound sperm populations in co-culture experiments are analysed after 24 h, the percentages of immotile and dead spermatozoa significantly increase in unbound populations, compared with time 0, whereas bound sperm remain live and motile. As differences between the medium without cells and OEC in unbound sperm viability and between LLC-PK1 cells and OEC in bound sperm viability are observed, it may be concluded that both OEC secretions and sperm-OEC binding preserve sperm viability. Other authors reported this event in OEC secretions (Yeung et al. 1994; Abe et al. 1995; Yao et al. 1999), in OEC-sperm binding (Dobrinski et al. 1997; Smith and Nothnick 1997), and in proteins derived from the oviductal epithelium (Boquest et al. 1999) that regulate bull sperm function, by

promoting sperm viability and delaying acrosomal damage. Related to this, Lapointe et al. (1998) proposed that the presence of catalases in oviductal fluid may play a key role in sperm function preservation within the mammalian female tract.

It is also interesting to examine the effects of oviductal fluid proteins and CM produced by OEC in culture on sperm function. It is known that in oviductal physiology, glycoproteins are secreted and accumulated in the caudal isthmus, the site of the preovulatory reservoir. These secretions prevent uterine and ampullary tubal fluids from entering the functional sperm reservoir and remove residual male secretions from the sperm's surface (Hunter 2002) and appear to affect spermatozoa (Wagh and Lippes 1989; Abe et al. 1995; Lapointe and Sirard 1996; Zhu et al. 2001; Quintero el al. 2005). In the case of in vitro co-culture studies, incubations with OEC-CM are slightly better for sperm motility and viability than those with culture medium alone in pigs (Yeste et al. 2009a), which is in agreement with other reports in cattle and humans (Ijaz et al. 1994; King et al. 1994; Abe et al. 1995; Zhu et al. 2001). Furthermore, several papers conducted in bovine and porcine species have shown that oviductal fluid regulates sperm function to facilitate fertilisation, capacitating spermatozoa and inducing their hyperactivation (Parrish et al. 1989; McNutt and Killian 1991; McNutt et al. 1994; Abe et al. 1995; Coy et al. 2010).

In conclusion, uncapacitated spermatozoa bind preferentially to OEC, the sperm-OEC binding being a basic process in forming a sperm reservoir and in the reproductive events leading to fertilisation. Furthermore, OEC-CM and EEC-CM affects sperm viability and motility less intensively than the combination of sperm-OEC binding and secretion during co-culture, as other studies dealing with the role of OEC secretions (Yeung et al. 1994; Abe et al. 1995; Yao et al. 1999) and sperm-OEC binding (Dobrinski et al. 1997; Smith and Nothnick 1997; Töpfer-Petersen et al. 2002) on sperm function have also shown. In fact, some reports have suggested that direct contact between sperm-OEC is the key mechanism for selecting high quality spermatozoa and preserving their fertilising ability (Dobrinski et al. 1997; Murray and Smith 1997; Petrunkina et al. 2001a; Yeste et al. 2009a) (see also Sect. 6.8). Intriguingly, the importance of direct binding between spermatozoa and OEC is also supported by other findings, since the incubation of spermatozoa with some proteins of solubilised oviductal APM also maintain sperm viability in rabbits (Smith and Nothnick 1997) and pigs (Fazeli et al. 2003).

6.12 Spermatozoa as Modulators of Gene Expression in the Oviduct

6.12.1 Introduction

The global profiling of the oviductal surface contains 270 proteins including aminopeptidase N and various members of the HSP family (Sostaric et al. 2006; Töpfer-Petersen et al. 2008). However, the presence of gametes, spermatozoa and

oocytes, alters the oviductal proteome/secretome profile (Ellington et al. 1993b; Georgiou et al. 2005, 2007). In cattle, spermatozoa have also been reported to modulate the gene expression of bovine EEC in homologous co-culture (Reyes-Moreno et al. 2008).

Classical views proposed that the female reproductive tract nourished spermatozoa in the sperm reservoir. However, today we know that this view is too simplistic as spermatozoa are also able to modulate proteome and secretome of the female genital tract in birds and mammals (Long et al. 2003; Fazeli et al. 2004; Georgiou et al. 2005, 2007).

Focusing on spermatozoa within the female tract, it must be stated that the dialogue between sperm cells and the oviduct is not univocal. Thus, not only is there a modulating effect of OEC on spermatozoa, as described in the previous section (Sect. 6.11), but fertilisation-competent spermatozoa also modulate oviductal gene expression and, therefore, the secretory proteome of the oviductal explants (Fazeli et al. 2004; Georgiou et al. 2005, 2007), and of the OEC in in vitro co-culture (Fazeli et al. 2004; Sostaric et al. 2006; Kodithuwakku et al. 2007; Lachance et al. 2007; Yeste et al. 2008, 2009b). This fact contributes to the creation of a favourable microenvironment for gametes and for the physiological events that take place in the oviduct, such as the formation of sperm reservoir and sperm capacitation, amongst others.

In recent years, many efforts have been made to define the oviductal cell surface proteome (Georgiou et al. 2005; Sostaric et al. 2006) and secretome (Georgiou et al. 2007) in order to provide a basis for understanding the processes that take place in the oviduct. In this section, these findings will be described based on the three different performed approaches: using oviductal explants, oviductal monolayers or surgical models. The present section ends with an overall view of male gametes as modulators of oviductal gene expression.

6.12.2 Experiments Using Oviductal Explants

In mice, spermatozoa trigger a signal transduction pathway that modulates gene expression in oviductal cells when they arrive at the oviduct after insemination (Fazeli et al. 2004), and in cattle, the motile spermatozoa upregulate the expression of prostaglandins in OEC (Kodithuwakku et al. 2007) after in vitro co-culturing.

In pigs, oocytes and spermatozoa alter the expression of some specific oviductal proteins after the co-incubation of gametes with oviductal fragments and OEC. According to Georgiou et al. (2005), co-incubation of oviductal explants with boar spermatozoa and/or porcine COCs regulates the expression of thirty-four oviductal proteins. From these proteins, twenty are regulated by the presence of spermatozoa, five by the presence of oocytes, and nine by the presence of both gametes. As far as the functional categories of these proteins are concerned, 41 % are related to production, maintenance and repair of proteins, 18 % perform antioxidant and radical-scavenger functions, 15 % are related to metabolism, and 25 % are categorised as miscellaneous. The induced-changes by sperm cells seem to provide a favourable microenvironment for gametes and also prepare the oviduct milieu for embryo arrival.

6.12.3 In Vitro Co-Culture Experiments of Spermatozoa and Oviductal Monolayers, and the Relevance of Some HSPs

6.12.3.1 The Culture and Co-Culture Approach

Another approach consists of studying how sperm affects the gene expression of OEC monolayers in in vitro co-culture, as several authors have done (Sostaric et al. 2006; Yeste et al. 2008, 2009b). In this regard, the expression of some genes encoding sAPM-oviductal proteins have been reported to be altered in cultured OEC monolayers in response to sperm presence. Accordingly, and as stated in Sect. 6.11, sperm survival is maintained when spermatozoa are incubated with sAPM extracts from the oviduct in rabbits (Smith and Nothnick 1997), pigs (Fazeli et al. 2003) and cattle (Boilard et al. 2004). In addition, these sAPM extracts have partially been characterised in pigs (Elliott et al. 2009), in an effort to identify which protein or proteins account for modulation of sperm function, and especially which one is responsible for the survival-maintaining effect in sperm. This approach may be beneficial for the swine sector, as more knowledge about which sAPM proteins extend the life of sperm may facilitate the development of long-term semen diluents, benefiting both agricultural industries and conservation practices (Yeste 2008; see also Sect. 10.2.1).

One of these studies focused on four genes encoding proteins identified in the sAPM extract. These proteins were clusterin (*CLU*), heat shock cognate protein related 70 kDa (*HSPA8*, also known as *HSC70*), HSP 90 kDa alpha A.1 (*HSP90AA1*) and glucose-regulated protein 78 kDa (*HSPA5*, also known as *GRP78*) (Yeste et al. 2008, 2009b).

On the one hand, the expression of these four genes in oviductal monolayers was investigated in OEC coming from sow reproductive tracts that were at the follicular and luteal stage, and also in LLC-PK1 cells. In this case, the expression of these four genes did not appear to be significantly different in follicular- and luteal-OEC (Yeste et al. 2008), so that it seems that the expression profiles of these four genes in vitro do not depend on the ovarian state. This is consistent with previous reports studying other aspects of OEC, since the ability of OEC in in vitro culture to affect sperm function does not depend on the ovarian state and oviductal region (Fazeli et al. 1999; Petrunkina et al. 2001a).

However, other studies in vivo referring to the human endometrium have shown differences depending on the menstrual cycle in gene expression patterns of HSPA8, HSPB1 and HSPD1. Furthermore, the human endometrium, in response to steroid hormones, undergoes characteristic cycles of proliferation and secretory changes expressing different HSPs such as HSPB1, HSPD1, HSPA1A, and alpha crystallin B chain. Specifically, the expression of HSPB1, HSPD1, and HSPA8 shows a sharp increase in the human endometrium after ovulation (Tabibzadeh and Broome 1999). In addition, Shah et al. (1998) showed that HSP90AA1 in humans decreases in decidual and placenta tissues during the course of normal gestation. On the other hand, although no changes in *CLU* expression have been observed

when comparing both ovarian states, an increase in CLU gene expression has been observed in corpus luteum when luteolysis is induced (Forni et al. 2003).

Despite of all the aforementioned, as changes in protein patterns have been observed in other organs in vivo, it is not clear whether the lack of the differences between the follicular and luteal stages is due to the nature of oviduct epithelium in respect of the endometrium, or is the result of the cell dedifferentiation of in vitro culturing itself. More research, including proteomic analyses, is therefore needed to better address this conclusion in vitro and understand changes in gene expression during the ovarian cycle (Yeste 2008).

On the other hand, in our study (Yeste et al. 2008, 2009b), spermatozoa were co-cultured with OEC or with LLC-PK1 monolayers (positive control), or incubated with co-culture medium but without cells (negative control). Furthermore, in the case of co-culture with epithelial monolayers (i.e. OEC or LLC-PK1), a membrane-diffusible insert was absent or present to prevent direct contact between spermatozoa and epithelial cells, to determine whether changes produced in gene expression in response to the presence of sperm cells were due to direct-binding between spermatozoa and OEC, or not (Yeste et al. 2009b). In this case, boar spermatozoa appeared to alter the expression of *HSP90AA1*, *HSPA5* and *HSPA8* in OEC when they did bind to epithelial cells, but not when direct binding was prevented by the presence of a membrane-diffusible insert (Figs. 6.10, 6.11, 6.12). This means that the observed upregulation-effect needs spermatozoa directly

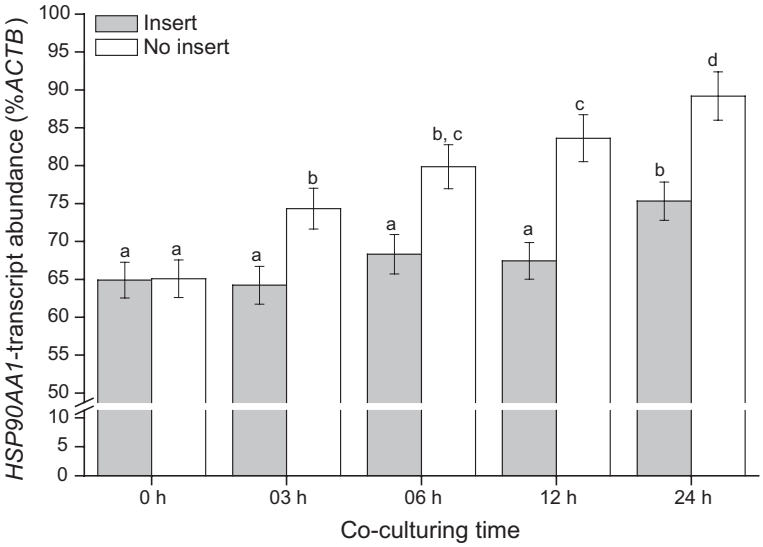

Fig. 6.10 Relative transcript abundances of *HSP90AA1* in OEC co-cultured with spermatozoa (with and without inserts). Different superscripts (*a*, *b*, *c* and *d*) mean significant differences ($P < 0.05$) between the presence and the absence of membrane inserts over the co-culturing time (Yeste et al. 2009b)

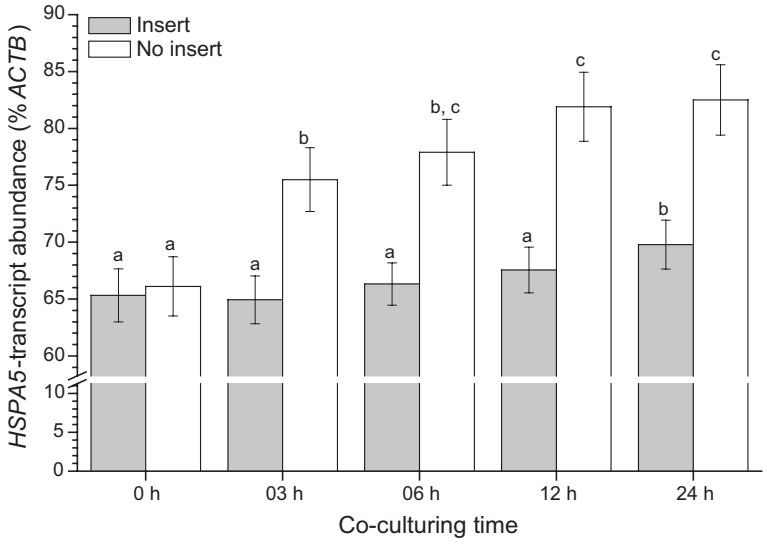

Fig. 6.11 Relative transcript abundances of *HSPA5* in OEC co-cultured with spermatozoa (with and without inserts). Different superscripts (*a*, *b*, *c* and *d*) mean significant differences ($P < 0.05$) between the presence and the absence of membrane inserts over the co-culturing time (Yeste et al. 2009b)

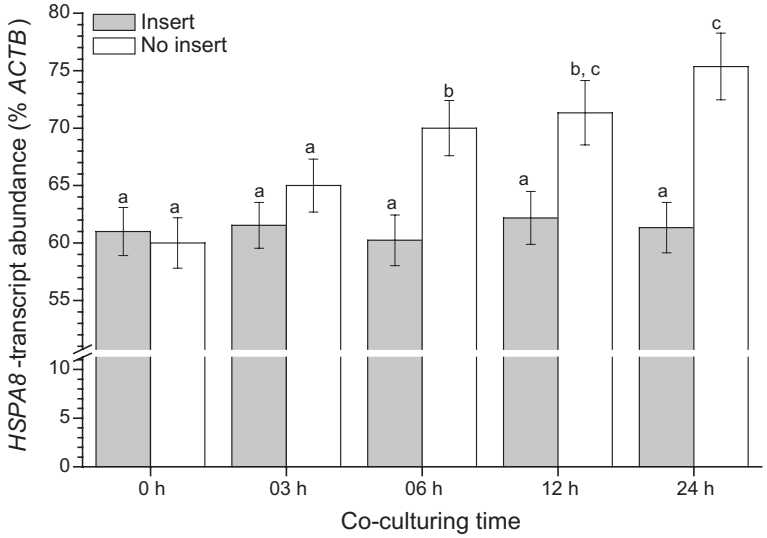

Fig. 6.12 Relative transcript abundances of *HSPA8* in OEC co-cultured with spermatozoa (with and without inserts). Different superscripts (*a*, *b*, and *c*) mean significant differences ($P < 0.05$) between the presence and the absence of membrane inserts over the co-culturing time (Yeste et al. 2009b)

bound to OEC (Yeste et al. 2009b); otherwise, they do not alter gene expression. Moreover, *CLU* expression was not affected by sperm presence and the upregulation-effect observed in the other three genes and mediated by spermatozoa seemed to be specific for OEC rather than for LLC-PK1 monolayers (Yeste 2008; Yeste et al. 2009b).

In the light of these data, it appears that HSP90AA1, HSPA8 and/or HSPA5 could be the sAMP-components involved in the modulation of sperm function. In addition, these results back other reports (Tabibzadeh and Broome 1999; Georgiou et al. 2005; Sostaric et al. 2006) and suggest a certain role for HSPs in the female reproductive tract.

6.12.3.2 Some Additional Information About HSPs and Reproductive Physiology in Mammalian Species

HSPs have been observed in all kinds of cells and organisms, and are members of highly conserved protein families consisting of both constitutive and inducible components (Jeremias et al. 1997). These proteins act as molecular chaperones (Arrigo 2005; Calderwood et al. 2007; Voellmy and Boellman 2007) and are mainly involved in protein folding in a wide range of physiological processes (Ciocca et al. 1993; Hendrick and Hartl 1993; Jakob and Buchner 1994; Mariani et al. 2000; Brown et al. 2007; Javid et al. 2007), as well as in the regulation of the function of steroid receptors (Burel et al. 1992; Rajapandi et al. 2000). In recent years, HSPs have also become therapeutic targets in pharmacology (Sõti et al. 2005; Arrigo et al. 2007). Related to reproductive physiology, changes in the amounts of HSPs have been observed during the menstrual (Wu et al. 1996) and the endometrial cycles (Komatsu et al. 1997; Tabibzadeh and Broome 1999).

To begin with, HSPs activate the transcription of several genes related to environmental stress, cell growth control and developmental signals (Morano and Thiele 1999), and their expression can be induced as a result of cell exposure to different physicochemical stresses (Burg et al. 2007). For this reason, HSPs are expressed at a relatively low level under normal conditions but are inducible by a number of several signals, including stress, steroid hormones, temperature changes, heavy metals, oxidative stress, viral or bacterial infection and cytokines (Jäättelä and Wissing 1992; Baniahmad and Tsai 1993; Jeremias et al. 1999).

As already noted, HSPs function as molecular chaperones. This term is applied to proteins that prevent incorrect interactions of proteins and participate in their assembly without being part of their structure (Ellis 1987; Craig et al. 1994). Different studies have demonstrated that the members of the HSPs family, such as HSP 60 kDa (HSPD1) and HSP 70 kDa (HSPA1A, also known as HSP70-1), act in this way by participating in the folding and unfolding of cellular proteins (Jäättelä and Wissing 1992).

Secondly, HSPs synthesised constitutively are involved in the regulation of the function of steroid receptors (Burel et al. 1992; Rajapandi et al. 2000; Havarinen et al. 2001; Azuma et al. 2004; Al-Madhoun et al. 2007). In this particular case,

and as aforementioned, these factors are expressed at a relatively low level under normal conditions but are inducible by a number of signals, including stress and cytokines (Jäättelä and Wissing 1992; Baniahmad and Tsai 1993).

Finally, HSPs are involved in the reproductive function. In point of fact, seminal fluid is rich in prostaglandins, polyamines, zinc, proteases and other enzymes that create stressing conditions, so that some HSPs are induced in endocervical cells in the female genital tract (Hang and Fox 1994; Al-Madhoun et al. 2007) (see also Sect. 5.8).

On the other hand, several proteins constitute the HSPs superfamily: the highly conserved HSP70 family (Hunt and Morimoto 1985), the conserved HSP 90 kDa (HSP90) family (Knoblauch and Garabedian 1999), the nucleolar HSP 110 kDa (HSPH1) (Subjeck et al. 1983), the mitochondrial HSPD1 (Jindal et al. 1989), the collagen-binding HSP 47 kDa (SERPINH1) (Hirayoshi et al. 1991), and the heterogeneous family of low molecular weight HSPs, ranging from 16 to 40 kDa (Miller et al. 2005; Sun and MacRae 2005; Al-Madhoun et al. 2007; Nakamoto and Vígh 2007; Shemetov et al. 2008).

Focusing on the effects of sperm on OEC gene expression, HSP 90 kDa alpha A.1 (HSP90AA1), heat shock cognate protein related 70 kDa (HSPA8) and glucose regulated protein 78 kDa (HSPA5) are constitutive and inducible HSPs. The role of HSPs in reproduction has been observed in steroid receptors, whose ligand binding domain includes a ligand recognition site and regions for receptor dimerisation, interaction with HSPs, nuclear localisation and ligand dependent transactivation.

Steroid hormones act by binding to their receptors, which regulate the transcription of the target genes in tissues responsive to these hormones (Smith and Toft 1993). These steroid receptors regulate a wide variety of physiological processes and their mode of action is well known: after hormone binding, they undergo a conformational change, translocating to the nucleus, where they modulate the transcription of target genes (Kakar et al. 2006; Grad and Picard 2007). Indeed, steroid receptors are transcription factors and their function is regulated by ligand binding (Havarinen et al. 2001). A large number of receptor-interacting proteins (co-activators and co-repressors) have been identified in several studies, suggesting that such cofactors act by mediating the regulation of transcription (Weigel 1996). The binding to ligands regulates their association and dissociation by modifying the structure of the receptors, and thereby affecting the surface properties of the receptor (Wurtz et al. 1996; Pratt and Toft 1997; Moras and Gronemeyer 1998; Torchia et al. 1998). In addition to these receptor-interacting proteins, several other proteins are associated with receptors in hypotonic cell extracts and in the reticulocyte lysate, forming an oligomeric complex (Dougherty et al. 1984; Johnson et al. 1994).

HSP90AA1 was the first identified receptor-associated protein (Dougherty et al. 1984), and it was initially suggested that it functioned as a repressor, because its association stopped DNA binding (Kost et al. 1989; Oñate et al. 1991). Later studies confirmed this repressing function on non-ligand binding domains, by showing that its deletion generated active receptors that did not form a stable oligomeric

complex with HSP90AA1 (Scherrer et al. 1993). Conversely, Lee et al. (1996) and White et al. (1997) established disagreement with the study of Scherrer et al. (1993) when they observed that even though the non-ligand binding domains of the oestrogen receptor did not interact with HSP90AA1, some mutants of oestrogen receptors did. The relevance of HSP90AA1 as a regulator of the activity of steroid receptors has been widely studied in several different experiments, for example using HSP90AA1-deficient yeast strains (Picard et al. 1990; Bohen and Yamamoto 1993), observing that ligand binding to a glucocorticoid receptor is compromised when HSP90AA1 is dissociated from the receptor complex (Bresnick et al. 1989).

Most receptor-associated proteins act as chaperones, as demonstrated by Inano et al. (1994) after they observed that HSP90AA1 can restore the DNA binding ability of partially denatured oestrogen receptors in vitro. However, attention must be paid as oligomeric complex formation in vitro does not correlate with the biological activity of HSP90AA1 in vivo (Dalman et al. 1991; Holley and Yamamoto 1995). Apart from HSP90AA1, other members of the HSPs, such as HSPA1A, FKBP4 (FK506 binding protein-59 kDa, also known as HSP56 or p59) and cyclophilin-related protein 40 kDa form complexes with non-liganded forms of steroid hormone receptors (Bagchi et al. 1991; Baniahmad and Tsai 1993; Kakar et al. 2006; Grad and Picard 2007).

Most nuclear proteins enter the nucleus via a process that requires energy and involves the recognition of specific sequences, known as nuclear localisation signals (NLS) (Richardson et al. 1988). Steroid receptors, which present multiple proto-signals for nuclear targeting (Ylikomi et al. 1992, 1998), shuttle between cytoplasm and the nucleus (Guiochon-Mantel et al. 1991, 1994; Perrot-Applanat et al. 1992). The mechanism of the nucleocytoplasmic shuttling of steroid receptors may be due to a distinct biological function of a nuclear compared with a cytoplasmic receptor (Verdi and Campagnoni 1990; Migliaccio et al. 1996), which seeks to use mechanisms that involve NLS instead of nuclear export signals (Tyagi et al. 1998; Weis 1998). Different experiments have shown that HSPs, such as receptor-associated proteins, are involved in the translocation of nuclear proteins. These experiments have dealt with different HSPs such as FKBP4 (Czar et al. 1995), HSPA1A (Jeoung et al. 1991; Imamoto et al. 1992; Yang and DeFranco 1994), and HSP90AA1 (Yang and DeFranco 1996).

It is interesting to note that in humans, the endometrium undergoes characteristic cycles of proliferation and secretory changes in response to steroid hormones, and has also been shown to express a molecular repertoire of proteins, including some HSPs such as heat shock 27 kDa protein 1 (HSPB1), HSPD1, HSPA1A, HSP90AA1, and alpha crystallin B chain (Koshiyama et al. 1995). The expression of HSPB1, HSPD1, and HSPA8 shows a peak after ovulation (Nip et al. 1994; Koshiyama et al. 1995) and the maximal expression of the alpha crystallin B chain is observed during the secretory phase. In view of the known functions of HSPs, these proteins appear to be involved in the protection of endometrial proteins against factors with the potential to lead to protein denaturation. Since tumour necrosis factor α (TNF-α) is a cytotoxic cytokine that is produced in progressive

amounts during the secretory phase, the function of the HSPs seems to be to protect the cells against the cytotoxic damage of TNF-α, especially during the implantation window (Tabibzadeh and Broome 1999).

Although different HSPs are expressed by the human endometrium, the expression of some is constant while for others it varies. For example, while the expressions of HSPA1A and HSP90AA1 show minimal changes during the menstrual cycle, the expression of HSPB1, HSPD1, and HSPA8 increases progressively during the late proliferative and early secretory phases and diminishes in the mid to late secretory and menstrual phases (Tabibzadeh et al. 1996). The expression of heat shock-related protein 20 kDa (HSPB6) is reduced during late pregnancy and labour in rats (Cross et al. 2007) and a reduction of HSP90AA1 in human decidua and placenta tissues is also observed during the course of normal gestation (Shah et al. 1998).

Additionally, with the exception of HSPA1A, which is found in the epithelial cells, HSPs are located in both the stroma and the epithelium. The HSPB1 has been found in the lymphoid aggregates within the endometrial stroma, and both HSPB1 and HSP90AA1 have been found in the endothelial cells. However, the expression of HSPA1A has also been found to be stronger in the epithelium than the stroma, whereas HSP90AA1 is present in both the epithelium and stroma (Tabibzadeh et al. 1996).

6.12.3.3 Spermatozoa Upregulate the Expression of HSPAA1, HSPA5 and HSP8 but not that of *Clusterin*

HSP90AA1

The HSP90 alpha A.1 (HSP90AA1) belongs to the HSP90 family, an abundant group of constitutively synthesised cytosolic proteins that are moderately stress-inducible (Jez et al. 2003; Neckers and Ivy 2003). As previously stated, HSP90AA1 interacts with steroid receptors (Catelli et al. 1999), actin, tubulin, and several protein kinases and prevents the aggregation of citrate synthase and casein kinase II (Tabibzadeh and Broome 1999). It plays a key role in the stabilisation and conformational maturation of many signalling proteins that are deregulated in cancer diseases (Kamal et al. 2004; Powers and Workman 2006).

This protein has also been related to reproductive functions, its expression being in the proliferative rather than in the secretory phase in the human endometrium (Komatsu et al. 1997). As far as spermatozoa are concerned, Huang et al. (1999) reported a decrease in HSP90AA1 before a reduction in sperm motility in boar sperm cooling. In addition, Ecroyd et al. (2003) showed that HSP90AA1 is involved in sperm capacitation both in humans and rats, while Hou et al. (2008) have shown that geldanamycin, an HSP90AA1-specific inhibitor, induces sperm capacitation in boars. All these data suggest that HSP90AA1 may be involved in sperm capacitation. It is worth bearing in mind that in pigs, as in other mammalian species, uncapacitated spermatozoa preferentially bind to OEC and then capacitate

quickly (Fazeli et al. 1999). Since sperm binding to LLC-PK1 cells does not induce sperm capacitation (Yeste et al. 2009a), and spermatozoa upregulate the expression of *HSP90AA1* in OEC (Fig. 6.10) more intensely than in LLC-PK1, we suggest that both phenomena could be related.

HSPA5 and HSP8

Both HSPA5 and HSPA8 belong to the HSP70 family, one of the most highly conserved HSP families that comprise several proteins, localised in distinct cellular compartments with overlapping and distinct functions (Hunt and Morimoto 1985; Daugaar et al. 2007), including HSPA1A (also known as HSP70-1), HSPA1B, glucose-regulated protein 78 kDa (HSPA5) and heat shock cognate protein 70 kDa (HSPA8), amongst others. Interestingly, HSPA1A is implicated in the mechanism of cell reaction to a variety of cytotoxic factors, and its protective function is related to its ability to promote the folding of nascent polypeptides and to remove denatured proteins (Guzhova and Margulis 2006). Regarding the reproductive function, Georgiou et al. (2005) showed that HSPA1A might have a beneficial effect on gamete development. These authors observed an up-regulated release in response to sperm in the oviduct that maintains the viability and function of spermatozoa. For this reason, HSPA1A not only acts as an inflammatory activator of innate and adaptive immunity (Javid et al. 2007) and as a cytoprotector in a variety of cell types, but is also involved in gamete function. Another member of this family, the heat shock 70 kDa protein 1B (HSPA1B, also known as HSP70-2) is down-regulated in semen from infertile men with idiopathic oligoteratozoospermia, suggesting that such anomalies of gene expression may be associated with some subtypes of male infertility (Cedenho et al. 2006).

Glucose-regulated protein 78 kDa, also known as immunoglobulin heavy chain-binding protein (BiP) or heat shock 70 kDa protein 5 (HSPA5), is the most abundant and the best characterised among glucose-regulated proteins (Munro and Pelham 1986; Lee 1992; Ma and Hendershot 2004). This protein is a major calcium-binding protein that resides in the endoplasmic reticulum (Macer and Koch 1988), where it functions as a molecular chaperone (Little et al. 1994) and in the translocation of nascent proteins across the membrane of endoplasmic reticulum (Kim et al. 1998). However, it is believed that HSPA5 does not only act as a molecular chaperone, assisting in the proper folding of *de novo* synthesised proteins, but also protects cells against a variety of physiological stresses. For this reason, HSPA5 can be induced in response to a multitude of stimuli including the interruption of proper protein folding, transport, or processing, the deprivation of glucose and oxygen, and the perturbation of intracellular Ca^{2+} stores (Little et al. 1994). In relation to this feature and using transgenic mice, Mao et al. (2006) demonstrated that the HSPA5 promoter is highly activated not only in early embryonic development but also as a consequence of others factors, such as low glucose levels, acid pH or hypoxia. All of these factors are known to create a microenvironment in which tumours grow (Jamora et al. 1996). Following this, a large

number of studies have demonstrated a correlation between induced expression of HSPA5 and resistance to apoptotic death in somatic cells, particularly in progressively growing tumours (Reddy et al. 2003; Miyake et al. 2000). The induction of HSPA5 has widely been used as an indicator of endoplasmic reticulum stress and the start of unfolded protein response, a defence mechanism that cells have developed and conserved (Mao et al. 2006).

HSPA5 is present in the uterus and the oviduct and it seems that it is required for the efficient biosynthesis and secretion of proteins related to the onset of uterine sensitisation in rats (Simmons and Kennedy 2000). Boilard et al. (2004) identified HSPA5 as one of the six major proteins in APM extracts isolated from bovine OEC. According to these authors, this protein is located in the luminal/apical surface of bovine OEC and binds to spermatozoa. However, even though a beneficial effect of oviductal-APM proteins has been observed on the maintenance of sperm viability and integrity, and HSPA5 forms part of this protein extract, its exact role on the maintenance of sperm function still remains unknown. Thus, even though incubation with exogenous HSPA5 does not affect sperm viability, motility or acrosomal integrity, this protein has been reported to be involved in the capacitation of human spermatozoa (Lachance et al. 2007). In addition, HSPA5 appears to perform an important function in the process of spermatogenesis, as a differential expression of this protein was observed in the physiological process of spermatogenesis (Huo et al. 2004).

When spermatozoa and OEC are in vitro co-cultured, *HSPA5* is also specifically up-regulated in OEC rather than LLC-PK1 cells in a similar fashion to *HSP90AA1* and only when spermatozoa directly interact with these epithelial cells (Yeste et al. 2008, 2009b) (Fig. 6.11). This suggests that HSPA5 could play a role in the physiological processes occurring within the oviduct (Yeste 2008).

HSPA8 (or HSC70) is the constitutive form of HSPA1A and is localised in the nuclei and the cytosol of cells. However, in most animal species, except in primates, the most inducible members of the family of HSP70 proteins are not found in the cytosol or in the nuclei of cells (Shaner and Morano 2007).

HSPA8 is also up-regulated-expressed in OEC in response to sperm (Yeste et al. 2008, 2009b) (Fig. 6.12) but not to the same extent as inducible HSPs (HSP90AA1 and HSPA5). This suggests that this gene is involved in reproductive processes and its upregulation is different from that of inducible HSPs (HSP90AA1 and HSPA5), even though more experiments concerning proteomics should further be addressed, since gene expression analyses are not sufficient to explain changes in protein expression patterns. In fact, it is interesting to note that Elliot et al. (2009) in porcine, and Lloyd et al. (2012) in ovine species, have found that this protein is the sAPM-protein that seems to exert the survival-maintaining effect observed in co-incubation of spermatozoa with apical plasma preparations.

Unfortunately, there are few reports studying this protein in the oviduct, the inducible form (HSPA1A) being the most documented in mammalian reproduction. Accordingly, spermatozoa induce the expression of HSPA1A in co-culture with a cultured human cervical cell line (HeLa cells) or with cells from the endocervicals of sexually active women (Jeremias et al. 1997). Nevertheless, in

other reports studying both HSPA8 and HSPA1A in the human endometrium, it has been observed that the changes in the expression of both proteins are different. Thus, whereas the levels of HSPA8 vary, increasing progressively during the late proliferative and early secretory phases and decreasing in the mid- to late secretory and menstrual phases, those of HSPA1A remain constant (Tabibzadeh and Broome 1999).

Clusterin

CLU is a multifunctional glycoprotein protein widely expressed in a variety of tissues undergoing apoptosis. It is a constituent of cell-adhesion complexes and is involved in the interactions between cell and cell, and cell and extracellular matrices (Lemansky et al. 1999; Jones and Jomary 2002; Wang et al. 2007).

Although its role in reproduction is not yet clear, it has been localised in human spermatozoa and in the male reproductive tract (Atlas-White et al. 2000; Plotton et al. 2006), and is also expressed by accessory sex glands in bulls (Moura et al. 2007). Also in bulls, Ibrahim et al. (2000) found that clusterin-positive spermatozoa sorting is a better way to predict fertility than sperm motility and morphology, and that in swine, CLU gene expression increases when luteolysis is induced (Forni et al. 2003). Han et al. (2007) have shown that styrene, which is a chemical product, may produce infertility in male rats by repressing the expression of CLU in the testis and by inducing the expression of another five genes (testis-specific expressed gene 101, protein kinase C, H^+-ATPase isoform 2, peroxiredoxin 1, and aquaporin 9). The infertility provoked by styrene could be due, therefore, to the suppression of CLU gene expression.

In contrast, our *in vitro* co-culturing experiments of spermatozoa with OEC and LLC-PK1 showed no effect of sperm presence on the expression of *CLU*, both when spermatozoa bound directly, or did not bind to the epithelial cells (Yeste et al. 2009b).

6.12.4 Observations Using Surgical Models

Studies using the pig as a surgical animal model (Georgiou et al. 2007) demonstrated that both spermatozoa and oocytes are able to modulate the gene expression of the oviduct around the time of natural mating, thereby changing the secretory proteome and thus the composition of oviductal fluid. Although a high variation in the composition of oviductal fluid exists among sows, and this may be explained by genetic differences among individuals (inter- or within-breeds), the presence of gametes has been reported to alter the expression profile of thirty-two proteins. To conclude this, a study performed by Fazeli's group compared the composition of oviductal fluid from inseminated and non-inseminated sows, submitted to the same hormonal influence (Georgiou et al. 2007).

From the overall total of 32 proteins, twenty appeared to be only regulated by the presence of spermatozoa, one protein was regulated only by oocytes (Ig kappa light chain, whose function in reproduction still remains unknown), and three proteins were regulated by both sperm and oocytes. Moreover, proteins regulated by the presence of gametes have been grouped into six categories depending on their reproductive function. Thus, Georgiou et al. (2005) distinguish the following categories:

1. Proteins influencing sperm function, like fibrinogen.
2. Proteins influencing oocyte function, like alpha-2-HS-glycoprotein.
3. Proteins involved in the fertilisation process, like oviductin (OSP/OVGP1) and alpha-1 acid glycoprotein, which is up-regulated by sperm and has been reported to influence sperm-ZP binding (Kratz et al. 2003) (see also Sect. 8.6.4).
4. Proteins influencing the establishment of pregnancy, such as OVGP1 and complement component C3.
5. Proteins associated with immune response, such as complement component C3.
6. Proteins with an unknown function. In this category are proteins up-regulated by spermatozoa, such as Ig gamma 2a and 2b chains, and others up-regulated by both sperm and oocytes, like Ig kappa variable region.

On the other hand, from these 32 proteins, spermatozoa up-regulate the expression of:

(a) Oviductin (OSP/OVGP1; see also Sect. 6.5 about oviductal fluid and Sect. 8.6.4 about ZP-modifications during the oviductal transit), which maintains sperm motility and survival (Kouba et al. 2000) and increases fertilisation rates and embryo development after IVF (McCauley et al. 2003).
(b) A retinol-binding protein that has been reported to be involved in retinol delivery to spermatozoa (Sundaram et al. 1998), oocytes (Brown et al. 2003) and embryos (Harney et al. 1994).
(c) Fibrinogen A α-chain, which attaches to sperm membrane and seems to confer resistance against phagocytosis (Reyes-Moreno et al. 2002).
(d) Complement component C3, which appears to be involved in removing non-viable spermatozoa (Riley-Vargas et al. 2005), sperm-oocyte binding (Anderson et al. 1993) and early embryonic development (Lee et al. 2004).

As for the three proteins up-regulated by the presence of both spermatozoa and oocytes, Georgiou et al. (2007) identified complement component C3, Ig kappa variable region and haemoglobin beta chain, which in vitro acts as a nitric oxide scavenger thereby improving fertilisation rates (Dinara et al. 2001) and embryo development (Lim and Hansel 1998). It is interesting to mention that complement component C3 is up-regulated by the presence of spermatozoa but down-regulated by the presence of oocytes. According to Georgiou et al. (2007), this observation suggests complement component C3 is needed before fertilisation, which is why spermatozoa would stimulate its release into the oviductal fluid, but at the same

time, its levels have to be low after fertilisation, which is why oocytes and early embryos appear to down-regulate it. This hypothesis is in agreement with levels of complement component C3 within oviductal fluid, which are highest at oestrus (time of mating/copulation), but decrease and are undetectable during the first days of pre-implantation embryonic development (Buhi and Alvarez 2003).

Finally, the up-regulation of some immunoglobulins in response to gametes raises serious doubts about their actual function within the oviductal environment. Thus, Georgiou et al. (2007) have speculated that these proteins could be involved in other functions related to reproductive processes rather than in the immunological response (see also Sect. 5.8 about Reproductive Immunology in sows). According to these authors, this is especially relevant in the case of oocytes because, since they belong to the female organism, it does not make sense to suppose they elicit a humoral response.

6.12.5 An Overall View of Spermatozoa as Modulators of Oviductal Gene Expression

Oviductal monolayers alter their transcriptome in response to the presence of gametes. Interestingly, up-regulation of gene expression in response to the presence of spermatozoa is not restricted to mammals, but it has also been observed in sperm storage tubules of turkeys (Long et al. 2003), where 214 novel gene sequences have been reported to be up-regulated by their presence.

By analysing both the transcriptome and proteome in in vivo (Fazeli et al. 2004; Bauersachs et al. 2003; Lee et al. 2002) and in vitro studies (Yeste et al. 2008, 2009b), as well as the composition of the oviductal fluid (Georgiou et al. 2005, 2007), oviductal responses to the presence of gametes can be regarded as a favourable response for maintaining their viability and facilitating their function (Figs. 6.10, 6.11, 6.12). This is quite clear when observing that HSPA8, a highly conserved protein present in the OEC that maintains sperm function and survival in porcine (Elliott et al. 2009) and ovine species (Lloyd et al. 2009, 2012), is up-regulated in response to the presence of sperm (Yeste et al. 2008, 2009b) (Fig. 6.12). This suggests that gametes are thus able to regulate the oviductal environment and to coordinate the key reproductive events preceding and during fertilisation and early embryonic development (Georgiou et al. 2007), by regulating the expression of both intracellular and the released proteins in OEC. Furthermore, the relevance of HSPs in the maintenance of sperm survival within the oviduct has been widely reported in porcine, bovine and ovine species (Boilard et al. 2004; Lachance et al. 2007; Elliott et al. 2009; Lloyd et al. 2009, 2012). According to Holt and Lloyd (2010), since these proteins are highly evolutionarily conserved, they may play a crucial role in sperm storage mechanisms through species.

Despite the fact that up-regulation of HSPs has been observed in co-culture experiments with oviductal monolayers that are not under hormonal influence, it must be noted that direct OEC-sperm contact is needed to observe the alteration

of HSPs levels in OEC (Yeste et al. 2009b). This data, together with the fact that the arrival of gametes in the oviduct triggers cell type-specific signalling pathways that alter the proteome of oviductal cells, would suggest that a mechanism of the female reproductive tract exists that is triggered by the presence of gametes (Georgiou et al. 2007). Therefore, it seems quite clear that not only hormones but also gametes modulate and regulate the oviductal function. In addition, although it may be likely that not all oviductal proteins require direct binding between sperm and oviductal epithelium, up-regulating the expression of a protein related to the maintenance of sperm viability like HSPA8 needs this direct contact, in a similar fashion to what occurs in the case of sperm reservoir in vivo.

6.13 Conclusions

Uncapacitated boar spermatozoa traverse the uterine body and cornuae of the sow after copulation or insemination and enter the UTJ and oviductal isthmus, where they are trapped by the OEC forming the sperm reservoir. Sperm-oviduct interaction is mediated by carbohydrate recognition mechanisms, the flexible high-mannose type glycans exposed by oviductal glycoproteins being recognised by the sperm surface-associated proteins AQN-1 and DQH. The Sperm reservoir maintains sperm viability and fertilising ability and keeps spermatozoa uncapacitated while they remain attached to OEC. When ovulation occurs, spermatozoa are released from the oviductal epithelium, quickly capacitate and move towards the ampullary-isthmic region, the venue of fertilisation. However, although it is widely known that the oviductal environment modulates sperm function during their stay in the oviduct, more recent studies have demonstrated that the presence of gametes (spermatozoa and oocytes) also intervenes in the modulation of the oviductal environment.

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Chapter 7
Boar Spermatozoa Within the Oviductal Environment (II): Sperm Capacitation

Marc Yeste

Abstract Around ovulation, a set of changes leads to a destabilisation of the sperm membrane that results in the release of spermatozoa from the oviductal reservoir (see Chap. 6). This destabilisation of the sperm membrane is an early step of the capacitation process, is mediated by bicarbonate, and allows AQN-1 to be shed from the surface. After being released from sperm reservoir, spermatozoa freely swim from the isthmus towards the ampulla/ampullary–isthmic junction where, amongst others, the following crucial and sequential events take place: (1) completion of sperm capacitation, (2) binding of spermatozoa to the ZP of the oocyte, (3) acrosome exocytosis and (4) further membrane fusion. The present chapter deals with the first issue, and thus focuses on the changes that the spermatozoon undergoes during capacitation. These changes, which can be separated between early/fast and late/slow, entail the activation of several signalling pathways, the increase of certain intracellular messengers, such as Ca^{2+} and cAMP, the reorganisation of proteins and lipids of sperm plasmalemma, and changes in motility patterns. Finally, destabilisation of the acrosomal sperm head membrane increases the sperm's ability to bind the zona pellucida of the oocyte.

7.1 Introduction

Uncapacitated and freshly ejaculated spermatozoa are not able to interact with ZP and fuse with the oocyte, but they need to undergo a prior priming sequence of events (Visconti 2009). This physiological process, known as sperm

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capacitation, was first discovered in 1951 in independent observations that Austin and Chang made in rats and rabbits, respectively (Austin 1951; Chang 1951, 1959). Indeed, both authors found that spermatozoa must reside in the female reproductive tract for a period of time prior to gaining fertilising ability (Signorelli et al. 2012).

We can define sperm capacitation as a complex and lengthy physiological process that involves a combination of sequential and parallel molecular changes that affect both the sperm head and the sperm tail (Suarez 2007). This process allows spermatozoa to acquire the ability to fertilise the oocyte, and mainly takes place when the spermatozoa are in the vicinity of the oocytes at the ampullary–isthmic junction (Rodríguez-Martínez et al. 2005; Rodríguez-Martínez 2007; Tulsiani et al. 2007). In fact, there are three sequential events that take place while the spermatozoa become capacitating/capacitated (Sutovsky 2009, 2011). All these events occur within the oviduct:

1. Detachment from the oviductal sperm reservoir (see also Chap. 6),
2. Remodelling of the acrosome surface in a required step prior to acrosome exocytosis mediated by ZP, and
3. Priming of sperm membranes for fusion with the oolemma.

It is worth noting that the ability of spermatozoa to bind to intact-ZP, only lasts for a relatively short period (from hours to days) and this depends on the species. In addition, the time required for spermatozoa to 'switch-on' and become fertile is species specific, being shorter for some species and longer for others (Fraser 2010). In the case of boar, capacitation of spermatozoa takes about 2 h on average to be completed (Botto et al. 2010). Some authors believe that events taking place immediately after ejaculation and before reaching the oviduct should also be considered as part of sperm capacitation, since they are required steps prior to the events occurring within the oviduct (Visconti 2009). This aspect will be taken up again when dealing with fast capacitation events.

Finally, we must mention that even though many biochemical and cell biological indicators of capacitation have been described, the sequence of steps to achieve full capacitation (i.e. the ability to interact immediately with the oocyte) has yet to be defined (Visconti 2009; Fraser 2010). However, it is quite evident that the ability of spermatozoa to bind ZP involves considerable remodelling of sperm plasmalemma, since, as mentioned before, uncapacitated spermatozoa are not able to recognise the oocyte and do not show innate fusibility.

7.2 A General Overview of the Changes that Sperm Undergo During Capacitation

During capacitation, spermatozoa undergo a wide array of changes and at the end of this process, i.e. when a spermatozoon is fully capacitated, the apical plasma membrane of the sperm head has become fusogenic (Harrison 1996, 2004).

Summarising, the most important are (Töpfer-Petersen et al. 2002; Tardif et al. 2003):

– First remodelling of plasma membrane architecture and cholesterol-efflux that increases membrane fluidity and provokes lipid scrambling and increases membrane fluidity (Harrison et al. 1996; Gadella and Harrison 2002; Harrison and Gadella 2005).
– Up-regulation of cellular signalling pathways (Kalab et al. 1998).
– Changes in sperm motility and kinematic parameters and changes in flagellar activity (Cancel et al. 2000; García-Herreros et al. 2005).
– Removal of AQN-1 from the sperm surface (Calvete et al. 1997; Töpfer-Petersen et al. 2008).
– Changes in permeability to extracellular calcium and increases in intracellular concentrations of this cation (Adeoya-Osiguwa and Fraser 2003).
– Phosphorylation of tyrosine residues of sperm head proteins (Petrunkina et al. 2004).
– A second reorganisation of membrane lipids and proteins, leading to sperm plasma and the outer acrosome membranes become fusogenic (Flesch and Gadella 2000; Tsai et al. 2007, 2010).

In short, the most significant changes during sperm capacitation entail the activation of several signalling pathways, such as sperm-specific adenylyl cyclase cAMP-dependent protein kinase A (PKA) and the reorganisation of proteins and lipids on the plasma membrane (Gadella et al. 2008). This change is facilitated by the removal of steroids (e.g. cholesterol) by acceptor proteins (such as BSA) and results in a more fluid membrane with an increased permeability to Ca^{2+}. This calcium influx produces, in turn, increased intracellular cAMP levels and, thus, an increase in motility (motility activation during fast and further hyperactivation during the slow capacitation events) (Baldi et al. 1996; Visconti et al. 1999a).

During the early stages of sperm capacitation, AQN-1, a seminal plasma protein that non-covalently attaches to the sperm membrane, is removed (Sanz et al. 1993; Dostàlovà et al. 1994; Calvete et al. 1997), thereby allowing AWN, AQN-3 and P47/SED1 to be accessible and able to interact with ZP-glycans (Flesch et al. 2001a).

Finally, when spermatozoa are fully capacitated, there is a destabilisation of the acrosomal sperm head membrane that allows greater binding ability of sperm to bind the zona pellucida of the oocyte (Tsai et al. 2007).

Some authors (Salicioni et al. 2007; Visconti 2009) have divided capacitation into two different types of events to facilitate consideration of the complex cascade of molecular events that occurs during this process (Fig. 7.1):

- Fast and early events, which comprise activation of sperm motility and start as soon as the sperm leave the epididymis, and
- Slow and late events that comprise changes in the pattern of movement (hyperactivation), the ability to carry out acrosome reaction stimulated by a physiological agonist and the phosphorylation of tyrosine in proteins (Baldi et al. 1996; Visconti et al. 1999a). These events take place within the oviduct.

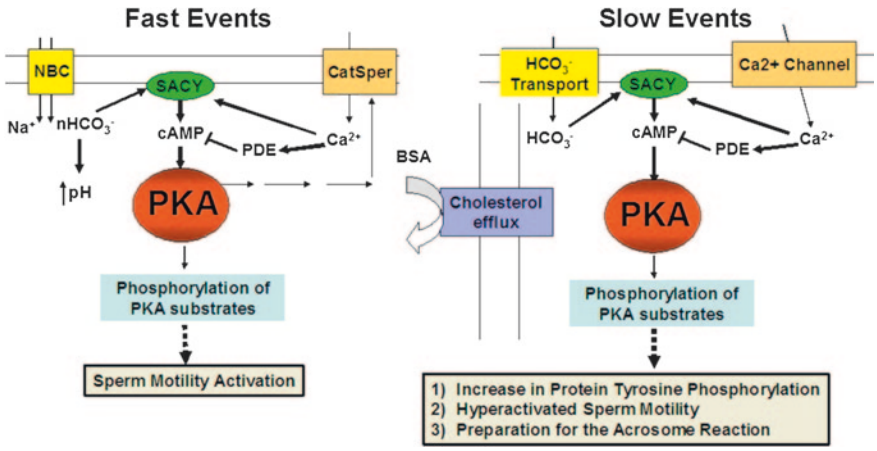

Fig. 7.1 Molecular basis of fast and slow events associated with sperm capacitation. In fast events (*left*), bicarbonate and Ca^{2+} stimulate sperm motility, through PKA and sACY. According to Visconti (2009), bicarbonate and Ca^{2+} are transported by a Na^{+}/HCO_3^{-} co-transporter (NBC) and a sperm-specific Ca^{2+} channel (CatSper). In slow events (*right*), spermatozoa acquire the ability to fertilise the egg. There are increases in tyrosine phosphorylation, sperm present hyperactivated motility and prepare to undergo the acrosome exocytosis (Visconti 2009; Reproduced with permission)

7.3 The Relevance of In Vitro Studies on Sperm Capacitation

According to Rodríguez-Martínez (2007), one inconvenience of investigating sperm capacitation is the difficulty in confirming in vitro experiments in in vivo conditions. In fact, it is quite difficult to study the intraluminal set of environments in the oviduct without disrupting the homoeostasis of this organ.

In recent years, however, many efforts have been made to study the events related to sperm capacitation. The set of molecular changes that occur during sperm capacitation as well as their regulation have been largely studied in in vitro conditions. This has provided relevant data about the pathways involved in this process, but with the inconvenience of lacking in vivo studies.

Thus, most of the studies developed for understanding which molecular mechanisms govern sperm capacitation have been performed using in vitro conditions. Before performing these in vitro experiments, seminal plasma proteins binding spermatozoa have to be eliminated, and this can be accomplished in the laboratory by density-gradient centrifugation of spermatozoa (Petrunkina et al. 2003; Yeste et al. 2009) or by washing and centrifugation of the sperm suspension (Fazeli et al. 1999). After washing, spermatozoa are in vitro capacitated by incubating in bicarbonate-enriched media (Holt and Harrison 2002; Puigmulé et al. 2011; Ramió-Lluch et al. 2012) (Fig. 7.2) which stimulates a series of downstream events (Gadella and Van Gestel 2004), including the stimulation of a special form of soluble adenylyl cyclase (sACY) (Litvin et al. 2003), which increases the intracellular

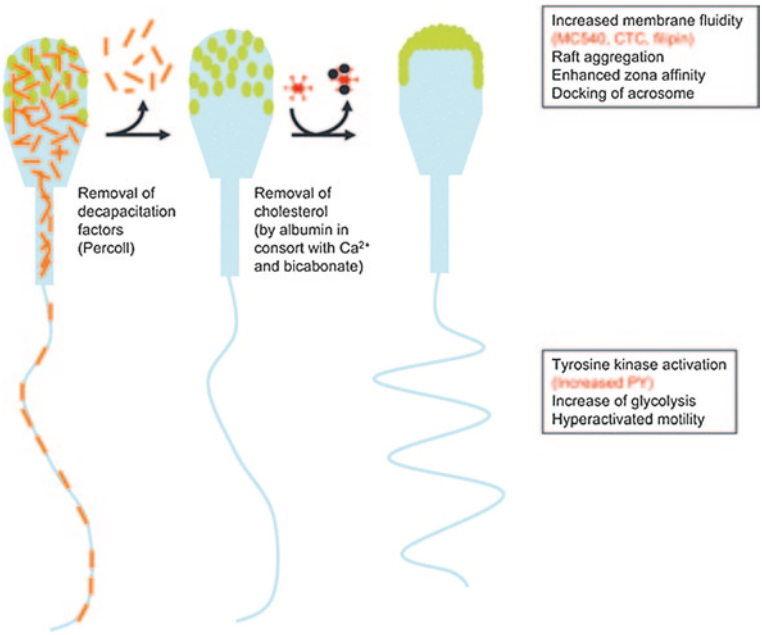

Fig. 7.2 Surface alterations during in vitro capacitation of boar spermatozoa. The decapacitation factors (in *orange*), originating from seminal plasma and adsorbed to the sperm surface during ejaculation, are removed by washing spermatozoa over a Percoll gradient. Subsequent incubation of spermatozoa in Tyrode's medium containing bicarbonate, fat-free albumin (in *red*) and Ca^{2+} cause the lateral redistribution, and partial removal, of cholesterol (in *black*). Consequently, lipid ordered domains (in *green*) aggregate into the apical ridge area of the sperm head. This allows the formation of a zona binding protein complex as well as the docking of the acrosome to the sperm head surface (Leahy and Gadella 2011; Reproduced with permission)

levels of cAMP and subsequently activates a signalling pathway dependent on PKA (Visconti et al. 1999a; Harrison 2004). Finally, in vitro capacitated spermatozoa are able to penetrate the cumulus layers and bind to ZP proteins.

Therefore, it is important to bear in mind that most of the data presented in this chapter about the molecular changes related to sperm capacitation have been obtained by conducting in vitro studies. This is undoubtedly a good approach to understand what happens in vivo, but can underestimate some important mechanisms of regulation. For this reason, further sections about the environmental conditions of the oviduct are expected to compensate this bias.

7.4 Role of the Oviduct in Sperm Capacitation

7.4.1 Lessons from In Vitro Studies

Direct contact of spermatozoa with oviductal epithelial cells (OEC), with oviductal explants, or with apical plasma membrane fractions, seem to delay rather than

promote sperm capacitation in porcine (Fazeli et al. 1999; Yeste et al. 2009), and other mammalian species, such as humans (Murray and Smith 1997), rabbits (Smith and Nothnick 1997) and sheep (Lloyd et al. 2009), thereby prolonging sperm function and survival (Rodríguez-Martínez et al. 2005). The viability-prolonging and capacitation-delaying effects of sperm are even observed when the male gametes are co-incubated with proteins isolated from apical plasma membranes (Satake et al. 2006), where HSPA8 plays a main role (Elliott et al. 2009; Lloyd et al. 2009, 2012). Indeed, although sperm motility is stimulated when uncapacitated boar spermatozoa are incubated with bicarbonate (Holt and Harrison 2002), the response to bicarbonate is reduced and the motility pattern of individual tracks is modified when these uncapacitated spermatozoa are co-incubated with apical plasma membranes (Satake et al. 2006). As will be further discussed, bicarbonate appears to be the key effector of sperm capacitation, and changes in the motility patterns are one of the features of this physiological process (Cancel et al. 2000). Thus, the non-response of spermatozoa to bicarbonate in terms of sperm motility clearly indicates that these apical plasma fractions delay sperm capacitation.

On the other hand, as widely reported in (Chap. 6), in in vitro co-culture, uncapacitated spermatozoa preferentially bind to OEC and then seem to capacitate quickly. In contrast, capacitation is not induced when uncapacitated sperm attach to non-reproductive cells (LLC-PK1) also in in vitro co-culture conditions (Fazeli et al. 1999; Yeste et al. 2009; see also Chap. 6).

All these data are not contradictory but need a careful explanation. In fact, it is worth noting that the storing function of sperm reservoir seems to be regulated by a concerted mechanism that is species-specific, since homologous co-culture is better than heterologous, and sperm-OEC binding is mediated by species-specific ligands (Pollard et al. 1991; Suarez et al. 1991; Suarez 2007).

7.4.2 Modulation of Sperm Capacitation Within the Oviduct

From the deposition site and upon their arrival at the ampullary–isthmic junction, where fertilisation takes place, spermatozoa are in contact with different environments within the female reproductive tract, spending a short time in the cervix and the uterus and staying longer in the oviduct (Rodríguez-Martínez 2007). In this context, the oviduct plays a relevant role in the modulation of sperm capacitation, in a process that occurs during sequential exposure of male gametes to these different female compartments (Yanagimachi 1994), and is actively and progressively coordinated in relation to ovulation (Hunter and Rodríguez-Martínez 2004; Rodríguez-Martínez 2007).

As discussed in the previous chapter, the oviduct provides a suitable environment for sperm transport, storage and capacitation, for oocyte collection, transport and maturation, for fertilisation and early embryo development; phenomena that occur during oestrus and metaoestrus (Rodríguez-Martínez 2007). Related to this, it is worth noting that the intraluminal fluid from the oviduct has been reported to

be involved in boar sperm capacitation (Rodríguez-Martínez et al. 2001; Tienthai et al. 2004), mediating the start of this process (Rodríguez-Martínez 2007).

As mentioned before, capacitation and molecular-related events have been hitherto investigated in in vitro conditions, with the inconvenience of having to confirm whether all these observations also take place in vivo (Rodríguez-Martínez 2007). One of the main problems that arises is trying to study intraluminal sets of environments without disrupting the homoeostasis of the organ. Thus, although the surface of ejaculated spermatozoa is known to be covered by spermadhesines and is accessible to lipid-binding components of uterine and oviductal fluids after mating and the sequence of capacitation-like changes has been largely addressed (Tardif et al. 2003; see Sects. 7.6 and 7.7), less is known about the modulation of sperm capacitation within the oviductal environment (Rodríguez-Martínez 2007).

Early studies showed that spermatozoa capacitate faster when they are exposed to the oviducts than when they are exposed to uterus and oviducts together (Hunter and Hall 1974). Under surgical conditions in pigs, spermatozoa are capacitated faster when they are first exposed to the caudal isthmus and then exposed to the cranial ampulla, than when they are only exposed to the latter (Hunter et al. 1998; Hunter and Rodríguez-Martínez 2004). In cattle, oviductal fluid from the isthmus has a greater ability to capacitate bull spermatozoa than the fluid collected from the ampulla (Killian 2004). These differences in time needed for capacitating male gametes could be related to the ability of the environment to clean the surface of the spermatozoa, so that the uterine and oviductal fluids modulate the velocity of sperm capacitation and allow the sequence of related events (Rodríguez-Martínez 2007).

During the pre-ovulation period, capacitation is not promoted by the sperm reservoir and spermatozoa remain attached (Hunter and Rodríguez-Martínez 2004; Rodríguez-Martínez et al. 2005). Around ovulation, spermatozoa are gradually released from the sperm reservoir (located at the isthmus) and go towards the venue of fertilisation, i.e. the ampullary–isthmic junction. However, it is worth to mention that the proportion of sperm leaving the isthmic sperm reservoir is always very small, probably only 5 % of them at most (Rodríguez-Martínez 2007). This finding suggests that capacitation events may occur at different times over several hours (see also Sect. 6.10).

Currently, the releasing mechanisms from sperm reservoir are not clear enough, as described in Chap. 6. Thus, on one hand, some authors have reported that hyaluronan (HA) could be involved in the detachment of spermatozoa from the sperm reservoir, by inhibiting the interaction between the spermadhesins covering the sperm surface and the oviductal cells (Liberda et al. 2006). On the other hand, it has also been hypothesised that release of spermatozoa from the sperm reservoir could be due to the capacitation-related changes themselves, which entail some proteins being removed. These changes would, in turn, involve a loss of binding sites in the sperm surface and thus diminish the affinity of spermatozoa to the epithelium (Fazeli et al. 1999). This would be supported by in vitro observations, since, as stated, spermatozoa bound to OEC capacitate sooner than spermatozoa bound to non-reproductive cells (LLC-PK1) (Yeste et al. 2009). Unfortunately, it is still not clear enough when capacitation starts and how spermatozoa are released from the reservoir.

To explain the modulating role of the oviduct on sperm capacitation, there is another interesting concept that we should bear in mind; that is, how sperm hyperactivation occurs in pigs. Sperm hyperactivation is defined as a late capacitation state in which spermatozoa exhibit vigorous and high amplitude flagellar beating. This phenomenon, which has been reported to occur in the oviduct of rodents and also during in vitro co-incubations of spermatozoa with oviductal explants in porcine and cattle, has been hypothesised as a putative mechanism by which mammalian spermatozoa detach from the reservoir (Suarez et al. 1992; Suarez 2007). However, no evidence has yet been reported that sperm hyperactivation also occurs in vivo in porcine (Rodríguez-Martínez 2007). One should note that in our description about molecular changes during late capacitation events (see Sect. 7.7), we repeatedly mention the term 'sperm hyperactivation'. This is due to the fact that the available knowledge about these changes mainly comes from in vitro studies, and care must thus be taken when trying to extrapolate this to what happens in vivo.

Considering all these aspects, Rodríguez-Martínez (2007) has proposed that the release of uncapacitated spermatozoa from the sperm reservoir is a constant and progressive process that occurs over a long time period, from pre- to post-ovulation, rather than massively at the moment of ovulation. This hypothesis is supported by other findings demonstrating that uncapacitated spermatozoa recovered from the seminal reservoir of inseminated sows can be in vitro capacitated by incubation in a medium containing bicarbonate, a capacitation effector, at concentrations similar to those recorded in vivo in the ampullary–isthmic junction and in the ampulla during the peri-ovulatory period (Tienthai et al. 2004).

On the other hand, the modulating function of the oviduct on sperm capacitation in vivo seems to be related to regional differences throughout the oviduct, apart from reported differences in the ovarian state. Indeed, as stated in Chap. 6, the oviduct can be anatomically divided into different parts. The first part of the oviduct forms the sperm reservoir, which involves the adhesion of spermatozoa to OEC and provides a safe environment for spermatozoa. Here, a portion of the inseminated spermatozoa undergoes a period of storage within a restricted tubal segment. The period of this storage time, when sperm cells are in a quiescent state within a restricted segment of the female oviduct, lasts from hours to days (Rodríguez-Martínez 2007).

The second part, the ampulla, would be the place where capacitation takes place. The suggestion of a regional difference in sperm capacitation through segments is supported by in vivo observations, since boar spermatozoa having capacitation-like motility (increase of VCL, decrease of LIN, etc.) are only retrieved from the ampulla (Suarez et al. 1992). In addition, the influence of the oviductal region on sperm capacitation is also supported by other studies conducted with oviductal fluid, since the peri-ovulatory fluid collected at the ampullary–isthmic junction increases the number of ejaculated spermatozoa showing hyperactivation-like motility (Nichol et al. 1997).

Finally, Fraser (2010) has recently suggested that oviductal fluid contains specific regulatory ligands, like adenosine, calcitonin and fertilising promoting peptide (FPP), which can accelerate sperm capacitation after release from the oviductal reservoir. This aspect will be extensively discussed in Sect. 7.11.

7.5 Effectors of Sperm Capacitation

7.5.1 Introduction: Relevant Molecules for Sperm Capacitation

The literature has provided extensive evidence that bicarbonate, calcium, and serum albumin (BSA) are molecules that are present in the female reproductive tract and play a critical role during sperm capacitation (Yanagimachi 1994; de Lamirande et al. 1997; Fraser 2010). These three molecules promote cytoplasmic and plasma membrane changes in spermatozoa, which must occur before cells can complete capacitation, undergo acrosome reaction, and fertilise the oocyte.

The mechanisms by which these molecules are able to promote capacitation at the molecular level, by modulating adenylyl-cyclases and cAMP levels, plasma membrane architecture, and protein phosphorylation (Fraser 2010), have been an active area of research in recent years (Salicioni et al. 2007).

7.5.2 The Crucial Role of Bicarbonate in In Vivo Capacitation

As explained above, most of the capacitation-related events have been observed in in vitro experiments, by using media containing higher concentrations of bicarbonate (Holt and Harrison 2002; Gadella and Van Gestel 2004). Although bicarbonate appears to be a key effector in these in vitro experiments, it is not known exactly whether it is the sole effector.

For the time being, bicarbonate has been considered to be the effector, or at least one of the key effectors, of sperm capacitation in porcine, bovine and equine species (Harrison and Gadella 2005; Tienthai et al. 2004; Rodríguez-Martínez 2007) within the oviductal environment. This is consistent with the variations of bicarbonate levels along the oviductal segments towards the site of fertilisation, and with the relationship between boar sperm motility and bicarbonate concentrations. In epididymal cauda, for example, spermatozoa are in a quiescent state because of lower levels of bicarbonate and pH when compared to ejaculated spermatozoa (Rodríguez-Martínez et al. 1990). In the case of the oviduct, bicarbonate levels are lower in the caudal isthmus than in the ampulla–isthmic and ampulla regions (Rodríguez-Martínez 2007), and also vary during the oestrous cycle. In this regard, this concentration could be optimal around ovulation, since, as mentioned, oviductal fluid collected during the peri-ovulatory stage elicits sperm capacitation (Rodríguez-Martínez et al. 2001).

Therefore, all these data support the role of bicarbonate in triggering the capacitation of boar spermatozoa in vivo (Tienthai et al. 2004). Indeed, according to this hypothesis, sperm capacitation would not be induced in the sperm reservoir because of the low levels of bicarbonate that are non-capacitating, while capacitation would occur at the ampullary–isthmic junction where the levels of

bicarbonate are higher. Thus, the progression of a spermatozoon through the oviduct is enough to elicit its capacitation, when it does not take place in the sperm reservoir, and when the concentration of bicarbonate in the oviductal fluid is adequate (Rodríguez-Martínez et al. 2005).

This hypothesis also suggests that, at least in vivo, the release from sperm reservoir is gradual and might occur prior to eliciting sperm capacitation-related events (Rodríguez-Martínez 2007). Therefore, if we assume that bicarbonate is the key effector of sperm capacitation and that the sole progression of spermatozoa through the oviduct leads to their capacitation, and if we bear in mind that spermatozoa die when they do not encounter any oocyte, it is likely that the gradual release of spermatozoa from the sperm reservoir acts as a mechanism to warrant the availability of capacitated spermatozoa for the long interval lasting from sperm deposition to ovulation (Rodríguez-Martínez et al. 2005).

Although the molecular changes during sperm capacitation will be taken up again in further specific sections (Sects. 7.6 and 7.7), we briefly describe now, to complement this section, the changes produced on sperm by the presence of bicarbonate. As such, bicarbonate induces the remodelling of the sperm plasma membrane by firstly increasing its fluidity (Harrison and Miller 2000; Rodríguez-Martínez 2007; Puigmulé et al. 2011), despite individual spermatozoon differences existing in terms of phospholipid scrambling (Gadella and Van Gestel 2004). After lipid scrambling and due to the presence of both bicarbonate and BSA, there is an efflux of cholesterol molecules, which are redistributed or removed from the sperm head domain (Flesch et al. 2001b; Gadella and Van Gestel 2004). This event consists of the activation of tyrosine kinases, and the subsequent tyrosine phosphorylation of sperm proteins (Ficarro et al. 2003).

Bicarbonate is also involved in the migration of seminolipid, a sperm-specific glycolipid, from the apical head to the equatorial domain (Gadella et al. 1995) (Fig. 7.3). When bicarbonate levels are low, seminolipid molecules stabilise the plasma membrane, thereby impeding the acrosome reaction in the apical head domain of uncapacitated spermatozoa. In contrast, when bicarbonate levels are higher, seminolipids are translocated to the equatorial region, which diminishes their protective role. The outer membrane is then destabilised and becomes fusogenic to undergo acrosome reaction after binding with ZP-proteins (Rodríguez-Martínez 2007; Tsai et al. 2007). In contrast to the apical head sperm region, the post-acrosomal domain that fuses with the oolemma when fertilisation occurs does not react to higher levels of bicarbonate and keeps its stability (Gadella et al. 1995; Rodríguez-Martínez 2007).

High levels of bicarbonate also stimulate a sACY, the unique sACY-type present in spermatozoa (Signorelli et al. 2012), which triggers the production of cAMP and activates, in turn, the PKA (Chen et al. 2000; Harrison 2004; Botto et al. 2010). The activation of this cAMP-dependent pathway activates phospholipid scrambling in the apical sperm membrane, increases lipid disorder and membrane fluidity, as merocyanine-540 staining can show (see Sect. 9.3.6.2), and facilitates cholesterol redistribution over the sperm head (Gadella and Harrison 2002), in one of the key early/fast events of sperm capacitation. After their redistribution, cholesterol molecules are extracted and are then taken by acceptor proteins (Gadella 2008a).

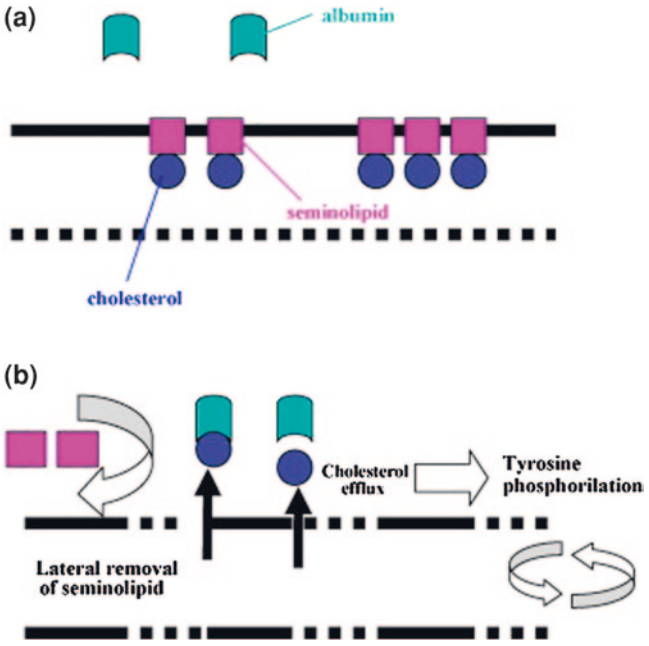

Fig. 7.3 Cholesterol efflux from the sperm plasma membrane during sperm capacitation. In ejaculated spermatozoa (**a**), the distribution of phospholipids is asymmetric and cholesterol levels are relatively high. The phospholipids scramblase is not activated and the cholesterol cannot be depleted by albumin. The sperm-specific glycolipid seminolipid is concentrated in the apical plasma membrane. In sperm capacitated in the presence of albumin, bicarbonate and Ca^{2+}, scramblase is activated via a bicarbonate adenylate cyclise-PKA signalling pathway. As a result, the phospholipids asymmetry of the plasma membrane collapses and this permits an albumin-mediated efflux of cholesterol and a lateral membrane removal of seminolipid (Witte and Schäfer-Somi 2007; Reproduced with permission)

In short, all these molecular changes modify the physicochemical properties of sperm membranes, thereby allowing further late changes of sperm capacitation, such as the fusion of plasma membrane and outer acrosome membrane (Gadella 2008a; Gadella et al. 2008). Therefore, the role of bicarbonate is crucial in sperm capacitation-related events since it primes the spermatozoon to interact with ZP, render the apical sperm head membrane fusogenic and undergo acrosome reaction/exocytosis (Gadella and Van Gestel 2004).

7.5.3 The Role of Other Oviductal Fluid Components (HA and BSA) on Sperm Capacitation

As already mentioned in this and Sect. 6.5.3, oviductal fluid also influences sperm capacitation. In bovine species, for example, the oviductal fluid collected during standing oestrus increases the percentage of capacitated spermatozoa (Bergqvist

et al. 2006). In porcine species, preovulatory fluid collected from the isthmus seems to maintain sperm viability without inducing sperm capacitation, while post-ovulatory fluid from the same region increases the percentage of capacitated spermatozoa, thereby indicating a different composition of oviductal fluid depending on the stage of the oestrus cycle. In fact, only the oviductal fluid collected at the peri-ovulatory period induces sperm capacitation (Rodríguez-Martínez 2007).

Oviductal fluid, as explained in Sect. 6.5.3, contains sulphated and non-sulfated glycosaminoglycans. Within sulphated glycosaminoglycans, dermatan sulphate induces capacitation, while the effects of HA appear to be in conflict with the literature because it delays capacitation after 3 days of storage at 15 °C (Yeste et al. 2008) and in vivo (Rodríguez-Martínez et al. 2001), but also seems to induce it in vitro conditions. Indeed, HA causes a slight capacitation in vitro, without triggering the acrosome reaction in bovine and boar semen after incubating at 38 °C (Bergqvist et al. 2006). In contrast, when boar spermatozoa are flushed from the sperm reservoir during the pre-ovulatory period (in vivo conditions) and they are further co-incubated with HA and bicarbonate, HA appears to prevent the induction of sperm capacitation (Tienthai et al. 2004). Similarly, when ejaculated spermatozoa are extended with a short-term extender and preserved at 15 °C, HA also delays sperm capacitation after 3 days of storage (Yeste et al. 2008).

Amidst this controversy, heparin and dermatan sulphate, both glycosaminoglycans like HA, have been regarded as sperm capacitation inductors in bull spermatozoa, but they increase the number of dead spermatozoa without increasing the percentage of capacitated spermatozoa in frozen-thawed spermatozoa (Bergqvist et al. 2007).

Finally, another component of oviductal fluid that is involved in sperm capacitation is BSA. This molecule is believed to function as a sink for the depletion of sperm plasma membrane cholesterol and can be replaced by other cholesterol-binding compounds such as cyclodextrins to induce capacitation (Salicioni et al. 2007).

7.6 Early/Fast Capacitation Events: Bicarbonate, Calcium and Activation of Sperm Motility

7.6.1 Introduction

As mentioned, controversy exists as to whether early/fast capacitation events have to be considered as part of the capacitation process or not. These rapid events occur a few seconds after ejaculation, and therefore not within the oviduct, and they are related to high concentrations of calcium and bicarbonate present in seminal fluid (Visconti 2009). Since capacitation requires several hours, and we are now referring to early/fast events, it is quite reasonable to consider that these changes are required to initiate capacitation prior to other events that continue and complete capacitation. For this reason, Fraser (2010) has rightly considered that these early/fast changes of sperm capacitation form the basis for eventual acquisition of fusibility characteristics required for acrosome reaction.

It is very important to emphasise that fast changes occur following release of spermatozoa into a bicarbonate-containing medium, also in in vitro conditions (Visconti et al. 1999a; Gadella and Harrison 2000; Holt and Harrison 2002; Harrison 2004). In this regard, we should also bear in mind what has previously been discussed about the main role of bicarbonate as a sperm capacitation effector (see also Sect. 7.5.2).

Bicarbonate enters the sperm through the co-transporter Na^+/bicarbonate (Demarco et al. 2003), producing an increase in intracellular pH and activating the sACY, which, as mentioned before, is the only type of sACY present in spermatozoa (Signorelli et al. 2012). Evidence has repeatedly indicated that this atypical cyclase is the main target of bicarbonate during sperm capacitation (Fraser 2010). In contrast to transmembrane adenylyl-cyclases, this soluble form is activated by bicarbonate and Ca^{2+} but does not respond to activators of Gs, the G protein stimulator of transmembrane cyclases, such as cholera toxin and non-hydrolyzable analogues of GTP (Visconti 2009).

During these fast/early capacitation events, there are two bicarbonate-mediated changes that occur almost simultaneously. One involves changes in the sperm plasmalemma (membrane fluidisation), while the other is known as sperm motility activation (Fraser 2010), and the latter event occurs faster than the former (Harrison 2004; Harrison and Gadella 2005). However, and despite both events taking place together to some extent, they will be described separately in the following two subsections.

7.6.2 Plasma Membrane Changes During Early Events

Bicarbonate activates a phospholipid scramblase, an enzyme that translocates membrane phospholipids between the outer and inner leaflets of the membrane and causes rapid changes in the phospholipid asymmetry of this membrane (Gadella and Harrison 2000; Harrison and Gadella 2005). Indeed, this scramblase-activation results in translocation of phosphatidylserine and phosphatidylethanolamine to the outer leaflet, rather than being mostly or exclusively located in the inner leaflet. It is worth noting that although translocation of these lipids in the outer leaflet mainly occurs in the acrosomal region, sACY has been identified in the midpiece of the tail (Hess et al. 2005). All these changes reduce membrane stability (Gadella and Harrison 2002), so that, in turn, this scramblase-initiated destabilisation makes cholesterol available to external receptors. Cholesterol can then be more easily removed, and this leads to a rapid collapse of sperm plasmalemma asymmetry (Salicioni et al. 2007; Visconti 2009) (Fig. 7.3). The cholesterol removal by acceptor proteins, such as BSA, is considered a late capacitation event (Gadella and Harrison 2002) and will be considered in greater detail in Sect. 7.7.

Botto et al. (2010) have studied the biochemical events involving membrane architecture in spermatozoa exposed to bicarbonate before the extraction of cholesterol molecules. These authors have paid special attention to the relocation of two

endocannabinoid system receptors (see also Sect. 6.9); cannabinoid receptor type 1 (CBR1) and transient receptor potential cation channel 1 (TRPV1) during early/fast capacitation events, since both proteins are known to be involved in capacitation-related spermatozoa signalling. Interestingly, these researchers have observed that bicarbonate promotes massive membrane remodelling in the absence of extracellular proteins. This membrane re-organisation entails a series of changes.

Firstly, when spermatozoa are in vitro incubated with bicarbonate, there is an increase in protein content in lipid microdomains, also known as detergent-resistant membrane-domains (DRMDs), since they are insoluble in non-ionic detergents and have light buoyant/floating density after centrifugation in a discontinuous sucrose gradient (Botto et al. 2008; Pike 2009). These lipid microdomains, which are rich in cholesterol and sphingolipids, compartmentalise the sperm membrane. Previous reports in porcine (Shadan et al. 2004; Van Gestel et al. 2005) and other mammalian species (Travis et al. 2001) have suggested that such microdomains are involved in the acquisition of fertilising capacity. In a study that we are conducting at present, it would appear that sperm DRMDs play a relevant role not only in capacitation but also in the changes in sperm function after boar sperm cryopreservation (Yeste et al. 2012, unpublished).

To date, caveolae and lipid rafts are the two proposed types of lipid microdomains (Botto et al. 2010). Anderson (1998) has reviewed the concept and function of caveolae, which are lipid raft-enriched domains present on the plasma membrane of many eukaryotic cell types (Anderson 1998). Caveolae can present different shapes, including flat, vesicular and tubular and they can be either open at the cell surface or closed off forming an exocytic/endocytic compartment. Although all cells have plasma membrane domains with the biochemical features of caveolae, only a subset of these membranes display the flask-shaped morphology typical of caveolae (Anderson 1998). They play a role in cell signalling, cholesterol homoeostasis, clathrin-independent endocytosis, transcytosis and potocytosis (Matveev et al. 2001; Parton and Richards 2003). With regard to their biochemical composition, caveolae contain lipids, such as glycosphingolipids and cholesterol, and proteins, such as caveolin, which is the main integral protein of these domains, presenting their carboxyl and amino termini located in the cytosol and a hydrophobic loop inserted into the membrane (Glenney and Soppet 1992; Rothberg et al. 1992). These caveolins bind to cholesterol (Murata et al. 1996) and oligomerise, thereby stabilising the membrane domain and defining the size and the shape of caveolae (Fernández et al. 2002; Parton and Simons 2007). Within caveolins, caveolin-1 and caveolin-2 are ubiquitously expressed in mammalian cells. As far as lipid rafts are concerned, it is important to note that they are flat domains containing high amounts of glycosphingolipids and glycosylphosphatidylinositol-anchored proteins (Brown 2006). The formation of these lipid rafts depends on lipid–lipid interaction (Lindner and Naim 2009).

The increase in protein content in DRMDs is associated with a significant increase in caveolae and CD55, but does not alter the percentages of cholesterol in these DRMDs, thereby suggesting that this reorganisation of the sperm membrane architecture is independent from cholesterol extraction. In addition, this finding

suggests, for the first time, that the reorganisation of DRMDs, which is one of the features of sperm capacitation, starts before cholesterol extraction. These results have led the sequence of capacitation events to be reconsidered, since until the report by Botto et al. (2010), membrane remodelling was believed to take place only when extracellular proteins or cholesterol-extracting molecules were present (Choi and Toyoda 1998).

Bicarbonate has also appeared to promote the migration of the two mentioned receptors of endocannabinoids (CBR1 and TRPV1). Thus, although the CBR1 content is low in DRMDs before membrane remodelling, the amount of this protein in these lipid rafts increases after bicarbonate exposure. In DRMDs extracts, CBR1 appears in a double band: one corresponds to the unglycosylated form (54 kDa), and the other corresponds to the glycosylated (active) form (65 kDa) (Botto et al. 2010). According to Maccarrone et al. (2005), CBR1 would slow the tendency of plasma membrane to become unstable, thereby avoiding the premature loss of acrosome integrity. The signal transduction of the activated receptor is a cAMP-dependent pathway.

In the case of TRPV1, its amount is low in DRMDs before membrane reorganisation, but it also increases after bicarbonate exposure (Botto et al. 2010). This receptor, modulated by ionic intracellular concentrations, is involved in the regulation of sperm transmembrane potential, in the intracellular calcium levels and in actin polymerisation (Bernabò et al. 2010a), all of them being capacitation-related events.

All these results lead these authors to hypothesise some role of these endocannabinoid receptors in the control of sperm–oocyte interaction. In addition, the role of these two endocannabinoid receptors in the acquisition of sperm fertilising ability does not depend on their action mechanism, but it relies on the membrane remodelling process itself, which affects the biochemical localisation of these molecules.

On the other hand, Botto et al. (2010), based on research by Asano et al. (2009) in mice and Cummerson et al. (2006) in humans, have proposed that during their formation, lipid rafts are also present in the acrosome membrane. These authors also hypothesised that, after bicarbonate exposure, some DRMDs component could originate from the outer acrosomal membrane and be later transferred to the sperm surface via an exocytotic process (Botto et al. 2010). This hypothesis seems to be reasonable given that plasma membrane and outer acrosomal membrane fuse with each other at the moment of acrosome reaction.

In short, Botto et al. (2010) have suggested that sperm capacitation entails a quite complex and integrated functional dialogue between the endocannabinoid system and the microdomain architecture of plasma and outer acrosomal membranes. All these events take place prior to the fusion of plasma and outer acrosome membranes, which will be further described in Sect. 7.7 about late/slow capacitation events.

Finally, we must mention that controversy exists as to what extent cholesterol removal is the signal that leads to membrane destabilisation. Thus, even though some studies have reported that loss of cholesterol is the initial event leading to

membrane destabilisation, to cAMP-increases and to the activation of PKA (Visconti et al. 1999b, c, 2002), other evidence suggests that the collapse of membrane asymmetry precedes and facilitates subsequent cholesterol removal (Flesch et al. 2001b). As stated, the other changes in the sperm membrane that occur during late/slow events will be reconsidered in a further specific section (Sect. 7.7).

7.6.3 Removal of AQN-1 from Sperm Surface During Early Capacitation Events

Another relevant change that is produced during an early stage of in vitro capacitation is the release of AQN-1 from the sperm surface (Ekhlasi-Hundrieser et al. 2005). This spermadhesin, which plays a relevant role in the formation of pig sperm reservoir as described in Sect. 6.7.3.3, masks other sperm surface associated proteins (AQN-3, AWN and P47/SED1) that are involved in the interaction of the spermatozoa with the oocyte's zona pellucida (Figs. 6.4 and 8.2).

At a very early stage of sperm capacitation, sperm membrane is destabilised and AQN-1 is removed from the sperm surface. This leads to the release of the spermatozoa from the oviductal reservoir and allows AWN, AQN-3 and P47/SED1 to become accessible and thus make their interaction with ZP possible (Töpfer-Petersen et al. 2008). Interaction of these three sperm surface associated proteins to ZP will be taken up again in Fig. 8.2.

7.6.4 Activation of Sperm Motility

Activated sACY increase cAMP levels, and the increased levels of this second messenger stimulate PKA. Once activated, PKA phosphorylates various target proteins that are presumed to initiate several signalling pathways. Notwithstanding, in sperm exposed to bicarbonate, cAMP-increases to a maximum within 60 s, and the rise in PKA-dependent phosphorylation begins within 90 s (Salicioni et al. 2007). In contrast, increases in protein tyrosine phosphorylation only occur later (Harrison 2004).

One of the PKA-activated pathways modulates the response of sperm-specific calcium voltage-gated channels (CatSper), which produces changes in the membrane potential and increases the intracellular concentration of Ca^{2+} (Wennemuth et al. 2003; Signorelli et al. 2012). All these molecular changes participate in the activation of sperm motility (Fig. 7.1). This can be explained by the role of bicarbonate and Ca^{2+} on sperm motility before and after ejaculation. Indeed, although sperm stored in the cauda epididymidis consume oxygen at a high rate, they are immotile. Conversely, when spermatozoa are released from the epididymis and they come into contact with high bicarbonate and Ca^{2+} present in the seminal fluid, there is an immediate activation of the flagellum movement.

7.7 Late/Slow Capacitation Events: Hyperactivation, Membrane Remodelling and Protein Phosphorylation

7.7.1 Introduction

In contrast to the fast activation of sperm motility, other capacitation-associated processes require longer incubation periods. These slower processes can be accomplished in vitro by using sperm incubated in defined media. In all cases, in vitro capacitation media must contain a protein source that is usually BSA; and an assortment of ions including bicarbonate and Ca^{2+} (Salicioni et al. 2007; Visconti 2009). The relevance of in vitro studies in sperm capacitation has been briefly mentioned in Sect. 7.3.

It is considered that late/slow sperm capacitation events start after cholesterol efflux from sperm plasma membrane mediated by BSA, and take place in the oviduct. This cholesterol removal increases membrane lipid disorder and fluidity, allows reorganisation of lipid-raft membrane domains and permits spermatozoa to maintain high levels of bicarbonate (Cross 2004). In addition during this phase, PKA phosphorylates serine and threonine residues of several proteins. Such Ser- and Thr phosphorylation results, in turn, in an increase in the phosphorylation of tyrosine residues, through the activation of protein kinases or the inhibition of protein phosphatases (Signorelli et al. 2012). Finally, this signal transduction pathway leads to a completion of sperm capacitation, which is manifested by the following events (Baldi et al. 1996; Salicioni et al. 2007):

1. Hyperactivation
2. Chemotactic behaviour
3. The ability of spermatozoon to trigger acrosome reaction induced by ZP or progesterone
4. The ability of spermatozoon to fertilise an oocyte.

7.7.2 Calcium and Hyperactivated Motility

Yanagimachi (1970), working with hamster sperm, was the first to report the existence of hyperactivated motility in mammalian spermatozoa. Hyperactivated motility consists of very active thrusting and asymmetric flagellar movements. In vivo, these changes in the specific motility/kinetic patterns occur in the oviduct. In addition, these changes can also be observed after proper stimulation in vitro, so that these findings have allowed more about this phenomenon to be learnt. We should bear in mind, as previously mentioned, that hyperactivation, without the so vigorous flagellar movement reported in mice, has been observed in vitro but not yet in in vivo conditions in boar spermatozoa (Rodríguez-Martínez 2007; see also Sect. 7.4.2). Moreover, under in vitro conditions, the percentage of hamster spermatozoa exhibiting hyperactivated motility (about 40 %) is quite higher than that of boar

spermatozoa, where only few sperm show this motility pattern. Therefore, and although the main data about sperm hyperactivation mentioned in this subsection come from rodents rather than from pigs as studies about the latter are scarce, it is worth remembering that this knowledge can not be directly extrapolated to pigs but care must be taken when trying to explain why happens in pigs from mice studies.

Hyperactivation is a calcium-dependent process and is required for successful fertilisation of zona-intact oocytes (Suarez 2008). Apart from motility activation in early-capacitation events, CatSper proteins (one, two, three and four), which we remember are sperm-specific calcium channels, have also been reported to be involved in motility hyperactivation (Kirichok et al. 2006; Qi et al. 2007; Fraser 2010). The main role of these calcium channels in hyperactivated motility is supported by previous studies made in male mice null for any one of the four CatSper proteins. These males are infertile and are not able to penetrate zona-intact oocytes (Ren et al. 2001; Quill et al. 2003; Jin et al. 2007; Qi et al. 2007). In addition, Ho et al. (2009), also working in mice, demonstrated that CatSper-null spermatozoa were unable to show hyperactivated motility and were unable to detach from the oviductal epithelium and thus reach the site of fertilisation.

7.7.3 Reorganisation of Sperm Membrane During Late/Slow Capacitation Events

As is widely known, one of the most relevant features of sperm capacitation is related to the changes and reorganisation of membrane lipids and proteins that affect all the subcellular compartments. This event concomitantly takes place with the activation of several signalling pathways such as sperm sACY, cAMP-dependent PKA and protein tyrosine phosphorylation (Flesch and Gadella 2000; Gadella et al. 2008). In a more accurate analysis, remodelling of sperm membranes during capacitation has been proposed as a two-step model (Flesch et al. 2001b):

- First, changes in sperm plasma membrane that involves a quick exposure of phosphatidylserine, detectable after 5 min and completed within 10 min; and a later exposure of phosphatidylethanolamine, which is completed after 30 min (Gadella and Harrison 2002). This would correspond to what occurs during the early/fast capacitation events (see Sect. 7.6.2).
- Second, and after between 2 and 4 h, cholesterol molecules are extracted with the involvement of acceptor proteins, and sperm plasma and outer acrosomal membranes become stable docked (Gadella 2008a; Gadella et al. 2008). This completes membrane remodelling and allows spermatozoa to acquire fertilising ability. These changes would correspond to what occurs during late/slow capacitation events and will be treated in more detail in the present section.

Both during early/fast and late/slow capacitation events, membrane remodelling entails the participation of specific DRMDs, as Gadella (2008a) and Botto et al. (2010) have proposed. The participation of DRMDs in early/fast capacitation

events has been previously discussed in Sect. 7.6.2. In the case of late/slow events, the completion of capacitation induces a close apposition/interaction of the apical plasma with the outer acrosome membrane. Indeed, when sperm membranes of capacitated and uncapacitated spermatozoa are isolated using nitrogen cavitation and differential centrifugation steps, it appears that membrane fractions of capacitated spermatozoa are bilamellar, while those of uncapacitated spermatozoa are unilamellar (Tsai et al. 2007, 2010). Furthermore, the outer acrosomal membranes isolated from capacitated spermatozoa present a significantly higher number of PNA-binding sites than those isolated from uncapacitated spermatozoa (Flesch et al. 1998).

In the formation of these bilamellar structures after capacitation as well as during acrosome exocytosis (see also Chap. 8), SNARE complexes play a key role. The acronym 'SNARE' stands for proteins from the Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor. These proteins are involved in calcium-mediated membrane fusion processes and in exocytosis-mediation in several cells types, such as neutrophils (Chen and Scheller 2001; Jahn and Scheller 2006; Verhage and Toonen 2007). Proteins belonging to the SNARE family have also been suggested to play a relevant role in mammalian fertilisation (De Blas et al. 2005; Ramalho-Santos et al. 2000; Tomes et al. 2002).

Both bilamellar membrane structures and trans-SNARE complexes formed after sperm capacitation remain stable after their post-cavitation isolation (Tsai et al. 2007). These trans-SNARE complexes appear to be highly stable, resistant to high temperature and to reducing conditions, and only fall apart into SNARE monomers when a treatment combining reducing agents and high temperature is provided. Related to this, Tsai et al. (2010) have suggested that SNARE-monomers gain extra protection when trans-SNARE complexes form large functional DRMDs to withstand the changes (i.e. temperature, pH etc.) in the surrounding environment.

The high stability of SNARE complexes can be explained because SNARE monomers are assembled prior to membrane fusion (Bennet and Scheller 1994; Jahn and Sudhof 1994). This assures the formation of stable SNARE complexes allowing successful acrosome exocytosis upon calcium stimulation. This idea is in agreement with Ackermann et al. (2008) and Heydecke et al. (2006), who suggested that a DRMD-associated scaffold protein governs the acrosome reaction in mammalian spermatozoa, and this may serve to direct the acrosome and plasma membrane SNAREs into the right membrane topology for the formation of stable ternary complexes (Tsai et al. 2010).

SNARE interactions have been related to the calcium-dependent acrosome reaction in mouse spermatozoa (Heydecke et al. 2006), while in boars Tsai et al. (2007) have observed that SNARE proteins cluster into DRMDs upon capacitation, forming a ternary trans-SNARE complex. Indeed, after capacitation but not before, there is a formation of an 80 kDa SNARE-complex, made up of SNAP23 (a specific protein of outer acrosome membranes) and VAMP3 (a specific protein of plasma membrane). Acrosomal SNAP23 is present in the outer acrosome membrane and in the plasma membrane of capacitated spermatozoa, while VAMP3, which has a molecular mass of 16 kDa, is specific for the plasma membrane and

is only present in monomeric form in uncapacitated spermatozoa. In contrast, it appears in the 80 kDa band in capacitated spermatozoa (Tsai et al. 2007, 2010). As a result of SNAP23/VAMP3 interaction, the acrosome membrane becomes stable docked to the apical sperm head plasma membrane, without the occurrence of fusions between the two interacting membranes (Fig. 7.4).

Both uncapacitated and capacitated spermatozoa present SNARE complexes containing VAMP-1 and -2 and syntaxins 2 and 2. This suggests that syntaxin 2/3 and VAMP 1/2 could be interacting SNARE proteins and form an additional pool of SNARE complexes (Tsai et al. 2010). These molecules do not seem to participate in membrane docking, but their exact role in the regulation of the acrosome exocytosis still remains unknown. Syntaxins 1B, 2 and 3 are predominantly present in sperm plasma membrane in uncapacitated spermatozoa, and syntaxin 2 and 3 are present in their monomeric form of 36 kDa (Tsai et al. 2010). However, the contribution of these two syntaxins after capacitation to the 80 kDa protein complex is marginal, so that it does not seem to be the primary cognate Q-SNARE with SNAP 23 and VAMP 3. In contrast, syntaxin 1B is present in the 80 kDa SNARE complex formed after capacitation, so that Tsai et al. (2010) have suggested that syntaxin 1B/SNAP 23/VAMP 3 are the interacting SNARE proteins in the 80 kDa trans-SNARE complex (Fig. 7.4).

From the previously mentioned SNARE proteins, it is worth noting that SNAP 23 shows a three-time higher DRMD-association affinity than SNAP 25 (Salaun et al. 2005; Tsai et al. 2010).

As mentioned, the stable docking of the outer acrosome membrane to the plasma membrane after sperm become completely capacitated is not accompanied by membrane fusions (Tsai et al. 2010). This entails the bilamellar structure remaining intact after docking and priming and the two interacting membranes persisting together as bilamellar membrane structure. Thus, formation of ternary trans-SNARE complexes is not sufficient to induce the AR but require additional stimuli or the removal of inhibitory components to allow fusion reaction. Therefore, binding of spermatozoa to intact-ZP is needed to increase calcium levels, via the opening of a transient receptor potential (TRP) channel (Florman et al. 2008). In this regard, it is worth noting that the area where the docking and priming of the plasmalemma and the acrosome occurs is the same area where the spermatozoon binds to the zona pellucida (Flesch and Gadella 2000). In addition, this area corresponds to the area where sperm membrane DRMDs have been shown to aggregate during capacitation treatments (Tsai et al. 2007; Van Gestel et al. 2005, 2007). According to Tsai et al. (2010), the stability and extended area of these docked and primed membranes might explain why acrosome membrane fusions after sperm bind to ZP. These interactions and fusions between the two membranes involved in the acrosome reaction (i.e. acrosome and plasma membrane), and which result in a generation of mixed vesicles, are thus separated processes in mammalian fertilisation. In addition, the formation and stabilisation of SNARE complex during docking and priming between plasma and acrosome membrane has also been suggested as a mechanism for preventing acrosome exocytosis in the absence of ZP-induced calcium influx (Tsai et al. 2010).

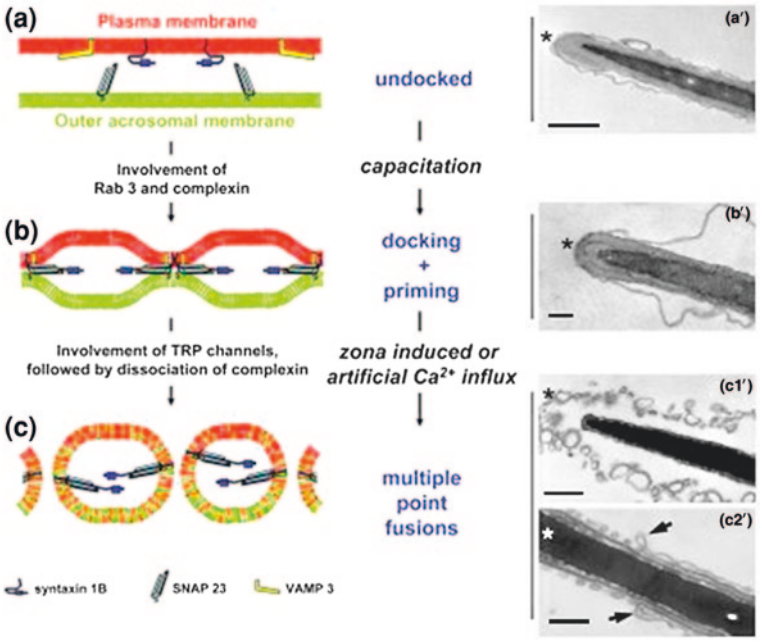

Fig. 7.4 Model for capacitation-induced stable docking of the acrosome to the sperm plasma membrane proposed by Tsai et al. (2010). In uncapacitated spermatozoa (**a**), plasma membrane (in *red*) and outer acrosomal membrane (in *green*) are not associated together. Syntaxin 1B (*purple blue*) and VAMP 3 (*yellow*) are located at the plasma membrane and SNAP 23 (*light blue*) is found at the outer acrosome membrane. The undocked plasma membrane appears as loose arrangement at the entire head area due to the osmotic effect by EM processing (**a′**). In capacitated spermatozoa (**b**), outer acrosomal membrane is stably docked to the apical plasma membrane and a stable ternary-SNARE complex is formed without membrane fusion (**b′**). Finally, when spermatozoa bind to the ZP (**c**) there is the fusion between membranes. However, although mixed vesicles of the apical plasma and outer acrosomal membranes are the result of the multipoint fusions characteristic for sperm acrosome exocytosis (**c1′**), there is no fusion at the equatorial sperm head area (distal from the arrows indicated in **c2′**) (Tsai et al. 2010; Reproduced with permission)

Roggero et al. (2007) suggested a model for acrosomal exocytosis that involved the introduction of extracellular calcium between the acrosome and plasma membrane. This, in turn, was proposed to form loose trans-SNARE complexes, and the intra-acrosomal calcium efflux was thought to trigger the SNARE-mediated acrosome reaction. In the light of their own results, however, Tsai et al. (2010) have refined this model, proposing the following features:

1. The acrosome is not associated with the plasma membrane in uncapacitated spermatozoa.
2. Sperm capacitation leads to stable docking of the outer acrosome membrane to plasma membrane at the apical region of the sperm head without the fusion of both membranes.

3. ZP-binding allows calcium to enter into the sperm (Arnoult et al. 1996; Breitbart 2002). The high levels of cytosolic calcium will induce a conformational calcium-dependent change from trans- to cis-SNARE complex. Then, the two membranes are fused and acrosome reaction occurs (Fig. 7.5).

We can thus conclude that sperm plasmalemma and the outer acrosome membrane become more unstable during late capacitation and stable dock between one another. However, actual fusion between these two membranes only takes place during acrosome reaction, i.e. after sperm bind to ZP. For this reason, this aspect will be specifically treated in the next chapter.

7.7.4 Tyrosine Phosphorylation of Sperm Proteins

Sperm capacitation is also associated with an increase in tyrosine phosphorylation of some sperm proteins (Töpfer-Petersen et al. 2002; Visconti 2009). This phenomenon is a late event that relies on the presence of BSA, Ca^{2+} and bicarbonate (Morgan et al. 2008). Specifically, the absence of any one of these media constituents prevents both tyrosine phosphorylation and capacitation.

Petrunkina et al. (2004), co-incubating spermatozoa with oviductal explants, observed an increase in the tyrosine phosphorylation of tail proteins and later of sperm head proteins in unattached sperm populations during sperm capacitation. In contrast, Töpfer-Petersen et al. (2002) reported spermatozoa bound to oviductal epithelium presented lower calcium uptake and their proteins do not become phosphorylated.

The increase in tyrosine phosphorylation residues of sperm proteins during capacitation is mediated by PKA within in a pathway that is unique to spermatozoa (Signorelli et al. 2012), and has been observed in different mammalian species (Bailey 2010). The involvement of PKA is illustrated by experiments demonstrating that cAMP-permeable analogues are able to induce the increase in tyrosine phosphorylation in the absence of BSA, bicarbonate or calcium.

To date, different substrate-proteins for tyrosine phosphorylation have been identified in mammalian spermatozoa. These substrates are ion channels, enzymes, structural proteins, members of the extracellular signal-regulated kinase family (ERK) family and a calcium-binding protein localised in the principal piece of the tail and associated with the fibrous sheath (Ficarro et al. 2003; Luconi et al. 1998a, b; Naaby-Hansen et al. 2002). One of the main Tyr-phosphorylated structural proteins is a member of the A-kinase-anchoring protein (AKAP) family (Ficarro et al. 2003). These proteins are located at the fibrous sheath and are involved in sperm motility (Muratori et al. 2011).

It is worth noting that phosphorylation of tyrosine residues in sperm proteins does require the presence of bicarbonate and BSA in the surrounding environment, but not that of extracellular calcium, as studies conducted on human spermatozoa have shown (Muratori et al. 2011). In addition, the presence/absence of cholesterol in the sperm membrane is related to the ability of sperm to undergo tyrosine phosphorylation of capacitation-involved proteins. Accordingly, Shadan et al. (2004)

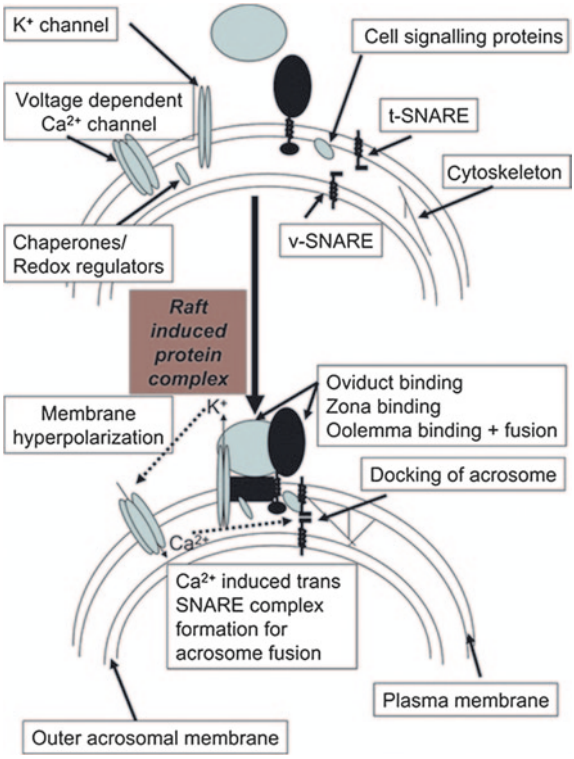

Fig. 7.5 Hypothetical assembly of a sperm-zona binding complex formed during sperm capacitation by raft induced protein clustering. This may result in a multifunctional protein complex known to play a role in diverse processes leading to fertilisation. (Gadella 2008b; Reproduced with permission)

working with boar spermatozoa observed that cholesterol removal from sperm plasmalemma promoted tyrosine phosphorylation of sperm proteins, and Buffone et al. (2009) working with human spermatozoa observed that high cholesterol levels and decreased fluidity of plasma membrane of spermatozoa coming from subfertile/infertile men appeared to be related to deficiencies in tyrosine phosphorylation of such proteins.

In short, the increase in phosphorylated tyrosine residues in sperm proteins is considered to be a marker of late capacitation events (Arcelay et al. 2008). In humans, the relevance of Tyr phosphorylation in sperm capacitation has been demonstrated because it increases the ability of spermatozoa to bind ZP (Liu et al. 2006).

7.8 Bicarbonate, Soluble Adenylyl-Cyclase and cAMP as the Key Mediators of Early/Fast and Late/Slow Sperm Capacitation Events

One of the regulation paradoxes in fast and slow capacitation-associated events is that both are mediated by the same molecules (bicarbonate, sACY and cAMP) and the same transduction pathway (PKA). Thus, although we have separated

fast/early from slow/late capacitation events, the aforementioned three molecules appear, in both cases, as the key molecules involved in their regulation (Esposito et al. 2004; Harrison 2004; Harrison and Miller 2000; Hess et al. 2005; Salicioni et al. 2007; Visconti 2009). Backing this, Morgan et al. (2008), working with mice that bear a mutation in the catalytic subunit of PKA, demonstrated that both fast/early and slow/late capacitation events were activated by the same pathways. When inhibiting this PKA in mutant mice, these authors blocked:

1. The increase in the HCO_3^--dependent flagellar frequency,
2. The phosphorylation of PKA substrates that occurs within 90 s of bicarbonate addition, and
3. The increase in Tyr phosphorylation.

These data led these authors to conclude that PKA has, at least, two independent roles in the regulation of sperm movement:

a. a 'quick' action that is required for the activation of the flagellar beat, and
b. a 'slow' action that allows changes in the pattern of movement of the sperm and that requires PKA to remain active for an extended period of time.

Thus, Morgan et al. (2008) have been the first in proposing a chemical-genetic switch approach to understanding the temporal action of this enzyme in sperm capacitation. However, and despite this ground-breaking study, little is known about how PKA-activity specifically mediates different aspects of sperm capacitation. In this regard, it must be mentioned that although activation of the PKA-pathway occurs immediately and does not need cholesterol acceptors, the increase in tyrosine phosphorylation and other late events are not immediately stimulated and require the presence of cholesterol acceptors (Visconti 2009).

7.9 Phosphorylation/Dephosphorylation of Sperm Proteins During Capacitation

7.9.1 Introduction

As stated, one of the most important changes that takes place during capacitation is the phosphorylation of some sperm proteins. This does not only involve tyrosine residues but also the serine and threonine residues of sperm proteins.

It is worth remembering that mature spermatozoa are transcriptionally quiescent and protein phosphorylation–dephosphorylation thus appears as a method for controlling protein function (Naz and Rajesh 2004; Urner and Sakkas 2003). In addition, it is widely known that regulation of cellular processes requires the coordinated action between kinases and phosphatases. In fact, in the case of mammalian sperm capacitation, protein kinases and phosphatases play a balanced role, regulating the phosphorylation state of sperm proteins. Although much work has

been performed in the field of sperm kinases, recent evidence indicates that phosphatases also play a significant role (Signorelli et al. 2012).

Therefore, and considering the relevance of protein phosphorylation–dephosphorylation in sperm capacitation, the present section exclusively focuses on this aspect and thus reviews the involvement of kinases and phosphatases during this process.

7.9.2 Phosphorylation of Tyrosine Residues in Sperm Proteins

7.9.2.1 Tyrosine Kinases

Tyrosine phosphorylation is mediated by tyrosine kinases, which are activated, directly or indirectly, by PKA (Signorelli et al. 2012). Tyrosine kinases can be divided into two classes:

– Receptor tyrosine kinases (RTKs)
– Non-receptor tyrosine kinases (PTKs).

The first class, RTKs, are transmembrane proteins with an extracellular binding domain to a ligand and an intracellular domain, while the second, PTKs, are found in the cytoplasm, nucleus, or the inner side of the plasma membrane (Fisher et al. 1998). Data on boar spermatozoa are scarce, so that in this subsection we follow currently available studies, which are mainly conducted on human and mouse spermatozoa.

Previous reports have shown the involvement of PTKs in the capacitation of human and mouse spermatozoa. Specifically, four members of the Src-family, *SRC*, *FYN*, *LYN* and *YES1* (*V-YES-1*; Yamaguchi Sarcoma Viral Oncogene Homologue 1) seem to play a relevant role in this regard, the first three members being mainly located in the sperm tail while the other is present in the head. Indeed, YES 1 has been found in the head of human sperm and its activity seems to be modulated by cAMP (Leclerc and Goupil 2002).

Although SRC is also present in the sperm head, only the form located in the mitochondrial/middle piece is active. In humans, SRC presents sites that are phosphorylated by PKA and the phosphorylation of Tyr_{416} leads to its activation during sperm capacitation. This activation leads to acrosome reaction but does not affect sperm motility (Varano et al. 2008). This is because, despite inhibition of PKA and SRC leading to a significant decrease in Tyr phosphorylation, only the inhibition of PKA, but not that of SRC, suppresses sperm motility (Mitchell et al. 2008). In fact, it seems that SRC is involved in the response to calcium triggered by progesterone (Varano et al. 2008).

SRC has also been found in the tail of mouse spermatozoa (Baker et al. 2006; Krapf et al. 2010) and it has also been suggested as a mediator of sperm capacitation in this species (Signorelli et al. 2012).

7.9.2.2 PKA, PKC and ERK/MAPK Pathway

PKA is a tetrameric enzyme that, in somatic cells, contains two regulatory subunits and two catalytic subunits. The activity of this enzyme relies on cAMP, since binding of cAMP to the regulatory subunits leads to tetramer dissociation and allows the activation of the catalytic subunit (Nolan et al. 2004). In the case of spermatozoa, PKA, located on the acrosomal region and in the flagellum, only presents a catalytic (α) subunit (i.e., $C_{\alpha 2}$) (Pariset and Weinman 1994; Visconti et al. 1995a). The critical role of this catalytic subunit in tyrosine phosphorylation and sperm capacitation has been reported, amongst others, by Visconti et al. (1995a) and Esposito et al. (2004). In the first case, inhibition of PKA also resulted in an inhibition of Tyr phosphorylation and sperm capacitation. Furthermore, Esposito et al. (2004), working with Cα2-null mice spermatozoa, observed that capacitating conditions did not induce Tyr phosphorylation and were infertile.

Another protein kinase that has also been found in mammalian spermatozoa, and for which a role in sperm motility and acrosome reaction has been suggested is the calcium-dependent protein kinase (PKC) (Breitbart and Naor 1999).

Apart from PKA, Awda and Buhr (2010) have recently identified three elements (RAF, MEK1/2, and ERK1/2) of the ERK/MAPK pathway in boar spermatozoa, which appears to regulate the phosphorylation of tyrosine residues of different sperm proteins. Awda and Buhr (2010) also observed other kinases from other pathways, like cAMP/PKA and PKC that seem to interact with the MAPK/ERK pathway.

In addition, ROS species play some role in regulating boar sperm capacitation and tyrosine phosphorylation. Awda and Buhr (2010) observed that the presence of ROS inhibited the tyrosine phosphorylation of high-molecular mass proteins, which are phosphorylated during sperm capacitation. According to these authors, inhibition could be explained by interactions with some elements of the MAPKs signal transduction cascade, affecting the phosphorylation of RAF1, MEK1/2, and ERK1/2. On the other hand, Awda and Buhr (2010) observed that the ERK1/2 element of the MAPK/ERK signal transduction pathway is significantly associated with tyrosin-phosphorylation of sperm proteins that takes place during capacitation.

7.9.3 Phosphorylation of Serine/Threonine Residues of Sperm Protein and Ser/Thr Kinases

Apart from the role of Tyrosine kinases and PKA, there are two other kinases that phosphorylate serine and threonine kinases and whose role has also been reported in mammalian species (Fernández-Novell et al. 2011). However, there are fewer reports dealing with this Ser/Thr than with Tyr phosphorylation (Signorelli et al. 2012).

The involvement of Ser and Thr protein phosphorylation in sperm capacitation is quite recent, as it was Naz (1999) who, working with human spermatozoa,

observed that at least four groups of proteins (43–55, 94, 110 and 190 kDa) were phosphorylated on serine residues and threonine residues, during capacitation and after exposure to ZP. Some of these proteins were also observed to be phosphorylated at Tyr residues.

Given the simultaneity of Ser, Thr and Tyr residues on the same proteins, Bedu-Addo et al. (2005), still working with human sperm, observed that phosphorylation on Ser/Thr residues took place earlier than phosphorylation on Tyr residues. This finding provided really novel information about capacitation pathways and about which residues phosphorylate and the chronology of these phosphorylations. In addition, these phosphorylation events were reversible and dependent on the presence of BSA and bicarbonate. In humans, Bedu-Addo et al. (2005) observed that sperm incubated in the absence of both bicarbonate and BSA showed extremely low levels of phosphorylation. In boars, Harrison (2004) observed that bicarbonate stimulation in the absence of BSA led to reduced Ser/Thr phosphorylation levels and lower levels of cellular cAMP than after stimulation of bicarbonate and BSA.

For the time being, and from the available studies, we can state that the dynamics of phosphorylation on Ser and Thr residues during capacitation is very complex, and that we are not currently able to describe an exact picture of what path this phosphorylation follows in mammalian spermatozoa. For instance, and focusing on boar spermatozoa, results are not clear. On one hand, Harayama and Nakamura (2008) have reported a large group of proteins that are phosphorylated on their Ser/Thr residues during capacitation. This phosphorylation presents a specific kinetic, reaching a peak after 80 s, then decreasing and increasing again slowly until the end of the incubation period. In addition, Harrison (2004) observed that phosphorylation of Ser/Thr residues of boar sperm proteins significantly increases when spermatozoa are incubated with calyculin-A, a Ser/Thr phosphatase inhibitor. On the other hand, Alnagar et al. (2010) observed dephosphorylation of five phosphorylated proteins in Ser/Thr residues within 15 min of capacitation. However, when spermatozoa are incubated with calyculin-A during sperm capacitation, such dephosphorylation is not observed.

All these data, together with data from Kong et al. (2009) obtained in human spermatozoa, suggest that the proteasome may be involved in the Ser and Thr phosphorylation that takes place during capacitation but not in Tyr phosphorylation.

7.9.4 Protein Phosphatases

As in the case of tyrosine kinases and serine and threonine kinases, more available information about protein kinases exists than about phosphatases. Phosphatases are classified into two groups depending on the residues they dephosphorylate, i.e. protein tyrosine phosphatases that dephosphorylate at Tyr residues, and protein Ser/Thr phosphatase that dephosphorylate Ser and/or Thr residues.

7.9.4.1 Protein Tyrosine Phosphatases

Tyrosine phosphatases are encoded by a large family of phosphatase genes and are divided into three classes (Stoker 2005; Tonks 2006):

- The classical phosphotyrosine (pTyr)-specific phosphatases, which include transmembrane receptor-like proteins and nontransmembrane cytoplasmic protein tyrosine phosphatases.
- Dual-specificity phosphatases (DSPs) and,
- The low-molecular-weight tyrosine phosphatases.

Transmembrane receptor-like proteins are associated with the plasma membrane and regulate signalling through ligand-activated dephosphorylation. These phosphatases are involved in the processes of cellular adhesion and communication. The non-transmembrane cytoplasmic tyrosine phosphatases PTPs are located in various subcellular compartments, such as the cytosol, plasma membrane and the endoplasmic reticulum and do not present transmembrane segments. Some of these phosphatases are also involved in mechanisms of cell adhesion and motility (Zhang et al. 2004).

Another type of tyrosine phosphatases are DSPs. These proteins present smaller catalytic domains than the classical tyrosine phosphatases and are called 'dual' because their activation site accommodates phosphoserine (pSer)/phosphothreonine (pThr) residues and pTyr residues in proteins. Within this class, we find mitogen-activated protein kinase (MAPK) phosphatases (MKPs). These phosphatases inactivate MAPKs through dephosphorylating tyrosine and threonine phosphorylation sites, and participate in MAPK-dependent signalling pathways (Kondoh and Nishida 2007; Tonks 2006). We must remember here that Awda and Buhr (2010) reported that cAMP/PKA and PKC interact with the MAPK/ERK pathway. Another member of this class, VHY, has been found in spermatocytes and in spermatids but not in mature spermatozoa (Alonso et al. 2004).

Finally, it is worth noting that some MKPs are degraded by the ubiquitin–proteasome pathway (Choi et al. 2006). For this reason, the proteasome pathway has been suggested to degrade sperm protein phosphatases (Signorelli et al. 2012).

7.9.4.2 Serine/Threonine Phosphatases

Three different gene families encoding Ser/Thr specific have been reported so far:

1. Protein phosphatases depending on Mg^{2+} or Mn^{2+} (PPM) (e.g. pyruvate dehydrogenase) (Barford et al. 1998)
2. Transcription-factor-IIF-associating C-terminal domain phosphatases (FCP) (e.g. FCP1 and small C-terminal domain phosphatases 1–3) (Gallego and Virshup 2005)
3. Phosphoprotein phosphatases (PPP) that share high homology in the catalytic domains but differ in their N- and C-terminal domains (Barford et al. 1998; Fardilha et al. 2011).

Interestingly, PPP bind to a variety of substrates, are found in various cell types and are involved in the regulation of many cellular functions (e.g. PP1 and PP2 are involved in regulation of the metabolism, cell cycle, cell signalling, muscle contraction, translation or apoptosis) (Cohen 1989, 2002; Millward et al. 1999; Janssens and Goris 2001).

PP4 has been found in the centrosomes of mammals (Sumiyoshi et al. 2002), and PP6 has been detected in the testis of men and bulls (Bastians and Ponstingl 1996).

7.9.4.3 The Specific Case of Phosphatases in Mammalian Spermatozoa

Sperm phosphatases play an important role in the acquisition of motility during sperm maturation (Chakrabarti et al. 2007a; Mishra et al. 2003) and in the acquisition of hyperactivated motility (Fardilha et al. 2011; Krapf et al. 2010) after ejaculation. However, in contrast to the vast knowledge about the involvement of protein kinases in sperm capacitation, little is known about whether protein phosphatases play any role during this process. Thus, even though it is well known that PKA-activation during capacitation leads to phosphorylation of Ser/Thr residues in sperm proteins, it has also been reported that regulating the phosphorylated state of these proteins also involves the participation of Ser/Thr phosphatases.

One of these Ser/Thr phosphatases is the calcium/calmodulin-dependent phosphatase, or calcineurin (PP2B), which was first identified at the postacrosomal region and tail of the spermatozoa of boar, dog, goat, mouse, bovine and sea urchin and is involved in the acquisition of sperm motility (Tash et al. 1988; Tash and Bracho 1994; Carrera et al. 1996). In addition, another phosphatase that has been identified in bovine and fowl spermatozoa is PP2A (Ashizawa et al. 2006; Vijayaraghavan et al. 1996). Despite the role of PP2B and PP2A still remaining unclear in sperm capacitation, Signorelli et al. (2011) recently suggested that, at least in humans, PP2A and PP2B may be involved in sperm capacitation (Signorelli et al. 2011).

Other studies have shown that another Ser/Thr phosphatase, known as PP1, is involved in sperm motility (Mishra et al. 2003). Specifically, this phosphatase has four isoforms of its catalytic subunit (PP1α, PP1β, PP1γ1 and PP1γ2), each one encoded by an individual gene. In the case of spermatozoa, Smith et al. (1996) were the first to observe that PP1γ2 was the main PP1 isoform in human sperm. Thereafter, other studies found PP1γ2 isoform was also present in mice, hamsters, and bulls (Chakrabarti et al. 2007b; Han et al. 2007).

In humans, PP1 is involved in sperm motility and capacitation, whereas PP2A only participates in capacitation, and PP2B regulates sperm motility and hyperactivation (Signorelli et al. 2012).

7.9.4.4 The Specific Case of Phosphatases in Boar Spermatozoa

Focusing on boar spermatozoa, we must say that data are quite scarce and the results that are available at the moment are very recent. Harayama and Nakamura (2008) conducted interesting experiments that compared the response in sperm

capacitation when spermatozoa were incubated with cBIMPS (a cAMP analogue), or with calyculin-A, which is a Ser/Thr phosphatase inhibitor. These authors observed an increase in the percentage of capacitated spermatozoa after incubation with cBIMPS, but not after incubation with calyculin-A.

In addition, Harayama (2003) also found that non-capacitated sperm incubated with cBIMPS presented Ser/Thr-phosphorylated proteins in the postacrosomal region. However, Ser-/Thr-phosphorylated proteins decreased at the postacrosomal region during capacitation, but increased towards the tail (Harayama 2003). In contrast, no changes in the fluorescence patterns associated to Ser-/Thr-phosphorylated proteins were observed in the postacrosomal regions and in the tail after incubation with calyculin-A.

Other reports have also demonstrated that one of the proteins located at the postacrosomal region of non-capacitated spermatozoa, which is phosphorylated when it is in an inactive state, is PP1. In addition, in the presence of cBIMPS, but not in that of calyculin-A, the rate of acrosome-exocytosed spermatozoa increases (about 50 %) (Adachi et al. 2008). Therefore, PP1 seems to play a critical role in boar spermatozoa by dephosphorylating proteins at the postacrosomal region. Specifically, it has been suggested that PP1 mainly suppresses premature acrosome reaction before and after ejaculation, and it is not directly involved in sperm capacitation.

7.10 The Role of the Ubiquitin–Proteasome System in Sperm Capacitation

7.10.1 Ubiquitin

The ubiquitin–proteasome system is the current mechanism of degradation in the most normal and abnormal intracellular proteins (Hochstrasser 1996; Goldberg 2003). In this mechanism, proteins are first marked for degradation by covalent linkages to multiple ubiquitin molecules.

Ubiquitin, an evolutionarily highly conserved 76-amino-acid protein, is covalently linked to proteins in a multistep process involving the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) enzymes. Polyubiquitin chains are assembled via an isopeptidic linkage between a lysine residue of the previous ubiquitin and the C-terminal Gly residue of the subsequent ubiquitin. Various multiubiquitin chains can be formed due to the presence of seven lysine residues in the ubiquitin molecule (Bedford et al. 2010). Chains of four or more ubiquitin moieties linked via Lys_{48} of ubiquitin are known to represent the usual signal for proteasome-mediated proteolysis (Hicke and Dunn 2003).

It is worth noting that the ubiquitination process is balanced with the process of deubiquitination, which is mediated by a number of enzymes. Once marked by polyubiquitin chains, proteins are rapidly degraded by the 26S proteasome. In addition, deregulation of ubiquitination/deubiquitination has been related to diseases in humans (Zhang et al. 2007a), and to male infertility (Martínez-Heredia et

al. 2008; Siva et al. 2010), since regulated ubiquitination marks sperm mitochondria for destruction in a current fertilisation process (see also Sect. 8.9).

Finally, we must also mention that ubiquitin also tags defective sperm during epididymal passage, in a mechanism that seems to mark the abnormal spermatozoa for proteolytic destruction (Sutovsky et al. 2001). This can be regarded as an ubiquitin-dependent sperm quality control that resides in the somatic cells of epididymal epithelium (Tengowski et al. 2007) and detects spermatozoa with fragmented DNA or with other defects (Sutovsky et al. 2002). Matching with this, increased sperm ubiquitin has been inversely associated with sperm concentration, motility and morphology in humans (Sutovsky et al. 2004a).

7.10.2 The Proteasome and its Regulation

The proteasome is involved in the degradation of the majority of intracellular proteins. Proteolytically active sites are within the 20S core particle, which is a cylindrically shaped structure formed by four stacked rings in a α7β7β7α7 pattern (Ciechanover 1998, 2005a, b, 2006; Ciechanover and Schwartz 1998). Subunits β1, β2 and β5 have proteolytic activity and display trypsin-like, chymotrypsin-like and caspase-like peptidase activity, respectively (Groll et al. 2001) (Fig. 7.6).

Apart from the core particle, proteasome presents a regulatory particle that contains six ATPases. This is important, since protein degradation is an ATP-dependent mechanism that needs the presence of ATP-hydrolysing proteins (Bedford et al. 2010). Although core particles can associate with one of two regulatory particles, there are also other activators that can bind the core particle-20S (Gallastegui and Groll 2010).

The function of 26S proteasome is modulated by phosphorylation/dephosphorylation of such ATPases, in a process that is mediated by PKA and phosphatases. Accordingly, phosphorylation of the ATPase subunits by PKA increases the chymotryptic and tryptic activity of proteasome and is reversed by phosphatase 1γ (PP1γ) (Zhang et al. 2007b). Working with cardiac proteasomes in murine species, Zong et al. (2006) observed an increase in their proteolytic activity after activation of PKA or inhibition of another phosphatase PP2A.

As stated above, tyrosine kinases (PKA) and phosphatases (PP1γ and PP2A) have been found in mammalian spermatozoa and phosphorylation, mediated by PKA and these phosphatases appear as a key mechanism for regulating the proteasome function. This background has led Signorelli et al. (2012) to suggest that the cAMP/PKA pathway regulates proteasome phosphorylation/activity in spermatozoa.

7.10.3 Phosphorylation and Proteasomes

The phosphorylation of a given protein can be a signal for its further degradation via the ubiquitin–proteasome pathway (Glickman and Ciechanover 2002;

Tanaka and Chiba 1998). Related to this, phosphatases have been reported to be targeted by the ubiquitin–proteasome system in somatic cells (Brush and Shenolikar 2008; Trockenbacher et al. 2001).

In the case of spermatozoa, Signorelli et al. (2012) have mentioned the relationship between protein phosphorylation and the proteasomal activity may also be interesting, for two reasons:

1. Proteasomes have been found in mammalian spermatozoa and they play a relevant role during fertilisation as discussed in the next Chapter (Morales et al. 2003, 2004, 2007; Sutovsky et al. 2000, 2004b; Zimmerman and Sutovsky 2009; Zimmerman et al. 2011).
2. Phosphorylation of some proteasome subunits exerts a significant regulatory role (Bose et al. 1999; Pardo et al. 1998; Rivett et al. 2001).

7.10.4 The Case of Sperm Capacitation

After identification of the sperm proteome, the ubiquitin–proteasome system has been reported to be involved in both capacitation and fertilisation (Baker et al. 2010; Sutovsky 2011; Zimmerman and Sutovsky 2009; see also Chap. 8). Indeed, proteasomes, which are protein complexes, have been located in the plasma membrane of the sperm head and neck in pig (Yi et al. 2007), human (Tipler et al. 1997; Wojcik et al. 2000; Morales et al. 2004), mouse (Pasten et al. 2005) and ascidian (Sawada et al. 2002) spermatozoa, and a function for this proteasome pool has been suggested by Yi et al. (2007) and Zimmerman et al. (2011).

As mentioned before, protein phosphorylation both on Ser/Thr and on Tyr residues is a hallmark of sperm capacitation (Bailey 2010). Related to this, proteins belonging to the ubiquitin–proteasome system have been seen to phosphorylate during the capacitation of boar, mouse and rat spermatozoa (Arcelay et al. 2008; Baker et al. 2010). These proteins were: the ubiquitin itself (Arcelay et al. 2008), the ubiquitin activating enzyme UBE (Baker et al. 2010), multiple proteasomal subunits (Arcelay et al. 2008; Baker et al. 2010), and the valosine-containing protein, which is involved in the ubiquitinated substrate presentation to the 26S proteasome (Geussova et al. 2002). In addition, Choi et al. (2008) observed differences in the electrophoretic patterns depicted by several 20S core subunits in capacitated and non-capacitated boar spermatozoa. Therefore, growing evidence clearly indicates that sperm proteasomes are involved in sperm capacitation.

In functional studies conducted with human spermatozoa, it was seen that when sperm became capacitated, the chymotrypsin-like activity of their 20S proteasomal core increased, and an increase of the phosphorylation of several proteasomal subunits both on Ser/Thr and on Tyr residues also took place (Kong et al. 2009). However, when inhibitors of ser/Thr and of Tyr kinases (PKA and PKC) were added to capacitation medium, the chymotrypsin-like activity of 20S diminished (Kong et al. 2009). Other complementary studies, also performed in human sperm,

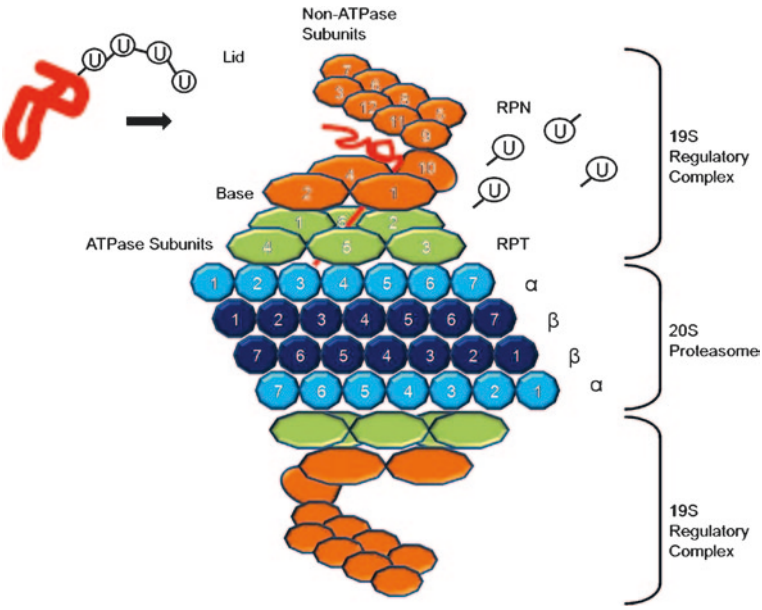

Fig. 7.6 Drawing of the 26S proteasome. This protein complex consists of the 19S regulatory complex and the 20S core, which contains two α- and two β-rings, each made of 7 subunits. On either side of the core resides there is a 19S regulatory complex; this complex recognises proteins bound to multiubiquitin chains, removes the multiubiquitin chains and primes the substrate proteins for degradation. Recognition of ubiquitinated proteins occurs primarily through subunit RPN10; upon recognition the ubiquitin molecules are released through the recruitment of deubiquitinating enzymes to the 19S complex, and the deubiquitinated protein is unfolded and transported into the 20S core (Zimmerman and Sutovsky 2009; Reproduced with permission)

demonstrated that epoxomicin, an inhibitor of proteasome activity, reduced Ser phosphorylation during sperm capacitation (Morales et al. 2007). Therefore, inhibitors of tyrosine kinases and PKA decrease the activity of the proteasome during capacitation, and proteasomes are phosphorylated during capacitation via tyrosine kinases and PKA (Kong et al. 2009; Morales et al. 2007).

According to Sutovsky (2011), all these findings open up the possibility that, although regulated itself by phosphorylation, proteasomal activity may directly or indirectly govern the activity of sperm-borne protein kinases. These events might not only occur at the sperm acrosome but also in the principal piece of the sperm tail, where proteasomes could exert an effect on the capacitation-related changes of sperm motility.

In short, it is quite clear that sperm proteasomes play an active role in the capacitation process and that proteasome activity is modulated by protein kinases. However, more research on the role of the ubiquitin–proteasome system in sperm capacitation is warranted since there are still several relevant aspects of this process that remain to be elucidated.

7.11 Regulation Mechanisms of Sperm Capacitation

7.11.1 Defective Regulation of Sperm Capacitation In Vivo

One of the most important aspects of the regulation of sperm capacitation within the oviduct is that spermatozoa have to remain uncapacitated and acrosome intact until they meet the egg. This crucial and well-coordinated event has been extensively described in the previous Chapter, when speaking about the importance of the sperm reservoir in mammalian and, thus, in swine reproduction (see Chap. 6).

In vivo, there are ligands that interact with G-protein coupled receptors (GPCRs) and inhibit the membrane-associated adenylyl-cyclases. This reduces the production of cAMP production in capacitated spermatozoa and prevents spontaneous/degenerative acrosome reactions (Fraser 2010). These ligands that inhibit spontaneous acrosome reaction have been found in mouse (Fraser et al. 2006), in boar (Funahashi et al. 2000a, b) and human (Fraser and Osiguwa 2004) spermatozoa. However, this inhibition of spontaneous acrosome loss does not prevent spermatozoa from undergoing acrosome reactions in response to either progesterone (Green et al. 1996) or the zona intact-oocytes (Fraser et al. 1997; Funahashi et al. 2000b).

Finally, it is worth mentioning that defective regulation of sperm capacitation may be related to infertility. Thus, in human sperm, Fraser and Osiguwa (2004) have observed higher percentages of capacitated and acrosome-reacted spermatozoa in infertile than in fertile men. This finding suggests that spermatozoa from infertile men may undergo accelerated capacitation and hence die before reaching an oocyte. Different causes could explain this phenomenon, such as a lack of decapacitation factors, lack of GPCR-modulators, or problems with GPCRs themselves (Fraser 2010).

7.11.2 The Role of 'Decapacitation' Factors

7.11.2.1 Introduction

One of the main disadvantages of studying sperm capacitation and fertilisation is that information about these crucial events comes from in vitro rather than in vivo studies. In vitro, some spermatozoa can undergo a degenerative/spontaneous acrosome reaction if damages like cryopreservation, short/cold shocks, or other environmental changes are infringed. However, acrosome-reacted spermatozoa have lost their fertilising ability, because they do not present the anterior plasma membrane that has the molecules needed for interacting with ZP-glycoproteins (Fraser 2010).

However, some aspects that avoid premature/degenerative acrosome reaction must be considered in vivo. Thus, despite spermatozoa not having intrinsic mechanisms to put a brake on this 'over-capacitation', seminal plasma and female tract

secretions contain several molecules that can interact with external receptors of sperm surface and inhibit spontaneous acrosome exocytosis (Fraser et al. 2003). This emphasises the relevance of spermatozoa keeping their fertilising ability by retaining intact their acrosomes while awaiting the oocyte (Fraser 2010).

Apart from the early sACY-dependent changes in sperm plasma membrane architecture, there are other changes that involve the loss, unmasking, or rearrangement of molecules on the sperm surface (Fraser 2010). Entities that are lost during process are usually referred to as 'decapacitation factors' (Bedford and Chang 1962; de Lamirande et al. 1997), because their addition to capacitated spermatozoa may reverse capacitation, causing cells to become 'decapacitated', i.e. they revert sperm to the non-fertilising state. With time, spermatozoa can recapacitate and regain fertility, indicating that capacitation itself is 'reversible'. Despite this, care must be taken when talking about 'decapacitation' and 'reversibility', since this often refers to early-capacitation events, and because some steps of this process, like membrane docking, are not reversible. Thus, it is worth noting that when capacitation is reversed by decapacitation factors there is no back addition of cholesterol to the plasma membrane, even though spermatozoa do not present fertilising ability (Fraser 2010).

To date, most of the knowledge about decapacitation factors has come from epididymal mouse spermatozoa. Indeed, Fraser (1984) reported more than two decades ago that when decapacitation factors were added to capacitating/capacitated spermatozoa, there was an inhibition of the sperm's fertilising ability. Spermatozoa could, however, regain fertilising ability when they were reincubated in a capacitation medium that did not contain decapacitation factors (Fraser 1984). Notwithstanding, DasGupta et al. (1994) observed that mouse decapacitation factors were also able to reverse the proportion of capacitated to uncapacitated status in human spermatozoa.

7.11.2.2 The Nature of Decapacitation Factors and Their Specific Receptors

According to Fraser et al. (1990), the decapacitation factor (DF) in mice is an anionic protein of about 40 kDa and stable to heating and proteolytic degradation. This molecule contains fucose residues, which are critical for the function that this factor exerts (Fraser 1998a). In fact, when fucose is added to a medium containing uncapacitated spermatozoa there is an increase in sperm capacitation and an increase in the sperm's fertilising ability. This has been observed both in mouse (Fraser 1998a) and human spermatozoa (Fraser and Osiguwa 2004).

More recent studies have demonstrated that the DF binds to a specific receptor that has fucose-binding sites and is attached to the sperm surface via a glycosylphosphatidylinositol (GPI) anchor (Fraser 2010). When the DF binds to its specific receptor, calcium ATPase is activated (Fig. 7.7). In contrast, when the DF is lost during capacitation, the activity of this ATPase is reduced and this leads to an increase in intracellular calcium levels (Adeoya-Osiguwa and Fraser 1996).

This explains why amounts of intracellular calcium increase during capacitation (Florman 1995).

A DF receptor (DF-R) presents high homology with phosphatidylethanolamine-binding protein 1 (PEBP 1) (Gibbons et al. 2005). In immunocytochemical studies, PEBP1 has been indentified on the head and the tail of both mouse and human spermatozoa. These studies have also shown that the fluorescence intensity in these regions varies, and this variation depends on the capacitation status of spermatozoa. In addition, these fluorescence patterns also change when decapacitation factors are added, and a reversion from capacitated to uncapacitated spermatozoa has, therefore, taken place (Gibbons et al. 2005).

7.11.2.3 Decapacitation Factors and Capacitation-Related Changes of Sperm Membrane

The presence/absence of decapacitation factors appears to cause conformational changes in the specific receptor (DF-R) of these factors (Fraser 2010). These conformational changes lead to alterations in the functionality of several membrane-associated proteins, such as GPCRs and calcium ATPase (Gibbons et al. 2005) (Fig. 7.7). This has been confirmed in a study conducted with mouse spermatozoa that assessed the role of adenosine receptors (Adeoya-Osiguwa and Fraser 2002). Related to this, we must indicate that mouse spermatozoa have two populations of adenosine receptors (GPCRs), stimulatory $A_{2\text{-}A}$ and inhibitory A_1, and that the function of these two receptors depends on their capacitation status. Thus, while the stimulatory $A_{2\text{-}A}$ is only active in uncapacitated spermatozoa, the inhibitory A_1 receptor only works in capacitated spermatozoa (Fraser and Adeoya-Osiguwa 1999).

Interestingly, Adeoya-Osiguwa and Fraser (2002) observed that when exogenous decapacitation factors are added to capacitated spermatozoa, A_1 receptors become unresponsive to A_1 agonists while $A_{2\text{-}A}$ receptors, previously unresponsive to $A_{2\text{-}A}$ agonists, are reactivated. Thus, when decapacitation factors are added to capacitated spermatozoa and they bind to their own specific receptors, there is an altered accessibility of adenosine binding sites in these receptors.

Other findings that support the idea that interaction of decapacitation factors with their specific receptors affects other membrane proteins are related to the organisation of sperm membrane. As previously mentioned, the membrane lipid microdomains (DRMDs) play a relevant role during docking/fusion between outer acrosome and plasma membrane. We should recall that these DRMDs are enriched in cholesterol and sphingolipids, and contain molecules that appear to mediate signal transduction pathways (Pike 2009; Tsai et al. 2007, 2010).

When spermatozoa are in vitro capacitated, there is a reorganisation of membrane architecture that affects these lipid rafts and the proteins that these DRMDs contain (Cross 2004; Van Gestel et al. 2005). In this regard, it is important to take into account that GPI-anchored proteins are frequently

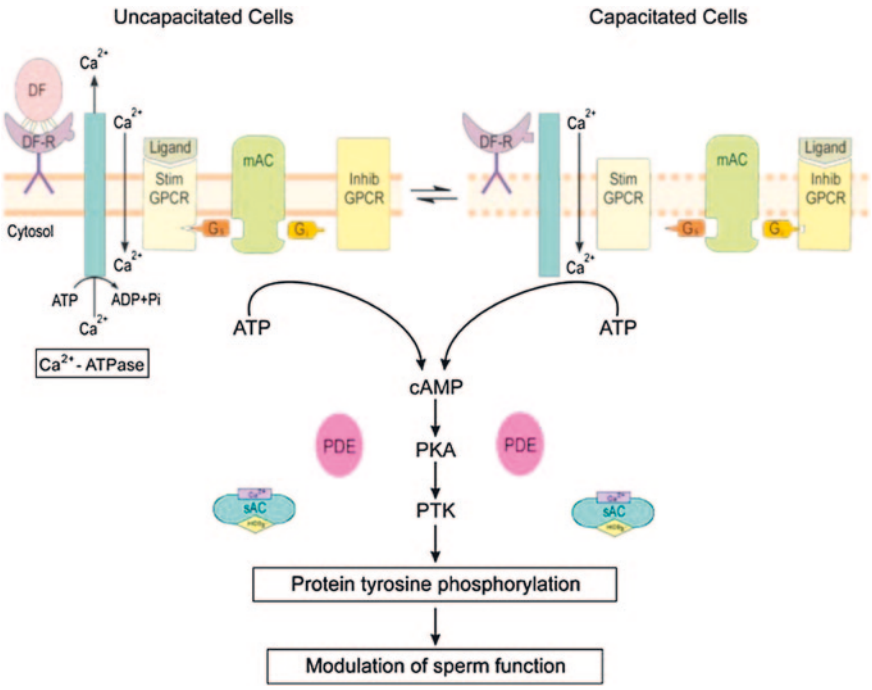

Fig. 7.7 Schematic diagram depicting the proposed mechanism of action of mammalian sperm DF during capacitation, resulting in altered availability of cAMP. The presence or absence of DF bound to its receptor DF-R (PEBP 1) and alters the conformation and function of various membrane-associated proteins, including Ca^{2+}-ATPase and GPCRs. If an appropriate exogenous ligand binds to a GPCR, the receptor will then interact with G_s or G_i to modulate the function of mAC/mACY, either stimulating or inhibiting cAMP production, respectively. In the cytoplasm, there is a sAC/sACY activated by Ca^{2+} and bicarbonate. The availability of cAMP within cells, reflecting the relative activities of mACs, sAC and phosphodiesterases (PDEs), will determine the activity of PKA and protein tyrosine kinase (PTK), which will in turn either stimulate or inhibit protein tyrosine phosphorylation and, thus, modify sperm function (Fraser 2010; Reproduced with permission)

associated with DRMDs (Lai 2003; Mayor and Riezman 2004; Pike 2009), and that a GPI anchor is involved in the attachment of the receptor of DF to the sperm plasma membrane (Gibbons et al. 2005). If we relate these two findings, we may explain the mechanism by which decapacitation factors act. Indeed, if the DF receptor is located in DRMDs and these lipid domains undergo conformation changes during capacitation, it is quite likely that the function of this receptor will be affected during capacitation. This hypothesis is supported by the more recent results provided by Asano et al. (2009). These authors have localised PEBP1/DF-R in DRMDs of sperm head and flagellum, which matches with previously mentioned results obtained in immunolocalisation studies and conducted with mouse and human spermatozoa (Gibbons et al. 2005).

7.11.3 Small Molecules that can Regulate Sperm Capacitation

7.11.3.1 Introduction

After being released from the sperm reservoir, the spermatozoa find an appropriate milieu to continue capacitation up to its completion (Fraser 2008). However, this step might lead some sperm cells to 'over-capacitation', resulting in spontaneous and undesired acrosome reactions, since only spermatozoa with an intact plasma membrane can interact with ZP and overcapacitated spermatozoa lose their fertilising ability. Accordingly, despite being important that spermatozoa become capacitated, the acrosome reaction has to be delayed until sperm do not interact with ZP. This issue will be discussed in the next subsections.

7.11.3.2 FPP, Adenosine and Calcitonin

The role of small molecules in sperm capacitation was first observed in the tripeptide pyroglutamylglutamylprolineamide (pGlu-Glu-ProNH_2) (Fraser 2008), later known as 'fertilisation promoting peptide' (FPP). This molecule is found in the seminal plasma at high concentrations (FPP is produced in the prostate gland), and comes into contact with spermatozoa upon ejaculation. FPP is structurally related to thyrotrophin-releasing hormone (TRH), elicits a biphasic response, since on the one hand it accelerates capacitation in uncapacitated sperm, but at the same time it inhibits spontaneous acrosome reactions in capacitated sperm (Fraser et al. 2003).

Apart from FPP, adenosine, calcitonin, and adrenaline have also been reported to be able to stimulate capacitation and also to inhibit acrosome reaction (Fraser 2008, 2010; Fraser et al. 2006; Adeoya-Osiguwa and Fraser 2005). In in vitro procedures, the addition of adenosine and FPP diminishes polyspermy in mouse and boar spermatozoa, since these two molecules also appear to accelerate sperm capacitation and to reduce the number of spontaneous acrosome reactions (Funahashi et al. 2000a, b).

Interestingly, the biological sperm response of these small molecules depends on their concentration. Thus, high levels of FPP, such as those found immediately after ejaculation, prevent sperm capacitation, but this concentration drops in the female reproductive tract after mixing with vaginal secretions and/or becomes less active due to the vagina-pH (Fraser 1998b, 2008). In addition, the concentration required to inhibit the spontaneous acrosome reaction is higher than that required for accelerating capacitation (Fraser and Adeoya-Osiguwa 1999). All these data also back the idea that these small molecules regulate sperm capacitation and acrosome reaction in a biphasic-manner accelerating capacitation but inhibiting acrosome reaction. The final effect of these molecules is the increase of fertilisation rates along with a decrease in monospermy rates, thereby helping the spermatozoa to maintain their fertilising ability until they reach the oocyte (Fraser 2010).

7.11.3.3 Action Mechanisms of Small Molecules that Regulate Sperm Capacitation

FPP affects cAMP levels, stimulating its production in uncapacitated sperm and inhibiting it in capacitated suspensions (Fraser et al. 2003). The mechanism of action involves the modulation of membrane-associated adenylyl cyclase (mACY) and cAMP production. In other studies, adenosine (Fraser et al. 2003), calcitonin (Fraser et al. 2006) and adrenaline (Adeoya-Osiguwa and Fraser 2005) also trigger the same response, i.e. stimulation of capacitation and fertilising ability, inhibition of spontaneous acrosome reactions, by regulating mACY and cAMP levels.

In the presence of the proper 'first messenger', the mammalian spermatozoon has a number of signal transduction pathways that can modulate the cAMP production (Fraser 2008). In the acrosomal cap region and in the flagellum, the mammalian spermatozoa present specific receptors for these small molecules, i.e. adenosine, calcitonin, adrenaline and FPP (Fraser et al. 2003, 2006; Adeoya-Osiguwa et al. 2006) (Fig. 7.8). Specific receptors for adenosine, calcitonin and adrenaline are GPCRs and are often involved in modulation of mACY activity in somatic cells, while the mechanism of action of the FPP receptor is not exactly known, but it has a synergistic stimulatory effect with adenosine that increases mACY activity in the sperm (Fraser 2010) (Fig. 7.8).

In the case of adenosine receptors (GPCRs), spermatozoa present both stimulatory $A_{2\text{-}A}$ and inhibitory A_1 receptors and work, as stated, in a capacitation state-dependent manner. Thus, stimulatory receptors only work in uncapacitated sperm while the inhibitory receptors only function in capacitated cells (Fraser 2008) (see also Sect. 7.11.3). Similarly, stimulatory β (β_1, β_2, and β_3) and inhibitory α (α_{2A}) adrenergic receptors are also present in the mammalian spermatozoa and their function changes also depend on capacitation status. Moreover, sperm cells only present one type of calcitonin receptor, even though they also function in a capacitation state-dependent manner (Fraser et al. 2003, 2006; Fraser 2008) (Fig. 7.8).

Although some authors considered that mammalian spermatozoa possess inhibitory-Gα subunits ($G\alpha_i$; required for inhibition of mACY-directed cAMP production), but lack stimulatory $G\alpha_s$ subunits ($G\alpha_s$; required for stimulation of mACY-directed cAMP production), biochemical, immunohistochemical and physiological studies have now shown that $G\alpha_s$ subunits are also present (Fraser et al. 2003; Spehr et al. 2004). In addition, cholera toxin enhances ADP-ribosylation of a protein that has a similar size to $G\alpha_s$ (Bentley et al. 1986; Kopf et al. 1986; Baxendale and Fraser 2003). Therefore, the signalling pathway components required for external ligands to activate GPCRs that regulate cAMP production via mACY are present in spermatozoa. Accordingly, stimulatory ($G\alpha_s$, $G\alpha_{olf}$) and inhibitory ($G\alpha_{i2}$) Gα subunits, $mACY_3$ and $mACY_8$ isoforms and several cyclic nucleotide phosphodiesterases (PDEs) have been detected close to the GPCRs (Fraser 2010). Specifically, these components are found both in sperm head and in sperm tail (Fraser et al. 2003; Fraser 2010), since PDE1 has been located in the flagellum and stimulates capacitation, and PDE4 has been identified both in the head and in the flagellum and inhibits capacitation (Fraser et al. 2006).

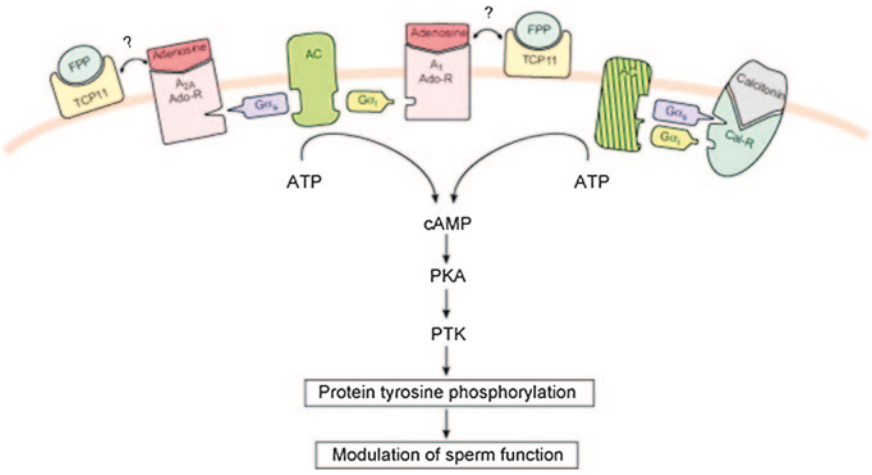

Fig. 7.8 Schematic diagram depicting the signal transduction pathways in sperm regulated by FPP, adenosine and calcitonin. These first messengers interact with their specific receptors to regulate mACY activity and cAMP production during capacitation (Fraser 2008; Reproduced with permission)

If we consider all these data, it appears that mammalian spermatozoa have several signalling pathways able to regulate mACY/cAMP. These pathways are triggered after binding of the specific receptors to the mentioned small molecules, which act as ligands (first messengers). In all cases, it seems that the activated pathway in uncapacitated spermatozoa leads to an increase in cAMP levels which, in turn, accelerates sperm capacitation with respect to the absence of the ligand (Fraser 2008). Later, when spermatozoa have become capacitated, the set of sperm membrane changes related to this process induce conformational alterations in the various receptors, so that the same ligand does not increase cAMP levels, but decreases them. This decrease leads to an inhibition of spontaneous acrosome reaction and ensures the maintenance of sperm fertilising ability, thereby maximising the number of potentially fertilising spermatozoa in the vicinity of unfertilised oocytes. As mentioned before, the first messengers involved in this mechanism would be the small molecules that are present in various body fluids and for which the spermatozoa present specific receptors (Fraser et al. 2006; Fraser 2008).

Finally, it is worth noting that in a possibly wrong approach, caffeine has been usefully added to IVF media to increase the fertilisation rate. However, the main problem of this methylxanthine is that it causes an unregulated cAMP increase by inhibiting PDEs, so that when intracellular cAMP reaches a threshold level, this can trigger spontaneous/degenerative acrosome reactions (Fraser 2010). In contrast, adenosine regulates cAMP and keeps cells acrosome-intact, suggesting that it should be possible to use lower sperm concentrations than those routinely used with caffeine-treated suspensions (Funahashi et al. 2000b). Thus, the addition of adenosine and FPP does not only increase fertilisation rates, like caffeine, but also

the rates of monospermic penetration (Funahashi and Nagai 2001; Funahashi and Romar 2004; Suzuki et al. 2005). This warrants the use of these small molecules to improve the success of assisted reproduction techniques in mammals.

7.11.4 Role of Dopamine Type 2 Receptor in the Modulation of Sperm Capacitation

In the oviduct of humans (Helm et al. 1982), swine (Chaud et al. 1983), rabbits (Khatchadourian et al. 1987), and cattle (Kotwica et al. 2003), there are high levels of catecholamines (the most abundant are adrenaline, noradrenaline and dopamine). These levels of catecholamine vary according to the oviductal region and to the phase of the oestrous cycle, thereby suggesting that they are under hypothalamo-pituitary control (Ramírez et al. 2009).

High levels of catecholamines have also been found in human semen (Fait et al. 2001), and mammalian spermatozoa also present receptors for these neurotransmitters (Meizel 2004). Indeed, male rat germ cells (Otth et al. 2007) and spermatozoa from boar, rat, mouse, human and bull express dopamine type 2 (D2)-like receptors dopamine type 2 receptor (DRD2) (Ramírez et al. 2009). This indicates that the presence of this dopamine receptor is highly conserved in mammalian spermatozoa and also suggests that DRD2 is a potential target for endogenous dopamine. In addition, Ramírez et al. (2009) have also demonstrated that boar, human, mouse, bull and horse spermatozoa have a catecholaminergic phenotype and are a sensitive cellular target for cocaine, amphetamine, and antidepressant and antipsychotic drugs.

One of the most abundant catecholamines in the body of swine and other mammals, together with adrenaline and noradrenaline, is dopamine. This catecholamine is a neurotransmitter within the mammalian central nervous system that has an important role in functions such as cognition, emotion and motor activity control (Aumann and Horne 2012). Alteration of certain elements of the dopaminergic system has been associated with neurological and psychiatric disorders such as Parkinson's disease (Picconi et al. 2012) and schizophrenia (Yin et al. 2012). In addition, dopamine seems to act as a physiological modulator of capacitation, viability and motility of mammalian spermatozoa, as studies conducted in porcine have shown (Ramírez et al. 2009).

Receptors of dopamine are seven-transmembrane trimeric guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors, classified into distinct subfamilies based on their pharmacological characteristics and their sequence homology. Examples of dopamine are the dopamine type 1 (D1)-like receptors (DRD1 and DRD4) and the D2-like receptors (DRD2, DRD3, and DRD4) (Missale et al. 1998). Stimulation of D1-like receptors activates adenylyl cyclase by coupling to stimulatory GTP-binding regulatory protein (G_s protein). This increases cAMP accumulation and activates the cAMP/PKA pathway (Himmler et al. 1993; Das et al. 1997). In contrast, stimulation of D2-like receptors that

show high affinity for antipsychotic drugs inhibits adenylyl cyclases by coupling to inhibitory GTP-binding regulatory protein ($G_{i/o}$). In this case there is a decrease rather than an increase in PKA activity (Missale et al. 1998).

Catecholamines and phenylpropanolamines, which act as potent and selective releasing agents of adrenaline, noradrenaline and to a lesser extent of dopamine and function as endogenous catecholamines, modulate sperm capacitation and acrosome exocytosis. Indeed, phenylpropanolamines have been found to accelerate capacitation but inhibit spontaneous acrosome reaction in mouse spermatozoa (Adeoya-Osiguwa and Fraser 2005). Catecholamines play a relevant role in the later stages of sperm capacitation, since adrenaline, noradrenaline and isoproterenol increase the number of hamster sperm with hyperactivated motility (Cornett and Meizel 1978) and the proportion of spontaneous acrosomal exocytosis in hamster (Cornett and Meizel 1978; Meizel and Working 1980) and in bull spermatozoa (Way and Killian 2002). The exact role of these catecholamines is, however, not exactly known, since at lower concentrations noradrenaline induces sperm capacitation, but at higher concentrations it prevents capacitation in bull spermatozoa (Way and Killian 2002). Related to this, Schuh et al. (2007) have demonstrated that adenosine (see also Sect. 7.11.3), adrenaline and noradrenaline increase the velocity of the flagellar beat by a non-receptor-mediated mechanism. This means that catecholamines could enter by sodium- and chloride-dependent transport mechanisms. These catecholamines might then inhibit phosphodiesterase that, in turn, would increase the levels of cAMP (Ramírez et al. 2009).

As previously mentioned, changes in tyrosine phosphorylation following increases in cAMP levels and the activation of PKA have been proposed as the key mechanisms by which sperm capacitation and acrosome reaction are regulated (Visconti et al. 1995a, b; Bajpai and Doncel 2003). In the case of boar spermatozoa, Ramírez et al. (2009) have observed that dopamine exerts an effect on sperm capacitation, specifically on tyrosine phosphorylation, but does not induce acrosome loss. Again, however, the role of this catecholamine is dual and depends on its concentration. Thus, at higher concentration, dopamine decreases sperm motility and the tyrosine phosphorylation pattern of sperm proteins, by a transporter-mediated mechanism for catecholamine uptake (Ramírez et al. 2009). Related to this, an excess of products from dopamine oxidation are toxic for sperm and appear to cause the inhibitory effects of dopamine on tyrosine phosphorylation and sperm motility (Ramírez et al. 2009). Conversely, at low concentrations, dopamine increases sperm viability and total and progressive motility at 2 h of capacitation through DRD 2 activation. The activation of DRD increases AKT-phosphorylation at Serine-473 (Ser_{473}) residue as occurs in neurons (Brami-Cherrier et al. 2002; Kihara et al. 2002; Nair and Sealfon 2003; Ramírez et al. 2009). This observation indicates that dopamine seems to play a significant role in maintaining sperm viability at early stages of the capacitation process.

Although it has been reported that the activation of DRD2 entails the reduction of cAMP levels, as has been seen in rat ovaries (Hall and Strange 1999), DRD2 also modulates $G\alpha_s$, a subunit protein that couples the cannabinoid receptor CB1 (Jarrahian et al. 2004), which is present in human (Rossato et al. 2005) and boar

(Maccarrone et al. 2005) spermatozoa. In this regard, Ramírez et al. (2009) have proposed that the modulation of the interaction of DRD2 and CB1 could affect the cAMP levels before and after the onset of sperm capacitation, in a manner that might be independent of the presence of progesterone.

Finally, it is worth noting that the association of DRD2 with tyrosine phosphorylated proteins, which still remain unidentified, increases during sperm capacitation, and this occurs independently from DRD2 activation. In fact, the localisation of both DRD2 and tyrosine phosphorylated proteins depends on the capacitation status (Tardif et al. 2001; Ramírez et al. 2009). Therefore, in uncapacitated spermatozoa, DRD2 is found in the flagella and pTyr proteins are predominantly localised in the post-equatorial area of the head. In capacitated spermatozoa, DRD2 is present in the flagella but also in the acrosomal region of sperm head, whereas the presence of tyrosine phosphorylated proteins increases in the midpiece and the acrosomal region co-localised with DRD2 (Ramírez et al. 2009). All these findings suggest the presence of a signalling complex that contains DRD2 and tyrosine phosphorylation target proteins and seems to play a relevant role in energy control and in the modulation of sperm capacitation and motility, and in acrosome reaction.

7.12 In Silico Studies and the Coordinating Role of Actin in Capacitation Network

To conclude this chapter, we focus now on recent and novel studies dealing with the coordination of the subcellular signalling process during boar sperm capacitation. These studies have used computational models, also known as in silico models, and have later been confirmed by in vitro experiments (Bernabò et al. 2011). From these results, the actin cytoskeleton has been suggested to play a key role in signalling capacitation, apart from mechanically supporting the cell. The actin cytoskeleton appears thus to be a node that coordinates signalling cascades of different intracellular compartments during capacitation.

By means of this model, these authors have also found the functional meaning and the localisation of all the molecules involved in sperm capacitation and the nodes that interconnect the intracellular compartments. They have identified three key nodes: the intracellular levels of calcium, the ATP levels and 'actin polymerisation'. From these three elements, they have observed that two of them (intracellular Ca^{2+} and ATP) were linked with a high number of links, thereby working as ubiquitous second messengers (as in the case of intracellular calcium; 28 links), or as metabolic sustainment (as in the case of ATP; 15 links). The same group previously reported that ablation from the network provokes the collapse of network structures (Bernabò et al. 2010b).

As far as the third node (actin polymerisation) is concerned, Bernabò et al. (2011) have found a lower number of links (eight) relating to all the subcellular compartments involved in capacitation (cytosol, cytoskeleton, mitochondria

and acrosome). In contrast, it is worth noting that these authors have not found actin polymerisation to be related to the sperm nucleus during capacitation. This seems logical since the nucleus is the only compartment of the spermatozoon that remains stable during capacitation. Interestingly, the actin polymerisation links during capacitation have also been indentified and they are the following (Bernabò et al. 2010b):

- Phosphatidic acid (i.e. the product of phospholipase D). This can be explained by the dependence of actin polymerisation on the activation of phospholipase D. This activation takes place via the bicarbonate/cAMP/PKA pathway or via the G-protein coupled receptor (GPCR)/PKC pathway (Breitbart et al. 2005).
- ATP. Actin polymerisation is an ATP-dependent process. Moreover, this molecule is the most important molecule of sperm energy metabolism. In the sperm cells, the energy production depends on glycolysis and/or on mitochondrial oxidative (Storey 2008; see also Chap. 2).
- F-actin and G-actin.
- Levels of intracellular calcium. Intracellular calcium is a key second messenger that is involved in sperm capacitation, amongst other processes.
- Protein synthesis. In the sperm cells when the mitochondrial-type ribosomes, which translate nuclear-encoded proteins, are blocked, actin polymerisation is inhibited (Gur and Breitbart 2006).
- The fusion of outer acrosome and plasma membranes (see also Sect. 7.7.3 and Chap. 8).

From the different nodes, actin polymerisation is the most linked node and interacts with three main nodes of sperm capacitation: phospholipase D, ATP and intracellular calcium levels. For this reason, Bernabò et al. (2011) have suggested that since the actin polymerisation node is the most linked node, it could reach all the subcellular compartments affecting the whole signal transduction system of spermatozoa. This hypothesis is backed by the effects of removing the actin polymerisation node in the in silico model. In this case, some nodes involved in sperm capacitation, such as membrane fusion, F- and G-actin and mitochondrial protein translation, are excluded from the capacitation network, while others are unaltered. Thus, blocking actin polymerisation during sperm capacitation would impede the fusion of plasma and outer acrosome membranes, thereby leading the spermatozoa unable to undergo acrosome reaction.

All these events predicted by the computational model were confirmed by the same authors, by inhibiting, under capacitating conditions, actin polymerisation in spermatozoa with cytochalasin D (Bernabò et al. 2011). This inhibition led the spermatozoa unable to undergo the acrosome reaction, in the presence of solubilised zona pellucida, which is well-known for inducing acrosome reaction, as described in Chap. 8.

In contrast, the presence of cytochalasin D did not inhibit other capacitation nodes. Thus, typical membrane remodelling which then enables the fusion of plasma and outer acrosome membranes, was not affected by the ablation of actin polymerisation in the presence of cytochalasin D. Furthermore, the inhibition of

actin polymerisation did not affect either the characteristic protein phosphorylation pattern of boar capacitated spermatozoa or the translocation of the phospholipase C-γ1 (PLC-γ1). Phospholipase C-γ1 is involved in coupling actin cytoskeleton and membrane dynamics with the calcium metabolism. Thus, upon activation, PLC-γ1 migrates from the cytosol to the plasma membrane (Spungin and Breitbart 1996), where it hydrolyses PIP2 (phosphatidylinositol 4,5-bisphosphate; PI(4,5)P2) to IP3 (inositol 1,4,5-triphosphate), a calcium mobilising second messenger, and DAG (diacylglycerol), which in turn activates the protein kinase C.

Finally, the inhibition of actin polymerisation did not affect calcium metabolism either. However, intracellular calcium plays a key role in sperm capacitation, as has been widely discussed in this Chapter. Indeed, we remember here that intracellular calcium participates in the capacitation-signalling cascade, translating the extracellular stimulation to activation of other molecular systems, such as PKA and protein kinase C pathways. When sperm cells are completely capacitated, they can respond with a high increase in intracellular calcium to the interaction with the zona pellucida of oocyte, thereby triggering acrosome reaction (see also Chap. 8).

Remodelling of plasma membrane permits its fusion with outer acrosomal membrane (Breitbart et al. 2005; see also Sects. 7.6 and 7.7), but the actin network impedes the fusion of both membranes. When sperm capacitation has been completed, and acrosome exocytosis has taken place induced by ZP-proteins, an induced high increase in intracellular calcium depolymerises the actin network, thereby allowing the fusion of plasma membrane with outer acrosome membrane. However, the main concern of the interesting study conducted by Bernabò et al. (2011) is that they did not observe an increase in acrosome-reacted spermatozoa when incubating the sperm cells with cytochalasin D. This should have been expected if the role of the actin network in separately maintaining both membranes had been crucial. In the future, further research should address this concern and other aspects that remain to be elucidated.

To date, and from the literature currently available, we can suggest that actin polymerisation appears to play a major coordination role in all the cellular districts involved in capacitation (Bernabò et al. 2010b, 2011). This hypothesis is in agreement with the newly emerging evidence that in different cellular systems the cytoskeleton not only plays a mechanical support function, but it also participates in cell signalling, especially if one considers the surface of cytoskeleton filaments on which proteins and other cytoplasmic components can dock (Janmey 1998). It is thus possible to hypothesise that another level of cell function regulation, through the diffusion along cytoskeletal networks, exists, this being an alternative to the other known route of intracellular signal transduction, such as intracellular calcium or ATP (Shafrir et al. 2000; Forgacs et al. 2004).

In short, spermatozoa behave as complex systems, also in terms of their capacitation and their ability to bind and fuse with the oocyte. Thus, spermatozoa acquire their fertilising ability when they are considered in their entire signalling network, in such a way that if a single element of the network is unperturbed by an external factor, but the coordination among all elements is broken, the spermatozoon is unable to respond to ZP-proteins.

7.13 Conclusions

Around ovulation, spermatozoa are released from the oviductal epithelium and become free within the oviductal fluid. Then, spermatozoa continue capacitation and, at an early step of this process, AQN-1 is shed from the surface. This allows the unmasking of three proteins (AWN, AQN-3 and P47/SED1) that are also attached to the sperm surface and mediate the interaction with zona pellucida. During capacitation, sperm cells undergo different changes that affect all the subcellular compartments, and can be divided into early/fast and late/slow events. Within these changes, we can find the increase in certain intracellular messengers, such as Ca^{2+} and cAMP, and the reorganisation of proteins and lipids of sperm plasmalemma. Finally, the sperm plasma and the outer acrosome membranes become more unstable and gradually acquire the ability to fuse with each other. Fusion between these two membranes will only take place when the spermatozoon interacts with the zona pellucida of the oocyte, as we will see in the next chapter.

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Chapter 8
Boar Spermatozoa Within the Oviductal Environment (III): Fertilisation

Marc Yeste

Abstract This chapter is the last of a series of four chapters about the transit of boar spermatozoa within the female reproductive tract and deals with the fertilisation events that take place during and after a capacitated spermatozoon interacts with the ZP of the oocyte. Thus, we will focus on the mechanisms of gamete binding and interaction, including acrosome exocytosis, ZP-penetration and fusion between sperm and oocyte plasma membranes. In addition, subsequent events that take place after sperm-oocyte fusion, such as sperm chromatin remodelling and sperm-mediated oocyte activation will also be dealt with. To conclude, other novel aspects about the role played by sperm in post-fertilisation and early embryo development events will also be discussed.

8.1 Introduction

Fertilisation is the process by which two haploid gametes, namely the spermatozoon and the oocyte, unite to produce a genetically distinct individual (Brewis et al. 2005; Signorelli et al. 2012).

In mammalian species, fertilisation involves a number of sequential steps (Yanagimachi 1994a, b) (Fig. 8.1):

1. Sperm migration through the female genital tract (see Chaps. 5, 6 and 7)
2. Sperm penetration through the cumulus cells
3. Sperm adhesion and binding to the zona pellucida (ZP)
4. Acrosome exocytosis
5. Sperm penetration through the ZP
6. Fusion of sperm plasmalemma to oolemma.

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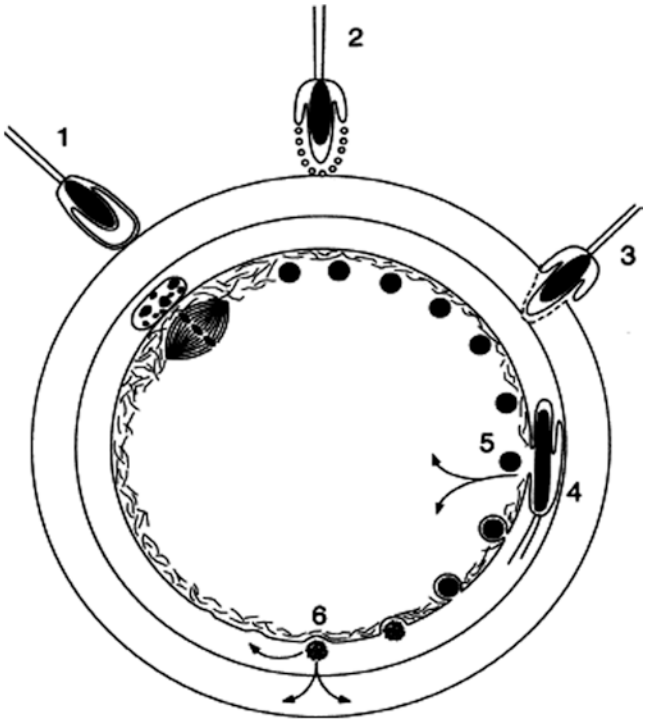

Fig. 8.1 Schematic representation of the sequence of interactions between the male and female gametes leading to fertilisation: (*1*) sperm binding to the ZP (restricted to the apical ridge subdomain area), (*2*) the acrosome reaction (fusions restricted to the apical ridge and pre-equatorial subdomain areas), (*3*) the penetration of the zona pellucida (equatorial membranes remain intact and that the mixed vesicles resulting from the acrosome reaction are shed of the penetrating sperm, see also Chap. 9), (*4*) binding and fusion with the oolemma (restricted to the equatorial subdomain area), (*5*) activation of the fertilised oocyte by a soluble sperm factors, and (*6*) polyspermy block by the cortical reaction (Boerke et al. 2008) Reproduced with permission

In short, at fertilisation, the capacitated mammalian spermatozoon binds in a specific manner to the ZP (outer coating of the egg), and in response to this contact exocytosis its acrosome (Töpfer-Petersen et al. 2008). It then penetrates the ZP by means of a combination of flexured ('hyperactive') motility and the activity of released or unmasked acrosomal hydrolases. Finally, the spermatozoon binds to the oolemma and enters the ooplasm (Barroso et al. 2009). Once fertilised, the oocyte is called a zygote.

Recently, Mugnier et al. (2009) identified four key elements of the fertilisation process from in vitro studies:

(a) During sperm-ZP binding, the composition and structure of the ZP play a critical role.
(b) The capacity of spermatozoa to undergo the acrosome reaction is essential in this step of fertilisation.

(c) The plasma membrane of the oocyte is crucial in the mechanism of gamete fusion.
(d) The competence of the ooplasm is a major element in pronuclei formation.

8.2 Ovaries and Ova Transport

8.2.1 The Ovaries

Ovaries are the primary structures of the female reproductive tract that perform two main functions: to produce the female germ cells (i.e. the oocytes) and to synthesise and release oestrogens (oestradiol, oestrone and oestriol) and progesterone. They are suspended from the abdominal roof by long mesovaria and each ovary is surrounded by a thin membrane called the infundibulum, which acts as a funnel to collect ova and divert them to the oviduct (Hafez 1993).

With regard to the hormonal control of the ovaries, the hypothalamus located at the basis of the brain secretes the gonadotropin-releasing hormone (GnRH) which regulates the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinising hormone (LH) into the blood. These two gonadotropins stimulate the production of the ovarian hormones, oestrogens and progesterone, which in turn regulate the reproductive process (Espey and Lipner 1994; Madej et al. 2005).

In the pig, ovaries are approximately 5 cm long and irregular in shape, due to numerous follicles and/or corpora lutea protruding from the surface, as occur in cyclic animals. The oocytes are contained within follicles in different stages of development—primary, secondary and antral follicles. Antral follicles may undergo further development into mature follicles that ovulate (Espey and Lipner 1994).

8.2.2 Ovulation and Transport of Ova

Ovulation is the rupture of a mature follicle at the surface of the ovary, thereby releasing an oocyte surrounded by cumulus cells (COCs) into the oviduct, and is initiated by an increase in gonadotropic hormones (Richards et al. 2002). Then, a set of structural changes in the follicular wall and vascular microcirculation takes place, due to the action of proteolytic enzymes and prostaglandins (Espey and Lipner 1994; Brüssow et al. 2008). After this, the oocytes are transported by the cilia-covered fimbria towards the ampulla, in a process that takes about 30–45 min (Brüssow et al. 2008), and the ova spend about 2 days in the oviducts (Mwanza et al. 2002a, b). Finally, the cumulus cells disperse within 6 h after ovulation (Hunter 1984; Brüssow et al. 2008). This process is influenced by the presence of spermatozoa in the oviduct (Rodríguez-Martínez et al. 2001).

The mechanism of ova transport and its regulation still remains unknown, but it has been hypothesised that myosalpingeal peristalsis (Rodríguez-Martínez

et al. 1982; Mwanza et al. 2002b), cumulus cells and extracellular matrix, which increase the size of the egg plug making ciliary beating, are involved (Talbot et al. 1999).

8.3 An overview of Fertilisation and Early Embryonic Development

8.3.1 A General View of Fertilisation

As occurs for all sexually reproducing species, the fertilisation of an oocyte is the completion of the role of the sperm and involves the creation of a new individual (Töpfer-Petersen et al. 2008). In mammalian species, fertilisation is an internal process that takes place in the upper third of the oviduct, specifically in the ampullary-isthmic junction. In swine, the success rate of this event is about 100 % when a fertile boar mounts the female at the correct time relative to ovulation (Hafez 1993).

As widely explained in Chap. 6, the oviduct plays a crucial role in the events leading to fertilisation, since it first stores the spermatozoa until an ovulated oocyte is released forming the sperm reservoir, and then capacitates the spermatozoa before they bind the ZP-glycoproteins (Darson et al. 2007; Salicioni et al. 2007). In Chap. 7, the importance of sperm capacitation before binding to ZP has been extensively discussed.

Binding and interaction of a capacitated spermatozoon with an ovulated oocyte start as a coordinated series of events that lead to the formation of the diploid zygote and initiates embryonic development. This process begins when a capacitated spermatozoon interacts with the ZP of the oocyte, which is a surrounding extracellular matrix that contains glycoproteins. This event, like sperm binding to oviductal epithelial cells, is mediated by carbohydrate interactions (see also Sect. 6.7). In this case, two different binding phases can be distinguished, and both involve carbohydrate mediation (Töpfer-Petersen 1999; Wassarman 2005; Wassarman et al. 2005; Töpfer-Petersen et al. 2008) (Figs. 8.1 and 8.2):

(a) Primary binding that consists of recognition between the capacitated spermatozoon and the ZP-oocyte and triggers the acrosome reaction.
(b) Secondary binding, when an acrosome-reacted spermatozoon interacts with the oolemma. After this secondary binding, the cortical reaction occurs and the ZP is hardened to impede polyspermia (Funahashi et al. 2000; Funahashi and Nagai 2001).

After the acrosome reaction, the equatorial region of the sperm membrane is exposed, adheres to and fuses with the oolemma, facilitating the introduction of the sperm nucleus into the oocyte. Once in the ooplasm, the sperm nucleus undergoes several structural changes that involve chromatin remodelling (Ajduk et al. 2006). This step leads the oocyte, stopped at Metaphase II, to complete the

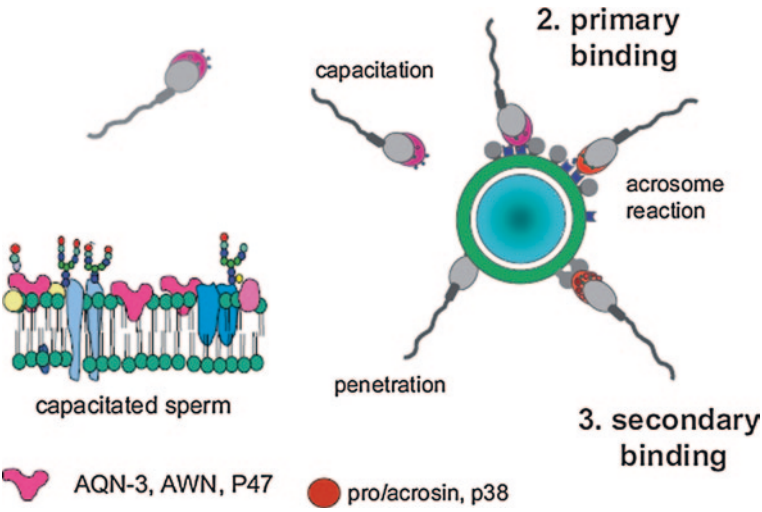

Fig. 8.2 Sperm-ZP binding. Capacitated spermatozoa binds to ZP by recognising a set of neutral complex N-glycans (primary binding). Upon binding, the acrosome reaction is induced and the acrosome reacted spermatozoa binds to polysulfated glycan structures of the zona pellucida via pro/acrosin, p38 and others (secondary binding) (Töpfer-Petersen et al. (2008) Reproduced with permission)

second meiotic division. The next step is the migration of pronuclei and chromosome alignment when the two haploid nuclei (called pronuclei) come together and combine their chromosomes into a single diploid nucleus (Yanagimachi 1994b; Töpfer-Petersen et al. 2008). In the case of mammals, the two pronuclei do not fuse directly as they do in many other species like *Xenopus laevis* (Edwards and Beard 1997). Instead, they approach each other (pronuclear apposition), but remain distinct until after the membrane of each pronucleus has broken down, preparing for the first mitotic division of the zygote (Alberio et al. 2001; Alberts et al. 2008; Snook et al. 2011).

8.3.2 Some General Concepts Related to Early Embryonic Development

Embryos remain for a long period of time (up to 26 h) at the one-cell stage, but only between 6 and 8 h in the 2- and 4-cell embryo stage (Hunter 1984; Brüssow 1985). The early cleavage of embryos takes place in the isthmus and embryos usually leave the oviduct about 50–60 h post-ovulation (Brüssow et al. 2008).

About 2 days after fertilisation, embryos enter the uterus. They are at the 4–8 cell stage and begin to space themselves at equal distances in the uterine horns. It is worth noting that blastocysts can migrate from one horn to the other during this stage (Hafez 1993).

One of the most critical periods of pregnancy is from approximately 11–16 days after mating. At this time, the blastocysts elongate into long, stringy masses and they begin to attach to the uterine wall. During this stage of development, the greatest potential loss in litter size may occur because of attachment failures. In fact, some factors have been suggested to limit the number of blastocysts that can attach in a given uterus. In this regard, embryonic survival might be related to certain uterine secretions which have yet to be elucidated. On the other hand, stressing factors, such as high temperatures or animal regrouping, can also negatively influence both implantation and embryo survival. In addition, a minimum number (four) of the implanted blastocysts in the uterus is required for maintaining pregnancy (Hafez 1993; Brüssow et al. 2008).

By days 25–35, the length of the embryo is between 2.5 and 3.8 cm, and the major body systems are well formed. Each embryo is surrounded by a separate series of fluid-filled membranes, the amnion and chorion, which comprise the placenta. These membranes help to protect and nourish the growing embryo. Nutrients, waste, gases and certain antibodies cross the membranes between the dam and embryo blood systems (Pusateri et al. 1990; Bazer et al. 2012).

The foetal period begins at approximately day 36. Gender may be easily determined by external examination and the main systems of the body are still better defined. Foetal orientation is random; some are head to head, some are tail to tail and some are head to tail. At farrowing, about half are born tail first and half are born head first. Embryos which die before day 35–40 are usually reabsorbed by the dam. However, progressive calcification of the skeleton begins to occur from day 36 onwards and deaths taking place after this point result in mummification (Hafez 1993; Knobil and Neil 1994; Bazer et al. 2012).

At day 109, the weight of foetuses is between 0.6 and 0.8 kg and at farrowing this weight reaches 1.2–1.6 kg. On the other hand, the uterus gradually enlarges over gestation from about 0.8–1.2 kg at mating to up to 24 kg, including foetal contents, during the last week of pregnancy. In fact, some females lose up to 10–11 % of their body weight at parturition.

8.3.3 Effect of 'Stressful' Events After Ovulation

The effects of stressful conditions on post-ovulatory events (fertilisation, ova transport, ova cleavage rate and number of spermatozoa attached to the ZP) have also been investigated by fasting or ACTH administration (Mburu et al. 1998; Mwanza et al. 2000a, b; Razdan et al. 2001, 2002; Einarsson et al. 2008).

Food deprivation for 2 days immediately after ovulation reduces the numbers of viable spermatozoa in the sperm reservoir, and delays the cleavage rate of fertilised ova (Mburu et al. 1998). Fasting for 2 days after ovulation elevates the plasma levels of cortisol and $PGF_{2\alpha}$, decreases oestradiol-17β levels and raises the isthmic intraluminal pressure and the frequencies of pressure fluctuations (Mwanza et al. 2000a). Fasting also delays ova transport through the isthmic part

of the oviduct, perhaps owing to a prolonged α-adrenergic response in the smooth circular musculature of the isthmus (Mwanza et al. 2000a, b; Razdan et al. 2001).

As far as induction of stress by ACTH-stimulation after ovulation is concerned, this does not affect the activity of the isthmus and frequency distribution of ova within the isthmus, but it does reduce the number of sperm cells attached to ZP and the number of embryos retrieved from the sow reproductive tract (Mwanza et al. 2000b; Razdan et al. 2002; Einarsson et al. 2008). In contrast, ACTH also increases the velocity of embryo transport along the uterus (Brandt et al. 2007).

8.4 Gamete Binding and Interaction: Ovum Surface

The mechanism of gamete recognition is conserved throughout the biological evolution from marine vertebrates to eutherian mammals. As previously mentioned, the oocyte is coated by the ZP in the case of mammals. This ZP plays a protective role, which modulates sperm function during fertilisation and prevents polyspermia (Töpfer-Petersen et al. 2008).

Since recognition and interaction between spermatozoa and oocyte involves sperm-surface associated proteins that recognise oligosaccharide ligands of the ZP-glycoproteins, this section and the next will focus on these molecules.

8.4.1 ZP-Glycoproteins: ZPA, ZPB and ZPC

In mammals, ZP is formed by more than one glycoprotein (Harris et al. 1994). All ZP-glycoproteins share a common domain of about 260 residues that play a critical role in the polymerisation of ZP-glycoproteins into filaments (Litscher and Wassarman 2007).

Glycoproteins from the ZP are encoded by seven gene families that have originated from a duplication of an ancestral gene during vertebrate evolution (Spargo and Hope 2003; Hughes 2007). Related to this, Goudet et al. (2008) used phylogenetic analysis to classify ZP-genes in six subfamilies: *ZP1*, *ZPA/ZP2*, *ZPB/ZP4*, *ZPC/ZP3*, *ZPAX* and *ZPD*. However, the mammalian-ZP is not always formed by the same number of ZP-glycoproteins, but differences exist among species. Thus, in pigs, cattle, dogs and cats, ZP is constituted by three glycoproteins: ZPA/ZP2, ZPB/ZP4 and ZPC/ZP3 (Noguchi et al. 1994; Goudet et al. 2008; Mugnier et al. 2009), while in humans (Lefièvre et al. 2004; Conner et al. 2005; Barroso et al. 2009), rats (Hoodbhoy et al. 2005), hamsters (Izquierdo-Rico et al. 2009), macaques, chimpanzees (Goudet et al. 2008) and horses, it is formed by four glycoproteins (ZPA/ZP2, ZPB/ZP4, ZPC/ZP3 and ZP1). Differences between the species presenting three or four ZP-glycoproteins have been attributed either to the loss of the gene in the case of pigs or to the 'death' of the ZP1 gene, as in bovine and canine species (Goudet et al. 2008). In addition, it is worth noting that ZPAX and ZPD proteins are not present in any of the mentioned species.

As stated, porcine-ZP is formed by three different glycoproteins that are encoded by the gene subfamilies *ZPA/ZP2*, *ZPB/ZP4* and *ZPC/ZP3*. From these three ZP-glycoproteins, two (ZPB and ZPC) are synthesised by the developing oocyte during the early phases of follicle development (primordial and primary follicles), while the other (ZPA) is later released and is not observed in immature oocytes. As follicles grow, the contribution of the oocyte in the synthesis of ZPB and ZPC decreases, while that of the follicular cells increases (Sinowatz et al. 2001). In tertiary follicles (also known as Graafian follicles), ZPA is present in the oocyte cytoplasm and ZPB and ZPC are found in ZP (Niemann and Rath 2001). This finding suggests spatio-temporal secretion of ZPA and ZPB, and ZPC is differently regulated in pigs during folliculogenesis.

ZPA has been reported to harden ZP, thereby preventing polyspermia. This occurs after fertilisation and is related with the formation of intra-/inter-molecular disulphide bonds within/between monomers of this protein, as has been observed in bovine (Iwamoto et al. 1999) and porcine species (Töpfer-Petersen et al. 2008). In mice, ZPA/ZP2 also mediates secondary binding of spermatozoa, and cleavage of ZPA/ZP2 by proteases released through cortical granule reaction causing ZP-'hardening' and thus preventing polyspermy (Barroso et al. 2009).

Both ZPB/ZP4 and ZPC/ZP3 are post-translationally modified. The carbohydrate chains in ZPB are involved in the recognition of spermatozoa (Kudo et al. 1998), and maximal sperm binding is observed when ZPB/ZP4 forms heteromultimeric complexes with ZPC/ZP3 (Yurewicz et al. 1998). In fact, it seems that it is the interaction of these two ZP-glycoproteins that induce the conformational changes in ZPB/ZP4 and allows its sperm-binding domains to be exposed (Töpfer-Petersen et al. 2008).

8.4.2 Morphological Structure and Organisation of ZP

The morphological structure of ZP in the pig consists of a meshwork with pores in a similar fashion to other species (Funahashi and Nagai 2001; Fléchon et al. 2004; Mugnier et al. 2009). In this case, ZPB/ZP4 and ZPC/ZP3 form heteromultimeric complexes that may be interconnected by ZPA/ZP2 (Yurewicz et al. 1998) and are organised forming a filamentous and homogenous ring around the oocyte (Mugnier et al. 2009).

In mice, early work by Greve and Wassarman (1985) showed that ZP consists of a matrix constituted by repeating units of ZPA/ZP2 and ZPC/ZP3 heterodimers that are cross-linked by ZP1 homodimers and form a fibrous pattern (East and Dean 1984). Related to this, a hypothetical model for those ZPs formed by four ZP glycoproteins has been proposed; its organisation consisting of ZPA/ZP2 and ZPC/ZP3 heterodimers arranged in filaments and connected by ZP1 and ZPB/ZP4. This organisation would appear to be conserved through species (Familiari et al. 2008).

In humans, ZP is formed by four different glycoproteins known as ZP1, ZP2, ZP3 and ZP4 (Lefièvre et al. 2004; Conner et al. 2005); ZPC/ZP3 forms an homogenous ring around the oocyte in a similar fashion to pigs (Hinsch et al. 1994).

In the case of equine species, Mugnier et al. (2009) have proposed that ZPB/ZP4 and ZPC/ZP3 form heteromultimeric complexes interconnected by equine ZPA/ZP2, so that equine-ZP might contain different types of filament cross-linkers such as ZP1 homodimers, ZPA/ZP2 homodimers and/or ZP1 and ZPA/ZP2 heterodimers. Thus, while in porcine species ZPB/ZP4 and ZPC/ZP3 are organised forming a filamentous meshwork, in equine species they form homogenous patches (Mugnier et al. 2009).

When comparing the size and the number of pores, Mugnier et al. (2009) have observed that porcine-ZP presents fewer pores than equine ZP, but their diameter is larger. This observation is related to differences in ZP-composition in both species and may explain why the penetration rate is lower in equine than in pigs after in vitro fertilisation (IVF) procedures. Taking into account this observation, a general model has been proposed on the basis of porcine and equine species assuming that polyspermy rates are higher when the ZP presents fewer and larger pores (Santos et al. 2008; Mugnier et al. 2009).

8.4.3 Glycosylation and Glycans in ZP-Glycoproteins

Among the most critical domains of ZP-glycoproteins are those formed by carbohydrate chains, since they are involved in the recognition of binding to the sperm surface.

In the case of ZP-glycoproteins, glycosylation consists of neutral and acidic O- and N-linked oligosaccharides that are arranged in N-acetyl-lactosamine residues (Töpfer-Petersen et al. 2008). Regarding O-linked glycans, these are mainly core-1 type (Galβ1-3GalNAc) but they can also be, to a minor extent, core-3 type glycans (GlcNAcβ1-3GalNAc) (Hokke et al. 1994).

As far as N-linked chains are concerned, they mainly belong to the complex type containing bi, tri and tetra antennae, which are all α(1,6)-fucosylated at the innermost N-acetyl-glucosamine residues (Noguchi and Nakano 1992; Noguchi et al. 1992; Von Witzendorff et al. 2005). Amounts of acidic N-glycans in ZP-glycoproteins are approximately two times higher than neutral ones.

On the other hand, it is worth noting that acidic N-glycans containing a single sulphate group (SO_3^-) is abundant. Accordingly, sulphation mainly occurs at the C-6 position of non-repeated GlcNAc residues, of N-acetyl-lactosamine repeating units, and of non-repeated residues of N-glycans (Noguchi and Nakano 1992; Noguchi et al. 1992; Mori et al. 1998). In addition, sulphation is also possible at the C-3 position of the innermost N-acetylglucosamine residues (Mori et al. 1998), but it is less frequent. In contrast, there is no sulphation of repeated N-acetylglucosamine residues (Töpfer-Petersen et al. 2008).

When the glycan profiles of ZP-glycoproteins are compared, high-mannose type glycans appear to be exclusively linked to ZPA/ZP2 (Töpfer-Petersen et al. 2008), while in ZPB/ZP4 and ZPC/ZP3, acidic N-glycans are elongated by up to six sulfated N-acetyl-lactosamine repeats carrying N-acetyl-or N-glycolylneuraminic acid residues at the non-reducing ends (Noguchi and Nakano 1992).

ZP-glycoproteins carry different numbers of potential N-glycosylation sites. In the case of ZPB/ZP4 and ZPC/ZP3, Töpfer-Petersen et al. (2008) have predicted that all N-glycosylation sites are located within the ZP-domain (ZPB/ZP4: N_{202}, N_{220}, N_{333}; ZPC/ZP3: N_{124}, N_{146}, N_{271}). However, only the asparagine residues located at position 220 (N_{220}) of ZPB/ZP4 and at position 271 (N_{271}) in ZPC/ZP3 carry tri-, and tetra-antennary complex N-glycans (Kudo et al. 1998; Yonezawa et al. 1999). In ZPA/ZP2, there are five glycosylation sites: N_{84}, which carry tri- and tetra-antennary glycans and are located at the N-terminal domain, and N_{268}, N_{316}, N_{323} and N_{530}, which are located in the ZP-domain and carry bi-antennary chains (Töpfer-Petersen et al. 2008). According to Von Witzendorff et al. (2005), glycosylation site N_{268} in ZPA/ZP2 also contains mannosyl residues (Man_5GlcNA_2).

Within the glycans linked to the ZP-glycoproteins, sperm-binding activity has been located at the neutral tri- and tetra-antennary complex N-glycans of ZPB/ZP4 expressing non-reducing terminal β-galactosyl residues (Kudo et al. 1998; Yonezawa et al. 2005). These carbohydrate ligands are found in the surface of the ZP and are recognised by the carbohydrate-binding proteins attached to the sperm surface (see also Sect. 8.5.2).

On the other hand, glycosylation is highly important because it regulates the function of ZP-glycoproteins. In mice, for example, sugars have been reported to regulate the ligand abilities of ZPC/ZP3, since O-glycosylation, and particularly terminal galactose residues of O-linked oligosaccharides, and the amino sugar N-acetylglucosamine play a critical role in the maintenance of gamete interaction. In contrast, the acrosome exocytosis triggering activity of ZPC/ZP3 does not depend on sugars but on the integrity of the protein backbone (Chapman and Barratt 1996). In humans, Chiu et al. (2008) observed that the activity of ZPC/ZP3 and ZPB/ZP4 in terms of sperm-ZP binding and of acrosome exocytosis-induced ability also depended on their glycosylation. These authors also reported that N-linked glycosylation appeared to be more significant than O-linked glycosylation.

Finally, it is worth noting that ZP-glycoproteins present considerable heterogeneity in carbohydrate-linked chains. This is related to different degrees of sialylation and sulphation, and depends on oocyte maturation, the age of the gilts/sows and even on breed and diet (Noguchi et al. 1992; Noguchi and Nakano 1992; Takasaki et al. 1999; Töpfer-Petersen et al. 2008). Specifically, differences in ZP-glycoproteins related to oocyte maturation are discussed in the next section (Sect. 8.4.3).

8.4.4 Maturation of ZP

Apart from the maturation of nucleus and cytoplasm in the pig oocyte, the ZP also undergoes a maturation process prior to achieving full fertilisation competence as observed in pigs, humans, mice or cats (Calafell et al. 1992; Familiari et al. 1992; Hermansson et al. 2007). As previously stated, the ZP of an immature and in vitro-matured oocyte differs from that of a mature one, since the former has a spongy

appearance with numerous large pores while the latter presents small pores and a more compact surface (Rath et al. 2005; Michelmann et al. 2007; Mugnier et al. 2009). In addition, kinetics of the acrosome reaction are faster in the ZP of mature oocytes than in the ZP of immature oocytes (Rath et al. 2006).

In fact, the relevance of ZP-maturation during oocyte development is seen in IVF procedures. Specifically for the case of porcine species, there is a low IVF success rate due to high polyspermic rates (Coy et al. 2008) that appear to be related to the defective in vitro maturation (IVM) of collected oocytes. Indeed, both immature oocytes from pre-pubertal animals and IVM-oocytes show a delayed secretion of ZPA/ZP2 from the oocyte. This incomplete secretion of ZPA/ZP2 may influence the three-dimensional architecture of the ZP, thereby accounting for the aforementioned high polyspermic rates after IVF (Niemann and Rath 2001; Töpfer-Petersen et al. 2008).

Maturation of ZP involves a set of changes, including modification in the carbohydrate antennae chains (Rath et al. 2005). For example, ZP-glycoproteins from the oocytes of adult animals or IVM are slightly more acid than those from pre-pubertal or immature animals (pI pre-pubertal: 3–7 vs. pI adult: 3–5.5) (Rath et al. 2005). In addition, ZP-glycans of the oocytes from adult animals are more N-sulphated than those from pre-pubertal gilts (Noguchi and Nakano 1992). Indeed, neutral N-glycans are dominant in immature oocytes, containing high-mannose type glycans with five mannosyl residues and a core made up of bi-antennary N-glycans with one and two terminal galactose residues (Töpfer-Petersen et al. 2008). Therefore, ZP-maturation implies an increase in sulphated N-acetyl-lactosamine repeated units in ZP-glycoproteins. The high occurrence of polysulphate structures in mature oocytes affects the sperm penetration of oocyte through ZP (Töpfer-Petersen et al. 2008).

8.5 Gamete Binding and Interaction (II): Sperm Surface

8.5.1 A General Overview of Sperm Receptors for ZP-Proteins in Mammals

Research on carbohydrate-binding mechanisms between spermatozoa and oviductal cells (see Chap. 6) and between sperm and ZP started more than 25 years ago (Töpfer-Petersen et al. 1985). In this regard, one of the most important efforts has been to identify whether an individual protein or a carbohydrate side chain working as ‘sperm receptor’ existed (Barroso et al. 2009). Accordingly, different studies have been conducted and have proposed the following primary sperm receptors for ZP:

1. A 95 kDa protein-kinase in mice (Leyton et al. 1992).
2. SP56 (Bookbinder et al. 1995) in mice.
3. Trypsin-like protein (Boettger-Tong et al. 1993) in humans.

4. β1,4-galactosyltransferase (GalTase) in mice (Shur 1994).
5. Zona-adhesins/Spermadhesins in mice (Gao and Garbers 1998) and ungulates (Töpfer-Petersen et al. 2008), such as pigs.

At present, none of these molecules have been unequivocally established as an active receptor, but growing evidence indicates that spermadhesins play a relevant role in ungulates, as will be further discussed. In addition, recent progress in mammalian spermatozoa has shown that two proteins that interact with ZP3 are zona-adhesin and PKDREJ (Polycystic Kidney Disease Polycystin) and REJ (sperm receptor for egg jelly homolog, sea urchin homolog). Evolutionary studies made within and between primate species have identified promising regions of the proteins that seem to interact and co-evolve with ZP3 (Bi et al. 2003; Sutton et al. 2006).

8.5.2 The Case of Porcine Species: Primary and Secondary Binding of Spermatozoa to ZP

In the case of sperm-ZP interaction in pigs, boar spermatozoa were first described as having fucoidan-binding proteins, especially at the apical region of the sperm head (Töpfer-Petersen et al. 1985; Friess et al. 1987). Within these proteins, identified as the major ZP-binding proteins of boar spermatozoa, we can distinguish between sperm-surface associated proteins (mainly spermadhesins and P47/SED1) that have a molecular mass of 12–16 kDa (Töpfer-Petersen et al. 1998; Petrunkina et al. 2003) (see also Sect. 6.7.3) and pro/acrosin, which is an intra-acrosomal serine protease that has a molecular mass of 53–55 kDa (Töpfer-Petersen and Henschen 1987) (Fig. 8.2).

The different localisation of sperm-surface attached proteins and proacrosin (the former are peripheral membrane-associated proteins while the latter is an intra-acrosomal protein) seems to be related to the sequence of carbohydrate-mediated events occurring within the oviduct, i.e. the formation of sperm reservoir and binding to the ZP (Töpfer-Petersen et al. 2008). Thus, sperm-surface associated proteins not only participate in the formation of sperm reservoir but also in the primary binding with ZP-proteins of the oocyte. After primary binding, proacrosin is activated to acrosin, leading to acrosomal exocytosis and subsequently allows secondary binding. In addition, sperm-surface attached proteins are thought to stabilise the plasma membrane over the acrosomal vesicle between ejaculation and fertilisation (see also Chaps. 1 and 6).

8.5.3 Sperm-Surface Attached Proteins Involved in Primary Binding to ZP

Different sperm-binding ZP proteins have been identified, which represent the contribution of multiple sperm proteins in recognition and binding events. These proteins are sperm-surface attached proteins (spermadhesins AWN and AQN-3,

and protein DQH; see Sect. 6.7.3), lactadherin (also known as P47 and SED-1), fertilin β (also known as ADAM-2) and peroxiredoxin 5 (PRDX5), which belong to a novel family of peroxidases and is usually intracellular (Blobel 2000; Brewis et al. 2005; Primakoff and Myles 2000; Van Gestel et al. 2007) (Figs. 6.4 and 8.2).

As extensively discussed in Chap. 6, there are different sperm-surface attached proteins, but the major ones are spermadhesins. The first interaction of spermadhesins to spermatozoa is mediated by non-aggregated AWN and AQN-3 proteins that directly bind the phosphorylethanolamine molecules (Calvete et al. 1996; Dostàlovà et al. 1995a; Ensslin et al. 1995). Then, there is another spermadhesin, AQN-1, which is located over AWN and AQN-3 and indirectly attaches to the sperm surface via binding to DQH. This DQH interacts, in turn, with the sperm membrane through phosphorylcholine-binding sites (Calvete et al. 1997; Ekhlasi-Hundrieser et al. 2007).

Three spermadhesins (AQN-1, AQN-3 and AWN) play a crucial role in the carbohydrate-mediated events that take place in the oviduct (i.e. sperm reservoir and fertilisation) (Töpfer-Petersen et al. 2008). These three proteins differ, however, in the carbohydrates they recognise, since AWN only interacts with α- and β-linked galactose residues, while AQN-1 also recognises Man α1-3(Manα1-6)Man structures (Ekhlasi-Hundrieser et al. 2005). This fact has been related to the role of these sperm proteins within the oviduct, since AQN-1 participates in the formation of the sperm reservoir by interacting with the glycoconjugates of the oviductal epithelium, while AQN-3 and AWN take part in primary binding of the spermatozoon to ZP-glycoproteins during fertilisation. In this latter case, it must be mentioned that AQN-3, AWN and P47/SED1 are masked by AQN-1 before capacitation. During capacitation, AQN-1 is removed from the sperm surface (Sanz et al. 1993; Calvete et al. 1997), so that AQN-3, AWN and P47/SED1 become unmasked, accessible and able to interact with ZP-glycans (Rodríguez-Martínez et al. 1998; Wagner et al. 2002; Petrunkina et al. 2003; Ekhlasi-Hundrieser et al. 2005). Related to this, and despite AWN and AQN-3 presenting a similar binding affinity for ZP-glycoproteins that contain Galβ(1-3)GlcNAc and Galβ(1-4)GlcNAc sequences either in N- or O-linked oligosaccharides, they differ regarding the recognition of tri-/tetra-antennary N-glycans (Dostàlovà et al. 1995b; Töpfer-Petersen et al. 2008).

Finally, there is another protein named lactadherin (the porcine homolog of SED1, also known as sperm-surface protein P47/SED1, MFGM or Milk fat globule-EGF factor 8 protein), which is expressed in the testis and in the epididymis of mammals (Ensslin et al. 1998), binds the sperm surface, and also seems to participate in the interaction between the spermatozoon and oocyte, as has been suggested in mouse experimental models (Shur et al. 2004). However, it still remains to be elucidated whether P47/SED 1 interacts with ZP by carbohydrate protein or protein–protein interactions.

8.5.4 Proacrosin and Secondary Binding

When the sperm-surface attached proteins primarily interact with the ZP-glycoproteins, a signal cascade is triggered leading to the exocytosis of the acrosome, which contains a large amount of hydrolytic enzymes like acrosin

(Jungnickel et al. 2001; see also Chap. 1). The acrosome-reacted spermatozoon is then able to penetrate the ZP by alternating cycles of sperm-ZP binding, limited proteolysis of the matrix and release from the ZP (Fig. 8.1). In these cycles, the force generated by the forward motility of hyperactivated spermatozoa also plays a crucial role (O'Rand et al. 1986; Green 1987; Suarez 2008).

Proacrosin has two forms, i.e. the inactive (zymogen) named proacrosin and the active (serine proteinase) called acrosin, and exerts a key function in the interaction between spermatozoon and oocyte. Genes encoding the proacrosin have been reported to be highly conserved in mammals during their biological evolution (Klemm et al. 1991).

Proacrosin, the inactive form, is stored in the acrosomal vesicle until the acrosome reaction is triggered and has a single chain molecule of 53/55 kDa that presents the catalytic triad (formed by residues of Histidine (His) at position 70, Aspartate (Asp) at position 124 and Serine (Ser) at position 222) like other serine proteinases, and a sequence of 125 amino acidic residues at the C-terminus that contain 23 consecutive prolines. Zelezna et al. (1989) suggested that this proline-rich domain could be responsible for the strong binding of proacrosin to biological membranes and possibly to the sperm inner acrosomal membrane.

Proacrosin presents a high affinity for sulphated oligosaccharide chains such as ZP-glycoproteins that, together with fucoidan and heparin, have been reported to accelerate the activation process from proacrosin to acrosin (Töpfer-Petersen and Henschen 1987; Töpfer-Petersen and Henschen 1988; Töpfer-Petersen et al. 1990a). Accordingly, this activation process is mediated by a basic peptide of 18 residues, between Isoleucine at position 43 (Ile_{43}) and Leucine at position 60 (Leu_{60}) that contains one polysulphate-binding site (Moreno and Barros 2000). In fact, activation from proacrosin to acrosin as well as control of enzyme activity depends on the spatial proximity of the proacrosin-active site and the polysulphate-binding sites, which are recognised by ZP-components and trigger the acrosome reaction and subsequent downstream events (Tranter et al. 2000).

After the activation mediated by Ile_{43}-Leu_{60} peptide, conversion of proacrosin to acrosin occurs via the autocatalytic-proteolytic cleavage between arginine and valine residues located at positions 23 and 24, respectively (Arg_{23} and Val_{24}). This leads to the formation of an active two-chain molecule of the same molecular mass in which the light chain (23 amino acids) remains linked to the heavy chain by two disulphide bonds (Cechova et al. 1988). Each chain presents one N-glycosylation site in asparagine residues, which are respectively located at position 3 (Asn_3) in the light chain and at position 10 (Asn_{10}) in the heavy chain (Cechova et al. 1988; Töpfer-Petersen et al. 1990b).

Apart from the activation-proteolytic site located between Arg_{23} and Val_{24} residues, proacrosin presents another processing site at C-terminal, which releases the last 77 amino acids originating β-acrosin, an mature acrosin form of 38-kDa (Baba et al. 1989; Puigmulé et al. 2011).

Although proacrosin may take part in all three stages of the mentioned cycles (i.e. binding, digestion and release and proacrosin reactions), its exact role is not clear, since proacrosin null mouse spermatozoa are able to penetrate the ZP and

to fertilise the oocyte (Honda et al. 2002) but at a lower velocity than the wild type (Adham et al. 1997; Nayernia et al. 2002). In addition, Jin et al. (2011) have recently observed that mice spermatozoa that undergo acrosome reaction before interacting with ZP are also able to fertilise the oocyte. We mention here data available in mice, as no similar studies have been conducted in pigs. Care must be taken, however, when extrapolating these findings in mice to pigs, since the thickness of ZP in pig oocytes is greater than in mice, so that acrosin may play a more critical role in pigs in the secondary binding and sperm penetration through ZP.

Finally and still related to the actual role of proacrosin, secondary binding also seems to involve other enzymes and binding proteins apart from proacrosin, which could act synergistically to achieve maximal fertilisation. In this regard, two other serine proteases have been identified in mouse sperm (Honda et al. 2002), and another ZP-binding protein (SP38, also known as ZPBP) has been found in boar spermatozoa (Mori et al. 1995; see also Chap. 1).

8.6 Gamete Binding and Interaction (III): Sperm-ZP Recognition and Acrosome Exocytosis

8.6.1 Introduction

Several studies have previously shown that sperm-ZP interaction plays a critical role for successful fertilisation (Yanagimachi 1994b). This interaction takes place through two sequential steps. The first one occurs when ZPC/ZP3 interacts with spermatozoa. Then, a sperm receptor that is coupled to an inhibitory regulative G-protein (G_i) activates phospholipase Cβ1 and regulates adenylyl cyclase leading to an increase in cAMP levels. Cyclic AMP activates, in turn, protein kinase A (PKA) to open a calcium channel in the outer acrosomal membrane, resulting in a small increase of cytosolic Ca^{2+}. Then, Ca^{2+} activates phospholipase C_γ, which is coupled to the second tyrosine kinase receptor. The two products of phospholipase C (PLC) activity, diacylglycerol (DAG) and inositol triphosphate (IP_3), activate protein kinase C (PKC) and IP_3 receptor. Upon activation, PKC opens a Ca^{2+}-channel in the membrane, and IP_3 activates the Ca^{2+}-channel in the outer acrosomal membrane that leads to a higher increase in cytosol calcium. This set of intracellular changes results in membrane fusion and completion of the acrosome reaction (Breitbart and Naor 1999; Breitbart 2002; Florman et al. 1989, 1992) (Fig. 8.3).

A second binding then follows which involves ZPA/ZP2 and the inner acrosomal-sperm membrane. This second binding finally leads to ZP-penetration (Wassarman 1995, 1999).

Cleavage of ZPA/ZP2 by proteases released through the cortical granule reaction causes the hardening of ZP and thus prevents polyspermy (Barroso et al. 2009). Related to this, uncleaved ZPA/ZP2 seems to be related with post-fertilisation persistence of mouse sperm binding to ‘humanised’ ZP (Castle and Dean 1999).

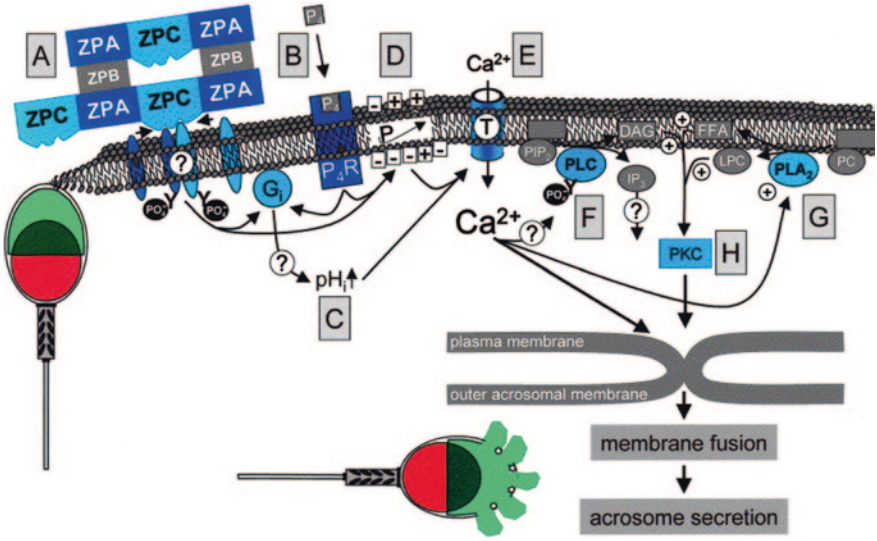

Fig. 8.3 Proposed sequence of the ZP and progesterone induced acrosome exocytosis. (*A*) ZP proteins (most likely ZPC) bind to sperm ZP receptors, leading to aggregation and tyrosine (Y) phosphorylation. (*B*) The direct environment of the ZP contains high levels of progesterone that can bind to its non-genomic receptor (P_4R) on the sperm surface. Both ZP and progesterone have a dual effect on sperm cells. (*C*) The intracellular pH (pH_i) is increased via (G_i) and (*D*) the plasma membrane potential depolarises. (*E*) Both the increased pH_i and depolarisation induce the entry of calcium via a T-type voltage dependent Ca^{2+} channel. (*F*) The higher intracellular Ca^{2+} levels activate PLC that has been translocated to the plasma membrane during capacitation. PLC converts PIP_2 to DAG and IP_3. (*G*) Increased Ca^{2+} levels activate PLA_2, which degrades PC to LPC and free fatty acids (FFA). (*H*) The role of IP_3 is unclear, but DAG, FFA and LPC activate PKC. Both the increased intracellular Ca^{2+} levels and PKC activation are necessary for the fusion of the plasma membrane with the underlying acrosomal membrane, which leads to the subsequent secretion of acrosomal enzymes (Flesch and Gadella 2000) Reproduced with permission

For this reason, it has been hypothesised that the supramolecular structure of ZP necessary for sperm binding is modulated by the cleavage status of ZPA/ZP2 (Rankin et al. 1998, 2003; Castle and Dean 1999; Hoodbhoy and Dean 2004).

8.6.2 Species Specificity of Sperm-ZP Recognition: Barriers to Penetration

Specificity of sperm-oocyte interaction exists that varies between vertebrate species and represents a barrier for fertilisation between some species. Some of these barriers are located at the ZP level, so that ZP often works as a barrier for heterologous crosses in vitro (Wassarman et al. 2005). Indeed, for example, complex N-glycans with terminal fucose and GlcNAc residues are involved in the sperm-ZP binding in *Xenopus laevis* (Vo and Hedrick 2000), O-linked oligosaccharides

of ZP-glycoproteins take part in sperm-oocyte interaction in mice (Wassarman et al. 2005), and the ZP of human oocyte presents mannose-binding sites that play a crucial role in the recognition mechanisms of gametes (Rosano et al. 2007).

However, there are also exceptions to this rule in those species presenting both four and three ZP-glycoproteins. Thus, for example, rat spermatozoa are able to bind mouse-ZP, but mouse spermatozoa are less able to adhere to rat-ZP (Hoodbhoy et al. 2005). Mouse spermatozoa are, in turn, able to bind human-ZP. In contrast, human spermatozoa cannot bind mouse-ZP (Hoodbhoy et al. 2005), but they are able to interact with porcine-ZP (Cánovas et al. 2007).

In cattle, sperm-oocyte binding involves the polyvalent presentation of terminal α-mannosyl residues of high-mannose N-glycans (Amari et al. 2001), while this interaction is mediated by terminal β-galactosyl residues of complex N-glycans (Yonezawa et al. 2005) in pigs. In both species, sugars mediating this interaction are linked to the ZPB/ZP4 glycoprotein, and despite receptor-ligand systems being different, porcine and equine spermatozoa can bind bovine-ZP in vitro, and equine spermatozoa are even able to penetrate the bovine-ZP and to enter the bovine oocyte (Sinowatz et al. 2003). When comparing equine and porcine species, Mugnier et al. (2009) observed that although the number of porcine spermatozoa bound to equine ZP is similar to that of equine spermatozoa, equine spermatozoa are less able to bind porcine ZP. Thus, it seems that that equine-ZP is less selective than porcine ZP.

All these data suggest that there are some ZPs that are not able to support heterologous sperm binding, whereas others are more permissive (Mugnier et al. 2009). This selective ability, apart from emphasising the key role of the composition and structure of ZP in sperm-ZP binding, seems to be related with the phylogenetic distance between species. In this regard, it is worth noting that tiger spermatozoa appear to be able to fertilise cat oocytes (Donoghue et al. 1992), while goat and ram spermatozoa are able to fertilise bovine oocytes (Cox et al. 1994; Kouba et al. 2001; García-Álvarez et al. 2008) and deer sperm can fertilise sheep oocytes (Soler et al. 2008).

This heterologous in vitro recognition and interaction may be explained by the existence of glycans with a similar structure that can be recognised by different ZP-binding proteins.

In addition, the heterologous interaction observed in vitro suggests that there are other mechanisms in vivo, apart from the specific recognition between spermatozoon and oocyte, which also act as inter-species barriers. These mechanisms are related to the anatomy and behaviour of males and females, species specificity of the sperm oviductal reservoir (see Chap. 6), fusion between sperm and oocyte membranes and post-fertilisation events (Töpfer-Petersen et al. 2008).

8.6.3 Lessons from In Vitro Studies: Porcine versus Equine Spermatozoa

As stated, penetration and polyspermy rates after IVF are higher in equine (Dell'Aquila et al. 1996; Alm et al. 2001; Hinrichs et al. 2002) than in porcine

species (Day 2000; Nagai et al. 2006; Mugnier et al. 2009). This fact can be related to two findings recently reported by Mugnier et al. (2009):

1. During sperm-ZP binding, equine-ZP is less selective than porcine-ZP.
2. During sperm penetration, equine ZP represents a barrier for both homologous (equine) and heterologous (porcine) spermatozoa, whereas porcine ZP represents a barrier for heterologous but not for homologous spermatozoa.

Since equine and porcine ZPs differ in the presence/absence of ZP1, Mugnier et al. (2009) have suggested that this protein might be involved in the different sperm attachment and penetrating ability in equine and porcine species, the former presenting a higher sperm binding but a lower penetration rate than the latter. Despite the fact that ZP1 does not seem to play a direct role in sperm-ZP interaction, the ability of ZP1 to form intermolecular disulphide bonds may allow it to be involved in the three-dimensional structure of the ZP. Since Hoodbhoy and Dean (2004) have proposed the supramolecular structure as a key component in sperm-oocyte recognition, ZP1 could take part in sperm-ZP binding via a role in this supramolecular structure (Mugnier et al. 2009).

On the other hand, ZP-glycoproteins are differently located in equine and porcine species, and this may be related to differences in ZP-composition in both species. Thus, in pigs, ZPB/ZP4 and ZPC/ZP3 are organised forming a filamentous meshwork and in equine species they form homogenous patches (Mugnier et al. 2009). Therefore, a different molecular organisation of ZP-glycoproteins exists in these porcine and equine species, since ZP1 is present in equine but not in pigs, and ZPA/ZP2 is differently located in equine and porcine species.

When comparing the size and the number of pores, Mugnier et al. (2009) have observed that porcine-ZP presents a lower number of pores than equine-ZP, but their diameter is greater. This observation may explain why the penetration rate is low in equine IVF, whereas the penetration and polyspermy rates are high in porcine IVF. Therefore, a general model has been proposed on the basis of porcine and bovine species assuming that polyspermy rates are higher when the ZP presents fewer and larger pores (Santos et al. 2008; Mugnier et al. 2009).

In short, the main differences between equine- and porcine-ZP are the number of ZP-glycoproteins, three in porcine, four in equine species, their localisation and the molecular organisation of ZP, including the number and the size of pores (Mugnier et al. 2009). These differences may underlie the different penetration and polyspermy rates in equine and porcine species.

8.6.4 ZP-Modifications Mediated by the Oviductal-Specific Glycoprotein to Prevent Polyspermia

Since polyspermy is an important anomaly of fertilisation in placental mammals, it is important to mention that there must exist mechanisms avoiding this polyspermy (Snook et al. 2011; Iwao 2012).

Although most studies have used rodents as models, ungulates may differ in their mechanisms to prevent polyspermy. Related to this, Coy et al. (2008), working with pigs, observed that periovulatory oviductal fluid from sows increased ZP resistance to digestion with pronase (a parameter commonly used to measure the block to polyspermy). These authors also reported an increase in monospermy and a decrease in sperm-ZP binding when the oocytes were previously exposed to oviductal fluid. In addition, Coy et al. (2008) also observed that an heparin-depleted medium abolished the resistance of oviductal fluid-exposed oocytes to pronase, but a medium with heparin did not alter this resistance. When these authors analysed the content released in the heparin-depleted medium after removing the oocytes, they isolated and identified the oviduct-specific glycoprotein (OVGP1, also known as OSP; see Sect. 6.5). Taking into account all these data, Coy et al. (2008) hypothesised that ZP-changes during the transit of oocyte along the oviductal ampulla modulated the interaction with spermatozoa. In this context, oviduct-specific glycoprotein-heparin protein complex would be the responsible for these ZP changes, thereby affecting sperm binding and contributing to the regulation of polyspermy.

We can thus conclude that, according to Coy and Avilés (2010), there are three groups of mechanisms that contribute to block the polyspermy in mammals:

1. Oviduct-based mechanisms, avoiding a massive arrival of spermatozoa in the proximity of the oocyte.
2. Egg-based mechanisms, including changes in the membrane and ZP in reaction to the fertilising sperm.
3. Modifications of the ZP in the oviduct before the oocyte interact with spermatozoa, known as 'pre-fertilisation ZP hardening'. As stated, this mechanism is mediated by the OVGP1 secreted by the oviductal epithelial cells around the time of ovulation, and is reinforced by heparin-like glycosaminoglycans (S-GAGs) present in the oviductal fluid (Coy and Avilés 2010).

8.6.5 Role of ZP in Triggering Acrosome Exocytosis

Solubilised ZP triggers the acrosome reaction of spermatozoa, not only in pigs, but also in humans (Cross et al. 1988) and other mammalian species. However, when human spermatozoa are treated with pertussis toxin, an inactivator of inhibitory G-like proteins, there is a decrease in the percentage of acrosome-reacted spermatozoa after incubation with solubilised ZP. In contrast, calcium ionophore A-23187, also used to induce acrosome reaction, is able to trigger the acrosome reaction of human spermatozoa, despite the latter having previously been treated with pertussis toxin (Lee et al. 1992). This acrosome reaction triggering effect mediated by ZP is dose-dependent and involves inhibitory G_i (Franken et al. 1996). Related to this, Schuffner et al. (2002) demonstrated that the AR mediated by homologous ZP depends on the activation of inhibitory G_is (toxin-sensitive)

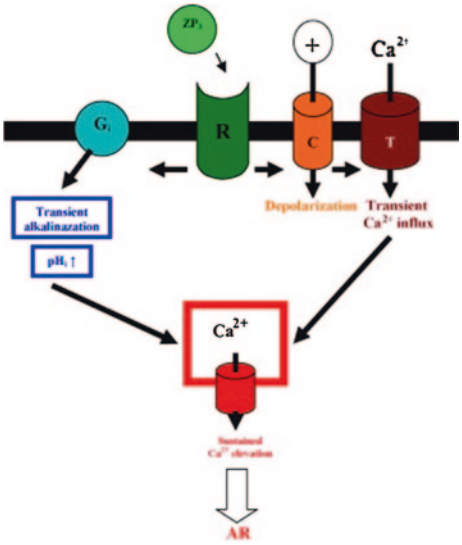

Fig. 8.4 Schematic representation of the proposed mechanism of sperm ion channel activation by the zona pellucida (ZP3/ZPC). One pathway consists of the activation of a cation channel (*C*), which leads to a depolarisation of the sperm plasma membrane; the result of membrane depolarisation is the activation of another, low-voltage-activated T-type channel (*T*). The other signalling pathway results from a transient alkalinisation of the internal pH (pH_i). Both pathways lead to a sustained Ca^{2+} elevation, necessary for acrosome exocytosis (Witte and Schäfer-Somi 2007) Reproduced with permission

and on the presence of extracellular Ca^{2+} (Fig. 8.4). In addition, the oviductal environment, particularly progesterone and follicular fluid, exert a priming role in the ZP-mediated induction of acrosome (Schuffner et al. 2002).

Although as is widely known, sperm binding to ZP triggers the acrosome reaction (Yanagimachi 1994b), Mugnier et al. (2009) have shown that ZP-mediated induction is not species specific. Thus, porcine ZP is able to induce acrosome exocytosis of horse spermatozoa and equine ZP is also able to induce the acrosome reaction of boar spermatozoa, thereby indicating that the ZP-ligand inducing acrosome reaction may be conserved between different species.

On the other hand, there are other interesting reports that have studied how acrosome exocytosis occurs when ZP is not present (i.e. in the case of zona-free oocytes). These reports have shown that despite porcine- and equine-ZP presenting a similar ability to induce acrosome exocytosis in boar and horse spermatozoa, equine spermatozoa are less able than boar sperm to undergo acrosome reaction regardless of the ZP-origin (Mugnier et al. 2009). This suggests that acrosome exocytosis depends on the capacity of the spermatozoa to undergo it, rather than the structure and composition of the ZP. Thus, the composition/conformation of ZP appears to be a critical element for sperm-oocyte binding and recognition but not for triggering acrosome reaction. The mentioned differences between boar and stallion spermatozoa in terms of acrosome reaction induced by ZP might be

related to differences in sperm capacitation, to the second binding of the spermatozoa, and/or to the penetration of the spermatozoa through the ZP.

8.6.6 SNARE Proteins and Acrosome Exocytosis

Yunes et al. (2000) were the first to hypothesise that the essential components involved in the membrane fusion of intracellular compartments in somatic cells, such as Rab3A, GTPase and SNAREs, are also present in mammalian sperm and can be involved in membrane fusion during the acrosome reaction. We must remember that conformational changes of SNARE proteins occur during sperm capacitation as has been described in Chap. 7 (Fig. 7.5), in a prior step to fertilisation (Tsai et al. 2010).

In the exocytosis of somatic cells mediated by SNARE proteins, the Q-SNARE proteins located in the cell membrane interact with the R-SNARE proteins located in the vesicle membrane and form a 7S or a 20S trans-SNARE protein complex (Chernomordik and Kozlov 2008). This complex then undergoes a conformational change depending on calcium levels, and this leads to membrane fusion (Martens and McMahon 2008; Sudhof and Rothman 2009). Therefore, it is worth noting that membrane fusion does not take place directly after the assemblage of SNARE proteins, but needs the appropriate stimulus (i.e. extracellular calcium influx) as well as other factors, such as RAS-associated protein RAB3A, N-ethylmaleimide-sensitive factor (NSF) or complexin, that mediate the fusion of the two membranes (Michaut et al. 2000; Maximov et al. 2009; Tsai et al. 2010; Vrljic et al. 2010). Furthermore, the formation of the trans-SNARE complex involves multiple regulatory pathways in a complicated process (Verhage and Toonen 2007).

Taking into account all the aforementioned, Verhage (2009) distinguishes three stages in the fusion between cell and vesicle membranes:

1. Membrane docking, when Q- and R-SNARE proteins interact to form the trans-SNARE complex (Jahn and Scheller 2006).
2. Priming or preparation of the fusion event when trans-SNARE complex pulls the interacting membranes into a close and tight apposition (Jahn and Scheller 2006; Verhage and Toonen 2007).
3. Membrane fusion.

Specifically for acrosome exocytosis, Tsai et al. (2007, 2010) have investigated how SNARE proteins are involved in the regulation of acrosome docking, priming and fusion in the male gametes. Accordingly, upon sperm capacitation, the redistribution of both Q- and R-SNAREs at the apical head region appears to be a bicarbonate- and albumin-dependent event (Tsai et al. 2007; see also Chap. 7). This redistribution step is required for further membrane fusion. In the case of acrosome reaction, the fusion points are located in the sperm head plasma (Q-SNARE) and in the outer acrosome membranes (R-SNARE) and form a trans-SNARE protein complex when interacting with ZP-glycoproteins (Tsai et al. 2010).

8.6.7 The Role of Glycodelins

As previously mentioned, carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates of the ZP (Miller et al. 1992; Clark et al. 1996, 1997; Oehninger 2001). In the case of humans, the sperm-binding proteins and ZP-glycans involved in this recognition system have been reported to be linked to natural killer cell inhibition (Clark et al. 1996; Oehninger 2001, 2003; Ozgur et al. 1998; Patankar et al. 1997), i.e. to immune response.

This hypothesis has been reinforced by the discovery of Glycodelin-A, a protein that is not present either on the sperm surface or in the ZP but is involved in the recognition and binding between gametes. Indeed, Glycodelin-A is an endometrial epithelial protein that in vitro inhibits sperm-ZP binding in a dose-dependent manner (Oehninger 2001; Oehninger et al. 1995; Seppälä et al. 2002). In addition, oligosaccharides associated with Glycodelin-A also block selectin-mediated adhesions. Therefore, carbohydrate binding specificity of the receptors mediating sperm-oocyte recognition seems to converge with that of lymphocyte/leukocyte adhesion, since Glycodelin-A exerts a contraceptive and immunosuppressive role, which depends on carbohydrates (Clark et al. 1996; Dell et al. 1995, 2003).

Glycodelin-A binds to N-glycans, specifically to high mannose bi-antennary bisecting type, and to biantennary, triantennary and tetra-antennary oligosaccharides, that terminate with Lewis-x (Lex) and Lewis-y (Ley) sequences (Dell et al. 1995, 2003). It is worth noting that, at least in humans, the major N-glycans of spermatozoa, which are Lex/Ley-terminated (Pang et al. 2007), are related to the inhibition of both innate and adaptive immune responses (Barroso et al. 2009). Following this, Clark et al. (1996, 1997) linked the expression of carbohydrate functional groups with the protection of gametes and the developing human embryo in the uterus, giving rise to the ‘fetoembryonic defense system hypothesis’ for eutherian mammals.

More recently, Chiu et al. (2007) have observed that a sperm protein identified as fucosyltransferase-5 (FUT5) is able to bind both to Glycodelin-A and to intact and solubilised ZP. For this reason, these authors have suggested that Glycodelin-A inhibits sperm-ZP binding, because it blocks the binding of sperm-FUT5 to ZP (Chiu et al. 2007).

8.7 Gamete Binding and Interaction (IV): Fusion of Sperm and Oocyte Membranes

8.7.1 Introduction

Fusion between a spermatozoon and an oocyte is a cell–cell membrane fusion event (Barroso et al. 2009; Sutovsky 2009) that is only possible when acrosome exocytosis has occurred (Yanagimachi 1994b). Thus, binding and fusion of spermatozoa

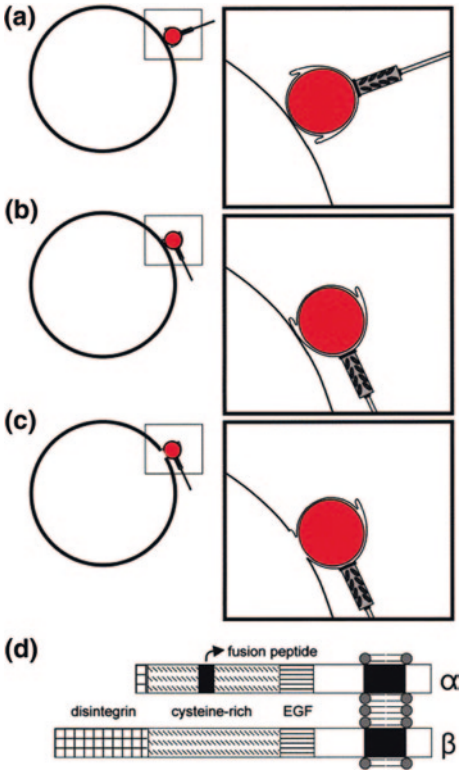

Fig. 8.5 Binding and fusion between both gametes. **a** Sperm initially binds with the apical tip of the inner acrosomal membrane to the oolemma. **b** After proper positioning, the apical binding is diminished and has facilitated a lateral binding of the sperm cell to the oolemma. The equatorial region of the sperm head plasma membrane is involved in this process. **c** The spermatozoon is now capable of fusing its equatorial plasma membrane with the oolemma. **d** The multidomain organisation of fertilin has been proposed to be directly involved in processes (**b**) and (**c**) (Flesch and Gadella 2000) Reproduced with permission

with the oolemma occurs at the post-acrosomal/equatorial region, since after the completion of acrosome reaction and ZP-penetration, plasma and inner and outer acrosomal membranes of this region remain intact (Stein et al. 2004) (Fig. 8.5).

As early studies conducted in mice showed, an acrosome-reacted spermatozoon fuses with numerous microvilli of the oolemma during fertilisation, and it is the whole spermatozoon (with the exception of part of the sperm plasmalemma, most of the outer acrosomal membrane and the acrosomal components) that is incorporated into the oocyte (Yanagimachi 1998).

8.7.2 Protein Candidates Involved in Sperm-Oocyte Fusion

As far as molecules involved in binding/fusion between sperm and oocyte membranes are concerned, different studies have suggested a list of candidate proteins that may mediate this mechanism (Snell and White 1996). However, the specific roles of these molecules still need to be validated (Vjugina and Evans 2008). These proteins are:

- Two cysteine-rich secretory proteins (CRISP), also known as epididymal protein CRISP1, and testicular protein TPX1 (CRISP2) that interact with egg-binding sites (Busso et al. 2005; Da Ros et al. 2007; Vadnais et al. 2008).

- A member of the ADAM family of trans-membrane proteins on sperm (ADAM-1 and ADAM-2, Fàbrega et al. 2011).
- IZUMO1, a family member of immunoglobulin proteins, in spermatozoa (Inoue et al. 2005, 2008).
- Equatorin, which takes its name from its localisation at the equatorial segment of the acrosome (Toshimori et al. 1998).
- Integrin $\alpha v\beta_1$ on oocytes (Linfor and Berger 2000).
- CD9, known to complex with integrins, on oocytes (Kaji et al. 2000; Miyado et al. 2000).

From all these candidates, we must mention that sperm binding to oocyte-integrinβ_1 is a necessary step prior to fusion between spermatozoon and oocyte (Blobel 2000; Cowan et al. 2001). Oocyte integrinβ_1 recognises fertilin-β (ADAM-2), a sperm-surface protein also known as PH-30 (Fàbrega et al. 2011) (Fig. 8.5).

Another protein candidate that has been reported to be involved in sperm plasmalemma- and oolemma-binding is P-selectin. This protein has been detected in the oolemma of human and hamster oocytes following sperm adhesion on the equatorial region of human spermatozoa (Fusi et al. 1996).

Finally, Inoue et al. (2005) identified a gene known as *IZUMO1* that seems to mediate sperm-oocytes fusion at least in humans and hamsters. However, the function of the protein product encoded by this gene does not seem to be dependent on glycosylation.

8.7.3 Membrane Fusion Between Gametes as a Barrier to Penetration

Apart from the role that ZP can play as a barrier to penetration between species, fusion between sperm plasmalemma and oolemma after secondary binding and acrosome reaction can act, in some species, as an intra/inter-specific barrier (Mugnier et al. 2009).

In several studies evaluating heterologous IVF and using ZP-free oocytes, horse and boar spermatozoa have been seen to fuse with equine and porcine oolemma (Mugnier et al. 2009). In addition, horse spermatozoa are able to fuse with bovine and hamster oolemma (Landim-Alvarenga et al. 2001; Matsukawa et al. 2002; Choi et al. 2003), and rat sperm have also been seen to fuse with porcine oolemma (Zhao et al. 2002). All these findings suggest that the ligand-receptor mediating sperm-oolemma binding may be conserved through some species (Sartini and Berger 2000; Mugnier et al. 2009). However, despite horse and boar spermatozoa being able to fuse with equine and porcine oolemma, both are less able to penetrate ZP-free equine oocytes than ZP-free porcine oocytes. This suggests that the oolemma may represent a barrier of equine oocytes to be penetrated by homologous or heterologous spermatozoa (see also Sect. 8.6; Mugnier et al. 2009).

On the other hand, it is worth noting that zona-free hamster eggs bind sperm from almost any species, including human (Tyler et al. 1981; Matson et al. 1987) and, even, birds (Samour et al. 1986), and allow sperm penetration (Maleszewski et al. 1995). However, while guinea-pig and human spermatozoa can activate hamster oocytes as efficiently as hamster spermatozoa, mouse spermatozoa cannot. This means that sperm-oocyte membrane fusion *per se* is not enough to trigger oocyte activation, but there is another species-specific barrier related to sperm-derived oocyte activating factors (Maleszewski et al. 1995).

8.8 Remodelling of Sperm Chromatin and Pronuclei Formation After Fertilisation

8.8.1 Organisation of Chromatin in Mature Spermatozoa

During spermatogenesis, DNA is progressively compacted by the replacement of nuclear histones for sperm-specific proteins known as protamines (see Chap. 3). These protamines contain a large amount of cysteine residues that establish inter- and intra-molecular disulphide bonds formed after the oxidation of the sulphydryl groups that these cysteine-residues contain. This forms a toroid shaped 'doughnut' of coiled chromatin loops that are much tighter than the normal solenoid loop typical of somatic cell nuclei (Calvin and Bedford 1971; Ward and Coffey 1991; Ward 1993).

Despite the aforementioned substitution, sperm chromatin also contains histones. Accordingly, studies conducted in humans have demonstrated that about 15–20 % of sperm DNA, which in contrast to porcine sperm (that only present protamine-1), contains two different protamine-types (i.e. protamine-1 and protamine-2), is structured around histones (O'Brien and Zini 2005). This finding is highly relevant, since the structure associated with histone-linked DNA domains is much less compact and rigid than the one related to protamine-linked DNA domains (Wykes and Krawetz 2003; O'Brien and Zini 2005). In addition, these histone-linked domains have been reported to be located at the telomeric sequences, so that they present a given function (O'Brien and Zini 2005), that may be related to fertilisation and/or early embryo development (Barroso et al. 2009).

Chromatin in spermatozoa does not only contain protamines but also histones, as has been reported in humans (Gatewood et al. 1990) and pigs (Flores et al. 2011), amongst other mammalian species. The presence of histone has been associated with telomeres (Gineitis et al. 2000; Zalenskaya et al. 2000). In addition, chromatin packaging in the nucleus is not homogeneous, since some zones are more condensed than others (Flores et al. 2011) and sperm chromosomes are arranged in territories (Foster et al. 2005). This might be related with the hypothesis that sperm chromatin retains some features of active chromatin, mainly acetylated histones, and the arrangement of certain chromatin domains in the nucleosomes.

On the other hand, Nazarov et al. (2008) have proposed a model for the organisation of human sperm chromatin, in which sperm chromosomes are organised at different structural levels in a non-random way. Similarly, Flores et al. (2011) have also proposed a model for the nuclear architecture of boar spermatozoa, of areas with higher or fewer chromatin condensation levels.

Finally, we must also mention that protamine-1 contains more cysteine residues in pigs than in other mammalian species (Gosálvez et al. 2011). As studies conducted in boar sperm cryopreservation have shown (Flores et al. 2011; Yeste et al. 2012), these elevated number of cysteine residues appear to be related with their contribution to the stability of nucleoprotein structure, through the disulphide bonds that such residues establish between them.

8.8.2 Remodelling of Sperm Chromatin

After fusion of sperm and oocyte membranes, a global remodelling of sperm chromatin takes place. This process entails the participation of different elements/events, such as reduced glutathione (GSH), histone-assembling, chromatin maturation, nucleosome positioning or addition of other DNA-binding proteins (McLay and Clarke 2003; Ajduk et al. 2006).

Early studies conducted in mice showed that sperm chromatin remodelling in ooplasm consisted of three phases: decondensation, recondensation and male pronuclei formation (Adenot et al. 1991).

Decondensation overlaps with the anaphase of the second meiotic division in the oocyte. During this stage, GSH present in the oocyte cytoplasm decondenses sperm DNA by reducing the protamine-disulphide bonds (Calvin and Bedford 1971; Kvist 1980; Perreault et al. 1984, 1988; Sutovsky and Schatten 1997). As the links are cleaved, the 'doughnut' loops unfold, the sperm chromatin is uncoiled, and protamines can be removed from this paternal chromatin (Perreault 1992). Interestingly, using intracytoplasmic injection (ICSI), it has been reported that equine and porcine cytoplasm decondenses equine and porcine sperm chromatin (Mugnier et al. 2009). In addition, bovine oocyte-cytoplasm decondenses sperm chromatin from human (Nakamura et al. 2001) and equine species (Li et al. 2003), and porcine ooplasm is able to decondense bovine, mouse and human sperm chromatin (Kim et al. 1999; Cánovas et al. 2007). Thus, the molecules involved in decondensing sperm chromatin again appear to be partially conserved throughout mammalian species.

After decondensation, sperm chromatin recondensation takes place that consists first of a protamine–histone exchange, in a process that is mediated by nucleoplasmin in *Xenopus* (Philpott and Leno 1992), and seems to be mediated by nucleoplasmin-3 (NPM3) and nucleosome assembly proteins in the case of mammals (McLay and Clarke 2003). To date, the exact time during which protamines are exchanged with histones still remains to be elucidated, since the literature has provided inconsistent data (Nonchev and Tsanev 1990; Shimada et al. 2000; van der

Heijden et al. 2005). The end of chromatin recondensation overlaps with the telophase of the second meiotic division in the oocyte and at that time sperm DNA is bound to histones and already presents a nucleosome-based structure (McLay and Clarke 2003; van der Heijden et al. 2005).

The last stage of sperm chromatin remodelling is the formation of the male pronucleus after decondensation of the tightly histone-recondensed sperm chromatin. During this phase, sperm chromatin interacts with proteins involved in kinetochore formation (Schatten et al. 1988) and/or proteins involved in DNA replication (McLay and Clarke 2003).

8.8.3 Centrosome and Pronuclei Migration

The process of pronuclear migration takes longer in mammals (about 12 h) than in other species, such as sea urchin (one hour). During this process, the mammalian male pronucleus enlarges while the oocyte nucleus completes its second meiotic division. This male pronucleus is associated with the centrosome, which acts as a microtubule nucleation centre to form the sperm aster. The sperm aster grows and drives the centrosome and the associated male pronucleus from the cell cortex towards the centre of the oocyte (Barroso et al. 2009; Snook et al. 2011). Thus, the role of the male pronucleus in pronuclear formation and chromosome alignment is crucial. This is supported by other observations made in humans that have related infertility with structural abnormalities and incomplete junctioning of the centrosome (Hewitson et al. 2002).

In contrast, the female pronucleus presents neither an associated centrosome nor microtubule activity (Barroso et al. 2009). As the female pronucleus is able to migrate along microtubules from the cell cortex towards the centrosome located in the centre of sperm aster, it is quite reasonable to suggest that this movement along the microtubule lattice involves a dynein motor, similar to organelle motility (Schatten 1994; Allan 1996). In fact, dynein and dynactin are involved in the union of both pronuclei. However, while dynein is exclusively located around the female pronucleus, dynactin has been observed around both pronuclei in association with nucleoporins, vimentin and dynein (Helfand et al. 2002; Barroso et al. 2009). Thus, a sperm aster is required for dynein to localise the female pronucleus and the microtubules are necessary to retain dynein, but not dynactin, at their surface. This emphasises the relevance of the integrity of the sperm nucleus beyond fertilisation and suggest that nucleoporins, vimentin and dynactin associate upon pronuclear formation (Payne et al. 2003).

Finally, the sperm aster contacts the female pronucleus allowing dynein to interact with nucleoporins, vimentin and dynactin (Helfand et al. 2002; Sheeman et al. 2003). Then, each pronucleus migrates towards the other and DNA is replicated as it travels. Upon meeting, the two nuclear envelopes break down. However in mammals, instead of producing a common syngamy (as happens in sea urchin fertilisation), the chromatin condenses into chromosomes that are disposed on a

common mitotic spindle. Therefore, a true diploid nucleus in mammals is first seen not in the zygote, but at the two-cell stage (Alberts et al. 2008).

To conclude with this section, we must mention that there are differences between species in terms of oocyte competence to form pronuclei. Accordingly, Mugnier et al. (2009) have reported that despite penetration rates being higher in porcine than in equine oolemma, the fertilisation rates after ICSI are higher in equine oocyte cytoplasm than in the porcine ooplasm. This finding indicates that, at least under ICSI conditions, the equine ooplasm is more competent for the formation of pronuclei than the porcine ooplasm.

8.8.4 The Relevance of Sperm Chromatin Integrity in Embryo Viability

In mammals, several reports have shown that sperm chromatin integrity is related to fertility and in vitro embryo development up to the blastocyst stage (Silva and Gadella 2006; Didion et al. 2009). Chromatin packaging has also been reported to be the main cause of ICSI failure (Nikolettos et al. 1999).

Esterhuizen et al. (2002); Zalensky and Zalenskaya (2007) have emphasised the relevance of sperm chromatin organisation during fertilisation, since such genomic architecture appears to govern the orchestrated chromatin decondensation and the ordered activation of the male genome. These authors have thereby suggested the paternal contribution to the epigenetic level of embryos. Related to this, the presence of the histone human testis/sperm-specific histone H2B (hTSH2B), which is expressed in spermatogenic germline cells and in some mature spermatozoa, has been related to chromatin decondensation, pronuclei formation and the activation of paternal genes after fertilisation and during early embryonic development (Singleton et al. 2007). On the other hand, Avendaño et al. (2009) have shown that infertile men can present DNA fragmentation in morphologically normal spermatozoa. The presence of these DNA-fragmented spermatozoa is negatively correlated with embryo quality and pregnancy outcome. Again, this finding highlights the relevance of sperm chromatin integrity in embryo viability.

Therefore, it is important to evaluate the integrity of sperm DNA due to its robust power to predict fertilisation in vitro (Duran et al. 1998; Larson et al. 2000), through tests such as TUNEL, Comet assay, SCSA or SCDt (see also Chap. 9). The origin of DNA damage in boar spermatozoa can be explained by different causes, such as defective packaging during the transition of histone to protamine complex during spermiogenesis, the result of oxidative damage or even apoptosis, although the actual existence of apoptotic mechanisms in mature spermatozoa is quite challenged (Tamburrino et al. 2012). In this regard, Koppers et al. (Koppers et al. 2011) have suggested that spermatozoa have a special kind of apoptosis that only involves the early stages of apoptosis but not DNA fragmentation. Indeed, these authors have identified the involvement of PI3 K (phosphoinositide 3-kinase)/AKT pathway in sperm apoptosis and they have observed that

the apoptotic cascade in spermatozoa is characterised by loss of rapid motility, generation of mitochondrial ROS, activation of caspases in the cytosol, annexin V binding to the cell surface, cytoplasmic vacuolisation and oxidative DNA damage. However, DNA fragmentation does not occur as a direct result of apoptosis in spermatozoa as it does in somatic cells.

Taking into account these findings, it has been suggested that this truncated apoptotic cascade prepares spermatozoa for silent phagocytosis within the female tract and prevents DNA-damaged spermatozoa from participating in fertilisation (Koppers et al. 2011). Intriguingly, phosphatidylserine externalisation is a late event in sperm apoptosis that may facilitate the silent phagocytosis of moribund cells in the female reproductive tract, i.e. the phagocytosis of senescent spermatozoa without generating an inflammatory response (Aitken et al. 2012). In this context, this process will culminate in a regulated cell death that will prepare the spermatozoa for phagocytosis by leucocytes in the female reproductive tract (see Sect. 5.8 about Reproductive Immunology in Swine).

In addition, some of these apoptotic changes have also been associated with capacitation, so that Aitken (2011) has hypothesised that capacitation and apoptosis might be part of a continuous process driven by peroxynitrite and oxysterol formation. According to this hypothesis, if the spermatozoon fails to find an egg, then the continued generation of peroxynitrite might promote oxysterol formation in the mitochondrial membranes, leading to activation of the intrinsic apoptotic cascade that ultimately results in cell death. (Aitken 2011).

8.9 The Ubiquitin–Proteasome System During Fertilisation

8.9.1 Introduction

One of the most important post-translational changes for protein is ubiquitination. Ubiquitination consists of a stable and covalent modification that marks defective or outlived intracellular proteins and alters their activity by proteolytic degradation through 26S proteasomes or lysosomes (Hochstrasser 1996; Sutovsky et al. 1999; see also Chap. 7). This route is essential for those events that require degradation of substrate-specific proteins, such as progression within the cell cycle, endocytosis of membrane receptors, presentation of antigens in the humoral response and notwithstanding fertilisation (Sawada et al. 2002; Sutovsky 2011; Yi et al. 2012). However, despite the relevance of sperm proteasome during fertilisation being currently better known, there are also indices that suggest the proteasome pathway could be involved in other regulation processes of sperm physiology. In this regard, the studies dealing with this issue to date have been scarce, and more evidence is still needed to learn about the actual role of ubiquitin proteasome in regulating sperm function.

The presence of proteasome has been reported in spermatozoa of different species, including invertebrates (Sakai et al. 2004; Yokota and Sawada 2007) and vertebrates (Zimmerman and Sutovsky 2009). In mammals, the components of

the 26S proteasome have been found in different species, such as pigs (Sutovsky et al. 2004a; Yi et al. 2012; Zimmerman et al. 2011), humans (Baker et al. 2007; Morales et al. 2003; Tipler et al. 1997; Wojcik et al. 2000), rats (Baker et al. 2008; Haraguchi et al. 2007; Pizarro et al. 2004) and mice (Baker et al. 2008; Pizarro et al. 2004; Tipler et al. 2007). Indeed, the presence of multiple ubiquitin-specific proteases (Baker et al. 2008) and ubiquitin-conjugating enzymes E2 (Fischer et al. 2005; Sutovsky et al. 2000; Tipler et al. 1997) and E3 (Rivkin et al. 2009; Rodríguez and Stewart 2007; Wong et al. 2002) have been found identified in the proteomes of mammalian spermatozoa.

Other findings that support the view of the ubiquitin–proteasome system being involved in sperm function are related to the presence of ubiquitinated proteins in the sperm nucleus (Haraguchi et al. 2007) and with the nature of proteasome α6 subunit which has been found as a substrate for Tyr phosphorylation during capacitation (Arcelay et al. 2008; Ficarro et al. 2003).

Ubiquitination–proteasome pathway is not restricted to capacitation and post-fertilisation events (see also Chap. 7), but it also plays a critical role during spermatogenesis, when ubiquitination allows the replacement of the spermatid's nuclear histones by transition nuclear proteins (TNP) and then by permanent substitution with protamines (Baarends et al. 1999a, b). Ubiquitination also participates in the dramatic reduction of sperm centrosome during spermatid elongation. In addition, and although protein ubiquitination typically occurs in the cell cytosol or nucleus, defective mammalian spermatozoa are ubiquitinated on their surface during sperm maturation in the epididymis (Sutovsky et al. 2001a). Related to this, morphologically abnormal ubiquitinated spermatozoa have been found in human ejaculates (Rawe et al. 2008; Sutovsky et al. 2001a), thereby indicating that they escaped from epididymal degradation. Baska et al. (2008) found these ubiquitinated spermatozoa mainly presented defects in head and/or axoneme. Finally, some reports have found a relationship between sperm ubiquitination and DNA fragmentation (Sutovsky et al. 2002, b) and have associated this degradation pathway with poor quality sperm parameters (Sutovsky et al. 2001b). However, this finding has been challenged by Marchiani et al. (2007) who found apoptotic bodies in human semen, so that it still remains to be firmly elucidated whether ubiquination and/or apoptosis pathways take place in mature spermatozoa.

8.9.2 The Sperm Proteasome During In Vitro Fertilisation and Acrosome Exocytosis

To date, the relevance of sperm proteasome during IVF and acrosome exocytosis has been documented in different species (Pizarro et al. 2004; Sutovsky 2011), and as mentioned before the presence of an extracellular pool of proteasomes has been observed in the plasma membrane of the head of pig (Yi et al. 2007), human (Morales et al. 2004), cattle (Sánchez et al. 2011), mouse (Pasten et al. 2005) and ascidian (Sawada et al. 2002) spermatozoa. Related to this, Zimmerman et al.

(2011) have demonstrated that the sperm acrosome-borne 26S proteasomes recognise and degrade ubiquitinated ZP proteins during pig fertilisation (Zimmerman et al. 2011).

One aspect that is well-known is the involvement of the sperm proteasome in the sustained phase of the Ca^{2+} influx that precedes acrosome exocytosis (Morales et al. 2003). Proteasome inhibitors block sperm capacitation (see also Chap. 7) and sperm-penetration through ZP but they do not affect sperm motility and ZP-binding (Morales et al. 2007; Kong et al. 2009). In addition, proteasome phosphorylation and activity are regulated by the extracellular matrix proteins, fibronectin and laminin (Díaz et al. 2007; Tapia et al. 2011). This supports the idea that COCs and their matrix play an important role in mammalian fertilisation (Jin et al. 2011).

We must finally mention other previous studies conducted on human sperm that have reported a relationship between proteasome and male fertility. Indeed, human spermatozoa of poor quality and/or with defective centriolar/pericentriolar structures present lower proteasomal activity. This deficiency in intrinsic proteasome activity seems to be related to a lower fertilising ability (Rawe et al. 2008; Rosales et al. 2011).

8.9.3 Mechanism of Ubiquitination During Acrosome Exocytosis and Sperm-ZP Interaction: UBA1

In the ubiquitination mechanism, ubiquitin plays a significant role. This protein is a small and highly conserved chaperone protein presents in all eukaryotic cells that binds covalently to substrate proteins via an isopeptide bond between the C-terminal glycine of ubiquitin and the E-amino group of a lysine in substrate proteins.

Ubiquitination follows with a multistep enzymatic pathway, catalysed by the enzymatic activities of ubiquitin-activating enzyme E1 (UBA1), ubiquitin-conjugating enzyme E2 and a substrate-specific ubiquitin ligase E3. During the initial step of ubiquitin-protein binding, mediated by the E1-type ubiquitin-activating enzyme (UBA1), one molecule of ubiquitin interacts with an internal lysine residue of the substrate protein (Fig. 8.6). Following this, additional molecules of ubiquitin are ligated in tandem forming a multi-ubiquitin isopeptide (Hershko and Ciechanover 1998; Glickman and Ciechanover 2002). The polyubiquitinated substrate proteins are then recognised and degraded by the 26S proteasome, which is composed of a proteolytic complex (20S) and one/two regulatory complexes (19S) (Glickman et al. 1998; Voges et al. 1999). Substrate proteins are degraded and the multi-ubiquitin isopeptide is finally released from the substrate protein and disassembled by deubiquitinating enzymes (Wilkinson 1997).

The presence and role of UBA1 in reproductive cells has also been investigated. In this regard, it has been reported to be necessary for mouse (Odorisio et al. 1996) and human spermatogenesis (Zhu et al. 2004), and highly expressed in rat spermatogonia (Du et al. 2008). Furthermore, an active form of UBA1 enzyme

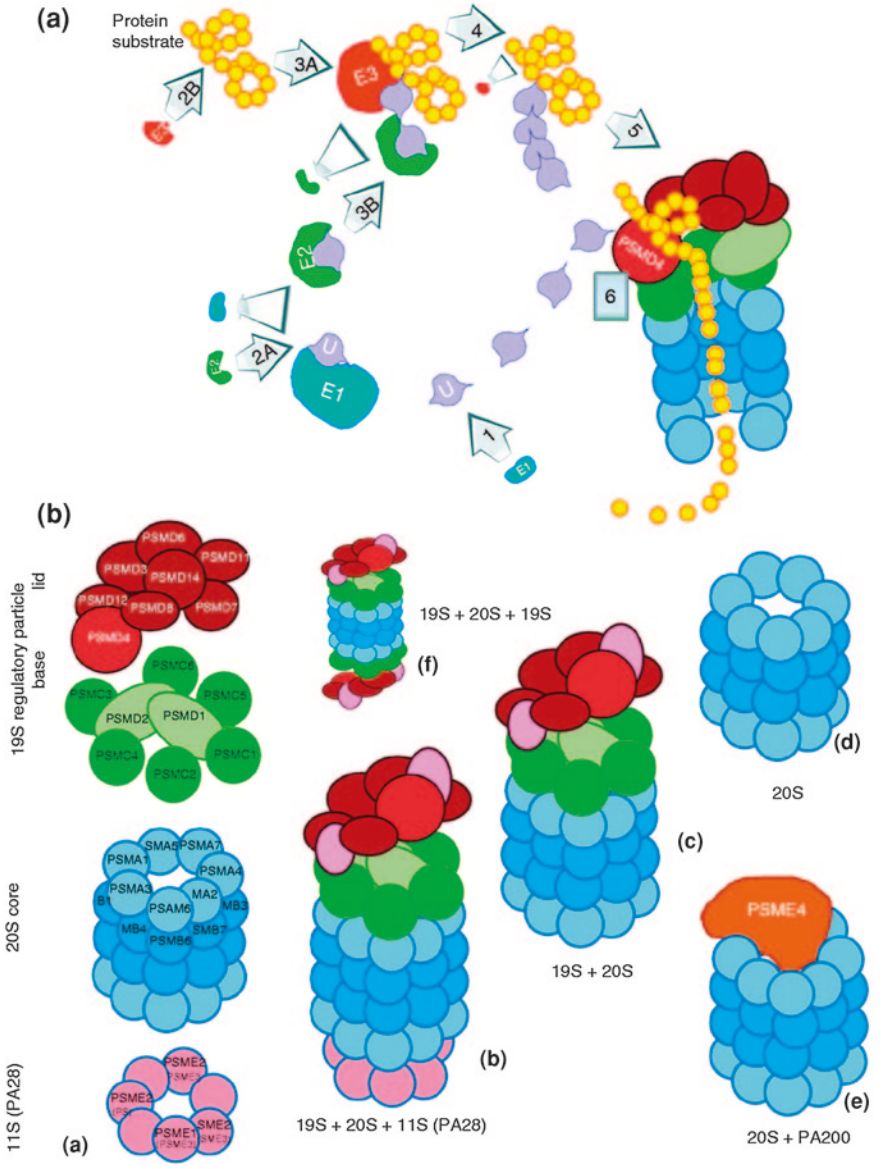

Fig. 8.6 **a** Diagram of protein ubiquitination and degradation by the 26S proteasome. **b** Variations on the subunit composition of the 26S proteasome (Sutovsky 2011) Reproduced with permission

has been found in bovine epididymal fluid, thus suggesting that it could also play some role in epididymal sperm maturation (Baska et al. 2008).

In mature rat spermatozoa, UBA1 is also present and it is phosphorylated during sperm capacitation (Baker et al. 2010). This protein also plays a key role

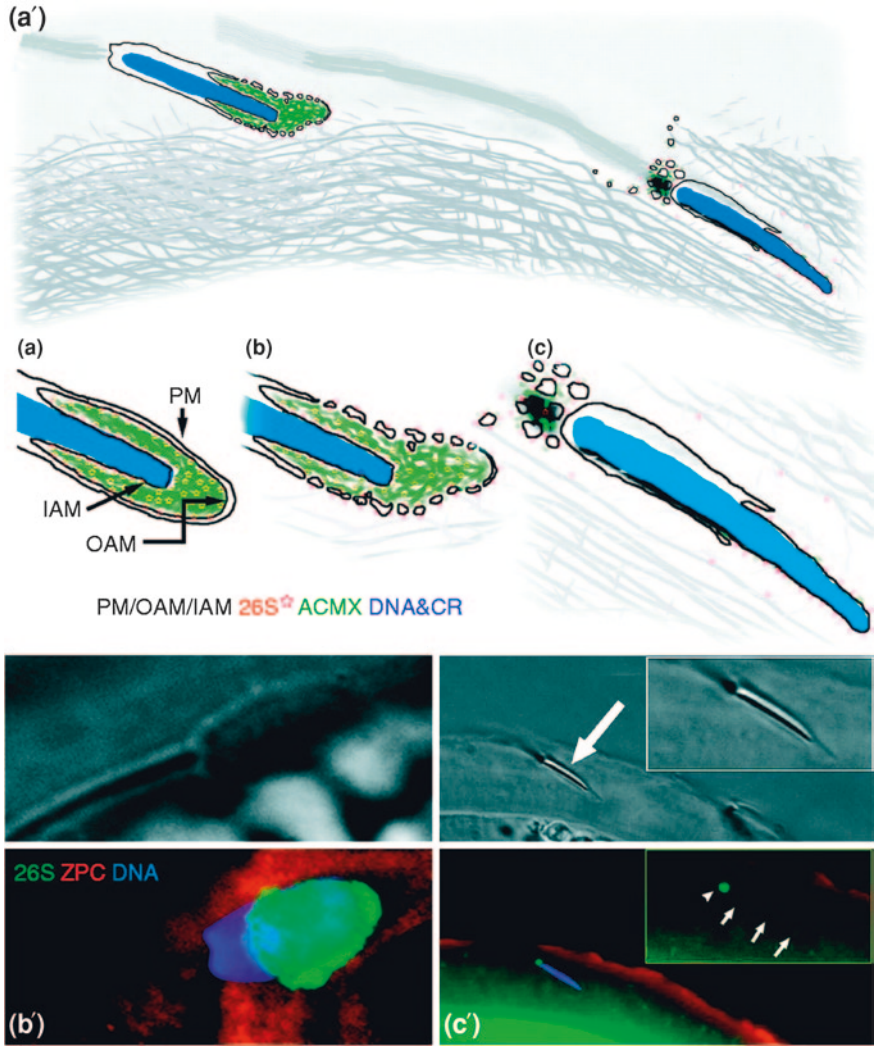

Fig. 8.7 Acrosome remodeling and proteasome localisation during the zona pellucida-induced acrosome reaction in the pig IVF system. *a′* Diagram based on immunofluorescence and transmission electron microscopy images of boar sperm acrosomal exocytosis. In an intact acrosome (**a**), proteasomes (*red star*) are bound to inner (*IAM*) and outer (*OAM*) acrosomal membranes and also dispersed throughout acrosomal matrix (ACMX; *green*). On sperm–zona binding, the *IAM* and *OAM* give rise to acrosomal membrane vesicles coated with proteasomes (**b**), which together with loosened ACMX form a zona-bound acrosomal shroud, a transient but structurally stable structure. During sperm–zona penetration, (**c**) remnants of the acrosomal shroud envelope the sperm tail connecting piece, while a subpopulation of proteasomes remain associated with *IAM*. (*b′*) Immunodetection of proteasomes (*green*) in the acrosomal shroud of a ZP-bound, acrosome-reacted boar spermatozoon. Sperm receptor protein *ZP*C is labelled red; DNA is counterstained blue with DAPI (*c′*) Proteasomes (*green*) are detected on the *IAM* (*arrows*) and in the cluster of acrosomal membrane vesicles associated with the sperm tail connecting piece (*arrowhead*) in a spermatozoon passing through ZP (*red*; anti-*ZPC* antibody) (Sutovsky 2011) Reproduced with permission

during fertilisation in porcine and other mammalian species, since different reports have shown that the ubiquitin–proteasome pathway participates in sperm–oocyte interaction (Sutovsky et al. 2004a; Yi et al. 2007a, b, 2012; Zimmerman and Sutovsky 2009, 2011) (Fig. 8.7). Intriguingly, proteasomal proteolysis of proteins from spermatozoa and oocyte takes place when sperm acrosome interacts with the ZP of the oocyte during fertilisation (Fig. 8.7). Related to this, Sutovsky et al. (2004a) observed that proteasome inhibitors impeded sperm penetration of ZP. On the other hand, sperm-acrosomal ubiquitin C-terminal hydrolases have also been reported to be involved in antipolyspermy defense (Yi et al. 2007).

Yi et al. (2012) have recently studied the requirement of UBA1 activity for sperm capacitation, acrosome exocytosis and spermatozoa–ZP penetration during porcine IVF. These authors sought to elucidate whether the substrates were solely ubiquitinated during gametogenesis or *de novo* ubiquitination also took place during acrosome exocytosis and fertilisation. In fact, during spermatogenesis in boars, UBA1 is accumulated in the spermatogonia, spermatocytes and spermatids, and in the acrosome regions of round and elongating spermatids, so that an active form of UBA1 is present in the acrosomal extracts of boar spermatozoa.

Against this background, Yi et al. (2012) assessed the effect of an UBA1 inhibitor (PYR-41) on sperm capacitation and oocyte fertilisation. The incubation of this inhibitor altered the reorganisation of the outer acrosomal membrane during sperm capacitation, and reduced the fertilising ability of spermatozoa. These authors also showed that PYR-41 altered the capacitation-induced modification of two acrosomal proteins: the spermadhesin AQN1 and the acrosin inhibitor SPINK2. In the presence of PYR-41, spermatozoa showed normal motility and sperm–ZP binding, but they were unable to penetrate the oocyte, thereby suggesting that the course of acrosomal exocytosis had been altered. These data have led them to conclude that *de novo* protein ubiquitination involving UBA1 contributes to sperm capacitation and acrosomal function during fertilisation.

8.10 Sperm Contributions Beyond Fertilisation

8.10.1 Introduction

The spermatozoon does not only act as a simple vector that delivers paternal DNA to the oocyte after fertilisation, but it also contributes to other events during the first stages of pre-implantation embryo development. Thus, the oocyte needs the presence of the spermatozoon for its activation, and at the same time the spermatozoon needs to fertilise the oocyte to decondensate its chromatin.

The spermatozoon has, therefore, a very dynamic and critical participation in normal embryogenesis that clearly extends beyond the fertilisation process (Barroso et al. 2009). Although the molecules involved in fertilisation and post-fertilisation developmental steps remain largely unknown, defective fertilising spermatozoa may cause arrest of development at multiple levels during embryo

pre-implantation development. In addition, evidence suggests sub-lethal effects can be carried over after implantation, resulting in untoward embryonic/foetal defects (Barroso et al. 2009).

To the authors' knowledge, there is no specific report on pigs that reviews sperm contribution beyond fertilisation. For this reason, this section essentially mentions the role of various human sperm components/molecules on early embryo development, since they are the most abundant so far.

The contribution of sperm tail to embryonic development remains largely unknown. Despite microinjection of isolated sperm fractions (heads or tails) allowing oocyte activation and pronuclei formation, these oocytes are not able to undergo normal mitotic division (Moomjy et al. 1999; Alberio et al. 2001). This finding points to the fact that the structural integrity of fertilising spermatozoon contributes to normal embryogenesis, since there are abnormalities of the mitotic apparatus when sperm integrity is damaged (Colombero et al. 1999).

8.10.2 Oocyte Activation as a Sperm-Mediated Step

As mentioned above, the contribution of a given fertilising spermatozoon in the formation of a new zygote after fertilisation is not only related to the paternal haploid genome, but is also involved in signalisation processes that lead to oocyte activation and to the microtubule assembly leading to the formation of mitotic spindles during the initial zygote development.

It is well-known that only fertilised oocytes are able to continue meiosis from metaphase II, where they are arrested at the time of ovulation (Alberio et al. 2001). This phenomenon, also known as oocyte activation, is related to a signalling mechanism related to spermatozoa. According to Barroso et al. (2009), three theories have been proposed to explain this event:

1. *The fusion theory* that proposes the presence of active calcium-releasing components in the sperm head (Dale et al. 1985; Fissore et al. 1999).
2. *The receptor theory* that suggests a signal transduction pathway mediated by a receptor located on the oolemma (Kline et al. 1988).
3. *The calcium pump theory* that hypothesises that Ca^{2+} enters the oocyte upon fertilisation, either from stores in the sperm itself or through channels of the sperm plasma membrane (Jaffe 1983).

Although a sperm factor located at the equatorial segment and known as oscillin was initially identified by Parrington et al. (1996) as being responsible for calcium oscillation in spermatozoa that leads to oocyte fertilisation in mammals, Tesarik (1998) later reported that oscillin was not responsible for these calcium oscillations in mammalian sperm cells.

Another interesting finding in sperm contribution to oocyte activation is related to sperm PLC-zeta (PLCζ) (Saunders et al. 2002, 2007). PLCζ is a novel form of PLC specific for mammalian spermatozoa that appears to trigger calcium

oscillations leading to oocyte activation (Kouchi et al. 2004). This observation was confirmed when recombinant PLCζ was injected into mouse oocytes and this was seen to lead to egg activation (Kouchi et al. 2004). Following this, it has been proposed that after fusion of sperm and oocyte plasma membranes, sperm-derived PLCζ diffuses into the oocyte cytosol that results in hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) from an unknown source to inositol 1,4,5-trisphosphate (IP_3) (Swann et al. 2004; Saunders et al. 2007).

Two of the earliest indications of oocyte activation are the extrusion of cortical granules by exocytosis and the resumption of meiosis. For the time being, we know that both events are triggered by Ca^{2+} transient oscillations, but it remains to be elucidated which pathways leading to the intracellular Ca^{2+}-release are involved. Indeed, $Ca2^{+}$ oscillations allow the resumption of meiosis as the activity decreases of both a M-phase-promoting factor and a cytostatic factor, as seen in *Xenopus* oocytes (Dupont 1998). The extrusion of cortical granules seems to be mediated either by Ca^{2+} transients and/or by PKC (Eliyahu and Shalgi 2002). Although the exact mechanism leading to the extrusion of cortical granules has not been completely understood, it is worth noting that kinases from Src-family have been suggested to be involved in calcium release and oocyte activation (Talmor-Cohen et al. 2004).

Finally, another interesting contribution about the role of sperm presence in oocyte activation concerns two different sperm-borne proteins that induce pronuclei formation in oocytes. These sperm proteins are:

1. The truncated c-Kit tyrosine kinase that activates the dormant/quiescent oocyte by eliciting intracellular Ca^{2+} oscillations. In this context, Ca^{2+} as a secondary messenger for downstream effectors/events of zygotic development (Sette et al. 1997, 2002).
2. The post-acrosomal sheath WWP domain-binding protein (PAWP), which is exclusively located in the post-acrosomal sheath of the sperm perinuclear theca (Wu et al. 2007). The WWP domain (also known as WW) is a protein domain with two highly conserved tryptophans that binds proline-rich peptide motifs.

Interestingly, Wu et al. (2007) observed that when recombinant PAWP or an extract of perinuclear theca is injected into metaphase-II-arrested porcine, bovine, macaque or xenopus oocytes, a high rate of pronuclear formation takes place. In contrast, these authors also observed that fertilisation was arrested and pronuclei formation prevented when ICSI of porcine oocytes was performed together with injection of PAWP-competitive peptide or of an anti-recombinant-PAWP (Wu et al. 2007). This finding thus points out the relevant role of sperm's PAWP in oocyte activation.

8.10.3 The Role of Paternal Mitochondrial DNA in the New Zygote

Contribution of sperm DNA in post-fertilisation events has been previously discussed in the specific section about the integrity of sperm nuclear chromatin and

its relevance in early embryo development (see Sect. 8.8.4). However, sperm mitochondrial DNA (mtDNA) also plays a relevant role in the development of the new zygote. This issue will be discussed in this Section.

To the best of our knowledge, paternal mtDNA inheritance has most assuredly not been reported so far. However, some studies have hinted at this possibility (Schwartz and Vissing 2002). Notwithstanding, Brenner et al. (2004) reported heteroplasmic or mixed mtDNA inheritance after ooplasmic transfusion (ooplasmic transfusion consists of injecting ooplasm from young oocytes to older oocytes from older women).

Most somatic cells of the body contain between 103 and 104 copies of mtDNA. Oocytes contain slightly higher copies of mtDNA (mtDNA), which can be related to the preparation for energetic demands of the new individual during embryogenesis (Shadel and Clayton 1997). As explained in Chap. 2, spermatozoa from porcine and other species switch between aerobic and anaerobic metabolism, depending on the oxygen tensions of the surrounding environment, from near anoxia in the testis and epididymis to ambient tension in the vagina and in vitro.

Sperm mtDNA, like somatic cells, is highly vulnerable to mutation (Cummins 1998) and in humans it has been reported that a high number of mtDNA deletions are found in at least 50 % of normospermic men (Cummins et al. 1998). This high vulnerability of sperm mtDNA to mutation is related to the length of the spermatogenic wave, which consists of spermatogenesis (34–36 days) and epididymal maturation (12–15 days) and lasts between 46 and 51 days (See also Chap. 3). During these processes, spermatozoa are exposed to mutagenic agents that can damage DNA. This is of particular importance, especially if we bear in mind that spermatozoa present few DNA-repair mechanisms.

In this context, defective sperm mtDNA needs to be eliminated to prevent this detrimental paternal inheritance contributing to the embryo. Therefore, mtDNA from mammalian embryo is mainly inherited from the maternal rather than paternal line. In this regard, Short (1998) proposed that this asymmetric inheritance could be the fundamental driving force behind amphimixis and anisogamy because of the need to conserve a healthy stock of mtDNA for embryo development by means of a long period of quiescence in meiosis. Indeed, the strictly maternal inheritance of mtDNA in mammals represents a paradox, especially when we take into consideration that a fertilising spermatozoon introduces up to 100 functional mitochondria into the oocyte cytoplasm at the time of fertilisation (Ingman et al. 2000).

However, it is quite likely that destruction of paternal mitochondria after fertilisation results from an evolutionary and developmental advantage (Ankel-Simons and Cummins 1996), since sperm mtDNA can be compromised by deleterious action of ROS that spermatozoa encounter in their surrounding environment during spermatogenesis, storage, transport along the female tract and fertilisation, as Aitken (1995) pointed out. Kaneda et al. (1995) found that membrane proteins from spermatozoa, rather than mtDNA, determined whether sperm mitochondria and mtDNA die or are degraded. Related to this, we must indicate that one of the most significant processes related to the ubiquitin–proteasome pathway in

fertilisation (see also Sect. 8.9) is the destruction of sperm mitochondria (Sutovsky et al. 1999, 2000). Thompson et al. (2003) found that a mitochondrial membrane protein known as prohibitin, was one of the ubiquitinated substrates that made the sperm mitochondria responsible for the egg's ubiquitin–proteasome dependent, proteolytic machinery after fertilisation. Notwithstanding, abnormalities of this recognition system seem to be related to deregulations of mitochondrial inheritance and leads them to escape from the 'sperm quality control' that oocyte manages.

8.10.4 'Early' and 'Late' Paternal Effects

Assisted reproductive technologies have been widely implemented in human and other mammalian species during the last 20 years, and some lessons about early and late paternal contributions can be obtained from these technologies.

Apart from the relationship between successful fertilisation and oocyte quality/characteristics in human (Van Blerkom 2000; Swain and Pool 2008) and other species, some evidence has supported that contribution of sperm to faulty fertilisation and abnormal embryogenes (Silva and Gadella 2006). Indeed, abnormal sperm morphology and/or function, which is related to oxidative damage, DNA fragmentation or chromatin defective packaging, can result in failed or delayed fertilisation and/or aberrant embryo development. This has been reported in human but also in bovine and other species (Oehninger et al. 1989, 1996; Ron-el et al. 1991; Parinaud et al. 1993; Grow et al. 1994; Gorczyca et al. 1993).

Early cleavage divisions of the new zygote depend on the endogenous machinery of the oocyte, and transcription of the embryo genome occurs between the four-cell and eight-cell stage of preimplantation development (Braude et al. 1998). For this reason, alterations of sperm nucleus (like DNA fragmentation) are not detected up to the eight-cell stage when a major expression of sperm-derived genes has begun, while deficiencies in sperm cytoplasm are detected at the 1-cell stage and during preimplantation embryo development (Tesarik et al. 2004).

From these observations, one can talk about 'early' and 'late' paternal effects depending on which pathologies are related to sperm cytoplasm and nucleus (Tesarik 2005):

– 'Early' paternal effects are related to abnormal embryo morphology and low cleavage speed, and are not associated with DNA fragmentation of spermatozoa. Early paternal effects may also be due to alterations of the oocyte-activating factor, which can be caused by no sperm delivery of this factor or by a dysfunctional oocyte-activating factor, and to deficiencies of the centrosome-cytoskeletal apparatus.
– 'Late' paternal effects are related to poor development competence and implantation failure and appear to be associated with the sperm chromatin integrity of spermatozoa (Barroso et al. 2009). In humans, ICSI with testicular spermatozoa has been regarded as an efficient treatment to avoid the 'late paternal effect, as ejaculated spermatozoa can contain fragmented DNA due to the ROS-mediated

effects of DNA damage during epididymal maturation (Greco et al. 2005). As more recent studies have reported, late paternal effects may also be related to sperm mitochondrial dysfunctions or abnormal sperm mRNA delivery.

Finally, alterations due to genomic imprinting anomalies may result in both early and late paternal effects.

8.10.5 Disorders Beyond Fertilisation due to Defective Spermatozoa

8.10.5.1 Disorders of Oocyte Activation

As mentioned above, early paternal effects can be provoked by a defective/absent oocyte-activation factor. Dysfunction of the oocyte-activation factor occurs when a fertilising-spermatozoon enters the oocyte but there is no stimulation for oocyte activation. This leads to failure to resume meiosis or to undergo extrusion of cortical granules. In humans, 40 % of failed fertilisation cases after ICSI are related to the absence or dysfunction of the sperm's oocyte-activating factor (Rawe et al. 2000). Yoon et al. (2008) have related this defective oocyte-activating factor with undetectable levels of PLCζ in sperm, which results in the inability of fertilising spermatozoon to induce calcium oscillations in the oocyte.

Apart from early paternal effects, there are other cases of non-response of the oocyte to the sperm's oocyte-activating factor that are due to multiple elements of the oocyte responsive system (PIP_2, IP_3 receptor, PKC, etc.), rather than the oocyte-activating factor itself.

8.10.5.2 Disorders Related to Microtubule Organisation

Another dysfunction that may result in fertilisation arrest is related to defective organisation of microtubules/centrosome (Asch et al. 1995; see also Sect. 8.8), as correlation between sperm aster formation and embryonic cleavage rate has shown (Terada et al. 2004). In a study conducted in humans, fertilisation arrest was observed to occur at various levels:

1. At metaphase II
2. After successful fertilisation
3. After formation of the sperm aster
4. During meiotic cell cycle progression
5. During mitotic cell cycle progression.

From the failed fertilisation procedures after human IVF, Rawe et al. (2000) reported that 55.5 % were due to no sperm penetration. In the case of ICSI, the same authors observed that failed fertilisation was due to incomplete oocyte activation in 39.9 % of cases. However, care must be taken when talking about

unsuccessful fertilisation and its underlying causes in ICSI procedures. Indeed, the presence of the sperm acrosome and apical perinuclear theca, structures normally removed at the oolemma during in vivo and IVF, may result in abnormal nuclear remodelling during sperm decondensation and a delay in DNA synthesis (Hewitson et al. 2000, 2003). In fact, as described in Chap. 1, sperm acrosome contains a variety of hydrolytic enzymes that are released into the ooplasm in ICSI, and may thus generate some kind of damage and physical disturbance when decondensing sperm chromatin (Tesarik and Mendoza 1999). Also related to this, Katayama et al. (2002) working in pigs, observed differences between ICSI and IVF in the RNA-binding properties of sperm head components introduced into the ooplasm.

8.10.6 The Role of Sperm mRNAs

Mature and ejaculated mammalian spermatozoa contain mRNA transcripts, as detected in human (Ostermeier et al. 2004, 2005; Zhao et al. 2006), boars (Yang et al. 2009), and other species. In addition, the amounts of mRNA in human spermatozoa are 600 times lower than somatic cells (Zhao et al. 2006), and Pessot et al. (1989) found RNA in sperm nucleus and residual DNA, and RNA-polymerases activity has also been early reported (Hecht 1974). After these findings, the main question is to determine the role of these mRNAs, and when they have been synthesised. There are different possibilities:

1. One possibility is that these mRNAs are residual and non-functional material.
2. Another possibility is that these mRNAs have a function during late spermiogenesis. As explained in Chap. 3, spermiogenesis is a complex process in which the round spermatids undergo deep changes, such as condensation of the nuclear chromatin, formation of the flagellum and development of the acrosomal cap (Wykes et al. 1997). Spermatids lose most of their cytoplasm when they have been transformed into spermatozoa. It is logical, therefore, to consider that mature spermatozoa are unlikely to transcribe novel RNAs and translate protein-coding RNAs (Grunewald et al. 2005). Many of the late post-meiotically transcribed genes are most likely synthesised during the final burst of transcription prior to histone-protamine exchange in haploid spermatids, and then later actively translated in elongated spermatids (Kramer and Krawetz 1997).
   In this second possibility, mRNAs would equilibrate imbalances in sperm phenotypes brought about by meiotic recombination and segregation. In addition, these mRNAs have also been hypothesised to be involved in early embryo development. Specifically, they could establish imprints during the transition from maternal to embryonic genes (Barroso et al. 2009).
3. Another explanation for the presence of mRNA in ejaculated spermatozoa could be related to the existence of translational activities that Naz (1998) reported during capacitation and acrosome reaction of human spermatozoa.

4. Another possibility is that mRNAs accumulated in the sperm nucleus play some role in early embryogenesis (Krawetz 2005). This more recent approach is backed by other observations made on ejaculated sperm in mice (Hayashi et al. 2003), humans (Ostermeier et al. 2004) and pigs (Yang et al. 2009) that emphasises the role of sperm-mRNA delivery to oocytes. In fact, sperm transcripts encoding proteins that participate in fertilisation and embryo development have been found in early embryos after IVF, but not in the non-fertilised oocyte (Signorelli et al. 2012). In heterologous experiments conducted with human spermatozoa and hamster oocytes, Avendaño et al. (2008) have observed that two human sperm-specific transcripts (PSG1 and HLA-E) involved in implantation are found in hamster-fertilised oocytes, fertilised after 24 h post-fertilisation. This finding clearly indicates that mature spermatozoa deliver mRNA into the oocyte, and provides specific transcripts that are needed for embryo development before the activation of its own genome, thereby contributing in embryo survival and development.

On the other hand, some of the transcripts present in ejaculated spermatozoa have been determined and although this list is, of course, incomplete, it provides us with some idea about the nature of the information about these mRNAs encode (Dadoune et al. 2005; Kumar et al. 1993; Miller et al. 1994; Ostermeier et al. 2002):

- Protamine 1 (PRM1) and protamine 2 (PRM2)
- Transition nuclear protein 1 (TNP1, also known as TP1 and STP1), which protein products replace histones and are subsequently replaced by protamines in mature spermatozoa
- Heat shock protein 70 (HSP1A1)
- Heat shock protein 90 (HSP90AA1)
- c-MYC (a protooncogen involved in several carcinogenic processes)
- HLA class 1
- β-integrins
- β-actin
- Variants of phosphodiesterase
- Sperm progesterone receptor
- Aromatase
- Transcript encoding factors (NFkB, HOX2A, ICSBP, JNK2, HBEGF, RXRb, and ErbB3).

In one of the scarce studies about RNAs in mature boar sperm, Yang et al. (2009) have evaluated the presence/absence of different rRNAs and mRNAs. These mRNAs are related to nucleic acid binding, structural modifications and transcriptional regulation, and can therefore be related to spermiogenic processes and/or later fertilisation events. In addition, these authors found that the two major rRNAs essential for 80S cytoplasmic ribosome assembly within eukaryotic cells, were absent in boar spermatozoa, in agreement with other studies conducted with other species, like human (Ostermeier et al. 2002; Lambard et al. 2004), and bovine species (Gilbert et al. 2007).

Yang et al. (2009) also found remnant spermatozoal mRNA transcripts within mature boar ejaculated spermatozoa. It is quite evident that these transcripts play relevant functions mostly of genes in various types of male germ cells (Kramer and Krawetz, 1997; Gilbert et al. 2007). However, the role and the mechanism of action for mRNAs within the mature spermatozoon during fertilisation and embryonic development have yet to be determined.

Despite Hayashi et al. (2003) reporting that spermatozoal mRNA transcripts in mice did not have any effect on embryogenesis and were degraded rapidly in embryos at late 1-cell stage, Yang et al. (2009) suggested that these spermatozoal mRNA transcripts were found to survive up to the 4-cell period. Thus, spermatozoa deliver not only genetic materials but also spermatozoal RNAs into the oocyte during the process of fertilisation. These authors suggested that mRNAs from sperm-specific antigen 2 (SSFA2), and Sestrin 1 (SESN1) could be involved in early embryo development.

Other mRNAs found in mature ejaculated spermatozoa, but less clearly involved in early embryo development, are protamine P1 (PRM1)-mRNAs. According to Yang et al. (2009), the importance of *PRM1* might be related to male infertility, but this should not rule out the possibility that these *PRM1* mRNA residues in spermatozoa may influence early embryo development indirectly. Related to this, Hwang et al. (2012), also working with pigs, have recently reported that the abundance of the following mRNA transcripts: *MYC, CYP19, ADAM-2, PRM1* and *PRM2* is related to early embryo development. In addition, these authors have also determined that *MYC*-mRNA is less abundant in capacitated than in uncapacitated spermatozoa, thereby suggesting that sperm-RNAs also regulate capacitation.

8.10.7 The Role of Sperm MicroRNAs

MicroRNAs (miRNAs) are endogenous or artificial single-stranded siRNAs capable of interfering with mRNA (RNAi) via complete or partial complementarities (Lagos-Quintana et al. 2001). Initially, miRNAs were named small temporal RNAs after being discovered in *Caenorhabditis elegans* and in *Drosophila melanogaster* as regulators of gene expression (Lee et al. 1993; Olsen and Ambros 1999; Reinhart et al. 2000).

Different types of miRNAs have been observed in diverse species. One class of these miRNAs are intron-derived miRNAs, which are derived from intron sequences, and differ from the other intergenic miRNAs in that they require RNA II polymerases and spliceosomal components for their biogenesis (Lin et al. 2007). Intron-derived miRNAs are firstly transcribed from introns as hairpin structures (primary miRNAs) and then matured as single-stranded RNAs by Dicer enzyme (mature miRNAs) in several organisms (Hutvagner et al. 2001; Ketting et al. 2001; Lau et al. 2001; Mourelatos et al. 2002; Tabara et al. 2002). Then, the mature miRNA binds to the three prime untranslated region (3'-UTR) of its corresponding mRNAs (Provost et al. 2002). As a general mechanism of RNAi, RISC-like is also involved in the miRNA pathway (Hutvagner and Zamore 2002).

In zebrafish, RNAi mediated by miRNAs regulates early development. In contrast, in the case of mammals, the exact role of miRNAs in mammalian fertilisation still remains unknown. To date, studies conducted in mice have demonstrated that although sperm-borne prototypical miRNAs are present in mouse spermatozoa and enter the oocyte at fertilisation, they play either a marginal role or no role in mammalian fertilisation and early preimplantation development (Amanai et al. 2006).

8.11 Conclusions

When spermatozoa become completely capacitated, they are able to recognise and bind to the ZP of the egg by recognising properly arranged neutral complex N-glycans of ZP glycoproteins. This interaction triggers the acrosome reaction and the acrosomal content is exocytosed. Then, proacrosin binds to the polysulfated glycans of the ZP and mediates secondary binding of the acrosome-reacted sperm to the matrix. The next steps are the fusion of sperm membrane with oolemma, sperm chromatin remodelling and pronuclei migration. Finally, chromosome alignment takes place and the zygote immediately starts the first and subsequent mitotic divisions. After fertilisation, some spermatozoal components, such as some specific sperm mRNAs can also influence early embryonic development.

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Part III
Artificial Insemination (AI) Technologies in Pigs

Chapter 9
The Boar Ejaculate: Sperm Function and Seminal Plasma Analyses

Sílvia Sancho and Ingrid Vilagran

Abstract A complete study of the boar ejaculate includes both routine sperm quality analysis and complementary tests of sperm function. Rigorous monitoring of both steps permits the fertilising ability of a given ejaculate to be predicted as accurately as possible. With regard to quality analysis and after successfully carrying out a macroscopic inspection of semen (volume, temperature, pH and osmolality), the technician proceeds with a microscope analysis that includes the study of the following relevant semen parameters: concentration, motility, morphology, plasma membrane integrity and osmotic resistance of spermatozoa. This chapter also reviews those tests that assess additional features of the sperm cell, such as its capacitation status, its nuclear integrity and that of its membranes, and also provides information on in vitro fertility assays as estimators of in vivo sperm fertility. Finally, the chapter outlines the main components of seminal plasma and the most important methods in their study.

9.1 Introduction

In the pig sector, the accurate prediction of the fertilising ability of an ejaculate is of great importance. To achieve this objective, we make use of the basic techniques of sperm quality analysis that are routinely applied in the centres of pig production and artificial insemination (AI). However, results from these techniques do not always positively correlate with fertility in vivo (Rodríguez-Martínez 2003; Yeste et al. 2010). That is why, thanks to new technologies that have been implemented in recent years, several complementary assays have been proposed and developed to facilitate reliable estimation of sperm fertilising ability.

Although many of them are still difficult to apply in the sector due to their high cost, turning some tedious practices into simple and standardised protocols are the

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key to the development of state-of-the-art techniques and their implementation in the industry. In this way, the prediction of the fertilising ability of an ejaculate will result in reduced production costs that will significantly offset the expenses incurred.

The total set of techniques is intended to provide a reliable approach to the structure and function of sperm and its surrounding secretions. Specifically, the study of the cell membrane appears to be a good way to evaluate the general viability of the male gamete. Many techniques nowadays resort to fluorochromes to understand processes and structures of sperm such as the acrosome reaction and capacitation, mitochondrial activity, membrane permeability, structure and stability of the nucleus, chromatin integrity, and so on. (Harrison and Vickers 1990; Garner and Johnson 1995; Silva and Gadella 2006).

A thorough approach to the physiology of the sperm cell is essential to establish corrective measures for deviations in fertility; metabolic and enzymatic studies of different chemical components present in the ejaculate help to understand the relationship established between seminal plasma and sperm fertility (Strzezek and Skaweta 1984; Gadea et al. 1998). Studies carried out on the membrane lipid composition of sperm, which broaden our knowledge of the sensitivity of boar spermatozoon to temperature changes (Shadan et al. 2004), and the impairment of nuclear integrity that could be related to fertility problems (Evenson et al. 2002), definitely contributes to the success of fertilisation. Additionally, the study of regulation systems governing sperm cell capacitation and acrosome reaction provide clues for handling ejaculates better.

Undoubtedly, the best in vitro method to predict the fertility of a given ejaculate is to prove its ability for penetrating oocytes (Brown et al. 1990). This method, mostly used in the form of heterologous penetration of zona-free hamster oocytes, has a good rate of correlation with in vivo pregnancy indexes, but it is very expensive to practice and does not provide enough information about the recognition process between gametes or, more concretely, about the molecular interactions occurred when binding oviductal membranes or penetrating the zona pellucida. Other methods, such as the homologous penetration test, provide more information about these processes; however, their reliability to estimate the fertilising ability of a boar ejaculate is still limited (Zhao et al. 2002; Foxcroft et al. 2008).

The information provided by all these techniques will also help to improve sperm quality results achieved for freshly diluted semen (see Chap. 10) and for cryopreserved sperm (see Chap. 11), the two semen storage systems that are necessary to maintain the complex machinery of the swine industry.

9.2 The Boar Ejaculate

In pigs, first ejaculations occur at the beginning of puberty, between 5 and 6 months of age. Boars are considered to be post-pubertal from 8 to 12 months, and mature from 1 year onward (Martín 1982; Hugues and Varley 1984; Sancho 2002; Cordova-Izquierdo et al. 2004). A boar ejaculation has a volume averaging

200–300 mL and contains between 10×10^9 and 100×10^9 sperm cells (Foote 2002; Casas et al. 2010) (see Sect. 9.2.3) immersed in the seminal plasma that mostly consists of secretions from the accessory sex glands and less from the epididymis and testis (see Chap. 3).

9.2.1 Parts of an Ejaculate: Sperm and the Seminal Plasma

Full discharge of semen occurs during the ejaculation process. The whole ejaculate appears as a viscous, creamy, slightly yellowish or greyish fluid. In boars, the ejaculation process takes approximately 10–30 min within which there are three phases (see Sect. 9.2.2). The boar ejaculate consists of two main parts or fractions: the sperm fraction containing the sperm cells, representing 10–30 % of the final volume, and the liquid fraction containing the seminal plasma and representing the remaining 90–70 % of the total ejaculate. In the last one, only up to 5 % of fluid comes from the secretions of the seminiferous tubules in the testes and from the epididymides; the remaining liquid comes from the secretions of the accessory glands (Table 9.1).

The seminal plasma, the fluid fraction of semen after removal of sperm by centrifugation or filtration, is the liquid nutrient medium that allows the maintenance of cell viability; that is, it is responsible for ejaculate survival along the first passages of the female tract after insemination, and helps/participates in the metabolism of spermatozoa. Seminal plasma mainly consists of organic and inorganic compounds, namely carbohydrates, lipids, amino acids and proteins of high and poor molecular weight (Pursel et al. 1973; Mann and Lutwak-Mann 1982). Their proportions vary among species and depend on the interval between ejaculations and on the health of the animal (Caballero et al. 2004; Strzezek et al. 2005; Maxwell et al. 2007).

In this sense, several studies show that seminal plasma contains specific protein factors that influence the function and fertilising capacity of sperm in several mammalian species including the pig. Furthermore, these factors appear to interact with the environment of the female genital tract during sperm transport to the site of fertilisation (Barrios et al. 2000; Centurion et al. 2003; Moura et al. 2006) (Table 9.2).

Boar seminal plasma contains fructose, glucose and sorbitol as its main sugar components. All these molecules are important energy sources, essential for sperm movement and metabolism. Additionally, the main sperm receptor proteins are glucose and fructose transporters (GLUT-3 and GLUT-5, respectively) belonging to a gene family encoding seven proteins with identical transmembrane domains

Table 9.1 Accessory sex gland contribution to seminal plasma (Martín 1982; Buxadé 1984; Sancho 2002)

Accessory sex glands	Contribution (%)
Testis and epididymis	2–5
Seminal vesicles	15–20
Bulbourethral glands	10–15
Prostate	45–60

Table 9.2 Seminal plasma content (Hugues and Varley 1984; Sancho 2002)

Seminal plasma compounds	Normal range values (units in mM)
Na^+	125–252
K^+	17–46
Ca^{2+}	1.5–4.6
Mg^+	2.5–2.4
Cl^-	85–105
Phosphate	0.4
Fructose	0.5
Glucose	0.06–0.3
Sorbitol	0.4
Inositol	28
Lactid acid	2.2
Citric acid	2.6–10.4
Glutamic acid	2
Glycerophosphocholine	4
Glycerophosphoinositol	0.26
Arginine	0.01
Creatinine	0.03
Ergothioneine	0.7
Proteins (mg/mL)	30

(Sancho et al. 2007; Casas et al. 2009). Other studies show the existence of a heterodimer protein consisting of the low molecular weight monomers PSP-I and PSP-II which, added to the dilution medium when concentration of the ejaculate is low, enhance in vitro membrane integrity, motility and mitochondrial activity of spermatozoa (Centurión et al. 2003; Garcia-Hernandez 2007).

9.2.2 Ejaculated Semen Fractions

In boars, three fractions can be obtained from sequence in the whole ejaculate as a result of testicular and epididymal activity, as well as different secretions coming from the accessory sexual glands (Fournier-Delpech and Thibault 1993; Garner and Hafez 1996; Sancho 2002; Cordova-Izquierdo et al. 2004; Peña et al. 2006).

Pre-spermatic fraction: This is formed by the secretions produced by prostate, seminal vesicles and Cowper or bulbourethral glands. The volume of this fraction is about 10–15 ml. It does not contain spermatozoa and normally presents a transparent or clear appearance.

Spermatic rich fraction: The volume of this fraction ranges approximately 70–100 ml and has a milky-white appearance. The testicular activity produces high sperm concentration, varying between 0.5×10^9 and 10^9 spermatozoa per ml. This fraction also contains secretions produced by both the prostate and seminal vesicles. This fraction is the only one collected to prepare seminal doses.

Post-spermatic fraction: The volume of this fraction is about 150–200 ml. It is pale white in appearance and contains few spermatozoa (the concentration is lower than 10^6 spermatozoa per ml). In addition, secretions of gelatinous consistency coming from the prostate and Cowper glands are found in it. This fraction contains a large amount of seminal plasma that acts in such a way as to stimulate the spermatozoa. As basal spermatic metabolism must be achieved for best storage, collection of the post-spermatic fraction when preparing seminal doses is not recommended.

9.2.3 Physiochemical Parameters of a Standard Ejaculate: Volume, Density, Viscosity, Temperature, pH and Osmolality

In terms of volume, the boar ejaculate varies between 150 and 300 ml and exceptionally it can reach 500 ml (Martín 1982; Garner and Hafez 1996; Rothschild 1990; Pinart et al. 1999; Sancho 2002).

The volume is subjected to considerable variations as a result of individual boar characteristics, such as breed, age, reproductive physiology and environmental conditions (Setchell 1991). In comparative terms, it is much higher than in other species of domestic mammals. In goats, for example, it is between 0.2 and 2.5 ml (Setchell 1991); in bulls it is between 5 and 30 ml (Garner and Hafez 1996; Rasbech 1975; Setchell 1991); in horses, it ranges from 30 to 150 ml (Rasbech 1975). In humans, standard values vary between 2 and 6 ml (Ludwig and Frick 1990; Andolz and Bielsa 1995; WHO 2000).

As stated before, the density (in terms of total number of cells) of a boar ejaculate usually varies between 10×10^9 and 100×10^9 sperm cells, which are high values when compared to other species of mammals (Crabo 1997; Sutkeviciene et al. 2005). The viscosity depends on the secretion of the accessory sex glands. The temperature of the ejaculated semen is 37 ± 1 °C and the pH is usually between 6.85 and 7.9. Variations in semen osmolality depend on the seminal composition that is in turn related to the rate of testosterone secretion resulting from testis activity (Martín 1982).

9.3 Assessment of Sperm Quality and General Viability

The first evaluation of semen, immediately after extraction, corresponds to a macroscopic control to confirm normal volume, colour, smell, viscosity and density. The normal ejaculate, consisting of the sperm-rich fraction, must be of considerable volume (70–100 ml), the intensity depending on the concentration, and should have a milky white colour. This colour can be somewhat yellowish but it normally has a similar appearance to that of skimmed milk. Occasionally, small amounts of

blood, usually originating from the urethra, may be present in the ejaculate, which gives the semen a pinkish hue. This does not reduce the fertility or the viability of the ejaculate, but when a darker red colour is associated with a pungent odour due to possible infections, the ejaculate is excluded from the commercial circuit. Hence, microbiological analyses are also required to ensure the hygienic status of semen (see Chap. 10).

Sperm quality analysis of semen can include both cell and biochemical parameters (Briz 1994; Knobil and Neil 1994; Sancho 2002). After measuring the volume, a careful microscopic examination of semen, including parameters of quality (concentration, motility, morphology, plasma membrane integrity and osmotic tolerance), is performed in order to assure the reproductive performance of a given ejaculate. Conventional semen evaluation is mainly focused on measuring volume, sperm concentration and, the percentage of spermatozoa that is progressively motile and morphologically normal (Amann and Hammerstedt 1993; Gadea 2005; Foxcroft et al. 2008). Even though microscopic evaluations are the standard for accepting or rejecting ejaculates or sperm doses, it is important not to forget obvious visual and olfactory characteristics of semen.

Although the parameters mentioned above are traditionally used for the evaluation of a given ejaculate, several authors affirm that these are not always accurately correlated with sperm fertility (Gillan et al. 2005; Holt 2005). Therefore, other parameters measuring the structural, physiological and metabolic status of the sperm cell are necessary to better predict fertilising ability; these are the following: plasma membrane integrity, osmotic tolerance, capacitation status, membrane fluidity, nuclear integrity, status of the mitochondrial sheath (MS), percentage of apoptotic spermatozoa, levels of reactive oxygen species (ROS), acrosome integrity and the activity of different metabolites and enzymes. All these assessments must be carried out using a large number of replicates because of the high level of variability within and between individual samples and the subjectivity of some assays (Woelders 1991). These will be dealt with in greater depth in the following corresponding sections.

9.3.1 Sperm Concentration

One step in the evaluation of boar semen is the determination of sperm concentration that, apart from contributing to the fertility diagnosis, establishes the number of doses that can be obtained from a single ejaculate. In this way, the concentration has a direct economic implication in porcine AI centres as well as in AI efficiency, depending on the number of spermatozoa used at each insemination (Camus et al. 2011).

Therefore, accurate assessment of sperm concentration is essential for AI organisation in boars, although there is no agreed method to use as a standard.

Various techniques are available to evaluate sperm concentration of mammalian species; in most laboratories it is routinely assessed by the use of a counting

chamber such as: Bürker (BT, Brand, Wertheim, Germany), Thoma (TH-50 and TH-100; Hecht-Assistant, Sondheim, Germany) and Makler (Sefi Medical Instruments, Haifa, Israel) (Prathalingam et al. 2006; Mrkun et al. 2007). This method consists of a microscope slide which can hold a specific volume of semen sample and a cover glass with a grid marked on its surface that makes it possible to calculate the sperm count (Fig. 9.1). This technique is widely used because of its simplicity, low cost and reproducibility (Mrkun et al. 2007); however, this method has some handicaps like time consumption, and high variability in results due to subjectivity (Knuth et al. 1989) and the use of different types of chambers (Christensen et al. 2005).

Spectrophotometers and colorimeters are widely used for determining sperm concentration not only in boars in many AI centres, but also in other mammalian species (Woelders 1991). Both instruments measure the sample opacity as the percentage of light transmittance through an aliquot of the diluted ejaculate. In a machine specially calibrated for seminal doses, this percentage is directly plotted against a standard curve performed by the manufacturer. These automated systems allow a quicker estimate of the concentration, but great variability exists in transmittance between different ejaculates because of the different compound profile and concentrations. So, to overcome this difficulty, calibration curves need to be precisely evaluated before use (Maes et al. 2010; Camus et al. 2011).

Recently, several novel techniques have been proposed which involve computer assistance to determine sperm concentration, namely Computer-Assisted Semen Analysers (CASA): Sperm Class Analyser (SCA, Microptics S.L., Barcelona, Spain), Integrated Semen Analysis System (ISAS, Proisers, Valencia, Spain), Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, United Kingdom) or Sperm Vision® (Minitüb, Tiefenbach, Germany). Several commercial companies offer integrated facilities, equipped with a phase contrast microscope, a digital

Fig. 9.1 Makler chamber counting (Sefi Medical Instruments, Haifa, Israel) where a 5 μl sample is loaded before the cover class is put in position to assess sperm concentration (TechnoSperm)

video camera and a specific program for image analysis. These devices improve the accuracy and repeatability of data collection, avoiding errors due to individual subjective evaluation and time saving in the analytical procedure (Maes et al. 2010). As a result, these systems are being used increasingly in AI centres, progressively replacing the counting chamber method.

Another instrument called Nucleocounter SP-100 (Chemomethec, Denmark) can be used for quick analysis of the sperm concentration and the sperm viability. It analyses the image from its integrated fluorescent microscope displaying accurate values. Nevertheless, this instrument is not fully implemented routinely in laboratories (Hansen et al. 2002; Anzar et al. 2009).

Finally, it is important to know that flow cytometry is a tool that also determines mammalian sperm concentration. This device can measure the number of spermatozoa in a sample utilising a variety of fluorochrome staining methods and it can be used as a reference procedure due to its velocity, sensitivity and objectivity in its measurements. However, controversy exists as to whether cytometry can accurately assess sperm concentration; it has been reported in studies using human sperm samples that this parameter could be determined correctly compared to other techniques, but there are investigations that conclude that flow cytometry overestimates sperm concentration (Lu et al. 2007).

9.3.2 Sperm Motility

It is well-known that motility together with concentration are two very important indicators of boar sperm quality for AI centres (Flowers 1997; Rigau et al. 2001; Sancho 2002; Vyt et al. 2008; Tejerina et al. 2008). Specifically, the progressive motility of spermatozoa indirectly indicates unimpaired metabolism as well as undamaged membranes (Johnson et al. 2000) and is the most frequent viability parameter assessed in andrology laboratories.

Visual estimation of motility by phase contrast microscopy is cheap and quick, but its reliability depends on the accuracy of the technician (Woelders 1991; Tejerina et al. 2008). The experience and training of the operator explain the large intra- and inter-assay variation documented in the literature (Dunphy et al. 1989; Yeung et al. 1997; Brazil et al. 2004). Moreover, the correlation between sperm motility and sperm fertilising ability, measured as the fertility rate and the non-returning rate to oestrus within 60 days (NRR_{60d}) is a controversial subject (Berger et al. 1996; Pérez-Llano et al. 2001; Yeste et al. 2010). Nevertheless, its assessment is expected to provide clues to the potential fertility of the spermatozoa.

Other tools to avoid subjectivity have been developed for the assessment of this parameter, such as turbidimetry, laser-Doppler spectroscopy and photometric methods. However, these systems do not take into account the evaluation of each individual spermatozoon and they only allow for a rough estimation of the whole population (Verstegen et al. 2002). There are other automated devices, belonging

to CASA systems, available for motility assessment of porcine spermatozoa. For example, the Hamilton-Thorne Ceros sperm analyser (HTR Ceros 12.1; Hamilton-Thorne Research, Beverly, CA, USA); this assesses the motion of individual spermatozoa by processing digital images of the trajectories over a certain time interval. The signal is digitalised and the information processed by a computer that reconstructs the trajectory of each sperm cell from the position of the head in subsequent frames (Rijsselaere et al. 2002; Vyt et al. 2008). Another homologous system is the sperm quality analyser (SQA). The first version, named SQA-IIC (SQA-IIC, Medical Electronic Systems Ltd, Tirad Carmel, Israel), was consistent and suitable for the estimation of boar semen quality (Vyt et al. 2008) but recently a device specifically designed for boars (SQA-Vp, A-Tech, Los Angeles, California, USA) has been introduced. This device analyses the signals of a mass of motile sperm traversing a light through a capillary tube, and the percentage of motile sperm is obtained by mathematical algorithms. However, it does not provide as many sperm motility and velocity parameters as the HTR system (Vyt et al. 2004; López Rodríguez et al. 2011).

In the last decade, these CASA systems have become commercially available for objective and accurate assessment of sperm motility in pigs and other species. The information provided by these devices is especially interesting with regard to the quality of sperm movement, which is classified into several sperm quality parameters of motility. It is interesting to mention the following: total motility (in percentage), circular trips, curvilinear velocity (VCL, determined by the average velocity measured over the actual point-to-point track followed by the spermatozoon in micrometers per second), straight-linear velocity (VSL, which corresponds to the average velocity measured in a straight line from the beginning to the end of one track in micrometers per second), average velocity (VAP, average velocity of the smoothed cell's pathway in micrometers per second), linearity index (LIN = VSL/VCL in percentage which estimates the proximity of the cell's track to a straight line), straightness index (STR = VSL/VAP, determined as the straightness of the average path), oscillation index (WOB = VAP/VCL, oscillation of the real trajectory with respect to the mean trajectory), amplitude of lateral head displacement (ALH, defined by the amplitude of lateral head displacement along its average trajectory in micrometers per second), beating frequency (BCF, which is the frequency at which the head of the spermatozoon crosses the sperm cell's average path trajectory in Hertz) and progressive motility (sperm showing a given percentage of STR) (Verstegen et al. 2002; Martínez-Pastor et al. 2011) (Table 9.3).

The CASA examination is considered faster than a visual assessment and it can provide much more data with such a degree of accuracy that it can reveal subtle differences in motility not perceivable to the human eye (Tejerina et al. 2008; Casas et al. 2010). Since subjectivity is avoided, there has been increasing interest in including this methodology in the routine analysis of sperm motility (Tejerina et al. 2008), but visual estimation is still the main practice in those AI centres that cannot sustain the expenses of such automated systems. Detailed CASA motility data in pigs can be related to fertility results to a certain extent, as explained in a study undertaken by Holt and collaborators (Holt et al. 1997).

Table 9.3 Semen characteristics and sperm quality parameters (Hugues and Varley 1984; Sancho 2002)

Parameters	Normal range values
Temperature (°C)	37 ± 1
pH	6.8–7.9
Volume (mL)	150–300
Density (number of cells)	10,000–100,000
Osmolality	Variable
Sperm concentration ($\times 10^6$/mL)	200–300
Sperm motility (%)	
Total	>80
Progressive	>60
Sperm viability	
Viables(%)	>75
Sperm morphology (%)	
Normal	>80

An ejaculate assessed by CASA with a minimum of 80 % of total sperm motility and 60 % of progressive motile spermatozoa (established as sperm cells showing more than 45 % of their STR) matches the values for good sperm quality in accordance with investigations carried out in our group. In a given ejaculate, this percentage is subjected to variations due to handling. Although this problem can be solved by incubating sperm samples at 37 °C between 15 and 20 min, which induces motility (Briz 1994), inter-replicate variability is very high (Yeste et al. 2010). Different reports have studied ejaculate structure by analysing subpopulations of spermatozoa in an attempt to explain this variability. In these studies, cluster analysis of sperm motility parameters plays a basic role and manifests the existence of spermatozoa groups with differential behaviour inside the ejaculate (Abaigar et al. 1999; Quintero-Montero et al. 2004; Satake et al. 2006; Rodriguez-Martinez et al. 2009). The relative percentages of these groups in each ejaculate sample could be a reason for the large data deviation observed.

In boars, as well as in other mammalian species, it has been noted that a structure of separate motile subpopulations exists in an ejaculate. This finding allows a better predictive ability of boar semen quality analyses because the ejaculate is not treated as a normal, uniform, distribution model of motility (Quintero-Moreno et al. 2004), permitting better assessment of the ejaculate and its fertility potential (Martínez-Pastor et al. 2011). Although total motility does not seem to be of great importance in vivo fertility, it has been observed that specific motile subpopulation structures can be strongly related to the fertilising ability of a sample (Quintero-Moreno et al. 2004).

Besides, it has been noted that great differences exist in the proportion of motile subpopulations among individuals (Quintero-Moreno et al. 2004), and no standard method exists to assess these different subpopulations. In addition, an improvement in the statistical methods used to disclose different motility patterns is needed, in order to enhance the results and facilitate comparison among studies (Abaigar et al. 1999; Quintero-Moreno et al. 2004; Martínez-Pastor et al. 2011).

9.3.3 Sperm Morphology

The assessment of sperm morphology is an effective tool to estimate the function of the seminiferous epithelium and the epididymal maturation in porcine and other mammalian species, and serves as an additional measure of ejaculate quality and fertility potential (Bonde et al. 1998; Gadea 2005).

Usually, a morphological examination of the boar ejaculate comprises a qualitative and also a quantitative classification of normal and abnormal sperm morphologies. This classification used to be assessed by optical microscopy and a simple staining techniques, such as Eosin nigrosin, Trypan Blue, Giemsa, Papanicolaou or Diff-Quick (Foxcroft et al. 2008). This manual approach has always been problematic because of large variations between technicians and laboratories, extensively reported in the literature (Comhaire et al. 1994; Root Kustritz et al. 1998; Eustache and Auger 2003).

Currently, a contrast phase microscope can provide better resolution without requiring staining the samples and, as in motility assessment; it can be coupled to a CASA system. In this case, the device identifies individual spermatozoa and allows easy classification, which is usually composed of the following main categories: normal or mature spermatozoa, immature spermatozoa with proximal cytoplasmic droplets and aberrant spermatozoa with head or tail abnormalities. In this last category, a more specific subclassification is also provided. Computerised systems help analysis by reducing the time of assessment, but require standardisation of the parameters that allow images of sperm cells and seminal debris and/or superimposed cells to be distinguished. This should be done to minimize variability among analyses performed with these systems in different laboratories (Fig. 9.2).

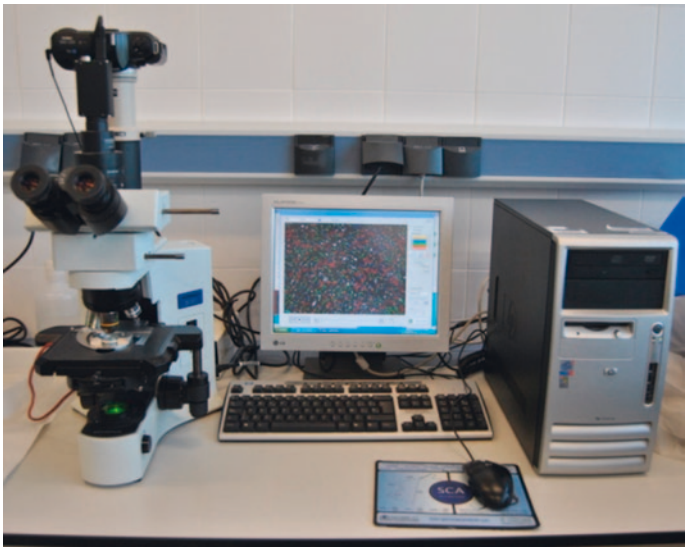

Fig. 9.2 Computer-Assisted Semen Analysis (CASA) system. In the screen a detail from morphology analysis is displayed (TechnoSperm)

A threshold of 75–80 % of mature spermatozoa has been set as the standard value (Rozeboom et al. 2000; Sancho, 2002). When the percentage of immature spermatozoa is higher than 30 %, an epididymal dysfunction is assumed, often caused by a high rhythm of collections (Thibault et al. 1993; Pruneda et al. 2005). Concerning the aberrant spermatozoa, teratozoospermia is considered when the percentage of abnormal cells in an ejaculate is higher than 50 % (Knobil and Neill 1994). Aberrant morphologies can affect the head and/or the tail of the spermatozoon. Depending on the origin, malformations can be primary, when produced in the testicle during spermatogenesis, or secondary, when occurring in the epididymis (Bonet el al. 1995). Head malformations can be presented as aberrations in number (two or more heads), shape (triangular, ovoid, pear formed, etc.) or size (micro or macro cephalic). Tail malformations can be presented as aberrations in number (two or three tails) and length or trajectory (folded or coiled tails) (Bonet et al. 1995; Sancho 2002).

9.3.3.1 Sperm Agglutination

Sperm agglutination is observed when a spermatozoon binds to another spermatozoon by head-to-head or tail-to-tail contact. Under normal conditions, agglutination does not occur and an immunological cause may be suggested when it is often present. It is well-known that bivalent and trivalent cations in boar seminal plasma, as well as long storage, induce sperm agglutination (Sánchez et al. 1991; Yeste et al. 2008b).

9.3.4 Sperm Plasma Membrane Integrity

Although sperm membrane integrity is often confused with terms like “viability”, “live” and “dead”, this nomenclature is not scientifically correct. Even though it is certain that the rupture of the membrane leads to the death of the spermatozoon, almost all dyes and fluorochromes that strictly provide information on the status of the membrane are wrongly believed to be the only indicators of sperm viability. Viability should be understood as a whole cell physiological status, and thus it can be approached with any quality parameter and not only by using membrane integrity; however, this is currently the best estimator.

In the assessment of sperm membrane integrity, porcine ejaculates should contain 85 % of spermatozoa with intact membranes to be considered of good quality (Martin 1982; Buxadé 1984; Briz 1994; Bonet et al. 1995; Briz et al. 1995; Pinart et al. 1999; Sancho 2002).

9.3.4.1 The Use of Stains as a Field Approach

The percentage of membrane integrity can be assessed by conventional optical microscopy with specific dyes including the eosin blue aniline (Shaffer and Almquist 1948) and eosin nigrosin (Dott and Foster 1972; Garcia-Artiga et al. 1994;

WHO 2000; Kvist and Björrndahl 2002; Sancho 2002). These stains are based on the fact that intact membranes are impermeable to the dye, whereas impaired ones are permeable to it. In the eosin-nigrosin test, sperm that is stained in pink red is classified as "intact", whereas sperm that has not been stained is labelled as "damaged" (Dott and Foster 1972; Kvist and Björndahl 2002; Sancho 2002). This approach is still utilised in on-field assessments as it is a cheap procedure that does not require the use of expensive microscopes. The use of stains, however, is not recommended when certain media (like freezing extenders) are used as they could mutually interfere, producing misleading results (Casas et al. 2010).

9.3.4.2 Fluorochrome Probes for Fluorescence Microscopy or Flow Cytometry

There are other methods to assess membrane integrity of boar spermatozoa more accurately than with conventional non-fluorescent dyes. These take advantage of fluorochromes by directly binding to the organelle to be tested either by covalent unions or in other molecules such as lectins (Serrano et al. 2001). Fluorochromes are not only efficient in binding the nucleus but also the acrosome, the MS and other membrane proteins (Gravance et al. 2000). For example, acridine orange is a nuclear probe that has been used in a wide range of mammalian species such as the porcine (Bonet et al. 1995; Pinart et al. 1999). It is based on the nucleic acid selectivity of the fluorochrome, which emits green fluorescence when it binds to non-altered (double stranded) DNA and orange fluorescence when it binds to RNA or denatured (single stranded) DNA. The degree of emission in the orange spectrum has been shown to correlate with male infertility in the sperm chromatin structure assay (Silva and Gadella 2006).

Sperm cells labelled with fluorochromes can be either assessed under fluorescence microscopy or by flow cytometry. Fluorescence microscopy is more subjective than flow cytometry, but the sharpness of fluorescence labelling reduces the incidence of mistakes coming from the observer. Although a flow cytometer is expensive and voluminous it processes samples quickly, automatically and objectively and therefore it is frequently found in clinical laboratories for routine assays. On the other hand, this apparatus requires expensive maintenance and cautious selection of the samples that are to be processed, as particles exceeding a certain size can result in obstruction of filters and pipes.

Double Fluorochrome Labelling

Since the beginning of the twenty-first century, the use of double labelling has been the most used quantitative method for assessing sperm membrane integrity. It is based on using two specific fluorochromes; one labels viable cells by binding enzymes, active only when the cell is alive, or by emitting fluorescence when ionic pumps are working (Shapiro 1998), and the other fluorochrome is specific for non-viable cells as it only enters cells whose plasmalemma is ruptured.

The most commonly used fluorochromes to label boar sperm cells with intact membranes are SYBR-14 (Garner et al. 1994; Gravance et al. 2000; Huo et al. 2002; Love et al. 2003; Puigmulé et al. 2011), Hoechst 33342 (Cai et al. 2005; Hallap et al. 2006) and Hoechst 33258 (Bussalleu et al. 2005; González-Urdiales et al. 2006; Pinart et el. 2006). The last two dyes have been developed by a German company from the molecule bisbenzimide, a membrane-permeable fluorescent dye that intercalates between Adenine–Thymine base pairs in DNA of both viable and non-viable cells. When excited at 346 nm it emits blue fluorescence (488 nm) if bound to DNA. The fluorochromes often used to stain damaged membrane of boar sperm cells are ethidium homodimer (EthD-1) (Yeste et al. 2009) and propidium iodide (PI) (Bussalleu et al. 2005; Puigmulé et al. 2011). The latter is a DNA-intercalating agent widely used as vital dye in cell biology (Garner et al. 2004). When intercalated with the nucleic acid helix and irradiated at 488 nm, it emits fluorescence in the red spectrum (615 nm) (Love et al. 2003; Nagy et al. 2003; Rowland et al. 2003).

A frequently reported combination of fluorescent stains is SYBR-14/PI (Garner and Johnson, 1995). In a flow cytometrical analysis the labelled sperm is excited by an argon ion laser emitting at 488 nm to measure both green SYBR-14 and red PI fluorescence. The green wavelength fluorescence is collected through different band pass filters and data are plotted on a bidimensional graph, each spermatozoon corresponding to a dot. For measuring SYBR-14 fluorescence, a 525 nm band pass filter is used (FL-1), while PI fluorescence is detected through a 635 nm band pass filter (FL-3). Depending on the percentage and quality of the fluorescence each cell is plotted up and down or right and left to the axes and counted in the population of sperm with intact membrane or damaged membrane, or classified as debris. Sperm cells depicting double fluorescence can be considered in the damaged membrane cluster (Nagy et al. 2003; Garner et al. 2004).

Multiple Fluorochrome Labelling

New tests have been developed to assess the integrity not only of the plasmalemma but also of other organelles and vesicles in sperm cells (Nagy et al. 2003; Bussalleu et al. 2005). A simple method of multiple staining with fluorochromes allows the status of the nucleus, the acrosome, and the MS of boar spermatozoa to be assessed objectively and accurately (Bussalleu et al. 2005; Pinart et al. 2006).

First, bisbenzimide and PI are added to the sample and penetrate the sperm membranes to bind DNA. Bisbenzimide penetrates all sperm membranes and produces blue fluorescence of 488 nm when bound to DNA, whereas PI penetrates only damaged membranes and emits red fluorescence of 617 nm (Love et al. 2003), which hides the bisbenzimide emission. Bisbenzimide is monitored through flow cytometry with a sensor using a 500 ± 30 nm band pass filter and PI labelled spermatozoa with a sensor using a 630 ± 20 nm band pass filter.

Second, two more fluorochromes are added to the sperm suspension, these fluorochromes being Mitotracker® Green FM (Molecular Probes Inc., Eugene,

OR) and Alexa Fluor® 488 Conjugate-SBTI (Molecular Probes Inc., Eugene, OR). Mitotracker® Green FM is excited at 490 nm and emits green fluorescence at the 516 nm wavelength. It passively diffuses across the plasma membrane and binds to membrane lipids of functional mitochondria (Fraser et al. 2001; Keij et al. 2000); in contrast, this dye is not retained in those mitochondria with altered membrane potential. Mitotracker® Green FM fluorescence is monitored through flow cytometry with a sensor using a 530 ± 300 nm band pass filter. Alexa Fluor 488 Conjugate-SBTI is formed by the Trypsin inhibitor from soybean (SBTI), which is an inhibitor of the catalytic activity of serine proteases that bind to and inhibit acrosin (Tollner et al. 2000; Fukami et al. 2003) and the fluorochrome Alexa Fluor 488. The fluorochrome is excited at 490 nm and emits green fluorescence of 519 nm. Alexa Fluor 488 Conjugate fluorescence can be monitored through flow cytometry with a sensor using a 530 ± 30 nm band pass filter. Both fluorochromes can also be checked under fluorescence microscopy.

From the results obtained by this technique, spermatozoa can be considered to have either intact membranes (blue-stained nuclei) or damaged membranes (red-stained nuclei). Spermatozoa with intact membranes are classified into three additional categories according to both acrosomal and mitochondrial staining: (1) spermatozoa with intact acrosome and mitochondria that display intense green fluorescence over the acrosomal cap and the MS; (2) spermatozoa with reacted acrosome and intact mitochondria that show intense green fluorescence of the MS. The acrosome displays a patchy disrupted green fluorescence on the equatorial segment, or it is not stained at all; (3) spermatozoa with intact acrosome and altered MS that exhibit intense green fluorescence over the acrosome and a partial or total lack of fluorescence in the MS. Spermatozoa with both the acrosome and mitochondria altered (lack of fluorescence on the acrosome and the MS) are classified into category 4 (Fig. 9.3) (Bussalleu et al. 2005).

9.3.5 Osmotic Tolerance of Sperm

Spermatozoa have different mechanisms of adaptation and resistance to sudden changes in the osmolality of the medium they are in. These mechanisms involve complex metabolic pathways, such as ion channels related to ATPase-dependent Na^+/K^+ type or exchanger of Na^+/K^+, all of them requiring precise regulation.

Boar spermatozoa are sensitive to osmotic changes in the environment, both hypotonic and hypertonic and the ability to respond to osmotic stress is related to cell functionality and can be a good indicator of its physiological state (Yeste et al. 2010). It has been reported that osmotic shock affects the diffusion of phospholipids in the bilayer membrane of spermatozoa (Christova et al. 2002). When sperm cells are exposed to an environment of high osmotic pressure, their motility decreases and the acrosome is damaged (Curry and Watson 1994; Revell and Mrode 1994; Liu and Foote 1998, Rossato et al. 2002; Chantler and Abraham-Peskir 2004; Yeste et al. 2010).

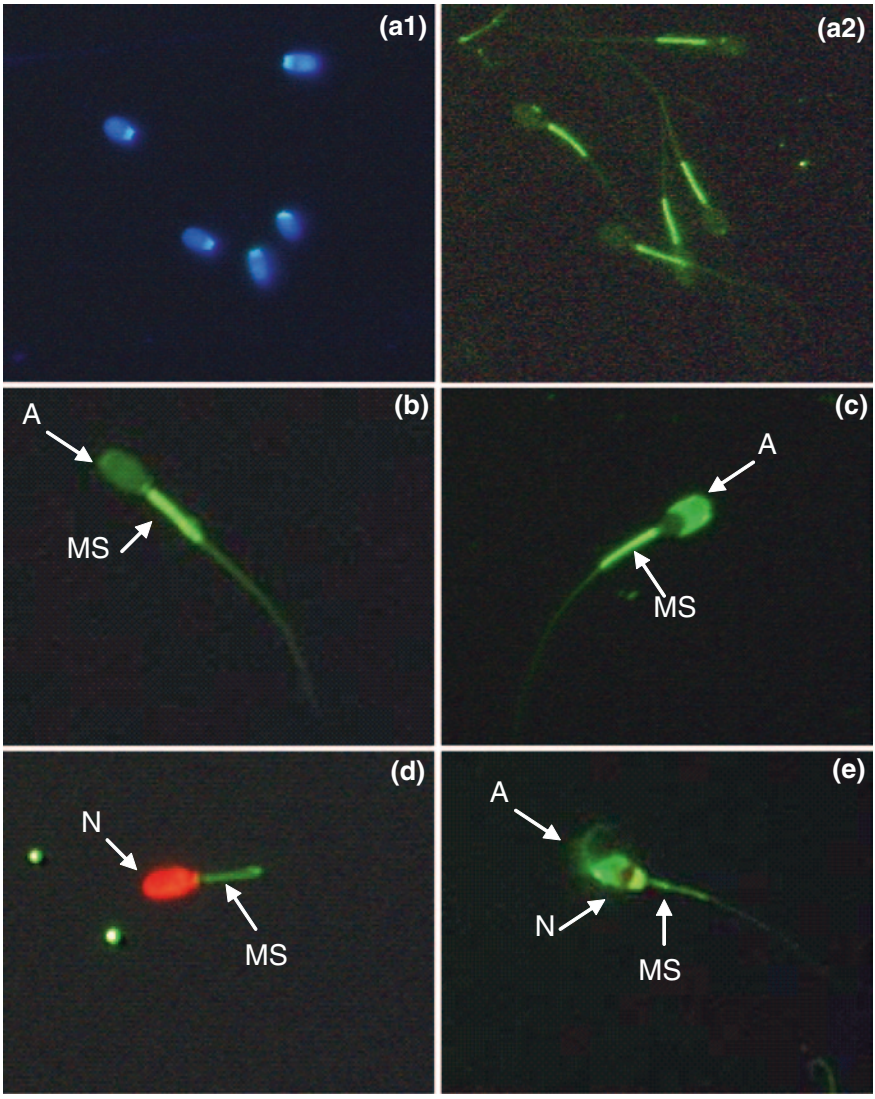

Fig. 9.3 Images of spermatozoa obtained from multiple staining methods in the membrane integrity assay. **a1**–**a2** Viable spermatozoa with intact acrosome and intact MS. The nuclei of viable spermatozoa emit intense blue fluorescence (**a1**), whereas intact acrosomes and intact MSs emit weak and moderate green fluorescence, respectively (**a2**). **b** Viable spermatozoon with reacting acrosome (*A*) and intact mitochondrial sheath (*MS*). Note that the reacting acrosome exhibits moderate green fluorescence. **c** Spermatozoon with altered acrosome (*A*) and intact mitochondrial sheath (*MS*). **d** Non-viable spermatozoon with intact acrosome and intact mitochondrial sheath (*MS*). The intense red fluorescence of the nucleus (*N*) masks the weak green fluorescence of the intact acrosome. **e** Non-viable spermatozoon with reacted acrosome (*A*) and altered mitochondrial sheath (*MS*). Note a weak red fluorescence of the nucleus (*N*) (×40) (Bussalleu et al. 2005)

In boars, an isotonic solution is established between 300 and at 400 mOsm Kg^{-1} (Petrunkina and Topfer-Petersen 2000; Petrunkina et al. 2000; Fraser et al. 2001).

Several tests have been developed to measure the response of boar sperm to osmotic changes as a parameter indicator of semen quality. These methods are the hypo-osmotic swelling test (HOST), the hyperosmotic resistance/swelling test (HRT) and the osmotic resistance test (ORT). All of them are focused on the response of the sperm membrane to sudden changes of osmolality depending on the media in which they are enclosed.

9.3.5.1 Hypo-Osmotic Swelling Test

This technique, which was first described in humans (Jeyendran et al. 1984), measures and evaluates the response of sperm to a hypo-osmotic solution (below 300 $mOsm \cdot Kg^{-1}$) based on the swelling capacity of these cells when trying to balance the ionic content of the external environment. This swelling is manifested in a characteristic curl of the tail that can vary depending on different factors such as individual tolerance or composition of the media (Gonzalez-Urdiales et al. 2006; Bonet et al. 2006) and represents a quick, easy technique to estimate the quality of a given ejaculate from several mammals including the boar.

9.3.5.2 Hyperosmotic Resistance/Swelling Test

This technique is also based on the resistance that spermatozoa exhibit to sudden changes of osmolality. In essence, this test consists of challenging sperm to a solution that has higher osmolality than seminal plasma (hyperosmotic or hypertonic medium) and to another at a similar osmolality (isoosmotic or isotonic medium). The result is the quotient obtained by dividing the percentage of osmotically tolerant sperm (sperm with intact membranes) in isoosmotic medium and the percentage of osmotically tolerant sperm in hyperosmotic medium. Sperm cells can be contrasted through the eosin-nigrosin staining technique (Rodriguez-Gil et al. 1994).

9.3.5.3 Osmotic Resistance Test

The ORT consists of checking the capacity of sperm to resist a hypoosmotic shock by challenging sperm to a hypoosmotic medium ($\leq$150 $mOsm \cdot Kg^{-1}$) and to a medium at a similar osmolality to seminal plasma (~300 $mOsm \cdot Kg^{-1}$). It tests the capacity of spermatozoa to resist changes in osmotic pressure like the ones they will encounter in the female tract. The result is the quotient obtained by dividing the percentage of osmotically tolerant sperm (sperm with intact membranes) in the isoosmotic medium and the percentage of osmotically tolerant

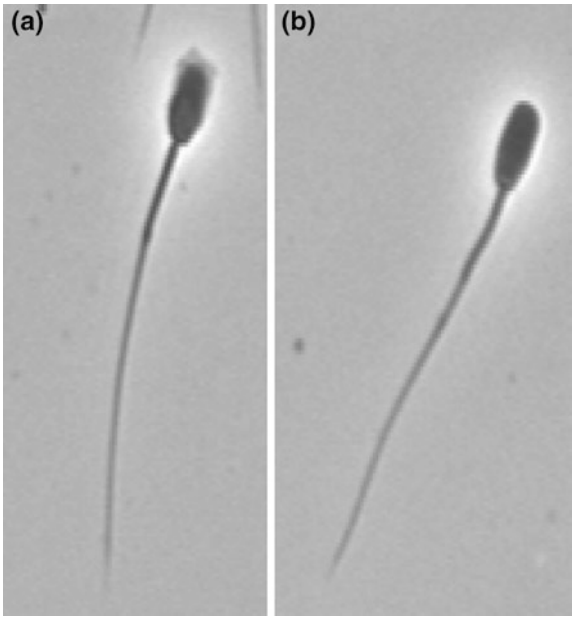

Fig. 9.4 Osmotic tolerance of sperm. **a** spermatozoa with altered acrosomal membrane. **b** Non-reacted spermatozoa (TechnoSperm)

sperm in the hypoosmotic medium. Osmotically, tolerant sperm must resist changes in osmolality and must show a low percentage of altered acrosomal membranes (Bonet et al. 1995; Briz et al. 1995; Sancho et al. 2004; Medrano et al. 2006) (Fig. 9.4).

9.3.6 Sperm Capacitation Status

Capacitation involves several physiological changes that the spermatozoon undergoes at the oviduct and that are essential for achieving its fertility ability. These changes alter its membrane and include reorganisation of proteins, metabolism of phospholipids and reduction in cholesterol levels. They also provoke hyperactivation in terms of sperm motility.

There are different techniques to assess the capacitation status of spermatozoa that analyse part of the physiological changes occurred in the sperm membrane during the capacitating process.

9.3.6.1 CTC Staining

One of the most used techniques to test capacitation status in boar sperm is chlortetracycline (CTC) co-staining with ethidium homodimer (EthD-1). This technique is based on the procedure described by Wang et al. (1995) and Mattioli et al. (1996)

and adapted in boars by Fazeli et al. (1999) and Yeste et al. (2008a, 2009). It consists of the assessment of calcium fluctuations by using the fluorescent antibiotic CTC, which traverses the cell membrane of spermatozoa and enters intracellular compartments containing free calcium (Tsien 1989). After its entrance, CTC becomes negatively charged and binds calcium, becoming more fluorescent; the CTC-calcium complex preferentially binds to hydrophobic regions, such as the cell membrane, resulting in characteristic staining patterns (Gillan et al. 2005). Labelled cells are observed under fluorescence light microscopy using blue-violet illumination. Viable spermatozoa (ethidium homodimer-1 negative) are counted, differentiating three fluorescence patterns: NC/F (uniform fluorescence over the whole head; uncapacitated spermatozoa), C/B (fluorescence-free band in the post-acrosomal region, capacitated spermatozoa) and AR (no fluorescence over the head or a thin fluorescent band in the equatorial segment, acrosome reacted).

A variant of this technique is combined Hoechst 33258/CTC labelling. This dual staining method is based on that described by Perez et al. (1996) and Hewit and England (1998). Spermatozoa are classified as dead (nuclei show bright blue fluorescence over the sperm head), live/non-capacitated (bright green fluorescence distributed uniformly over the entire sperm head with or without a stronger fluorescent line at the equatorial segment), live/capacitated (green fluorescence over the acrosomal region and unlabelled postacrosome), or live/acrosome reacted (sperm showing mottled green fluorescence over the head or no fluorescence at all and green fluorescence only in the post-acrosomal region) (Maxwell and Johnson 1999).

9.3.6.2 M540 and Plasma Membrane Fluidity

Detection of plasma membrane fluidity of boar sperm can be performed using the fluorescent dyes Merocyanine-540 (M540), based on the technique introduced for boar sperm by Harrison et al. (1996). The results analysed using flow cytometry are expressed as the percentage of membrane intact sperm (Yo-Pro-1 negative). In this category, two populations of Merocyanine-540 positive cells are typically observed; low fluorescence/low fluidity and highly ordered, and high fluorescence/high fluidity and greater disorder, which are associated with capacitating and/or acrosome-reacted sperm (Januskauskas et al. 2005; Purdy 2008; Puigmulé et al. 2011).

9.3.6.3 Levels of Intracellular Calcium as an Indicator of Sperm Capacitation (Fluo-3 AM)

Changes in membrane properties and enzyme pathways occurred during capacitation are facilitated by the activation of cell signalling cascades (Salicioni et al. 2007). One result of capacitation in mammalian sperm is an increase in membrane permeability to calcium, which allows rapid movement of calcium into the spermatozoa that is required for the fusion of the plasma membrane and the outer

acrosomal membrane to occur, initiating sperm acrosome reaction (Yanagimachi and Usui 1974; Triana et al. 1980; Landim-Alvarenga et al. 2004). This property could be used to evaluate the capacitating status by the assessment of intracellular levels of calcium (Okazaki et al. 2011) using the fluorescent calcium indicator Fluo-3 AM (Harrison et al. 1993; Kardivel et al. 2009).

9.3.6.4 Assessment of Proacrosin-Acrosin System

Another technique has been developed recently that allows a large number of samples and most of the parameters involved in the capacitating process to be examined.

The capacitated status of spermatozoa can be checked by measuring sperm viability, membrane fluidity and Ca^{2+} influx using flow cytometry (Puigmulé et al. 2011). This set of sensitive assays comprises a reliable method to study the molecular changes occurring in vitro capacitated spermatozoa, using SYBR-14 and PI for the assay of sperm plasma membrane integrity (see Sect. 9.3.4), Merocyanine 540 (M540) and Yo-Pro-1 for the assay of sperm membrane fluidity, and Fluo-3 AM and PI for the estimation of intracellular calcium levels (Harrison et al. 1993; Kardivel et al. 2009; Puigmulé et al. 2011).

9.3.7 Nuclear Integrity of Sperm: DNA Fragmentation

It has been observed in humans that the existence of abnormal semen parameters is associated with the presence of DNA strand breaks (Sakkas et al. 1999). The structure of sperm chromatin in mammals is unique compared with that of somatic cells. In spermiogenesis, chromatin condensation begins when the main histone complexes of round spermatids in DNA are replaced first by transition proteins and finally by small basic protamines. During the passage of sperm through the epididymis, the chromatin condenses fully by forming disulfide bonds between cysteine residues of protamines, which gives a very rigid structure to the nucleus. Therefore, to study the state of the DNA of mature sperm, that structure must be broken to release the chain of nucleotides (Garcia-Macias et al. 2006). Results obtained by Flores et al. (2011) suggested that the boar-sperm nuclear structure is heterogeneous and it is possible to differentiate a zonated pattern with different DNA density and compactness of the precise nucleoprotein structures.

This heterogeneity in chromatin structure, which is associated with disturbances of spermatogenesis, could be related to the variation observed in the susceptibility of DNA to denaturation leading to reduced fertility (Evenson et al. 1980; Januskauskas et al. 2000).

The status of sperm DNA in mammals is analysed by fluorochrome-based techniques, which have specific and complex interactions with chromatin or DNA: single cell gel electrophoresis (COMET) assay (Abu-Hassan et al. 2006; Enciso et al. 2011),

terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay (Lopes et al. 1998; Sun et al. 1997), in situ nick end translation assay (ISNT) (Gorczyca et al. 1993) or in situ ligation (ISL) assay (Hornsby and Didenko 2011), sperm chromatin structure assay (SCSA) (Evenson and Lorna 2000; Evenson and Jost 2001) and, less used due to its inaccuracy is the acridine orange test (AOT) (Evenson et al.1980). All of them are based on the detection of possible breaks in the DNA chain of the mature sperm nucleus using enzymes or induced lysis by heat or acid and subsequent staining of the treated sperm. The need for the use of flow cytometry limits the routine application of these techniques (Garcia-Macias et al. 2006; Gadea et al. 2008; Pérez-Llano et al. 2010; Matás et al. 2011).

Although some studies in humans have shown that the percentage of sperm containing fragmented DNA may be an independent parameter of semen quality and fertilising capacity (Evenson et al. 2002), other studies in boars using the SCSA technique suggest a relation with fertility (Garcia-Macias et al. 2006; Didion et al. 2009). However, it must be said that controversy exists about the extent of DNA fragmentation in boars (Fraser and Strzezek 2007; Flores et al. 2008, 2011) and some studies sustain this phenomenon is rarely observed in this species owing to the tight packaging mediated by protamines although alterations in the overall protamine-DNA structure have not been ruled out (Flores et al. 2008).

Use of these techniques is of great interest in boar semen analysis, although they are not adapted to routine monitoring due to complex matters.

To overcome such inconveniences, a quick, easy technique has been developed in recent years based on the detection of sperm nuclei presenting fragmented DNA, which can be performed in any conventional analysis laboratory. This technique is the sperm chromatin dispersion test (SCDt) and was originally developed for human semen (Fernández et al. 2003) and later adapted to boar semen (Enciso et al. 2006). It presents two possible variations depending on whether staining for bright field microscopy or labelling for fluorescence microscopy are used (Enciso et al. 2006; Flores et al. 2008). This technique involves immersing intact spermatozoa in an agarose matrix, smearing it on slides, dropping an acid solution to denature the DNA and lysing membranes and proteins to freed nucleotides, forming a peripheral halo of dispersion in those spermatozoa containing fragmented DNA. By staining, halos can be visualised by light or fluorescence microscopy and can be counted manually or by software image processing to find the percentage of sperm with fragmented DNA, called the DNA fragmentation index (DFI). In boar, the extremely tough of the sperm in relation to DNA fragmentation makes this assessment less useful than in other species (Gosalvez et al. 2011).

9.3.8 Detection of Apoptotic Spermatozoa

The spermatozoon is a highly specialised cell whose main function is to transport the male haploid genome into the female genital tract and to deliver it during fertilisation of the oocyte. During their genesis in the testis, spermatozoa lose most

of the organelles, except the acrosome (derived from the Golgi vesicle) and a certain subset of mitochondria, which are allocated in the midpiece of the sperm tail and play critical roles in sperm function prior to fertilisation (Peña et al. 2009). However, the spermatozoon should not be interpreted as a simplified version of a somatic cell because its whole physiology is complex like any other. Conventional quality parameters reflect dysfunctions in sperm, but the precise identification of their origin is only achieved after dissection of the mechanisms that hold spermatic activity.

Apoptosis is a complex phenomenon that consists of a fine regulation of cellular proliferation and programmed cell death as a normal component of development. At the earlier stages of apoptosis, plasma membrane becomes slightly permeable and loses asymmetry. When the cell membrane is disturbed phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane and this is identified as a signal for phagocytes (Desagher and Martinou 2000; Peña et al. 2003). The process of apoptosis can be divided into three phases: induction, execution, and degradation (Peña et al. 2009).

When induced apoptosis mitochondrial pores are opened, leading to a decrease in the mitochondrial membrane potential (Peña et al. 2009). During the execution phase, the mitochondrial pores open and trigger the release of pro-apoptotic factors into the cytoplasm, where they are activated leading to the degradation phase (Peña et al. 2009). During this latter phase an increase in the permeability of sperm plasma membrane and the externalisation of the constituent phospholipid PS are produced, which triggers a non-inflammatory recognition of the apoptotic cell by phagocytes (Peña et al. 2009).

According to Peña et al. (2009), when permeability of the inner mitochondrial membrane increases for solutes smaller than 1.5 kDa, the permeability transition pore related to that membrane is opened (Grimm and Brdiczka 2007) and when this occurs, the mitochondrial membrane potential, which relies on the impermeability of the inner mitochondrial membrane for protons, breaks down together with the ability of the cell to synthesise ATP. The blocking of the respiratory chain generates ROS intermediates (Grimm and Brdiczka 2007) that increase the concentration of solutes in the mitochondrial matrix. The increase in osmotic pressure provokes the expansion of the inner mitochondrial membrane, which could even affect the outer membrane, as well as the release of pro-apoptotic factors that leads to cell death (Peña et al. 2009).

One of the techniques used to identify apoptotic cells is the calcium-dependent binding of Annexin-V, which detects the externalisation of PS in plasma membrane (Martin et al. 1995). The Annexin-V binding assay is more sensitive to detecting changes in sperm plasma membrane than other membrane integrity assays since, during the process of apoptosis, the translocation of PS precedes the loss of membrane integrity by several hours; this early sign of apoptosis can be monitored by using Annexin-V conjugated with fluorescein isothiocynate (FITC) and PI in a cytometer equipped with standard optics (Peña et al. 2003).

Another recent, accurate technique is the use of YO-PRO-1 dye and PI together, which provides a sensitive indicator of apoptosis (Trzcinska et al. 2011).

In this, the green fluorescent YO-PRO-1 dye can enter apoptotic cells which exhibit a slight permeability of its membrane, whereas other dyes such as the red fluorescent PI cannot (Trzcinska et al. 2011).

9.3.9 Levels of Reactive Oxygen Species

Seminal plasma is a powerful source of antioxidants (Saleh and Agarwal 2002) and evidence suggests that as long as spermatozoa are suspended in it they are protected from oxidative damage. The most common ROS with potential implications in sperm oxidative damage include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and affect sperm function, both in vivo and in vitro (Aitken 1995). In order to counteract the toxic effect of these ROS, seminal plasma contains the enzymes superoxide dismutase (SOD) and peroxidases, such as catalase (CAT). The SOD converts the O_2^- to H_2O_2, and the CAT converts the H_2O_2 to H_2O and O_2, eliminating potential ROS toxicity (Aitken 1995, Aitken et al. 2010).

One of the most useful techniques for estimating ROS level in boar spermatozoa is the use of the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent dye (Gadea 2005; Lampiao et al. 2006; Guthrie and Welch 2008; Awda et al. 2009). This dye is a fluorogenic probe commonly used to detect cellular ROS production. Non-ionised H_2DCFDA is membrane permeable and therefore is able to diffuse readily into cells. Within the cell, the acetate groups are hydrolysed by the intracellular esterase activity forming 2′,7′-dichlorodihydrofluorescein (DCFH), which is polar, and thus stays trapped in the cell. The 2′,7′-Dichlorodihydrofluorescein fluoresces when it is oxidised by H_2O_2 (or lipid peroxides) to yield 2′,7′-dichlorofluorescein (DCF). Measurements by flow cytometry are expressed as mean green intensity fluorescence units as an estimator of ROS generation (Guthrie and Welch 2008; Matás et al. 2010).

Other techniques to assess the production of ROS levels of sperm cell are chemiluminescence methods using luminol (amino-2,3-dihidro-1,4-phthalazinedione; A-8511) and lucigenin (bis-N-methylacridinium nitrate; M-8010) described by Aitken et al. (1992). Levels of ROS are assessed by measuring the luminol- and lucigenin-dependent chemiluminescence with a luminometer.

The ROS levels can also be evaluated by Mitotracker Red/proxylfluorescamine double staining (Wenzel et al. 2005; Flores et al. 2010).

9.3.10 Acrosomal Integrity

Several methods can be used to evaluate acrosome integrity; among them, the most common is the use of plant lectins labelled with a fluorescent probe (Vázquez et al. 2005; see Sect. 9.3.4.2). Lectins are glycoproteins, mainly of non-immune origin and without enzymatic activity, which specifically detect sequences of oligosaccharides

present in the membrane of cells and, in the case of sperm, are involved in primary recognition at the time of oocyte fertilisation. Lectins have two binding sites: specific sugars and glycosylated molecules (Hernández et al. 2006). The first authors to study the cell membrane using lectins were Kashiwabara et al. (1965). To view the location of the lectins these can be conjugated to Alexa Fluor® 488 Conjugate-SBTI (Molecular probes Inc., Eugene, OR) (Bussalleu et al. 2005), fluorochromes such as TRITC and FITC (Nicolson et al. 1977), to peroxidase or to ferritin (Nicolson et al. 1977), to hemocyanin (Kinsey and Koeheler 1976) and to colloidal gold using ultrastructural techniques (Sinowatz and Friess 1983).

Techniques using lectins allow us to locate changes in the distribution of sugars in the sperm membrane. Jimenez et al. (2002) suggested that the membrane glycoconjugates play an important role in the recognition of gametes during fertilisation. Others, such as Schwarz and Koehler (1979), observed changes at the site of attachment of lectins during sperm capacitation.

9.3.11 The Status of the Mitochondrial Sheath

The role of mitochondria in sperm physiology involves two main aspects: the first is related to motility acquisition by the spermatozoon to be able to swim along the female genital tract until reaching and fertilising the oocyte (Peña et al. 2009), and the second is the regulation of sperm death by being a major source of ROS (Lesnefsky and Hoppel 2006; Ott et al. 2007; Erkkila et al. 2006).

Spermatozoa are able to generate energy (in the form of adenosine triphosphate, ATP) by either aerobic or anaerobic metabolic pathways. Monosaccharides are often the main available substrate for spermatozoa both in vivo and or during in vitro handling; by transforming to glucose-6 phosphate (G6-P), they enter the glycolytic pathway to generate pyruvate. Later, pyruvate can either produce extracellular lactate or enter the mitochondrial Krebs cycle. The equilibrium between glycolysis and glycolysis-oxidative phosphorylation depends on factors such as O_2 pressure, the intracellular levels of ATP and the number of intracellular factors, such as nitric oxide (Peña et al. 2009). Moreover, there are possible species-specific differences among mammals, with boars having the highest glycolytic activity (Marin et al. 2003). Mitochondria are found only in the midpiece; thus, oxidative phosphorylation occurs exclusively at this level. However, flagellar kinases and dynein-ATPases need large amounts of ATP to maintain sperm motility (Cao et al. 2006). It has therefore been suggested that the amount of ATP produced in the mitochondria is not enough to diffuse all along the flagellum to provide energy to support motility (Turner 2003) and that other energy sources may exist. Several studies have identified glycolytic enzymes in the principal piece of mammalian spermatozoa, including hexokinase, lactate dehydrogenase and glyceraldehydes 3-phosphate dehydrogenase (Nagdas et al. 2006; Perl et al. 2006).

The second major function of sperm mitochondria is the regulation of cell death (Lesnefsky and Hoppel 2006; Ott et al. 2007; Erkkila et al. 2006).

Mitochondria are a major source of ROS, mainly generated at complexes I and III of the respiratory chain (Krahenbuhl et al. 1991; Gonzalvez and Gottlieb 2007). The most common ROS with potential implications in sperm oxidative damage include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The concentration of superoxide anion in the mitochondria is about 5 to 10-fold higher than in the cytosol or nucleus (Cadenas and Davies 2000), and thus mitochondria might also be a primary target of the damage generated by ROS. Mitochondrial-generated ROS play an important role in the release of Cytochrome-C and other pro-apoptotic proteins, which can trigger caspase activation and apoptosis (Mishra and Shaha 2005; Chan 2006; Tsujimoto and Shimizu 2007; Peña et al. 2009). Mitochondria also perform other diverse cellular functions essential for sperm viability such as modulation of redox status and osmosis, and regulation of Ca^{2+} homeostasis, this cation being involved in metabolic pathways that allow epididymal sperm maturation and fertilisation of the oocyte (Peña et al. 2009).

Several fluorochromes are available to assess the status of the MS in pigs, among them the rhodamine-123 (Fraser et al. 2001) and the iodide of 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbicianine (JC-1) (Huo et al. 2002; Ramió-Lluch et al. 2011). Since fluorochromes that stain the MS are cationic and lipophilic, they are able to passively diffuse across the plasma membrane and accumulate in the negatively charged mitochondrial matrix. Thus, depending on the membrane potential of mitochondria, fluorochromes are accumulated in a greater or lesser degree (Gilmore and Wilson 1999; Fraser et al. 2001).

Another interesting fluorochrome mentioned before (see Sect. 9.3.4.2) is the Mitotracker® Green FM, which passively diffuses across the plasma membrane and binds to membrane lipids of functional mitochondria (Keij et al. 2000; Fraser et al. 2001); it is included in multiple staining methods for assessing sperm cell integrity (Kavac et al. 2003; Bussalleu et al. 2005) and for the identification and localisation of mitochondria (Keij et al. 2000; Fraser et al. 2001). Another fluorochrome that can be used is the Mitotracker Red (Flores et al. 2010).

9.3.12 Metabolic Activity in Boar Spermatozoa: Methods Involving Signal Pathway Transduction and Kinase Activity

In mammals, sperm function is critically controlled through the phosphorylation of specific proteins (Aparicio et al. 2007). Specifically, sperm capacitation involves tyrosine phosphorylation of several proteins (Galantino-Homer et al. 1997; Visconti and Kopf 1998; Baldi et al. 2002) and this phenomenon is also observed in boar spermatozoa (Kalab et al. 1998; Flesch et al. 1999; Bravo et al. 2002).

Various specific signalling pathways mediating phosphorylations during sperm capacitation include intermediate metabolites like protein kinase (PK) A and C (Thundathil et al. 2002), cAMP/PKA (of fibrous sheath protein) and protein tyrosine kinase (PTK) (Visconti et al. 1995). On the other hand, sperm motility appears to depend on the level of phosphorylation of certain specific proteins that is mediated by protein kinases, especially the PKA, and inhibited by protein phosphatases (PP), especially of the PP1 type (Si and Okuno 1999).

Modulation of phosphorylation by PKA is achieved by alterations in the intracellular concentration of the major sperm second messenger- molecule, cyclic adenosine monophosphate (cAMP), brought about by changes in the adenylyl cyclase activity (Holt and Harrison 2002). Two important effectors of motility in natural environment of sperm, bicarbonate and calcium, have been identified as direct activators of adenylyl cyclase (Morton et al. 1974; Okamura et al. 1985; Holt and Harrison 2002). Moreover, numerous studies have shown that pharmacological agents that promote a rise in intracellular levels of cAMP (e.g. inhibitors of cyclic nucleotide phosphodiesterase, such as caffeine or pentoxifylline) can stimulate motile sperm samples poorly. It is therefore supposed that motility shortcomings may often be due to the inability of the spermatozoa to produce and/or maintain sufficient levels of cAMP to stimulate PKA (Magnus et al. 1993; Holt and Harrison 2002). Moreover, studies carried out by Rivlin et al. (2004) show that high concentrations of H_2O_2 inhibit tyrosine phosphorylation, thus corroborating that high levels of ROS are implicated in damaging sperm in the bull (Lopes et al. 1998) and also in the boar (Awda et al. 2009). Because capacitation is regulated by kinases, an indirect way to assess kinase activity is the assessment of the capacitating status of sperm (see Sect. 9.3.6).

9.4 In Vitro Methods for Predicting the Sperm Fertilising Ability

The methods for assessing sperm quality are inaccurate for predicting the fertilising capacity of a given ejaculate even if it displays acceptable sperm quality parameters (Rodríguez-Martínez 2003). Since it is not always possible to carry out in vivo trials, useful in vitro techniques are available that let us know an approximation of the real sperm fertilising ability. These tests are focused on the binding and penetration of the zona pellucida that spermatozoa must overcome to fertilize the oocyte. Therefore, the study of gamete interaction allows sperm fertility to be predicted more accurately.

A number of in vitro assays are available to estimate the capacity of boar sperm to fertilize the oocyte. Some of them are focused on the ability of sperm to develop the specific functions involved in the fertilising process and others are centred on assaying the successful recognition between gametes and the penetration of the oocyte. The most useful tests for predicting boar sperm fertilising capacity are explained in the next sections.

9.4.1 Sperm Binding Assays

Gamete recognition, binding and fusion are highly regulated processes that involve a number of biochemical mechanisms in which a great number of specialised molecules act, even though their particular function is not always known. Sperm binding assays are focused on the study of the molecules that participate in the interaction between gametes prior to penetration and fertilisation. The co-culture of spermatozoa with oocyte substrates or oviductal extracts allows the evaluation of sperm indicators that validate the success of the process. These tests are often contrasted by the same techniques used in the evaluation of the status of spermatozoa (see Sect. 9.3.6).

9.4.1.1 Sperm-Zona Pellucida Binding Test

The zona pellucida (ZP) of the oocyte is the major selective barrier that spermatozoon encounters after its release from the oviductal reservoir (Yanagimachi 1994). The initial contact between spermatozoa and the ZP induces the acrosome reaction, allowing secondary ligands and receptors to act (Rath et al. 2005). The ZP-binding test evaluates the ability for biochemical recognition between the sperm membrane and ZP-receptors, testing the co-culture of spermatozoa with isolated ZP from oocytes and performing an ulterior count of acrosome-reacted spermatozoa (Yanagimachi 1994). Specific recognition, binding and fusion are necessary prior to the penetration of the oocyte and this in vitro test acts as an indicator of the fertilising capacity of the sperm cell, as variations in the number of sperm cells that bind to the ZP between fertile and subfertile boars have been described (Ivanova and Mollova 1993; Lynham and Harrison 1998; Waberski et al. 2005; Collins et al. 2008).

9.4.1.2 Sperm-Oviduct Binding Test

This in vitro test challenges the capacity of the sperm to bind to the oviductal epithelium (Green et al. 2001; Fazeli et al. 2003; Holt et al. 2006) (see also Chap. 6). The sperm population in the oviductal reservoir depends on the initial sperm quality of each ejaculate from each boar, on the site of sperm deposition, and on the number of inseminated sperm (Foxcroft et al. 2008). This assay is interesting since it allows better understanding of sperm-oviductal interactions and can also be proposed for fertility prediction as a complement to other tests designed for the same purpose (Petrunkina et al. 2000; Waberski et al. 2005).

9.4.2 Sperm Penetration Assays

These assays are designed to evaluate in vitro the potential of spermatozoa to overcome the different barriers that participate in the fertilising process.

9.4.2.1 Cervical Mucus Penetration Test

The ability of spermatozoa to penetrate the cervical mucus has been considered essential for fertilisation for a long time (Yanagimachi 1972; Lorton and First 1977; Cupps 1991; Zhao et al. 2002). Since cervical mucus exerts a qualitative selection of sperm entering the cervix (Suarez and Pacey 2006; Martínez-Rodríguez et al. 2012), the cervical mucus penetration test (CMPT) has been proposed as an in vitro laboratory assay for analyzing the fertility of a given ejaculate in many mammalian species. Generally, it consists of a visual assessment of the linear distance covered by the foremost sperm cell in a capillary tube with natural mucus, using the number of spermatozoa accumulated in different segments of the capillary tube as the parameter of analysis (Cox et al. 2002; Martínez-Rodríguez et al. 2012).

Furthermore, Tas et al. (2007) have developed another variant of this technique by using transparent plastic straws instead of capillary tubes. In this test, the total number of penetrating spermatozoa that predetermined distances in cervical mucus is measured on slides. The major problem with this method is the difficulty to standardise the quality of cervical mucus, that is, to obtain large volumes of natural homogeneous cervical mucus. This has been mainly solved for in vitro sperm penetration tests in humans, bulls and rams by formulating synthetic media such as acrylamide, methylcellulose and, hyaluronic acid as substitutes of the natural cervical mucus (Ivic et al. 2002; Tas et al. 2007; O'Hara et al. 2010), but it has not been tried yet in boars.

9.4.2.2 Oocyte Penetration Test (Sperm Penetration Assay–SPA)

This in vitro technique includes two variants: the homologous and the heterologous penetration tests, both based on the assay of successful sperm penetration of oocytes from the same or different species. In boars, the heterologous penetration in vitro test was first described by Imai et al. (1977) by using hamster oocytes; subsequent studies showed that this test correlates positively with semen quality (Clarke and Johnson 1987; Berger and Horton 1988). Nevertheless, this technique has not received widespread attention in porcine species, because it is time consuming and involves high economical costs. Conversely, the heterologous penetration in vitro test has an extra value in human as it avoids using human oocytes which entails ethical implications (Rogers 1985). The inconvenience of these techniques is that they obviate the recognition and attachment of sperm to the oocyte membrane as well as the process of penetration through the ZP.

SPA Using Zona-Free Hamster/Bovine Oocyte Test

A variant of this technique, the zona-free hamster/bovine oocyte test—sperm penetration assay (SPA) has been described as an in vitro heterologous penetration test, which is useful for predicting sperm fertilising capacity in several mammals

including the boar (Yanagimachi et al. 1976). This test requires in vitro preparation of sperm and oocytes in different steps including sperm capacitation, acrosomal reaction, superovulation of females, removal of the zona pellucida from the recovered oocytes, incubation of gametes and finally evaluation of sperm penetration. Nonetheless, Brown et al. (1990) suggested that it might provide a better prediction of fertility than homologous in vitro fertilisation using oocytes with intact zona pellucida. The use of homospecific spermatozoa and oocytes with its zona pellucida intact in in vitro techniques allows all phases of the fertilisation process to be analysed in great detail, including those related with the acquisition of hyperactivity required for in vivo fertilisation. However, it is not always possible to harvest oocytes from the corresponding species and the induction of sperm capacitation (Nagai et al. 1994; Martínez et al. 1996) which, together with the optimal oocyte stage (Mattioli et al. 1990; Martínez et al. 1993), are crucial events when developing this technique. Moreover, if the standardisation and simplification of in vitro fertilisation protocols, including the preparation of cell gametes and the reduction of the economical cost of the whole process were achieved, this would encourage routine use of this technique.

The heterospecific in vitro sperm penetration into zona-intact oocytes is difficult regardless of the phylogenetic distance between species (Yanagimachi 1972; Hanada and Chang 1978). However, if oocytes are freed from the zona pellucida, heterospecific sperm penetration into oocytes is facilitated between some species, including rodents and porcine (Imai et al. 1977). Thus, zona-free hamster oocytes have been widely used for evaluating the fertilising ability of spermatozoa from different species (Shibahara et al. 1998; Brahmkshtri et al. 1999).

SPA Using Zona-Free Hamster/Bovine/Pig Oocyte Test

This assay is the most classical sperm penetration test. It uses zona-free hamster/bovine oocytes for an in vitro assessment of sperm competence required to fuse with the oocyte membrane and to undergo decondensation after penetration (Foxcroft et al. 2008). High positive correlation between the penetration rate and male fertility has been observed in several studies (Berger and Parker 1989; Ivannova and Mollova 1993; Martinez et al.1993) performed a similar assay using zona-free pig oocytes (a homologous in vitro penetration assay) to study the relationship between penetration and boar fertility and they were able to discriminate fertile and sub-fertile boars. However, it must also be stated that other authors have reported that the ability to identify differences in relative fertility among fertile boars is limited (Foxcroft et al. 2008).

9.4.3 In Vitro Fertilisation Assay

The in vitro fertilisation assay (IVF) allows us to evaluate the potential of spermatozoa to complete several biochemical and biophysical changes, such as capacitation, acrosome reaction, sperm-oocyte binding and penetration, and

sperm decondensation, which are all required for successful fertilisation. This test assesses the ability of sperm to fertilize a homologous zona-intact oocyte, generally using immature oocytes collected from fresh ovaries of prepubertal gilts and matured in vitro, a process termed oocyte in vitro maturation (IVM). Immature frozen oocytes (germinal vesicle stage) have been used to reduce time and costs involved in the oocyte maturation process, and also to minimize variation in oocyte quality between replicates of IVF within a same experiment (Martínez et al. 1993; Foxcroft et al. 2008). After 48 h of IVM, the oocytes are freed from cumulus cells by gentle pipetting and are then washed with equilibrating medium and transferred to four-well plates containing fertilisation medium. Sperm diluted to an appropriate concentration of 1×10^5 spermatozoa $\times$ mL^{-1} in IVF medium is added to each fertilisation-well containing the oocytes. Gametes are co-incubated at 39 °C in a humidified atmosphere of 5 % CO_2 for 16–18 h and the success of the IVF assay is tested by evaluating stained oocytes with acetic acid and lacmoid under phase contrast microscopy (Gil et al. 2007), or either by Hoechst staining under fluorescence microscopy (Coy et al. 2005). The parameters evaluated are the number of sperm per oocyte and the following rates: penetration, polyspermy, monospermy, male pronuclear formation (MPN) and potential embryo production (Xu et al. 1998; Rath et al. 1999; Gil et al. 2007).

9.5 Assessment of Seminal Plasma Composition

9.5.1 Common Elements in Seminal plasma and Methods for their Detection

Seminal plasma is a complex mixture of secretions originated in the testes, epididymis and in the male accessory reproductive glands (Yanagimachi 1994). Seminal plasma is not only a vehicle for spermatozoa; there is growing evidence demonstrating that this fluid plays other roles including modulation of sperm function and interaction with the epithelium and secretions of the female genital tract (Rozeboom et al. 2000; Rodríguez-Martinez et al. 2011).

Seminal plasma composition mainly consists of inorganic and organic compounds, such as carbohydrates, lipids, amino acids and proteins (Kordan et al. 1999; Strzezek et al. 2002). Among proteins, spermadhesins represent more than 90 % of their bulk (Calvete et al. 1995; Calvete et al. 1996; Petrunkina et al. 2000; Assreuy et al. 2003). X-ray crystallography has been used to determine the structure of this important group of boar sperm proteins (Varela et al. 1997; Nimtz et al. 1999). The technique consists of the study of the angles and intensities of the diffracted beams produced when an X-ray beam strikes the crystal structure of a protein. Thus, information such as the arrangement of the atoms, their chemical bonds or their disorder can be determined.

Moreover, in these proteomic investigations, affinity chromatography is a frequent resource used to separate heparin-binding proteins from the rest, because these bind to the surface of spermatozoa during ejaculation and are implied in sperm modulation as well as in interaction with the female reproductive tract; in the non-heparin-binding fraction some spermadhesins can be isolated as well (Holody and Strzezek 1999; Centurion et al. 2003; Strzezek et al. 2005). This technique consists of coupling heparin to a water insoluble copolymer in a column using carbodiimide reaction; the boar seminal plasma is loaded into the column with a buffer. Afterwards, non-adsorbed proteins are washed out with a diluent, such as PBS, and heparin-binding proteins attached to the copolymer are eluted with NaCl (Manásková et al. 2003). Chromatography columns can also be used to study protein aggregates by carrying out size exclusion chromatography, which involves size separation and it is usually applied to large protein complexes. Two solutions of proteins are mixed to study their tendency to form aggregates. Afterwards they are eluted through a Sephadex® column or similar and the protein complexes obtained in the fractions are analysed (Manáskova et al. 2003).

If the aim of the study is to analyse the whole proteome, the most used strategy is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). This type of electrophoresis is based on the separation of the pool of proteins by using two features of the peptides: their Isoelectric point (pI) and their size. Hence, there is a first separation or a first dimension called isoelectric focusing (IEF) in which the sample is dropped onto a pH gradient gel. The charge of a protein is affected by its surrounding pH and when an electric potential is applied, making one end of the gel positive and the other negative, proteins in the sample migrate to one or another pole depending on their electrical charge. They stop when they reach their own pI, i.e. the pH point at which their overall charge is 0. The second separation or dimension is made 90° from the first. It consists of a conventional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) that allows mass-dependent separation. Since proteins are denatured and bound to sodium dodecyl sulphate (SDS), which is negatively charged, they move to the positive pole at different velocities depending on their size. The 2D-PAGE permits an effective separation of the proteome since it is difficult, if not impossible to find two proteins sharing the same pI and mass; furthermore, this method presents a high resolution so it is not difficult to understand why it has become the base of proteomic analyses (Strzezek et al. 2005). Often, the 2D-PAGE is coupled to mass spectrometry (MS), specifically designed to characterize the protein spots obtained. There are different MS methods, one of the most popular being the matrix-assisted laser desorption/ionisation source with a time-of-flight mass analyser (MALDI-TOFF). In brief, the sample is adsorbed in a solid matrix and is irradiated with a UV laser beam. Therefore, ions and neutral molecules are expelled from the matrix, which form a cloud on the sample and finally protein is ionised by the collisions with these little cations. The system, using an electric field, accelerates the ions and measures the time they take to arrive to the detector and, in this way, it can identify peptide sequences.

A wide range of immunological techniques to study seminal plasma proteins are also available. An example, the enzyme-linked immunosorbent assay (ELISA), is frequently carried out to evaluate the presence of a protein in a sample as well as to determine its concentration. The sample is loaded in the wells of the assay plate and is incubated with a coating buffer; the wells are deactivated to prevent unspecific unions and the sample is incubated with an antiserum against the antigen studied, which is in turn coupled to a fluorescent or colorimetric substrate (Manásková et al. 2003; Nishita et al. 2011).

Regarding the other compounds of seminal plasma, biochemical analyses, such as enzymatic methods to determine the concentrations of lactate, citrate or phosphorus, have been the most used in the last few years (Kamp and Lauterwein 1995). More recently, however, automated equipment has substituted these time-consuming techniques, given their high accuracy and speed. For instance, the DRI-CHEM® analyser (Fuji Film, Tokyo, Japan) fits a wide range of colorimetric and electrolyte tests for measuring molecules like sodium, potassium, chloride and calcium as well as proteins such as albumin (Murase et al. 2007).

Studies using multinuclear magnetic resonance (NMR) spectroscopy have also been performed for boar seminal plasma (Kamp and Lauterwein1995; Kalic et al. 1997). These approaches, apart from allowing an identification of seminal plasma molecules, permit the concentration of these different compounds to be determined (Kamp and Lauterwein 1995).

NMR unveils physical and chemical parameters of molecules like proteins, carbohydrates and nucleic acids that have nuclei with magnetic properties. Concretely, it studies the atomic nucleus of these molecules when they are aligned in a constant magnetic field while they are perturbed with another magnetic field with an octagonal orientation.

Finally, gas chromatography is applied, which consists of vaporising the seminal plasma sample and injecting it in a chromatographic column containing a liquid stationary phase. Then, the sample is transported through this column by an inert, gaseous mobile phase such as helium or an unreactive gas. Thus, the identification of the different compounds is based on the comparison of the retention time of the seminal plasma sample with standards analysed under same conditions.

This type of chromatography is widely applied in order to study fatty acid and carbohydrate content in boar seminal plasma samples (Johnson et al. 1972; Pizzi et al. 2005; Am-in et al. 2011).

9.6 Conclusion

The study of ejaculate quality is obviously a key factor for the successful development and applicability of AI techniques in pigs. The results obtained from the different macroscopic and microscopic analyses of sperm quality, routinely performed in AI centres, sustain the economical yields of reproductive strategies. Conventional techniques can be complemented with more complex methods that

provide detailed information about sperm physiology and a more precise approach to the fertilisation aptitude of a given ejaculate. These latter techniques require specialised equipment and experienced technicians and are therefore still relegated to research purposes or specifically requested when ejaculates coming from high-value genetic boars are required for specific trials.

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Chapter 10
Quality Improvement of Boar Seminal Doses

Eva Bussalleu and Eva Torner

Abstract The implementation of artificial insemination techniques (AI) has been a turning point in the swine industry. To prepare doses for AI, semen must be collected by following a serial procedure involving high hygiene measures handling in order to minimise microbiological risk. One of these practices is the inclusion of antibiotics in the extenders, the aqueous media used for packing seminal doses that contain elements for assuring the survival of sperm cells for a short or long time period. However, a certain degree of microbial contamination cannot always be prevented, and in this case sperm quality and sanity standards of AI are better preserved if, prior to selling or freezing the doses, microbes are removed by applying methodologies, such as sperm filtration and sperm washing. Additionally, the demand for doses with a high ratio of X- or Y-bearing sperm is increasing due to the particular structure of commercial pig production; hence, it is also necessary to optimise current sex-deviation techniques. All these topics will be fully discussed in the present chapter.

10.1 Introduction

10.1.1 The Concept of Seminal Dose

The first time that artificial insemination (AI) was attempted in boars was at the beginning of the twentieth century. Nowadays, the use of AI has become widespread all over the world, and in developed countries it sustains the entire pig production system (see Chap. 12). The biological basis of AI is the high amount of spermatozoa present in an ejaculate, which entails the possibility of fertilising a high number of sows (from 15 to 40), whereas in natural mating there is only one recipient (Domínguez et al. 2006).

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To ensure the success of AI, ejaculates must be diluted in the appropriate medium to prolong their viability, so that a commercial dose can be defined as liquid extended semen. Typically, different ejaculates from a single or multiple boars are collected, and then processed individually or otherwise pooled. Pooling semen takes advantage of the high fertility of some boars to minimise the effects of subfertile boars (or ejaculates). Thus, semen doses can be treated in two different ways:

- Homospermy: Doses are from a single ejaculate and inseminations are carried out with this single ejaculate.
- Heterospermy: Doses that contain sperm from at least two different animals in the same dose.

Heterospermy allows the processing of different ejaculates at the same time (the ones with the best quality are chosen) and it increases prolificity (number of piglets born alive) (Martin and Dziuk 1977; Dziuk 1996; Haugan et al. 2005).

The number of total spermatozoa in seminal doses is an important parameter in fertilisation (see Sect. 12.2.1.3). Normally, the doses destined to AI contain approximately 2 to 4 $\times$ 10^9 spermatozoa in a volume of 80–100 ml, although the threshold varies with semen quality (Saacke et al. 1991).

10.1.2 From Ejaculates to Doses in Commercial Farming: Collection Procedures, Packaging, Refrigeration and Transport

Semen can be collected from boars mainly in two ways: using an artificial vagina, or by a simpler practice commonly known as the "gloved-hand" method. Although in other species electrical stimulation (electroejaculation) is widely used, it is not predominant in pigs (King and Mcpherson 1973; Basurto-Kuba and Evans 1981; The PigSite 2011). During the collection stage and the entire process, it is mandatory to use hygienic techniques and to control temperature fluctuations. The routinely common procedure for semen collection in the "gloved-hand" technique is the following (adapted from Hancock and Howell 1959; Singleton 2002; Althouse 2007):

1. A filter is placed on a collection flask pre-warmed to 37 °C.
2. The extender is prepared at least 1 h before its use and pre-warmed at the same temperature before being dropped into the flask.
3. The ejaculate is collected. It can be collected from the boar while it mounts a sow or gilt in oestrus. This methodology has two main drawbacks: (1) the boar may be too big to be supported by females; (2) females may begin to walk around the pen, making it difficult to collect semen. Both inconveniences have made breeders decide to train boars to mount a dummy sow. Boars must be trained when they are aged 8–10 months. The trainers must

be patient and the boar should be moved to the collection room two or three times daily to get used to it. If it becomes difficult to make the boar mount the dummy, it can be sprayed with boar scent as this awakens the instinct of the animal. Dummies can be purchased or made in the farm, assuring that they are solid, well-secured on the floor and have no sharp edges, and their height should be adjustable to fit different boar sizes. When boars mount the dummy their sheaths must be visible below. During training, sudden movements and loud noises should be avoided. Semen is collected using polyvinyl, not latex, gloves; once the gloves are on, nothing must be touched except the boar's penis.

4. The penis is grasped and directed inside the collection flask. The first part of the ejaculate (pre-spermatic fraction) is discarded as this watery fluid does not contain sperm but a high bacteria count.
5. The sperm-rich fraction, which is chalky in appearance and contains 80–90 % of all sperm cells in the ejaculate, is collected.
6. The boar is allowed to complete his ejaculation (5–8 min) until the jellified last secretion is observed.
7. The filter is removed and discarded.
8. Within the first 15 min after collection the 37 °C extender must be added to semen. The collection container is placed on a scale to check the volume and the extender should be added slowly.
9. Sperm quality is evaluated (motility, morphology and concentration). The minimum requirements for use of fresh boar semen are the following: the consistency should be milky to creamy; the colour, from grey–white to white; the gross motility of unextended semen should be ≥70 % (if doses are used within 48 h) or ≥80 % (if doses are used later than 72 h after ejaculation); and total abnormalities should not exceed 25 %, including the cytoplasmatic droplets (Althouse 2007).
10. Semen is further diluted and packed into doses. Each standard dose contains about 2 to 4 billion sperms and 80–100 ml of fluid depending on the AI centre. The final concentration should be determined on the basis of the expected duration of storage before use. For example, if the doses are stored for 2 days, a 2 billion sperm/dose is recommended; if the doses are stored for 3 days, a 3 billion sperm/dose, etc. (Althouse 2007). The final number of packed doses depends on the characteristics of the boar, the frequency of collection and semen quality. It also depends on the opaqueness and chalkiness of the ejaculate (Table 10.1). There are different containers for sperm packaging: bottles with screw cap, bottles with clip-clap, plastic blisters, twist-off plastic tubes, and so on. (Fig. 10.1).
11. After packaging, it is advisable to monitor motility for some days as a quality control step for the whole process.
12. Doses are placed into a cooling chamber at a temperature of between 15 and 18 °C and are rotated twice a day to resuspend the sperm cells.
13. Finally, transport must be made using appropriate containers at 15–20 °C to avoid temperature fluctuations.

Table 10.1 A short guide table for boar sperm dilution based on opaqueness (adapted from Singleton 2002)

Semen volume (gram/ml)	Opaqueness	Dilution semen/ extender	Ml of semen + extender	Total amount of extended semen	Number of bottles (100 ml each)
150	Watered down milk	1:3	150 + 450	600	6
150	Milky	1:6	150 + 900	1050	10
150	Creamy	1:10	150 + 1500	1650	16

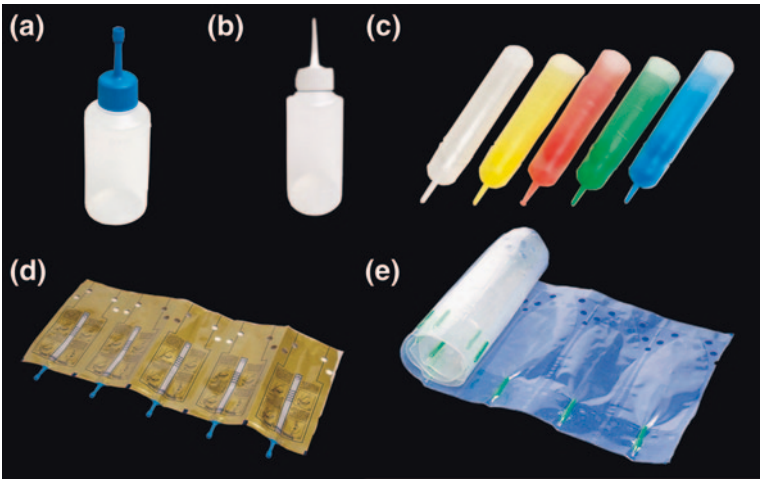

Fig. 10.1 Some of the products used for seminal dose packaging: **a** bottle with twist off cap; **b** bottle with clip clap; **c** coloured tube flexible 90 cc; **d** twist off bags for semen doses; **e** blister for semen doses (http://www.kubus-sa.com/products.php?id=1

10.2 Techniques for Quality Improvement

10.2.1 Formulation of Extenders

10.2.1.1 History and Basic Formulation of Extenders

With the term "extender", we define the aqueous solution used to increase the volume of the ejaculate to that of the required dose (Gadea 2003). The extenders used in AI are a mixture of compounds that are physiologically, biochemically and biophysically equilibrated with the spermatozoa to maintain their integrity and fertilising ability until insemination is performed. The extenders reduce sperm metabolism to prolong its lifespan and also avoid the development of microorganisms. Early diluents for boar semen were proposed by Milovanov in the 1930s and were composed of glucose sulphate and glucose tartrate (Milovanov 1962; Johnson

et al. 2000). In 1965, the Kiev extender, developed by Plisko, introduced an ionic chelator to neutralise ions that could damage the cell membrane structure or trigger a premature acrosomal reaction. In 1975, Pursel and Johnson initially developed the Beltsville Thawing Solution (BTS) for thawing cryopreserved boar sperm but its beneficial properties permitted its development as a conventional extender for refrigerated semen (Johnson et al. 1998). The BTS extender, in comparison with its predecessors, contains a small amount of potassium that allows the maintenance of sodium–potassium pumps in sperm, so it prevents intracellular reduction, and consequently there is no impairment of motility (Alvarez and Storey 1982).

Basically, extenders provide nutrients for the maintenance of sperm metabolism, and substances to control osmotic pressure. It is also necessary to add compounds that reduce the metabolic activity of sperm, whose action is enhanced if the storage temperature is reduced. Due to the characteristic lipid content of boar sperm cell membranes, boar spermatozoa are highly sensitive to temperature changes; so, stabilising the temperature to 15–17 °C also permits keeping a controlled environment. The most common ingredients in extenders are glucose for the sperm metabolic activity, BSA (Bovine Serum Albumin) for cold-shock protection and for the compensation of protein loss, bicarbonate, TRIS (tris(hydroxymethyl) aminomethane) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for pH control, antibiotics to prevent microbial growth and substances like NaCl or KCl for osmotic pressure control. However, most companies keep the exact composition of their extenders secret for commercial reasons.

10.2.1.2 Long-Term and Short-Term Extenders

Extenders are actually divided into two groups: short-term extenders (those that allow preservation from 1 to 3 days) and long-term extenders (those that allow a preservation period higher than 4 days).

Short-term extenders are mainly used when semen is distributed within a local commercial network (Gadea 2003); in contrast, long-term extenders are used when semen production and insemination sites are far apart. The use of long-term extenders allows the possibility of performing analyses for sanitary controls, and more in-depth quality assessment. As stated before, extenders (both long term and short term) must contain nutrients for the metabolic maintenance of the spermatozoa, substances against cold shock, elements for controlling pH, substances for controlling osmotic pressure and antibiotics for bacterial growth inhibition. Long-term extenders such as DiluPorc™ (Sinus, Germany) have a protein mixture that, apart from helping the maintenance of osmotic pressure and pH, neutralises toxic substances secreted by bacteria and also proteins resulting from dead sperm cells. The pH of all these diluents is set between 6.8 and 7.2 and osmolarity ranges from 240 to 380 mOsm kg^{-1}. Sperm metabolism is reduced and viability maintained at temperatures ranging from 15 to 17 °C. However, when doses are warmed sperm activity is stimulated once again (Huo et al. 2002) and the sperm cells begin to consume high amounts of sugars in exchange for releasing free radicals (Parrish

et al. 1999; O'Flaherty et al. 1997). As boar sperm cell membranes have many unsaturated phospholipids, which are vulnerable to lipid oxidation, it is necessary that extenders also include antioxidants (Aitken and Curry 2011; Am-in et al. 2011; Martín-Hidalgo et al. 2011).

Vyt et al. (2004) performed a study comparing three long-term and two short-term extenders for a period of 7 days. They pointed out that motility was dependent upon the time of storage and fell into acceptable values in the two short-term extenders during 3–4 days. After that, motility decreased until being out of the quality range on day 7, although it was still noticeable by this time point. They concluded that the choice of a long-term extender is essential for long-term preservation of extended semen. Likewise, a study by Silva et al. (2011) suggests that the addition of an insulin-like growth factor-I (IGF-I) may be beneficial to semen stored for long periods of time. In their study, they found that the addition of 150 ng/mL IGF-I improved the quality of semen stored for 24 h, and the addition of 78 ng/mL IGF-I improved the quality of semen stored for 72 h.

Antimicrobials are usually added to semen extenders to inhibit bacterial contamination and growth, so that sperm stays viable for days or at maximum for 2 weeks when it is stored at 15–17 °C (Sone 1990). Some studies point out that the presence of antibiotics in the extenders at the appropriate concentration enhances sperm survival, and consequently fertility results (Colenbrander et al. 1993). However, there are certain resistant bacteria that can survive in the extended semen despite the presence of antimicrobials, thereby reducing the fertilising ability of this sample (Sone 1990). Some studies have revealed that the majority of bacteria isolated from extended semen are resistant to the most common antibiotics (Althouse et al. 2000; Althouse and Lu 2005; Bolarín 2011). New studies and strategies are being developed to avoid or reduce the use of antimicrobials. For example, Morrell and Wallgren (2011) tested single layer centrifugation (SLC) as a tool to reduce bacterial contamination, with successful results. There has been great controversy concerning the utilisation of antibiotics in boar semen extenders, so their use is currently legislated.

10.2.1.3 Legislation

In Europe, the addition of antibiotics in boar semen extenders and the sanitary requirements for intra-community trading and importations are regulated by the Council Directive 90/429/EEC. This directive states that "an effective combination of antibiotics, in particular against leptospires and mycoplasmas, must be added to the semen after the final dilution. This combination must produce an effect at least equivalent to the following dilutions: not less than 500 IU per ml streptomycin, 500 IU per ml penicillin, 150 mg per ml lincomycin and 300 mg per ml spectinomycin. Immediately after the addition of the antibiotics, the diluted semen must be kept at a temperature of at least 15 °C for a period of not less than 45 min."

There is another reference body for the legislation of semen extenders and semen collection: the World Organisation for Animal Health (the former Office

International des Epizooties, OIE). This organisation regulates the criteria to be applied to semen extenders, as stated in its International Animal Health Code (OIE 2011). According to these guidelines, diluents must be pathogen-free or sterilised if they contain milk, egg yolk or other animal proteins.

10.2.2 Column Filtration

10.2.2.1 Methodology

Sperm morphology anomalies in boar ejaculates account for considerable economic losses in farms and AI centres. Furthermore, the presence of dead or aberrant spermatozoa in ejaculates can have toxic (Shannon and Curson 1972) and lytic (Lindemann et al. 1982) effects on adjacent spermatozoa. In several species of productive interest, different methods of filtration have been used to improve the sperm quality of seminal doses. The use of such techniques is also advisable before freezing-thawing procedures, especially in those ejaculates from asthenozoospermic or teratozoospermic males (Sieme et al. 2003). Filtration serves to increase the number of viable and motile spermatozoa, although its effects vary considerably depending on the nature of the filtration matrix and the species (Johnson et al. 1996; Adiga and Kumar 2001).

Gel-filtration columns allow the separation of molecules by different parameters such as size, hydrophobicity, charge or ligand binding. The matrix used for filtration is poured into a column to form a packed bed and is chosen for its chemical and physical stability and inertness (lack of reactivity and adsorptive properties). The bed is equilibrated with a buffer, the optimal pH varying depending on the type of matrix. The principle of filtration is that non-viable spermatozoa tend to be more entrapped by the matrix than motile sperm and seemingly functional spermatozoa (Jeyendran 2002). In addition, viable spermatozoa are able to cross the filtration barrier without modifying their functional characteristics (Adiga and Kumar 2001). Immotile and dead spermatozoa tend to agglomerate because of changes in their surfaces charges (Ahmad et al. 2003) or in the proteins after capacitation (Januskaukas et al. 2005). It has been reported that filtration methods eliminate leukocytes (sources of reactive oxygen species, ROS) and select motile and morphologically normal sperm (Ibrahim et al. 2001). It is believed that they are also selective for acrosome intact spermatozoa (Anzar et al. 1997).

There are several matrices available for column filtration. The most common ones are the Sephadex™ neuter column matrices (Sephadex™ G-15, Sephadex™ G-25, Sephadex™ G-50Fine, Sephadex™ G-50Medium, Sephadex™ G-75), with different porous sizes ranging in diameter from 60 μm in Sephadex G-15 to 280 μm in Sephadex™ G-75 (in each type of Sephadex there are different particle sizes, the ones mentioned here are the smallest and the biggest in all the range) (GE Healthcare 2012). Apart from neuter Sephadex™, there are ionic Sephadex™: anionic Sephadex™ (DEAE-50), cationic Sephadex™ (CM-50),

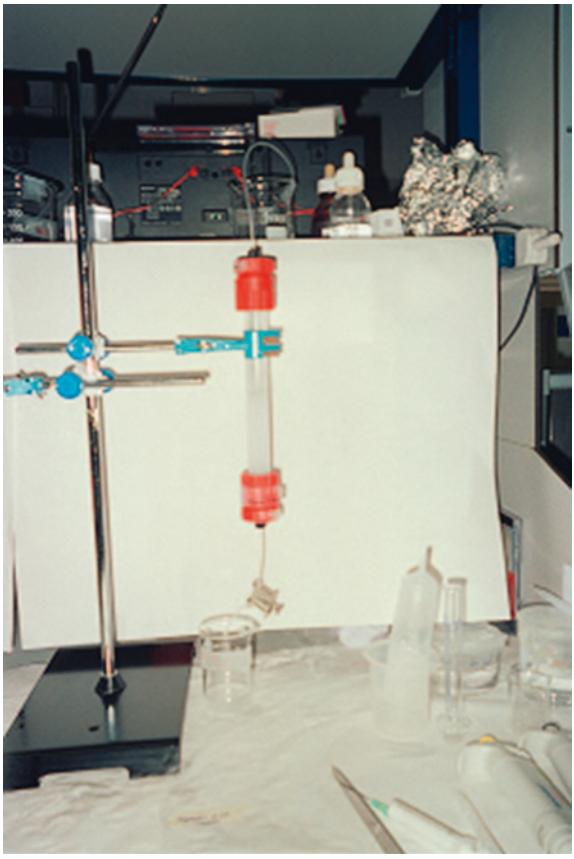

Fig. 10.2 Sephadex™ column packed and ready for sperm filtration

glass wool, glass beads, Leucoscorb™, and so on. Sometimes it is possible to use mixed columns of different matrices (Fig. 10.2).

10.2.2.2 Column Filtration Versus Other Methods

Apart from filtration alternative methods to improve boar sperm quality exist and are gathered under the name of purification or washing practices. They basically consist of sequential centrifugations to remove poor quality sperm.

In the simplest of these methods, the ejaculate in its seminal plasma and extender is centrifuged once and the pellet is resuspended with an appropriate volume of new fresh extender (Morrell and Rodriguez-Martinez 2010). This method only seeks the separation of the spermatozoa from the seminal plasma (Björndahl et al. 2005), so that no separation of dead, moribund or abnormal cells is achieved (Hallap et al. 2004). Another method consists of colloid or density centrifugation, which comprises some variants: Density Gradient Centrifugation (DGC),

SLC and swim-up (see Sect. 10.2.3). In these colloidal systems only sperm that is motile, viable and with intact chromatin is separated from the seminal plasma and the non-viable sperm (Pertorft 2000).

Filtration is not considered the cleanest technique since some cellular debris remains in the sample, but fewer spermatozoa are lost in comparison with other methods (Januskaukas et al. 2005).

10.2.2.3 Implementation of Column Filtration at AI Centres

Experiments conducted so far have established suitable filtration protocols for different types of pathologies affecting boar sperm quality (Bussalleu et al. 2006, 2008, 2009a, b). Each type of column needs different elution conditions for optimal sperm separation and is recommended for a given pathogeny affecting the male ejaculate (Tables 10.2 and 10.3). Filtration experimental tests showed high efficiency in enhancing boar sperm quality (Bussalleu et al. 2008, 2009a, b). Other authors (Ramió et al. 2009) also pointed out that the use of matrices like Sephadex™ G-15 and C-50 are good tools, not only for the improvement of boar semen quality but also for in vivo fertility. In contrast, the election of an inappropriate matrix could diminish boar sperm quality.

Column filtration could be easily implemented at AI centres since no heavy or expensive equipment is required. However, for large-scale filtration it would be necessary to develop a commercial prototype that suited large volumes, which is why research is ongoing in this respect.

Table 10.2 Different characteristics and filtration conditions for matrices used in semen filtration at 23 °C

Type of matrix	Particle diameter (μm) (approximately)	Matrix length (cm) (using a column of 20 cm length and a diameter of 2 cm	Elution velocity
Sephadex™ G-25 medium (neuter)	85–260	10 ± 0.5	1 ml/20 s
Sephadex™ G-50 fine (neuter)	40–160		
Sephadex™ G-50 medium (neuter)	100–300		
Sephadex™ G-75 (neuter)	90–280		
Sephadex™ DEAE-50 *(anionic)	182–214	2.5 ± 0.5	1 ml/40 s
Sephadex™ CM-50 *(cationic)	221–250	5 ± 0.5	
Glass beads	150–212	10 ± 0.5	
Glass wool	–	2 ± 0.5	

*The use of peristaltic pump is mandatory

Table 10.3 Application of different filtration matrices in relation to different sperm pathologies

Type of males	Best matrix to use	Advantages	Disadvantages
Non-pathological males	Sephadex G-50 fine, Sephadex G-50 medium, Sephadex G-75	Increase in the frequency of mature spermatozoa	
	Sephadex G-25 medium	Increase in the frequency of mature spermatozoa. Decrease in the frequency of spermatozoa with distal droplet	
	Sephadex CM-50, glass wool	Decrease in the frequency of spermatozoa with distal droplet	Decrease in the frequency of motile spermatozoa
	Glass beads	Increase in the frequency of mature spermatozoa. Decrease in the frequency of spermatozoa with distal droplet	Decrease in the frequency of motile spermatozoa
	Sephadex DEAE-50	Decrease in the frequency of spermatozoa with distal droplet	Decrease in the frequency of motile spermatozoa. Decrease in the frequency of viable spermatozoa. Increase in the frequency of spermatozoa with head abnormalities
Pathogenic males			
Asthenospermia (ejaculates with high frequency of immotile spermatozoa)	Glass beads	Increase in the frequency of viable spermatozoa	–
	Glass wool	Decrease in the frequency of spermatozoa with the tail folded in the connection piece	–
Astenoteratonecrospermic (ejaculates with high frequency of immotile, morphologically abnormal and death spermatozoa)	Sephadex G-25 medium, Sephadex G-50 fine, Sephadex G-50 medium, Sephadex G-75	Decrease in the frequency of spermatozoa with proximal droplet, spermatozoa with distal droplet, spermatozoa with tail folded in Jensen annulus and agglutinated spermatozoa. Increase in the frequency of morphologically mature spermatozoa and in the frequency of viable spermatozoa	Decrease in the frequency of motile spermatozoa
	Glass beads	Increase in the frequency of viable spermatozoa	

(continued)

Table 10.3 (continued)

Type of males	Best matrix to use	Advantages	Disadvantages
Teratospermia (ejaculates with high frequency of morphologically abnormal spermatozoa)	Sephadex DEAE-50	Increase in the frequency of mature spermatozoa and decrease in the frequency of spermatozoa with proximal droplet and spermatozoa with broken tails	Decrease in the frequency of motile spermatozoa and in the frequency of viable spermatozoa
	Sephadex CM-50	Increase in the frequency of morphologically mature spermatozoa and decrease in the frequency of spermatozoa with distal droplet	Decrease in the frequency of motile spermatozoa
	Glass wool	Increase in the frequency of morphologically mature spermatozoa and decrease in the frequency of spermatozoa with proximal droplet and in the frequency of isolated heads	
	Glass beads	Increase in the frequency of morphologically mature spermatozoa and in the frequency of viable spermatozoa	
Teratospermia (ejaculates with high frequency of morphologically abnormal spermatozoa)	Glass beads	Decrease in the frequency of spermatozoa with proximal droplet, agglutinated spermatozoa and spermatozoa with abnormal heads. Decrease in the frequency of isolated heads and in the frequency of broken tails	
Astenonecrospermia (ejaculates with high frequency of immotile and dead spermatozoa)	Sephadex G-25 medium	Decrease in the frequency of spermatozoa with tails folded in the connection piece	
	Sephadex G-75	Increase in the frequency of viable spermatozoa	

10.2.3 Purification (Washing) Sperm Procedures

10.2.3.1 Applications

As explained in Sect. 10.2.2.2, simple washing and density centrifugation are good techniques to separate motile sperm from other cell types (Agarwal and Ranganathan 2001), but only the use of colloids permits to discard sperm with different pathologies, namely immature, aged and dying sperm (Morrell et al. 2001, 2004), morphologically abnormal sperm (Tomlinson et al. 2001), and sperm with damaged chromatin (Morrell et al. 2001, 2004; Sakkas et al. 2000; Tomlinson et al. 2001). Removal of bacteria and viruses is also possible with density centrifugation (Nicholson et al. 2000; Guibert et al. 2001; Cassuto et al. 2001, 2002; Levy et al. 2001; Bujan et al. 2001, 2002; Englert et al. 2004) as well as the elimination of ROS (Agarwal and Ranganathan 2001).

10.2.3.2 Density Gradient Methods

The DGC has been used for the separation of different types of cells, not only spermatozoa. The principle of this technique is that under the centrifugal force applied to a colloidal density gradient the cells move to the isopycnic point; that is, the point at which the gradient matches their density (Mortimer 1994). At this point, they can be easily aspirated and dropped into another tube with buffered medium. As spermatozoa have diff erent density from leucocytes, bacteria, viruses and cell debris, they can be separated using density gradient methods (Morrell 2006). When centrifuged, motile spermatozoa move faster in the direction of the centrifugal force than immotile spermatozoa and are separated. Motile spermatozoa remain at the bottom of the tube, whereas immotile spermatozoa and other cells are retained in the upper layers together with seminal plasma.

Normally, when DGC is applied it uses at least two layers of different density (and up to 12 layers in some protocols). A variant of this methodology is SLC, which only uses one layer. There are several substances used as colloids for gradient centrifugation, most of them commercial, although BSA is applied in some cases. The most common ones are Percoll™ (Sigma, St. Louis, MO), Ficoll™ (GE Healtcare, Salt Lake City, Utah, USA), PureSperm™ (Nidacon, Goteborg, Sweden), Isolate™ (Irvine Scientific, Santa Ana, CA, USA), Ixaprep™ (Medicult, Copenhagen, Denmark), Optiprep™ (Nycomed Pharma, AS, Majorstua, Oslo, Norway), Androcoll™ (SLU, Uppsala, Sweden) and Nycodenz™ (Sigma-Aldrich, St. Louis, MO). However, in 1996 there was great controversy about the use of Percoll™ (colloidal silica coated with polyvinylpyrrolidone (PVP)). The manufacturer withdrew it from human-AI because of possible adverse effects on sperm survival and development of fertilised eggs. Nowadays, this substance is only used for research purposes and not for the preparation of sperm for AI (Mortimer 2000). This product has been replaced by alternative colloidal silica preparations covalently bound to silane molecules (for example, PureSperm™ and Isolate™) or

others (Nycodenz™ is based on the iodinated cyclic hydrocarbon iohexol), which also give great separation yields.

The main inconvenience of density gradients is dealing with the high volume of semen that boars produce, which makes it difficult to use this procedure in routinely AI dose packaging. However, Morrell et al. (2009) proposed that the SLC procedure, with the colloid Androcoll™, is suitable for processing large volumes. Moreover, this technique has also been shown to be highly efficient at removing bacteria from boar ejaculates (Morrell and Wallgren 2011).

10.2.3.3 Swim-Up

Although it can be considered a density gradient method, this technique deserves to be given individual attention owing to its particular features. The swim-up technique has been widely used in andrology laboratories for the selection of motile spermatozoa since at least the 1950s. This migration procedure is considered to be functionally equivalent to the process by which spermatozoa escape from the ejaculate and colonise the cervical mucus (Mortimer 2000). It is based on the fact that fully motile spermatozoa swim from the pellet, after the ejaculate is centrifuged, to the top layer of the swim-up medium, leaving the cellular contents and seminal plasma behind. The technique is quite simple and only requires an incubator. Briefly, the fresh diluted semen is concentrated by centrifugation (600 × *g* for 5 min) and then the sperm pellet is overlaid at the bottom of the tubes containing the swim-up diluent. Afterwards, the tubes are incubated at 37–39 °C for between 40 and 60 min and the medium and top layers, where motile spermatozoa have swum, are removed and processed (Holt et al. 2011). This technique is not highly efficient and the total recovery rate of motile spermatozoa does not exceed 10 or 20 % (Hallap et al. 2004). Moreover, some studies have criticised that this selection method is only based on sperm motility and does not select for general morphology, chromatin or acrosome/membrane integrity (Somfai et al. 2002). For this reason, and despite its simplicity, swim-up is not widely used for preparing spermatozoa for AI (Morrell and Rodriguez-Martínez 2011).

10.3 Sanitary Control

10.3.1 How can Semen be Microbiologically Contaminated? Economic and Sanitary Implications for the Swine Industry

The presence of microorganisms in boar sperm is currently one of the problems that the swine industry, and especially artificial insemination (AI) centres, deal with. In the farm industry, AI and the international market of germoplasm require a high sanitary control to avoid the spread of diseases caused by viruses

or bacteria and to avoid the sale of infected doses (also with probably decreased quality due to the presence of microorganisms). Microbial contamination of semen can be due to systemic and/or urogenital tract infections of the boar, or can occur during collection, processing and storage. Sources of bacterial contamination of extended semen can be of animal origin (faeces, preputial cavity fluids, skin/hair, respiratory secretions and personnel), or non-animal origin (water, plant matter, sinks/drains, air and ventilation systems or inanimate objects) (Althouse 2008).

The source of fungi and yeasts is basically individuals and air ventilation systems. In the case of viruses, their presence in semen samples has its origins in the infected blood that goes to the genital tract, in preputial contamination via faeces (this venereal contamination affects semen collection and processing steps) and in aerosol contamination during semen collection and processing (Guérin and Pozzi 2005).

There are several recommendations for avoiding or minimising semen contamination (adapted from Althouse 2008):

Personnel:

- Follow good hand hygiene and use protective gloves.
- Avoid contact of hands with products that will be used in semen processing.
- Avoid sneezing or coughing into material in contact with semen.
- Use caps and hair nets (especially people who perform semen collection).
- Cleanness of overalls and shoes/boots.

Animal housing/handling:

- Regular sanitary maintenance, including the removal of organic material and application of broad-spectrum disinfectants.
- The boar abdomen must be clean and dried prior to semen collection.
- Clean preputial opening and surrounding area with convenient material.
- Preputial fluids can sometimes contain a high number of bacteria, so these must be eliminated prior to the semen collection process.
- When semen collection is done using an artificial vagina or the gloved-hand method, the collector should direct the penis in such a way that gravitational contamination of the semen collection flask with preputial fluids is minimised.
- When collection is done by the gloved-hand method, separation of the pre-sperm fraction helps to reduce the bacterial load.
- At the end of each collection, the working area must be thoroughly cleaned and disinfected.

Laboratory:

- Usage of disposable products whenever possible.
- If recycled laboratory material cannot be sterilised it must be cleaned using a laboratory-grade detergent (residue-free) with water, followed by a distilled water rinse. Allow sufficient time and proper ventilation for complete evaporation of residual alcohol.
- Laboratory purified water should be checked regularly. When there is bacterial growth, the source of contamination must be identified.

- Daily disinfection of the equipment using a residue-free detergent and thorough rinsing.
- The floor should be mopped with disinfectant at the end of the day.
- Break down bulked products into aliquots.
- It is advisable to install ultraviolet lighting in the laboratory surfaces.

The presence of microorganisms in semen can result in poor sperm quality, embryonic or foetal death, endometritis, systemic infection and/or disease in the recipient female (Maes et al. 2008). However, the transmission of pathogens does not always occur. The conditions required for the establishment of infection in the sow are complex, and lack of transmission might be explained by factors such as the sow's innate immunity, or concentrations of the pathogen below the minimum infection dose (Maes et al. 2008). Another example is the results reported by Maroto Martin et al. (2010), in which litter size was significantly reduced when semen was contaminated with *Escherichia coli* above a threshold value of 3.5×10^3 CFU/ml.

In relation to the regulation of the presence of microorganisms in semen, the Office International des Epizooties (OIE 2011) has defined some guidelines in the annexes of the International Zoosanitary Control (OIE 2011), and different countries all over the world have also established their own regulations.

10.3.2 Bacteria (and Fungi) Transmitted Via Semen

A variety of bacteria can be found in boar semen under pathological conditions; most of them are gram negative and belong to the enterobacteriace family (Althouse and Lu 2005). The most common ones are *E. coli*, *Staphylococcus* spp., *Proteus* spp., *Pseudomonas* spp, *Klebsiella* spp, *Bacillus* spp and *Actinomyces* spp. There are some differences in the genera found depending on the source consulted, but the ones mentioned above are common in all the studies. Other genera of bacteria identified in boar semen are: *Enterobacter* spp, *Pasteurella* spp, *Citrobacter* spp, *Providencia* spp, *Neisseira* spp, *Corynebacterium* spp, *Streptococcus* spp, *Bacteroides* spp, *Lactobacillus* spp, *Acinetobacter* spp, *Actinobacillus spp, Flavobacterium spp, Serratia* spp and *Micrococcus* spp. (Tamuli et al. 1984; Dagnall 1986; Danowski 1989; Sone et al. 1989). Another bacterium prevalent in boar semen used for AI is *Chlamydia* spp (Kauffold et al. 2006). Maroto Martin et al. (2010) have also mentioned the presence of anaerobic bacteria in some seminal samples. Their incidence in semen has also been reported in equine livestock (Corona and Cherchi 2009) but it has not been widely studied in boars. *Clostridium perfringens* can survive in extended boar semen and can also cause lethal damage in sperm quality depending on the bacterial load (Sepúlveda et al. unpublished data).

Bacteria and spermatozoa are unable to regulate their temperature but the latter are much more sensitive to temperature changes than bacteria, so that exposure to a few degrees above body temperature causes sperm death. As environmental temperature decreases, cell plasma membrane fluidity changes and growth rates and metabolism decrease; at a certain point, growth and metabolism stop and cells become

dormant; this phenomenon, used to reduce metabolism and to induce dormancy in extended semen (and, consequently, increase sperm longevity), can be beneficial for contaminant bacteria, which can adapt to low environmental temperatures (Althouse 2008). To avoid their growth, antimicrobials are widely included in the formulation of extenders, but some studies have demonstrated that over 90 % of bacteria isolated from extended semen are resistant to common antibiotics (Bolarín 2011).

One of the most studied bacteria in boar semen is *E. coli* since its presence in semen induces sperm agglutination and motility impairment (Auroux et al. 1991; Diemer et al. 1996), which may be explained by the adhesion of *E.coli* to sperm cell membranes. Moreover, this bacterium produces spermicidal effects without an acidic environment (Althouse et al. 2000). Its presence in doses destined to AI is frequent and compromises sperm quality (Bussalleu et al. 2011a, b). This was demonstrated in an experiment in which different concentrations of enterotoxigenic *E.coli* (ETEC) and verotoxigenic *E.coli* (VTEC) strains ranging from 10^2 to 10^8 colony forming units (cfu) per ml, were inoculated in doses destined to AI and left during 11 days at 15ºC (Bussalleu et al. 2011a, b). Both bacteria (ETEC and VTEC) were selected for their high prevalence in farms (Thomson 2001). As shown in Fig. 10.3, the percentage of progressive motile spermatozoa significantly decreased in the tube inoculated with 10^8 cfu/ml after 24 h of inoculation. This tendency was maintained until the end of storage time, so that the highest inoculation dose dramatically affected the percentage of progressive motile spermatozoa from the very beginning of the experiment. Regarding the percentage of viable spermatozoa (intact nucleus, intact mitochondrial sheath and intact acrosome) (according to Bussalleu et al. 2005), it significantly decreased in the tube inoculated with 10^8 cfu/ml after 2 days of refrigeration when compared with other treatments

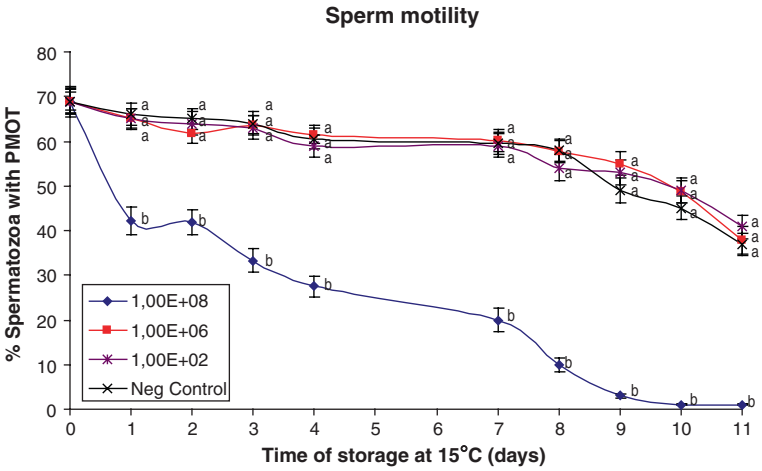

Fig. 10.3 Percentage of viable spermatozoa (mean ± SEM) after 11 days of storage at 15 °C. Different superscripts (*a*, *b*, *c*) mean significant differences ($P < 0.05$) among treatments at the same given time point

(negative control, inoculation with 10^6 cfu/ml and inoculation with 10^2 cfu/ml) (Fig. 10.4). The percentage of morphologically normal spermatozoa, analysed under light microscopy, was not affected by the different concentrations of *E.coli* (Bussalleu et al. 2011a, b). However, previous studies have demonstrated that *E.coli* adhere to the sperm surface via mannose binding sites (Wolff et al. 1993; Monga and Roberts 1994) and produces ultrastructural changes at the level of the midpiece, plasma membrane and acrosome (Diemer et al. 1996), thereby altering sperm function (Villegas et al. 2005). These differences may be explained by the fact that it was not possible to see the ultrastructural damages induced by *E.coli* under light microscopy. On the other hand, Yániz et al. (2010) have reported that deleterious effects in ram semen samples are only evident if the sperm: bacterial ratio is 1:1 or greater. The same authors have pointed out that, during storage at 15 °C, multiplication of bacteria causes the reduction of the ratio, thus increasing the adverse effects on spermatozoa. It would seem that the same process occurs in boar ejaculates. Furthermore, the presence of factors released by bacteria, like α-hemolysin, Shiga-like toxin, lipopolysaccharides and peptidoglycan fragments, may also have important deleterious effects on spermatozoa (Schulz et al. 2010).

Regarding fungi and yeasts, their presence in the reproductive tract and in the semen of boars has not been widely studied. Only an investigation conducted by Cioreni et al. (2008) reveals that different fungi species can also infect boar semen samples. Species such as *Cladiosporum* sp., *Penicillium* sp., *Fusarium* sp., *Aspergillus* sp., *Mucor* sp., *Alternaria* sp and *Geotrichum* sp., apart from different yeasts, have been found in boar semen (Cioreni et al. 2008).

The harmful effects of contamination on sperm quality are not observed immediately, but, as mentioned before, appear after one (in the case of the highest

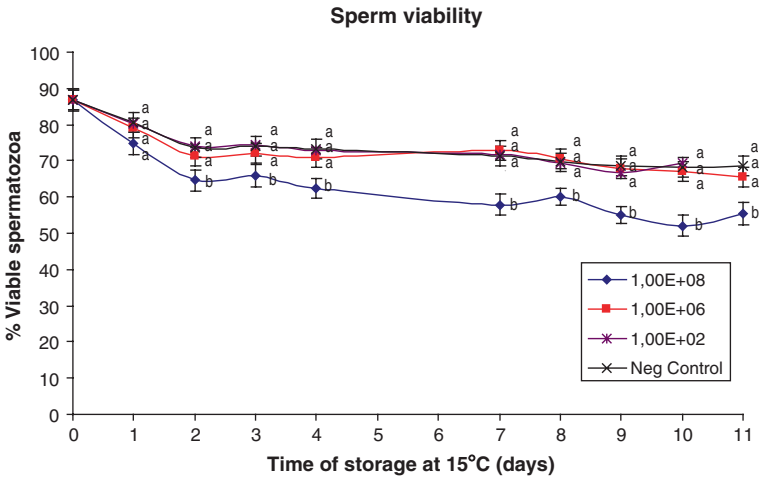

Fig. 10.4 Percentage of viable spermatozoa with intact acrosome and mitochondrial sheath (mean ± SEM) after 1, 2, 3 and 4 days of storage at 37 °C. Different superscripts (*a, b, c, d*) mean significant differences ($P < 0.05$) among treatments at the same given time point

infective concentration) or more days of storage. This finding may be explained by the fact that once the extended semen is contaminated, bacteria must adapt to the novel environment by recovering from the physical damage or shock of initial transfer (Althouse 2008). Bacteria, fungi and yeasts that can be found in semen samples are susceptible to being transmitted via AI. Thus, it is necessary to control the microbiological sperm quality to avoid the spread of illnesses and the use of semen doses with low quality due to the presence of bacteria, fungi or yeasts.

10.3.3 Viruses Transmitted Via Semen

Regarding viruses, many of them have been recovered from the semen of infected boars (Table 10.4; adapted from Maes et al. 2008). Apart from the strains mentioned in Table 10.4, there are others that have been isolated: adenovirus, reovirus, influenza, transmissible gastroenteritis and swine papilloma virus (Guérin and Pozzi 2005). The presence of these viruses in semen is related to reduced reproductive performance and infertility (Guérin and Pozzi 2005).

Many of the viruses can be transmitted venereally with or without clinical signs of disease. The most likely period to find virus in semen is in the stage of clinical illness of the host (Larson et al. 1980; Wittman 1989). If symptomatology exists, the breeder immediately removes the ejaculate from the AI circuit. The danger comes when the animal does not show any clinical sign, so that its infected semen is used. For this reason, it is advisable to perform analyses to detect the presence of viruses prior to the use of doses. Moreover, the conditions required for the appearance of clinical illness in sows after insemination with infected semen are complex, and the sows do not always become infected or show clinical signs (Guérin and Pozzi 2005).

Guérin and Pozzi (2005) have categorised the viruses that can be transmitted via semen into four types:

- Category I: viruses or viral diseases with proved evidence of transmission via semen, but without any sanitary risks for AI because of the existence of an official eradication policy. In this category there are the following viruses: Foot and Mouth Disease (FMD) virus, Aujezky's disease virus, Classical Swine Fever (CSF) virus, African swine fever virus and swine vesicular virus.
- Category II: viruses or viral diseases with proved evidence of transmission via semen and which can be associated with sanitary risk for AI unless they are accompanied by control measures and/or an official eradication policy. Porcine reproductive and respiratory syndrome (PRRS) virus, Japanese B-encephalitis virus and porcine parvovirus are included in this category.
- Category III: viruses or viral diseases that seem to be associated with sanitary risks for AI. In this case, more research to ensure the risk of transmission via semen is required. In this category viruses Porcine circovirus 2 (PCV-2), rubulavirus, enteroviruses and picornaviruses.
- Category IV: viruses or viral diseases for which there are no proof of their presence in semen or transmission via semen and which are dangerous for AI.

Table 10.4 Important pathogenic viruses transmitted via semen

Organism	Boar infection type	Timing of detection (test used)	Potential risk for contamination	Reference
Classical swine fever virus (CSFV)	Experimental inoculation	7 and 11 DPI (virus isolation)	High	de Smit et al. (1999)
	Experimental inoculation	7-63 DPI (RT-PCR); 11, 18, 21 and 53 DPI (virus isolation)		Choi and Chae (2003)
Foot-and-mouth disease virus (FMDV)	Exposure to experimentally inoculated pen mates	Up to 9 d post-exposure (virus isolation)	Low	McVicar et al. (1978)
Japanese encephalitis virus	Experimental inoculation	35 DPI	High	Ogasa et al. (1977)
Porcine circovirus type 2 (PCV2)	Natural infection	Detected (multiplex nested PCR)	High	Kim et al. (2001)
	Natural infection	Detected (nested PCR)		Hamel et al. (2000)
	Experimental inoculation	Intermittently between 5 and 47 d DPI (nested PCR)		Larochelle et al. (2000)
Porcine enterovirus	Experimental inoculation	45 DPI (virus isolation)	High	McAdaragh and Anderson (1975)
	Natural infection	Detected (virus isolation)		Phillips et al. (1972)
Porcine parvovirus	Natural infection	Detected (virus isolation)	High	McAdaragh and Anderson (1975)
	Natural infection	Detected (multiplex seminested PCR)		Kim et al. (2003)

(continued)

Table 10.4 (contniued)

Organism	Boar infection type	Timing of detection (test used)	Potential risk for contamination	Reference
Porcine reproductive and respiratory syndrome virus (PRRSV)	Experimental inoculation	2-57 DPI (nested PCR)	High	Shin et al. (1997)
		12-21 DPI (nested RT-PCR)		Christopher-Hennings et al. (1998)
		Up to 47 DPI (nested RT-PCR)		Christopher-Hennings et al. (1995a, b)
		Up to 92 DPI (nested RT-PCR)		Christopher-Hennings et al. (1995a, b)
		7 and 8 DPI (swine bioassay-sero-conversion)		Swenson et al. (Swenson et al. 1994a, b)
		43 DPI (swine bioassay seroconversion)		Christopher-Hennings et al. (1995a, b)
		Up to 43 DPI (swine bioassay seroconversion and virus isolation)		Swenson et al. (Swenson et al. 1994a, b)
		7 DPI (virus isolation)		Prieto et al. (1996), Shin et al. (1997)
		11 DPI (virus isolation)		Christopher-Hennings et al. (1995a, b)
Pseudorabies virus (Aujezky)	Natural infection	Detected (virus isolation)	High	Medveczky and Szabó (1981)
	Experimental infection	Detected (virus isolation)		Vannier and Gueguen (1979)
Rubula virus	Experimental infection	2-49 DPI (virus isolation)	High	Solis et al. (2007)
Swine vesicular disease virus (SVDV)	Exposed to experimentally inoculated pen mates	Up to 4 DPI (virus isolation)	Low	McVicar et al. (1978)

*Adapted from Maes et al. (2008)

10.3.4 Methods for the Sanitary Control of Semen

In the farm industry, AI and the international germplasm market require a high sanitary control to avoid the spread of diseases caused by viruses and bacteria. This control must be based on rapid, sensitive and specific diagnostic tests for certifying semen free from pathogenic agents (Afshar and Eaglesome 1990; Eaglesome and Garcia 1992). Currently, the isolation of bacteria from semen is based on culture procedures, which are complex and laborious and can easily be altered by the presence of antibiotics and inhibitors in semen (Gradil et al. 1994). This fact makes it necessary to use sensitive and rapid techniques, such as PCR (Polimerase Chain Reaction), which allows the amplification of a specific DNA sequence of a microorganism present at a low concentration (Mermin et al. 1991; Gradil et al. 1994; Eaglesome et al. 1992). Nowadays, the use of the PCR method is widespread and there are plenty of primers available to match a wide range of known viruses and bacteria. Some laboratories also apply the qPCR (quantitative PCR), which permits, apart from detection, the quantification of the microbial load. Due to its high efficiency and to the fact that results are obtained in a few hours, the use of this rapid technique allows quick detection of microbes, and infected doses can be immobilised on time before they are marketed.

Some PCR techniques are available for the detection of viruses and bacteria in boar semen. For example, the PCR techniques from Zhang et al. (2007) and Yilmaz et al. (2006) were adapted for the detection of ETEC and VTEC in boar semen. Concretely, two multiplex PCR (a type of PCR which allows the simultaneous detection of several genes under the same PCR conditions), and a conventional PCR were used (Bussalleu et al. 2011a, b). These PCRs permit the detection of 14 virulence factors (K88, K99, 987P, F18, F41, AIDA-I, Stx2e, STa, STb, LT, EAE, EAST1, VT1, VT2) present in different pathogenic *E.coli* strains (Fig. 10.5). Furthermore, a multiplex PCR technique developed by Ferrarezi et al. (2008) and adapted to boar semen, combined with a conventional PCR (developed by Bussalleu et al., unpublished), allows the detection of the following virulence genes from *Clostridium perfringens*: *cpa, cpb2, cpb*, *cpe*, *iA* and *etx* (Bussalleu et al., unpublished).

Regarding viruses, the methods used for their isolation are poorly sensitive, time consuming and highly expensive (every virus needs a different cell line to grow; sensitive cells lines for each virus are recommended in the OIE standards (OIE manual 2000) and they generate toxicity to host cells) (van Rijn et al. 2004).

To overcome these problems there are plenty of conventional PCR, nested PCR or quantitative PCR (qPCR) techniques available (more sensitive and less time-consuming than conventional virus isolation), like the two nested PCR techniques for the detection of PRRSV (from a sequence of the ORF7) (Bussalleu et al. 2009a, b) and Aujezky disease (from the gene *gB*) (Figs. 10.6 and 10.7). The nested PCR consists of two sequential PCR, in which the product of the first PCR is the template for the second. With this type of PCR the sensibility of the technique increases in comparison with the conventional one.

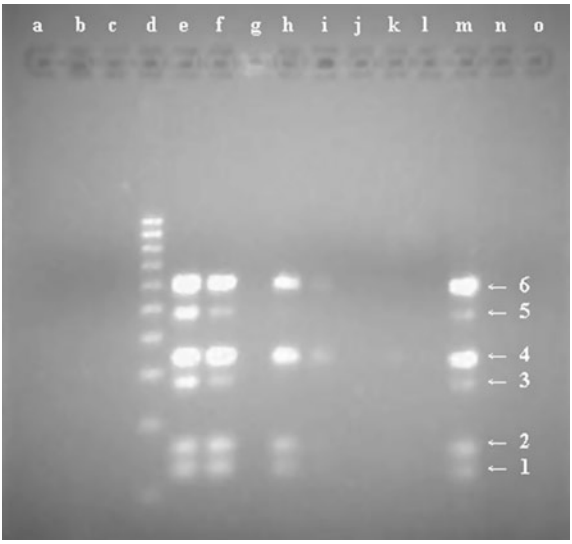

Fig. 10.5 PCR multiplex assay for the detection of genes *K88*, *F18*, *F41*, *987P*, *K99*, *LT*, *STa*, *STb* and *Stx2e* of different infective loads of *E.coli* in boar semen. Lanes *a*, *b*, *c* and *o*, empty; lane *d*, 100 bp ladder; lane *e*, sample from an infective dose of $\times 10^8$ bacteria ml^{-1}; lane *f*, an infective dose of $\times 10^7$ bacteria ml^{-1}; lane *g*, reagent control (negative control); lane *h*, an infective dose of $\times 10^6$ bacteria ml^{-1}; lane *i*, an infective dose of $\times 10^5$ bacteria ml^{-1}; lane *j*, an infective dose of $\times 10^4$ bacteria ml^{-1}; lane *k*, an infective dose of $\times 10^3$ bacteria ml^{-1}; lane *l*, an infective dose of $\times 10^2$ bacteria ml^{-1}; lane *m*, direct cell culture of $\times 10^9$ bacteria ml^{-1} (positive control); lane *n*, negative control. Band 1 corresponds to gene STb (125 bp), band 2 to STa (146 bp), band 3 to LT (280 bp), band 4 to gene F18 (334 bp), band 5 to gene K88 (440 bp) and band 6 corresponds to gene Stx2e (599 pb)

10.3.5 How can the Microbiological Contamination of Boar Semen be Eliminated?

There are different strategies to maintain microbial concentration, of bacteria in particular, below a threshold level to preserve sperm fertility (Althouse et al. 2000). The first and most viable option is to enhance hygienic measures during semen collection and processing. The second option is to establish a shelf-life time limit on the extended semen product in order to limit the amount of time available for bacterial multiplication and to prevent overwhelming the buffering capacity of extenders. The third and most used option is to select antibiotics as preservatives with a broad-spectrum bactericidal or bacteriostatic activity to include them in the semen extender formulation (Althouse et al. 2000; Yániz et al. 2010). The Council Directive 90/429/EEC stipulates the antibiotics and the minimum concentrations that must be added to extended semen doses for their sale within the European Union (Morrell and Wallgren 2011). The most common antibiotics used in porcine extenders are spectinomycin, gentamicin, neomycin, amoxicillin, penicillin,

Fig. 10.6 Detection of the ORF7 from PRRVS using a nested PCR. Lane *a*, product of first PCR (310 pb); lane *b*, negative control from the first PCR. Lane *c*, ladder of 100 pb. Lane *d*, product of the second PCR (162 pb); lane *e*, negative control of second PCR; lane *f*, negative control of first PCR after second PCR; lane *g*, negative control of reagents used for RNA extraction

lincomycin, tylosin, polymixin and enrofloxacin (Althouse 2008). Antimicrobials must be present at a concentration that provides enough readily-available active product; they must be able to permeate bacteria to a certain degree, and they must also occupy a sufficient number of active sites of the microorganism, and during enough time, to ensure their detrimental effect. If there are interferences in this process, this leads to the apparition of antimicrobial resistance (Althouse and Lu 2005).

Nowadays, some studies show promising results to improve some aspects of sperm quality and to reduce the use of antibiotics, thus minimising the development of antibiotic-resistant strains. Morrell and Wallgren (2011) have reported that it is possible to obtain bacteria-free sperm samples, or sperm samples with reduced bacterial load, without detrimental effects on sperm quality, using the SLC boar-specific colloidal system Androcoll™-P just after collection. Moreover, this technique not only reduces the bacterial load, but also improves the sperm quality of the samples by increasing the proportion of spermatozoa with linear motility and with normal morphology.

Still, more research must be done in the development of antibacterial and antiviral elements, as well as in the packaging systems. Nevertheless, the most important thing is to follow the recommended hygiene rules (see Sect. 10.3.1) during all the collection and processing of AI doses to minimise microbial contamination.

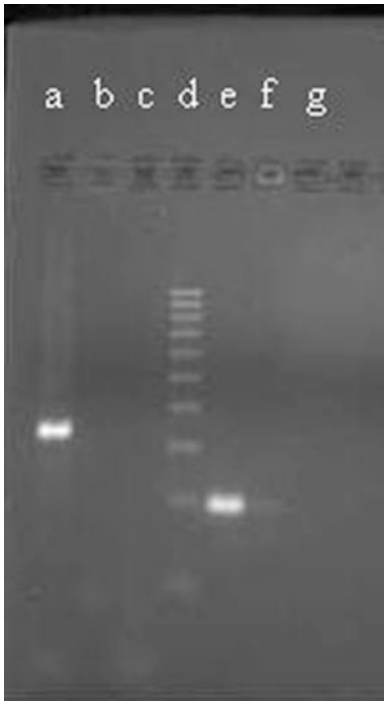

Fig. 10.7 Detection of Pseudorabies (Aujezky) virus using a nested PCR that allows the amplification of g*B* gene. Lane *a*, product of first PCR (334 pb); lane *b*, negative control from the first PCR. Lane *c*, ladder of 100 pb. Lane *d*, product of the second PCR (195 pb); lane *e*, negative control of second PCR; lane *f*, negative control of first PCR after second PCR; lane *g*, negative control of reagents used for RNA extraction

10.4 New Trends: Sex-Ratio Deviation of Doses

10.4.1 Why Utilise Sexed Sperm?

Selection of the sex of the offspring is really important for the improvement of AI management in pigs (see Sect. 12.2.3). In the swine industry, the main purpose of sex sorting is to maintain the elevated sow-to-boar ratio required in commercial breeding. In farms using natural mating, a sow-to-boar ratio of 16:1 is fairly typical. Thus, for a 300-sow unit, 19 boars would be required. In on-farm AI programmes, the sow-to-boar ratio is increased to 100:1 and only 3 boars are required for the same 300-sow unit (Estienne 1999). Another application of sex-sorted semen is the production of male and female crossbred lines for different meat characteristics; depending on the breed and the gender there will be differences in meat quality. Control of the sex ratio also permits faster genetic progress, higher productivity, improves animal welfare (avoids castration) and produces less environmental impact due to the elimination of the unwanted sex before growing to adulthood (Rath and Johnson 2008). The major demand of sex-sorted semen is for the production of females destined to consumption. Sows escape from the effects of androstenone and skatol, the metabolites responsible for the sexual smell in boars that give a particular bad taste to

pork. Moreover, in some countries in Europe the prohibition of castration will be soon implemented, so the production of sex-deviated offspring will be more necessary.

However, application of sex-sorting technology in the livestock industry is dependent on economics, efficiency and easiness of use (Martinez et al. 2005). An alternative to sexed semen is the use of sexed embryos. This technique, which is under development, requires embryos obtained in vitro that can be vitrified and used upon commercial demand.

10.4.2 Current Sex-Sorting Methodology

The most effective and the most used technology for sex-sorting nowadays is flow cytometry, a technique that has been used since the 1980s (Johnson et al. 1987; Garner 2006). Basically, the sperm DNA from a semen sample is quantified with a sorting cytometer that retrieves two populations, one of X- and the other of Y-bearing sperm. Sex-sorting through flow cytometry requires four main steps (Rath and Johnson 2008):

a. Modification of a commercially available flow cytometer sorter into a sperm cell sorter by adding a forward fluorescence detector and a bevelled sample injection needle to accommodate sperm orientation and minimise DNA variability (Johnson and Pinkel 1986).
b. Development of a method to stain sperm cells with intact membranes with a vital fluorescent dye just to ensure the maintenance of sperm viability throughout the sorting process (Johnson et al. 1987).
c. Merging the analytical and sorting capacity of the machine for the production of separated populations of living X and Y sperm based on the differential DNA content (it has been demonstrated that there is a difference of 3.6 % DNA content between X- and Y-chromosomes) (Bathgate 2008).
d. Development of a method for re-analysis of sorted X and Y viable sperm populations to verify their purity in the laboratory (Welch and Johnson 1999). Some studies have used in situ hybridisation to check the purity of sex-sorted sperm (Parrilla et al. 2003).

The efficiency of this technology depends on the number of sexed sperm produced per unit of time, on the fertilising ability of the samples after sorting, on the number of spermatozoa required per insemination and on the percentage of piglets born with the expected gender (Martinez et al. 2005).

Among boars, there is a high variability in the identification and sorting of X- or Y-spermatozoa (Vazquez et al. 2009) There are differences in sperm staining, maybe related to the stability of chromatin (Bathgate 2008), which depends on the number of chromatin disulfide bridges, highly variable between species and within the same species (Rodriguez-Martinez et al. 1985). Other factors are also involved in the variability of sex-sorting results in boars (Vazquez et al. 2009).

Due to the fact that sperm sorting requires handling of the sample, it entails a certain degree of physical damage to the sperm. As will be detailed in Chap. 12, AI requires spermatozoa to be deposited as close as possible to the site of fertilisation and also an accurate control of ovulation time to achieve acceptable fertility (Martinez et al. 2005). The deep intrauterine insemination technique (in which 50–70 million spermatozoa are deposited in the anterior third of the uterine horn), combined with a high accurate hormonal control of ovulation is useful to enhance the success in AI with sex-sorted semen (Rath et al. 2003; Vazquez et al. 2003; Grossfeld et al. 2005). Inseminations in swine require a high number of spermatozoa which still represent a limit if considering the speed of sorting (Johnson et al. 2005; Vazquez et al. 2009). Unfortunately, the speed of sorting and the purity of populations obtained are inversely correlated. Both parameters are defined on a graph by sorting windows, which are regions defined to match the cell populations that will be separated (on the basis of their DNA content, for example). The narrower a sorting window is, the more pure the population obtained, and the fewer cells sorted per unit of time. Increasing the size of the sorting window by 15 % (in terms of sperm DNA content) increases the speed of sorting to 20 %, but in contrast, decreases the purity of X-/Y- sperm populations by 10 % (Johnson 1997). Moreover, inseminations with sex-sorted sperm run the risk of inducing alterations on the expression patterns of mRNA blastocysts, as observed in bovine livestock (Morton et al. 2007), and on the distribution of some heat-shock proteins in boar sperm (Spinaci et al. 2006).

Another technique tested for the separation of X- and Y-chromosomes is the use of density gradients. The separation of X- and Y- bearing sperm with this method was first reported in human sperm in 1977 (Shastry et al. 1977) and is based on the theory that: (a) a small fraction of X-sperm is the fastest of all spermatozoa, (b) the next fastest spermatozoa are the Y-sperm, and (c) the slowest are the majority of X-spermatozoa. Despite this, this technique is not completely suitable for gender separation (Upreti et al. 1998).

Other techniques like Sephadex™ gel filtration have been revealed to be unsuccessful in the separation of X- and Y-bearing sperm (Schilling et al. 1978). Nor do washing procedures enrich the population of Y-bearing spermatozoa, as observed in humans (Flaherty and Matthews 1996). There are also some products available on the market that are said to alter the sex-ratio both in fresh and frozen/thawed semen, like PigPlus™ (Emlab Genetics, Arcola, IL). The formulation of this product is under patent and its principle is to make Y-bearing spermatozoa become dormant. Six or eight hours after the addition of PigPlus™ in a dose, sperm bearing the Y-chromosome recover their motility. Up to the present, there are no available data about the effectiveness of this product and only flow cytometry produces a significant enrichment of X- or Y- bearing spermatozoa.

New research is ongoing to improve the efficiency of the flow cytometry procedure and to experiment with new techniques. For example, it has been suggested that it may eventually be possible to use other genetic markers for mammalian sex selection besides the sex chromosomes (Holt et al. 2007).

10.5 Conclusion

The development of AI has represented a great improvement in the swine industry, since it has allowed the multiplication of litter production with a sole ejaculate. Another important point to take into account in the development of the AI industry is the formulation of extenders, which increase the longevity of doses and assure the survival of spermatozoa. Currently, there are two major types of semen extenders: short and long term, and their utilisation will depend on the preference of the centres where AI doses are prepared and their final destination. Nowadays, the majority of developed countries are running AI programmes (more than 90 % of pig inseminations in some countries). Collection, manipulation and packaging of semen require high hygienic measures to prevent microbial contamination due to intrinsic or extrinsic factors. Unfortunately, this contamination is not always avoidable, so that it is necessary to take some measures to eliminate or minimise it. As a common principle, antibiotics are added to the extenders under different legislations (depending on the country). However, a large list of bacteria is becoming resistant to them, so it is necessary to continue research based on the development of alternative or complementary methods. The presence of bacteria and viruses in doses destined to AI is a double-edged sword, since not only does semen become a vector of transmission, but sperm quality diminishes. Several methods are available to improve a poor quality sample, namely, column filtration, density gradients and swim-up. Each technique has its advantages and disadvantages and depending on the farmer or on the AI centre, one or other methodology is used. The improvement of seminal doses is a suitable practice before AI and also before sperm cryopreservation. Finally, there is an increasing requirement for sex-sorted doses in the swine industry. To date, the most suitable methodology for sex-sorting in boars is flow cytometry but research is ongoing in order to improve its efficiency and for the development of new methods better adapted to AI requisites.

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Chapter 11
Gene Banking: The Freezing Strategy

Isabel Casas and Eva Flores

Abstract Genetic resource banking (GRB) or ex situ conservation in livestock species is the storage of genetic material for breeding purposes. Genes are present, with few exceptions, in every cell within an organism but only germ cells have the ability to transmit this information from one individual to another. Germplasm banking is the main tool for directing and enhancing this genetic flow in intensive farming, as it is for preserving the genetic diversity of a livestock population. The only current technique for the storage of germ cells is freezing them in liquid nitrogen (cryobanking). Traits in the breeding goals of a genetic program are retained in this way from the moment of insemination; otherwise, these genetic resources would be lost. Sperm from boars displaying high genetic values can be preserved following either rapid or slow freezing, as it is explained in this chapter together with a complete introduction to the legal issues of animal cryobanking. A new way of sperm preservation in pigs is also discussed.

11.1 Introduction

11.1.1 Freezing Living Cells

Stopping biological time while maintaining viability is the main objective of freezing strategies, thus permitting long-term storage of cells. Whereas some organisms have evolved to cryobiosis, that is, entering into a state of lethargy in response to decreased temperatures (Lubzens et al. 2010), most isolated cells are not capable of this unless they are manipulated to withstand freezing. These manipulation

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strategies are applied in a wide range of disciplines, from ecology to medicine (Miller et al. 2009). Preservation of germ cells or even embryos is a powerful tool in biomedical research (Mazur et al. 2008) and the ultimate resource of some critically endangered species (Watson and Holt 2001), whereas preserving organs, blood cellular elements or other tissue and cell types assures a continuous stock in hospitals (Fuller and Grout 1991; Chian and Quinn 2010). Moreover, freezing is the strategy of choice in most laboratories that maintain transgenic cell lines with biomedical aims (Baust and Baust 2007).

Regardless of the applications of freezing, all cells share similar behavior at low temperatures. Cells are preferably stored indefinitely in liquid nitrogen at −196 °C instead of being kept in an ultralow freezer at −80 °C. This preference is due to the higher stability of ice below −132 °C, which is the glass transition temperature of water (Rapatz and Luyet 1959). Also, apoptosis may occur when temperature fluctuates around −132 °C or when cells are stored at −80 °C (Lopaczynski et al. 2002). The metabolic activity of enzymes is only possible in an aqueous medium so when water crystallizes no reaction to maintain cellular processes is achievable and cells enter into a state of lethargy. This state is natural in cells that have evolved to resist desiccation, but most cells lack the ability to survive long periods of starvation even if no energy is demanded.

Freezing is a phase change in which a liquid turns into a solid. The cytosol of living cells is a fluid mainly constituted by water (the solvent), and by molecules (the solutes). Freezing the solvent poses a problem for the cell integrity. In a fluid, ice expands like a wave from a focus whose temperature is near freezing point and from which the first ice crystal appears (nucleation) and propagates (Zhmakin 2009). Because the cell membrane diffuses water ice can access the cytosol, shrinking organelles and membranes as if it was a blade. At the same time, the cell meets with another challenge: the expansion of crystallization progressively densifies the solution where the cell is suspended causing the water to flow out of the cell due to osmosis, which are the so-called "solution effects" (Mazur 1963; Dayong and Critser 2000). This is a complex physical event (Han and Bischof 2004) that basically consists of cell dehydration and membrane collapse beyond a critical water loss point.

The amount of water loss depends on factors that affect the water permeability of the cell. Although it is not the scope of this chapter to study in depth the physics of water flow, it is interesting to recall research by Muldrew and McGann (1994) to understand how the osmotic rupture hypothesis of intracellular freezing injury works. Several experimental approaches are described in equations that define the complex dependence relationship among the parameters driving osmosis: pressure P (N m^{-2}) on the membrane due to water flow, temperature T (K), surface area of the cell A (m^2), diffusion coefficient of water within the hydrophobic region of the bilayer D_w (m^2 s^{-1}), water flow J_w (molecules s^{-1}), width of the hydrophobic region of the membrane Δ_x (m), water permeation coefficient of the membrane Lp (μm^3 $\mu m^{-2}{\cdot}min^{-1}{\cdot}atm^{-1}$), isotonic volume V_{iso} (μm^3), osmotically inactive fraction of cell volume V_d (x V_{iso}), etc.

The essential point to protect a cell during freezing is to ensure that the expansion of ice does not damage the cell structure and that osmotic shock is prevented

after the solute concentration increases due to a drop in water content. The cooling rate is critical for the control of both events (Mazur 1984). Moreover, freezing protocols also require the use of cryoprotectants as substitutes for conventional extenders and, in most cases, these are harmful at high concentrations, so a balance between benefits and damage must be established. Needless to say that the success of freezing will not only depend on our protocols but also on the nature of the cell we are dealing with.

An optimal cooling rate must fit the complex osmotic properties of living cells. The Two-Factor Hypothesis of freezing injury, put forward by Mazur et al. (1972), states that with too fast a cooling rate water in the cell has no time to flow out and intracellular ice is formed. On the contrary, with too slow a cooling rate water flows out of the cell so that ice crystal formation is prevented, but the cell is dehydrated and exposed to pH changes and to cryoprotectant toxicity for too long. Thus, the osmotic response of cells is considered to be a determining factor of cell resistance. Meryman (1971) proposes that cells can be dehydrated to a minimal volume (osmotically inactive volume, V_d) beyond which a cell cannot respond to osmotic pressure and the plasma membrane breaks. In boar sperm, the osmotically inactive volume is achieved under 210 mOsm Kg^{-1} (Gilmore et al. 1996; Curry et al. 2000). The optimal cooling curve differs depending on cell types but in most cases it consists of different rates following the physical changes in the extracellular solute concentration, and in the permeability of the cell membrane through decreasing temperatures (Leibo and Mazur 1971; Morris et al. 1999).

Not only the cooling rate but also the addition of cryoprotectants seek to avoid either dehydration or large ice crystal formation through modeling some of the parameters described by Muldrew and McGann (1994). Afterwards, the cell can be safely stored in liquid nitrogen if no irreversible lesion has occurred during freezing. But there is another challenge to overcome before the sample is used: the thawing process (Mazur 1963). The return of cells to physiological conditions when they are being thawed is accompanied by mechanical and osmotic stress, and thus an optimal thawing rate is also mandatory to maintain our cells viable. During thawing, water flows into the cell because there is an abrupt decrease in extracellular concentration due to ice melting. Cells are exposed again to osmotic pressure, their survival depending on the mechanical response of the membrane to volume increase (Rodríguez 2005). The toxicity of the cryoprotectant is not a common problem during thawing, since cells are further diluted in the thawing medium. Rapid warming rates (>1,000 °C min^{-1}) are generally applied to prevent enlargement of ice crystals when temperature rises above 0 °C.

11.1.2 Why Freeze Gametes? Germplasm Banking in Swine

In the livestock industry, the efficient transmission of genetic characters is managed through artificial insemination (AI) or through transfer of viable embryos

after in vitro fertilization (IVF). The latter procedure, although crucial for the swine industry and far beyond for human xenotransplantation, is in the experimental phase in pigs due to the high rates of polyspermy and the poor quality of IVF-derived pig embryos (Koo et al. 2005; Coy et al. 2008; Lloyd et al. 2009; Isom et al. 2011). On the other hand, AI is routinely performed to inseminate a group of sows with semen from one boar, thus spreading the genes from one individual to the maximum number of offspring (See Chap. 12). Swine farms develop exhaustive selection programs to obtain new lines of boars of commercial interest that improve the characteristics of established breeds. However, the genetic erosion makes it difficult to maintain these traits from one generation to another and the solution is to create frozen germplasm banks (also named cryobanking, gene banking or ex-situ preservation) for stabilizing the new characters.

A germplasm bank is a collection of germ cells, which contain the genetic resources of an organism (namely germinal tissues, seeds, pollen, embryos, oocytes or sperm). Although in first experiments scientists used ice for freezing, the introduction of liquid nitrogen in 1938 permitted indefinite storage of viable cells (Jahnel 1938; Fuller et al. 2004).

In the case of pigs, some constraints exist in the implementation of frozen-thawed (FT) sperm. Still today, the sensitivity of some boar ejaculates to cold-shock, the success of long-term extenders, the cost of FT sperm doses, and the timing accuracy required for this kind of AI have restrained the expansion of cryopreservation in the swine market and its use is still limited to special breeding programs. The availability of genetic material in a frozen state, however, provides full-time access to high-value genetic resources for reconstituting populations, introducing genetic variability, improving rates of genetic progress and profitability, developing new breeds, supplying periods of low production and for increasing sanitary control and safety exchange of samples across long distances, among others. These features satisfy the food demand in developed countries and help alleviate food deficiencies in developing ones (FAO 2007).

The Food and Agriculture Organization (FAO) urges the introduction of gene banks or biological resource centers in developing countries to counteract the decrease in farm animal diversity that follows both an excess of outcrossing and inbreeding practices. Cattle, rabbits, horses, and pigs are, in this order, the livestock species that have the highest proportions of breeds at risk (FAO 2009). The cost of frozen samples is relative taking into account that it is calculated as a long-term inversion and so investments are recouped. Compared to the budget for the maintenance of living populations or in situ preservation, the FAO indicates that, in some cases, it is less costly (FAO 2007, 2011; Groeneveld et al. 2008).

Cryopreservation of animal sperm has been carried out since the 1950s when the first cattle cryobanks were set up, but the first swine offspring obtained from frozen semen was achieved in 1970 (Polge et al. 1970). Swine germplasm banks can be found worldwide nowadays, both from private and public funding, and most of these are registered in databases like FABISnet (FABISnet 2011).

11.1.3 Legislation on Animal Germplasm Banking

Gene banking is still a relatively new concern in policy, and legislation does not entirely cover all aspects of animal genetic resources (AnGR) management that stakeholders might encounter. Despite recent efforts, the management of AnGR is still behind the legal achievements of crop banking, which runs under the policies of the Governing Body of the International Treaty on Plant Genetic Resources for Food and Agriculture. Even though there is an incomplete framework, the responsible of the gene bank must be aware of the national and international policies currently regulating this activity.

To put some light on the subject, there exist international organisations that directly refer to AnGR cryobanking (e.g. Rare Breeds International, RBI) (Hiemstra 2011) and almost 40 percent of countries have developed some kind of regulations for AnGR trading (Boettcher and Akin 2010). Most regulations are intended for wild animal populations or for domestic breeds with interest in developing countries, but they have also become a roadmap for swine cryobanking.

A good global framework for the management of AnGR was developed in September 2007 at the first International Technical Conference on AnGR for Food and Agriculture held in Interlaken, Switzerland (FAO 2007). The FAO presented the Global Plan of Action for AnGR that was adopted by the 109 participating countries. The strategic areas have yet to be implemented in different regions worldwide (Hoffman and Scherf 2010) and have been updated in the Guidelines for the cryoconservation of AnGR presented at the FAO conference in Rome (FAO 2011). The latter is a thorough comprehensive manual including recommendations and guidance on all aspects of cryopreservation practices in different animals, including pigs. It also contains a list of suggestions (rather than legislation itself) for better germplasm administration, and a chapter dedicated to financial issues.

The global Convention of Biological Diversity (CBD) also provides tools for germplasm banking management. The convention started in 1993 with the objectives of conserving biological diversity and assuring sustainable use and fair utilization of genetic resources. Two main protocols have arisen from the different meetings of the CBD: the Cartagena protocol (CBD 2000), intended to enhance biosafety, and the Nagoya protocol (CBD 2010), specific for genetic resources. The latter formalized a handbook of legal requirements for access to genetic resources and benefit-sharing (IISD 2007). Although most of the information addresses negotiation practices for exchanging crop genetic resources, it is also a tool for managing AnGR. These Guidelines provide voluntary guidance on ABS practice for companies, researchers, communities, and governments to comply with the CBD Bonn Guidelines (CBD 2002).

Resulting from the Cartagena protocol on Biosafety, a platform, the Biosafety Clearing-House, was established in which information from the CBD is registered and any law from any country can be more easily searched and retrieved (SCBD 2001–2011). The World Association of Zoos and Aquariums (WAZA 2005), the International Union for Conservation of Nature (IUCN), the Convention on

International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the World Wildlife Fund (WWF) are involved with the CBD. Because regulations from the CBD are aimed at the conservation of biodiversity the implementation of ex-situ measures is intended as a support to in situ practices. There are independent charitable organizations that also subscribe to AnGR ex-situ preservation and carry out valuable work in the field. The Frozen Ark project is a global consortium of research and conservation bodies that aims to strengthen the cryopreservation of material from endangered species (www.frozenark.org).

Besides the CBD, another important organism outlining international standard-setting in relation to AnGR conservation is the World Organization for Animal Health (Office International des Epizooties, OIE). Regarding measures relevant for germplasm banking, the OIE delegates agreed to a code for safeguarding the international trade of animals, which was published in two volumes under the name of "Terrestrial Animal Health Code" (Terrestrial Code). The official version of the document is revised annually and published in English, French, and Spanish. The 20th edition (OIE 2011) incorporates modifications agreed at at the 79th OIE General Session in May 2011, and aims to prevent sanitary conflicts during animal trade. Some chapters directly affect the import/export of germplasm banking in swine, and also indicate the appropriate preparation of frozen semen extenders (Table 11.1).

The committee for Companion Animals, Non-Domestic and Endangered Species of the International Embryo Transfer Society (IETS-CANDES) is an advisory board that provides guidance to international governmental regulatory agencies, recognized animal specialty groups and organized conservation programs about different issues related to reproductive biotechnologies, for what it also submits recommendations about the conservation and genetic management of species. A link to import/export regulations for biological materials in different countries can be found on its web (IETS-CANDES 2012).

Table 11.1 Chapters and articles from the 2011 © OIE Terrestrial Animal Health Code relevant for swine germplasm banking

Chapter	Title	Article	Content
4.6	Collection and processing of bovine, small ruminant and porcine semen	4.6.7	Conditions applicable to the handling of semen and preparation of semen samples in the laboratory
5.4	Animal health measures applicable before and at departure	5.4.2	Semen, embryo/ova, and hatching eggs
		5.4.4	Certificate
5.10	Model veterinary certificates for international trade in live animals, hatching eggs, and products of animal origin	5.10.1	Notes for guidance on the veterinary certificates for international trade in live animals, hatching eggs, and products of animal origin
		5.10.3	Model veterinary certificate for international trade in embryos, ova, and semen (Fig. 11.1)

Additional information available in the OIE website: http://www.oie.int/publications-and-documentation/general-information/

Each country should establish its own legislation on behalf of these international organisms, thus ensuring that the principal guidelines on gene banking are implemented and fair trade is applied in all territories. For consultation of country-based policies, the germplasm owner must apply to the competent organism in the country of origin and, in case of transboundary movement, to the one in the country of importation. As examples, the Department of Justice in Canada has established regulations to control germplasm importation (DJC 2011), and the New Zealand government demands an export certification (MAF 2011). In Europe, apart from national laws there is the European Regional Focal Point for AnGR (ERFP). This regional platform supports the in situ and ex situ conservation and sustainable use of AnGR and facilitates the implementation of FAO's Global Plan of Action for AnGR in Europe. It published the Guidelines for the Constitution of National Cryopreservation Programmes for Farm Animals (ERFP 2003). It is a good complement to the FAO's guidelines for developing a cryopreservation program for swine from the European perspective, but it is also a useful tool for other regions and it contains a full list of the national germplasm banks in Europe.

Because of gaps in germplasm regulation national governments are free to agree to policies that are not contemplated by international or regional organisms. On the opposite, germplasm stakeholders must refer to international or regional organisms (or even make their own decisions) when a case is not stated in national policies. The backflow of information that characterizes relations among the parts implicated in AnGR banking is shown in Fig. 11.1. In any case, at least four major issues should be addressed for correct practices (please consult the FAO and ERFP guidelines for extended information).

11.1.3.1 Germplasm Property

All nations consider livestock as private property, which includes any product derived from the animal. Setting up a germplasm bank requires strict control of the personal/institutional access to the material stored. Since private property is defined by contract, the owner stipulated in the contract must be consulted for permission to gain access to the collection. In the case of boar germplasm banking, the sperm stored are, in most cases, property of the breeder. Instead of transferring the entire property rights from the breeder to the responsible germplasm bank, a material transfer agreement (MTA) is desirable. Under this agreement, the responsible germplasm bank is granted the right to manipulate the collection, whereas the breeder maintains ownership. The MTA document should stipulate the ownership rights of the breeder and the responsible germplasm bank (including intellectual property rights or benefit sharing and access to confidential data), the economical terms of storage, the conditions before releasing material to a third party, a health veterinary certificate from the boar whose sperm have been collected, and a commitment to best storage practice. Equally, a material acquisition agreement (MAA) should be formulated as a standard contract to stipulate the conditions of use of the material after its transfer.

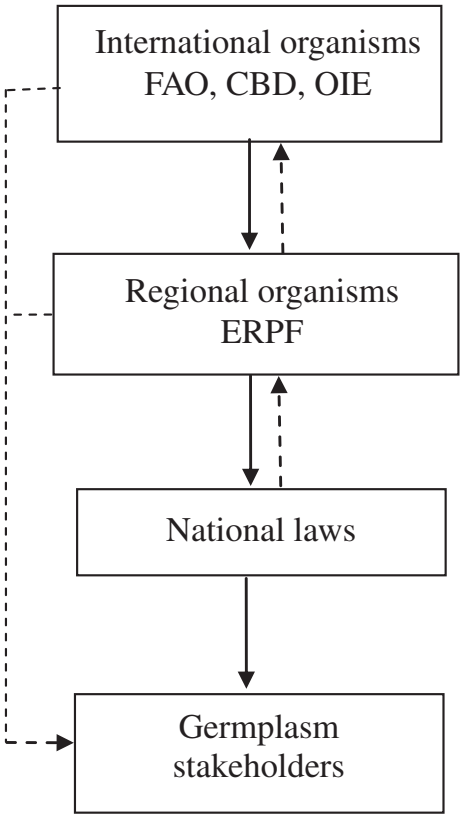

Fig. 11.1 Flow chart resuming information backflow among the parts implicated in animal germplasm banking management

It is important the germplasm bank does not become an isolated entity. The FAO advises inviting different stakeholders to participate in the project with the aim of maximizing management efficiency (the Ministry of Agriculture, breed associations, individual farmers, private companies, the FAO National Coordinator for AnGR). Making contact with the corresponding Ministry of Agriculture to obtain information about legal duties and public funding is recommended as a first step in the creation of a germplasm bank.

11.1.3.2 Biosecurity

The material stored, including the freezing diluents, must be free from pathogena (expedition of a health veterinary certificate is compulsory) and isolated from external hazards. There are OIE guidelines about this concern and according to

FAO recommendations (FAO 2011) the chamber where samples are stored should be closed to unauthorized personnel, maintain its structural integrity in case of major challenges, assure the availability of nitrogen and electricity, and be set up for biological level-2 protocols to maintain health standards. Nonetheless, personal security must be guaranteed when handling liquid nitrogen by monitoring oxygen levels and wearing protective gloves and glasses. Basic security tips can be found in a safety data sheet and in different supplier webpages. Certifications on biosecurity can be expedited by external certification bodies and permit international recognition of good practices of the germplasm bank.

11.1.3.3 Exportation/Importation

The germplasm bank must be protected from non-certified new entries and also must conform to health standards for semen shipment. In this respect, the OIE Terrestrial Animal Health Code and the national regulations of countries participating in the exchange must be consulted. The OIE permits public access to veterinary health certificate templates (Fig. 11.2) and customized models are also found online at a national level, like the Canadian (CFIA 2011). The general safety standards for transport of compressed nitrogen, classified as chemical hazard class 2 (identification number UN1066), must also be observed.

11.1.3.4 Databasing

Registry of data is mandatory in a germplasm bank. Such a need for tracking samples has led to the development of archives, containing all kinds of information from the material stored. As cryobanks have gradually increased, common databases have appeared to centralize the management of different repositories simultaneously. One of the first common databases was developed by the European Association for Animal Production (EAAP). Later, the FAO launched a different database for non-European countries called Domestic Animal Diversity Information System (DAD-IS). Both entities were incompatible until the development of the European Farm Animal Biodiversity Information System (EFABIS) project from the European Union. The two databases are currently linked under the name of FABISnet, creating a global system that permits the flow of information between both entities and others that request to join (Groeneveld et al. 2007).

The objective of FABISnet is to coordinate and synchronize the management of AnGR around the world through an integrated network involving regional and national germplasm programs. As commented above, major regional programs in FABISnet are EFABIS at the European level (http://efabis.tzv.fal.de/), and DAD-IS at the non-European level (http://dad.fao.org/). An example of a national database is the National Animal Germplasm Program (NAGP) in the

COUNTRY :

Part I: Details of dispatched consignment		
	I.1. Consignor: Name : Address:	I.2. Certificate reference number: I.3. Veterinary Authority:
	I.4. Consignee: Name: Address:	
	I.5. Country of origin: ISO code*	I.6. Zone or compartment of origin**:
	I.7. Country of destination: ISO code*	I.8. Zone or compartment of destination**:
	I.9. Place of origin: Name: Address:	
	I.10. Place of shipment:	I.11. Date of departure:
	I.12. Means of transport: Aeroplane ☐ Ship ☐ Railway wagon ☐ Road vehicle ☐ Other ☐ Identification :	I.13. Expected border post: I.14. CITES permit No(s).**:
	I.15. Description of commodity:	I.16. Commodity code (HS code): I.17. Total quantity:
	I.18.	I.19. Total number of packages:
	I.20. Identification of container/seal number:	I.21.
	I.22. Commodities intended for use as: Artificial reproduction ☐ Other ☐	
	I.23.	
	I.24. Identification of commodities: Species (Scientific name) Breed* Donor identity Date of collection Approval number of the centre/team Identification mark Quantity	

Fig. 11.2 Model veterinary certificate for international trade in embryos, ova, and semen (2011 © OIE Terrestrial Animal Health Code, Article 5.10.3). Reproduced with the kind authorization of the World Organisation of Animal Health (http://www.oie.int/eng/en_index.htm)

COUNTRY :

Part II. Zoosanitary Information

II.a. Certificate reference number

II. The undersigned Official Veterinarian certifies that the embryos, ova and semen described above satisfy(ies) the following requirements:

Official Veterinarian:

Name and address (in capital letters):

Official position:

Date:

Signature:

Stamp:

Fig. 11.2 (continued)

USA (http://www.ars-grin.gov/), which at the same time is part of the Germplasm Resources Information Network of the Department of Agriculture.

FABISnet links individual databases maintaining the same restricted access to certain contents. National databases can include the information they consider relevant and keep other information to be accessed only through their national web. Moreover, some data regarding European samples are much more complete

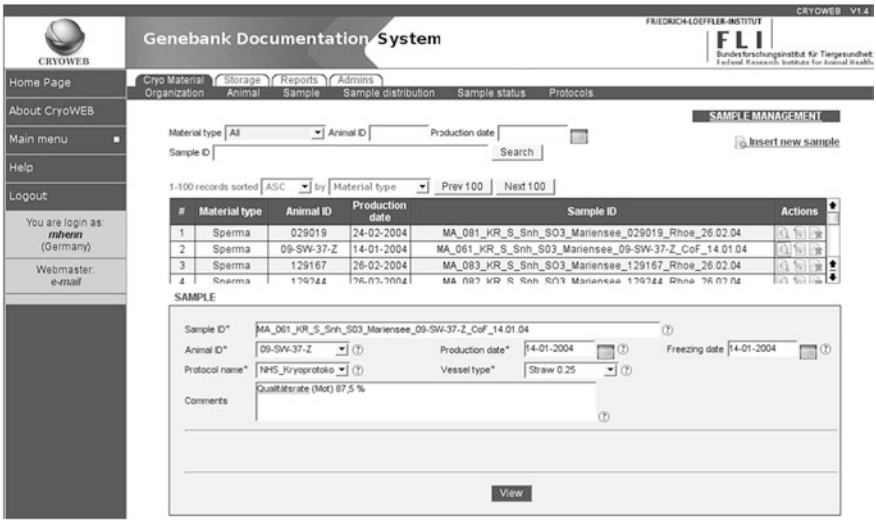

Fig. 11.3 Cryoweb open source software for Genebank documentation. Reproduced with the kind authorization of Dr. Zhivko Duchev, Institute of Farm Animal Genetics, Neustadt, Germany. The software can be downloaded at http://cryoweb.tzv.fal.de/

in EFABIS than in DAD-IS, and the opposite. Any country can create a national web-based information system integrated in FABISnet. The list of countries and AnGR regional and national coordinators enrolled in FABISnet can be consulted both in EFABIS and DAD-IS webs and private breeders should contact their national coordinator to join. FABISnet works with the free licensed CryoWEB software that facilitates introducing and managing cryopreserved sample data (Fig. 11.3). The software can be downloaded at http://cryoweb.tzv.fal.de/download.html.

A fundamental key to databasing is the identification of samples. Semen straw labeling according to ERFP guidelines should include species, breed, and identification of animal, country, collection center, and production date. These data are often requested in health certificates for trading and can be entered in the CryoWEB.

11.2 Preserving the Genetic Heritage in Pigs

11.2.1 Slow Versus Rapid Freezing: Which is the Best Method?

Sperm cells can currently be preserved for up to 15 days in refrigeration (liquid storage; see Chap. 10) or for longer by freezing water inside them. There are two methods for freezing sperm: slow freezing (ice-equilibrium freezing) and

rapid freezing (ice-free freezing). According to the two-factor hypothesis (see Sect. 11.1.1) both slow and rapid freezing induce damage to cells through different mechanisms. Then, how can we manage to freeze sperm successfully?

Since two major conflicts affect frozen sperm cells (dehydration and shrinkage), two major answers have been proposed depending on the characteristics of freezing. Both rely on the use of molecules called cryoprotectants, discovered in the fluids of ectothermic animals living in cold environments (Eastman and DeVries 1986; Storey and Storey 1990) and used for sperm since the 1930s (Milovanov and Selivanova 1932; Bernstein and Petropavlovsky 1937). These are added to the freezing extender and interfere with the expansion of ice by increasing the solute concentration (colligative or permeating cryoprotectants) or by stabilizing the biological membranes (non-permeating cryoprotectants).

If cooling is carried out at slow rates dehydration is minimized by using a mixture of colligative and non-permeating cryoprotectants at low concentrations. In contrast, if cooling is performed at fast rates nucleation is completely avoided by using high concentrations of colligative cryoprotectants. But since the viability after thawing depends not only on the freezing protocol but also on the sample only 50 % or more sperm in porcine ejaculates can usually survive under optimal freezing conditions (Green and Watson 2001). In the slow freezing of boar sperm the mixture of cryoprotectants together with progressive cooling permits the cell to become entrapped in non-frozen (glassy) areas surrounded by ice veins, the reason this process has been called "ice-equilibrium freezing" or "cryopreservation". In rapid freezing, the cell is entirely surrounded by a matrix of glassy material. In this case, the use of toxic percentages of colligative cryoprotectants is not a problem since the cell has no time to respond. The osmotic conditions are controlled by adding these substances in the freezing extender in a multiple step sequence consisting of increasing concentrations. The poor availability of water in this extender stops the expansion of ice and the medium turns into a glassy or vitrious phase instead of being frozen, which is why this process has been called "ice-free freezing", "non-equilibrium freezing" or simply "vitrification".

Scientists still do not agree on which method is the best for freezing biological samples. The literature is controversial on that point and many different outcomes on cell viability are retrieved even for the same method. The use of one or the other depends on personal preferences, on the background of the researcher, on the equipment available, and, mainly, on the suitability of the cell type (See Sect. 11.2.4). In fact, the selection of a method is not crucial for the survival of the sample. It all depends on the personal ability for fitting the procedure to the biological and physical characteristics of the sample in question and the application, in all cases, of a rapid thawing rate (>1,000 °C min^{-1}) to avoid the effects of ice crystals and hyperosmosis (Mazur 1963).

For both slow and rapid freezing some considerations about the storage of samples should be taken into account. In Fig. 11.4 the basic material required when owning a germplasm bank is shown. A monthly fee may be paid to the research center for storage of the straws in case the owner prefers not to deal with it. Frozen samples are stored in tanks of different capacities, the biggest usually containing

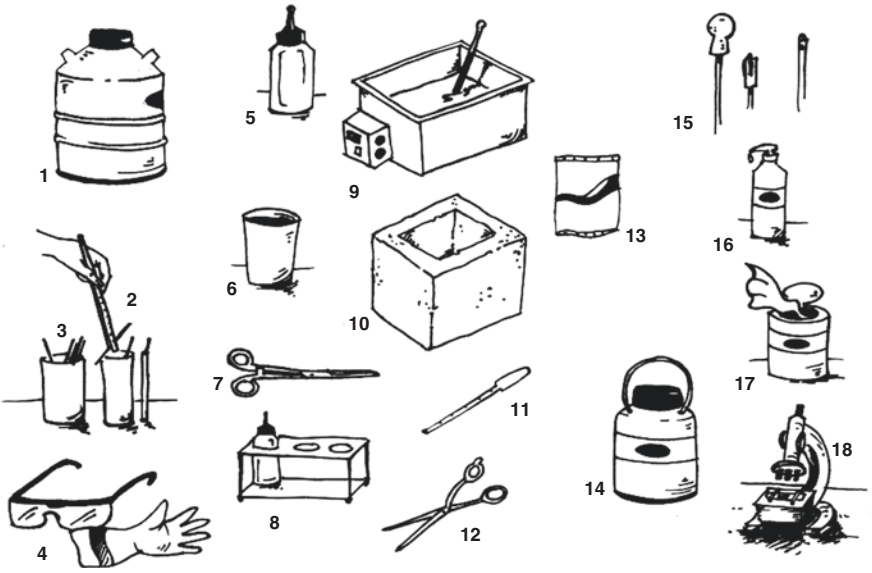

Fig. 11.4 Owning a germplasm bank: basic tools. *1* Dewars for refilling purposes and liquid nitrogen containers with canisters for storage of sperm samples; *2* long tweezers; *3* Goblets for the straws; *4* eye and hand protection for handling liquid nitrogen; *5* dose bottles for dropping thawed sperm; *6* rigid plastic cup for nitrogen transvasing; *7* long Halstead mosquito forceps for holding goblets and cups; *8* rack for dose bottles; *9* Water bath at 37 °C that fits the rack (*8*); *10* foam box for liquid nitrogen; *11* Disposable Pasteur pipettes; *12* Scissors; *13* Thawing extender; *14* transportable Dewar for straws; *15* Catheters for intrauterine insemination with dose bottles (*5*) adapters; *16* non-spermicidal lubricant for catheters; *17* cleaning and drying wipes; *18* microscope for verifying the motility of sperm before insemination

50 L of liquid nitrogen. A periodic maintenance consisting of frequent refillings with liquid nitrogen is important, since the maximal evaporation rate in a closed tank can be of three liters per week. The narrower the neck of the tank, the lower the evaporation rate, so the size of the tanks should fit the number of samples to be stored.

11.2.2 Characteristics of Boar Sperm Relevant to Freezing

The ability of boar sperm to survive freezing has been always questioned. Such bad reputation is mainly owed to the composition of the sperm membrane, with a low cholesterol to phospholipid molar ratio (0.26) (Parks and Lynch 1992; Cerolini et al. 2001), and a high content of unsaturated phospholipids (De Leeuw et al. 1990; Maxwell and Johnson 1997; Maldjian et al. 2004; Chen and Liu 2007). Unsaturated phospholipids give the cell membrane a high fluidity and enhance its permeability whereas cholesterol produces the opposite effect (Alberts et

al. 2008). Cooling under the 5 °C onsets a phase transition in plasma membrane lipids, from liquid-crystalline to a jellified state, at a given time point depending on the nature of the lipid so that some types of unsaturated phospholipids become jellified earlier than others. The result is a mixture of phases that compromise bonds among lipids so that ruptures appear (Drobnis et al. 1993). Such a phenomenon is not completely restored even in the presence of cryoprotectants (Holt and North 1984). Cholesterol is believed to interfere with the condensation into jellified phases and hence helps to maintain the integrity of the membrane (Quinn 1985; Blesbois et al. 2005). If the membrane is broken, sperm functions are compromised and obviously sperm cannot correctly respond to the challenges posed by freezing. In this respect, it has been demonstrated that boar sperm become more sensitive to freezing after their passage through the epididymis, where there is an important loss of cholesterol, (Nikolopoulou et al. 1985; Simpson et al. 1987).

Although the aforementioned features are true for the porcine species, certain genetic and ambiental factors also give particular traits to the sperm that help in overcoming these handicaps, thus producing notable differences among breeds and individuals for sperm resistance to freezing (Medrano and Holt 1998; Holt 2000; Hernández et al. 2006). For instance, slight differences in the shape and volume of the boar sperm head interfere with osmotic regulation during exposure to hypertonic conditions, as it occurs in ice formation (Thurston et al. 2001; Peña et al. 2005; Petrunkina et al. 2005a; Pesch and Bergmann 2006; García-Herreros et al. 2008). The amount of long-chain polyunsaturated fatty acids in the sperm plasma membrane (Waterhouse et al. 2006) and the presence of certain proteins in sperm before freezing, like the HSP90AA1 (Casas et al. 2009, 2010b), also participate in acquiring resistance. Genetics is thus believed to be crucial in the freezing success of boar sperm (Thurston et al. 2002a, b; Medrano et al. 2009) but it is not the only factor. Ejaculates from a same individual sometimes display different tolerance to freezing, which is why the presence of good freezability ejaculates (GFEs) and poor freezability ejaculates (PFEs), depending on their post-thaw viability, is generally accepted (Casas et al. 2009, 2010a, b). This means that ambiental factors must have a role in the matter together with genetics, both producing direct alterations to the whole ejaculate or to sperm populations. The combination of genetic and ambiental factors depicted by GFEs is still under research.

The characteristics of boar sperm that influence dehydration and ice-mediated injuries during freezing also give rise to other dramatic adjustments under the name of “cold-shock”. The sensitivity of the membrane to form jelly phases under 5 °C leads to its destabilization and the loss of its selective permeability. Together this triggers the influx of ions in the cytosol, which switches to a metabolic cascade whose effects resemble the events of true capacitation (Buhr et al. 1994; Bailey et al. 2000; Green and Watson 2001; Petrunkina et al. 2005b): increase in membrane permeability to ions, reorganization of the sperm plasma membrane, cholesterol release, signalling cascade for protein phosphorylation and sperm motility hyperactivation, release of reactive oxygen species

(ROS) (Guthrie et al. 2008; Awda et al. 2009) and, apoptotic-like features (Peña et al. 2009). Collateral damage includes decreased mitochondrial membrane potential (Flores et al. 2009, 2010), membrane lipid peroxidation (White 1993), chromatin unstability (Courtens et al. 1989; Flores et al. 2008a, 2011) and motility and viability impairment (Watson 2000; Cremades et al. 2005; Flores et al. 2008b, 2009). This capacitation-like status, called cryocapacitation, shortens the lifespan of sperm and interferes in their response to the signalling events required for fertilization and in their survival in the oviducts (Buhr et al. 1994; Green and Watson 2001; Petrunkina et al. 2005b). As stated before, frequently and even under the most optimal conditions half of the boar spermatozoa die after freezing, which results in 20 % less farrowings after AI (Johnson et al. 1981).

Although post-thaw sperm quality is thoroughly checked by the research center that processes the ejaculate for freezing, sperm motility should be rescreened on-farm at the moment of thawing, just before insemination. A basic microscope would suit this purpose. The minimal value for acceptance of a boar ejaculate in a freezing program is 80 % for parameters such as total sperm motility, morphology, osmotic tolerance (acrosome intactness) and membrane integrity, whereas for progressive sperm motility it is 60 % (Casas et al. 2009, 2010a, b) (see Chap. 9 for details on quality assessment). These values must be displayed by the ejaculate to account for the decrease in viability that usually occurs after freezing. It is reported that boar ejaculates with the best freezability (GFEs) are those that present a value of sperm membrane integrity and progressive motility that is at least 40 %; otherwise it is considered a PFE. The post-thawing parameters are usually assessed just after thawing and after 240 min to ensure sperm are viable within the insemination-to-ovulation interval (Casas et al. 2010a).

Developing new tests to detect ejaculates with poor freezability before the freezing process will be useful to increase the confidence of farmers. The motility values for linearity and straightness in boar sperm at 5 °C have been reported to be inversely related to levels of cold-shock, and the combination of both can predict around 70 % of sperm cryosurvival. Similarly, the abundance of HSP90AA1 in the sperm cytosol is a good predictor, since it distinguishes between good and poor freezability ejaculates before freezing (Casas et al. 2009, 2010b). However, when investing in the creation of a frozen sperm bank the reproductive performance of a boar and its ejaculate freezability could take second place to other traits that the germplasm owner wishes to preserve.

The improvement of the freezing protocol has been a major advancement in the last decades (Pursel et al. 1973; Hernández et al. 2007) to lessen the intrinsic weakness of frozen sperm, but it is also important to consider the optimization of AI by synchronizing the ovulation of sows to establish proper insemination timing (not earlier than four hours before ovulation) (Waberski et al. 1994) and by using intrauterine insemination techniques (see Sect. 12.2.2) (Gadea 2004; Vázquez et al. 2008). Polge and collaborators were the first to achieve farrowings with FT boar sperm (Polge et al. 1970).

11.2.3 Cryopreservation of Boar Sperm: Slow Freezing

Slow or ice-equilibrium freezing is characterized by the coexistence of two phases or fractions in the sample: an unfrozen fraction and a crystal fraction. When cooling, the temperature of the sperm solution decreases until nucleation occurs at freezing point. At this moment, the temperature stabilizes reaching a temperature plateau, as there is equilibrium between cooling and the formation of ice (latent heat of ice fusion). The more ice crystals, the more concentrated the solution, and the more difficult to attach water molecules to form ice. This causes the freezing point to progressively decrease and this is the reason the plateau is slightly inclined in solutions compared to pure water. When all water in the solution has solidified and the latent heat of ice fusion has dissipated, the equilibrium breaks and the temperature decreases again. At a given point, called the glass transition temperature, the remaining non-frozen solution turns into an amorphous metastable net around the ice crystals (Morris 2007; Taiz and Zeiger 2010). It is in this vitrified structure where the sperm are concentrated (Zavos and Graham 1983; Mazur and Koshimoto 2002; Morris 2007; Casas 2010).

Cryopreservation must ensure that the combination of the freezing extender, packaging properties, and cooling rates permits rapid nucleation, uniform expansion of the ice wave, and progressive dissipation of the latent heat of ice fusion (Zavos and Graham 1983; Berger and Fischerleitner 1992). If not, the process of supercooling may take place, which consists of a lack of nucleation at the freezing point of the sample solution (Debenedetti and Stillinger 2001; Giovambattista et al. 2004). This usually happens when applying high cooling rates in samples not prepared for vitrification, especially those packed in low surface-to-volume ratio containers. Consequently, the solution cools far below its freezing point with increasing possibilities for nucleation. When nucleation occurs, there is a sudden warming of the sample up to the freezing point to abruptly decrease again after the latent heat of ice fusion has dissipated (Morris 2007). Such an event is not desirable as ice formation is not equilibrated with the vitrified parts of the solution. For this reason, induced nucleation can eventually be performed at the start of cryopreservation.

Cryopreservation was the first method implemented for freezing boar sperm and is the most widespread nowadays. Yet in the eighteenth century, Spallanzani demonstrated that fertilization with frozen sperm was possible (Spallanzani 1776), but it was not until the 1970s that two successful models of cryopreservation were developed: the American or Beltsville method (Pursel and Johnson 1975), and the German or Hülsenberger method (Westendorf et al. 1975). Both are based on the use of freezing extenders containing the following substances as cryoprotectants: egg yolk, glycerol, and Orvus-ES® or Equex STM® Paste (commercial concentrated synthetic detergents, in paste form, to emulsify lipids in the egg yolk). The difference relies on the kind of sugar added and on the mode of cryopreservation. The Beltsville method adds glucose and freezes sperm in the shape of round pellets on carbonic ice, whereas the Hülsenberger method uses lactose and packs semen into straws that are frozen over vapors of liquid nitrogen. The latter

Table 11.2 Cryopreservation slope set for boar sperm in a programmable freezer

Step	Initial temperature (°C)	Final temperature (°C)	Cooling rate (°C/min)	Time
A	5	−5	−6	1 min 40 s
B	−5	−80	−39.82	1 min 53 s
C	−80	−80	0	30 s
D	−80	−150	−60	1 min 10 s

provides better sanitary conditions as sperm are enclosed in a package, for what it is possibly the most popular.

Cryopreservation requires removal of the seminal plasma to dilute sperm in a freezing extender, which mainly contains low density lipoproteins (LDL) from the egg yolk as non-permeating cryoprotectants. These egg yolk LDLs have been proved to be the best cryoprotectants, possibly because they compensate the lack of cholesterol in the boar sperm membrane and model the shape of ice crystals so they are less damaging (Andreeva et al. 2008). To allow the interaction of the egg yolk with the boar sperm membrane, it is preferable to follow a slow descent in a water bath from 17 °C, the temperature of commercial doses, to 5 °C. Although higher cooling rates are tolerated in most ejaculates (Juarez et al. 2011), some could not withstand the cold shock. Temperatures below 5 ºC would impair sperm if any colligative cryoprotectant was included in the freezing extender. Although lactose acts as a colligative cryoprotectant in the Hülsenberger method, it does not permit alone the formation of an unfrozen fraction large enough to host sperm. Glycerol is the colligative cryoprotectant of choice, although it is toxic at elevated concentrations and so no more than 6 % (v:v) is added. Together with glycerol, the addition of a synthetic detergent will facilitate the emulsion of the egg yolk lipids at low temperatures.

The addition of glycerol precedes a slope from 5 to −150 °C at different precise cooling rates, for which the use of a programmable freezer (Thurston et al. 2003) is required. Before its introduction, researchers used to suspend sperm on vapors of liquid nitrogen and cooling rates could not be controlled. One of the current freezing programs for boar sperm consists of 5 min and 13 s of cooling (Table 11.2). It first provides a slow cooling rate to permit uniform nucleation and the accommodation of sperm to the osmotic conditions. Thereafter, rapid cooling rates avoid dehydration of the cell, and a few seconds standing at −80 °C allows the correct vitrification of glycerol (Thurston et al. 2003; Zondervan et al. 2007). Once at −150 °C, the samples can be plunged into liquid nitrogen (−196 °C) and stored indefinitely.

Certain modifications from the original cryopreservation methods are adapted by different laboratories. A common one is the retention of boar sperm ejaculates at 17 °C from 3 h (Eriksson et al. 2001) to 24 h (Tamuli and Watson 1994) before cryopreservation, the so-called “holding time”. This period gives higher tolerance to low temperatures by making sperm membranes rather insensitive to cold shock, although the mechanism behind this is still not clear (Pursel et al. 1973; Tamuli and Watson 1994; Johnson et al. 2000). In this respect, it is thought that effects

of cold shock on the plasma membrane architecture are prevented or reversed by certain factors in seminal plasma (Muiño-Blanco et al. 2008; Okazaki et al. 2009). The benefits of seminal plasma on sperm fertility have also been reported when it is added to the thawing solution that recovers sperm after cryopreservation, as first reported by Larsson and Einarsson (Larsson and Einarsson 1975).

Another modification introduced respect the original methods is the use of different kind of packaging. Once sperm are at 5 °C, the sample must be packed inside a container resistant to low temperatures and with a high surface/volume ratio to ensure uniform freezing and rapid thawing. Suitable packages are the 0.25 or 0.5 mL plastic straws, engineered by the Danish Sørensen (Sørensen 1940), and the 5 mL flat plastic polyethylene terephthalate bags (FlatPack) (Ekwall 2009; Eriksson and Rodríguez-Martínez 2000). Adoption of one or other container requires different machinery for filling, sealing, and storing.

Some companies are developing media free from animal-origin compounds because of legal compliances on extenders (see Chap. 10). Among other options, the use of egg yolk phospholipid synthetic liposomes is a good alternative to egg yolk in sperm freezing procedures. Liposomes modify the composition of cellular membranes by saturated lipid and cholesterol transfer, which can moderate the response of cells to low temperatures (Parks et al. 1981; Watson 1981; Holt and North 1988; Wilhelm et al. 1996; He et al. 2001; Zeron et al. 2002; Pillet et al. 2011; Röpke et al. 2011).

Thawing cryopreserved boar sperm is a simpler procedure than freezing, but not less important, so its correct performance is essential recovering quality parameters and avoiding ice crystal enlargement. Sperm samples are directly transferred from liquid nitrogen to a 37 °C water bath where they are left for at least 20 s. Afterwards, the content of the package (i.e. straw or bag) is poured inside a recipient containing thawing extender at the same temperature and further processed for AI or for research purposes. In the former case, the sample is thawed on-farm at the very same moment of insemination.

11.2.4 Vitrification: Rapid Freezing

Vitrification is considered an alternative to standard cryopreservation and has been used so far in mammals for freezing embryos, oocytes, stem cells, and organs (Tucker and Liebermann 2007). Compared to the slow freezing method, vitrification has economic advantatges and is cost effective because there is no need for freezing instruments and because vitrification/warming requires only a few seconds (Palermo et al. 1992; Saki et al. 2009). Unfortunately, the different insemination techniques used in pigs require large sperm doses and effective vitrification demands very low sample volumes, so it is scarcely performed in the species. At the same time, pig oocyte and embryo rapid freezing are still in an experimental phase. As this freezing system has been relatively unexplored in boar sperm compared to cryopreservation we may have to wait some years before it develops its

full potential. In spite of, we have considered worth mentioning vitrification in this Chapter given that some trials have been carried out with sperm in other species.

Pioneer works that set the basis for the development of vitrification as a technique for freezing biological material involved embedding cells in supercooled water; that is, water that exists in liquid state below its freezing point. This happens when there is no element in contact with water that could trigger crystallization. Since it is a delicate equilibrium (metastable state) the probabilities of ice formation increase as temperature decreases (Debenedetti 1996), so researchers sought for methods to reduce its incidence.

Luyet (1937) first mentioned the possibility of using the vitrification technique and described it as a process by which a liquid turns into a solid in the absence of ice crystals. A good physical definition is the solidification of a solution at low temperatures not by ice crystallization but by extreme elevation of its viscosity during cooling (Isachenko et al. 2008). Vitrification is thus the supercooling of a high concentrated liquid so that the intercellular and intracellular fluids metamorphose into a glassy matrix that hinders the spontaneous triggering of nucleation. Phase transition from water into ice is replaced by glass transition from supercooled water into glass at a much lower temperature. Chemical reactions are slowed down in this matrix, stabilizing molecules inside and preserving their activity. One of the conditions for vitrification is the occurrence of agents in the solution that protect against the denaturation of proteins, which take in turn an active part in the formation of the matrix by increasing the viscosity of the solution (Debenedetti 1996). This role is played by cryoprotectants and these are naturally present in certain animals living in cold environments. These molecules prevent chilling injuries by depressing the freezing point of corporal fluids (Knight et al. 1984), which is the same working principle for vitrification protocols.

Luyet and Hodapp (1938) were the first to demonstrate, in frogs, that it was possible to freeze sperm by vitrification. A few years later, Schaffner (1942) vitrified fowl spermatozoa using a modification of Luyet's technique, while Hoagland and Pincus (1942) directly plunged raw human and rabbit sperm in liquid nitrogen. All subsequent attempts to vitrify mammalian spermatozoa did not result in satisfactory survival (Parkes 1945; Polge et al. 1949; Smith 1961). Despite obtaining results difficult to reproduce, these systems have established the bases of most current vitrification technologies. It was not until some decades later that Rall and Fahy (1985) successfully applied the vitrification technique to the preservation of mouse embryos. Since that first report this method has been investigated extensively and applied to female gametes and embryos of different mammalian species (Chen et al. 2001; Cervera and Garcia-Ximénez 2003; Isachenko et al. 2004a; Silva and Berland 2004). However, it has been challenging to standardize it because of the need for high concentrations of permeable cryoprotectants and the osmotic and cytotoxic effects they produce (Gilmore et al. 1997; Holt 1997; Katkov et al. 1998; Mazur et al. 2000).

Briefly, three factors affect the probability of vitrification: cooling rate, viscosity, and volume of the sample. Contrary to what occurs with cryopreservation, the

vitrification of cells demands elevated cooling rates and viscosity, and a high surface-to-volume ratio, which is achieved by different techniques.

The critical cooling speed for the vitrification of pure water varies dramatically depending on the method used (Karlsson and Cravalho 1994). The general technique is rapid non-equilibrium cooling (>10,000 °C/min; Leibo 1989; Leibo and Songsasen 2002; Shaw and Jones 2003; Nawroth et al. 2005), which differs from traditional cryopreservation protocols in that dehydration takes place before cooling begins. Such elevated cooling rates have risks of their own, nonetheless. In particular, glass fractures may form within the sample at temperatures below the glass transition temperature. Moreover, to prevent ice crystal formation vitrified samples must be "warmed" (a term preferred to "thawing" when talking about vitrification) as fast as they have been cooled, so thermal shock may cause fracture formation either during the cooling process or during the warming process (Arav 1999); after storage, warming is achieved by direct melting of the frozen suspension in a water bath, as in cryopreservation. Hence, achieving vitrification depends on a reciprocal relationship between cooling and warming rates, but the cryoprotectant concentration also matters: the lower the concentration the higher (by an exponential factor) the required cooling and warming rates (Fahy et al. 1987). Thus, partial or total intracellular vitrification can eventually be observed even during slow cooling (Vajta et al. 2009) as cells such as spermatozoa display a high content of soluble macromolecules (such as proteins and sugars) that make the intracellular matrix highly viscous (Isachenko et al. 2003, 2007).

Increasing viscosity is the role of cryoprotectants in order to compromise nucleation, as viscosity hinders accession to water molecules. The typical aim of a vitrification protocol is to increase the speed of temperature descent to keep the concentration of cryoprotectants, although high, as adjusted as possible (Nawroth et al. 2005). In this rapid non-equilibrium cooling, apart from high cooling rates, elevated concentrations of cryoprotectants are used (40–60 %; Leibo 1989; Leibo and Songsasen 2002; Shaw and Jones 2003). Cryoprotectant permeation takes place before cooling begins together with dehydration. Given that high concentrations of cryoprotectants have a marked toxic effect (Fahy 1986; Pegg and Diaper 1988; Shaw et al. 2000), it is possible to decrease toxicity by using a combination of two of them (e.g. ethylene glycol and DMSO), and/or to expose cells to precooled concentrated solutions in a stepwise manner (Fahy et al. 1984; Fahy 1986). Another strategy is to reduce the amount of cryoprotectants and simultaneously increase the cooling and warming rates (Liebermann et al. 2002). Some of the deleterious effects of cryoprotectants on mammalian sperm can be avoided by adopting optimal regimes of addition and removal (Sherman 1973; Watson 1979; Critser et al. 1988; Pérez-Sánchez et al. 1994; Gao et al. 1995; Leffler and Walters 1996; Katkov et al. 1998; Katkov 2002). These regimes are, however, ineffective for animal spermatozoa treated with high concentrations of cryoprotectants.

Reduction in the use of some permeable and osmotically active non-permeable cryoprotectants has been suggested as an alternative (Nawroth et al. 2002). More recently, the use of carbohydrate supplements (glucose, sucrose, and trehalose) before directly plunging samples into liquid nitrogen has also been studied

in human spermatozoa (Schulz et al. 2006; Isachenko et al. 2004a, b and 2008). The dogma has been established that vitrification of large cells, tissues, and even organs can only be effective by using high concentrations of permeable and non-permeable cryoprotectants (Fahy 1988). The total concentration of such substances in the sample must be at least 50 % to reach the threshold of stable vitrification. Concurrently, the speed of cooling and warming can be lowered although they are still relatively high. These conditions can be very damaging for cells and lead to subsequent biochemical alterations and lethal osmotic injury (Fahy et al. 1984).

Nonetheless, survival without cryoprotectants is also possible, at least in the case of vitrified sperm, and could obey to the occurrence of large amounts of osmotically inactive water bound to macromolecular structures such as DNA and protamines, or to the presence of high weight components in sperm that affect the viscosity and glass transition temperature of the cytosol (Isachenko et al. 2004a; Rama Raju et al. 2006). This kind of cryopreservation is useful on sperm due to their scant cytoplasm and low tolerance to cryoprotectants, unlike other larger cells such as oocytes and embryos or those of embryonic tissues (Nawroth et al. 2002; Isachenko et al. 2004b). Sánchez et al. (2011) confirm that vitrification without the use of cryoprotectants on dog sperm is able to preserve >95 % of DNA integrity.

The importance of the sample volume is stressed in freezing protocols as it accounts for homogeneous freezing of the entire specimen. For example, small flattened spermatozoa display more optimal surface-to-volume ratio compared to oocytes and embryos (Isachenko et al. 2003, 2007). However, the volume does not only refer to the cell itself but also to the amount of fluid in which it is immersed as it must provide rapid transfer of the external temperature to the cell. Nowadays, vitrification protocols only permit the use of very small specimen volumes to attain efficient glassification.

The sample size is minimized by using different carrier systems, as reviewed by Saragusty and Arav (2011): open-pulled straws (OPS; Vajta et al. 1997), Flexipet® denuding pipettes (FDP; Oberstein et al. 2001; Liebermann et al. 2002), micro drops (Papis et al. 2001), electron microscopy copper grids (Martino et al. 1996; Hong et al. 1999), hemi-straws (Vanderzwalmen et al. 2000), the Cryotop® (Kuwayama and Kato 2000), the CryoLoop™ (Lane et al. 1999; Mukaida et al. 2001), or, nylon meshes (Matsumoto et al. 2001), among others. In daily practice, OPS, carrying up to 10 μL spermatozoa suspension, is a common method of choice (Vajta et al. 1998; Isachenko et al. 2005). Depending on the biological material, however, other techniques should also be considered (Liu et al. 2008). In the case of OPS, current industrial technology does not yet enable the manufacture of a standard diameter of the pulled part of the straw. This shortcoming is reflected in a non-uniform vitrification regime. On the other hand, industrial suppliers offer plastic capillaries of regular cylinder shape with stable (fixed) diameter for medical applications. The vitrification process can be standardized using these capillaries, which have a great potential for assisted reproduction (Isachenko et al. 2011). Other of the aforementioned practices have been patented and are commercialized as straightforward methods with great success.

11.3 New Trends in Boar Sperm Preservation

Cryopreservation is up to now the only practical resource for long-term preservation of boar sperm. Some research groups have started seeking alternatives to conventional freezing protocols mainly because of the awkwardness and cost of handling liquid nitrogen, and of the space requirements of tanks, which would still be a matter in case vitrification was standardized in the species. Lyopreservation is currently a promising option and provides a new exciting field to explore.

Lyopreservation defines the art of preserving biomaterials at ambient temperature and it follows the principles of the well-known technique of lyophilization, otherwise called freeze-drying or cryodessication, with the purpose of becoming a long-term storage solution more affordable than freezing (Holt 1997; Lovell-Badge 1998; Meyers 2006; Kawase and Suzuki 2011). Most eukaryote cells (sperm included) do not withstand dessication (Day and Stacey 2007) and so their manipulation is sought to mimic the anhydrobiotic strategies of those organisms that tolerate extremely dry periods (Potts 2001; Alpert 2005).

Although as yet few publications exist on the topic, there is increasing interest in this technique and its related literature has grown in the last decade. The major concern in the lyopreservation of sperm is to find a process that optimizes the percentage of cell dehydration so that it does not affect its viability. To date, there are three major protocols tested: freeze-drying, convective drying (also named "passive", "air" or "evaporative drying"), and spin-drying. Although they all preserve the genetic integrity of the sample, thus being suitable for intracytoplasmatic sperm injection (ICSI); to date none of them is able to preserve the motility of the cell, which has become one of the biggest challenges to overcome.

Freeze-drying is a pioneering protocol as it comes directly from the original lyophilization procedure (Nail et al. 2002; Oetjen and Haseley 2004). Polge et al. (1949) laid the foundations of sperm lyopreservation by freezing fowl sperm in highly concentrated media, so that the sample vitrified and lost water. Sherman (1954, 1957), Saacke and Almquist (1961) and Meryman and Kafig (1963) did the same with human and bull sperm but the procedure was difficult to reproduce. The first protocol was based on cryopreservation of sperm followed by sublimation of ice into gas using a vacuum freeze-dryer. The current protocol is almost the same and consists of a freezing step followed by two holding times at different temperatures and pressures (primary and secondary drying) to sublimate the ice and to remove unfrozen water molecules (Kawase et al. 2007). However, almost 50 years elapsed before obtaining the first living foetuses from lyophilized sperm and that was done in mice (Wakayama and Yanagimachi 1998). Live offspring from freeze-dried spermatozoa were successfully obtained after ICSI from mice, rabbits, rats, hamsters, and horses (Hirabayashi et al. 2005; Kaneko et al. 2007; Hochi et al. 2008; Li et al. 2009; Choi et al. 2011; Muneto and Horiuchi 2011) but the procedure is still under optimization for other species, including humans and porcine livestock (Larson and Graham 1976; Jeyendran et al. 1981; Hoshi et al. 1994; Keskintepe et al. 2002; Kwon et al. 2004; Liu et al. 2004, 2005; Poleo et al. 2005; Lee and

Niwa 2006; Martins et al. 2007a, b; Nakai et al. 2007; Kusakabe et al. 2008; Loi et al. 2008; Sánchez-Partida et al. 2008; Abdalla et al. 2009; Czarny et al. 2009; Meyers et al. 2009; Watanabe et al. 2009). The problem of freeze-drying is that the porous spaces left by ice induce the collapse of the cell (Yang et al. 2010). Research related to this technique is focused on drying the sample below its collapse temperature and its critical moisture without altering its viability (Fonseca et al. 2004).

Convective drying was performed for the first time by Bhowmick et al. (2003). Sperm is air-desiccated inside a chamber either at room temperature or through injection of highly purified nitrogen gas. The major inconvenience is that a thin glassy skin forms on the surface of the sample, which hinders its homogeneous dehydration and subsequent rehydration (Biggers 2009). Compared to freeze-drying the simplification of dessication permits saving on specialized machinery. The optimal design of convective desiccation protocols requires accounting for the size of the cell, its membrane permeability (Lp), and the starting thickness of the solution (Chen et al. 2006).

Spin-drying is a variant of convective drying and was introduced by Chakraborty et al. (2011). It consists of spinning sperm until the water content is expelled by centrifugal forces, reducing the thickness of the glassy surface layer and permitting fast dehydration. It shares the advantage of the convective protocol plus an improvement in membrane integrity due to osmotic shock reduction, even though sperm motility is not yet recovered. Current studies aim to prevent alterations in the different organelles of sperm, which may be the key to motility maintenance.

Scientists introduce lyoprotectants in the lyopreservation medium in order to simulate the mechanisms of anhydrobiosis. Because most of the lyoprotective substances cannot be found inside the sperm and they are non-permeable, they require different techniques to be internalized, namely, induced poration, transgenesis and lipofection (Garrett et al. 1999; Shirakashi et al. 2002; Stoll and Wolkers 2011).

Lipofection is the fusion of phospholipid bilayer vesicles (liposomes) with the plasmatic membrane for drug and acid nucleic delivery inside cells or for modification of the lipid composition of the membrane (Felgner et al. 1987). This procedure was tested on boar, bull, and ram sperm in 1978 (Evans and Setchell 1978) and was subsequently applied in the eighties and nineties in different experiments involving spermatozoa (Davis and Byrne 1980; Graham et al. 1987; Baranov et al. 1990; Bachiller et al. 1991; Padilla et al. 1991; Wilhelm et al. 1996; Arts et al. 1997; Gamzu et al. 1997; Garrett et al. 1999). The use of egg yolk phospholipid liposomes as an alternative to egg yolk in sperm cryopreservation (see Sect. 11.2.3) is a preview of the potential benefits of lipofection both in freezing and in lyopreservation procedures. Lipofection can effectively reduce chilling sensitivity in boar sperm (He et al. 2001) and may also be used to mimetize the intracellular content of dehydration-resistant species. Indeed, liposomes are themselves resistant to freeze-drying (Shulkin et al. 1984), which suggests sperm could be also resistant to desiccation with the aid of lyoprotectant-loaded liposomes that would preserve both the membrane and the intracellular content.

Lyoprotectants can be non-reduced disaccharide sugars (Kawai et al. 1992; Oliver et al. 2004; Mc Ginnis et al. 2005), chelating agents (Kaneko and Nakagata 2006), antioxidants (Sitaula et al. 2009) or other proteins (Brockbank et al. 2011). Some dehydration responsive proteins have recently been discovered in desiccation-tolerant plants (Choudhary et al. 2009), insects (Cornette and Kikawada 2011) and prokaryotes (Potts et al. 2005), and a number of them encode for mitochondrial-related ones, which may account for protecting this organelle and, in turn, the sperm motility. Deferoxamine, a chelator that entraps ions to repress metabolic reactions, also aids in protecting cells during dehydration stress in certain species (Potts et al. 2005; Buitink and Leprince 2008; Choudhary et al. 2009; Farrant et al. 2009; Sitaula et al. 2009; Tejedor-Cano et al. 2010; Tolleter et al. 2010). On the other hand, the dissacharide trehalose is one of the main players in anhydrobiosis and is naturally present inside cells in desiccation-tolerant organisms (Erkut et al. 2011; Hengherr et al. 2011).

Disaccharides bind to residual water found in the inner and outer membrane layers, where water binds to the phospholipid head groups and is retained while moisture is above a critical point. Under this point disaccharides maintain membrane integrity and minimal moisture to overcome dehydration. Together with other lyoprotectants they also provide a vitrified state in which metabolic processes are limited and the cell is thus preserved in a latent state without energy consumption (Stoll and Wolkers 2011). In 2005, an optimized protocol for freeze-drying red blood cells was established by loading them with trehalose via lipofection (Kheirolomoom et al. 2005). Trehalose has been included in lyopreservation protocols for mice (Mc Ginnis et al. 2005; Li et al. 2009; Elmoazzen et al. 2009), boars (Meng et al. 2010), bovine livestock (Martins et al. 2007a, b; Sitaula et al. 2010) and *rhesus macaque* (Klooster et al. 2011), and it is also beneficial as a non-permeating cryoprotectant for it is thought to avoid protein denaturation (Jain and Roy 2009).

The combination of different strategies found in nature is pinpointed as key to the success of sperm lyopreservation (Crichton et al. 1994; Brockbank et al. 2011). The introduction of different components in the liposomes, not only trehalose but also stabilizers of mitochondrial membranes, could be the solution for preserving both membrane integrity and motility during sperm desiccation and rehydration.

11.4 Conclusion

Gene banking is the most effective strategy of the pig industry for preserving characters of interest from the genetic drift after intensive selection work has been carried out. The development and enhancement of breeds and pig lines is greatly facilitated by cryopreservation and, possibly in a near future, by lyopreservation of sperm cells, embryos and oocytes. At the same time, gene banking allows uniform availability of sperm samples throughout the year, regardless of poor quality semen production periods, and takes advantage of isolation in case of infectious diseases in livestock. However, the low endurance of certain boar ejaculates to

freezing protocols demands more refined handling techniques together with early detection of poor freezability. The definition of molecular markers of freezing resistance is stressed for this latter purpose and there are candidates under research and development. Last but not least, proper management of a gene bank requires the legal definition of property arrangements, biosecurity, sample shipment, and databasing to assist good practices and international trading.

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Chapter 12
Artificial Insemination in Boar Reproduction

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Abstract This chapter is focused on the main aspects related to utilizing artificial insemination (AI) in pig production. The importance of AI in pig farming is discussed, as well as the main techniques used in its application. The first section is devoted to a brief description of the historical development of AI and its application in the pig industry, followed by a brief justification of its current importance. Subsequently, the application of AI with frozen-thawed and sex-sorted semen samples is discussed. A last section regarding current legislation in AI pig farming has also been included.

12.1 Introduction

12.1.1 A Brief History of Artificial Insemination: The Basis of the Current Porcine Industry

The use of artificial insemination (AI) in pig farming is relatively recent, especially when compared to other farmed species like dairy cattle. However, the first reported experiments with AI in swine were carried out by Ivanow in Russia at the beginning of the twentieth century (Ivanow 1907, 1922). More trials were developed by several investigation centres in the USA, Japan and the United Kingdom during the first half of the twentieth century (McKenzie 1931; Milovanow 1938; Anderson 1945; Ito et al. 1948; Polge 1956; Niwa 1958; Maule 1962; Nishikawa 1964). In all these cases, semen was stored under refrigerated conditions at a temperature that varied from 7 to 20 °C. Extenders were mainly composed of glucose solutions that included sodium potassium tartrate or sodium sulphate and peptone, substances aimed at keeping a low concentration of electrolytes (Milovanov 1938; Anderson 1945; Polge

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1956; Maule 1962). The in vivo fertility and prolificacy data obtained in those tests, however, were not good enough to exploit the introduction of AI in standard pig farming. Following this, other research groups tested AI with frozen semen making use of the freezing techniques developed by Polge in dairy cattle (Polge et al. 1949), with minor modifications (Nishikawa 1964; Graham 1978). However, in vivo results were even worse than those obtained in previous attempts using refrigerated doses.

The commercial application of AI in swine production, therefore, did not start until the 1980s. There were two main factors responsible for this change. The first factor was the development of more efficient semen extenders for liquid storage. The grandsire of all extenders is the Beltsville Thawing Solution (Pursel and Johnson 1975), initially designed as a thawing solution, but further developed for refrigerated semen (Johnson et al. 1988). This extender provided veterinarians and farmers with greater time availability for AI practice as it increased the lifespan of semen doses stored at 15–17 °C. Storage was improved with the introduction of long-term extenders such as the Zorlesco medium (Gottardi et al. 1980) and, especially, the MR-A extender (Martín-Rillo 1984), which can be considered the first commercial extender due to its high performance at a reasonably low economic cost. Since then, many other extenders have been produced to allow pork producers to store boar semen doses for 14–15 days without great impairment of sperm fertilizing ability. The amelioration of extenders, however, was not the only factor underlying the expansion of AI techniques in pig farming. The second factor was the standardization of these techniques to be used on-farm. Intracervical insemination (CAI) together with appropriate insemination catheters provided breeders with a fast, effortless method that did not even require special skills (Reed 1985; Crabo 1990; Johnson et al. 2000), and AI became an economically profitable system that replaced natural mating in commercial farms. In recent years, the establishment of the post-cervical AI (PCAI) technique has further reduced the number of sperm per insemination and increased the importance of AI in the pig industry in the context of a globalized world.

12.1.2 The Current Role of AI in Pig Production

The utilization of AI has grown exponentially since the 1980s and intensive pig farming has become completely dependent on its performance. It is practised in around 90 % of exploitations in European countries and in over 90 % of pig farms in the USA and Canada (Lowe and Gereffi 2008; Reportlinker 2011). Such wide deployment of AI is easily understood when the multiple advantages this practice offers to pork producers are considered. In brief, the boar-to-sow ratio has dramatically decreased in parallel with the time invested in each service. A high number of semen doses are now available from a selection of genetic lines, hence multiplying the possibilities of crossbreeding to match consumer demands. Additionally, the production of semen doses for AI allows better sanitary control of services, which in turn facilitates the implementation of the hygienic measures that pig farms must follow by law. Taking into account all these advantages, it is not surprising that the exponential increase in the mean number of sows on a farm runs in parallel with the

progressive adoption of AI as the service method. The Autonomous Community of Catalonia (Spain) is an example of this. In 1975, the total estimated number of pigs in this community was two million (Observatori 2009). At that time, the percentage of farms that utilized AI in the region was practically zero. Ten years later, the total number of pigs had risen to three million. In 2005, when AI was practised in almost all intensive pig farms in Europe, Catalonia was breeding more than six million animals (Observatori 2009). This tendency is also observed in other areas in which AI has been implemented, emphasizing its importance in modern farming.

Adoption of AI as a routine technique could not have been possible without the establishment of farms exclusively devoted to semen collection; farms entrusted with the task of breeding adult boars selected for their genetic values. The emergence of specialized semen farms had two very important features. The first was the release of private farmers from the tedious task of collecting and diluting boar ejaculates to obtain AI doses. This was important since many were not ready to take on this responsibility and AI outcomes were variable and even erratic depending on their expertise. The second feature, which has been previously mentioned, was the exponential increase of the genetic offer. Farmers were able to perform faster and more refined genetic improvements on their farms through the introduction of new genetic lines by purchasing semen from outstanding studs.

12.1.3 Future Prospects of AI: What are the Next Steps?

Making a forecast of what will happen in the future is a task far beyond the scope of this book; however, certain approximations can be made taking into account the evolution of AI in developed countries. The AI procedure experiences a constant turnover as new improvements are launched by the different manufacturers, but its basis has remained the same since its introduction. The high degree of optimization achieved in terms of reproductive indexes and economic costs makes replacement of current AI practice unlikely in the immediate future. However, frozen semen is expected to be adapted to AI in the next few years to bring higher flexibility to international trading of boar sperm samples. As yet, it still has not overcome the efficiency of liquid semen and the revenue this generates. This would indicate a clear case of technological inhibition, in which the development of new approaches is precluded by the existence of a well-consolidated technique. Under these conditions it is risky to make long-term predictions and we can only hope that a sudden, unexpected system improves the results of current AI.

12.2 Artificial Insemination Practices

12.2.1 Artificial Insemination with Refrigerated Semen

As indicated above, AI with refrigerated semen is the most important tool in pig farming at this moment. Since several modifications are emerging in a relatively short period of time, any up-to-date approximation to the topic will be unavoidably

surpassed by the facts. This is an essential caveat when putting the following data into their real context in pig farming. The principle of AI, however, has remained unchanged since the technique was first established. Therefore, even though certain features have changed, a complete understanding of the procedure remains.

12.2.1.1 Current Practice

AI Practices vary depending on factors such as the type of extended semen utilized and the precise form of pig farming. Generally speaking, the CAI technique is the most important at this moment and consists of depositing semen inside the cervix with an appropriate catheter. This AI technique is being gradually replaced in many farms by PCAI, which, as its names indicates, deposits semen at the entrance of the uterus. A succinct explanation of both techniques is needed at this point.

CAI consists of the introduction and deposition of semen doses by using a specifically designed intracervical catheter, also named "spirette". This technique requires several steps for optimal performance. The vulva should be first cleaned with a hygienic wipe to avoid contamination of the catheter. Afterwards, the tip of the spirette has to be lubricated using a nonspermicidal lubricant or a few drops of extender, taking care to prevent the lubricant from entering the spirette. There are many types of spirettes depending on the commercial firm and the size of the sow being inseminated. For instance, there are separate spirettes for gilts and sows, which are different in their size and tip form (see Fig. 12.1). The technician must guide the catheter gently, pointing the tip up through the vagina into the cervix. An adequate position of the tip will minimize the possibility of introducing the catheter into the bladder, which would cause a backflow of urine into the spirette and kill the sperm. This also explains why the semen dose has still not been flushed at this point. Another reason for this is to safeguard doses from environmental conditions that can be adverse to sperm, such as extreme temperatures or excessive light (Chap. 4). Once the technician localizes the entry of the cervix with the tip of the spirette the catheter is inserted by applying a gentle, counter-clockwise rotational force. If some resistance is detected it can be overcome by gently pulling back the

Fig. 12.1 Separate models of spirettes for classical, intracervical insemination in sows. The photograph compares separate commercial spirettes currently utilized for gilts (*A*) and sows (*B*)

spirette. Those with a foam tip cannot always be inserted far into the cervix and are placed at the cervix external os instead or, in other cases, are introduced only up to the first cervical ring. After the tip of the catheter has been securely locked in the cervix there is noticeable resistance to any attempt to rotate the spirette. This is the moment to insert the semen dose in the catheter, after previously mixing it by gentle inversion shaking. An imperative for this technique is that semen must be discharged slowly in order to send a mechanical signal to the sow that she is being inseminated. This signal is among the stimuli required for the induction of ovulation, as will be detailed below. Initially, introduction of semen might need a gentle squeeze; notwithstanding, breeders might allow semen to be taken up by uterine contractions. This step will be carried out slowly, over at least three minutes, since depositing semen too rapidly will cause a backflow out of the vulva. Taking into account that the number of sows that breeders inseminate at any given time is usually high, breeders cannot afford to dedicate three minutes to each sow. Differences in this time could cause serious problems in the final fertility results. To avoid this, semen doses are usually bottled in 90 ml semi-rigid containers (see Fig. 12.2). This allows semen to be introduced into the sow's genital tract only by using the aspirating forces caused by uterine contractions, thus permitting the breeder to handle different sows at a time. Despite all precautions, if the semen were to flow out, the catheter must be repositioned by rotating it a quarter of a turn, or by gently moving it back and forth in order to reinitiate semen flow. Another possible, if rare, accident is semen flow stopping because of vacuum build-up. This circumstance can be solved by cutting a small hole in the semen dose to break the vacuum. Finally, there is sometimes a great deal of resistance to the flow of semen, which is caused by an incorrect positioning of the catheter in relation to the cervical folds. This can easily be solved by repositioning the spirette in the cervix.

It is mandatory to control sow behaviour and the surrounding environment during the entire process. We must remember that environmental stress and/or any other circumstance that affects the oestrous behaviour of the sow can also affect AI results (Chap. 6). In CAI, the breeder must mimic the boar to provide correct stimulus needed to synchronize ovulation. It is achieved by combining different tips, such as presenting a healthy, adult boar to the sow, applying some back pressure and/or massaging the female's flank during AI. In turn, these stimuli increase

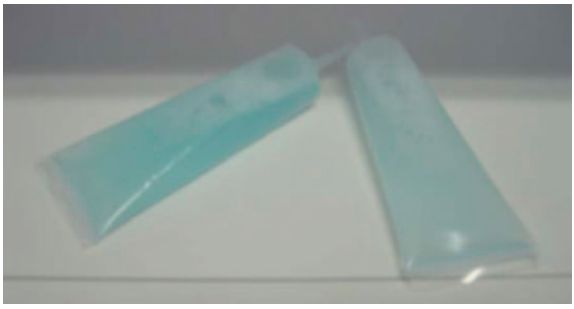

Fig. 12.2 Doses prepared for AI with refrigerated semen in pigs. The semen volume of each container is around 90 mL

the number or intensity of uterine contractions to help semen access the uterus, especially in gilts. If the female responds badly to stimuli, breeders must remove the female from the boar's presence for at least one hour and then try again. It should be taken into account that the sow initiates the standing reflex while being inseminated, which is essential in order to elicit the uterine contractions that are crucial for sperm transport. Finally, it is mandatory to keep the animal in a quiet environment for 20–30 min after AI. This time will avoid any possible disruption of semen transport and subsequent fertilization. After the introduction of semen has been completed, the spirette has to be removed by rotating it clockwise while gently pulling. The catheter can also be put in place for several minutes after the completion of AI in order to prolong cervical stimulation. The common practice is to inseminate a sow once within intervals of 24 h after the detection of a completely established oestrous. This procedure is followed during the entire oestrous cycle, generally resulting in two or three inseminations per sow. However, it must be stressed that an increased number of inseminations per oestrous does not enhance the probabilities of fertilization. On the contrary, a number of inseminations greater than three must be avoided, since it may lead to extremely serious reproductive problems, such as metritis and pyometra, which are two of the most important reasons for culling a sow in a farm. Obviously, a new catheter must be used for each insemination to eliminate the possibility of transmitting a disease or infection from one female to the other (Chap. 10). If all these precautions are taken, the probability of success in terms of pregnancy rates is very high.

The PCAI technique shares most of the steps described for CAI. In PCAI, the catheter consists of two parts (Fig. 12.3). The first part is a spirette very similar to the intracervical one. However, this spirette is utilized only as a guide for the true catheter through which semen is introduced into the sow. The inner catheter is made of a flexible material and has a diameter that facilitates its insertion in the inner space of the spirette. This implies that AI starts with the introduction of the spirette in the sow's cervix following the steps described for the intracervical

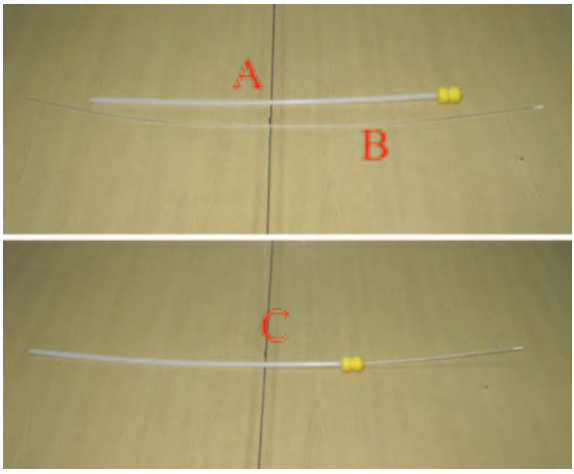

Fig. 12.3 Description of the system utilized for AI with the post-cervical technique. The photograph shows the outer spirette (*A*) and the inner catheter (*B*) that forms a complete commercial post-cervical insemination system, as well as the aspect that the device presents when completed (*C*)

technique. Afterwards, the operator passes the flexible catheter through the hole of the spirette into the distal area of the uterus, at which moment the semen dose is injected. The final procedures are the same as those described.

Several reasons may explain why PCAI is currently displacing CAI. The main reason is possibly the easiness of application with comparable fertility outcomes. Thus, the introduction of both the spirette and the flexible catheter poses no more challenges than introducing the spirette alone. The key point, however, is that semen is deposited in a much more cranial position in the genital tract. This increases the chances of sperm reaching the oviduct and makes it unnecessary to mechanically or visually stimulate the sow. Moreover, with the post-cervical method, semen can be introduced more rapidly than with the intracervical technique. This phenomenon seems to be linked to a decrease in resistance against semen introduction mediated by the lower volume of liquid flushed into the uterus. All these advantages will save breeders a great amount of time, thereby facilitating the service of more sows, and also a substantial saving of time and money. There is another important advantage in PCAI. The amount of sperm that is needed is far smaller than that utilized in CAI. This can be explained by the fact that semen is placed closer to the fertilization site, thus a smaller amount is needed to colonize oviducts in optimal conditions. Semen doses are not of 90 mL, but of 40 mL, and the total number of semen per dose decreases from about 3×10^9 to about 1×10^9 (Roca et al. 2006). Consequently, semen collecting farms will produce many more semen doses per ejaculate, with the economic benefit derived.

It is reasonable to foresee that PCAI will completely replace CAI in the near future if no other satisfactory technique is found. Regardless of the AI technique, it is noteworthy that success is, in any case, dependent on three factors: the precise timing of AI, the location of sperm deposition after AI, and semen quality.

12.2.1.2 Synchronization of Artificial Insemination and Ovulation: Physiological Bases and Optimization Techniques

In vivo fertilization is the result of a well-coordinated sequence of events, which requires precise timing between the arrival of spermatozoa to the oviducts and ovulation itself. This coordination allows oocytes to reach the fertilization site in the oviduct at the very moment at which the spermatozoa present in this region are fully able to fertilize. Females of different species have different, specific mechanisms that control this event. In the case of sows, these mechanisms are mainly three; the first mechanism involves olfactory, auditory, and mechanical stimuli induced by the boar during mating; the second is the mechanical stimulation induced by the penis inside the cervix during copula and by the ejaculate inside the uterus; the third mechanism involves signalling compounds in the ejaculate that accelerate ovulation (see Part 2). These three mechanisms should be taken into account for the development of new AI techniques.

Stimuli induced by the boar during mating are highly important in the standard CAI routine, since they significantly increase the success of this practice.

This effect seems to be related to neural activation of the reproductive response of the sow. Thus, it has been described that the boar's presence increases uterine contractions in sows, especially in those with low uterine contractibility, through an increase in the hypophyseal y oxytocin release (Langendijk et al. 2005). Uterine contractions during mating are a key factor for sperm progression to the oviduct (Harper 1988; Langendijk et al. 2005) as they optimize transport of sperm in synchronization with oocytes. However, the importance of boar stimulation seems to be relative, since PCAI performed in the absence of a boar has similar fertility results to the classical CAI routine (Gil, personal communication). This fact would indicate the existence of mechanisms other than the boar's presence, which increase the rate of uterine contractions in sows during mating.

Little is known regarding the intimate mechanisms of stimulation induced by the penis inside the cervix and the ejaculate inside the uterus, although it is obvious that it has a neuro-endocrine basis. Mechanical dilatation of both the cervix and the first segment of uterus induced by the penis and the ejaculate during mating may stimulate local neuronal terminations, in a similar manner to that described for the utero-hypophysary reflex induced by the foetus during the first steps of the expulsive phase of partum (Ludmir and Sehdev 2000). In fact, the hormonal situation of the sow during oestrous has some similarities with that observed during peri-partum, including high levels of oestrogens and the induction of uterine prostaglandin secretion (Still 2000). This hormonal equilibrium induces several simultaneous effects. The first effect is an increase in the basal contractility of the myometrium. The second effect is the increase in uterine sensitivity to the stimulatory and contractile actions of prostaglandins and estrogens. The third effect is the parallel boost of uterine sensitivity to oxytocin (Still 2000). This latter effect, of course, is not as intense as that induced during partum. This is due to the fact that the intensity of the utero-hypophysary reflex is directly related to the degree of dilation reached by the cervix and the caudal area of the uterus during penetration (Mitchell et al. 1977). This dilation is much greater during partum than during mating and, in this way, the intensity of the reflex is very different between both situations. Mechanical stimulation of cervix and uterus during mating is fundamental. Its importance is demonstrated during PCAI, in which the presence of a boar is not required and mechanical stimulation of the cervico-uterine area significantly increases its effectiveness (Roberts and Bilkei 2005).

The third mechanism that coordinates ovulation and mating is the stimulatory action caused by separate components of the seminal plasma in the cervico-uterine area. In fact, little is known regarding this mechanism although it was initially suggested a long time ago. It has been shown that boar seminal plasma contains substances like estrogens and prostaglandins (Reiffstek et al. 1982; Robertson 2007). These compounds could have two separate effects when kept in contact with the female genital tract. The first effect would be to reinforce the response of the uterus to mechanical stimuli induced by both the penis and the ejaculate. The second effect would be more complex in that it would consist of direct stimulation of ovulation in a similar way to that described for the luteolytic action of prostaglandins at the end of the diestrus, most probably related to seminal

plasma-induced synthesis of prostaglandins in the sow uterus (Madej et al. 2011). In fact, this system would be a signalling mechanism indicating to the ovaries that sperm are on their way to the oviduct, and signalling the onset of ovulation. Under this premise, the addition of both estrogens and prostaglandins to AI doses has been shown to increase the number of healthy foetuses (Willenburg et al. 2003). Following these results, several additives for AI doses containing these substances have been commercially developed, although their on-farm application has not been recommended due to the variability of results obtained.

12.2.1.3 Sperm Deposition: Does the Total Sperm Number Matter?

In all species one of the most important factors that modulate AI effectiveness is the precise deposition of semen inside the female genital tract. Generally speaking, semen must be flushed as near as possible to the oviduct. This can be explained by the fact that sperm that is introduced inside the female has been previously stored in either refrigerating or freezing/thawing conditions that impair its overall functionality to a greater or lesser extent. Thus, sperm packed in doses has worse fertilizing abilities and is less resistant to the environmental challenges than sperm that are used immediately after collection, as occurs in natural mating. This also implies that whereas non-stored sperm has enough resistance to reach the oviducts after crossing the entire female genital tract, refrigerated sperm lack this capacity. Thus, stored sperm must be delivered to a location closer to the oviduct than that in natural mating in order to reach an acceptable fertilizing rate.

The natural deposition site of semen after ejaculation is the caudal area of the uterus (Suarez and Pacey 2006). This place suits the anatomical penis-cervix unit formed during mating and the considerable volume of the ejaculate, which assures the availability of viable sperm (Suarez and Pacey 2006). The CAI mimics natural mating by depositing semen in this same placement, whereas PCAI places semen at a slightly more cranial point and, consequently, closer to the oviducts. This small difference is not important when utilizing freshly obtained semen or with seminal doses of high viability. However, when semen samples are of poor quality or spermatozoa have suffered strong environmental stresses, as in the case of frozen-thawed spermatozoa, it can make a difference in the success of fertilization. We do not know exactly if sperm quality is the only basis for this phenomenon, but it does significantly condition its proper transport to the oviduct. Whatever the reason, theory indicates that the poorer the quality of sperm, the nearer to the oviduct it must be placed. This theory has been the basis for the development of the deep intrauterine AI technique (DUAI). This technique, which has been specially developed for frozen-thawed doses, places semen at a very cranial position in the uterus, close to the uterotubal junction (UTJ). This is possible through the insertion of a semi-rigid catheter longer than the one used in post-cervical practice. The catheter is guided as cranially as permitted, thus depositing semen as near as possible to the oviduct [see Martínez et al. (2001, 2002)] for a precise description of this technique). With DUAI, researchers obtain fertility values with frozen

semen as high as 82.9 %, significantly higher than those reached with CAI techniques (Martínez et al. 2002). In this way, the hypothesis of the close relationship between semen deposition and fertility in sub-optimal conditions is sustained. However, other researchers get similar results with good freezability of frozen semen when doses are applied with a post-cervical device (Casas et al. 2010). This seems to indicate that, in pigs, semen deposition is of relative importance for good quality sperm, obtaining optimal results with a simple, post-cervical deposition.

The relative value of semen deposition could indicate that the most important factor involved in AI success would be semen quality itself. In fact, on-farm management clearly indicates that optimal semen doses yield optimal fertility results, regardless of the specific AI technique that breeders apply. On the contrary, fertility results with sub-optimal doses are highly dependent on the AI technique that breeders apply as well as on the individual ability that they have. The minimal requirements for a boar ejaculate intended for AI are classically defined as following (Martín Rillo et al. 1996):

Volume of the sperm-rich fraction (mL): $\geq$40.
Concentration of the sperm-rich fraction ($\times 10^9$): >300.
Progressive motility (%): >65.
Morphological abnormalities (%): <25.
Reacted acrosomes (%): <20.

The above parameters are very stringent; however, field results seem to indicate that these high quality values are not really needed to obtain good results after AI, at least with refrigerated semen. Quintero-Moreno et al. (2004) showed that samples with a mean percentage of proximal cytoplasmic droplets higher than 46 % had higher in vivo fertility and prolificacy results than other ejaculates with mean values of proximal cytoplasmic droplets of 3 %. This seems to imply, at least, two separate consequences. The first is that semen quality is only one factor influencing in vivo fertility results. In this sense, Quintero-Moreno et al. (2004) also showed that the statistical importance of semen quality in the overall explanation of in vivo fertility was at its best at 30 %. This implies that at least 70 % of in vivo fertility has to be explained by other factors such as the sow's health or the AI technique itself. In consequence, an optimal application of poor quality boar semen doses in a healthy, adult sow can yield optimal results of in vivo fertility. The second consequence is that current boar semen quality analyses do not define all of the functional characteristics of boar sperm. Thus, sperm viability can be affected in functional aspects that are not registered by breeders in a routine analysis. In relation to this point, the combined study of the statistical strength of the separate tests included in a conventional boar semen quality analysis indicates that the only tests that were related, although only weakly, with in vivo fertility results were the percentage of membrane integrity and the different osmotic resistance tests (Quintero-Moreno 2004; Yeste et al. 2010). Strikingly, parameters like the objective determination of motility or the percentage of morphological abnormalities were of little importance in the overall explanation of in vivo fertility (Quintero-Moreno et al. 2004). Thus, we lack an exact and complete definition of sperm quality and this information will not be complete until a more accurate test

array has been developed. Taking into account all these limitations, it is easy to understand the practical point of view of breeders, who prefer AI techniques that promote savings in the volume of semen doses without decreasing fertility results.

12.2.2 Artificial Insemination with Frozen-Thawed Sperm

The implementation of frozen semen in AI has occurred in several domestic species such as dairy cows and horses, but pig farming is a case that stands apart due to several practical problems. One major problem is that fertility results and prolificacy after the use of frozen-thawed boar semen are, generally speaking, clearly below those obtained after AI with refrigerated semen. Hence, whereas mean fertility with refrigerated semen can be set at a percentage equal or above 90 %, fertility after using frozen-thawed semen usually reaches values, in the best of cases, of about 80 % (Martínez et al. 2002; Casas et al. 2010). Taking into account that pig farming is based on obtaining the maximal number of piglets, it is understandable why AI with frozen-thawed semen is not considered a profitable technique in pig farming. Another important problem is the rise in the cost of frozen doses due to processing and storage requirements. Moreover, current insemination techniques are also less expensive than when handling frozen-thawed samples. Taking these points together, it becomes obvious that AI with frozen-thawed semen is more expensive than with refrigerated samples, worsening thus the economic profitability of this technique. However, AI with frozen-thawed semen has several important indications, which will be developed later (Chap. 11).

12.2.2.1 Current Practices

There are currently two main techniques involving AI with frozen-thawed semen in pigs that yield reasonable results, both based on the intrauterine deposition of sperm. The oldest technique is DUAI (Martínez et al. 2001, 2002; Roca et al. 2003; Bathgate et al. 2005; Wongtawan et al. 2006). The most recent is an adaptation of PCAI applied in refrigerated semen doses (Casas et al. 2010).

DUAI consists of a device closely related to that described before for PCAI; however, the inner catheter utilized for DUAI is longer and slightly more rigid. The preparation of the sow, the introduction of the spirette and, subsequently, of the inner catheter, are the same as for PCAI. The main difference, however, resides in that in DUAI, the inner catheter reaches a deeper point in the uterus than in the PCAI technique so that sperm are placed closer to the oviduct. A deeper introduction of the catheter implies, on the other hand, that the breeder must be more cautious in manipulations since the probabilities of injuries to the uterine wall increase and compromise the success of insemination. All these reasons make PCAI simpler and easier to apply.

Another consideration when performing AI with frozen semen is prior dose preparation. This is of utmost importance in order to achieve optimal results but there are still too many instances of negligence during sample handling. In both DUAI and

PCAI, treatment of semen doses is similar, consisting of a rapid thawing of sperm (i.e., 37 °C during 20 s) (Roca et al. 2003; Casas et al. 2010) and immediate dilution in a standard extender. However, another difference, although small, between DUAI and PCAI, is the total number of sperm inseminated. As a general rule, the former technique demands a lower total sperm number, which is established at around 10^8 spermatozoa per insemination (see as examples Martínez et al. 2002; Roca et al. 2003; Bathgate et al. 2005), although there are reports in which breeders use as much as 10^9 spermatozoa (Wongtawan et al. 2006). In the post-cervical system, the total number of spermatozoa utilized in each insemination is around 10^9 (Casas et al. 2010). This difference can be related to the sperm deposition point. Since DUAI deposits semen closer to the oviduct than PCAI, it is logical to assume that it will work with a lower total sperm number. However, the difference in total sperm number is not a major differential factor between both techniques.

12.2.2.2 Sperm Deposition, Artificial Insemination/Ovulation Synchronization and Optimization of Sperm Fertilizing Ability

The specific sperm deposition site is an important factor, albeit not the only one, to be considered for optimization of AI with frozen-thawed boar semen. It is supported by the fact that AI field results with frozen-thawed semen are very similar when comparing DUAI with PCAI (Martínez et al. 2001, 2002; Roca et al. 2003; Bathgate et al. 2005; Wongtawan et al. 2006; Casas et al. 2010). Other factors in the success of AI with frozen-thawed semen have in fact already been described for refrigerated semen (Sect. 12.2.1). Thus, the timing of the insemination-to-ovulation interval is also important in frozen-thawed samples and is usually resolved through repeated inseminations during the oestrous. However, depending on the protocol adopted on-farm, some modifications can be found. Thus, whereas in several cases AI is performed only once per oestrous (Roca et al. 2003), in other cases the sows are inseminated twice, with a maximal elapsed time between both inseminations of eight hours (Wongtawan et al. 2006). It is very important to estimate the ovulation time point since an optimal insemination period of four to eight hours before this moment has been recommended due to the short lifespan of frozen-thawed sperm (Wongtawan et al. 2006). Since this estimation is difficult, if not impossible, several authors have tried to synchronize ovulation and AI aided by hormonal treatments. However, results obtained after the combined use of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) do not show any significant improvement in fertility rates when compared to AI performed in spontaneously ovulating sows (Roca et al. 2003). The most up-to-date protocol for PCAI indicates that AI should be performed twice, with a time lapse between inseminations of four hours, taking into account that the second insemination should be performed around the predicted ovulation time (Casas et al. 2010). The two-step insemination technique seems to be sounder than the one-step system, and good results have been published elsewhere (Watson and Beham 2002; Gil 2006; Casas et al. 2010). However, as indicated above, this technique is based upon a reliable forecast of ovulation time. Unfortunately, although there are

several theoretical rules to calculate ovulation time in a sow (Waberski et al. 1994), there are many random factors that may alter our prediction. In this respect, many more efforts are needed to completely optimize AI field results with frozen-thawed boar semen, regardless of the specific technique adopted by breeders.

12.2.2.3 Does Pig Artificial Insemination with Frozen Sperm have a Future?

When starting this section devoted to the utilization of frozen-thawed boar sperm, we painted a dark picture regarding the practical application of processed sperm in pig farming. What, then, is the actual interest in frozen-thawed samples? In our opinion, the interest does not come from production farms, but from a specific niche in the pig industry–breeding farms. In these farms the final objective is not to obtain the maximal number of animals but to select a population with very specific genetic values that will be disseminated downstream at production farms. In these farms, frozen-thawed semen is of great utility for several reasons. Firstly, sperm freezing allows the storage of the complete genetic information of a boar for a time period longer than its life expectancy. Boar genetics can be stored practically indefinitely in a gene bank in this way, and farmers can have access to it at any time to obtain sperm from the most valuable studs. The second important reason has a sanitary basis. The storage of frozen semen is a guarantee that in case of epidemics that oblige the stud to be sacrificed, the genetic information is safeguarded, thus avoiding the complete loss of this most valuable resource. Last but not least, frozen semen allows breeders to transport doses worldwide easily and securely (Chap. 11).

12.2.3 Artificial Insemination with Sex-Sorted Sperm

As described in Sect. 10.4.2, sex-sorted semen technologies are spreading all over the world. Sexed semen is becoming an important tool in the future strategies of the pig industry and AI techniques are also being adapted to this reality.

12.2.3.1 Current Practices

Sexed semen has several similarities to frozen-thawed spermatozoa, since in both cases sperm is subjected to procedures that strongly affect its function. Sorting induces structural and functional damage that interfere with sperm storage and fertilizing ability. This is particularly true in pigs, while other species such as bovine and ovine livestock, seem to be less susceptible (De Graaf et al. 2009; Rath et al. 2009; Vázquez et al. 2009). Sperm damage is provoked by a variety of factors like fluorochrome staining, pressure of the flow (about 40 psi), exposure to UV laser, electrical

charge, centrifugal forces, slow current sorting rate and high dilution or centrifugation (Maxwell and Johnson 1997, 1999; Johnson 2000; Vázquez et al. 2002; Parrilla et al. 2005; Spinaci et al. 2006; Rath et al. 2009). As a result, sex-sorted sperm is prone to losing its fertilizing ability and AI must therefore be performed with great care. This is specialy true when considering that sorted semen is often subjected to a subsequent freezing-thawing procedure. This means that sorted sperm must often endeavour the troubles originated by sorting with those caused by the freezing/thawing procedure. This double combination of effects difficults the practical utilization of sorted semen even more.

Apart from the sensitivity of sex-sorted sperm, the doses obtained have a low total sperm count, which is why the deep intrauterine technique is preferred for AI. Frozen-thawed semen doses for DUAI contain around 10^8–10^9 sperm per insemination, whereas sorted sperm doses contain around 10^7 sperm, with a sorting yield of 96 % X-bearing spermatozoa (Rath et al. 2009). While results are promising, more field studies are needed to establish the optimal conditions for routine use of sex-sorted semen.

12.2.3.2 Future Prospective of Sexed Boar Sperm in Artificial Insemination

The organization of the current pig industry demands a high sow-to-boar ratio to maintain the number of litters and this is the perfect background for the introduction of sexed semen technologies. However, the sorting technique is tedious and its yields low, which are constraints for its practical application. Finding a solution to both points is necessary to reduce the economic cost of sex-sorting, and it is mandatory if producers aim to obtain sexed semen doses adapted to current AI systems. Moreover, the semen obtained must be more competent and similar in its functional characteristics to refrigerated semen. This would permit farmers to apply simpler and cheaper AI techniques than with DUAI and, in this way, sorted semen could easily be introduced in farms. To date, these points are far from being solved and sex-sorted boar semen is limited to specific applications in experimental farms.

12.2.4 AI with Washed Sperm

Artificial Insemination with washed sperm is not a common technique in pigs. Under conventional farming conditions, the elimination of substances that affect semen quality is conducted by diluting ejaculates in appropriate extenders. In this sense, the utilization of washed semen for routine AI in pigs is not a reality. On the contrary, it is utilized to prepare doses for intrauterine insemination in human or for in vitro fertilization in the majority of species, including pigs (Nagai et al. 1984; Abeydeera and Day 1997; Abeydeera 2002). A review of washing techniques is detailed in Sect. 10.2.3. In any case, AI practices with washed sperm do not greatly differ from that of frozen-thawed or sexed samples.

12.3 Legislation on AI Centres

The legislation that provides the framework for the correct management of an AI centre is a complex matter that displays noticeable heterogeneity depending on the particular legislation of the different countries. The lack of homogeneous legislation hinders worldwide trade and the exchange of semen doses.

Generally speaking, there are two main world legislative corpuses, although, as indicated above, each country has its own rules. The first involves the entire European legislation on AI centres. The second corpus is the one developed in the USA and Canada. European legislation is based on the "Code of Good Practice for Farm Animal Breeding and Reproduction Organisations" (EFABAR Code). The EFABAR Code "addresses the issues of food safety and public health, product quality, genetic diversity, efficiency, environmental impact, animal health, animal welfare, and breeding and reproduction technologies" (EFABAR 2000). For these purposes, this Code follows the principles of sustainable breeding previously developed in the European laws. Regarding boars, the EFABAR Code establishes broad guiding principles for farming and breeding. These guiding principles are organized in three chapters. The first chapter is devoted to six statements that guide the general lines that breeders must follow, involving aspects of biosecurity, health care and welfare. The second chapter is devoted to sustainability, which includes regulation of aspects such as food safety and public health, product quality, genetic diversity, efficiency, environment and sustainable breeding. In this chapter, aspects like animal health and welfare achieve the greatest importance. The third chapter is devoted to the development of technologies. It is focused on the description of current and prospective breeding and reproduction technologies, for which the EFABAR code establishes general lines that breeders must follow in order to perform them in a sustainable manner.

The EFABAR Code is a very important document, since the entire European legislation regarding pig breeding is being developed under its criteria. Hence, European breeders seeking to trade their products among the European Union must comply with all the guidelines described in the EFABAR Code, subsequently implemented in the legislative corpus in each country.

However, the legislative situation is different in countries outside the European Union. Thus, pig breeding laws are developed in the USA following the criteria of the National Agricultural Law Centre (NAGLC 2011). Similarly, Canadian breeders must follow the guidelines of the Canadian Swine Health Board (CSHB 2011). Both USA and Canada follow different guidelines than those presented in the EFABAR Code. American guidelines put less emphasis on sustainability and welfare aspects than those presented in the EFABAR Code, whereas biosecurity and health care are of paramount importance. These differences mean that the specific laws developed from the American guidelines oblige American breeders to make greater efforts in biosecurity than in welfare, in contrast to their European partners. In addition, these differences sometimes lead to conflicts when European breeders want to export their products to American countries and vice versa, since the specific trade laws that both producers must follow are different. This also

happens when breeders seek to export their products to other countries in which legislation often lacks precision. In these cases, export and import of breeding products can be awkward, with breeders being obliged to formalize too many, sometimes contradictory, rules. Thus, for easier trading among pig breeders in the context of a globalized world, reaching a consensus on the same legislation is an imperious necessity.

12.4 Conclusion

AI plays a key role in the development of modern and optimized pig farming strategies. In fact, the intensive pig industry cannot be developed without its utilization. At this moment, current AI practices are changing with the introduction of new semen deposition techniques such as PCAI, which maintains optimal in vivo fertility records by applying lower sperm numbers and in a simpler form than cervical AI. Furthermore, methods such as PCAI and DUAI facilitate new practices involving frozen and sexed semen and, although at this moment these strategies form a great minority in current pig farming practices, further development of PCAI and DUAI could be instrumental in their implementation.

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