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William V. Holt *Editors*

Periconception in Physiology and Medicine

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Preface

Reproductive science is undeniably important for the survival of all species on earth, but attempting to understand reproductive processes in animal species is a dauntingly complex task. The topic ranges from details of gametogenesis, to fertilisation and the subsequent processes of embryonic development, to growth and sexual differentiation, to endocrinology to aspects of behaviour and brain function. As if this list were not broad enough, evolution introduced many unexpected twists and turns as species colonised almost all potential habitats on earth and had to adapt to local conditions. Although such adaptations have been studied for many years, modern scientific advances have enabled us to drill down into the intricate details of gene expression, protein synthesis and the immune system, and it is increasingly clear that many unsuspected details of adaptation are now being uncovered. All animals evolve and adapt to their environment to optimise fitness, and the science of understanding these interactions has led to the realisation that phenomena, such as temperature, photoperiod and seasonality, have massive impacts on reproductive success. Reproductive success itself is not simply a matter of producing the maximum number of offspring, but ensuring that those offspring have the best chance for surviving and continuing to adapt in the future.

It is increasingly realised that parental diet, behaviour, exposure to stress and other influences experienced during the weeks and months leading up to, and including, the time of conception (known as the periconception period) can both help and hinder reproductive success. This is the key message presented in nearly every chapter of this book, and the motive for producing this book was to bring together a collection of authoritative articles that, while focused on different aspects of the periconception period, collectively emphasise the important influence of this period on health and the future well-being of offspring. To demonstrate that the periconception period is not solely a topic of interest in human medicine, we have included articles on a broad range of species in an effort to show that the topic is of universal relevance for reproductive science.

The editors are confident that this book is the first of a series of books that will try to answer some of the unanswered questions in the field and will further highlight the research needed to improve our knowledge regarding the periconception

period and its associated events. We hope this book will be followed by other books dedicated to advances in periconception research and will further shed light on the events and mechanisms taking place during periconception period.

Aside from the editors, other important players in the conception and production of this book were the colleagues and friends gathered during the two successful EU-funded COST Actions, Gemini (FA0702; www.cost-gemini.eu) and Epiconcept (FA1201; www.cost-epiconcept.eu). Discussions held during the course of these two Actions, fostered close partnerships, collaborations and lasting friendships between colleagues of different nationalities and inspired new ideas forming the main structure of the current book. We would like to acknowledge the contributions and support of Ann Van Soom (Chair of Epiconcept) and Laszlo Tecsi (Administrator of Gemini and Epiconcept) who helped us during the production of this book. Your friendship is much appreciated. We are also grateful to Gill Burkinshaw (Departmental secretary) for her support and patience during the preparation of this book. Here we would also like to acknowledge the COST Organisation for supporting these actions as well as the help and support of Ioanna Stavridou (Science Officer of Gemini and Epiconcept) for her support and dedication to our actions.

Finally we would like to thank a great many colleagues that took part in the preparation of the manuscripts that form different chapters of this book and have helped the editors and authors with their critical and constructive comments.

Last but not least, we would like to thank our families and loved ones for sacrificing the quality time that we could have spent with them and allowing us to spend it on production of this book.

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Introduction: A Brief Guide to the Periconception Environment

Alireza Fazeli and William V. Holt

Abstract Definition of the periconception period is not an exact science and is probably somewhat arbitrary. One can define it as spanning the period from the final stages of gamete maturation until formation of the embryo and the stages of embryonic development and implantation. Hence, the periconception period includes periods when spermatozoa are in the female reproductive tract, oocytes are matured and ovulated into the oviduct, fertilization occurs and the embryo undergoes development. By definition the implantation process and the early stages of placenta formation are also regarded as a part of the periconception period. In this article we highlight a few of the major advances which have transformed this topic over the last two decades. It is now clear that the fitness and wellbeing of developing mammalian embryos, including the human, are highly dependent on the health status, diet and habits of both parents especially in the months and weeks that precede the formation of oocytes and spermatozoa.

Keywords Development • Sex determination • Environment • Inheritance • Aquatic organisms • Fishes • Reptiles

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1 Introduction

Our current understanding of the events that take place during the periconception period, such as sperm capacitation and early embryonic developmental processes, were gained during the course of the twentieth century. The majority of research on the periconception environment during the second half of the twentieth century focused on understanding the main constituents of the periconception environment, and how this environment could be replicated in vitro in order to support gamete maturation, in vitro fertilization (IVF) and embryonic development. The birth of Louise Brown (the first baby born by IVF) was a major indication of achieving this goal and signalled the ability of human kind to replicate the periconception environment in vitro to a level sufficient to support the final stages of gamete maturation, conception and early embryonic development to such an extent that successful conception and the production of a conceptus capable of further development to term in vitro was achieved.

Without doubt, the success of IVF was a landmark achievement in reproductive research history and helped with the conception of babies for many infertile couples. However, it created a belief among reproductive physiologists/experts that the periconception environment could be replaced by a relatively simple, buffered balanced salt solution media containing an adequate energy source. As IVF became more and more successful and widespread, inevitably its success resonated the belief that, just as IVF media is a simple and static environment, the periconception milieu in vivo should also be a passive milieu. Once gametes enter this environment, they should doubtless initiate the final stages of maturation that lead to fertilization and early embryonic development. It became generally accepted and constituted a common dogma that the contributions of reproductive physiology towards the preconception milieu must be relatively negligible and limited in providing an energy source, the right pH, temperature and osmotic pressure. This view characterized the oviduct/Fallopian tubes and upper parts of the female reproductive tract as passive conduits, and the processes of fertilization and embryonic development as autonomous events with minimal need of cues or signals conveyed from their surrounding environment. This view supposed that once these processes have been initiated they can progress on their own.

In this chapter, we will present a summary of the work that challenged this view and established our current knowledge and modern physiological understanding of the periconception environment. We will first address the historic perspectives and experiments challenging the view that the periconception environment is a static environment. Thereafter we will explain some seminal experiments describing the major elements involved in forming the periconception milieu. We will then continue to bring examples of how a dynamic periconception milieu can either influence function and physiology of gametes as well as the development of embryos. Finally we will focus our attention on different possibilities to understand how the periconception milieu is regulated.

The majority of current advances in understanding the periconception environment resulted from work performed on defining the composition of embryo culture media for optimum embryonic development. This information is reviewed in the manuscript by Dimitrios Rizos et al. that can be found elsewhere in this volume.

1.1 Discovery of a Dynamic Periconception Milieu

After sexual intercourse, the ejaculated spermatozoa must focus their efforts upon reaching the uterus, oviducts and ultimately the newly ovulated eggs. Their uniquely adapted cellular structure gives them the ability to propel themselves forwards through the viscous milieu of the female reproductive tract, but their journey towards the egg is not as straightforward as driving a car from one place to another, where the car itself is not changed by its environment and in turn it does not change the road surface or the prevailing weather conditions. The spermatozoa have an entirely different prospect before them when they begin their journey. They must survive the rigorous selection processes imposed by the female reproductive tract (Holt and Fazeli 2016) before they reach the vicinity of the egg, and most of them fail their task (Holt 2009). There are many reasons for this: different regions of the female reproductive tract, i.e. the vagina, cervix, uterus and oviducts, create their own local environments. These are known to differ in simple characteristics such as pH or osmolarity, but also in more complex attributes such as ionic composition, presence or absence of hormones and the local identity of proteins in the luminal fluids. Spermatozoa are known to respond sensitively to changes in their environment by changing their motion pattern, sometimes becoming quiescent (Overstreet and Cooper 1975; Suarez et al. 1992), more active (Wennemuth et al. 2003) or changing the shape of their swimming trajectories (Armon and Eisenbach 2011; McNutt et al. 1994; Suarez and Osman 1987). Besides changing their motion characteristics, the spermatozoa also respond to external stimuli by adjusting their ability to undergo membrane fusion. Achieving an appropriate degree of sperm plasma membrane fluidity is crucially important both for the prevention of inappropriate and premature acrosome reactions and allowing the acrosome reaction to occur when a spermatozoon is poised and ready to commence the fertilization process.

Since Overstreet and Cooper's (Overstreet and Cooper 1975) original observations concerning sperm motility suppression by the oviduct, there has been considerable interest in many aspects of sperm-oviduct interactions especially the formation of a sperm reservoir within the oviduct, where a highly selected cohort of spermatozoa is able to reside for a few hours or days prior to the events of fertilization. Many in vitro experiments had shown that if spermatozoa were co-incubated with oviductal epithelial cells, even if they were from a different species (Ashizawa and Nishiyama 1983a, b), their survival was enhanced considerably. It was also observed that the spermatozoa usually became firmly bound to the epithelial cell surface, demonstrating the importance of membrane-membrane contact in the maintenance of sperm viability within the oviductal sperm reservoir (Dobrinski et al. 1997; Smith and Nothnick 1997; Boilard et al. 2001; Suarez and Pacey 2006).

The membrane-membrane contact was shown to be involved in prolonging the duration of sperm survival and initiated protein synthesis (Ellington et al. 1993; Thomas et al. 1995). Collectively these studies have revealed that these interactions are more complex than hitherto suspected and that cell signalling mechanisms play a major part in the sperm-oviduct dialogue.

That spermatozoa are able to elicit de novo gene expression in oviductal cells was first noticed in experiments carried out by Ellington and her colleagues (Ellington et al. 1993) using bovine tissues. The sophisticated proteomic methods available today had not yet been developed and therefore the observations were based on the appearance of new protein spots in electrophoresis gels, but only if the oviductal cells had been co-incubated with spermatozoa. At the time, these results were somewhat controversial and some researchers attributed them to various technical artefacts. However, later research vindicated the authors and confirmed their observations in a variety of ways. Fazeli and colleagues (Fazeli et al. 2004) used microarrays to show that novel gene transcription occurred in the mouse uterus/oviductal complex after natural mating. Using as controls a line of mutant male mice (T145H) that could not produce spermatozoa, but could mate naturally and produce seminal plasma, confirmed that the novel gene expression was a response to the spermatozoa. Although it was not possible at the time to identify all of the novel transcripts, two were identified as adrenomedullin and prostaglandin endoperoxidase synthase 2. It is of interest that adrenomedullin has since been implicated in the modulation of ciliary movement both in epithelia, such as the oviduct and nasal passage, as well as the sperm flagellum itself (Liao et al. 2011, 2012; Li et al. 2010; Chiu et al. 2010).

These studies were followed by experiments showing that the production of 19 novel proteins was identifiable if spermatozoa alone were introduced into isolated porcine oviducts and then incubated at body temperature for 2 h (Georgiou et al. 2005). A highly significant aspect of the data from this study was the identification of heat shock protein 70 (Hsp70) among the soluble components of the luminal fluid. Until relatively recently it was believed that mammalian HSPs are exclusively intracellular molecules and that they are only present in extracellular compartments in pathological conditions such as necrotic cell death. However, there is now extensive evidence to support the view that stress proteins can be released under non-pathological conditions and exert protective roles. The presence of heat shock proteins within oviductal fluid is now well established and they are known to exert influence on sperm-oocyte binding in humans (Marin-Briggiler et al. 2010).

Once these pathfinding experiments had been carried out, the methodology for investigating sperm-oviduct interactions could be characterized (Aldarmahi et al. 2012; Yeste et al. 2014) in order to answer specific questions. It is now evident that if direct contact between spermatozoa and epithelia is prevented, the induction of novel gene expression does not take place (Yeste et al. 2009a), and this correlates with the observation that the improved sperm survival seen in coculture no longer happens (Yeste et al. 2009b).

2 How Does a Dynamic Periconception Milieu Affect Events During Periconception?

The significance of sperm-induced gene expression in the oviduct is still poorly understood with respect to its effects upon the periconception environment and the future fitness of the developing embryo. A recent review of oviductal function (Li and Winuthayanon 2017) emphasized that the oviductal environment has a crucially important role in providing embryos with appropriate metabolic support and protecting the embryo from oxidative and other stresses. As fertilization itself can take place in vitro, it is clear that the presence of these novel proteins is not obligatory for this process; however, they may help to prepare the oviduct for the future arrival and development of the newly formed embryo.

Studies of the proteins found in the oviductal fluids of domestic animals have produced a long list of candidates (see, e.g. Coy and Aviles 2010; Coy et al. 2008a, b; Das et al. 2013; Seytanoglu et al. 2008). Some of them have been functionally characterized and are known to affect processes such as the acrosome reaction, sperm motility and sperm-oocyte binding. Exposure to oviductal fluid of newly ovulated oocytes causes hardening of the zona pellucida and inhibition of polyspermy (Coy and Aviles 2010). One protein worthy of special mention is oviductin, or oviduct-specific glycoprotein (OVGP1) (McCauley et al. 2003; Yang et al. 2015; Zhao et al. 2016). This major component of oviductal fluid is a high-molecular-weight, oviduct-specific and oestrogen-dependent glycoprotein that has been identified in a variety of species, including the mouse, hamster, rabbit, cow, pig, baboon, rhesus monkey, goat and human. It is one of the set of proteins that are upregulated by the arrival of spermatozoa, and logically therefore, it may be hypothesized that it may have a role over and above the modulation of sperm function. This proposal is supported by studies in which embryo cultures have been supplemented with recombinant oviductin (Coy and Yanagimachi 2015) and shown to enhance aspects of embryonic development. For example, in a study of feline embryonic development, the relative mRNA abundance of GJA1, a gene, whose expression level is known to be a marker of embryo quality, was significantly increased in blastocysts after oviductin treatment (Hribal et al. 2014). In contrast to this, expression of OCT4, HSP70, DNMT1, DNMT3A, BAX, IGF1R and GAPDH was not significantly affected.

These studies provide useful clues to the roles and importance of oviductal proteins in relation to embryo quality and development, but in order to understand their exact physiological function, we need to know the details, such as the amount of protein released to the periconception milieu and how much of these proteins come into contact with gametes and embryos.

3 Spatial Formation of Oviduct and Uterine Cavity Affecting the Periconception Milieu and Its Dynamic Nature

In nearly all mammals the periconception milieu concerns mostly the upper parts of the female reproductive tract. In the past some investigators have investigated the nature of secretions produced by the female tract at different stages of the reproductive cycle (Grippo et al. 1992; Killian 2011; Leese et al. 2008; Hunter 2012), and some investigators have examined the way in which the cellular proteins change during the oestrous cycle (Sostaric et al. 2005; Seytanoglu et al. 2008). Few precise measurements have been carried out to determine how protein production and other potential elements produced by the oviduct and uterine cavity change in response to gametes and embryos. Two recent studies are worthy of note, however. Changes in pig oviductal gene transcription in vivo following artificial insemination (López-Úbeda et al. 2015) found that 17 genes were upregulated following insemination and 9 were downregulated. Analysis of the upregulated genes, carried out by examining them in the context of functional networks revealed that the pathways affected by insemination were related to the inflammatory response, the immune system, to molecular transport, protein trafficking and developmental disorder and to cell-to-cell signalling and interactions. The authors concluded that the changes probably represent a degree of preparation for the imminent events of fertilization and early embryo development. At this stage it is difficult to go further than this and focus on more detail. However, a complementary in vivo study (Almiñana et al. 2014) showed if pig oviducts were surgically inseminated with sex-sorted spermatozoa, where the separate populations of X- and Y-bearing spermatozoa were inseminated into different oviducts in the same animals, the gene upregulation responses were different. The significance of the latter study is difficult to interpret, although it shows clearly that the oviductal epithelium has a sophisticated ability to scrutinize the cell surfaces of the inseminated spermatozoa and react in different ways.

4 Periconception Milieu and DOHAD

The relationships between the intrauterine environment and the embryo during the early life of mammals and the onset of adult diseases, such as cardiovascular disease, hypertension and diabetes were initially identified from epidemiological observations on human populations, and have since been confirmed in experimental mammals (Barker et al. 2010; Thornburg et al. 2010). These observations, often collectively known as the Barker hypothesis, repeatedly show that if embryos undergo different forms of ‘stress’ during early development, children are likely to be underweight at birth and will then show phenotypic symptoms of disease as they develop into adults. Many of the articles presented in this book underline both the mechanisms and consequences that underpin the hypothesis that maternal nutritional status has long-lasting impacts on the future health and wellbeing of her

offspring. Most attention has been paid to dietary stress, where the embryo initially seems to adapt its metabolic functions to make the most of the limited resources available. If conditions improve later in life, the individual cannot cope with the better lifestyle and tends to become obese and develop a suite of late-onset diseases.

However, it is also worth asking whether a suboptimal periconception environment might have negative consequences for embryonic development from as early in development as spermatogenesis (i.e. the paternal periconception environment) and the events surrounding fertilization. A recent study conducted within the context of a programme aimed at investigating possible links between diet and human fertility (Faure et al. 2015) compared semen parameters from 92 subfertile men with 91 fertile men and found that the extent of sperm DNA fragmentation was positively associated with birth weight and inversely correlated with total sperm count. As birth weight in the fertile population was significantly lower than in infertile patients, the authors suggested that paternal characteristics may even have affected visceral fat deposits in the newborns. Paternal body weight itself has been implicated as a determinant of deleterious epigenetic effects. Soubry and colleagues (Soubry 2015; Soubry et al. 2014, 2016) found significant differences in DNA methylation at differentially methylated regions (DMRs) of several imprinted genes if the father was obese.

Logically the presence of endocrine disrupting chemicals (EDCs) within the foetal environment, embryo, newborn or juveniles and the female reproductive tract could exert additional stress or have important influences on embryonic growth and development. This topic was recently reviewed in detail (Ho et al. 2017). A range of toxic persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), polychlorinated dibenzop-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dichlorodiphenyldichloroethylene (p,p'-DDE) and polybrominated diphenyl ethers (PBDEs), continue to contaminate the environment due to past and present human activities. Recent evidence from a Swedish human population showed that prenatal exposure to polychlorinated biphenyls (PCBs) was associated with higher birth weight, and PBDE exposure with lowered birth weight (Lignell et al. 2013). Although these effects are complex and difficult to clarify, such human population studies suggest that wild species, whose body burden of such chemicals is often higher, will also be affected. Extensive and detailed data on contaminant concentrations in arctic wild species, including mammals, birds and fishes, reported by (Letcher et al. 2010) lend support to this idea. In fact, these authors commented that evidence of defective neurological development in some polar bears might be attributed to such long-term effects, but they could not be sure because of the difficulties involved in obtaining relevant data.

Although still sparse, evidence from wild species should not be overlooked when considering the likely impacts of pollutants on human reproduction. Chemical analyses of several large mammals (seals, porpoises, whales and polar bears) have demonstrated high body burdens of hydrophobic contaminants, such as PCBs and brominated flame retardants (for review, Sonne 2010; Sonne et al. 2006). Typically, these species acquire pollutants via their diet and bioaccumulate EDCs, which tend

to be lipophilic compounds, within their body fat so that the concentrations increase together with increasing age. When females begin to suckle their offspring, their milk is enriched with EDCs, and the EDCs are transferred to their newborns, with probable effects on survival and reproductive development. Surprisingly, however, it has proven difficult to demonstrate that PCBs and other lipophilic compounds actually cause impaired reproductive development (Letcher et al. 2010), although population and pharmacokinetic modelling studies of East Greenland polar bears, backed up by experimental studies of Greenland sledge dogs, predict the imminent occurrence of negative population impacts (Sonne 2010). Evidence for reduced female reproductive efficacy has also been found in studies of the European harbour porpoise and short-beaked common dolphin (Munson et al. 1998). In harbour porpoises, high persistent organic pollutant burdens tended to be associated with few ovarian scars, suggesting that high contaminant levels may be inhibiting ovulation; however, the significance of ovarian scars has recently been re-evaluated (Pierce et al. 2008).

5 Periconception and Epigenetic Mechanisms

Epigenetic markers are enzyme-mediated chemical modifications of DNA and of its associated chromatin proteins. These modifications play key roles in regulating genomic functions, without changing the primary DNA sequence, and are then passed down through many cell generations. Epigenetic marks influence the way in which chromatin is transcribed and are therefore key determinants of embryogenesis and development. The main epigenetic mechanisms involve DNA methylation, histone modifications and microRNA species, which are involved in genomic activity states.

5.1 DNA Methylation

DNA methylation is the most extensively-studied epigenetic modification and changing DNA methylation patterns in key elements of a gene, such as promoters and enhancers, can have a profound effect on gene function. The best characterized examples of methylation-mediated silencing mechanisms are genomic imprinting, X-chromosome inactivation and silencing of retrotransposons. Imprinted genes specifically have one or more transcripts that are expressed preferentially or exclusively from one parental allele. This monoallelic expression is likely to be initiated by differential methylation of the oocytes and spermatozoa. Shortly after fertilization (the periconception period) this differential methylation leads to additional molecular changes, including allele-specific methylation, which ultimately results in gene expression exclusively from one allele.

Maintenance of DNA methylation is mediated by DNA methyltransferases (DNMTs), and this process is relatively well understood at a biochemical level. In contrast, much remains to be learned about the establishment of DNA methylation (de novo methylation) and the mechanisms underlying its deregulation.

As these mechanisms operate in strictly-defined stages of development, it is possible that the periconception period is split into several particularly sensitive windows of vulnerability. In the context of maternal nutrition and early development, it is notable that DNA methylation is dependent on the one-carbon metabolism pathway. This system relies on a variety of enzymes, whose activities, in turn, depend on micronutrients supplied through the diet. For example, methionine is used by DNA methyltransferases to attach methyl groups to the carbon-5 position of cytosine bases, thus generating 5-methyl cytosine. Different nutrients involved in one-carbon metabolism, such as folate, vitamin B6, vitamin B12, choline and methionine, play an important role in DNA methylation through their influence on S-adenosylmethionine and the methyltransferase inhibitor S-adenosylhomocysteine.

5.2 microRNAs

The human genome contains only 20,000 protein-coding genes, representing <2% of the total genome, whereas a substantial fraction of the human genome can be transcribed, yielding many short- or noncoding-RNAs (ncRNAs) with limited protein-coding capacity. Among these, the most extensively-studied ncRNAs are microRNAs (miRNAs), which are evolutionarily conserved and located within the introns and exons of protein-coding genes or in intergenic regions. MicroRNA species have been detected in spermatozoa where their functions are only now being elucidated (Miller et al. 2010; Miller and Ostermeier 2006); it is, however, interesting to note that differences between fertile and infertile men have been detected in the complement of sperm miRNA (Miller et al. 2005; Ostermeier et al. 2002, 2004). Dietary factors have been shown to modify miRNA expression profiling, notably those associated with maternal nutrition, insulin resistance and inflammation (for review, see Lee 2015).

Small RNAs are believed to direct DNA methylation machinery or chromatin/histone modification complexes for targeting specific genomic loci. The unique sequences of ncRNAs ensure the site specificity of de novo epigenetic silencing (Stephane and Robert 2013). The sperm-borne miRNA profiles are sensitive to various environmental exposures/conditions including paternal obesity in mice, chronic stress in male mice and smoking in humans (Marczylo et al. 2012), and given that miRNA enters the oocyte during fertilization (Rivera and Ross 2013; Amanai et al. 2006), it would be surprising if they did not exert any impacts upon the next generation. Changes in sperm miRNA content are indeed correlated with transgenerational behavioural effects in experimental animals (Guerrero-Bosagna et al. 2012; Anway et al. 2006) and metabolic outcomes (Vrijens et al. 2015). Exposure to the fungicide

vinclozolin has been shown to induce transgenerational changes in specific miRNAs in primordial germ cells in F1 to F3 generations.

Like the miRNA of spermatozoa, it is becoming clear that miRNAs also play important roles in oocytes and developing embryos (Zhao and Srivastava 2007). Optimum regulation of genes or critical gene regulatory events associated with early embryonic development have been shown to be controlled by miRNAs. For example, miR-430 has been shown to promote deadenylation and clearance of maternal mRNAs in zebrafish oocytes (Mishima et al. 2006; Schier and Giraldez 2006).

This brief summary has illustrated how the field of miRNA biology has been transformed over the past 25 years or thereabouts, from a topic that was largely mysterious, or even treated with scepticism, into a massively important subject that has important implications for many aspects of developmental biology and also for the treatment or prevention of disease. There may also be opportunities to modify miRNAs in the context of assisted reproductive technologies, in an effort to correct some aspects of inherited disease (Martin et al. 2017; Bouckenheimer et al. 2016).

6 Conclusions

The impact of the periconception environment on the future health of newborns was not widely appreciated until about 25 years ago, but nowadays it is increasingly recognized. The outcomes can be both positive and negative for the individuals involved. As income and living standards in some populations around the world have increased, it is apparent that they are accompanied by a significantly increased incidence of diabetes; in fact, India has earned the nickname ‘diabetes capital of the world’ (Wells et al., 2016). A likely reason for this outcome is an inability to cope with high glucose intakes in adulthood, which fail to match the way that the population is historically and biologically adapted to survive on a poorer quality diet. On the other hand, the studies on pollutant exposure and smoking discussed above, and possibly also some of the pharmaceuticals used in human medicine, indicate strongly that the reproductive system is a sensitive target likely to be affected by changing environments in ways that might be unexpected and unpredictable. These observations pose ethical questions for governments, especially in terms of agriculture and public health, where pesticide use is widely regarded as an essential way to improve crop yields and prevent the spread of malaria and other insect-borne diseases. It is likely that the cure also causes problems further down the line! Answering these questions is outside the scope of this book, but the chapters we present should at least help to raise relevant questions.

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Epigenetic Influences During the Periconception Period and Assisted Reproduction

Akwasi A. Amoako, Tamer M. Nafee, and Bolarinde Ola

Abstract The periconception period starts 6 months before conception and lasts until the tenth week of gestation. In this chapter, we will focus on epigenetic modifications to DNA and gene expression within this period and during assisted reproduction. There are two critical times during the periconception window when significant epigenetic 'reprogramming' occur: one during gametogenesis and another during the pre-implantation embryonic stage. Furthermore, assisted conception treatments, laboratory protocols and culture media can affect the embryo development and birth weights in laboratory animals. There is, however, an ongoing debate as to whether epigenetic changes in humans, causing embryo maldevelopment, placenta dysfunction and birth defects, result from assisted reproductive technologies or are consequences of pre-existing medical and/or genetic conditions in the parents. The periconception period starts from ovarian folliculogenesis, through resumption of oogenesis, fertilisation, peri-implantation embryo development, embryogenesis until the end of organogenesis. In men, it is the period from spermatogenesis to epididymal sperm storage and fertilisation. Gametes and developing embryos are sensitive to environmental factors during this period, and epigenetic modifications can occur in response to adverse lifestyles and environmental factors. We now know that lifestyle factors such as advanced parentage age, obesity or undernutrition, smoking, excessive alcohol and caffeine intake and recreational drugs used during gamete production and embryogenesis could induce epigenetic alterations, which could impact adversely on pregnancy outcomes and health of the offspring. Furthermore, these can also result in a permanent and irreversible effect in a dose-dependent manner, which can be passed on to the future generations.

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1 Introduction

Embryogenesis is a sequence of events that begins with fertilisation to form a single-cell zygote and ends in an organism with more than 200 different cell types. Gene expressions are controlled through two principal mechanisms; the best understood is mediated through the DNA sequence of our genome. The second, not directly related to DNA sequencing, is epigenetic (Nafee et al. 2008). There are at least two critical periods during the periconception window when significant epigenetic 'reprogramming' occurs: one during gametogenesis and another during the pre-implantation embryonic stage. Besides, it is now evident that assisted conception protocols, manipulations and associated culture media can affect the embryo development and birth weights (Dumoulin et al. 2010a; Nelissen et al. 2012; Vergouw et al. 2012). In this chapter, we will focus on how epigenetic modifications to DNA and gene expression within the periconception period and during assisted reproduction affect embryo development. See Fig. 1.

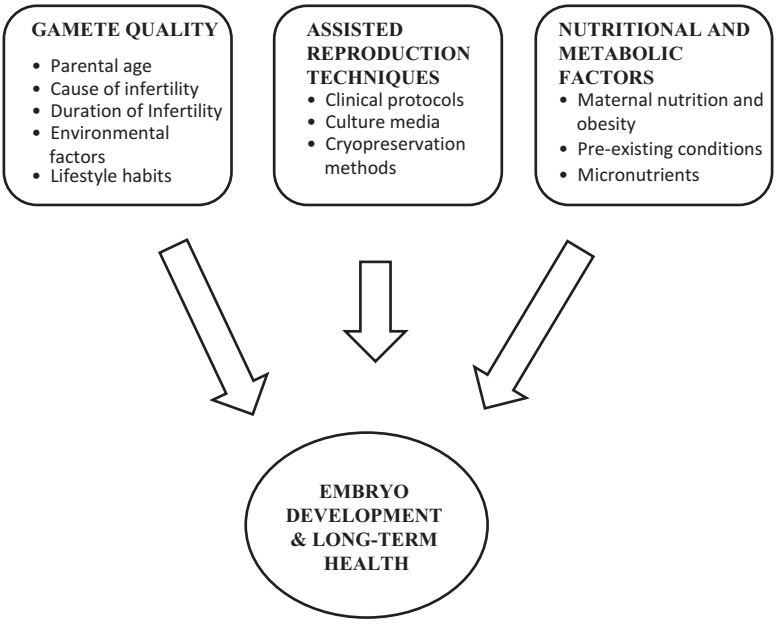

Fig. 1 Periconception epigenetic factors

The periconception period is the 6-month period preceding conception and embryo development to the tenth week of gestation. This period in women is considered to cover ovarian folliculogenesis, resumption of oogenesis, fertilisation, peri-implantation embryo development, embryogenesis and organogenesis. The preconception period in men is the time interval between the start of spermatogenesis to ejaculation and encompasses the processes of spermatogenesis, spermiogenesis, epididymal sperm maturation, epididymal sperm storage and fertilisation. It is estimated that in humans, the onset of spermatogenesis to epididymal sperm maturation typically lasts about 10 weeks (Griffin et al. 2006). Much is known about the adverse impact of periconception lifestyle on gametogenesis, embryo development and reduced reproductive potential (Homan et al. 2007; Hammiche et al. 2011). In women, oogenesis starts during embryonic life and is arrested during first meiotic metaphase until mid-cycle LH surge after puberty. The oocytes are extremely sensitive to environmental factors during this period, and epigenetic modification can occur in response to exposure to adverse lifestyle and environmental factors (Griffin et al. 2006).

There is mounting evidence that periconception lifestyle factors such as advanced parentage age, obesity or undernutrition, smoking, excessive alcohol and caffeine intake and use of recreational drugs during gamete production induce genetic and epigenetic alterations. These changes could impact on pregnancy outcomes and offspring health (Hutt and Albertini 2007; Hutt et al. 2008; Surén et al. 2013). Furthermore, adverse lifestyle factors have been associated with infertility, recurrent implantation failure, miscarriage, intrauterine growth restriction, prematurity and congenital malformations (Steegers-Theunissen et al. 2013). Exposure to adverse lifestyle factors can result in a permanent, irreversible effect in a dose-dependent manner, which can also be passed on to the subsequent generations.

In the last 40 years, enormous advances have been made through assisted reproductive technologies to help infertile couples who would have otherwise remained childless to achieve conception. It is currently estimated that about 4% of all births in the Western world is through in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) (Sutcliffe and Ludwig 2007; Zegers-Hochschild et al. 2009; De Mouzon et al. 2010).

Assisted reproductive technologies involve in vitro handling and manipulation of gametes and peri-implantation embryos, and concerns about the short-term impact on offspring have been raised since its inception in the 1970s. There is an ongoing debate as to whether techniques and processes involved in assisted reproductive technologies (ART) such as controlled ovarian hyperstimulation, in vitro fertilisation, intracytoplasmic sperm injection, embryo culture, embryo cryopreservation, pre-implantation genetic diagnosis (PGD) and pre-implantation genetic screening (PGS) could alter gamete, embryo and foetal developments.

2 The Impact of Periconception Parental Age on Human Reproduction

2.1 Paternal Age and Fertility

The impact of advanced paternal age on reproductive outcomes remains controversial. Epidemiological studies examining the impact of advanced paternal age on fertilisation, pregnancy rates, miscarriages and live birth have produced conflicting results (Dain et al. 2011). Studies addressing the impact of paternal age on reproductive performance often use semen parameters as the outcome of interest which, in themselves cannot differentiate between fertile and infertile men (Dunson et al. 2004). Although the current quantitative and quality analysis of semen based on current diagnostic techniques may not be different in men of different age groups, advanced paternal age is associated with increased risk of genetic and chromosomal disorders and may account for the poor reproductive function in men of advanced age. Ford et al. (2000) demonstrated that time to conception doubled after the age above 25 years. It is, however, not uncommon for men of advanced age to father offspring and therefore the impact of advanced paternal age on conception is less significant than that of maternal age. Age-related decline in semen volume, sperm motility and morphology has been described. Sperm parameters including semen volume, motility and morphology decrease with age, although a decline in sperm concentration was not demonstrated (Kidd et al. 2001).

Advanced paternal age has been shown to have an adverse impact on conception whether natural (Dunson et al. 2004; Ford et al. 2000; De la Rochebrochard and Thonneau 2003) or through assisted reproductive technologies. A decline in monthly fecundity associated with advanced paternal age has been described, although most of these studies do not account for the impact of maternal factors. Women with male partners aged >45 years have a fivefold increase in the time to achieve natural conception (Hassan and Killick 2003), and this effect was mainly attributed to advanced paternal age after controlling for maternal factors (Hassan and Killick 2003; Stewart and Kim 2001).

Studies exploring the effects of advanced paternal age in the context of assisted reproduction have also produced conflicted results and most often do not account for female factors (Klonoff-Cohen and Natarajan 2004; de La et al. 2006). A significant decrease in fertilisation rate (Luna et al. 2009; Aboulghar et al. 2007), the number of embryo reaching the blastocyst stage and the number of supernumerary embryos available for cryopreservation were reduced in partners of older men undergoing IVF treatment (Luna et al. 2009; Frattarelli et al. 2008). However, another study demonstrated that the live birth rate was not shown to be affected by advanced paternal age (Aboulghar et al. 2007). A retrospective study of 441 couples who underwent 558 oocyte donation cycles did not identify any impact of advanced paternal age on fertilisation and live birth rates (Paulson et al. 2001) suggesting that the effect of paternal age on fertilisation rates and pre-implantation embryo development is small (Dain et al. 2011).

Proponents of an association between advanced paternal age and adverse reproductive outcomes such as infertility and miscarriage have attributed these to the increased risk of epigenetic mutations, DNA mutations and chromosomal aneuploidies. It has been suggested that teratogenic effect of environmental pollutants accumulates as men age and could be linked to increased DNA damage in human spermatozoa (Jafarabadi 2007; Kovac et al. 2013). It is also suggested that DNA damage may result as a consequence of oxidative stress due to increased free oxygen radicals in the semen of older men (Cocuzza et al. 2008). Paternal age was shown in one study to be associated with increased risks of spontaneous abortions and Down's syndrome. Other studies have however found little or no association between advanced paternal age and risk of spontaneous abortion after controlling for maternal age (Kazaura and Lie (2002); Kleinhaus et al. 2006; Maconochie et al. 2007).

2.2 Maternal Age

Maternal age remains the most significant factor influencing conception and offspring health in human reproduction. The natural age-related decline in female fertility is a well-established association with natural conceptions and assisted reproductive technologies. After the age of 35 years, female fertility declines, and time to conception increases. The decline in female fertility arises as a result of ovarian ageing associated with increased maternal age (Broekmans et al. 2004). A decline in ovarian function occurs towards the end of women's reproductive life due to exhaustion of primordial follicles (Hansen et al. 2008; Broekmans et al. 2009; Johnson et al. 2006).

The decline in ovarian reserve means that as women age, fewer follicles are available for selection and follicles of poor quality which would otherwise undergo atresia will be selected. Oocyte quality also decreases with age as a result of cumulative damage to the oocyte, spindle fibres and declining quality of its surrounding granulosa cells (Warburton 2005).

Women of advanced age with fertility problems resort to assisted reproductive technologies for help. Although assisted reproductive technologies offer hope to these women by decreasing time to conception, live birth rate decreases with all forms of assisted reproduction as maternal age increases (Dovey et al. 2008; Harris et al. 2010; Gunby et al. 2010; Leridon 2004).

Reports from various studies and national databases show that the current live birth rate for women aged >42 years old undergoing IVF treatment is in the region of 5% (Gunby et al. 2011; Hourvitz et al. 2009).

Advanced maternal age is associated with an increased risk of pregnancy loss in addition to infertility. Effects of age are largely attributed to the increased risk of aneuploidy as a result of ovarian ageing which results in an increased rate of chromosomally abnormal conceptions. In a study of oocytes that failed to fertilise or cleave, chromosomal aneuploidy resulting from meiotic errors increased with age, although the study suffered from a lack of control (Pellestor et al. 2003). It would

have been useful if there was a control group of fertilised oocytes from women undergoing PGD; in order to investigate whether aneuploidies also increased more or less with maternal age in this group. For example, in another study, aneuploidies were significantly more common chromosomal abnormalities found in recurrent miscarriages compared with control group of couples undergoing PGD for sex-linked diseases (Rubio et al. 2003).

3 The Impact of Periconception Parental Obesity, Nutrition and Smoking Status on Conception

3.1 Paternal Obesity and Nutrition

The current global obesity epidemic has had some impact on semen quality and male reproductive potential. There is no agreement on the link between body mass index (BMI) and sperm concentration or total sperm count (Paasch et al. 2010; Aggerholm et al. 2008; Duits et al. 2010; MacDonald et al. 2010). Nevertheless, a trend towards paternal obesity and decreased sperm concentration resulting in infertility and reproductive failure has been observed. An updated systematic review by Sermondade et al. (2012) showed that obese men are more likely to present with oligozoospermia or azoospermia, compared with normal-weight men. The decline in sperm quality in obese men may be attributable to alterations in reproductive hormones such as oestrogen, serum testosterone and sex hormone binding globulin levels (Pasquali 2006; Qin et al. 2007; Pasquali et al. 2007; Hammoud et al. 2008a).

A recent systematic review and meta-analysis exploring the impact of BMI on sperm count found an association between obesity and abnormal semen parameters. Sermondade et al. (2013) compared sperm parameters of normal men with underweight, overweight, obese and morbidly obese men. The odds ratios (95% CI) for oligozoospermia or azoospermia were 1.15 (0.93–1.43), 1.11 (1.01–1.21), 1.28 (1.06–1.55) and 2.04 (1.59–2.62), respectively. The conclusion was that overweight and obesity were associated with an increased prevalence of azoospermia or oligozoospermia (Sermondade et al. 2012). Epidemiological studies have also revealed that women who have overweight, obese male partners or heavy cigarette smokers have elevated risk of infertility (Sallmén et al. 2006; Nguyen et al. 2007; Ramlau-Hansen et al. 2007a, 2007b).

A systematic review and meta-analysis on the effect of obesity on reproductive potential concluded that obese men were more prone to infertility (OR = 1.66, 95% CI 1.53–1.79), reduced live birth rate after ART (OR = 0.65, 95% CI 0.44–0.97) and a 10% increase in miscarriages (Campbell et al. 2015).

Possible explanations for the poor performance of spermatozoa from obese men have included low mitochondrial energy potential, increased DNA fragmentation, abnormal morphology and altered DNA methylation, which reduces their fertility potential (Soubry et al. 2013).

3.2 Maternal Obesity and Nutrition

Obesity in women of reproductive age has become prevalent worldwide, and the negative consequence on reduced fertility is well recognised. Obesity is associated with ovulatory disorders, menstrual disorders, increased time to conception, infertility, miscarriage and congenital abnormalities (Pasquali et al. 2007; Practice Committee of American Society for Reproductive 2008; Brewer and Balen 2010; Grindler and Moley 2013). Oocytes depend on their mitochondria for energy and cellular homoeostasis. Environmental and nutritional factors can adversely affect mitochondrial DNA copy number and function. These explain why obesity associated reproductive disorders can result from dysfunction of oocyte mitochondria leading to poor oocyte quality (Grindler and Moley 2013). Furthermore, obese women undergoing fertility treatment have poorer outcomes, have lower pregnancy rates and more likely to miscarry (Maheshwari et al. 2007). In IVF cycles, obese women require much higher doses of gonadotrophins, produce poor-quality oocytes and have reduced success rates compared to normal-weight controls (Maheshwari et al. 2007; Dokras et al. 2006; Erel and Senturk 2009; Depalo et al. 2011; Luke et al. 2011). Furthermore, gestational diabetes and pre-eclampsia are more common in obese women compared with normal-weight controls (Maheshwari et al. 2007).

3.3 Maternal Micronutrients: Folate and Vitamin D

The role of preconception folic acid supplementation in the prevention of neural tube defects has been established since 1991, through a double-blind, randomised, controlled trial, performed across seven countries (MRC Vitamin Study Research Group – 1991). Since then, many other studies in human populations demonstrated a beneficial effect of periconceptional folic acid in the prevention of preterm labour (Li et al. 2014); pregnancy-induced hypertension (Yang et al. 2016), small for gestational age babies (Hodgetts et al. 2015) and the growth velocity of the human embryonic cerebellum (Koning and Steegers-Theunissen 2015). In a large study of the association between maternal plasma folate during pregnancy and epigenome-wide DNA methylation in 1988 newborns from two European cohorts, 48 genes were significantly altered in babies with maternal serum folate levels (Joubert et al. 2016), implying an epigenetic mechanism for the action of folate. There is increasing attention on non-skeletal benefits mediated by periconceptional Vitamin D supplements. The mode of action is not fully understood, but the evidence is mounting that these effects are mediated via epigenetic controls (Hossein-nezhad and Holick 2012).

3.4 The Impact of Lifestyles on Fertility

3.4.1 Effects of Maternal Periconception Use of Caffeine, Alcohol and Smoking on Fertility

Evidence from some studies has shown that maternal caffeine use in the periconception period negatively impacts on fertility (Klonoff-Cohen et al. 2002; Homan et al. 2007; Anderson et al. 2010a, b; Wesselink et al. 2016) in a dose-dependent manner. The current recommendation is that women attempting to conceive should limit caffeine intake to 1–2 cups of coffee per day (Homan et al. 2007). The exact mechanism by which caffeine impacts on fertility is unclear, but it is purported to affect ovulation and corpus luteum function and to alter female reproductive hormone levels. Studies have suggested a relationship between caffeine intake and increased time to conception (Anderson et al. 2010a, b). Increased caffeine consumption in the periconception period decreases implantation and live birth rates and increases miscarriages in women undergoing assisted reproduction (Klonoff-Cohen et al. 2002).

Maternal alcohol use also has adverse consequences on fertility, and the current evidence recommends that women planning to conceive should stop drinking alcohol (Anderson et al. 2010a, b). There are no dose-effect studies, and there is no recommended safe limit of alcohol intake in the periconception period. The mechanism by which alcohol impairs fertility is still unclear. High maternal alcohol consumption reduces chances of live birth and increases the risk of miscarriage in both spontaneous and assisted conceptions (Klonoff-Cohen et al. 2002).

There is substantial research evidence that maternal smoking adversely affects female fertility. Female smokers take a longer time to conceive (Hull et al. 2000) and are more likely to be infertile compared to non-smokers (Homan et al. 2007). The impact of smoking on female fertility is dose dependent, and heavy smokers are more likely to be affected although low levels of smoking can have a significant adverse effect on female fertility. Female smoking accelerates loss of ovarian function (ESHRE Task Force on Ethics and Law 2010) and decreases endometrial receptivity (Soares et al. 2007). It also increases spontaneous miscarriage in both natural and assisted conception cycles (Winter et al. 2002) and increases the risk of ectopic pregnancies (Anderson et al. 2010a, b). In assisted reproduction treatment, maternal smoking has been shown to affect success rates significantly by causing a 50% reduction in implantation rate (Cooper and Moley 2008) compared to non-smokers (Waylen et al. 2009; Meeker and Benedict 2013). Maternal smoking is also associated with increased risk of intrauterine growth restriction, low birth weight, preterm birth (McCowan and Horgan 2009; Juárez and Merlo 2013) and birth defects (Hackshaw et al. 2011).

3.4.2 Effects of Paternal Periconception Use of Caffeine, Alcohol and Smoking on Fertility

There is good evidence that paternal preconception smoking adversely affects male fertility (Homan et al. 2007). The impact of smoking on male fertility is dose dependent, although light smoking is still associated with impaired male fertility (Ramlau-Hansen et al. 2007a, b). Men who smoke have lower sperm concentration (Aryanpur et al. 2011), have reduced sperm motility and viability (Gaur et al. 2010) and have a higher proportion of abnormal sperm (Gaur et al. 2010; Ramlau-Hansen et al. 2007a, b). Besides, smoking increased sperm aneuploidy and, overall, reduced fertilising capacity (Said et al. 2005; Sofikitis et al. 2000). Smoking increases oxidative damage to sperm DNA by decreasing seminal fluid antioxidant activity (Pasqualotto et al. 2008). Exposure to the toxicants in cigarette smoke has been shown to increase free oxygen radicals in semen, causing oxidative damage to sperm plasma membranes and sperm DNA integrity and inducing genetic and epigenetic abnormalities. The integrity of the paternal genome plays a crucial role in successful fertilisation and normal embryo development. Damage to the paternal DNA by tobacco smoke results in the transmission of abnormal genetic information into the oocyte during fertilisation, which results in abnormal embryo development. Consequently, this may result in abnormal pregnancy outcomes such as recurrent implantation failure, miscarriage and congenital malformations. Emerging evidence suggests that the negative impact of environmental toxicants induces permanent heritable genetic and epigenetic alterations in spermatozoa which can be passed on to subsequent generations. Individual susceptibility to the negative effect of toxicants in cigarette smoke depends on other lifestyle factors, genetics and the duration and dose of exposure (Marczylo et al. 2012).

Paternal preconception alcohol consumption is associated with deterioration of semen parameters in a dose-dependent manner (Gaur et al. 2010; Muthusami and Chinnaswamy 2005; Jensen et al. 2014) and reduces live birth rates as well as increasing miscarriage rate in assisted reproduction treatments (Klonoff-Cohen et al. 2003). Moderate alcohol consumption does not appear to impair semen parameters significantly (Jensen et al. 2014). However, heavy and chronic alcohol intoxication induces oligo-, astheno- and teratozoospermia and eventually azoospermia (Sermondade et al. 2010).

Paternal preconception caffeine intake seems to confer some protection to sperm by increasing sperm motility both as dietary and in vitro supplement (Sobreiro et al. 2005), although other studies have not demonstrated any beneficial effect (Jensen et al. 2010; Klonoff-Cohen et al. 2002). Emerging evidence suggests that high caffeine consumption may even have harmful effects on sperm and Sertoli cells by decreasing the antioxidant capacity and increasing oxidative damage (Dias et al. 2015).

4 Risk of Assisted Reproductive Technologies

The short-term impact of assisted reproductive technologies has been a subject of intense debate for the last three decades. So far no substantial evidence exists to suggest that the use of assisted reproduction technologies is associated with short-term adverse consequences such as low implantation rate, decreased conception rates or increased risk of miscarriages. There are, however, reports of increased incidence of birth defects, chromosomal abnormalities (Bonduelle et al. 2002), prematurity, intrauterine growth retardation (Schieve et al. 2004; Schieve et al. 2007; Romundstad et al. 2008), epigenetic changes and imprinting disorders (Khosla et al. 2001a, b). However, the unanswered question is whether these are due to the underlying cause of the couple's infertility or due to assisted reproductive technologies. There is an argument that parental background during the periconception period, as well as the underline causative factors in infertility, may be responsible for these changes and not the techniques used (Myklestad et al. 2012; Belva et al. 2012).

Controlled ovarian hyperstimulation plays a major role in ART, but concerns have been raised about its detrimental effects on oocyte maturation, embryo quality, endometrial receptivity, post-implantation embryo loss and perinatal outcomes. Ovulation induction using supraphysiological doses of gonadotrophins creates an artificial oviductal and endometrial environment as well as alteration of oocyte maturation (Santos et al. 2010; Ozturk et al. 2016). It has been proposed that controlled ovarian hyperstimulation may alter gene expression related to oocyte and early embryo development (Ozturk et al. 2016).

In IVF/ICSI cycles, gametes and embryos are subjected to artificial culture media which alters the hormonal and chemical environment with potential consequences such as alterations in DNA methylation, mRNA-mediated abnormal expression of genes and epigenetic modifications. These were some of the reasons used to explain findings in studies showing that different culture media caused significant changes in mean singleton birth weights (Dumoulin et al. 2010b) and placental weight (Eskild et al. 2013). Furthermore, extended embryo culture to the blastocyst stage was shown to increase the incidence of large for gestational age babies (Mäkinen et al. 2013).

In mammals, in vitro embryo culture was associated with aberrant genomic imprinting, altered intrauterine growth pattern and defective placentation (Khosla et al. 2001a, b). Epigenetic alterations of some imprinted genes were shown to vary with the method of fertilisation and the type and constituents of the culture medium used and to be tissue specific in bovine embryo. In vitro culture of mouse embryos up to the blastocyst stage, using some commercially available sequential media, resulted in a pronounced permanent shift in the expression of some non-imprinted genes, despite an apparently normal growth, development and morphometric measurements of the animals born (Morgan et al. 2008). The reports of susceptibility of such mice to hypertension despite their normal post-natal growth raises concerns (Watkins et al. 2007, 2008). So also, the potential trans-generational persistence of some of these adverse effects into the progeny of the next generation after normal

mating is possible (Mahsoudi et al. 2007). Although these observations were in animal studies, they raise an alarm about the possible end-points for babies born to current assisted conception techniques.

Intracytoplasmic sperm injection (ICSI) also raises some issues of concern. Genetic and chromosomal abnormalities are commonly found in spermatozoa from men with severe male factor infertility. As ICSI bypasses the natural selection of sperm in spontaneous conceptions and conventional IVF, there is a risk of injecting sperm with genetic or chromosomal disorders which may impact on embryo development or cause transmission of chromosomal abnormality in the case of sperm aneuploidy (Bonduelle et al. 2002).

5 Consequences of ART

5.1 Perinatal Outcome

Several studies and data from national and international birth registries have shown an increased risk of adverse perinatal outcome in singleton infants conceived by IVF and ICSI (Schieve et al. 2004, 2007). The offspring are more prone to intrauterine growth restriction, low birth weight, preterm delivery and perinatal deaths compared with infants born to fertile couples (Helmerhorst et al. 2004; Jackson et al. 2004; Sutcliffe and Ludwig 2007). Iwahata and colleagues (2015) compared the closed and open vitrification systems in a retrospective study and showed no significant differences in post-implantation embryo development, gestational age, birth weight, sex ratio, Apgar scores or neonatal congenital anomalies.

Other researchers have found an increase in the rate of adverse outcomes in IVF singleton pregnancies. These include an increase in the incidence of low birth weight (LBW) (Schieve et al. 2002), preterm birth (PTB), hospitalisation during pregnancy and perinatal mortality (Gissler et al. 1995). Others include pregnancy-induced hypertension, placenta praevia (Tanbo et al. 1995; Romundstad et al. 2006) and, more recently, stillbirth (Wisborg et al. 2010; Zhu et al. 2006). A recent and comprehensive meta-analysis used pooled data from 17 studies to determine the rate of obstetric complications in IVF singletons and found an association with adverse perinatal outcome, with an increased risk of PTB (RR 1.84, 95% CI 1.54, 2.21) and LBW (RR 1.60, 95% CI 1.29, 1.98) (data from McDonald et al. 2009). Therefore, it appears that the underlying causes of infertility, ovarian stimulation or the IVF method are attributable to adverse outcomes in various populations.

Other studies have, however, refuted these claims (Belva et al. 2012) and associated the increase in adverse perinatal outcomes to maternal and other factors rather than the assisted reproductive technologies (Myklestad et al. 2012). A recent systematic review and meta-analysis found an association between length of time to conception and increased risk of preterm labour in women who conceived spontaneously: pooled crude odds ratio (OR): 1.38 (95% CI: 1.25–1.54) (Messerlian et al. 2012).

5.2 Placental Dysfunction

The increased risk of adverse perinatal outcomes in ART-conceived pregnancies is largely attributable to placental dysfunction resulting from epigenetic alterations due to in vitro gamete and embryo handling. Although the epigenetic marks of the developing trophoblast are very distinct from those of the inner cell mass and the developing mammalian embryo (Santos and Dean 2004; Santos and Dean 2006), it may be epigenetically as vulnerable. Human trophoblastic cell migration and invasion are epigenetically regulated processes (Rahnama et al. 2006). Loss of imprinting, altered epigenetic marks and gene expression patterns were observed in small for gestational age (SGA) babies (Guo et al. 2008). Placentae of babies born to in vitro conception demonstrated altered DNA methylation patterns and gene expression profiles when compared to those resulting from spontaneous conception (Katari et al. 2009) in humans.

5.3 Birth Defects

Children from pregnancies occurring, either spontaneously or following induced folliculogenesis, after a period of subfertility, are also at higher risk of congenital malformations (Sutcliffe and Ludwig 2007). Chung et al. (2006) found no effect of aetiology of subfertility, the type or dose of stimulation drugs, use of embryo manipulation techniques or embryo quality on perinatal outcomes. The evidence of an increased risk of congenital anomalies and assisted conception has however always been contradictory (Hart and Norman 2013a, b). Anthony et al. (2002) found no significant associations between IVF and an increased risk of overall congenital malformation of different types and severity. However, an increased risk of congenital abnormalities in singleton IVF boys and congenital heart abnormalities in girls conceived via alternative ART procedures was demonstrated (Klemetti et al. 2005), in addition to an association between musculoskeletal defects and intrauterine insemination (IUI) (Olson et al. 2005). Pooled data from some studies has also found a significant increase in 45 cohort studies comprising 92,671 ART babies compared with 3,870,760 naturally conceived infants R.R. 1.32 (95% CI 1.24–1.42) (Hansen et al. 2013). The overall risk of anomalies could have arisen from either IVF procedure or the super-ovulation medications or intrinsic factors associated with infertility (Ludwig 2009; Hammoud et al. 2008a).

More recently, Kissin et al. (2015) showed that approximately 0.8% of ART children (P for trend 0.19) had autism; which was lower following ART in parents diagnosed with unexplained subfertility, compared with ART for other causes of infertility (adjusted hazard risk ratio [aHRR]; 95% CI: 0.38; 0.15–0.94). In the same study, the incidence of autism was higher when ICSI, rather than IVF, was used (aHRR 1.65; 1.08–2.52). Heisey et al. (2015), in a large retrospective cohort study, found that relative risk of congenital malformations was 1.43 (95% CI 1.19–1.72)

for singleton infants conceived through ART compared with natural conceptions. The particular ART-associated defects were hypospadias, patent ductus arteriosus and obstructive uropathies.

In a case-control study involving 380 cases comparing foetal anorectal malformations in IVF/ICSI and fertile patients, Wijers et al. (2015) found increased risks of anorectal malformations in singleton ART pregnancies following IVF (OR = 2.4; 95% CI = 1.0–5.90 and ICSI (OR = 4.2; CI = 1.9–8.9), respectively. Anorectal malformations were also more common in offspring of subfertile parents conceived by IVF treatment, compared with those of subfertile parents who eventually conceived naturally (OR 3.2; CI = 1.4–7.2).

Some studies associate congenital anomalies with assisted conception treatments; others do not. Seggers et al. (2015) studied offspring with congenital anomalies from a registry-based study in Northern Netherland. A total of 4185 malformed cases recorded in fertile couples and 340 in subfertile couples. Of these, 139 had conceived after IVF/ICSI and 201 naturally after >12 months. Seggers et al. (2015) linked subfertility to an increased risk of ventral wall defects (adjusted OR [aOR] 2.43, 95% CI 1.05–5.62) and penoscrotal hypospadias (aOR 9.83, 95% CI 3.58–27.04). Other associations were methylation defects causing imprinting disorders (aOR 13.49, 95% CI 2.93–62.06) and right outflow tract obstruction (aOR 1.77, 95% CI 1.06–2.97). In the same study (Seggers et al. 2015), in vitro fertilisation/ICSI was associated with an increased risk of polydactyly (OR 4.83, 95% CI 1.39–16.77) and more specifically polydactyly of the hands (OR 5.02, 95% CI 1.43–17.65).

A contrary view is supported by Chen et al. (2015) who conducted a large retrospective study, including 2060 live-born infants, corresponding to 1622 frozen-thawed (FET) cycles. There were 587 live-born infants from long protocol stimulation (LPS) (including 458 FET cycles) and 1257 live-born infants from the short protocol (SPS) (including 984 FET cycles) and 216 live-born infants from mild ovarian stimulation (including 180 FET cycles). Chen et al. (2015) found that multiple pregnancies, gestational age, baby weight and length and early neonatal deaths were comparable in all groups. Live-birth defects in the LPS group (1.02%) and SPS (0.64%) were not significantly different compared to the mild ovarian stimulation group (0.46%). Congenital malformations were, however, significantly increased for the infertility-duration and multiple pregnancies (the adjusted OR were 1.16 (95% CI =1.1–1.335 and OR 3.89 (95% CI = 1.18–12.89), respectively. No links were found between congenital birth defects and types of ovarian stimulation regimens, BMI, maternal age, parity, insemination method or infant gender.

5.3.1 Epigenetic Impact of Assisted Reproductive Technologies

Major epigenetic reprogramming in human reproduction occurs during gametogenesis and peri-implantation embryo development. The periconception period is, therefore, a critical stage for epigenetic events, and epigenetic disruptions may occur during gametogenesis, fertilisation and early embryo development (Reik and

Walter 2001; Strachan and Read 2003). Biological and lifestyle factors can, therefore, influence epigenetic reprogramming of offspring and induce permanent heritable modifications in gene expression without altering DNA sequence (Goldberg et al. 2007; Marczylo et al. 2012). On fertilisation, this extensive reprogramming effectively combines the two highly differentiated, transcriptionally silent gametes into a totipotent embryo and then extends further beyond embryonic genome activation to lay the cell lineage-specific imprints of the differentiating blastocyst (Fulka et al. 2004; Fulka et al. 2008). The process involves extensive modifications involving several inter-dependent layers of epigenetic controls, like covalent histone modifications, chromatin remodelling, DNA methylation and post-translational inhibition through microRNAs (Tang et al. 2007; Yang et al. 2008). The integrity of these processes is shown, in mammalian models, to be crucial for a smooth maternal-to-zygotic transition, through a timely embryonic genome activation, appropriate gene expression and, hence, proper embryo differentiation and development (Bell et al. 2008; Santos et al. 2010; Schier 2007; Shi and Wu 2009; Tang et al. 2007). It is during this extremely labile phase that the gametes and embryos are subjected to the unnatural stresses of ovarian hyperstimulation, in vitro culture and maturation and micromanipulation.

Exposure of gametes and embryos in vitro to artificial conditions and non-physiological media may lead to epigenetic mutations. Controlled ovarian hyperstimulation has been associated with increased risk of epimutations. Oocytes retrieved from mice and humans following controlled ovarian hyperstimulation have altered methylation of imprinting genes (Sato et al. 2007; Fauque et al. 2007). IVF culture media can also modulate DNA methylation and epigenetic mutation. Fauque et al. (2007) showed that culture media can influence DNA methylation and kinetics of cleavage stage embryos. Animal studies demonstrate an impact of assisted reproductive techniques on various aspects of embryonic DNA methylation, histone modifications, miRNA populations and gene expression studies (Fauque et al. 2007; Mtango et al. 2009; Qiao et al. 2010). Fewer than 50% of IVF embryos reach the blastocyst stage of development, with many of these unable to sustain development following embryo replacement (Betts and Madan 2008). It has been clearly documented that aberrant methylation and histone deacetylation during meiosis (Akiyama et al. 2006) result in embryonic abnormalities, aneuploidies and developmental arrest. The possibility that in vitro conception may stretch the epigenetic plasticity of some human embryos beyond their adaptive capacity cannot be ignored.

5.3.2 ART and Imprinting Disorders

Imprinting disorders like Beckwith-Wiedemann and Angelman syndromes appear to be increased in offspring from assisted reproductive technologies (Maher 2005; Sutcliffe et al. 2006; Amor and Halliday 2008). Most studies reporting an association between ART and imprinting disorders do not, however, control for parental

genetic and epigenetic factors like paternal origins of chromosome 15 deletions or maternal disomy 15, infertility, obesity and high family income (Chang et al. 2005). Other studies have not found any associations between the incidence of either the Angelman or Prader-Willi syndrome and IVF or ICSI treatments when corrected for parental subfertility (Vermeiden and Bernardus 2013; Bowdin et al. 2007; Sutcliffe et al. 2006). This raises the possibility that the association is more likely to be related to parental subfertility rather than ART (Ludwig et al. 2005). Altered DNA methylation in sperm from men with oligozoospermia, teratozoospermia or oligoasthenoteratozoospermia has been found, (Boissonnas et al. 2010; Marques et al. 2004) and preliminary data suggest that oligospermic patients may carry a risk of transmitting primary incorrect imprints to the offspring (Kobayashi et al. 2009).

5.4 Mitochondrial Replacement Therapy

There are many uncertainties about the genetic and epigenetic risks to babies born after mitochondrial replacement therapy. There are ethical and medical concerns relating to the offspring conceived after mitochondrial DNA replacement therapy (MRT); but many of these have not yet been studied. Human mitochondria contains only about 37 genes, and these contribute less than 0.1% of genomic DNA, yet thousands of women all over the world are at risk of transmitting mitochondrial diseases to their children, with most babies dying shortly after birth. For example, it was estimated that 1 in 4000 children were born in the USA with this condition (Schaefer et al. 2004). Recently, mitochondrial DNA replacement therapy (MRT), commonly referred to as 3-parent IVF, has become accepted in the UK and declared permissible ethically by the FDA.

Although abnormal mutations have been identified in 13 mtDNA genes, which are linked to mitochondrial diseases, it is however less clear how specific genetic defects are exactly linked to disorders of tissues, organs or systems (Koopman et al. 2012). Furthermore, we do not yet know of possible negative impacts of MRT on progressive enhancements of mitochondrial-nuclear allelic interactions, which are evolutionary responses to natural selection (Reinhardt et al. 2013). It is also suspected that mitochondrial replacement may have unknown effects on subsequent epigenetic programming during embryo and foetal development (Ishii 2014). Admittedly, however, it may take a long time before the risk-harm balances of these concerns are resolved by scientific studies.

5.5 Recreational Drugs (Licit and Illicit Drugs of Abuse)

Recreational drugs form a large spectrum, including alcohol and cigarette smoking which have already been covered. Others include amphetamine-group stimulants (e.g. amphetamine and methamphetamine, fenethylline, methcathinone,

methylphenidate, ephedrine, pseudoephedrine, 'ecstasy' and other hallucinogens), tranquilisers, opioids (heroin, morphine, codeine, oxycodone, hydrocodone and methadone), cocaine and cannabis. The use of recreational drugs is a worldwide problem. This is illustrated by the report of the US Department of Health and Human Services agency, the Substance Abuse and Mental Health Services Administration (SAMHSA 2015), which reported that 10.2% of Americans aged over 12 years had used illicit drug in 2014. This figure surpassed previous years since 2002 and was mainly accounted for by cannabis and non-medical pain relievers. Recreational drugs may lead to maternal, foetal and neonatal complications, depending on timing, dosing and duration of use. Teratogenic effects may occur if the foetus is exposed in the periconception period. However, long-term disorders of foetal growth and neuro-developmental impairment can also occur, as well as neonatal sequelae like congenital anomalies, sudden infant death syndrome, neonatal abstinence syndrome, respiratory distress syndrome and neurobehavioral changes (Miller and Hyatt 1992; Huestis and Choo 2002). Oxidative stress and genetic and epigenetic mechanisms have recently been implicated in affected offspring of addicted mothers (Kovatsi et al. 2011; Neri et al. 2015). 'Transgenerational epigenetics' have also been proposed as the cause of developmental abnormalities, impairment in learning and memory and attention deficit associated with periconceptional use of recreational drugs (Neri et al. 2015).

Huestis and Choo (2002) showed that over 75% of exposed infants developed major medical problems compared with 27% of unexposed. They also showed that 20% of exposed babies were born prematurely, compared with 6% of unexposed neonates prematurely. Antenatal use of amphetamine may cause foetal demise, prematurity, low birth weight, growth retardation, birth defects and development disorders. Neonates may also be jittery and hypersensitive to touch. Otero et al. (2004) found poor social adjustment, cognitive deficits and learning disabilities in older children manifesting long-term sequelae of antenatal methamphetamine use. Periconceptional and prenatal abuse of cocaine or marijuana has been linked with miscarriage, premature birth, low birth weight and decreased head circumference (Phibbs et al. 1991 and Zuckerman et al. 1989). In a study of 125 children followed up from 3–6 months until their fourth year, it was demonstrated that long-term sequelae of prenatal cocaine abuse include irritability, attention deficit and behavioural problems (Vogel 1997).

6 Conclusion

In conclusion, the period from 24 weeks preceding a pregnancy to 10 weeks after conception has significant impacts on human reproduction. This periconception period includes gametogenesis, gamete maturation, fertilisation, peri-implantation embryo development, embryogenesis and organogenesis. Advanced parentage age, lifestyle habits such as obesity or under nutrition, smoking, excessive alcohol and caffeine intake and use of recreational drugs can induce genetic and epigenetic

alterations, which could cause infertility, implantation failure, recurrent miscarriages and adverse pregnancy and perinatal outcomes. The short-term impact of assisted reproductive technologies has been a subject of intense debate for the last three decades. So far, no substantial evidence exists to suggest that the use of assisted reproduction technologies is associated with short-term adverse consequences such as low implantation rate, decreased conception rates or increased risk of miscarriages. Although there are reports of increased incidence of birth defects, chromosomal abnormalities, prematurity, intrauterine growth retardation, epigenetic changes and imprinting disorders, it is unclear whether these are due to the underlying cause of the couple's infertility or side-effects of assisted reproductive technologies.

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The Importance of the Periconception Period: Immediate Effects in Cattle Breeding and in Assisted Reproduction Such as Artificial Insemination and Embryo Transfer

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Abstract In livestock breeding, the successful outcome is largely depending on the "periconception environment" which, in a narrow sense, refers to the genital tract, where gametogenesis and embryogenesis occur. During these early stages of development, gametes and embryos are known to be particularly sensitive to alterations in their microenvironment. However, as the microenvironment somehow reflects what is going on in the external world, we must widen our definition of "periconception environment" and refer to all events taking place around the time of conception, including metabolic state and health and nutrition of the dam. In modern dairy cows that have to manage an optimal reproductive performance with continued growth and high milk yield, the periconception period is particularly challenging. The metabolic priority for growth and lactation is known to generate adverse conditions hampering optimal ovarian function, oocyte maturation, and development of embryo/fetus. In addition, by using artificial reproductive technologies (ARTs), gametes and/or embryos of livestock are exposed to unnatural conditions outside the male and female genital tract. Artificial insemination, the most widely used technique, is currently yielding pregnancy rates similar to natural mating, and calves

Focus: Discussing the immediate effects that the periconception environment can have on conception success, the rate of fertility, and the establishment of pregnancy in livestock.

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produced by AI are equally viable after natural mating. In contrast, other ART, such as multiple ovulation and embryo transfer, have been reported to induce changes in gene expression and DNA methylation patterns with potential consequences for development.

Finally, the "periconceptional" environment has been shown to not only influence the successful establishment of pregnancy but also the long-term health and productivity of the offspring. Hence, the optimization of management around the time of conception might open doors to improve animal production and product quality.

Keywords Epigenetics • Artificial insemination • Embryo transfer • Periconception environment • Farm animals

1 Introduction

Livestock breeding is seemingly not very difficult. Unlike in human reproduction, dairy and beef cattle can be selected for fertility traits (Bowley et al. 2015), and animals suffering from suboptimal fertility are eventually removed from the breeding system, unless they are of very high genetic value. However, cattle breeding is nowadays affected by several factors, such as animal management conditions (Shahinfar et al. 2014) and climate change (Burthe et al. 2011; Gauly et al. 2013). These factors can influence or change the "periconception environment." Periconception environment can be interpreted in a broader sense, and all the events that take place during this period are extensively discussed in the various chapters of this book. In the present chapter, we will use the expression "periconception environment" in the narrow sense of the word, first to specifically refer to the male and female genital tract, where gametogenesis and embryogenesis occur. In the second phase, we will also discuss "periconception environment" in a broader sense, when referring to the physiology of the animal and its environment.

At first sight, it may appear that the oocyte, maturing in the ovarian follicle, and the embryo, developing within the oviduct and uterus, are well isolated from issues related to the external environment and therefore obviously benefit from being in a protected environment. However, there is a growing realization that the period during which the oocyte is developing in the ovarian follicle, and also during the first few weeks of embryo development, is one in which gametes and embryos are particularly sensitive to changes occurring in their "protected" environment. Apparently, this "protected environment" somehow reflects what is going on in the external world. Features such as dietary changes; general health issues and inflammation, heat stress, and metabolic stress; and environmental pollution may affect the intrinsic quality of gametes, preimplantation embryos, or even fetal development and

thus negatively impact on conception rates and the chance of a successful establishment of pregnancy in livestock. Furthermore, there is currently more and more evidence, both from studies in humans and in a variety of animal species, that factors influencing the periconception environment not only have consequences on the short term, deteriorating pregnancy results and neonatal survival, but may have a distinct effect on the long term by impairing animal health and productivity later in life. Hence, in livestock there is a considerable interest in this concept, also known as developmental programming, since it may open doors to improve animal production and product quality when implemented in general management strategies (Wu et al. 2006).

However, with the advent of assisted reproductive technologies (ARTs), we must further widen our definition of the periconception environment. "Assisted reproductive technologies" refer to four generations of technologies, which all have an impact on the periconception environment in the broader sense: (1) artificial insemination (AI), (2) multiple ovulation and embryo transfer (MOET), (3) in vitro embryo production (IVP), and (4) cloning and transgenesis (Thibier 2005). In this chapter, we will only focus on the first three, since they are routinely used in practice, whereas cloning and transgenesis are not, although this may soon change with the construction of an animal cloning factory in Tianjin, China (ref http://www.theguardian.com/world/2015/nov/24/worlds-largest-animal-cloning-factory-can-save-species-says-chinese-founder).

By using ART, gametes and/or embryos of livestock are being exposed to unnatural conditions outside the male or female genital tract, such as exposure to ambient air, to changes in temperature and pH, to nonphysiological hormone balances, to synthetic culture media, and even to cryopreservation or to breaches in the cellular membrane. In this chapter, we will reiterate how these changes in periconception environment may affect fertility in livestock and even health of the offspring, with a focus on dairy cattle.

2 Gametogenesis, Embryogenesis, Establishment of Pregnancy, and Fetal and Placental Development in Cattle

Female calves are born with hundreds of thousands of oocytes (Erickson 1966, cited by Britt 2008), which remain in the ovaries as primordial and primary follicles until puberty. The moment of puberty is significantly affected by breed and body growth rates. Furthermore, a cow is a year-round breeder. When heifers start to cycle at about 12 months of age, about every 3 weeks, a single mature oocyte, surrounded by some expanded cumulus cells, is released into the oviduct. The estimated time course of folliculogenesis in the bovine is about 80–100 days (Britt 2008), from the

stage of the primary follicle until the mature Graafian follicle. During this period of oogenesis, follicular waves emerge, but only one dominant follicle is selected to ovulate. Dairy heifers are usually bred at the age of 15 months, just after ovulation.

Bull calves start to produce semen at puberty (9–10 months) and are sexually mature at the age of 2–4 years (Rawlings et al. 2008). In each ejaculate, they produce billions of spermatozoa, and the estimated time course of spermatogenesis is 62 days. Conception rates are high in beef cattle mated by a bull (up to 80%) but significantly lower (40%) in modern high-producing dairy cattle that are currently traditionally bred by AI. In both cases, fertilization of the oocyte occurs within the oviduct. The bovine embryo enters the uterus at about 4–5 days after ovulation, at the early morula stage. During the period preceding embryo attachment, the embryo is free floating and is dependent upon endometrial secretions into the uterine lumen, termed histotroph, both for energy and proteins. The critical period of maternal recognition of pregnancy occurs between days 15 and 18 after ovulation, followed by the initial stages of implantation and early placentation (starting gradually after day 19 (Lonergan and Forde 2015)). In the cow, the placenta attaches to discrete sites of the uterine wall called caruncles. These caruncles are arranged in two dorsal and two ventral rows throughout the length of the uterine horns. The placental membranes attach at these sites via chorionic villi in specific areas called cotyledons. The caruncular-cotyledonary unit is called a placentome and is the functional area of exchanges between the cow and calf. In association with the formation of the placentome, the caruncular area is progressively vascularized to meet the increasing demands of the conceptus. Cattle have been shown to have the synepitheliochorial type of placentation (Wooding and Burton 2008). Fetal survival is dependent upon proper placental growth and vascularity early in pregnancy, while further intrauterine growth is mainly dependent on the placental supply of maternal nutrients and oxygen to the fetus. Therefore, establishment of a functional fetal/placental vascular system is one of the earliest requirements during conceptus development (Vonnahme et al. 2008). However, during pregnancy, the placenta is exposed to a variety of environmental insults which can alter fetal organogenesis and growth, leading to improper pre- and postnatal growth and eventually lower life performance.

Calving rates are typically high in beef cattle after natural mating (80%), and in dairy cattle after artificial insemination (65%) and embryo transfer of in vivo derived embryo (60%), but decrease toward less than 50% after transferring in vitro produced bovine embryos. Interestingly, in the high-producing dairy cow, calving rates after AI have decreased over the last decades to about 40%. In the next paragraphs, we will discuss how these differences can be explained, with an emphasis on dairy cattle.

3 Factors Affecting Periconception Environment Both in Natural Breeding and Assisted Reproduction

3.1 A Dairy Cow Is Not a Typical Cow

To maximize milk production, currently, farmers are stimulated to breed their stock at young age in order to have a first calf at 24 months and subsequently have their cows calved with intervals no longer than 385–400 days. The latter implies dairy cows to be rather atypical since they have to manage the compatibility of optimal reproductive performance and (early) gestation with continued growth or the production of large quantities of milk. To assure a high level of milk production, heifers should be raised to weigh 350–375 kg at 15 months of age, the age at which they should be inseminated in order to allow calving at 24 months (Wathes et al. 2014). A pregnant lactating cow's capacity to care for her embryo is largely determined by the way she partitions nutrients to support embryonic, placental, and fetal development together with her own maintenance and milk production (Wathes 2012). Continued growth, production status, and energy balance are known to have a significant effect on how nutrients are partitioned. Rather than being an absolute shortage of energy substrates per se, this metabolic priority for growth and lactation (after calving) is known to generate adverse conditions hampering optimal ovarian functioning, follicular growth, oocyte maturation, and early embryo development (Leroy et al. 2008a, b). In the next paragraph, we will further focus on "reproductive success" in dairy cattle, which can be defined as the ability to produce a healthy offspring in a timely way.

3.2 Physiological Factors Affecting Reproductive Success in Dairy Cattle

3.2.1 Continued Growth in Adolescent Animals

In general, reproductive capacity of nulliparous heifers is higher when compared to that of multiparous animals. While this finding can be attributed to the fact that oocytes and embryos of the nulliparous heifers have not been challenged by the metabolic stress of milk production, one should also not forget the decisive role of the uterus in terms of pregnancy success. Uteri of nulliparous heifers have not been confronted yet with a parturition event which is, in the vast majority of cases, associated with bacterial infection. Besides the better reproductive performance of nulliparous heifers, significant differences have been noted in terms of production, reproductive capacity, longevity, and resilience against metabolic challenges, between offspring of first and higher parity animals (Banos et al. 2007; González-Recio et al. 2012), with, in most cases, the offspring of first parity animals being in a more favorable condition. The latter results may be interpreted as an indication of

the deleterious effects of lactation during conception and early pregnancy since in contrast to multiparous dairy cows, first-parity animals do not lactate.

All too often, however, researchers have used first-parity heifers as non-lactating and hence "negative" controls when examining the effect of lactation and its concomitant metabolic consequences on the animal's reproductive capacity. However, when reproduction has to coincide with continued growth of the first-parity dam, the fetus may face intense competition for nutrients from its mother's own metabolic needs while still growing. Hence, the normal hierarchy of nutrient partitioning between maternal body growth and fetal growth may be altered (Wallace et al. 2006). In sheep, for example, there is a general consensus nowadays that overnutrition during gestation in adolescent ewes gives rise to a lighter progeny, while the dam generally experiences a significant increase in body condition. In this paradigm, rapid maternal growth results in placental growth restriction and often premature delivery of low birth weight lambs when compared with moderately nourished ewes of equivalent age (Wallace et al. 2006).

Since farmers are stimulated to maximize daily growth in their growing young stock in order to maximize milk production in the first and subsequent lactations, they accentuate the mismatch between the milieu for which the offspring is prepared and the milieu the neonates actually experience, which may lead to even more deleterious effects. Examples hereof are well known in human medicine, where it has been shown that babies that had experienced intrauterine growth retardation and thereafter experience a catch-up growth are more prone to reproductive disorders such as polycystic ovarian syndrome (Ibánez et al. 2008). Epidemiological studies both in beef (Funston and Deutscher 2004; Funston et al. 2012) and in dairy cattle (Brickell et al. 2009; Swali and Wathes 2007) have indeed shown that heifers growing fast in the first months of life have a significantly earlier pubarche but need more inseminations to become pregnant, ending up with a similar age at first calving in comparison with their slower growing peers. In this light, we may refer to the "thrifty phenotype hypothesis," which proposes the epidemiological associations between poor fetal and infant growth and the subsequent development of type 2 diabetes and metabolic syndrome. Both of these outcomes result from the effects of poor nutrition in early life, which produces permanent changes in glucose-insulin metabolism (Hales and Barker 2001). This hypothesis may also apply to high-producing dairy cattle.

3.2.2 High Milk Yield and the Concomitant Metabolic Stress in Lactating Animals

The genetic drive to produce large quantities of milk makes modern dairy cows more vulnerable to factors generally known to impair overall health and fertility. Hence, since dairy cows are challenged by such a variety of environmental factors during the period when they should also reproduce, they represent a "natural" model to describe the effects of periconception environmental challenges on their reproductive capacity. Furthermore, the modern dairy cows' reproductive capacity is

under extreme pressure especially because of very high rates of (early) embryonic mortality (Wiltbank et al. 2016). The latter might be a reflection of the high number of insults the gametes and early embryos are confronted with during the periconception period (Ribeiro et al. 2016; Leroy et al. 2015).

Modern dairy cows have been predominantly selected for high milk yield in early lactation, which is associated with a very high capacity to mobilize body reserves during this period. Calculations have shown that cows can produce as much as between 120 and 550 kg of milk from body reserves on the basis of energy (average 324 kg). Most cows can cope with this metabolic load, which is defined as "the total energy burden imposed by the synthesis and secretion of milk, which is met by mobilization of body reserves." Metabolic load has, however, been opposed to metabolic stress, which is defined as "the amount of metabolic load that cannot be sustained by body mobilization, leading to the down-regulation of some energetic processes, including those that maintain general health" (Knight et al. 2000). Hence, the "over"-mobilization of body reserves during the period of negative energy balance is a key factor for disease susceptibility in modern dairy cattle. The genetically and hormonally driven body mobilization is further significantly aggravated by the mismatch between the energy need and the cow's capacity to take in energy, the latter often being even further negatively affected by an inadequate adaptation of both the gastrointestinal tract and the overall intermediary metabolism. Maximal feed intake in dairy cows occurs commonly at 6–8 weeks in lactation, which is much later than peak production, causing cows typically to be in negative energy balance for 5–7 weeks postpartum (Tamminga et al. 1997). High milk production per se is not the primary cause to elicit negative effects on health and fertility traits, since the effect mainly seems to depend on the overall farm management and production environment.

Typically, the negative energy balance and concomitant body fat mobilization are characterized by specific alterations in peripheral plasma metabolite concentrations such as high non-esterified fatty acids (NEFAs), low glucose and insulin, and high levels of ketone bodies. In our laboratory, ovum pickup (OPU) has been used to demonstrate that these alterations not only occur in the peripheral circulation but are also reflected in the follicular fluid of the ovaries (Leroy et al. 2004). Applying OPU allowed us to get a better insight into the environment in which both the follicular cells and the oocyte have to mature. The effects of the elevated/lowered concentrations of metabolites associated with high milk yield on follicular cells (Vanholder et al. 2005b, 2006) and oocytes (Leroy et al. 2005, 2006) were subsequently evaluated in the laboratory. Main conclusions were that saturated NEFAs at concentrations found in vivo were able to impair proliferation of granulosa and theca cells (Vanholder et al. 2005b, 2006), while oocytes that had to mature in media in which elevated levels of saturated NEFAs were added were associated with lower fertilization and blastocyst rates (Leroy et al. 2005). Furthermore, when oocytes were matured in vitro in media mimicking ketotic situations, low glucose rather than high levels of ketone bodies seemed to be detrimental for subsequent fertilization and blastocyst formation (Leroy et al. 2006, 2008c).

Based on the above, it is clear that the periconception microenvironment of the oocyte affects its developmental competence and thus the subsequent pregnancy success. Whether it can also induce pertinent changes in the offspring's metabolism and body functions, and hence has an effect on its health later in life, is still a matter of debate. Farmers can influence this periconception environment by adequate measures (such as nutrition, health programs, and breeding technologies), as reviewed by Santos et al. (2010). It has been demonstrated that the altered microenvironment gives rise to altered patterns of gene expression in the offspring (Lillycrop and Burdge 2012). Epigenetic phenomena such as DNA methylation or histone modification are crucial in the regulation of gene expression, and intense epigenetic modifications have been shown to take place at a very high rate both in germ cells and in the preimplantation embryo. However, the question still remains whether the abovementioned changes, such as high NEFA levels, which affect the microenvironment of the oocyte and later of the young embryo, can elicit epigenetic changes like DNA methylation or histone modifications.

3.3 Nutrition

In dairy cattle, the potential influence of nutrition on the periconception environment of the gamete and young embryo can be evaluated at three different levels: undernutrition, overnutrition, and diet composition.

3.3.1 Undernutrition

In modern dairy cows, undernutrition as such should be considered as a very rare or even nonexistent phenomenon, since animals that are to be inseminated (both nulliparous heifers and lactating multiparous cows) are generally fed according to their requirements. In beef cattle held under extensive conditions, undernutrition may, however, still occur, especially in specific seasons when animals are outdoors and the development of crops and grass is far below what is needed for animal feeding. Therefore, in lactating dairy cows, undernutrition is mainly regarded as incompetence to cope with the negative energy balance (NEBAL) during the immediate postpartum period. As mentioned earlier, the main challenge for the cows at that time is to optimize their dry matter intake in order to let the NEBAL not become too serious nor to last exceptionally long. As outlined by LeBlanc (2010) and (Mulligan et al. 2006), an inadequate management is the main underlying reason why cows fail to handle the NEBAL and finally experience severe metabolic stress. All too often, the latter leads to subclinical metabolic disease like subclinical ketosis or eventually even clinical ketosis and fatty liver.

Undernutrition in the postpartum dairy cow should therefore be seen as insufficient dry matter intake leading to inabilities for the cows to cope with NEBAL. In terms of reproduction, the latter will first of all be accompanied by a delayed

resumption of normal ovarian cyclicity. Modern high-yielding dairy cows that do experience NEBAL have been shown to resume ovarian activity significantly later in comparison to cows in which the NEBAL was lower. Furthermore, significantly, more ovarian disturbances have been demonstrated in modern high-yielding dairy cows (Opsomer et al. 1998, 2000). In addition, the expression of heat symptoms has been shown to be significantly lower in those cows. The latter often necessitates farmers to inseminate cows based on secondary heat symptoms (such as restlessness and a decreased feed intake and milk yield), which are known to be associated with lower pregnancy results.

Most dairy cows develop the first postpartum dominant follicle approximately within 2 weeks after calving, but only about 40% of these follicles produce sufficient estradiol to stimulate ovulation, despite having normal ultrasound appearance and growth (Cheong et al. 2015). The mechanism leading to a correctly timed ovulation of a fertile oocyte is based on well-orchestrated cross talk within the hypothalamic-pituitary-ovarian axis (Cheong et al. 2015). In dairy cows selected for a high level of milk production, peripheral levels of glucose, insulin, and insulin-like growth factor-1 (IGF-1) are known to be substantially reduced (Marett et al. 2015; Bossaert et al. 2008). Lower peripheral insulin levels have been associated with non-ovulation of the dominant follicle, finally giving rise to cystic ovarian disease (Vanholder et al. 2005a). The underlying reason has shown to involve compromised theca cell function, finally leading to estradiol levels that are inadequate to provoke an ovulatory LH peak and hence ovulation. The reason for the compromised theca cell function has been attributed to the elevated levels of non-esterified fatty acids concomitant with overall fat mobilization (Vanholder et al. 2005b, 2006), although not all authors support this hypothesis. Overall, Cheong et al. (2015) recently concluded that cows that fail to ovulate the first postpartum dominant follicle are characterized by lower periparturient energy balance, increased insulin resistance, lower LH pulsatility, and lower intrafollicular concentrations of androstenedione and estradiol.

Undernutrition or, particularly for the dairy cow, insufficient dry matter intake and thus a more extensive NEBAL may cause adverse changes of metabolites in the ovarian follicular fluid. Although altered metabolite concentrations give rise to lower oocyte quality and hence lower fertilization rates as outlined above, this is less important for embryo growth because of the limited nutrient requirements of the early embryo and fetus for growth and development during the complete first half of gestation. It is furthermore well known that 75% of the growth of the ruminant fetus occurs during the last 2 months of gestation (Robinson et al. 1977). However, it is during the early phase of fetal development that maximal placental development and growth, differentiation, and vascularization occur, as well as fetal organogenesis, all of which being critical events for normal conceptus development. Nutrient restriction for the fetus is broadly defined as any series of events that reduce fetal and/or perinatal nutrient supply during critical windows of development. Basically, nutrient restriction can result from altered maternal nutrient supply, placental insufficiency, deranged metabolism and regulation, physiological extremes, and environmental conditions. From a practical standpoint, maternal nutrient supply

and environmental conditions leading to stress responses are the most likely observed causes of nutrient restriction in ruminant livestock (Reynolds et al. 2006).

3.3.2 Overnutrition

Excessive energy intake particularly from high carbohydrate diets in cattle can reduce fertilization and embryo quality in some, but not all, circumstances. The latter has been attributed to increased circulating insulin levels during the final week of follicle growth, although the underlying mechanisms are still not clear (Wiltbank et al. 2014). Adamiak et al. (2005) demonstrated that high feeding levels were beneficial to nulliparous heifers in low body condition, but detrimental to oocytes from animals of moderately high body condition. Also here, elevated levels of insulin were considered as the underlying reason for this negative influence. Later, these findings were confirmed by Rooke et al. (2009) by feeding heifers diets high in starch, although they were able to avoid the adverse effects on oocyte quality when leucine intake was increased.

3.3.3 Composition of the Diet

Management strategies for transition cows (i.e., 3 weeks before calving until 2 weeks after calving) are mainly focused on helping the cows to cope with the metabolic load by optimizing health, minimizing stress (e.g., by minimizing the changes in group or ration), and stimulating dry matter intake and immune function. These are great opportunities for the veterinary practitioner to regularly monitor and adapt the herd management (Mulligan et al. 2006; LeBlanc 2010). A relatively new approach is to implement rather short-term changes in the quantity or composition of the diet at key stages in the reproductive process. Therefore, the term focus feeding, which refers to implementing short periods of nutritional supplements that are precisely timed and specifically designed to ameliorate the reproductive process including embryonic and fetal growth and development, has been introduced (Martin and Kadokawa 2006). In this context, Wiltbank et al. (2014) discussed possibilities to supplement rumen-protected fats in the ration of dairy cows. Of special interest herein is the supplementation of methionine since this is a rate-limiting amino acid for milk production and is known to be a methyl donor, which may potentially affect the status of DNA methylation and hence the expression level of certain genes.

Furthermore, application of diets specifically designed to improve fertility by counteracting mechanisms related to the NEBAL or by supporting a specific pathway necessary for successful fertility has always been a very attractive way to circumvent the impairment of reproduction during early lactation. Although the reproductive system is known to be influenced by multiple hormones that are also involved in the adaptation toward high milk production (growth hormone, IGF-1 and leptin), only insulin is known to be relatively sensible to the composition of the

ration. Ovarian follicles have been shown to bear insulin receptors (Bossaert et al. 2010), and cows with lower peripheral insulin levels in the immediate postpartum period have been demonstrated to suffer from delayed postpartum ovarian resumption and are at higher risk to suffer from cystic ovarian disease (Vanholder et al. 2005a). Therefore, glucogenic diets have been advocated in the immediate postpartum period aiming to enhance the peripheral insulin concentrations and advance normal ovarian resumption (Gong et al. 2002). However, insulin has been shown to have detrimental effects on oocyte and embryo competence (Fouladi-Nashta et al. 2005) and to stimulate enzymatic catabolism of progesterone in the liver (Lemley et al. 2008). The latter suggests that glucogenic diets are only of advantage when offered in the immediate postpartum period and are to be avoided when cows are inseminated. In addition, low saturated fat diets around conception, reducing insulin levels, lead to increased conception rates (Garnsworthy et al. 2009).

Soybean meal contains isoflavones in concentrations that are able to induce increases in the blood concentration of estrogenically active isoflavone metabolites (equol, O-desmethylangolensin, dihydrodaidzein) in high-yielding dairy cows postpartum, even when supplemented in relatively low amounts (1.72 kg per day on average) (Cools et al. 2014). When compared with rapeseed meal, soy supplementation was furthermore associated with a decreased angio- and steroidogenesis at the level of the corpus luteum (CL) based on biopsy sampling at day 9 of the estrous cycle. However, no effect on the peripheral progesterone concentration during the first three estrous cycles after calving could be demonstrated. Therefore, although the results of the study suggest negative effects of soy feeding on CL function in recently calved dairy cows, the contribution of this effect on the peripheral progesterone concentration and consequently on overall fertility of supplemented cows warrants further research (Cools et al. 2014).

Adding fats to the diet has been another strategy that has been extensively tested to reduce the impaired reproductive capacity of dairy cows. In a study aiming to minimize the negative energy balance by decreasing the milk fat synthesis, and hence limiting energy output via milk by supplementing the ration with exogenous fats, we were not successful since cows simply produced more milk when reducing the NEBAL (Hostens et al. 2011). Omega-6 fatty acids are believed to have pro-inflammatory, and thus prostaglandin F2α-stimulating, properties rendering them of extra value early postpartum, while omega-3 fatty acids can weaken this inflammatory potency, leading to a higher chance of survival of the embryo when supplemented during the periconception period. However, the consequences of these fat feeding strategies on oocyte and embryo quality remain an intriguing issue for debate. Fat feeding may alter the microenvironment of the growing and maturing oocyte of the early and older embryo and thus may affect reproductive outcome. Dietary-induced hyperlipidemic conditions can thus also be harmful for embryo development and metabolism (for review, see Leroy et al. 2008a, b, c, 2014). Furthermore, peripheral blood in lactating dairy cows will contain a mixture of fatty acids of dietary origin and from body tissue breakdown, the latter being largely abundant in the immediate postpartum period and containing a high proportion of

saturated fatty acids. Especially, the latter have been shown to have a significantly detrimental effect on both the oocyte and embryo quality (Leroy et al. 2005).

Supplementation of extra vitamins and minerals to the diet has often been suggested as the golden bullet solution to reduce the fertility decline. Usually, farmers are highly sensitive to this kind of advice since it does not involve extra labor, which is their paramount constraint nowadays. In herds in which cows are given a high amount of concentrates to sustain peak yield during the immediate postpartum period, the risk of suffering from such deficiencies is lower due to the fact that concentrates are usually highly supplemented with vitamins and minerals. In terms of their effect on immune response and embryo quality, special attention should be given to vitamin E and selenium. The latter was supported by the recent finding that during the dry period, treatment of tocopherol-deficient cattle with injectable vitamin E of 1000 IU each week for the last 3 weeks of gestation not only reduced the incidence of retained placenta and stillbirth but also significantly decreased pregnancy loss (20.5% vs. 12.5%; $P < 0.01$) (Pontes et al. 2015).

3.4 Heat Stress

Embryos are known to be very sensitive to the transient increases in body temperature arising as a result of elevated environmental temperature (heat stress), and dairy cows are very susceptible to heat stress since increasing milk yields interfere with body temperature regulation during warm weather, further exacerbating the deleterious effects on fertility. Heat stress is known to affect many components of the reproductive system including gonadotrophin profiles, follicular growth, granulosa cell function, steroidogenesis, and oocyte and embryo quality (for review see Roth 2008). Interestingly, observations of impaired fertility of dairy cattle in the autumn subsequent to a hot summer have been reported. The latter suggests a clear carry-over effect of heat stress. It seems that heat stress not only affects antral follicles emerging in the follicular wave but probably also affects the ovarian pool of small antral follicles resulting in a carry-over effect on follicular function and oocyte developmental competence.

3.5 Health Problems and Inflammatory Reactions

Forty to seventy percent of dairy cows across different levels of milk production, breeds, and management systems develop metabolic or infectious diseases in the immediate postpartum period (Dobson et al. 2007; Ribeiro et al. 2013). The calving-to-pregnancy interval is extended for at least 7, 8, 26, and 31 days in cows treated for mastitis, retained fetal membranes, hypocalcemia, and endometritis, respectively, compared with healthy herd-mates. Lameness is associated with even worse reproduction performance, as up to 40 days can be lost to get lame cows in-calf

again even though the lameness has been treated (Dobson et al. 2007). In part, these poor fertility data may be related to delayed resumption of ovarian cyclicity after calving and on a lowered expression of heat symptoms. On the other hand, some events seem to have more long-lasting effects. Signs of dystocia or immediate postpartum hypocalcemia, endometritis, or mastitis can be "cured" within days by clinical treatment, but the cows are subfertile many weeks later during the breeding period. Obviously, inflammatory diseases taking place in the first weeks of lactation are associated with a reduced fertilization of cows inseminated between 50 and 60 days postpartum (Santos et al. 2010).

In a recent study, Ribeiro et al. (2016) showed that the carry-over effects of disease on reproduction of dairy cows cannot be explained simply by the nutritional status and its consequences to body condition score and estrous cyclicity at the onset of breeding postpartum. The inflammatory mediators produced by the injured or infected tissues can reach the reproductive tract including ovaries and uterus, but also the brain, which ultimately affects the physiological processes that control normal reproductive cyclicity. For example, cows that suffered from uterine disease postpartum had delayed growth of the first dominant follicle postpartum and reduced concentrations of estradiol (Sheldon et al. 2002). The presence of lipopolysaccharide (LPS; i.e., an endotoxin) in the follicular fluid of cows with uterine diseases has been postulated as a potential reason for compromised steroidogenesis, follicle growth, and impaired oocyte developmental competence (Bromfield et al. 2015).

3.6 Timing of Insults Affecting Reproductive Success and Embryonic Development

More and more evidence is currently indicating that the periconception environment is not only decisive for the fertility of the cow on the short term, but may be as important for general health of the offspring both in the immediate postnatal period and in later life. Specific approaches to improve management of dairy cattle during the periconception period and during pregnancy may therefore not only enhance the reproductive success of the dam but also the growth potential, health, and performance of her offspring later in life. If such innovative approaches could be available for use on the farm, producers might be able to increase animal health concomitantly with an improvement of the quality of the product while reducing costs of production.

The time at which insults are exposed upon dairy cattle will definitely influence reproductive outcome. During the earliest stages of pregnancy, the nutrient requirements of the embryo and young fetus are considered to be very low, causing undernutrition, for example, to be of lesser importance at that stage. Later, in the first trimester, however, development of specific organ systems (e.g., mammary gland, ovaries, liver, and pancreas) takes place. Hence, deleterious insults taking place at that time during pregnancy might be associated with impaired production and

reproductive capacities and a lowered ability to ensure homeostasis in later life. During the second trimester of pregnancy, the fetus continues to develop and grow but, at the end of this stage, will still only reach about 25% of its size at birth. Therefore, and because the dam in most cases won back positive energy balance at that time, the risk of major metabolic challenges is lower during that stage. On the other hand, the development of major organ systems is still going on, so that major insults might still have pronounced effects on later health and productivity. The largest increase in fetal tissue size (75% of fetal growth) generally takes place during the final trimester of pregnancy, insults taking place at that time being mostly reflected in a significantly lowered birth weight.

4 Factors Affecting Periconception Environment in Assisted Reproductive Techniques, Such as Artificial Insemination and Embryo Transfer

4.1 Artificial Insemination (AI)

Artificial insemination (AI) consists of the introduction of sperm into the female genital tract for the purpose of achieving a pregnancy. At present, AI is a mature technique in dairy cattle breeding and established worldwide. Moreover, it yields pregnancy rates similar to natural mating. In a large study comparing AI with natural mating, no difference was found in fertility between both groups (Buckley et al. 2003). Among the adjustment variables in the model, those significantly associated with the likelihood of conception rate to first service included the herd, calving period, calving to first-service interval, and peak milk yield (as discussed above).

In general, frozen semen is used for AI in cattle; semen samples are thawed just before use and are deposited into the corpus uteri. Sperm numbers introduced vary around ten million live sperm cells (20 million frozen per straw), but it has been shown that numbers as low as two million give equally good pregnancy rates (49–53%) and that deep insemination into the uterine horn is not necessary (Verberckmoes et al. 2005). Deep insemination with normal dose of semen did not improve fertilization rates or embryo production in superovulated cattle (Carvalho et al. 2013). In one study in beef cattle, however, deep insemination gave better results (67% vs 49%) with low-dose (four million) semen than conventional insemination (Meirelles et al. 2012). Deep insemination of frozen-thawed semen into the uterine horns gives also better results in pigs (Vazquez et al. 2008), and in sheep, laparoscopic insemination with frozen-thawed semen deposition into the uterine horns has been the routine procedure for many decades, since vaginal or even deep cervical insemination is yielding much lower results (Evans 1988).

Detection of estrus is very important when performing AI, and results can easily be influenced by this aspect. In order to make the timing of artificial insemination (AI) relative to ovulation less critical, methods for prolonging shelf life of

spermatozoa in vivo after AI have been developed. Encapsulation of sperm cells is a documented technology, and recently, a technology in which sperm cells are embedded in alginate gel has been introduced and commercialized. A blind field trial has been performed in Norway using standard processed semen with the Biladyl extender (control) in comparison with semen processed by sperm immobilization technology developed by SpermVital AS (Standerholen et al. 2015). Here it was demonstrated that fertility was not affected by encapsulation (NRR in both groups of 73%), although higher percentages of acrosome-damaged sperm were present in the encapsulated group. This shows that AI is a mature technique and can be used with different types of semen.

However, differences in bull fertility remain a problem. Nowadays, differences between bulls in field fertility have been related to the presence of "compensable" and "uncompensable" effects (Saacke 2008). Males requiring more sperm to be fertile after AI are considered to have compensable seminal deficiencies. This was elegantly shown by Den Daas et al. (1998), in a field trial where it became clear that the minimum sperm numbers required to achieve maximum pregnancy rates differ between bulls. These so-called compensable seminal deficiencies include a number of known sperm viability and morphology traits preventing fertilization to occur at all. Differences in fertility among males independent of sperm dosage are considered "uncompensable." These seminal deficiencies are associated with fertilizing sperms that are incompetent to maintain the fertilization process or subsequent embryogenesis (once initiated), leading to early embryonic mortality (Saacke 2008). It is obvious that compensable differences between males are more important in the AI industry than in extensive breeding systems, since AI with low doses of semen is economically more profitable, and hence bulls with compensable traits, which do not perform well at low doses, are less attractive and therefore are in less demand. Also interesting though are the uncompensable traits: can they be induced by environmental influences, with stressors having deleterious effects upon spermatogonia of the young or even prepubertal bull, or even earlier, when a male fetus was being exposed in utero to an adverse periconception environment? This may seem farfetched and has not been investigated in cattle yet, but in mice there have been several studies that show an effect of in utero exposure on future male fertility of offspring, even for several generations. In some recent animal studies, mostly in mice and rats, transgenerational inheritance of acquired traits has been demonstrated. One such study examined the effect of endocrine disruptors on pregnant rats and their offspring (Anway et al. 2005). To this end, rats were injected with vinclozolin (an antiandrogenic endocrine disruptor) at 8–15 days post-coitum, which is the period of gonadal sex determination. Transient exposure of a gestating female rat to endocrine disruptors induced an adult phenotype in the F1 generation of increased incidence of male infertility. These effects were transferred through the male germ line to nearly all males of all subsequent generations examined (i.e., F1 to F4) (Anway et al. 2005). The effects on reproduction correlate with altered DNA methylation patterns in the germ line (Anway and Skinner 2008). However, when a similar study was performed by orally exposing pregnant rats to vinclozolin, to test

a more natural route, no effect on male fertility in F1 or subsequent generations was found (Schneider et al. 2008).

Similar data are more difficult to achieve in large animal models, because of the large generation interval. Most large animal studies focus on the F1 generation to evaluate an effect of the environment on the offspring – it is important that such studies are also being performed on bulls and other large male mammals.

At present, there is no indication that calves produced after AI are less viable or less fertile than calves produced after natural mating, but this has apparently not been investigated yet (no hits on PubMed with calves * natural mating * insemination). It has been argued however that sex ratio may be skewed after artificial insemination, leading to more male calves (Berry and Cromie 2007). This was not confirmed in another study performed in Ethiopia, where a normal sex ratio of 50:50 was found after AI, but with odds ratio 1.38 for female calves after natural mating (Delesa et al. 2014). This was probably due to the fact that cattle that came into estrus during the harsh season were more likely to give birth to a female calf, according to the Trivers and Willard hypothesis (Trivers and Willard 1973) which states that as maternal condition declines, the adult female tends to produce a lower ratio of males to females.

However, the perception that AI gives more males than natural mating, which was obvious from both studies, needs further investigation, since such assumptions may prevent AI from being introduced at a large scale in Africa, where female cattle are much preferred over male calves.

4.2 Multiple Ovulation and Embryo Transfer (MOET)

Multiple ovulation and embryo transfer has been used in farm animals since the early 1980s (Thibier 2005). It consists of the use of multiple hormone injections to induce the release of multiple oocytes from the ovaries (so-called superovulation), followed by AI, flushing of the embryos from the uterus and transfer of the resulting embryos into synchronized recipients. Therefore, this technique increased offspring from genetically valuable females, while reducing also the time between generations. The major disadvantage of MOET is the unpredictable response of the donor female to exogenous superovulation treatment: 20% do not react sufficiently, whereas in *Bos taurus* an average of five to six embryos can be expected.

Multiple ovulation or superovulation has been reported to induce changes in gene expression and DNA methylation patterns in several species. In addition, the use of high dosages of gonadotropins induced spindle and chromosomal abnormalities in bovine oocytes (Liu et al. 2011). In mice, *H19*, *Snrpn*, *Peg3*, and *Peg1* showed loss of DNA methylation in mouse blastocysts obtained after superovulation, and this alteration was dose dependent, with aberrant methylation more frequent at high hormone dosage (Market-Velker et al. 2010b). Importantly, the loss of DNA methylation of *H19*, *Snrpn*, and *Peg1* could be observed in the sperm of the mouse off-

spring during two generations (Stouder et al. 2009). Superovulation in mice also resulted in biallelic expression of *Snrpn* and *H19* imprinted genes in the placenta (Fortier et al. 2008).

4.3 In Vitro Embryo Production (IVP)

In vitro embryo production entails the combination of three steps that need to be performed following a strict timing. It covers all steps from the maturation and fertilization of the oocyte to the embryo development. The oocytes can be derived both from a living (by transvaginal ovum pickup) or dead animal (slaughterhouse-derived ovaries). It is also combined with embryo transfer in many species.

4.3.1 In Vitro Embryo Production Affects Embryo Development and Quality

Cleavage and blastocyst rates are the main noninvasive parameters to assess bovine embryo quality. However, over time, it has become evident that high cleavage/blastocyst rates do not necessarily correlate with excellent quality of IVP embryos. Therefore, other criteria have been introduced, to compare with in vivo-derived cattle embryos. It is clear that IVP embryos show a darker cytoplasm due to their higher lipid content (Pollard and Leibo 1994), a more fragile zona pellucida (Duby et al. 1997), differences in metabolism (Khurana and Niemann 2000), a reduced intracellular communication (Boni et al. 1999), higher incidence of chromosome abnormalities (Viuff et al. 1996; Lonergan et al. 2004), errors of imprinting (Doherty et al. 2000), slower growth rate, higher thermal sensitivity, lower ICM/TE cell ratio (Van Soom et al. 1997a, b), and differences in gene expression compared to their in vivo counterparts (Driver et al. 2012). Additionally, higher apoptotic rates have been reported in IVP embryos compared to their in vivo counterparts (Gjørret et al. 2003), with an increased incidence of apoptosis as the culture time increases (Vandaele et al. 2006). Surprisingly, in cattle, some media used for in vitro culture are reported to have an influence on the sex ratio of the produced embryos, with a shift toward male embryos (Massip et al. 1996; Gutierrez-Adan et al. 2001).

4.3.2 Effects of In Vitro Embryo Production on Gene Expression

Gene expression is definitely different in IVP embryos. Before the development of wide screening techniques (such as microarray and RNA-seq), the effort was focused on genes known to play important roles during pre- and postimplantation development. The expression of *DNMT1*, *3A*, and *3B* has been demonstrated to be upregulated in bovine oocytes after in vitro maturation (IVM) compared to

in vivo-matured oocytes (Heinzmann et al. 2011). In vitro embryo culture was shown to have a major effect on gene expression, which is logical since embryos spend 7–8 days in that environment. A microarray study showed that approximately 85% of differentially expressed genes was downregulated in IVP bovine blastocysts compared to their in vivo counterparts (Corcoran et al. 2006). Most of these genes are involved in transcriptional and translational events suggesting that a deficient machinery associated with transcription and translation is behind the inferior quality of IVP embryos (Corcoran et al. 2006). Furthermore, different culture media had a different impact on genes associated with transcription and translation (Corcoran et al. 2007). This is not a unique case; genes involved in blastocyst formation such as cell-to-cell adhesion (E-cadherin, connexins, TJ genes), cell communication (gap junctions), differentiation marks (Miller et al. 2003; Lonergan et al. 2003a, b; Boni et al. 1999), and genes related to apoptosis and oxidative stress (*BAX*, *SOX*, *HSP70*) had different expression patterns between different culture media (Sagirkaya et al. 2006; Rizos et al. 2002).

When comparing in vivo-derived IVP bovine embryos, genes related to metabolism, growth, and differentiation (*GLUT-5*, *CX43*, *IGF-II*, *LIF*) were upregulated in embryos derived in vivo, while genes related to stress (*SOX*, *MnSOD*, *BAX*, *HSP70.1*, *PRDX5*) were upregulated in IVP embryos. The significant increase in expression of those genes supports the hypothesis that current in vitro culture systems are associated with a considerable amount of oxidative stress (Rizos et al. 2003; Lazzari et al. 2002). Additionally, in embryos produced in vitro in the absence of fetal bovine serum (FBS), the expression of genes involved in the cholesterol biosynthesis pathway was upregulated compared to in vivo-derived embryos (Driver et al. 2012). In addition to culture media composition, culture conditions such as oxygen concentrations were shown to have an impact on gene expression (Harvey et al. 2004).

Other ARTs also induce alterations on the transcriptome. Some studies reported differences in the mRNA expression profile of several genes between embryos produced with sex-sorted and unsorted semen (Morton et al. 2007), while other studies failed to find differences (Bermejo-Alvarez et al. 2010). Nevertheless, offspring from sex-sorted spermatozoa did not display more abnormalities than the controls (Seidel and Garner 2002). Vitrification of mouse oocytes arrested at MII induced downregulation of *Dnmt1*, *1o*, *3A*, *3B*, and *3 L* in MII and of *Dnmt3B* in blastocysts (reviewed by Anckaert and Fair 2015). Furthermore, blastocyst vitrification altered the microRNA transcriptome of mouse embryos. Four miRNAs (mmu-miR-199a-5p, mmu-miR-329-3p, mmu-miR-136-5p, and mmu-miR-16-1-3p) were upregulated, and one (mmu-miR-212-3p) was downregulated in vitrified compared to fresh mouse blastocysts (Zhao et al. 2015). Additionally, superovulation induced alterations in the gene expression of bovine oocytes (Chu et al. 2012). Despite all this, a similar expression of developmentally important genes was observed between in vivo and IVP embryos carried to term (Ghanem et al. 2011).

4.3.3 Effects of ARTs on Epigenetic Marks

The study of the effects of ARTs on the epigenetic pattern of the embryos and resulting offspring has gained more attention in recent years. IVP increased the levels of DNA methylation compared to embryos derived in vivo, in rats and mice (Zaitseva et al. 2007). In bovine blastocysts, IVP altered the DNA methylation profile, with longer in vitro culture being translated in higher alteration compared to in vivo-derived embryos (Salilew-Wondim et al. 2015). Cloned embryos also showed aberrant DNA methylation patterns in several species, including cattle (Dean et al. 2003) and sheep (Beaujean et al. 2004). However, in rabbits no differences in DNA methylation status were observed between cloned and IVP embryos (Shi et al. 2004).

Alterations of imprinting have been observed in embryos, placenta, and offspring produced by ARTs. A loss of DNA methylation in *Igf2R* and *Peg1* and a gain of methylation in *H19* were found after IVM compared to in vivo maturation in mice. This gain of methylation in *H19* was also reported in humans after IVM in five out of 20 oocytes analyzed (reviewed by Ventura-Juncá et al. 2015).

In the mouse, loss of DNA methylation was reported in *H19*, *Snrpn*, and *Peg3* in IVP embryos using different culture media (Market-Velker et al. 2010a; Doherty et al. 2000). Additionally, serum supplementation induced alterations on the DNA methylation pattern of various imprinted genes (*H19*, *Igf2*, *Grb7*, *Grb10*, and *Peg1*), faster rates of development, and long-term behavioral consequences in mouse embryos (reviewed by Velker et al. 2012a). Vitrification also altered the methylation status of imprinted genes, causing a loss of methylation in *H19* in murine embryos (Wang et al. 2010).

A condition of overgrowth can be induced by ART in ruminants and is referred to as large offspring syndrome (LOS). It is characterized by large size at birth, breathing difficulties, reluctance to suckle, and sudden perinatal death (Young et al. 1998). LOS is caused by the exposure of pre-elongation ruminant embryos to unusual environmental conditions. It is not exactly clear what environmental changes are important, but a major cause is the use of serum in the culture media (Sinclair et al. 1999). Recent studies provide evidence for epigenetic changes in LOS as this syndrome shows dysregulation of several similar imprinted genes such as *IGF2R*, *KCNQ1OT1*, or *CDKN1C* (Chen et al. 2013, 2015). Furthermore, loss of maternal-specific *SNRPN* methylation was found in the placentae from in vitro-fertilized and in vitro-cultured bovine embryos (reviewed by Velker et al. 2012b). We have recently used RNA sequencing to examine the effect of in vitro embryo production, in either serum-containing or serum-free media, on the global gene expression pattern of individual bovine blastocysts. Compared to in vivo-derived embryos, embryos produced in serum-containing medium had five times more differentially expressed genes than embryos produced in serum-free conditions (1109 vs. 207). Importantly, in vitro production in the presence of serum appeared to have a different impact on the embryos according to their sex, with male embryos having three times more genes differentially expressed than their female counterparts (1283 vs. 456). On the contrary, male and female embryos produced in serum-free conditions showed the same number (191 vs. 192) of genes expressed differentially;

however, only 44 of those genes were common in both comparisons. Interestingly, the pathways affected by in vitro production differed depending on the presence or absence of serum in the medium. For example, embryos produced in serum-containing conditions had a lower expression of genes related to metabolism, while embryos produced in serum-free conditions showed aberrations in genes involved in lipid metabolism (Heras et al. 2016).

5 Conclusion

In conclusion, the periconception environment plays an important role in natural and assisted breeding of cattle. Changes in nutrition, temperature, metabolic status, or health status during natural breeding can affect reproductive success and offspring health. Likewise, changes in medium composition or the presence of stressors to which gametes or embryos are exposed can also influence reproductive success and offspring health in assisted reproduction. Recent research in herd health management and follow-up on offspring will help us to improve management of high-producing dairy cattle, and recent advances of "omics" technologies may enable the application of new molecular methods for the identification of signaling molecules which are imposing epigenetic marks upon gametes and embryos during early life, thereby affecting also reproductive success and offspring health.

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The Consequences of Maternal-Embryonic Cross Talk During the Periconception Period on Subsequent Embryonic Development

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Abstract The periconception period comprises the final maturation of sperm and the processes of fertilization and early embryonic development, which take place in the oviduct. The final goal of these important events is to lead to establishment of pregnancy leading to the birth of healthy offspring. Studies in rodents and domestic animals have demonstrated that environmental conditions experienced during early development affect critical aspects of future growth, metabolism, gene expression, and physiology. Similarly, in vitro culture of embryos can be associated with changes in fetal growth, gene expression and regulation, and postnatal behavior.

In the oviduct, the cross talk between the mother and gametes/embryo begins after ovulation, between the oocyte and the female reproductive tract, and continues with the sperm and the early embryo after successful fertilization. These signals are mainly the result of direct interaction of gametes and embryos with oviductal and endometrial cells, influencing the microenvironment at the specific location. Identifying and understanding the mechanisms involved in this cross talk during the critical period of early reproductive events leading to pregnancy establishment could potentially lead to improvements in current in vitro embryo production systems in domestic mammals and humans. In this review, we discuss current knowledge of the short- and long-term consequences of in vitro embryo production on embryo development.

Keywords Embryo development • Embryo quality • In vivo • In vitro • Embryo-maternal interaction

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1 Periconception Environment In Vivo

In vivo, mammalian oocytes and embryos develop in a complex and dynamic environment. First, in the ovarian follicle, the oocyte grows and matures, achieving full developmental competence (Fair et al. 1995). Prior to ovulation, the luteinizing hormone (LH) surge leads to the resumption of meiosis and final oocyte maturation. Oocyte maturation involves (i) nuclear maturation, i.e., progression from prophase I to metaphase II with extrusion of the first polar body, (ii) cytoplasmic maturation which involves organelle redistribution, and (iii) molecular maturation that involves the accumulation of specific mRNAs (Sirard 2001). It has been hypothesized that the quality of an oocyte is based on the presence of the appropriate set of mRNA and proteins stored during folliculogenesis (Wrenzycki et al. 2007). A defined oocyte-specific gene expression pattern arising during folliculogenesis is crucial for the acquisition of oocyte developmental competence; conversely, deficiencies in gene expression or dynamics that occur during follicle development may be linked to impaired oocyte competence (Eichenlaub-Ritter and Peschke 2002; Sirard et al. 2006).

After ovulation, in the oviduct, the oocyte undergoes fertilization and the first mitotic or cleavage divisions. Finally in the uterus, the blastocyst forms, hatches from the zona pellucida, and, depending on the species, either implants immediately [day 4.5 in rodents (Wang and Dey 2006) and days 6–10 in humans (Cha et al. 2012)], forms a large free-floating spherical structure which initiates implantation around day 40 (horses), or elongates and progressively attaches to the uterine wall (initiation between days 18–22 in cows, 15–18 in ewes, and 14–18 in pigs) (Senger 2003) (Fig. 1). These events must be properly orchestrated for successful pregnancy establishment and the delivery of a healthy offspring.

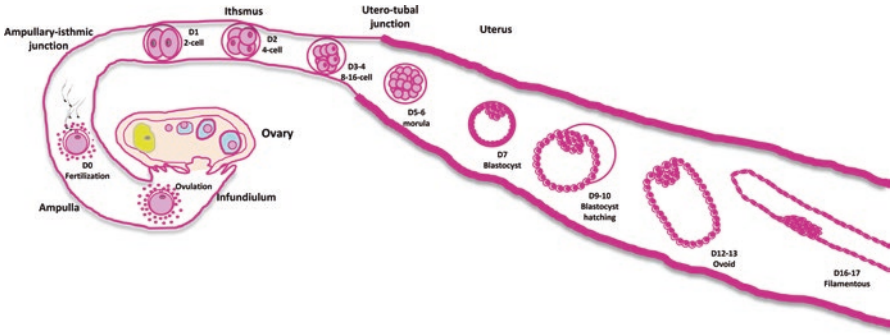

Fig. 1 Schematic representation of the process of early embryo development in vivo in cattle. After ovulation, the matured oocyte is fertilized (*D0*) at the ampullary-isthmic junction, while the first cleavage division takes place around 24–30 h later in the isthmus (*D1*) followed by subsequent mitotic divisions up to the 16-cell stage (*D4*). At this stage, the embryo passes into the uterus through the uterotubal junction and forms a morula (*D5–6*) and then a blastocyst (*D7*). After hatching from the zona pellucida (*D8*), the morphology of the embryo changes to ovoid (*D12–13*), then tubular and filamentous (*D16–17*) before implantation begins on D19

Following copulation, semen is deposited in the female reproductive tract which, depending on the species, will be in the cranial vagina (cow, sheep, primates, and cat) or into the uterine lumen (pig, horse, and dog) (Senger 2003). Typically, billions of sperm are deposited; however, during transit through the female reproductive tract, this number is dramatically reduced as sperm are selected by different barriers [cervix and uterotubal junction (UTJ)]. Once in the oviduct, the sperm are held in a storage reservoir in the isthmus which preserves sperm fertility, reduces the incidence of polyspermy by releasing sperm gradually (Suarez 2008), and constitutes the immediate source of viable sperm at the time of ovulation (Hunter and Wilmut 1984). It has been suggested that the sperm storage site recognizes and selects a fertile sperm population in mammalian species (Teijeiro and Marini 2012). After ovulation, the fimbria of the infundibulum that surrounds the ovary allows the passage of the ovulated oocyte into the oviduct. At this point, both muscle layers and ciliated cells mechanically guide the oocyte into the lumen of the ampulla to the site of fertilization (Hunter 1988). In addition, the oocyte loses the cumulus cells, and the zona pellucida (ZP) becomes exposed directly to the oviductal fluid (OF) which prepares it for fertilization and minimizes polyspermy (Coy et al. 2008, 2012).

Once fertilization occurs, the embryo spends the first 3–4 days in the oviduct, depending on the species. In cattle, the embryo remains in the oviduct until approximately day 4 of pregnancy, by which time it is at about the 16-cell stage. Then, the embryo enters the uterus and by day 7 forms a blastocyst consisting of an inner cell mass, which gives rise to the fetus, and the trophectoderm (TE), which forms the placenta. On days 9–10, the blastocyst hatches from the ZP and soon begins the process of elongation, which involves transitions from a spherical blastocyst on day 7 of gestation, through ovoid (days 12–13), tubular (days 14–15), and finally filamentous forms around days 16–17 (Fig. 1) (Degrelle et al. 2005). During elongation, the conceptus increases in size, more than 1000-fold, mainly through expansion of the trophectoderm (Betteridge et al. 1980) associated with an increase in protein content (Morris et al. 2000). After day 19, the elongated conceptus begins implantation with firm apposition and attachment of the trophectoderm to the endometrial luminal epithelium.

Proper communication between the conceptus-endometrium-corpus luteum (CL) is vital for pregnancy establishment. Progesterone (P4) synthesized by the CL acts indirectly via the endometrium to stimulate embryonic growth (Forde et al. 2009; Spencer et al. 2016). Conceptus elongation initiates interferon-τ (IFNT) production by TE cells (Roberts et al. 1999; Spencer and Bazer 2004; Robinson et al. 2008), which in cattle is the key signal for maternal recognition of pregnancy (Spencer and Bazer 2004). During maternal recognition of pregnancy, the mononuclear cells of the conceptus trophectoderm synthesize and secrete IFNT between days 10 and 21–25 with maximal production on days 14–16 (Bazer 1992; Roberts et al. 1999).

2 Periconception Environment In Vitro

In 1978, the first baby conceived by in vitro fertilization (IVF), Louise Brown, was born. Three years later, in 1981, the first calf obtained from IVF was born (Brackett et al. 1982). Although nearly 40 years have passed since then and many improvements have been made in in vitro embryo production (Paramio and Izquierdo 2016), even today in vitro systems are not as efficient as in vivo embryo production.

The goal of in vitro embryo production is to simulate as closely as possible the conditions that occur in vivo, to obtain high-quality embryos capable of continued development and implantation, and to result in viable births. Nowadays, in cattle, approximately 90% of oocytes cultured in vitro undergo nuclear and cytoplasmic maturation from which 80% are fertilized and cleave at least once (Lonergan et al. 2003a). Nevertheless, only between 30% and 40% reach the blastocyst stage (Rizos et al. 2008). In vitro, embryos are typically cultured until day 7 or 8 after fertilization, which corresponds to the blastocyst stage, when they are usually transferred into recipients. In heifers, the pregnancy rate following transfer of in vitro produced blastocysts is approximately 40–50% compared to about 70% when it comes to in vivo derived embryos (Hasler et al. 1995; Hoshi 2003). Thus, the challenge today is to improve current in vitro procedures providing high-quality embryos capable of continuing development and implantation after transfer to recipient and resulting in viable births.

2.1 In Vitro Maturation and Effects on Oocyte Developmental Competence

Cumulus-oocyte complexes (COCs) for research use are usually aspirated from ovaries recovered in the slaughterhouse, while those for commercial embryo transfer are recovered by transvaginal follicle puncture from live animals. In both cases, COCs are selected for in vitro maturation (IVM) based on morphological criteria as the compactness and thickness of cumulus and ooplasm homogeneity (Blondin and Sirard 1995). The cumulus cells play a critical role in the development of the oocyte by providing metabolites and nutrients, like pyruvate, oxaloacetic acid, and amino acids, thus stimulating them to resume meiosis and progress to metaphase II. Furthermore, cumulus cell expansion is an important marker for oocyte maturation, which is induced by gonadotrophin stimulation in vivo and in vitro leading to massive production of mucoid extracellular matrix protein (Chen et al. 1990). Recent evidence has shown that hyaluronic acid (HA), an important component of the extracellular matrix, plays an important role not only in cumulus expansion but also in oocyte maturation and further embryo development (Marei et al. 2012). Furthermore, transcriptome studies have identified a large number of genes in both oocytes and their associated cumulus cells that are involved in oocyte maturation (Regassa et al. 2011). Thus, the gene expression patterns in the cumulus cells have

been used as an indicator of oocyte quality (Tesfaye et al. 2009; Bunel et al. 2015). Also, the detection of glucose-6-phosphate dehydrogenase activity by brilliant cresyl blue (BCB) staining can be used as a predictor of oocyte quality, where BCB-positive oocytes are more competent to form blastocyst than BCB-negative oocytes (34.1% vs. 3.9%, respectively, $P < 0.05$) (Alm et al. 2005).

Oocyte developmental competence, often defined as the ability of the oocyte to mature, be fertilized, and develop to the blastocyst stage, has been associated with (i) the size of the antral follicle from which it is recovered, (ii) the stage of the follicular wave, and (iii) the site of maturation – in vivo or in vitro [for review, see Lonergan and Fair 2016]. Oocytes matured in vivo are of better quality than those matured in vitro, and this is reflected in the number of embryos obtained subsequently. Indeed, it has been shown that irrespective of whether in vitro culture (IVC) occurred in vivo or in vitro, when oocytes were matured in vivo, the resultant blastocyst rate was almost 80%, while when oocytes were matured in vitro, it was limited to about 35% (Rizos et al. 2002). In relation to in vitro maturation, oocytes originating from follicles bigger than 6 mm resulted in significantly more blastocysts than those from 2 to 6 mm follicles and those recovered prior to the LH surge (Rizos et al. 2002).

The environment to which the oocyte is exposed during maturation can influence the abundance of transcripts in the matured oocyte (Watson 2000; Lonergan et al. 2003a) and in the resulting blastocyst (Knijn et al. 2002; Russell et al. 2006). Recent evidence has shown that suboptimal conditions during oocyte IVM have an effect at the epigenetic level and on genomic imprinting (Anckaert and Fair 2015) in bovine (Heinzmann et al. 2011) and ovine (Colosimo et al. 2009) oocytes. In vitro models that mimic the in vivo situation of high-yielding dairy cows after calving, i.e., in which nonesterified fatty acid (NEFA) concentrations are elevated, indicate that not only the oocyte developmental capacity is affected but also that the phenotype of the resulting embryos is altered (Van Hoeck et al. 2011, 2013). Maturing oocytes for 24 h under high saturated NEFA conditions significantly altered metabolic footprints of day 7 embryos at the level of both gene transcription and gene function (Van Hoeck et al. 2011). Nevertheless, additional studies are required to investigate whether the expression and DNA methylation of imprinted genes in blastocysts, fetuses, and placental tissue derived from IVM oocytes are unaltered [for review see (Lonergan and Fair 2016)].

Supplementation of putative growth-promoting substances to the maturation media (e.g., gonadotrophins, steroids, and growth factors) typically results in a modest improvement in the proportion of oocytes reaching the blastocyst stage (Thompson 2000). Normally, oocytes submitted to in vitro maturation are recovered from small- to medium-sized follicles (2–8 mm) which are capable of nuclear maturation but did not have sufficient time to undergo normal cytoplasmic maturation. A range of cellular and chemical methods have been successfully employed to artificially inhibit the meiotic resumption of oocytes following removal from the follicle, thereby allowing cytoplasmic maturation in vitro in the absence of nuclear maturation [for review, see Sirard 2001]. However, none of these in vitro approaches substantially improved oocyte developmental competence.

2.2 *In Vitro Fertilization and Effects on Oocyte Developmental Competence*

The fate of an embryo is determined at fertilization. Delays in fertilization or fertilization by a damaged spermatozoon could conceivably lead to oocyte aging or the formation of a defective embryo, respectively (Tarin et al. 2000). Any damage to the sperm after ejaculation can lead not only to a reduced fertilization rate but also to the formation of embryos with reduced ability to develop to the blastocyst stage. This phenomenon has been demonstrated for embryos formed from sperm exposed to gossypol (Brocas et al. 1997), oxidative stress (Silva et al. 2007), and sorting for gender by flow cytometry (Wheeler et al. 2006; Wilson et al. 2006; Bermejo-Alvarez et al. 2008; Bermejo-Alvarez et al. 2010). In addition, the nature of the sire itself can affect cleavage and the ability of the resulting embryos to develop to the blastocyst stage and to establish pregnancy after transfer [for review see (Hansen et al. 2010)]. Studies in trout have shown that the oocyte is able to partially repair sperm with damaged DNA during the first cleavage; however, when DNA repair is inhibited, damaged sperm is able to fertilize the oocyte but leads to embryo loss (Perez-Cerezales et al. 2010; Perez-Cerezales et al. 2011).

Cryopreserved semen is the main source of sperm for in vitro fertilization, although the proportion of fully functional sperm in a frozen-thawed sample is quite low (Holt 1997). Therefore, before in vitro fertilization, it is necessary to separate a motile fraction of sperm using one of a variety of methods such as centrifugation on a density gradient. During this process, seminal plasma is washed away and thereby much of the antioxidant protection is lost (Marques et al. 2010). In sperm, moderate levels of reactive oxygen species (ROS) are necessary for sperm maturation, capacitation, hyperactivation, acrosome reaction, and sperm-egg fusion (Kothari et al. 2010). Nevertheless, in vitro the sperm are often exposed to supraphysiological levels of ROS (Du Plessis et al. 2008) which can affect cell membranes, DNA, and mitochondria (Agarwal et al. 2006), leading to low fertilization rates and poor embryo quality (Silva et al. 2007; Jang et al. 2010). Antioxidants have been used to decrease the impact of oxidative stress, thereby improving sperm quality and their ability to fertilize an egg and consequently increasing the number of embryos obtained (Roca et al. 2004; Roca et al. 2005; Sapanidou et al. 2015) and their quality (Jang et al. 2010; Pang et al. 2016). However, other studies have failed to demonstrate any beneficial effects following the use of antioxidants during IVF and have even reported impaired fertilization and blastocyst rates (Ali et al. 2003; Goncalves et al. 2010; Marques et al. 2010). Therefore, more studies are needed to elucidate which antioxidants are best and when they should be added to improve the efficiency of IVF.

2.3 *In Vitro Embryo Culture: Short- and Long-Term Effects*

Embryo culture is the longest step during the process of in vitro embryo production and the step during which the greatest reduction in development occurs, achieving only 30–40% of blastocyst rate. The presumptive zygotes are recovered around 18 h

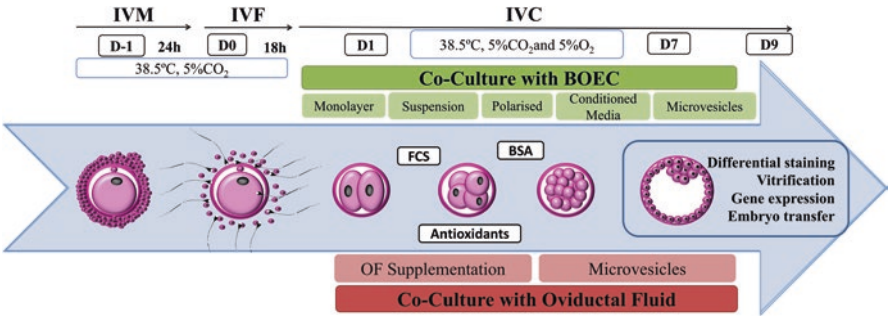

Fig. 2 Schematic representation of in vitro embryo production in cattle. In vitro maturation (*IVM*) of cumulus-oocyte complexes (COCs) is carried out for 24 h under an atmosphere of 38.5 °C, 5% CO_2, and maximum humidity. In vitro fertilization (*IVF*) is accomplished by co-incubation of matured COCs with selected motile sperm at a final concentration of 1×10^6 for 18 h, under the same conditions as IVM. In vitro culture (*IVC*) of the presumptive zygotes is maintained until D7–D9 at 38.5 °C, 5% CO_2, 5% O_2, and maximum humidity. The embryos can be cultured in different systems including coculture with BOECs in monolayers, suspension or polarized, and with products derived from BOECs such as microvesicles and conditioned media. Oviductal fluid recovered from the oviduct may also be used as well as its microvesicles

after fertilization and usually are cultured until days 7–9 (Fig. 2). It has been clearly shown that the intrinsic quality of the oocyte is the main factor affecting blastocyst yield, while the postfertilization culture environment affects the quality of the produced blastocysts (Rizos et al. 2002).

Culture conditions in vitro produce embryos of inferior quality compared to those derived from in vivo in terms of morphology, cryotolerance, transcript expression profiles, and pregnancy rate after transfer [for review, see Lonergan 2007]. To highlight the importance of developing in vitro systems that are as close as possible to the in vivo situation, different experiments have been carried out alternating in vitro with in vivo embryo culture. Thus, the culture of in vitro produced bovine zygotes in vivo in the sheep (Rizos et al. 2002), cow (Tesfaye et al. 2007), or mouse (Rizos et al. 2007) oviduct increases the quality of the resulting blastocysts so that it resembles that of totally in vivo produced embryos. Conversely, the in vitro culture of in vivo produced bovine zygotes results in blastocysts of low quality (Rizos et al. 2002). In addition, we have demonstrated that in vitro produced bovine zygotes cultured either in vitro or in vivo in the sheep oviduct at different stages of development up to the blastocyst stage exhibit a clear temporal sensitivity to their culture environment on their transcriptome and cryotolerance (Lonergan et al. 2003b). Thus, blastocysts cultured for the first 2 days in vivo and the last 4 days in vitro had the lowest survival rates after cryopreservation; those cultured only for the last 2 days in vitro had intermediate rates of survival, and those which spent the last 4 days of culture in vivo had high rates of survival, compared with those cultured entirely in vivo. Based on these studies, it was concluded that the period around the time of embryonic genome activation (EGA) is critical to the quality of the embryo. Similarly, Gad et al. (2012) investigated the consequences of culture conditions

before and during the EGA on bovine embryonic developmental rates and global gene expression patterns using the homologous cow oviduct. Embryo development was similar irrespective of where culture took place; however, the transcriptome of the blastocysts was clearly influenced by culture conditions, confirming once more the significant influence of culture conditions during EGA.

In vitro embryo culture has also been associated with epigenetic alterations in the embryo. In vivo, after fertilization the embryo's genome is epigenetically reprogrammed. This process involves the creation of the methylation patterns needed for normal development by activating and silencing specific genes (Reik et al. 2001). During this period, the embryo is especially vulnerable to in vitro induced epigenetic defects (El Hajj and Haaf 2013). Indeed, animal studies have revealed a link between different assisted reproductive technologies (ARTs) and imprinting disorders, via altered DNA-methylation patterns and histone codes (Urrego et al. 2014). In addition, imprinting disorders are more prevalent in gametes and embryos after ARTs than in their counterparts derived from in vivo production (Urrego et al. 2014). In vitro embryo culture has been associated with abnormal reprogramming of imprinted genes such as *SNPRN*, *IGF2*, or *H19* in cattle (Nowak-Imialek et al. 2008; Curchoe et al. 2009; Suzuki et al. 2009) and mice (Khosla et al. 2001). These alterations to the epigenetic profile may have a direct effect on the subsequent embryo and fetal development.

From the above, it can be concluded that the actual in vitro culture conditions provide a suboptimal environment for early embryonic development and have short- and long-term consequences. The short-term consequences include alterations in morphology, cryotolerance, and gene expression patterns, while the long-term consequences are reflected in abnormal offspring development and behavior (Rizos et al. 2008).

Embryos can be cultured in defined or semi-defined media, cocultured with oviduct epithelial cells or their conditioned media and with extracellular vesicles (Maillo et al. 2016). Nowadays, one of the most commonly used media for the culture of bovine embryos is synthetic oviductal fluid (SOF) which is frequently supplemented with fetal calf serum (FCS) and/or bovine serum albumin (BSA) (Tervit et al. 1972; Holm et al. 1999). The presence of serum in the IVC media has a stimulatory effect on the speed of development, with more blastocysts on day 6 of culture than either in its absence or with BSA (Gutierrez-Adan et al. 2001; Rizos et al. 2003). However, serum can have a negative effect on embryo quality as manifested by reduced cryotolerance and altered gene expression (Lazzari et al. 2002; Rizos et al. 2003; Wrenzycki et al. 2005) and pregnancy rate after transfer. Lazzari et al. (2002) evidenced that IVC of bovine embryos in the presence of serum or BSA significantly increased the number of cells in day 7 blastocysts and the relative abundance of transcripts for several genes including *HSP70.1*, *Cu/Zn-SOD*, *GLUT-3*, *GLUT-4*, *bFGF*, and *IGFI-R* compared with embryos cultured in vivo (either in the sheep oviduct or produced entirely in vivo). Moreover, these deviations were linked to gestation length and birth weight of the derived calves. Both in vitro systems, FCS and BSA, were associated with a significantly elevated incidence of deviations in embryonic development and a higher proportion of calves with

increased birth weight (Lazzari et al. 2002). In a recent study, it was shown that BSA removal over a 24 h period (from D6 to D7) in an individual embryo culture system decreased embryo development and cell counts in the inner cell mass, although the embryos tended to improve their survival after vitrification and also to result in a lower incidence of miscarriage (Murillo-Ríos et al. 2016).

Culture of embryos with FCS has been associated with alterations in the phenotype of newborn offspring in cattle (Farin et al. 2006), sheep (Walker et al. 1996), and mice (Fernandez-Gonzalez et al. 2004) named "large offspring syndrome (LOS)." LOS is characterized by aberrant placental development, extended gestation length, sudden perinatal death, breathing difficulties, more male calves, and large size at birth (Farin et al. 2006). Insulin-like growth factor 2 (IGF2) is an imprinted gene that regulates fetal and placental development in cattle and other species (Constancia et al. 2002). In sheep, the altered expression of *IGF2R* has been correlated with the incidence of LOS (Young et al. 2001) suggesting that IGF2 could be sensitive to epigenetic disorders during in vitro conditions. In addition, fetuses and placental tissue derived from IVP embryos presented an aberrant expression of imprinted and non-imprinted genes (Perecin et al. 2009; Farin et al. 2010). These phenotypic alterations may be the result of failure to properly establish or maintain DNA methylation and histone modifications during in vitro conditions (Farin et al. 2006). This also reflects the likelihood that epigenetic alterations during early embryo development are maintained in subsequent embryo and fetal development.

Oxidative stress is also important for the developing embryo during the embryo culture step. Substances such as ammonia, oxygen radicals, or growth factors can produce lipid peroxidation, membrane injury, and structural damage, leading to decreased cryotolerance and apoptosis (Somfai et al. 2007). Thus, antioxidant supplementation of the culture media has been shown to protect the embryo against oxidative stress. It also helps to maintain intra- and extracellular redox balance, which is necessary to reduce the toxicity of ROS, improving embryo development, increasing cryotolerance and cell differentiation, and inhibiting apoptosis during culture (Guerin and Menezo 2011; Takahashi 2012). Vitamins such as tocopherol, ascorbic acid, folic acid, or cyanocobalamin play a key role in reducing oxidative damage and improving blastocyst development rate in mouse embryos (Wang et al. 2002). Recently, it has been demonstrated that the supplementation of culture media with other antioxidants, such as cocretin which has a high antioxidant capacity, leads to an improvement of cryotolerance of bovine embryos (Zullo et al. 2016). Another antioxidant, retinol, seems to have a positive effect on early embryo development and quality (Livingston et al. 2004). Similar results were reported by Lawrence et al. (2004) suggesting that retinol protects early bovine embryos against damaging effect of heat stress during in vitro culture.

Despite the many efforts to improve in vitro culture media, conditions within the oviduct have not been fully recapitulated in vitro. As knowledge concerning embryo requirements during early embryonic development increases, in vitro culture systems have evolved to mimic more precisely what occurs in the oviduct during this period. Therefore, at this moment, it is known that the embryo requires an evolving

array of energy substrates (Gardner et al. 1996; Quesenberry et al. 2015); consumption of pyruvate and glucose is low until the 16-cell stage and increases significantly with morula compaction and blastocyst formation (de Souza et al. 2015).

As previously mentioned, in an attempt to mimic in vivo conditions and improve the quality of the embryos produced, different systems of embryo culture have been developed. They offer unique advantages such as the gradual change of culture media to suit the specific requirements of the developing embryo, thus overcoming limitations of conventional culture systems. Coculture in vitro with bovine oviductal epithelial cells (BOECs) has been considered to help with the production of good quality embryos (Ulbrich et al. 2010). These cells can be grown as monolayers, cell suspensions, or as polarized cultures (Fig. 2). A study by Cordova et al. (2013) showed that the use of BOEC in in vitro embryo culture at the early stages of embryo development, up to day 4, improves embryo development and embryo quality in terms of expression of specific gene transcripts. This period of culture coincides with the in vivo period when the embryo is still in the oviduct. However, the drawback of monolayers is that they dedifferentiate, losing important morphological characteristics (Rottmayer et al. 2006) including reduction of cell height, loss of cilia, and loss of secretory granules and bulbous protrusions (Thibodeaux et al. 1992; Walter 1995). Recently, we demonstrated that an established BOEC monolayer can be used successfully for coculture with no differences in embryo development when compared either with coculture with fresh recovered cells or normal culture in SOF (Lopera-Vásquez et al. 2016). Furthermore, the quality of the produced blastocysts in terms of cryotolerance and number of TE and ICM cells was higher than those produced using media supplemented with FCS. This method gives an advantage over the classical coculture systems as it provides homogeneous results. An alternative to monolayers is the short-term (24 h) epithelial cell suspension culture, which maintains morphological characteristics as well as the gene markers present in the cell in vivo such as *OVGP1*, estrogen, and P4 receptors (Rottmayer et al. 2006). Preliminary results from our group showed that BOECs in suspension cultures are closer to in vivo controls than monolayers in terms of morphology and oviductal epithelial cell markers *OVGP1*, *GPX4*, and *FOXJ1* (Hamdi et al. 2015). Finally, polarized cell cultures maintain the polarized asymmetrical structure of the oviductal epithelial cells, and it seems that this system preserves detailed morphological features of the porcine oviduct as well as oviduct-specific markers (Miessen et al. 2011).

Oviductal fluid (OF) has been used as a supplement during in vitro embryo production. Its composition is very complex, containing simple and complex carbohydrates, ions, lipids, phospholipids, and proteins (Avilés et al. 2010). Porcine oocytes treated with OF before fertilization showed significantly increased cleavage rates and blastocyst yield, suggesting that OF protects the embryo against adverse effects on mitochondrial DNA transcription or replication and apoptosis (Lloyd et al. 2009). In contrast, Cebrian-Serrano et al. (2013) evidenced that the exposure of cattle oocytes to OF before fertilization had no effect on embryo development and morphology of the resulting blastocysts; however, blastocysts produced from oocytes treated with OF showed differences in specific transcripts (Cebrian-Serrano

et al. 2013). Recently, we showed that low concentrations of OF (<5%) in bovine embryo culture media as a substitute for serum had a positive effect on development and quality in terms of cryotolerance, cell number, and expression of qualitatively related genes (Lopera-Vasquez et al. 2015).

Extracellular vesicles (EVs) is a general term encompassing several different vesicle types, released by somatic cells, that are present in body fluids and contain bioactive molecules (i.e., proteins, RNAs, mRNAs, and miRNAs) (Simons and Raposo 2009; Silveira et al. 2012) and lipids (Raposo and Stoorvogel 2013). The EV denomination is commonly size and origin associated, being exosomes (30–200 nm) from endosomal origin and microvesicles (MV) (100–1000 nm) from the plasma membrane. EVs are important in intercellular communication and play a key role in the regulation of physiological and pathological processes. It has been demonstrated that EV can horizontally transfer mRNAs to other cells, whereupon the mRNA can then be translated into functional proteins in the new location (Hergenreider et al. 2012). EVs have been identified in vivo in all body fluids including amniotic fluid, urine, and blood (Simpson et al. 2008). Currently, in reproduction, knowledge of the role of these secreted vesicles is limited to those from follicular fluid (Silveira et al. 2012), the endometrial environment (Ng et al. 2013), seminal plasma (Piehl et al. 2013), and uterine fluid (Burns et al. 2014). Recently, Burns et al. (2016) demonstrated that EVs emanate from both the conceptus trophectoderm and uterine epithelia and are involved in intercellular communication between those tissues during the establishment of pregnancy in sheep. Therefore, EV can be used as a supplement during in vitro embryo culture. Thus, Saadeldin et al. (2014) showed that the addition of exosomes isolated from the conditioned medium of parthenogenetic embryos increased the developmental competence of cloned embryos. In vitro derived embryos are also known to secrete EVs into culture media, where they may play a role in promoting development (Saadeldin et al. 2015). Recently, we provided evidence that EVs derived from BOEC conditioned media improve blastocyst quality and induce cryoprotection in in vitro cultures to the same extent as classical coculture with fresh BOEC monolayers (Lopera-Vásquez et al. 2016). Thus, the presence of EV in OF and their effect on early embryonic development may be of great importance and may provide information and new insights on early embryo-maternal communication and improve embryo quality in our current IVP systems.

3 Conclusion

During the last few decades, many advances have been achieved in our understanding of early reproductive events based on in vivo and in vitro studies, and assisted reproductive technologies are commonly used in humans and many animal species with success. Nevertheless, the quality of in vitro embryos is still inferior compared to their in vivo counterparts, and in vitro conditions have short- and long-term effects on the resulting embryo, fetus, and offspring. Therefore, a better understanding of how the embryo develops physiologically in the reproductive tract (oviduct

and uterus) will provide the knowledge to develop new strategies to decipher the mechanisms involved in oocyte developmental competence that help and improve the current systems of in vitro embryo production. Together, such improvements will lead to the production of better quality embryos. Moreover, proper in vitro models that mimic the physiological situation as closely as possible will be developed.

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The Role of Maternal Nutrition During the Periconceptional Period and Its Effect on Offspring Phenotype

Tom P. Fleming, Judith J. Eckert, and Oleg Denisenko

Abstract The early preimplantation embryo has been rigorously studied for decades to understand inherent reproductive and developmental mechanisms driving its morphogenesis from before fertilisation through to and beyond implantation. Recent research has demonstrated that this short developmental window is also critical for the embryo's interaction with external, maternal factors, particularly nutritional status. Here, maternal dietary quality has been shown to alter the pattern of development in an enduring way that can influence health throughout the lifetime. Thus, using mouse models, maternal protein restriction exclusively during the preimplantation period with normal nutrition thereafter is sufficient to cause adverse cardiometabolic and neurological outcomes in adult offspring. Evidence for similar effects whereby environmental factors during the periconceptional window can programme postnatal disease risk can be found in human and large animal models and also in response to in vitro conditions such as assisted conception and related infertility treatments. In this review, using mouse malnutrition models, we evaluate the step-by-step mechanisms that lead from maternal poor diet consumption though to offspring disease. We consider how adverse programming within the embryo may be induced, what nutrient factors and signalling pathways may be involved, and how these cues act to change the embryo in distinct ways across placental and foetal lineage paths, leading especially to changes in the growth trajectory which in turn associate with later disease risk. These mechanisms straddle epigenetic, molecular, cellular and physiological levels of biology and suggest, for health outcomes, preimplantation development to be the most important time in our lives.

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1 Introduction

Manipulation of reproduction during the periconceptional period is now commonplace across mammalian species. Domestic farm animals are bred to maximise milk or meat production and quality through in vitro oocyte maturation, in vitro fertilisation (IVF), embryo culture and transfer together with other contributing biotechnologies such as cloning and cryopreservation (Li et al. 2013). Similarly, overcoming human infertility, or the passage of adverse genetic risk to children, has facilitated related reproductive treatments (but not cloning!) to be developed clinically. Such assisted reproductive treatments (ART) have been largely successful in promoting efficient food production on one hand and safe delivery of several million babies globally on the other (Nardelli et al. 2014). Whilst development of such *direct* in vitro reproductive technologies has flourished, our understanding of more subtle in vivo influences on the periconception gametes and embryos has also increased, largely with the same goal, to improve reproductive outcome and health in the next generation. Thus, sustained, high quality maternal nutrition is important in enhancing reproductive performance in domestic animals (Diskin and Kenny 2016) and provision of such nutrition close to the time of conception has been recognised (Fleming et al. 2012; Leroy et al. 2015). Overcoming human metabolic disorders such as obesity and diabetes by healthy diet before conception is now advised to improve fertility and reproductive outcomes (Barker et al. 2013; Dodd et al. 2015). The same is true for women undergoing ART treatment to increase fertility success (Sim et al. 2014).

Periconceptional reproductive manipulation therefore provides an opportunity to overcome some of the obstacles we face in food security, clinical infertility and the wish for healthy offspring. But such optimism must be checked; recent research suggests that environment and manipulations around the period of conception can also cause *adverse* long-term outcomes, the opposite of the desired goal. In fact, the periconceptional period is a complex stage that can act as a 'double-edged sword' in terms of desired outcomes. One of the first inklings of this came following the early manipulation of sheep embryos in vitro and the unexpected consequence of 'large offspring syndrome' – the occurrence of abnormal excess growth at foetal and neonatal stages after extended culture of preimplantation embryos before transfer, commonly a lethal condition (McEvoy et al. 2000). Close scrutiny of the health of IVF children has revealed a small but increased incidence of perinatal complications (such as growth restriction and prematurity), imprinted gene disorders, and congenital abnormalities (Hart and Norman 2013; Brison et al. 2013; Lazaraviciute et al. 2014). For in vivo maternal nutrition and physiology, the pioneering

epidemiological studies from David Barker and colleagues (Southampton) on what has become known as the Developmental Origins of Health and Disease (DOHaD) hypothesis has demonstrated poor maternal nutrition during pregnancy closely associates with increased risks of adult non-communicable cardiometabolic disease in offspring (Barker 2007; Barker and Thornburg 2013). These studies across human clinical and animal models have further shown that poor maternal nutrition particularly during the periconceptional period in vivo can programme a broad spectrum of adult offspring comorbidities associated with cardiovascular, metabolic and neurological disease (de Rooij and Roseboom 2013; Turner and Robker 2015; Fleming et al. 2015). Moreover, human ART studies indicate increased prevalence of cardiometabolic disease risk in IVF children compared with spontaneously conceived children from parents with infertility problems (Ceelen et al. 2008).

The focus of attention on periconceptional reproduction, both from in vitro ART and in vivo maternal nutrition and physiology, leads us to consider two related questions. *Why might the period around conception be so susceptible to manipulations that it can alter long-term health outcomes throughout the lifespan and across species?* This question concerns the biological characteristics of the egg and early embryo, how the events of early development, both molecular and morphogenetic, may be instrumental in permitting manipulative conditions to impose immediate and enduring change on the developmental programme. In this review, we first briefly discuss the biological characteristics of the periconceptional period with these issues in mind. The second question to consider is – *How might environmental conditions experienced by the egg and early embryo change long-term potential affecting health across the lifespan?* This question concerns firstly the interface between the egg/embryo and different external factors, the sensing mechanisms that may 'read' this interface, and then how these conditions may cause permanent change in the way the embryo develops. The review will secondly discuss this multistep process using mainly a rodent maternal undernutrition model that has revealed some understanding of the plethora of mechanisms at work here.

2 Early Embryo Biology and Its Susceptibility to Developmental Programming

From fertilisation, the embryo has a multitude of tasks to achieve within a brief period of a few days. The highly specialised gametes must 'de-differentiate' and transform into a totipotent zygote and reinitiate cell cycling (cleavage divisions) coupled with cessation of expression of the parental genomes and activation of the new and unique embryonic genome (Li et al. 2013). Early blastomeres must begin the processes of expression and post-translational modifications regulating intercellular adhesion at compaction (8-cell stage in mouse) and subsequent epithelial differentiation in outer cells forming the extra-embryonic trophectoderm of the blastocyst (32-cell stage in mouse) (Eckert and Fleming 2008; Eckert et al. 2015).

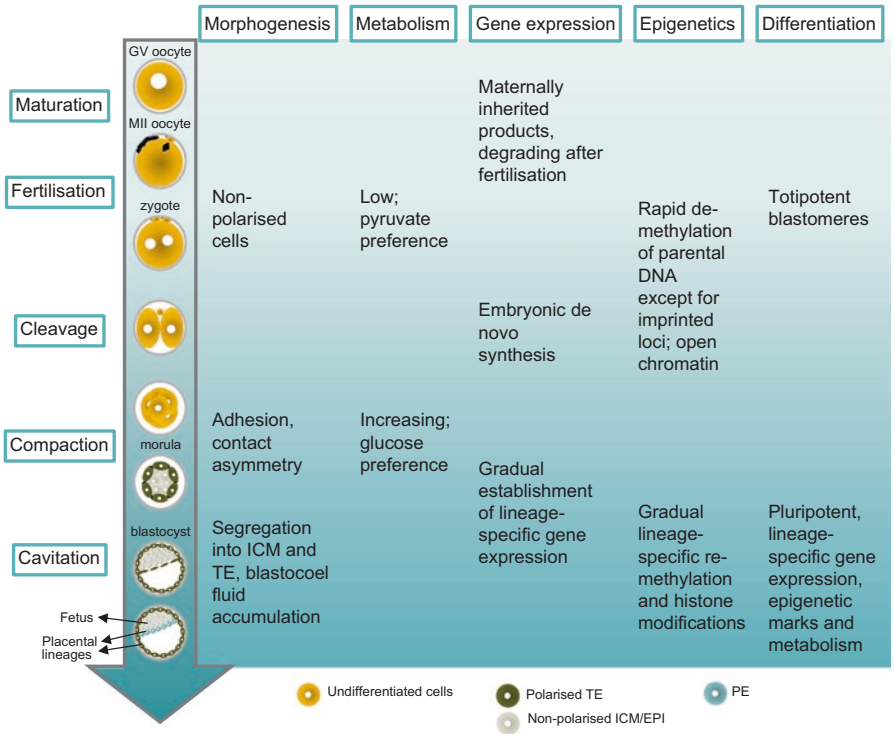

Fig. 1 A summary of the multiple events underway simultaneously in the early embryo activated upon maturation of the oocyte and its fertilisation. The proximity of these intrinsic events make the early embryo a suitable developmental stage for 'sensing' environmental conditions and optimising its future development (plasticity) dependent upon these conditions, including the quality of maternal nutrition. For details and references, see the text

Concomitant with embryo cleavage is the diversification and segregation of embryonic and extra-embryonic cell lineages (forming inner cell mass (ICM) internal to trophectoderm), achieved essentially through coordinated asymmetric cell divisions of polarised cells and through differential expression of regulatory transcription factors (Cockburn and Rossant 2010; Bedzhov et al. 2014; Sozen et al. 2014). Embryo morphogenesis further includes maturation of metabolic and nutrient uptake pathways (Leese 2012; Gardner and Harvey 2015), Trophectoderm transepithelial transport activity to form the blastocoel cavity (Eckert and Fleming 2008; Chen et al. 2010), and later hatching from the zona pellucida and the coordinated attachment to the uterine endometrial surface to achieve implantation (Seshagiri et al. 2009; Cha et al. 2012). See Fig. 1 for a summary of these events.

This complex series of biological processes, activated by fertilisation and rapidly accomplished, is essential for continuation of the developmental programme. The switch from gamete to embryo stages and the emergence of distinct cell lineages

requires a major epigenetic reorganisation of the genome. This comprises an initial global DNA demethylation of paternal and maternal genomes in blastomeres whilst maintaining the allele-specific pattern of imprinted gene expression and, from the blastocyst stage, the gradual re-methylation of genes that is lineage-specific and essential for their explicit patterns of gene expression (Chen et al. 2010; Rivera and Ross 2013; Zhou and Dean 2015).

Early embryo biology therefore comprises the coupling of several molecular, genetic and cellular activities over a strict time course and must coordinate the completion of maturation with maternal endometrial receptivity for development to continue. Thus, small changes or aberrations in any of these formative events may act to block or perturb the progression of a healthy pregnancy. However, we believe this is only half the story! In addition to the *intrinsic* series of biological processes governing the onset of gestation, the periconceptional period offers a unique 'opportunity' for *developmental plasticity*, a process to optimise developmental phenotype from a single genotype according to *extrinsic* conditions experienced by the embryo. The concept of embryo plasticity means that diverse environmental conditions can be 'factored' into the developmental programme as a conserved mechanism to promote offspring survival and competitiveness. Environmental conditions including maternal nutrition, metabolic health, sickness and infection could therefore act via appropriate embryo 'sensing' mechanisms to modify or adapt the intrinsic developmental programme. The epigenetic restructuring of the genome in the early embryo (Chen et al. 2010; Rivera and Ross 2013; Zhou and Dean 2015) provides a way for environmental sensing signals to induce modifications in gene expression and for these to persist in appropriate cell lineages long after the sensing event has occurred. In the context of maternal nutrition and its effect on development, we have found such adaptations can be distinct between embryonic and extra-embryonic cell lineages during the periconceptional period, to be mediated through epigenetic changes, and to confer an advantage in offspring fitness (Sun et al. 2015; Denisenko et al. 2016). We discuss such examples below. However, developmental plasticity mediates advantage usually only if environmental conditions persist and can represent the 'double-edged sword' referred to above in the Introduction when conditions change and adaptations no longer match conditions experienced in utero. Here, the long-term consequences can be increased susceptibility to disease comorbidities in offspring and can occur not only from in vivo conditions but also those experienced in vitro through ART manipulations.

3 Maternal Periconceptional Nutrition and Embryo Developmental Plasticity and Potential

The major animal studies on maternal periconceptional nutrition have utilised the rodent low protein diet (LPD) model where protein restriction (9% casein vs control 18%, normal protein diet (NPD)) is limited exclusively to the preimplantation

period (E0, 3.5 in mouse; E0, 4.25 in rat) with normal healthy nutrition thereafter for gestation and throughout postnatal life (known as Emb-LPD treatment). The maternal Emb-LPD diet leads to adult offspring comorbidity of abnormal cardiovascular, metabolic, growth and behavioural phenotype (Watkins et al. 2008a, 2010, 2011; Kwong et al. 2000). This profound legacy from a single dietary challenge mediated to their mothers when they were cleavage stage embryos is a powerful demonstration of adverse developmental programming that we find in this model is more acutely sensitive in female offspring. In more detail, outcomes include hypertension throughout life accompanied by attenuated arterial capacity to dilate and increased angiotensin converting enzyme activity; high adiposity and acquisition of a storage-type metabolism adapted to retain fats; increased growth and weight throughout life; and an abnormal hyperactive behaviour pattern (Watkins et al. 2008a, 2010, 2011). A similar yet distinct adult offspring phenotype can occur if the maternal LPD window is targeted to the period of oocyte maturation before fertilisation rather than of the early embryo (Watkins et al. 2008b), suggesting the periconceptional window extends before conception but that the presence of the paternal genome is contributory to the full phenotype, further supported by administration of paternal LPD alone (Watkins and Sinclair 2014). The influence and legacy of poor maternal nutrition during the periconceptional period has also been observed in domestic mammals using related models. Thus, ewes fed an undernutrition diet during the periconceptional period give rise to offspring with increased risk of cardiovascular, metabolic and neurocognitive disorders (Gardner et al. 2004; Hernandez et al. 2009; Todd et al. 2009; Torrens et al. 2009).

There is also good evidence that similar consequences occur in the human. People *conceived* during the 5-month Dutch Hunger Winter during the Second World War had increased risk of coronary heart disease, hypertension, high BMI, glucose intolerance, schizophrenia and depression and poorer cognition in adulthood compared to those experiencing the famine *later* in pregnancy (Painter et al. 2006; Roseboom et al. 2011; de Rooij and Roseboom 2013). Similarly, maternal nutritional profile at the time of conception in women in The Gambia has been associated with lifelong health of offspring born (Moore et al. 1997). In both of these human models, epigenetic modifications in a number of genes have been identified in offspring that associate with the periconceptional environmental challenge mediated through maternal nutrition (Heijmans et al. 2008; Waterland et al. 2010; Dominguez-Salas et al. 2014). Below, we will consider the stepwise mechanisms including epigenetic processes linking maternal periconceptional nutrition with long-term health outcomes, focusing mainly on the rodent Emb-LPD model.

4 Stepwise Mechanisms in Emb-LPD Periconceptional Developmental Programming

4.1 From Maternal Environment to Embryonic Induction

To understand why preimplantation embryo nutritional programming may lead to disease in later life, we need to consider the sequence of events that we think take place. The diet of the mother first modifies metabolite composition within her circulatory system, resulting in changes evident within serum. In response to short duration Emb-LPD in mice and rats, serum amino acids and insulin levels reduce within the period of preimplantation development whilst glucose increases in concentration (Kwong et al. 2000; Eckert et al. 2012). For the embryo to 'perceive' the maternal nutritional condition, such changes must be transmitted into the female reproductive tract where the embryos are located in the lumen before implantation. Careful collection and biochemical analysis of the uterine tract fluid from Emb-LPD and NPD dams at the time of blastocyst formation has shown that, at least for amino acids, the reduction in their concentration after Emb-LPD is also evident here (Eckert et al. 2012), indicating maternal dietary activity can modify tract secretions. A similar dietary influence on human uterine fluid amino acid composition has been shown recently (Kermack et al. 2015). Given the close juxtaposition of altered tract amino acid composition following Emb-LPD and the blastocyst, we must consider what signalling mechanisms might transmit this information to the embryo to initiate developmental programming. The mammalian target of rapamycin complex 1 (mTORC1) signalling pathway is the well-recognised regulator of cellular growth and translation rate, and its activity is mediated through extracellular insulin (acting through the insulin receptor and downstream phosphoinositide 3-kinase, PI3K) together with select amino acids of the branched chain group (BCAAs; leucine, valine, isoleucine) that contribute to cellular signalling (Avruch et al. 2009; Dibble and Cantley 2015). Indeed, BCAAs, both collectively and individually, are depleted in the serum and uterine fluid following Emb-LPD and changes in several amino acids have also been recorded in Emb-LPD blastocysts (Eckert et al. 2012). Moreover, quantitative analysis of mTORC1 in Emb-LPD blastocysts has revealed a significant reduction in activity of the downstream effector S6 ribosomal protein, a translation activator and regulator (Kim 2009). Collectively, these data suggest that maternal dietary Emb-LPD can alter nutrient composition within the uterus and that blastocysts can recognise this through sensing mechanisms that reduce mTORC1 signalling, representing the inductive event underlying developmental programming.

4.2 Programming of the Growth Trajectory

Following receipt of the reduced maternal nutrient environment, the blastocyst undergoes a series of responses or adaptations in the way it develops that act to compensate for the maternal nutrient restriction. These changes (discussed in 5.3–5.4) are distinct between the embryonic and extra-embryonic lineages, occur at different times during gestation, but act co-ordinately to promote survival and competitiveness. Perhaps most critical with respect to a mechanistic understanding of the long-term outcomes affecting growth, cardiovascular, metabolic and behavioural consequences are those responses that influence the growth trajectory of the embryo and foetus. We have found that the weight of pups around birth from Emb-LPD (or LPD, when the nutrient challenge is administered throughout gestation) is positively correlated with offspring comorbidity risks of adult weight, cardiovascular and behavioural phenotypes (Watkins et al. 2008a). The increase in weight caused by Emb-LPD treatment is evident at birth and, in females, is maintained relative to NPD offspring throughout life. This weight advantage is also evident during earlier foetal life and associates with an increased placental efficiency (foetal-placental weight ratio) in both Emb-LPD and LPD foetuses at E17.5 (Watkins et al. 2008a, 2015). This prenatal response reflects different adaptations occurring within embryonic and placental lineages activated from the blastocyst stage (see below). In LPD foetuses (untested in Emb-LPD foetuses), placental-foetal nutrient transfer activity is increased during mid/late gestational periods (Coan et al. 2011). The changes in blastocyst developmental path triggered by Emb-LPD become programmed at this time point because transfer of Emb-LPD and NPD blastocysts into NPD foster mothers results in enhanced growth of the Emb-LPD conceptuses in late gestation even in the absence of a continued Emb-LPD maternal background (Watkins et al. 2008a). Thus, we consider the Emb-LPD amino acid and metabolite induction mechanism involving mTORC1 signalling causes programming of blastocysts which initiates the early responses found within embryonic and extra-embryonic lineages to protect gestational growth.

4.3 Extra-Embryonic Compensatory Responses

The blastocyst outer trophectoderm layer shows several cell biological changes in response to maternal Emb-LPD that collectively contribute to the stimulation in growth discussed above. First, the Emb-LPD trophectoderm displays an increase in cellular endocytosis that likely reflects a response to increase nutrient uptake quantitatively from the maternal uterine fluid using histiotrophic means. This response appears to be comprehensive in that fluid uptake, protein ligand uptake, lysosome production and endocytosis receptor (megalin) expression are all significantly enhanced although no effect on cellular autophagy is evident (Sun et al. 2014). Mechanistically, trophectoderm endocytosis stimulation is brought about by

reorganisation of the actin cytoskeleton, mediated at least in part by Rho-A GTPase activity. In vitro studies suggest the trophectoderm endocytic response to maternal protein restriction is activated by the depleted uterine amino acid environment that the diet causes and is brought about rapidly and irreversibly in a culture medium with low concentration of protein (Sun et al. 2014). The mouse trophectoderm also undergoes a slight increase in cellular proliferation in response to Emb-LPD although this is not the case in the rat trophectoderm (Eckert et al. 2012; Kwong et al. 2000). Lastly, the cellular motility that accompanies the transition of late blastocyst trophectoderm (after hatching and attachment) into migratory trophoblast cells is significantly increased by maternal Emb-LPD. This response has been demonstrated in vitro in a normalised culture environment to mimic the implantation process, several days after blastocyst collection from mothers, indicating that its induction does not require continued protein restriction (Eckert et al. 2012). Indeed, programmed changes in Emb-LPD and especially LPD trophoblast motility persist in vivo and can be detected by in vitro assays at E8.5 (Watkins et al. 2015).

The primitive endoderm extra-embryonic lineage forms on the blastocoelic face of the ICM in the late blastocyst and gives rise to parietal and visceral endoderm, the latter contributing to the histiotrophic uterine-foetal nutrient transfer activity of the yolk sac placenta (Beckman et al. 1997; Zohn and Sarkar 2010). Examination of the mouse primitive endoderm phenotype in response to Emb-LPD has been conducted using embryonic stem cell lines (ESCs) generated from blastocysts collected from diet-treated mothers. Here, primitive endoderm in embryoid bodies grown in vitro from Emb-LPD ESCs show the same increased endocytosis phenotype as described above for the trophectoderm (Sun et al. 2014). This phenotype is also evident in vivo in the isolated yolk sac collected from E17.5 LPD conceptuses (Watkins et al. 2008a).

A common compensatory strategy in both trophectoderm and primitive endoderm lineages in response to maternal protein restriction is, therefore, to increase histiotrophic nutrition through endocytosis. The capacity to 'programme' these responses from preimplantation environment through to late gestation, likely requires epigenetic modifications as discussed above. With respect to Emb-LPD induced changes in primitive endoderm, we have found epigenetic modifications to occur within the gene encoding the Gata6 transcription factor that is known to activate primitive endoderm specification and differentiation through several downstream target genes (Rossant et al. 2003; Schrode et al. 2014; Artus et al. 2011; Morrisey et al. 2000). We noted that the embryoid bodies with outer primitive endoderm-like layer derived from Emb-LPD ESCs grew in normalised culture conditions to a significantly increased size (~15% larger) compared with NPD embryoid bodies yet exhibit reduced expression of Gata6 at mRNA and protein levels, as occurs in the in vivo Emb-LPD yolk sac visceral endoderm (Sun et al. 2015). Reduced Gata6 expression has been observed in other cellular systems regulated by this transcription factor such as in ovarian cancer models and coincides with increased growth promotion and malignancy (Caslini et al. 2006; Capo-Chichi et al. 2010; Cai et al. 2009). Increased proliferation of primitive endoderm and suppression of differentiation in response to Emb-LPD may facilitate histiotrophic capacity

in later gestation. Upon examination of the *Gata6* promoter in Emb-LPD ESCs, the reduced expression of the *Gata6* gene coincided with histone H3 and H4 hypoacetylation and reduced RNA polymerase II recruitment (Sun et al. 2015), features expected to cause reduced gene expression (Bartova et al. 2008). These epigenetic changes were coupled with increased expression of the histone deacetylase, *Hdac-1*, in Emb-LPD embryoid bodies. Interestingly, Hdac-1 expression in early embryos is sensitive to culture environment (Liu et al. 2014) and so may be a good candidate to reorganise targeted histone epigenetic reprogramming.

4.4 Embryonic Compensatory Responses

We anticipate the distinct extra-embryonic compensatory modifications discussed above, initiated in response to maternal protein restriction and mediated at least in part by epigenetic modifications, will act to enhance nutrient delivery from mother to embryo/foetus during gestation and minimise the effect of the dietary challenge. We have further investigated whether other modifications may occur within embryonic cell lineages that, mechanistically, may promote environmentally mediated control of offspring growth and thereby survival. Earlier human studies within the DOHaD literature recognised the close association that existed between foetal and postnatal growth and nutrient availability such that common characteristics for adversely programmed offspring following maternal undernutrition were low birth weight and excessive postnatal 'catch-up' growth when nutrients became unrestricted and therefore a mismatch with in utero conditions (Barker 2007; Barker and Thornburg 2013). This 'patterning' of the growth trajectory led to the 'thrifty phenotype' hypothesis which proposed poor maternal conditions would promote foetal adaptations to restrict growth of somatic tissues, modify metabolic homeostasis to conserve energy for essential organs such as the heart and brain, yet be permissive for opportunistic nutrient storage if conditions improved. However, these responses would become maladaptive if nutrient levels were significantly enriched postnatally leading to excess growth and adiposity and adult cardiometabolic disease risk (Hales and Barker 2001; Gluckman et al. 2005). This concept could apply to the Emb-LPD model since growth promotion and a storage metabolism are evident after release from the maternal nutrient challenge (Watkins et al. 2008a, 2011). In this case, however, the 'catch-up' growth is activated after implantation rather than postnatally, mediated at least in part by the compensatory responses from the extra-embryonic lineages discussed above. This demonstrates the importance of the periconceptional period for initiating long-term programming. However, despite an expansive literature associating the growth trajectory with nutrient availability and the relevance of a thrifty metabolism, clear mechanisms at genetic and epigenetic levels to explain this phenomenon have been underexplored.

In pig and sheep models we have found that protein restriction (pig) and nutrient/oxygen deprivation (sheep) triggered global downregulation of transcription in foetal kidneys (Denisenko et al. 2011). Recently, we have investigated whether a

unifying mechanism might directly regulate cellular growth across different somatic tissues in response to nutrient provision using the mouse Emb-LPD and LPD model. We have found foetal and postnatal liver and kidney global transcriptional activity per cell (as reflected by cellular RNA content) to be intimately linked with nutrient availability. Thus, maternal LPD throughout gestation lead to reduced transcriptional activity in foetal somatic tissues compared with NPD controls whilst in the adult, after release from nutrient challenge, expression was increased above control levels. In the Emb-LPD tissues, both foetal and postnatal time points revealed elevated transcriptional activity beyond the NPD control (Denisenko et al. 2016). Thus, transcriptional activity per cell is linked to maternal nutrient availability with activity suppressed during nutrient challenge and stimulated after release from challenge relative to NPD controls. Looking for molecular mechanisms that regulate offspring growth in response to changes in maternal diet, we examined expression of ribosomal RNA, the major constituent of cellular RNA, and found rDNA transcriptional rate (estimated by measuring pre-rRNA levels and RNA Pol I binding to rDNA) to match the pattern of total RNA expression, implicating ribosome biogenesis as a key regulator of growth, coordinated by maternal nutrition. Epigenetic analysis of the rDNA gene promoter in liver and kidney revealed increased DNA methylation relative to controls during periods of nutrient challenge (LPD, foetal tissues) and the converse, reduced DNA methylation and increased RNA Pol1 binding after release from challenge (Emb-LPD foetal and adult tissues; LPD adult tissues). Moreover, we find some evidence that cell sensing of nutrient availability in this mechanism may be controlled through the ribosomal transcription factor, Rrn3, whose cellular levels are low during nutrient deprivation and high after release (Denisenko et al. 2016). Thus, for example, over-expression of Rrn3 in an in vitro assay stimulates cellular rDNA transcription and reduces rDNA methylation. These findings provide strong evidence that somatic tissue growth throughout the life-course can be directly regulated by nutrient availability in accordance with the thrifty phenotype hypothesis mediated through epigenetic modification of rDNA genes and the dynamics of ribosome biogenesis. We speculate, therefore, that the pattern of rDNA expression during nutritional challenge and after release fulfils the requirements of a ‘thrifty gene’ (Denisenko et al. 2016).

4.5 Integration of Different Mechanisms and Their Consequences

The Emb-LPD model demonstrates that an important interaction occurs between the early embryo and its maternal environment. Thus, dietary nutrient levels of the mother change the chemical milieu of the uterine fluids and embryos can sense these changes via signalling pathways. Sensing dietary nutrient levels allows the embryo to exercise some plasticity with regard to optimal phenotypic state, in particular affecting the efficiency of extra-embryonic lineages for nutrient delivery

during gestation and the metabolic and biosynthetic features of the embryo. These changes appear compensatory and targeted to select a growth trajectory that is both sustainable and best fits the conditions experienced. See Fig. 2 for a summary of these integrated events.

Developmental programming following the preimplantation interaction between mother and embryo is broadly an *enduring* response, persisting throughout gestation and beyond, yet some features retain a dynamic character, and are modified by changing conditions. We demonstrate that epigenetic changes may contribute to the persistence of the programming response, for example either to key transcription factors regulating lineage diversification such as *Gata6* in the PE or to core genes

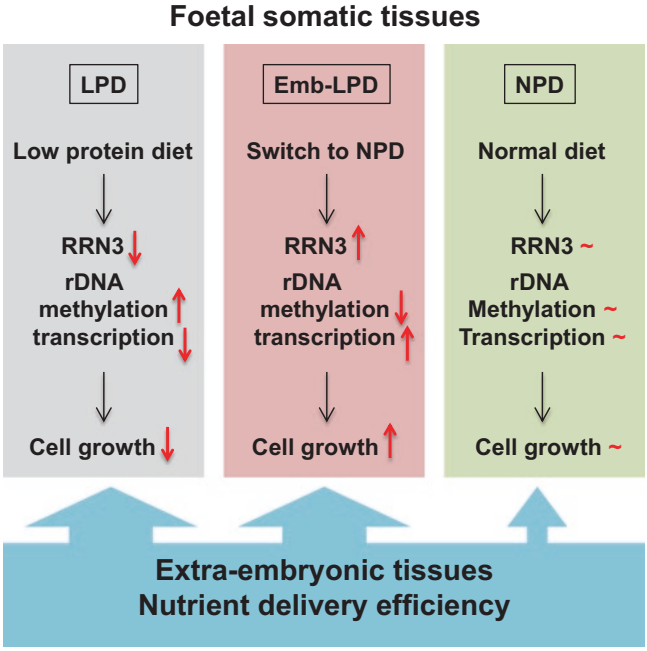

Fig. 2 The effect of maternal protein restriction on developmental plasticity in the early embryo is distinct between embryonic and extra-embryonic lineages and collectively sets the growth trajectory of the foetus. Foetal somatic tissues (e.g. liver, kidney) respond to LPD by epigenetic modification of ribosome biogenesis to restrict growth during nutrient deprivation relative to control (*NPD*). However, after release from maternal nutrient challenge (*Emb-LPD*), ribosome biogenesis is upregulated to stimulate foetal growth beyond that of NPD. In contrast, the extra-embryonic lineages (progenitors of chorioallantoic and yolk sac placentas) of the early embryo undergo several adaptations in response to maternal protein restriction that collectively promote nutrient delivery from the mother throughout gestation. These adaptations act as compensatory responses to promote growth and protect offspring fitness during dietary alterations and may involve epigenetic regulation, and most are maintained even if nutrient challenge is lifted (*Emb-LPD*). The combination of these embryonic and extra-embryonic mechanisms activated in the preimplantation embryo influence organism growth and disease risk over a lifetime. *Red arrows* represent either increased or decreased expression/activity relative to NPD control (~)

involved in biosynthesis such as rDNA in somatic tissues. The persistence of early embryonic programming after the decisive preimplantation period is most evident in the extra-embryonic lineages where compensatory responses are maintained in later gestation even if the dietary challenge is reversed (Emb-LPD) and responses become unnecessary, leading to larger offspring by birth. However, continued environmental interactions must persist to fine-tune the programming response. Thus, the increase in secondary trophoblast migratory activity at E8.5 induced by Emb-LPD is more substantial if the dietary challenge is maintained beyond implantation (LPD) (Watkins et al. 2015). Similarly, the epigenetic programming of rRNA production in somatic lineages is dependent upon continued sensing of nutrient levels via Rrn3 to generate the thrifty phenotype (Denisenko et al. 2016). Collectively, the balance between responses occurring within extra-embryonic and embryonic lineages drive the growth trajectory and later disease risk in adulthood. The maternal protein restriction model has therefore pinpointed the periconceptional period, and particularly the preimplantation embryo, as the critical stage coordinating developmental programming.

5 Maternal Obesity and Overnutrition

Whilst our understanding of how maternal undernutrition during the periconceptional period or even exclusively during the preimplantation period influences postnatal health is growing (see above), we know virtually nothing about the impact of overnutrition during this time. The chronic state of overnutrition, obesity, is on the increase worldwide and it is well-established that maternal obesity affects not only the individual's health but also their fertility and offspring health. By nature, maternal obesity and associated diverse physiological alterations will affect the oocytes before conception, the developing organism throughout pregnancy and lactation and even the gametes of the next generation which are laid down prenatally. But how sensitive is the periconceptional period to overnutrition? Considering that approximately 20% of weight-conscious women admit to binge on poor foods even if lean (this figure can rise to up to 55% in overweight women of reproductive age) (Bertoli et al. 2016), this is a relevant question which has been overlooked. With regard to maternal obesity, very few data are available with focus on the impact on the early embryo. Restricting exposure to obesity and overnutrition to around conception involves embryo manipulations before implantation (oocyte/embryo recovery, in vitro techniques, embryo transfer) which are technically demanding. Perhaps best characterised is the sensitivity of the oocyte. In oocyte donation programmes applied in human ART, recent evidence has shown that donor BMI plays a dominant role in achieving pregnancy and live birth outcomes when data are adjusted for recipient BMI and various other confounders. Chances to achieve a pregnancy and live birth progressively reduced with increasing oocyte donor BMI and were less than half if the donor was in the highest BMI quartile (average BMI 27.7) compared to the lowest BMI quartile (average BMI 19.7). Similarly, human embryos derived from obese mothers after IVF and culture display morphological

and metabolic abnormalities (Leary et al. 2015; Cardozo et al. 2016; Gu et al. 2015). In bovine models, replicating conditions of an obese maternal environment during oocyte maturation in vitro resulted in similar embryonic metabolic dysfunction (Van Hoeck et al. 2011, 2013). Overall, such data indicate compromised oocyte developmental capacity of oocytes derived from obese mothers. Among mechanisms that cause these alterations, a combination of spindle abnormalities and meiotic defects, accumulation of lipids and reactive oxygen species, epigenetic aberrations and overall metabolic disturbances as a result of dysfunction of organelles such as mitochondria and ER are suggested (Dunning et al. 2014; Gu et al. 2015; Igosheva et al. 2010). In rodents, mitochondrial malfunction in the oocyte or early embryo as a consequence of maternal obesity is linked to compromised foetal and placental growth, altered mitochondrial copy number and expression of imprinted genes or nutrient transporters, even if the embryo is transferred to a non-obese foster mother (Sasson et al. 2015; McPherson et al. 2015; Luzzo et al. 2012).

The impact on offspring health of exposure to overnutrition *exclusively during the preimplantation period* has not been examined yet in vivo. Data available through approaches using in vitro culture of embryos derived from lean mothers in the presence of molecules known to be elevated systemically in maternal obesity followed by embryo transfer to lean foster mothers suggest the preimplantation period alone may be sensitive to overnutrition, similar to undernutrition (see above). For example, brief exposure of mouse morulae or blastocysts to palmitic acid in vitro results in reduced blastocyst cell number and insulin signalling. When in vitro exposed blastocysts have been transferred to control foster mothers, foetal growth restriction and postnatal catch-up growth were observed (Jungheim et al. 2011). However, we only have very limited knowledge how maternal overnutrition is translated to conditions within the reproductive tract, the environment the embryo develops in. Further open questions relate to the 'toolkit' available to the early embryo: *which sensors trigger sequences of events, are these reversible or adaptable?* An important goal, therefore, for maternal overnutrition research will be to reveal the molecular participants of communication between mother and embryo and the range of responses that the embryo can adopt and their consequences for health.

6 Conclusion

The preimplantation embryo has a full programme of developmental 'objectives' to achieve over just a few days, to establish both an intrinsic plan of multi-lineage morphogenesis and a capacity to interrogate extrinsic cues including maternal nutritional status to set the growth trajectory for pregnancy. This important responsibility can impose an imprint on offspring phenotype and health that can last a lifetime. A better understanding of both maternal and embryonic mechanisms involved in setting up the developmental trajectory early on in response to maternal nutrition is

critical to develop effective intervention strategies. Perhaps even more pressing is to raise public awareness of the potential consequences of maternal malnutrition for the next generation at a time when the mother is still unaware of her pregnancy. It is this same early period in development that is the subject of numerous reproductive technologies that can also cause adverse long-term effects. Clearly, this research area should remain a priority in biomedicine to protect public health across the generations.

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The Long-Term Effect of the Periconception Period on the Embryo's Epigenetic Profile and Phenotype: The Role of Maternal Disease Such as Diabetes and How the Effect Is Mediated (Example from a Rabbit Model)

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Abstract Maternal metabolic diseases such as diabetes mellitus with diabetogenic hypoinsulinemia and hyperglycemia change periconceptional developmental conditions in utero. In preimplantation rabbit embryos, all major metabolic pathways are affected. Alterations in protein, lipid and glucose metabolism, adipokines, advanced glycation end products (AGEs) and reactive oxygen species (ROS) are described in this review. The embryonic metabolism is characterized by a high plasticity which enables survival of most preimplantation embryos under the non-physiological developmental conditions in diabetic mothers. Adiponectin, for example, compensates for the missing insulin-driven glucose supply and stimulates intracellular lipid accumulation in embryonic cells. AGEs and ROS are clear indicators of metabolic stress. The price paid for survival, however, needs to be taken into consideration. It is an increase in lipogenesis and proteinogenesis, leading to metabolic stress and with potentially negative long-term health effects.

Keywords Diabetes • Embryo metabolism • Adiponectin • Advanced glycation end products (AGE) • Reactive oxygen species (ROS)

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1 Introduction

In Germany more than 35.350 newborns are affected by maternal diabetes mellitus each year, as approximately 6% of all pregnancies occur in diabetic women (Deutscher Gesundheitsbericht Diabetes 2015). Amongst these women, 18% suffer from a preconceptional diabetes. The majority is affected by gestational diabetes. In the USA, 15% of pregnancies in women are diabetic (International Diabetes Foundation Atlas 2015). On an international scale, similar numbers are reported with approximately 17% of all births being affected by hyperglycemia during pregnancy (International Diabetes Foundation Atlas 2015). Again, gestational diabetes stands in most frequently (approximately 85%) followed by pre-existing type I diabetes (7%) and type II diabetes (5%) (Aceti et al. 2012). In the UK, between 1996 and 2004, the number of pregnancies complicated by pre-existing diabetes increased by 50% and the prevalence of gestational diabetes mellitus doubled (Aceti et al. 2012).

Diabetes during pregnancy is a significant medical challenge. It is associated with pregnancy complications, perinatal pathologies and a higher risk for the offspring to suffer from metabolic diseases later in life. Diabetic women are sub-fertile as indicated by ovulation disorders and higher risks for early spontaneous miscarriages (Casson et al. 1997, Penney et al. 2003, Verheijen et al. 2005, Yang et al. 2006). On average, the rate of pre-term births amongst women with pre-existing diabetes is about four times higher compared to women without diabetes. It is well known to obstetricians and pediatricians that about half of the newborns from diabetic mothers suffer from metabolic and adaptational dysfunctions after birth. The less controlled diabetes is, the more babies are born with congenital anomalies in women with pre-existing diabetes. Children born to diabetic mothers have higher risks of congenital malformations and childhood obesity. They have a predisposition to develop type II diabetes and cardiovascular diseases (metabolic syndrome) later in life. The National Institute for Health and Clinical Excellence in the UK advised diabetic women whose glycated haemoglobin A (HbA1c) is above 10% to avoid pregnancy. It is noteworthy that diabetes type II is no longer the disease of the elderly (Diabetes Atlas Update, International Diabetes Federation 2015). An increasing number of younger women who have not given birth so far develop diabetes. Therefore the number of diabetic pregnancies will rise in the future.

These data clearly demonstrate that a diabetic pregnancy requires special medical care and attention—for both the mother and her newborn. The health consequences for children from diabetic mothers raise the questions about when the metabolic and developmental disorders start. That the periconceptional (and preimplantation) period is a specifically critical period of pregnancy has convincingly been shown by the group of T.P. Fleming from Southampton. They have fed mice a protein-reduced diet during the short periconceptional time and describe numerous health effects in offspring later in life (Watkins et al. 2008a, b, 2010, 2011). The current review focuses on diabetogenic perturbations of preimplantation embryo

development in a rabbit diabetic model. We asked which potential mechanisms are involved in diabetogenic disorders and when they become noticeable in embryonic development.

2 The Diabetic Rabbit Model

The study of diabetogenic disorders in embryos cannot be performed in women. Therefore, valid experimental animal models are needed. Basically, diabetes mellitus can be experimentally induced by destruction of the pancreatic beta cells by chemicals (such as alloxan) or in genetic models, i.e. in animals with a naturally occurring diabetes, or by genetic modification, for example, in pigs as recently reported (Wolf et al. 2014). The described rabbit model is a diabetes type 1 model as maternal endogenous insulin production rapidly ceases after alloxan treatment. The review will summarize data raised in the rabbit model with a specific focus on the pre- and peri-implantation period.

The diabetic model is shown in Fig. 1. Sexually mature female rabbits are treated with alloxan. This chemical specifically destroys the pancreatic B cells leading to an experimentally induced type I-like diabetes (Ramin et al. 2010, Thieme et al. 2012a, b, Schindler et al. 2013, 2014, Gürke et al. 2015, 2016). The rabbit is used as an experimental model as its embryology resembles that of the human in many aspects (Fischer et al. 2012). The rabbit is a reflex ovulating species allowing easy scheduling of experiments involving stage- and age-specific maternal tissues and embryo analyses, even as close as in hours post coitum (pc). Stimulation of folliculogenesis (superovulation) leads to, on average, blastocyst numbers above 20. However, the number of corpora lutea and embryo recovery rates at day 6 pc are lower in diabetic than in healthy donor rabbits (Ramin et al. 2010), reflecting the lower fertility of diabetic females compared with healthy ones.

Blastocysts can be flushed out of the uteri up to the primitive streak stage (stage III), as implantation starts late on day 6 pc at stage IV. At day 6 pc, the rabbit blastocyst is 2.5–3 mm in size, allowing retrieval of substantial material for analyses and isolation of the embryonic cell lineages by microdissection. In a day 6 pc blastocyst, approximately 2,000–7,000 embryoblast cells, depending on the stage of development, and at least ten times more trophoblast cells (based on protein measurements), can be separated and recovered for further processing. This is a unique advantage of the rabbit model, saving on one side animal experiments and donor rabbits and allowing, on the other, subtle analyses in individual blastocysts.

After establishment of diabetes for 6–10 days, follicle stimulation and mating, preimplantation embryos are recovered, usually in the blastocyst stage at day 6 pc. Maternal blood glucose levels are kept between 19 and 30 mM by insulin supplementation thrice daily. Each time, insulin supplementation is adjusted according to the measured blood glucose level.

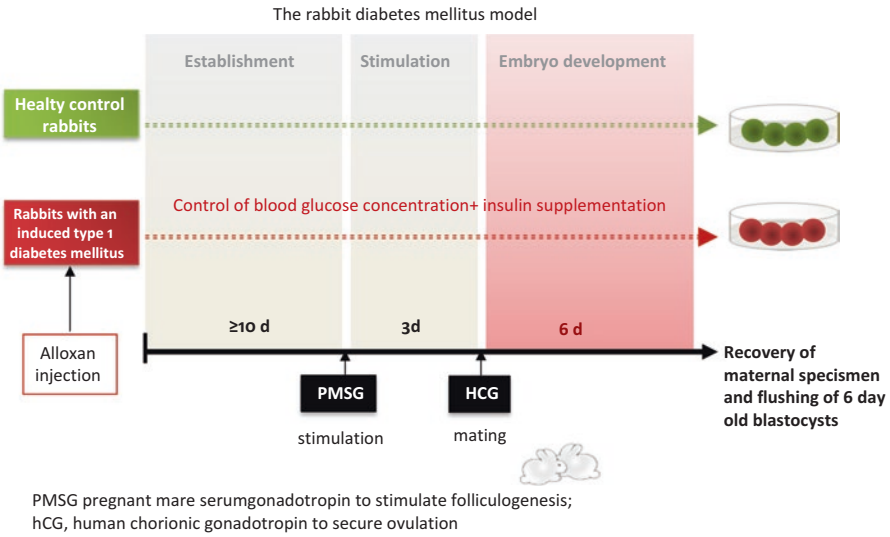

Fig. 1 The diabetic rabbit model. Experimental schedule. Blood glucose levels in alloxan-treated rabbits were kept between 25 and 30 mmol/l by insulin supplementation twice daily. Blood glucose in healthy rabbits is about 5 mmol/l

3 Metabolic Changes in Diabetic Rabbits and Their Blastocysts

3.1 Insulin and Glucose

First, we studied the insulin and glucose concentrations in the alloxan-treated females and their offspring. In diabetic rabbits, the plasma insulin concentrations were decreased by 85% to 15 pM. As rabbit blastocysts do not express insulin and due to the dramatic decrease in maternal insulin, no insulin could be identified in day 6 pc blastocysts recovered from diabetic mothers (Ramin et al. 2010). The measured uterine glucose concentrations reflected the plasma glucose concentrations in healthy and diabetic rabbits, respectively, albeit on a different scale. In diabetic rabbits, the plasma glucose concentration was kept to about 25 mM (see Fig. 1; glucose levels in controls approximately 5 mM). In the uterine cavity of such females, the glucose levels were clearly lower than in plasma but about three times higher than in healthy controls (1.75 mM compared to 0.5 mM in controls; Ramin et al. 2010). Therefore, metabolic disorders like missing insulin and hyperglycemia do reach the embryo and its developmental milieu right from the start at conception.

In plasma and uterine fluid and in blastocysts from diabetic rabbits, various significant metabolic changes are noticeable. As a more general observation, two major findings should be mentioned first. Blastocysts grown in a diabetic mother are

retarded in development (Ramin et al. 2010, Thieme et al. 2012a, b). Secondly, diabetes changes major metabolic pathways in the mother and in the embryo. So far, all pathways being investigated were altered. These changes, however, were associated with various compensatory actions by the mother and the embryo. For example, the preimplantation embryo compensates the loss of insulin and systemic IGF I by an enhanced endogenous IGF I and IGF II expression (Thieme et al. 2012 b). It needs to be emphasized that most embryos survive and continue development. However, further development is based on an altered embryonic metabolism compared to normal development.

3.2 Adipokines

Adipokines (such as adiponectin; Schindler et al. 2013) and triglycerides (Schindler et al. 2014) are clearly increased in the blood of diabetic rabbits. In their blastocysts, adiponectin and the adiponectin receptor 1 (AdipoR1) expression is enhanced. If the blastocysts' cell lineages are compared, adiponectin expression is particularly stronger in trophoblast cells of diabetic blastocysts. Adiponectin stimulates blastocyst glucose uptake in vitro and may substitute insulin action in glucose uptake, for example, by GLUT4 translocation into the apical cell membrane in trophoblast cells (Fischer et al. 2010).

3.3 Lipid Metabolism

An astonishing and striking observation in our studies was a strongly increased accumulation of intracellular lipid droplets and lipogenic marker genes in embryoblast and trophoblast cells from diabetic blastocysts (Schindler et al. 2014). The increase in adiponectin expression could be involved in this effect. Adiponectin may play a crucial role in glucose uptake and altered lipid metabolism in diabetic rabbit blastocysts. Looking at lipogenic genes involved, for example, FABP4, FATP4, CPT1, SREBP1 and PPARs (Schindler et al. 2014, Schindler et al. unpublished), it becomes obvious that major lipid metabolism pathways such as fatty acid uptake and beta-oxidation are affected. Adiponectin may serve as a failsafe regulation for blastocysts surviving in a diabetic uterine milieu. However, survival is not the same as in normal development, as shown by metabolic changes and intracellular accumulation of lipids due to compensatory actions required for embryo survival. We consider these observations as good experimental proof of evidence for the developmental origin of health and diseases (DOHaD) theorem. The rabbit model has provided new and constant results. All metabolic pathways studied so far in rabbit embryos were changed. Importantly, these changes ("origins") start at a very early stage of development.

3.4 Amino Acids and AGEs

The branched chain amino acids (BCAA) leucine, isoleucine and valine were increased by a factor of two in maternal plasma, uterine fluid and in blastocysts compared to healthy controls (Gürke et al. 2015). The higher availability of BCAA induced embryonic mTORC1 (mechanistic/mammalian target of rapamycin complex 1) signalling (Gürke et al. 2015, 2016). mTOR is a cellular nutrient sensor and master regulator of protein metabolism. Enhanced mTORC1 signalling increased protein synthesis in day 6 rabbit blastocysts, particularly in trophoblast cells (Gürke et al. 2016), leading to another important consequence. These findings demonstrate that non-physiological developmental conditions affect embryo development in a cell lineage-specific manner. As the ontogenetic impact of embryoblast (ICM) and trophoblast varies stage-specifically during further development, a precise description of these changes is needed to better understand diabetogenic disorders in pre- and postnatal development.

An increase in advanced glycation end products is a well-known phenomenon in diabetics. AGEs are formed non-enzymatically by reactions between reducing sugars and amine groups on proteins. A diabetic environment leads to an increased protein oxidation and AGE formation (Buongiorno et al. 1997, Guosheng et al. 2009), as confirmed in our model. In rabbits, AGEs were increased in blood plasma of diabetic females (up to 50%), correlating closely with an AGE accumulation in the endometrium and in blastocyst cavity fluid. These increases were accompanied by an increased RAGE (receptor of AGE) mRNA expression in blastocysts (Haucke et al. 2014). The increased AGEs constitute a remarkable metabolic stress for embryonic cells. A proposed mechanism of AGE-induced cellular damage is the release of reactive oxygen species, particularly superoxide and hydrogen peroxide. ROS are naturally occurring in mammalian cells; however, an excess of ROS generation overloads the cells' anti-oxidant defence mechanisms and in turn leads to oxidative stress. This, again, was detected through the use of the indicator 2′, 7′-dichlorofluorescein-diacetate (DCF-DA). Employing the diabetic rabbit model, we demonstrated clearly higher ROS levels in blastocysts from diabetic than from healthy females (Fig. 2). Recently, it has been shown that offspring from mothers with polycystic ovary syndrome (PCOS) and gestational diabetes showed increased oxidative stress markers (Boutzios et al. 2013).

These metabolic changes occur at a very early and vulnerable ontogenetic developmental stage and may persist. An increase in AGEs would implicate long-term effects on further prenatal and postnatal development of embryos from diabetic mothers.

To conclude, maternal metabolic diseases such as the diabetogenic hypoinsulinemia and hyperglycemia significantly change periconceptional and preimplantation developmental conditions in utero. In preimplantation rabbit embryos, all major metabolic pathways are affected on various regulatory levels (microRNA (not discussed), mRNA, protein). Preimplantation rabbit embryos adapt and compensate

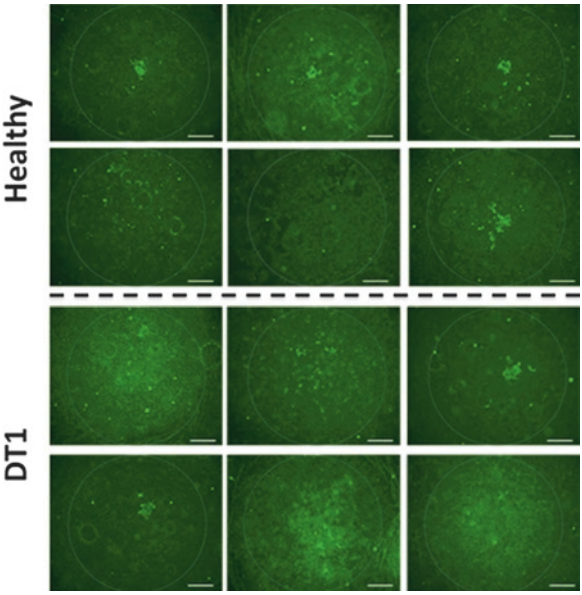

Fig. 2 Intracellular reactive oxygen species (ROS) in diabetic rabbit blastocysts. ROS measurements are shown in 6-day-old rabbit blastocysts developed under maternal diabetes mellitus type 1 (*DT1*) compared to the healthy control reference group (healthy). Blastocysts were stained with 2′, 7′-dichlorofluorescein-diacetate (DCFH-DA) and examined by fluorescence microscopy. Twelve individual blastocysts are shown ($N = 2$, $n = 6$). The area inside the circle marks embryoblast cells, outside trophoblast cells. Bars = 200 μm

for the non-physiological developmental conditions. The embryonic metabolism is characterized by a high plasticity which allows metabolic adaption and enables survival of most preimplantation embryos in a diabetic mother. Mechanisms identified are, amongst others, a hormonal switch from an insulin to an adiponectin-regulated metabolism with consequences for embryonic lipid and amino acid metabolism and accompanied by increased AGE and ROS levels.

The rabbit model shows that most mammalian embryos survive a diabetic pregnancy due to the high metabolic plasticity of embryonic cells, successfully overcoming cellular stress and damage, however, with potential long-term negative effects on pre- and postnatal development and the offspring's health.

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Long-Term Effects of the Periconception Period on Embryo Epigenetic Profile and Phenotype: The Role of Stress and How This Effect Is Mediated

James Ord, Alireza Fazeli, and Penelope J. Watt

Abstract Stress represents an unavoidable aspect of human life, and pathologies associated with dysregulation of stress mechanisms – particularly psychiatric disorders – represent a significant global health problem. While it has long been observed that levels of stress experienced in the periconception period may greatly affect the offspring's risk of psychiatric disorders, the mechanisms underlying these associations are not yet comprehensively understood. In order to address this question, this chapter will take a 'top-down' approach, by first defining stress and associated concepts, before exploring the mechanistic basis of the stress response in the form of the hypothalamic-pituitary-adrenal (HPA) axis, and how dysregulation of the HPA axis can impede our mental and physical health, primarily via imbalances in glucocorticoids (GCs) and their corresponding receptors (GRs) in the brain. The current extent of knowledge pertaining to the impact of stress on developmental programming and epigenetic inheritance is then extensively discussed, including the role of chromatin remodelling associated with specific HPA axis-related genes and the possible role of regulatory RNAs as messengers of environmental stress both in the

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intrauterine environment and across the germ line. Furthering our understanding of the role of stress on embryonic development is crucial if we are to increase our predictive power of disease risk and devise-effective treatments and intervention strategies.

Keywords Stress • Behaviour • Periconception • Hypothalamic-pituitary-adrenal (HPA) axis • Glucocorticoids • Psychiatric disorders • microRNAs

1 Introduction

Psychiatric disorders such as anxiety, depression, schizophrenia, post-traumatic stress disorder (PTSD), and autism spectrum disorder (ASD) represent an enormous source of human suffering and are one of the leading causes of disability (Kalueff et al. 2014; Vos et al. 2015). While these conditions have a broad range of effects on our cognition, awareness, mood, and our perception of reality, they all implicate dysregulation of our biological stress response system: the hypothalamic-pituitary-adrenal (HPA) axis. Responding appropriately to stressful situations is integral to our survival, but the mechanism by which we do so can be impaired, to the detriment of both our psychological and physical health.

Both genetic and environmental factors are likely to contribute to the risk of developing psychiatric disorders. The environmental contribution to risk is not only affected by our own experiences but, as a growing body of evidence now suggests, by our parents' experiences either during pregnancy or even before conception. Epidemiological data reveal that children whose mothers experience stress during pregnancy are at a higher risk of psychiatric disorders (Khashan et al. 2008), whilst rodent models show that gestational stress increases anxiety-like behaviour in adulthood (Lupien et al. 2009). The topic is further complicated by the interplay between stress and other aspects of our health; physical and psychological health often seem to go hand in hand, with patients suffering from psychological disorders at higher risk of detriment to their physical health, and vice versa (Bradley and Dinan 2010). For example, while children who were in gestation during the Dutch famine of 1944–1945 were found to be at an increased risk of metabolic syndrome (obesity and diabetes), their risk of psychiatric disorders, such as schizophrenia, also increased (Brown et al. 2000).

Due to the complexity of the human brain, in which psychiatric disorders manifest, to say that their underlying mechanisms and aetiologies are difficult to elucidate is a gargantuan understatement. Nevertheless, significant advances have been made in the past few decades in piecing together the developmental basis of the HPA axis and psychiatric disorders, using both epidemiology and model organism approaches. The following sections will address what we currently know about

stress, its underlying mechanisms, how stress may be dysregulated in disease, and the crucial relevance of stress and the HPA axis to the periconception period and disease risk.

2 What Is Stress?

Although its definition is somewhat ambiguous in the biological literature, the consensus becoming increasingly accepted is that stress entails a state of disrupted homeostasis which is counterbalanced by adaptive mechanisms known as *stress responses* (Barton 2002; Chrousos 2009). As aspects of the biotic and abiotic environment are in constant flux, organisms must continually respond to environmental changes through homeostasis mechanisms. However, excessive stimuli pose a threat to homeostasis and require a stress response to restore balance – a process also referred to as *allostasis* (Schulte 2014). Stimuli which induce a stress response may be referred to as *stressors*.

The stress response underlies the body's often extraordinary ability to respond to unexpected danger, colloquially known as the *fight or flight* response (Sorrells et al. 2009). However, stress, by definition, is something we are not adapted to cope with excessively or repeatedly. Thus, excessive environmental stress impairs an organism's fitness due to 'wear and tear' referred to as *allostatic load* (Schulte 2014). The stress response is best geared towards restoring homeostasis in the face of single or *acute* stressors, while allostatic load accumulates in the face of prolonged or *chronic* stress, which may constitute several exposures, perhaps over a substantial duration of an organism's life cycle (Sorrells et al. 2009). Arguably the most significant source of chronic stress relevant to modern life is psychological, resulting from negative socioeconomic factors such as job insecurity, financial problems, bereavement, and other personal struggles (Nargund 2015). The harsher end of chronic stress may include a prolonged state of real or perceived danger, such as domestic abuse or severe resource detriment (i.e. famine).

This chapter will adhere to the concept of stress relating to activation of the HPA axis. It is to be considered distinct from oxidative stress (excessive exposure to reactive oxygen species), which is a ubiquitous mechanism underlying several biological processes, but does not necessarily implicate the HPA axis.

2.1 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

When the sensory systems detect a threat to homeostasis, a stress response is initiated in order to evoke endocrine and behavioural responses to enhance survival in the face of stress and ultimately restore homeostasis. Biologically, the stress response entails the initiation and regulation of a suite of endocrine pathways

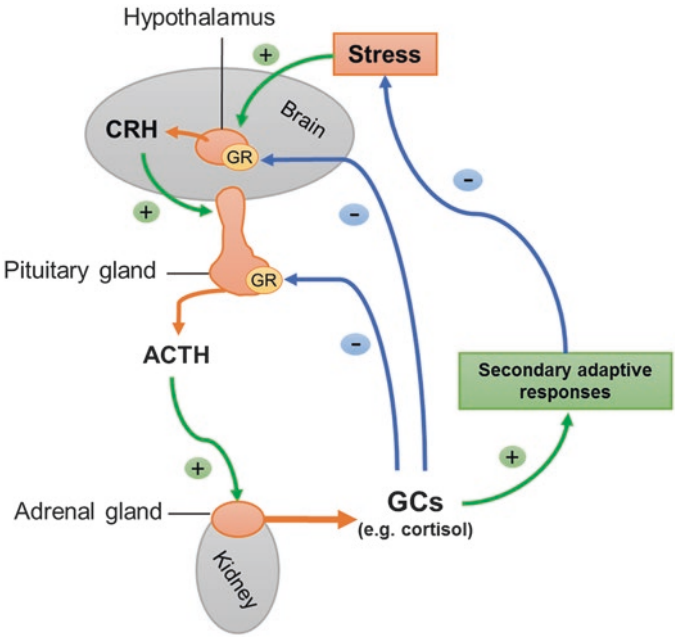

Fig. 1 Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis. Upon registration of a stress stimulus by sensory neurones, information is relayed to the paraventricular nucleus (PVN) in the hypothalamus. The PVN continuously synthesises corticotropin-releasing factor (*CRF*) which, in response to sensory stimuli, is secreted into portal blood vessels which lead to the pituitary gland. Here, binding of CRF to its receptors induces the release of adrenocorticotropic hormone (*ACTH*), which enters the systemic circulation. Circulating ACTH reaches the adrenal cortex, situated along the perimeter of the adrenal gland, and upon reception stimulates the production and release of glucocorticoids (*GCs*) from the adrenal gland. GCs interact with glucocorticoid receptor (GR) to enact secondary adaptive responses, as well as the inhibition of the HPA axis via negative feedback. Plus signs: stimulatory effects, minus signs: inhibitory effects

embodied by the hypothalamic-pituitary-adrenal (HPA) axis (Smith and Vale 2006; Bradley and Dinan 2010) (Fig. 1). As the name would suggest, the principal structures of the HPA axis are the hypothalamus (within the brain), pituitary gland (at the base of the brain), and adrenal glands (above the kidneys). In short, registration of a stress stimulus triggers a cascade of neuronal and endocrine events, culminating in the release of glucocorticoids (GCs) as the primary stress response, which interact with glucocorticoid receptors (GRs) to enact a variety of secondary adaptive responses.

The name ‘glucocorticoid’ derives from early observations that the hormones are involved in glucose metabolism. The primary GC hormone in humans is cortisol, which initiates and regulates a suite of adaptive responses: it interacts with the central nervous system to induce changes in cognition and awareness; stimulates increased glucose production, providing readily available energy for responding to an immediate threat (i.e. *fight or flight*); and inhibits costly immune functions. Since the discovery of their immunosuppressive properties in the 1940s, GCs have provided useful anti-inflammatory drugs, which have been used to treat inflammatory

diseases such as rheumatoid arthritis and asthma (Lupien et al. 2007). To regulate the stress response, cortisol also has an inhibitory effect on HPA activity in the hypothalamus, which establishes a negative feedback loop essential to healthy HPA axis functioning. The effects of cortisol and other GCs are mediated by the glucocorticoid receptor (GR) and a cytosolic protein complex composed of heat-shock proteins (HSPs) and expressed in almost every cell type in the body. Following stress, GCs extensively occupy GRs, which enact transcriptional modifications either via binding with transcription factors or as transcription factors themselves via direct interaction with glucocorticoid response elements (GREs). Thus, cortisol induces up- or downregulation of several genes, leading to the synthesis of enzymes responsible for glucose production, neurotrophic factors, and immunosuppressive factors. Cortisol dampens the stress response via the suppression of corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH) following GR binding in the hypothalamus and pituitary gland, respectively (Fig. 1).

Abnormal HPA axis functioning is associated with numerous pathologies, including both physical and psychiatric disorders. Both genetic and environmental factors may contribute to HPA axis dysfunction, which usually implicates imbalances of GCs, GRs, or both. Within the brain, GRs occur at high concentrations in the hippocampus, which is concerned with learning, memory, and attention (Lupien et al. 2007), and in the limbic system, which is responsible for emotion (Harris et al. 2013). Therefore, imbalances in levels of GCs or GRs have the potential to adversely affect attention span, emotional state, and other aspects of cognition. The association between GCs and psychiatric disorders first became evident in the 1950s through the increased incidence of psychosis in patients receiving GC therapy. These patients displayed gradually rising euphoria or dysphoria culminating in manic episodes, a condition which became known as ‘steroid psychosis’ (Lupien et al. 2007). Since then, imbalances in GCs and GRs have been implicated in major depressive disorder (MDD) (Alt et al. 2010), schizophrenia (Bradley and Dinan 2010), post-traumatic stress disorder (PTSD) (Palma-Gudiel et al. 2015a, b), and almost all anxiety disorders (Faravelli et al. 2012). The development of psychiatric disorders is frequently associated with chronic stress, such as childhood trauma, suggesting that HPA axis dysregulation may be induced by prolonged allostatic load at critical developmental stages (Heim and Nemeroff 2001; Lupien et al. 2009). This is possible due to the neuroplasticity of the early brain – its ability to reorganise its structure in response to intrinsic and extrinsic stimuli (Fenoglio et al. 2006; Cramer et al. 2011) – and is now thought to be mediated by chromatin remodelling associated with GR. In rodents, for instance, repeated psychological stress leads to increased phospho-acetylation of histone H3 in the hippocampus, but this is prevented by treatment with GR antagonists (Kolber et al. 2009). Furthermore, hypomethylation of the *NR3C1* gene (encoding GR) is found in PTSD patients (Palma-Gudiel et al. 2015a, b), while long-term alterations in DNA methylation in *NR3C1* promoter regions have been suggested to mechanistically link MDD with childhood trauma (Alt et al. 2010).

Psychological illness is frequently associated with physical ill health. This may owe partly to the fact that the HPA axis does not only regulate the response to stress

but also influences many other bodily processes including cardiovascular function, energy provision, fat deposition, and immune responses (Kolber et al. 2009; Sorrells et al. 2009; Bradley and Dinan 2010). Thus, as well as affecting psychological health, disruption of HPA axis function through stress may have consequences for physical health. For example, excessive production of glucose resulting from over-exposure to GCs may result in metabolic disorders such as type 2 diabetes (Bradley and Dinan 2010). Furthermore, the immunosuppressive properties of GCs leave the body open to infection in states of chronic stress. In mice, for instance, chronic psychological stress and subsequent increase in endogenous GCs induce downregulation of antimicrobial peptides, increasing the severity of a bacterial skin infection (Aberg et al. 2007). In contrast, however, in some cases of acute stress, GCs may also enhance the immune response in the central nervous system (Sorrells et al. 2009).

The HPA axis comprises an ancient mechanism which is largely conserved across the vertebrate subphylum; however, some interspecies differences (and similarities) are worthy of note. Importantly, rodents utilise corticosterone instead of cortisol as their primary GC hormone. Teleost fish possess an equivalent to the HPA axis called the hypothalamic-pituitary-interrenal (HPI) axis, although the core stress response mechanism is virtually identical to its mammalian counterpart, and it is also noteworthy that fish, like humans, utilise cortisol as their principal GC hormone. In fact, some of the core endocrine components of the HPA axis are so deeply rooted in the evolutionary substrata that they form part of equivalent stress response systems in invertebrates (Ottaviani et al. 1994; Couto-Moraes et al. 2009).

Because of the conserved nature of the stress response apparatus, methods for robustly quantifying behavioural and physiological stress responses have been developed in order to study HPA axis dysregulation in model organisms, including rodents and, more recently, fish. Rodent models of chronic stress typically entail a cocktail of stressful procedures administered daily, such as physical restraint or crowding, electric shock, exposure to fox odour, constant light, or loud noises (Takahashi et al. 1998; Aberg et al. 2007; Jensen Peña et al. 2012; Howerton et al. 2013). Cortisol or corticosterone concentrations in plasma or whole body samples (in the case of fish) can be quantified using enzyme-linked immunosorbent assay (ELISA) (Cachat et al. 2010), while several behavioural paradigms have been developed to quantify anxiety-like behaviour (Fig. 2). These include the open field test, which relies on rodents' innate aversion to an unfamiliar environment and generally uses the time spent at the edge of the test arena (a behaviour called thigmotaxis) as a measure of anxiety (Prut and Belzung 2003), and the light-dark preference test, which relies on rodents' aversion to bright light (referred to as scototaxis) and uses time spent in darkness as a measure of anxiety (Bourin and Hascoët 2003; Arrant et al. 2013). Tests designed for rodents have been successfully adapted for use with zebrafish (*Danio rerio*) and other teleosts (Champagne et al. 2010; Ariyomo et al. 2013), while unique assays to measure anxiety-like behaviour in fish have also been developed, such as the novel tank diving test, which uses the depth of a fish in an unfamiliar tank as a measure of anxiety (Egan et al. 2009). For more extensive coverage of behavioural tests used to assess stress phenotypes in model organisms, readers are directed to Kumar et al. (2013) for rodents and Stewart et al. (2012) for fish.

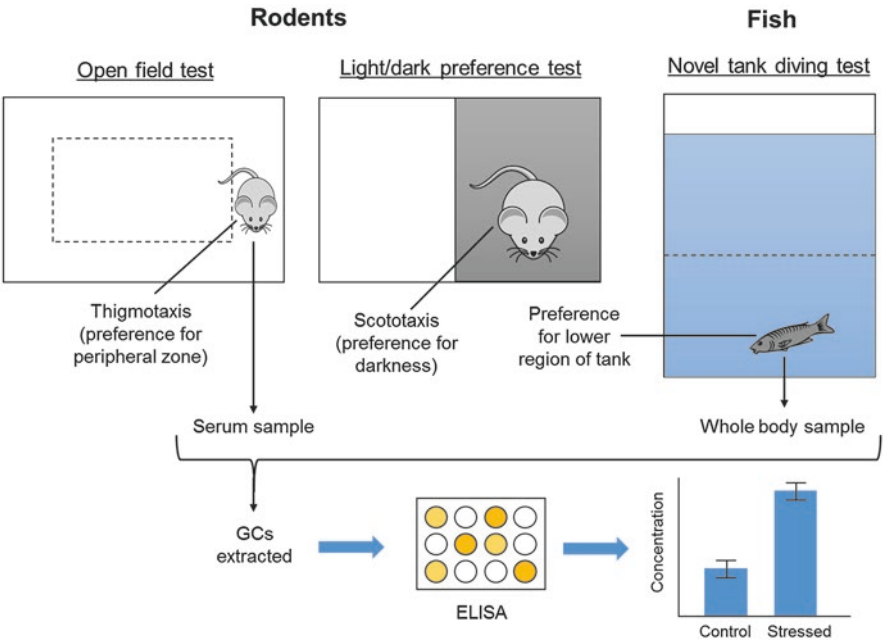

Fig. 2 Examples of quantitative behavioural and physiological stress phenotypes in rodents and fish. Strong anxiety-like behaviour (e.g. excessive thigmotaxis, scototaxis, or time spent in the lower region of a novel tank) is generally exhibited in response to HPA (mammals) or HPI (fish) axis activation by a stressor. Abnormal levels of anxiety-like behaviour detected using these measures may be indicative of dysregulation of the HPA or HPI axis, which may result from chronic stress. Following behavioural testing, cortisol may be extracted from serum or whole body samples and quantified using ELISA

3 Stress Dysregulation and Periconception

We have so far defined stress and are aware of the core components of the stress response (the HPA axis) and the pathologies that may arise from its dysregulation and how these can be translated into measurable phenotypes using model organisms. The remainder of this chapter outlines how HPA axis dysregulation, either by stress or other influences, during the periconception period may exert long-term effects on disease risk, via epigenetic alterations enacted during embryonic (or gametic) development. Pathologies associated with maternal stress, malnutrition, and alcohol exposure during pregnancy implicate HPA-axis dysregulation, which may be induced via long-term alterations to chromatin structure, in turn mediated by complex placental transduction pathways. There is also evidence to suggest that paternal stress influences embryonic development via epigenetic factors transmitted in sperm, although much remains unknown regarding the underlying mechanisms. Figure 3 presents a visual summary of molecular and phenotypic effects of periconception stress which have been identified in rodent models.

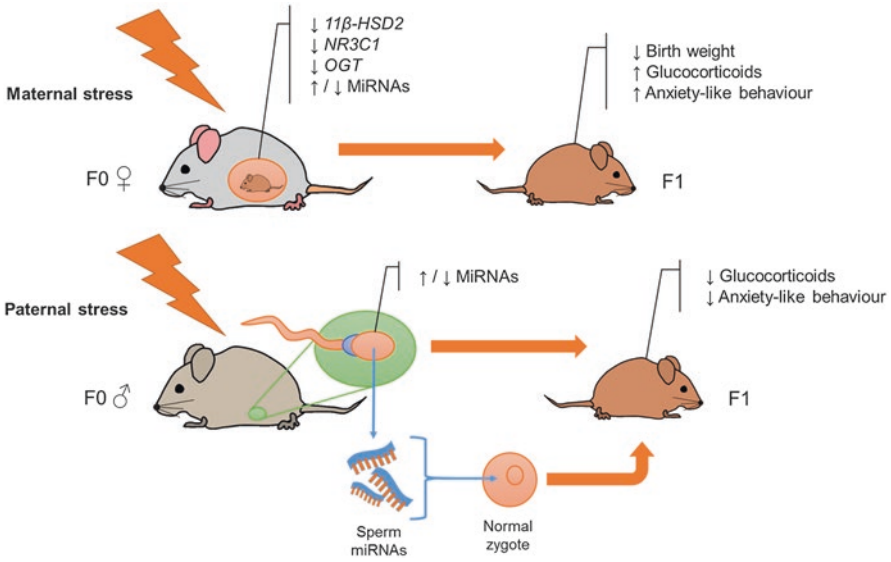

Fig. 3 Summary of molecular pathways altered by chronic stress in the periconception period and phenotypic effects in offspring. Chronic maternal stress in the prenatal period induces downregulation of *11β-HSD2* (Conradt et al. 2013), *NR3C1* (Cottrell and Seckl 2009; Conradt et al. 2013), and *OGT* (Howerton and Bale 2014) in the placenta and/or foetus and postnatal phenotypes indicative of HPA axis hyperactivity (increased glucocorticoids and anxiety-like behaviour) (Lupien et al. 2009). Chronic paternal stress in early life (Gapp et al. 2014), adolescence, or adulthood (Rodgers et al. 2013) alters sperm RNA composition and induces phenotypes indicative of suppressed HPA axis activity (decreased glucocorticoids and anxiety-like behaviour) in subsequent offspring. Insertion of sperm RNAs from stressed males into normal zygotes by microinjection recapitulates the paternal stress phenotypes in resultant pups (Gapp et al. 2014; Rodgers et al. 2015)

3.1 Maternal Influences

The prenatal period is now understood to be one of the most crucial stages of the human life cycle in terms of our future health and wellbeing, both physically and psychologically. Prenatal stress, which may include domestic abuse, is associated with increased risk of adverse birth outcomes, such as preterm birth (Lilliecreutz et al. 2016) and growth retardation (Cottrell and Seckl 2009), while evidence has grown over the past few decades to link psychological stress during gestation with longer-term developmental outcomes. Depression during pregnancy, which affects up to 10% of women in the UK (Vigod and Wilson 2016), with similar statistics reported in the USA (Kinsella and Monk 2009; Melville et al. 2010; Stewart 2011), has been shown to be a predictor of neurodevelopmental disorders in children and adolescents, while maternal stress during the first trimester of pregnancy is associated with increased risk of schizophrenia (Khashan et al. 2008), suggesting neurodevelopment is sensitive to stress during this early window. Prenatal famine

exposure, studied in the Dutch famine cohort, has been associated with an increased risk of psychiatric disorders, including a twofold increase in schizophrenia and related conditions (Brown et al. 2000), while foetal alcohol exposure is associated with later onset of depression and anxiety (Hellemans et al. 2010). Although several factors (e.g. postnatal influences) may play a role in these observed effects, there is extensive interest in, and growing evidence for, the impact of stress on prenatal development (particularly in relation to the HPA axis) via alterations to in utero physiology and epigenetic programming (Kinsella and Monk 2009; Palma-Gudiel et al. 2015a, b). Such alterations undoubtedly involve complex interactions between the maternal environment, the placenta, and the developing embryo (Howerton et al. 2013).

GCs play several essential roles in embryonic development, particularly of the neural tissues (Harris and Seckl 2011), but overexposure to GCs resulting from stress has adverse consequences for prenatal development (Lupien et al. 2009). In rats, chronic stress during pregnancy increases corticosterone in both mother and foetus (Takahashi et al. 1998), which mediates increased anxiety-like phenotypes in adult offspring (Barbazanges et al. 1996; Lupien et al. 2009). GCs, which are employed for glucose production, are also increased in both mother and foetus during the state of chronic stress induced by undernutrition (Blondeau et al. 2001) and as a result of alcohol exposure (Liang et al. 2011). Thus, HPA axis dysregulation resulting from overexposure to GCs may underlie pathologies associated with maternal stress and undernutrition (Brown et al. 2000), as well as foetal alcohol syndrome (Hellemans et al. 2010).

The molecular aetiology of developmental programming of the HPA axis in response to prenatal stress is likely to include epigenetic alterations to target chromatin, as chromatin organisation affects the levels of expression of associated gene sequences (Cottrell and Seckl 2009). Differential expression of three key placental genes has been implicated in prenatal stress: 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*), glucocorticoid receptor (*NR3C1*) (Conradt et al. 2013), and O-linked N-acetylglucosamine transferase (*OGT*) (Howerton et al. 2013). In addition, a host of regulatory RNAs have been implicated in developmental programming. However, these are likely only a few of the factors contributing to the byzantine dialect between the environment, placenta, and developing embryo, in which much remains to be elucidated.

11β-HSD2 regulates foetal GC levels by converting cortisol into inert cortisone, thus protecting the foetus from GC overexposure. Maternal stress, anxiety, and undernutrition induce downregulation of *11β-HSD2*, which has been shown to correlate with reduced birth weight, as well as HPA axis dysregulation and anxiety-like behaviour (Cottrell and Seckl 2009; Conradt et al. 2013). Similar outcomes are observed in homozygous knockout mice (*11β-HSD2−/−*) (Cottrell and Seckl 2009). In rats exposed to chronic prenatal stress, foetuses possess reduced expression of *11β-HSD2* and increased CpG methylation in the *11β-HSD* promoter region in hypothalamic tissue (Jensen Peña et al. 2012), while human mothers who report

anxiety during pregnancy possess greater placental methylation of *11β-HSD2* (Conradt et al. 2013). Collectively, the evidence suggests that *11β-HSD2* is an important component of the molecular interface between the maternal environment and the developing foetus and thus significant to the aetiology of stress-induced developmental pathologies.

NR3C1 is the gene encoding the glucocorticoid receptor (Conradt et al. 2013). Like GC, GRs are essential for normal development. For example, homozygous GR knockout mice die in the first few hours of life due to severely impaired lung development (Kolber et al. 2009). Likewise, reduction in *NR3C1* expression by 30–50% in transgenic mice leads to exaggerated HPA axis responses to stress (Michailidou et al. 2008). There is now evidence to link this differential expression to targeted epigenetic reprogramming in response to prenatal stress. For example, mothers who report depression during pregnancy have higher methylation of placental *NR3C1* (Conradt et al. 2013), while domestic abuse during pregnancy is significantly associated with methylation in the *NR3C1* promoter in adolescent offspring (Radtke et al. 2011). A recent meta-analysis of human DNA methylation data from 977 individuals revealed that methylation of a single CpG site in the promoter region of *NR3C1* was significantly associated with prenatal stress (Palma-Gudiel et al. 2015a).

Another factor recently implicated in the link between prenatal stress and disease risk is O-linked N-acetylglucosamine (O-GlcNAc) transferase (Ogt). The enzyme is a key cellular regulator which modifies, by addition of O-GlcNAc, protein targets responsible for chromatin remodelling (e.g. RNA polymerases, histone deacetylases) (Howerton et al. 2013). Ogt also preferentially associates with TET proteins (regulators of DNA methylation state) in close proximity to CpG-rich transcription start sites (Vella et al. 2013). Maternal stress leads to reduced expression of placental *OGT*, and *OGT*-knockout mice develop HPA axis dysregulation characteristic of that induced by stress in early pregnancy (Howerton and Bale 2014). Deficiency of Ogt is hypothesised to underlie observations of male-biased risk of neurodevelopmental disorders, as it escapes X chromosome inactivation in the placenta and is thus expressed at higher levels in females (Howerton et al. 2013). Furthermore, because O-GlcNAc is produced from glucose, Ogt is a potent sensor of cellular nutritional status and is thought to be similarly responsive to other aspects of the environment (Zachara and Hart 2004; Love and Hanover 2005; Vella et al. 2013). Because of this, and because of its interaction with TET proteins and other factors associated with chromatin remodelling (Vella et al. 2013; Howerton et al. 2013), it is plausible that Ogt is a key mediator of stress-induced epigenetic alterations associated with *11β-HSD* and *NR3C1*.

In addition to, and very likely in conjunction with, DNA methylation, small noncoding RNAs are now believed to be essential regulators at the crossroads of genes, development, and environment. MicroRNAs (miRNAs) are small noncoding RNA molecules (~22 nucleotides) which modulate gene expression by either repressing translation or inducing degradation of target mRNAs (Hollins and Cairns 2016). They are abundant in the brain and exhibit brain region-specific expression patterns in response to acute and chronic stress in animal models (Hollins and Cairns 2016), suggesting they are important in neuroplasticity. Subsequently, there is now evidence

that miRNAs are key mediators of stress-induced neurodevelopmental pathologies. In response to gestational stress, one study revealed that the brains of new-born mice exhibit differential expression of over 336 miRNAs (Zucchi et al. 2013). Several of these miRNAs are involved in neurodevelopment and have been implicated in psychiatric disorders, including miR-219, which is upregulated in patients with schizophrenia. This differential miRNA expression was subsequently demonstrated to persist into the F2 generation, suggesting miRNAs may play a role in transgenerational programming of the oocyte (Yao et al. 2014) and thus may mediate epigenetic inheritance of disease risk. Interestingly, among the downregulated miRNAs were miR-200b, which is implicated in uterine contractibility, and thus may provide a putative mechanistic explanation for preterm birth associated with gestational stress (Yao et al. 2014).

When considering long-term implications of prenatal stress on HPA axis development, the neuroplasticity of the early postnatal brain (Cramer et al. 2011) must also be considered, as some lines of evidence suggest that alterations to HPA axis development in the prenatal period can be attenuated by intervention in the neonatal period. For example, rats exposed to handling during the preweaning period exhibit permanent reductions in corticosterone secretion and GR expression (Welberg and Seckl 2008), and consequently, neonatal handling has been found to eliminate some of the adverse effects of foetal alcohol exposure, such as increased weight gain (Weinberg et al. 1995), and HPA axis hyperactivity (Ogilvie and Rivier 1997). However, subsequent experiments have produced conflicting results in this regard (Gabriel et al. 2000).

3.2 Paternal Influences

The vast majority of literature on parental environmental influences on HPA axis development has focused on maternally mediated effects. Understandably, given that humans are confined to the maternal environment for the first 9 months, it was long thought that the paternal environment was of little importance. However, it has since become apparent that the spermatozoon provides to the embryo more than simply a haploid genome, and subsequently the paternal environment (particularly paternal stress) is becoming increasingly implicated in offspring disease risk, including HPA axis dysregulation.

Chronic psychological stress has long been perceived to be a potential risk factor in male infertility, and, although epidemiological studies have produced conflicting conclusions regarding the association, evidence is building that chronic psychological stress can significantly impair aspects of male fertility (Nargund 2015). Several clinical studies have now demonstrated an inverse relationship between psychological stress and semen parameters. For example, a recent analysis revealed an association between perceived stress or recent stressful life events and a reduction in sperm concentration, motility, and normal morphology (Janevic et al. 2014). Mediated by GCs, stimulation of the HPA axis is now believed to have a direct inhibitory effect

on the hypothalamic-pituitary-gonadal (HPG) axis, which drives key reproductive functions in both sexes, including spermatogenesis (Nargund 2015). Specifically, GCs inhibit the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, the downstream consequences of which include a reduction in testosterone, which is an essential regulator of spermatogenesis at several stages (Smith and Walker 2014; Nargund 2015).

In contrast to maternal effects, in which germ line-mediated effects are difficult to discern from in utero effects on development, paternal effects on phenotype are more likely to represent the germ line transmission of environmental information. Germ line epigenetic inheritance has long been a puzzle due to the problem of erasure: the DNA methylation status of the parental genomes is reset during the first few cell divisions, and thus it is widely thought that most alterations to methylation acquired during the parents' lifetime are erased (Cantone and Fisher 2013). However, acquired methylation changes may escape erasure in some cases, and other types of transmissible epigenetic factors are not subject to erasure, including a host of regulatory RNAs. Specifically, miRNAs have been heavily implicated, while much is yet to be deciphered regarding the possible role of sperm histones and protamines in the conveyance of environmental information.

A handful of studies have identified heritable alterations in measurable phenotypic aspects of HPA axis activity induced by paternal stress at different developmental stages. Male mice subjected to a chronic stress paradigm (maternal separation) in early life develop depression-like symptoms, as well as phenotypes consistent with dampened HPA axis responsivity such as reduced anxiety-like behaviour and reduced corticosterone in response to stress. These phenotypes were found to be inherited by the offspring, even when fertilisation was carried out in vitro. RNAs were found to be integral to this environmental inheritance, as the injection of sperm RNA from traumatised males into normal zygotes recapitulated the observed phenotypes in the resulting pups (Gapp et al. 2014).

In addition to stress in early life, chronic stress experienced both in adolescence and during spermatogenesis (approx. 42 days in mice) in adulthood has been found to induce heritable alterations in measurable aspects of HPA axis activity. One dramatic example is the inheritance of a Pavlovian response, in which adult male mice were conditioned to associate the odour of acetophenone with an electric shock. The offspring of these mice, when presented with the same odour, exhibited a startle response without ever experiencing the electric shock. *Olfr151*, the gene encoding the odorant receptor for acetophenone, was found to possess CpG hypomethylation in the sperm of both F0-conditioned and F1-naïve males (Dias and Ressler 2014). However, whether the methylation state escaped erasure or was inherited by another mechanism is not clear.

A similar study (Rodgers et al. 2013) reported that male mice subjected to a 42-day chronic stress paradigm in either adolescence or adulthood sired offspring with dampened HPA axis activity, characterised by significantly lower corticosterone in response to stress compared to controls. These offspring also exhibited altered transcriptional profiles in the hypothalamus, including enriched expression of GC-responsive genes and gene sets associated with chromatin remodelling

(e.g. histone acetyltransferases). The researchers identified nine miRNAs exclusively expressed in the sperm of stressed males, the predicted targets of which included DNA methyltransferase 3a (*DNMT3a*), a critical regulator of de novo DNA methylation (Rodgers et al. 2013). Remarkably, in a subsequent study, these authors reported that inserting only these nine miRNAs into normal zygotes was sufficient to induce the same phenotype indicative of paternal stress (Rodgers et al. 2015). Similarly, it has been reported that injection of candidate miRNAs into normal zygotes recapitulates hereditary metabolic syndrome associated with paternal obesity (Grandjean et al. 2015). Thus, taken together, the evidence provides a strong case for miRNAs as a principal language of environmental inheritance.

Another interesting observation derived by comparing the published experiments is that similar hereditary HPA axis dysregulation occurs in response to paternal stress, irrespective whether stress is experienced in early life (Gapp et al. 2014), adolescence (Rodgers et al. 2013), or adulthood (Rodgers et al. 2013; Dias and Ressler 2014). This suggests that even though the phenotypes were induced in response to stress in different developmental stages, the underlying mechanism may be very similar if not the same. Extracellular vesicles are hypothesised to be important for intercellular communication via the exchange of genetic information in plants and animals (Mittelbrunn and Sánchez-Madrid 2012) and may be responsible for the transport of stress-induced miRNAs into sperm. It is also possible that stress-induced testosterone deficiency (Nargund 2015) may play a role in miRNA-mediated inheritance, as testosterone is known to regulate the expression of several miRNAs in Sertoli cells in the testes (Panneerdoss et al. 2012; Smith and Walker 2014). Interestingly, two of the nine stress-responsive sperm miRNAs discovered by Rodgers et al. (2013) (miR-25c and miR-375) are also regulated by testosterone, as shown using a mouse model of testosterone deprivation (Panneerdoss et al. 2012). MiR-375, well-characterised in terms of function, is important for the development of the pancreas and pituitary gland, while little is known about the miR-25 family except that they are implicated in cardiac function (Wahlquist et al. 2014).

Another possibility is that stress may influence offspring phenotypes via post-translational modification to sperm chromatin structure, specifically histones and protamines. Chromatin undergoes extensive reorganisation during spermatogenesis, in which most histones are supplanted by protamines (Luense et al. 2016). Numerous unique protamine modifications, particularly acetylation and methylation, have been discovered in human and mouse sperm (Brunner et al. 2014), prompting the hypothesis that these decorations may play an important role in the epigenetic regulation of embryonic development following fertilisation and furthermore may represent mediators of germ line epigenetic inheritance (Luense et al. 2016). Although most paternal histones and protamines are believed to be replaced by maternally inherited histones soon after fertilisation (Cantone and Fisher 2013), sperm histone marks retained at fertilisation have recently been reported to be essential for correct gene expression in *Xenopus* embryos (Teperek et al. 2016). There is still very little known regarding sperm histone and protamine post-translational modifications, including the extent to which they may be subject to external environmental influences, and thus more attention is needed in this area of research.

Although the underlying mechanisms remain elusive, it is clear that environmentally induced reprogramming occurs not just in the developing embryo but in developing germ cells. The observation that the same phenotypes induced by paternal stress in early life and adolescence is also induced by stress during spermatogenesis suggests that, rather than resulting from long-term alterations to germ cell precursors, modifications to maturing germ cells occur transiently in response to long-term alterations to HPA axis functionality. If this is the case, effective therapy and restoration of normal HPA axis function may halt or at least reduce the modification of maturing germ cells, preventing the inheritance of pathologies. Alternatively, if miRNAs do indeed constitute the principal language of environmental inheritance, blocking those miRNAs upregulated by paternal stress (or supplementing those downregulated) may prevent this differential expression from manifesting in pathologies in the offspring.

So far, paternal effects mediated by miRNAs have been identified only in rodent models, with some evidence of similar mechanisms existing in *Caenorhabditis elegans* (Grossniklaus et al. 2013). There is evidence that environmental exposures can influence the miRNA content of human sperm (Marczylo et al. 2012), and it has been suggested that paternal trauma or experience of violence, such as in the case of war veterans and holocaust survivors, may be paternally transmitted and influence offspring mental health (Vaage et al. 2011). However, little evidence has emerged from epidemiological studies to suggest that such paternal exposures are transmissible down the human germ line (Yehuda et al. 2001; Vaage et al. 2011), and such associations may be more likely to arise due to behavioural influences on children, rather than epigenetic transmission. Whether such mechanisms exist in distantly related vertebrates, such as fish, is not known, although non-genetic transgenerational phenomena associated with environmental stress have been observed in teleost fish (Miller et al. 2012), and miRNAs are known to play an essential role in teleost spermatogenesis (Babiak 2014). If the mechanisms of inheritance in other vertebrates are similar to those being delineated in rodents, it would hint at the evolutionary significance of miRNA-mediated environmental inheritance, and it is possible that the mechanism may hold an ancient adaptive function (Grossniklaus et al. 2013).

4 Conclusions and Future Directions

The aetiologies of psychiatric disorders remain frustratingly elusive, making efforts to devise effective treatments still difficult. However, recent studies in both humans and animal models have shown promise in uncovering the molecular basis of these conditions, including altered epigenetic states resulting from exposure in early life, gestation, or preconception. As high-throughput sequencing technologies and other molecular tools become more affordable and accessible, it will be possible to further address knowledge gaps pertaining to the mechanisms underlying long-term effects of periconception stress. For instance, although specific chromatin marks

and regulatory RNAs have been implicated in long-term effects of parental stress, there remain several such entities, such as long noncoding RNAs (lncRNAs), the functions of which we know very little about (Morris and Mattick 2014).

A well-established toolset already exists for studying stress dysregulation in model organisms, which continue to help further our understanding of this complex set of processes. There is now increasing interest in non-mammalian models, specifically zebrafish, which present an increasingly attractive avenue for the exploration of periconception stress. The rapid life cycle and easily manipulated transparent embryos of the zebrafish (*D. rerio*) have already made them one of the most powerful vertebrate tools available to embryologists, and there exists a well-developed toolset for studying their behavioural and physiological stress phenotypes (Cachat et al. 2010; Stewart et al. 2012). Zebrafish may also present a unique, high-throughput model for epigenetic effects associated with spermatogenesis, the duration of which is a mere 6 days in this species (Leal et al. 2009).

In addition, having uncovered previously unknown mechanisms of environmental inheritance in model organisms, further attention may be directed to epidemiology to determine the significance of these mechanisms in human populations. Unique miRNA profiles have already been identified in the sperm of smokers versus non-smokers (Marczylo et al. 2012), suggesting that other environmental influences, particularly stress, may affect gametic chromatin, with consequences for subsequent embryos. There is therefore a need to characterise miRNAs from gametes derived from humans suffering from chronic stress, as these may provide valuable molecular markers for risk of HPA axis dysregulation in subsequent generations.

In conclusion, an improved mechanistic understanding of environmental predisposition to HPA axis-related pathologies will have major benefits to public health, in the interests of both treatment and prevention. Increased knowledge of molecular pathways underlying disease risk may provide important biomarkers, such that those already at risk of psychiatric disorders may be identified, enabling early intervention to minimise long-term suffering. Increased knowledge of disease processes may also pave the way for the development of therapeutic agents to counteract the adverse effects of parental stress on offspring disease risk. Finally, increased awareness of environmental influences on development will help to further inform human lifestyles and behaviour, such that risk to subsequent generations is minimised.

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The Long-Term Effects of the Periconceptional Period on Embryo Epigenetic Profile and Phenotype; The Paternal Role and His Contribution, and How Males Can Affect Offspring's Phenotype/Epigenetic Profile

Emma S. Lucas and Adam J. Watkins

Abstract The number of adults afflicted with heart disease, obesity and diabetes, central components of metabolic disorder, has grown rapidly in recent decades, affecting up to one quarter of the world's population. Typically, these diseases are attributed to lifestyle factors such as poor diet, lack of exercise and smoking. However, studies have now identified strong associations between patterns of growth during foetal and neonatal life and an increase predisposition towards developing heart disease, obesity and diabetes in adult life. While the connection between a mother's diet and the long-term health of her offspring has been studied in great detail, our understanding of whether offspring health might be affected by a father's diet remains limited. Greater insight into the impact that paternal nutrition has on sperm quality, epigenetic status and potential offspring programming mechanisms is needed to redress this parental-programming knowledge imbalance. Disturbances in paternal reproductive epigenetic status represents one key mechanism linking paternal diet with the programing of offspring development and adult health, as many enzymatic processes involved in epigenetic regulation use metabolic intermediates to modify DNA and histones. Here, poor paternal nutrition could result in perturbed sperm and testicular epigenetic status, impacting on post-fertilisation gene transcriptional regulation and protein expression in offspring tissues, resulting in increased incidences of metabolic disorder in adult life.

Keywords Paternal nutrition • DNA • Histones • Metabolic syndrome • Reproductive fitness

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1 Introduction

It is estimated that up to 60% of global mortality is attributed to non-communicable diseases such as the metabolic syndrome (Wang et al. 2011). Typically, such diseases have been attributed to adult lifestyle characteristics such as poor diet, sedentary behaviour and smoking. However, research from world-wide human cohort data and a diverse range of animal models have established clear links between the size of individuals at birth, altered growth during infancy and an increased risk of developing cardiovascular disease, type 2 diabetes and obesity in adult life. Similarly, adult-onset degenerative diseases such as hypertension, osteoporosis and obstructive airway disease have also been linked to perturbed patterns of growth during foetal development and early life.

Studies have demonstrated that deficiencies or excess in a range of maternal macro- and micronutrients impact negatively on reproductive fitness, embryonic and foetal development, offspring growth and long-term health. Animal model studies, in conjunction with retrospective analyses of human cohort data, have shown that all stages of foetal and embryonic development, as well as the development and maturation of sperm and oocytes, are influenced directly by suboptimal environmental conditions, whether in vivo (parental diet) or in vitro (embryo culture) (Lee 2015; Yeung and Druschel 2013). Historically, studies have focused on the impact of maternal dietary deficiencies on long-term offspring growth. These have centred on the use of diets low in protein (Fleming et al. 2015), reduced in caloric content (Reynolds et al. 2015) or deficient for specific micronutrients such as iron (Radlowski and Johnson 2013). However, as the world today is faced with the dual burden of both excessive under- and overnutrition in human populations (Kelly et al. 2008; Martin et al. 2010b), a new focus into the impact of energy-dense diets on parental reproductive fitness and offspring long-term health has emerged.

While the significance of optimal maternal nutritional status during specific periods of gamete maturation and post-fertilisation development has received detailed investigation, it has only been over the past several years that research has begun to focus on the importance of male physiology and nutritional status at the time of conception. In men, obesity impacts negatively on testosterone levels, sperm number, sperm motility, embryonic development and live birth rates (Bakos et al. 2011a; Chavarro et al. 2011a; Kort et al. 2006b). In animal models, paternal nutritional manipulation has been shown to impact on embryonic metabolism, foetal growth and skeletal formation as well as long-term cardiovascular and metabolic health (Binder et al. 2012; Carone et al. 2010; Lambrot et al. 2013; Ng et al. 2010; Watkins and Sinclair 2014). Indeed, our increased understanding of potential mechanisms through which impaired paternal health may programme offspring long-term health has resulted in a new interest in defining semen quality and assessing sperm epigenetic status.

Global reproductive fitness is not only impacted on by the burden of increased levels of energy dense diets of low micronutrient content, but is faced also with the increase used of Assisted Reproductive Techniques (ART) such as IVF. The potential

long-term impacts of routine human embryo culture and manipulations were brought into question following observations that the in vitro manipulation, culture and transfer of cattle, sheep and mouse embryos results in significant changes in offspring growth, rates of neonatal mortality, blood pressure and gluconeogenic regulation and life span (Wrenzycki et al. 2004; Watkins et al. 2007; Rexhaj et al. 2013; Grace and Sinclair 2009; Calle et al. 2012). As a consequence, retrospective analyses of human children conceived through ART procedures have identified some associations between human embryo in vitro manipulation and low birth weight, pre-term delivery, altered epigenetic status, elevated blood pressure and glucose intolerance (Yeung and Druschel 2013; Scherrer et al. 2012; Nelissen et al. 2013; Dumoulin et al. 2010; Ceelen et al. 2008). With infertility affecting between 8% and 12% of reproductive-aged couples globally, and an estimated 1–3% of all births arising from some form of ART in developed countries (Inhorn and Patrizio 2015; Ombelet et al. 2008), more detailed and mechanistic studies are required to draw definitive conclusions regarding potential long-term health impacts of parental physiology, gamete quality and human embryo culture and manipulation on long-term health of resultant children.

With these topics in mind, the aim of this chapter is to provide insight and contemporary overview of mechanisms linking paternal environmental and nutritional status with early developmental processes and ultimately adult cardiovascular, metabolic and ageing health. Initially, we will summarise briefly current understanding of the impact of maternal nutrition during the periconceptional period, before assessing the role of paternal nutrition on male reproductive fitness and offspring development. This chapter will detail the impact of parental diet and physiological status on gamete quality, embryo development, foetal growth and long-term offspring health. The use of animal models has become a key tool in understanding the underlying mechanisms linking environmental factors with the programming of offspring health. Rodent models form a large proportion of studies due to their quick generation times, well characterised genomes and similar patterns of early embryo development to humans. However, larger animal models afford the opportunity to study foetal development over a longer period of time and, depending on strain, often only carry singleton foetuses (more reflective of human gestation). Therefore, this chapter will refer to data from human, rodent and, where appropriate, livestock species studies.

2 Developmental Programming

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that the maternal reproductive and uterine environment experienced during gestation can impact on offspring non-communicable disease risk susceptibility in adult life. Human clinical and epidemiological studies have accumulated a wealth of correlative data linking the quantity and/or quality of maternal gestational nutrition, foetal and placental development, birth weight, neonatal growth and chronic disease

risk in later life. These studies reproducibly demonstrate significant associations between altered birth weight (both increased and decreased) and the development of cardiovascular dysfunction, metabolic syndrome, psychological disorders and degenerative conditions such as osteoporosis (Barker et al. 1993; Hanson and Gluckman 2014). The first direct evidence that an adverse intrauterine environment may impact on long-term health came from the analysis of middle aged men and women in a Hertforshire cohort (Barker et al. 1993). Here, individuals who had a low weight at birth had increased death rates from coronary heart disease in adult life. Similar associations have been observed in separate human cohorts from around the world (Cheung et al. 2000; Dabelea et al. 1999; Forsen et al. 1997; Rich-Edwards et al. 1997).

From these diverse human epidemiological studies came the proposition that maternal nutritional status during gestation induces physiological changes in foetal development and growth, resulting in significant long-term changes in adult health. Nutritional signals received by the developing foetus promote adaptive responses within developing tissues and organism to maximise foetal and neonatal development for a perceived postnatal nutritional environment. However, if the pre- and postnatal nutritional environments are mismatched, these adaptations can become inappropriate and maladaptive for postnatal and adult life, resulting in increased risk for adult ill-health. With the link established between maternal diet, gamete quality and post-fertilisation development, it is easy then to apply these observations and mechanistic insights onto paternally mediated programming effects.

3 Maternal Nutrition

3.1 Obesity and Overnutrition

In the UK, between 1989 and 2007, the rates of maternal obesity have increased from 7.6% to 15.6% 9 (Heslehurst et al. 2010), while in the USA, reports indicate that the prevalence of pre-pregnancy obesity has risen to 28.5% (Hinkle et al. 2012). The negative impacts of obesity on female reproduction are well established; studies have shown that oocyte quality in obese women can be affected by elevated levels of triglycerides and free fatty acids within the follicular fluid surrounding the oocyte (Robker et al. 2009). Increases in insulin and androgen levels, ovarian dysfunction, type 2 diabetes, polycystic ovarian syndrome (PCOS) and endometrial hyperplasia are also observed as direct effects of maternal obesity (Kulie et al. 2011).

Mirroring observation in women, feeding female mice a high fat diet results in increased ovarian lipid accumulation, elevated levels of apoptosis within the ovary, reduced fertilisation rates and altered embryo mitochondrial reactive oxygen species (ROS) generation (Igosheva et al. 2010; Jungheim et al. 2010). Maternal high fat diet also perturbs mitochondrial metabolism, altering DNA content and membrane potential, inducing endoplasmic reticulum stress and caspase activation

(Igosheva et al. 2010; Jungheim et al. 2010; Wu et al. 2011). Those embryos that do develop form smaller foetuses which then display postnatal 'catch-up-growth', a well-established marker for adverse adult health in human epidemiological studies (Barker et al. 1993).

3.2 Nutrient-Deficient Diets

Epidemiological observations in adult offspring of women exposed to the Dutch Hunger Winter famine (1944–1945) have provided an opportunity to assess the long-term impact of maternal undernutrition restricted to specific period or stages of pregnancy. Maternal nutrient restriction during the first trimester of pregnancy was linked to increased prevalence of coronary heart disease, raised lipids and obesity in offspring (Ravelli et al. 1999; Roseboom et al. 2000; Roseboom et al. 2001), whereas famine occurring during late gestation led to decreased glucose tolerance in adult life (Ravelli et al. 1998).

As with maternal overnutrition, animal models have also identified maternal undernutrition during early development as a critical factor in programming offspring ill-health. Feeding female mice a low protein diet (LPD; 9% protein) exclusively during preimplantation development (first 3.5 days of gestation) induces offspring overgrowth and elevated systolic blood pressure in addition to vascular dysfunction and elevated patterns of offspring activity within an open field test (Watkins et al. 2008, 2011). Similar to the impact of a maternal high fat diet, LPD has been shown to alter both mitochondrial localisation and membrane potential (Mitchell et al. 2009). In addition, significant changes in maternal uterine amino acid profiles, insulin and glucose levels have been observed in female mice in response to periconceptional LPD (Eckert et al. 2012). These dietary-induced changes in maternal metabolic status may therefore induce metabolic stress within the preimplantation embryo, thereby altering foetal growth and development and, ultimately, programming life-long offspring ill-health (Fleming et al. 2015).

4 Paternal Programming of Offspring Development

In light of the significant impacts maternal periconceptional nutrition has on offspring development and life-long health, increased interest is being given to exploring the influence that paternal environmental manipulation can have on the development of their offspring. As with maternal mechanisms of offspring programming, animal studies have revealed themselves to be essential for the development of human-relevant models with which the underlying biological mechanisms can be investigated. For example, in Southeast Asia and Polynesia, the chewing of betel nuts is very popular. However, individuals who do so are at increased risk of developing metabolic syndrome (Lin et al. 2008) as well as increasing the risk in their

own offspring (Chen et al. 2006) irrespective of offspring betel nut consumption. Interestingly, in a mouse model in which males were exposed to betel nuts in their diet prior to mating, their offspring developed hyperglycaemia, mirroring effects observed in humans, and remained manifest for up to three generations (Boucher et al. 1994).

In many species, offspring development occurs in the physical absence of their fathers. Therefore, in order for paternal experiences to influence the development of their offspring, information must be faithfully transmitted at the point of fertilisation and stably incorporated into the offspring's genome. Sperm mediated programming through epigenetic transmission of heritable changes in chromatin structure offers one potential mechanism by which altered paternal physiology can impact on offspring development. The observation that a grandfather's prepubertal (8–12 years of age) nutritional status and growth can impact on the health and mortality risk of their grandsons, but not granddaughters, identifies the potential transgenerational impact of paternal programming (Kaati et al. 2002; Pembrey et al. 2006b). Such observations also indicate potential differential programming effects of the inherited X and Y chromosomes, dependent on the sex of the offspring inheriting them. In addition to a sperm-genomic contribution, seminal plasma constituents can also interact with maternal oviductal and uterine tissues, modulating the female reproductive environment during early embryo development, a critically sensitive period in development (Sinclair and Watkins 2013). This section will, therefore, highlight animal model and human studies demonstrating paternal programming of post-fertilisation development and discuss the role of epigenetics in mediating paternal influences.

4.1 Paternal Nutrition and Reproductive Fitness

Mirroring female reproductive fitness, male fertility is closely linked to nutrition, physiological status and body size. Daily rates of sperm production and sperm quality (i.e. sperm count and sperm motility) are decreased by undernutrition in rams (Parker and Thwaites 1972; Robinson et al. 2006), while in bulls, levels of nutrition affect testicular development and sperm production (Gauthier and Berbigier 1982; Vandemark et al. 1964). Male nutritional status can also affect testicular morphology (area and diameter of seminiferous tubules and the seminiferous epithelium (Martin et al. 2010a). In humans and rodents, elevated male BMI is associated with reduced sperm motility (Hammoud et al. 2009), increased incidences of sperm abnormality (Kort et al. 2006a) and sperm DNA fragmentation (Chavarro et al. 2011b), increased sperm reactive oxygen species levels, reduced serum testosterone and increased estradiol concentrations (Tunc et al. 2011). Consumption of a 'Western-style' diet high in sugar, fat and processed food associate with reduced sperm motility in men (Eslamian et al. 2012; Gaskins et al. 2012), while the consumption of energy dense diets in men and rodent models has been associated with poor sperm motility, morphology and DNA integrity (Agbaje et al. 2007), disturbed

testis metabolism (Rato et al. 2013) and impaired fertility (Bener et al. 2009). Reduced sperm DNA integrity, as associated with obesity and diabetes, correlates with reduced embryonic development and decreased pregnancy rates (Bakos et al. 2008; Bertolini et al. 2002; Seli et al. 2004).

4.2 Paternal Nutrition and Offspring Development

In men undergoing IVF treatment, obesity is associated with reduced blastocyst development and live birth rates (Bakos et al. 2011a). In mice and rats, the induction of paternal obesity, through the feeding of a high fat diet, increases sperm DNA damage (Bakos et al. 2011b), elevates sperm and testicular oxidative damage (Zhao et al. 2014) and reduces blastocyst development and implantation rates (Mitchell et al. 2011). Paternal dietary-induced obesity has also been shown to induce subfertility in both male and female offspring for up to two generations in mice (Fullston et al. 2012, 2013; McPherson et al. 2014). Here, male offspring displayed insulin resistance and hyperleptinaemia, while female offspring became obese with additional insulin resistance. Analysis of F2 generation offspring development revealed increased adiposity and insulin resistance in females, but increased adiposity and glucose intolerance in males. Interestingly, these negative effects on offspring development were prevented through paternal dietary and exercise interventions (McPherson et al. 2013; McPherson et al. 2014; Palmer et al. 2012), indicating sperm-mediated programming may be relatively transient and even reversible. In rats, the feeding of a high fat diet (41% fat) for up to 10 weeks prior to mating affected female offspring pancreatic β-cell function but not males, as well as increased body weight, glucose intolerance and impaired insulin secretion (Ng et al. 2010). Analysis of pancreatic gene transcript levels revealed differential expression of 61 genes involved in ATP binding, intracellular transport, calcium signalling, cell-cycle regulation, apoptosis and MAPK and Wnt signalling pathways. Offspring of male mice overnourished during neonatal life demonstrate glucose intolerance, fasting hyperglycaemia and insulin resistance, mirroring the metabolic disturbance seen in their fathers (Pentinat et al. 2010). Interestingly, F2 generation offspring only displayed mild hyperglycaemia, while neither F1 nor F2 offspring displayed the increased body weight observed in the overfed fathers. As the severity of offspring metabolic programming diminished with subsequent generations, these findings are suggestive of epigenetic mechanisms of gene expression regulation rather than stable, heritable modifications to DNA sequences (Gallou-Kabani and Junien 2005). Recently, Soubry et al. (2013) demonstrated a negative correlation between paternal obesity and the DNA methylation status of the IGF2 differentially methylated region in offspring.

Similar to the impacts of paternal obesity, paternal low protein diet (LPD) programmes the expression of genes involved in offspring hepatic lipid and cholesterol biosynthesis in offspring mice (Carone et al. 2010). Analysis of offspring tissue epigenetic status revealed genome-wide changes in DNA methylation, including the

key lipid regulator PPARα. In a rodent model of repeated paternal fasting prior to mating, significantly lowered offspring serum glucose, IGF-1 and corticosterone levels were observed (Anderson et al. 2006). We have shown that offspring from male mice fed LPD display increased weight at birth, reduced male:female offspring ratio, increased adult adiposity, hypotension, glucose intolerance and elevated serum TNF-α levels (Watkins and Sinclair 2014). Recently, the impact of a paternal low folate diet on offspring development has been demonstrated (Lambrot et al. 2013). Sperm from the low folate diet males displayed altered DNA methylation at genes associated with development, apoptosis, autism and schizophrenia. In the placenta, over 300 genes were differentially expressed in response to paternal low folate diet, while increased incidences of offspring craniofacial and musculoskeletal malformations were observed. The impact of parental undernutrition has typically been addressed through the use of supplementary dietary regimens with antioxidant compounds such as folate, zinc and iron (Das et al. 2013). In a recent mouse study (McPherson et al. 2016), the negative impact of a paternal undernutrition diet on sperm quality, testicular oxidative stress, fertility and offspring fat accumulation and dyslipidaemia were reversed through dietary vitamin and antioxidant supplementation. These differing models of paternal dietary manipulation all identify the suboptimally nourished father as a novel source of offspring health programming. In addition, they identify potential intervention strategies which could help alleviate adverse offspring health. Interestingly, some studies demonstrate paternal mediated programming may be reversible through simple dietary supplements of exercise regimens. These observations suggest the process of sperm maturation may be the critically sensitive stage during which paternal programming is established rather than becoming programmed permanently into the spermatogonial stem cells.

4.3 Paternal Toxicant Exposure

In humans, paternal and grand-paternal dietary and smoking behaviours have been shown to influence offspring and grand-offspring phenotype and mortality risk (Pembrey et al. 2006a), while paternal alcoholism is associated with reduced birth weight in offspring (Little and Sing 1987). Exposure of male mice and rats to alcohol impacts on offspring litter size, birth weight, developmental trajectory, mortality, immunity and behavioural characteristics such as spatial awareness, aggression, risk taking and anxiety-like behaviours (Abel 2004; Abel and Bilitzke 1990; Ledig et al. 1998; Meek et al. 2007). Similarly, males exposed to other drugs and environmental toxicants including opiates, ethylene dibromide, lead and cyclophosphamide sire offspring with a range of behavioural impairments, many transmitted to second and third generations via the male lineage (Hales and Robaire 2001).

Similar to nutritional and drug specific effects on paternal programming, endocrine disruptors such as the anti-androgenic compound vinclozolin, have been shown to induce changes in offspring development of exposed fathers.

Offspring from pregnant rat dams exposed to vinclozolin during gestation are at increased risk of tumour formation, kidney disease, immune dysfunction and infertility, transmissible down the male, but not female, lineage for up to four generations (Anway et al. 2005, 2008; Anway and Skinner 2008).

4.4 *Molecular and Epigenetic Mechanisms of Paternal Programming*

Changes in the normal patterns of sperm DNA (methylation) and histone modifications (methylation, acetylation) or RNA content, delivered at the time of fertilisation, are the prime candidate mechanisms through which altered paternal physiology could impact on offspring development. However, for any paternally established changes in epigenetic status to be faithfully transmitted to adult offspring, any established modifications must escape two major phases of genome-wide epigenetic reprogramming (see Fig. 1). The first phase occurs shortly after fertilisation in

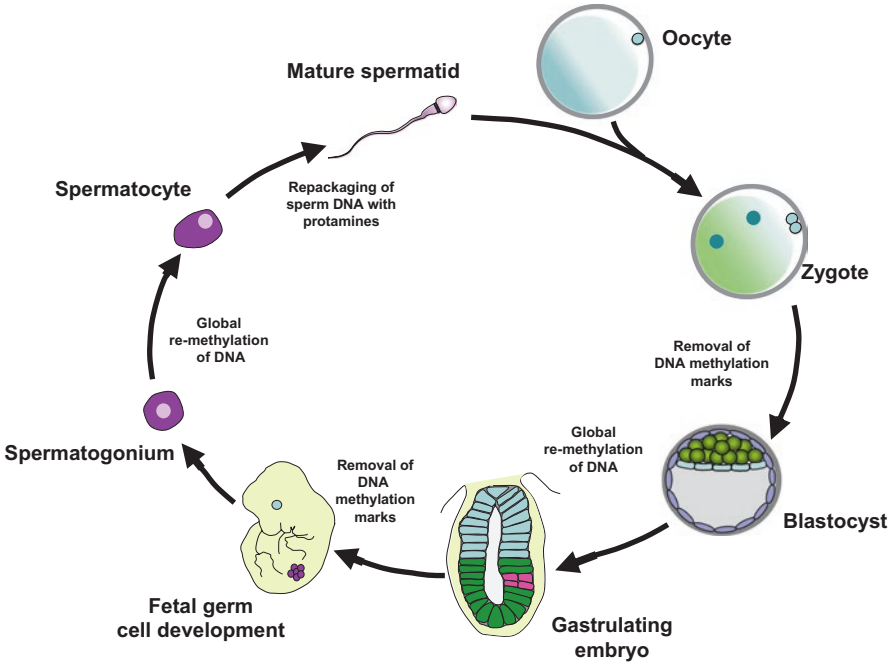

Fig. 1 Diagram representing the major developmental and epigenetic remodelling events during embryo development and sperm maturation. Any paternally derived epigenetic marks must be maintained during two separate phases of genome-wide epigenetic remodelling. Current research is attempting to understand which sperm-derived epigenetic marks are retained during post-fertilisation and how they may affect offspring development

the zygote, when the paternal genome undergoes extensive and active demethylation and remethylation as well as protamine to histone reorganisation (Shi and Wu 2009). The second phase occurs within the migrating primordial germ cells during foetal development. Here, as the primordial germ cells descend down the genital ridge towards the early gonads, genome-wide DNA demethylation occurs in a sex-specific manner (Ly et al. 2015). However, some classes of genes such as imprinted genes and retrotransposable elements have an ability to evade these phases of remodelling, retaining their original epigenetic marks.

Imprinted genes are expressed in a parent-of-origin specific manner, achieved through epigenetic silencing of either the maternal or paternal allele (typically by DNA methylation). Studies in mice have shown that paternal alcohol exposure can modify the DNA methylation status of imprinted genes in sperm (Ouko et al. 2009). Similarly in mice, sperm from male offspring generated through assisted reproductive techniques displayed significant differences in imprinted gene (*Snrpn, H19*) DNA methylation status (Stouder et al. 2009). Exposure of pregnant rat dams to the endocrine disruptor, vinclozolin, results in significant changes in offspring tissue gene expression patterns, for up to four generations (Anway et al. 2005; Stouder and Paoloni-Giacobino 2010). Exposure to drugs such as cocaine and alcohol has been shown to correlate to brain tissue DNA methylation, chromatin conformation and gene expression patterns (Bielawski et al. 2002; Ouko et al. 2009; Pandey et al. 2008) as well as reduced testicular and sperm DNA methyltransferase enzyme transcript levels in exposed male rodents (Bielawski et al. 2002; He et al. 2006; Ouko et al. 2009). Sperm from infertile men have been shown to display significant changes in DNA methylation levels (Aston et al. 2012).

A second transgenerational paternal programming mechanism lies in the extensive population of RNAs (mRNA, siRNA, piRNA) within the mature sperm. Analysis of human sperm has revealed the presence of several thousand coding transcripts (Ostermeier et al. 2002) with their expression profiles linked with male infertility (Garrido et al. 2009; Jodar et al. 2012; Montjean et al. 2012; Platts et al. 2007). Recent studies have employed next-generation sequencing techniques (RNA-Seq) to identify previously uncharacterised and novel coding and small non-coding RNAs (Krawetz et al. 2011; Sendler et al. 2013). The broad spectrum of non-coding RNAs that appear within the mature sperm is suggestive of a role in subsequent development. However, the overall functional significance of these sperm-derived RNA molecules remains to be fully elucidated.

Currently, histones seem the best candidates for transmission of paternal programming effects into the offspring at fertilisation, through their extensive potential for epigenetic modifications and influence on chromatin structure. Infertile men display significant changes in sperm histone populations (Hammoud et al. 2011) and histone methylation (Steilmann et al. 2011; Yap et al. 2011). In mice, haploinsufficiency of sperm protamines lowers sperm counts and induces DNA damage in mice (Cho et al. 2001; Perez-Crespo et al. 2008), while, in humans, altered protamine (P1:P2) ratio associates with reduced fertility rates (Aoki and Carrell 2003; Carrell et al. 2007). Recent analyses of human and mouse sperm active histone

(i.e. H3K27me3) localisation revealed significant enrichment at key developmental and pluripotency genes (Brykczynska et al. 2010; Hammoud et al. 2011). While it has yet to be determined whether any of the 2–15% of histones retained within the mammalian sperm contribute directly to zygotic gene expression regulation, studies have revealed that sperm-derived histones are transferred into the oocyte and become incorporated within the zygotic chromatin (van der Heijden et al. 2006; van der Heijden et al. 2008).

In addition to sperm DNA integrity, mRNA populations and histone modifications, sperm are able to influence the developmental programme through the initiation of oocyte calcium oscillations at the point of fertilisation. Upon fertilisation, sperm-derived phospholipase C zeta (PLC-ζ) initiates a series of intracellular Ca^{2+} oscillations that sweep across the egg, lasting for several hours (Swann et al. 2006). Manipulation of the number and amplitude of these Ca^{2+} oscillations has been shown to alter blastocyst cell number (Bos-Mikich et al. 1997) and foetal development (Ozil and Huneau 2001). RNAi knockdown of sperm PLC-ζ in mice has been shown to reduce the number of Ca^{2}+ transients at fertilisation and affect litter size (Knott et al. 2005). Separate to sperm-specific mechanisms of developmental programming, seminal plasma composition (i.e. granulocyte-macrophage colony-stimulating factor) has also been shown to influence embryonic, placental and offspring development (Sjoblom et al. 2005) as well as initiate maternal reproductive tract immunological responses, essential in the establishment and maintenance of pregnancy (Sharkey et al. 2007; Stewart et al. 2009). However, the exact impact of paternal nutrition and physiological status on these additional programming mechanisms, and the long-term offspring cardiovascular and metabolic health risks remain unknown.

5 Conclusions

The concept that parental nutrition during the periconceptional period can have a lasting legacy influencing fertility, offspring health and well-being is now firmly established for a wide range of mammalian species including humans. The study of paternal-mechanisms of transgenerational consequences for offspring health has naturally included an epigenetic perspective. While much of the evidence and support for paternal programming is fundamentally descriptive, advances in sequencing technology are providing scope for analysing epigenetic mechanisms of gene expression regulation and offspring development with increasing detail. However, new models and a greater understanding of the interaction between maternal and paternal genomes during the earliest stages of development are required to explore fully developmental programming mechanisms.

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Exploitation of Non-mammalian Model Organisms in Epigenetic Research

William V. Holt

Abstract Model organisms are widely used in research that is ultimately aimed at understanding the causes and consequences of human disease. It may seem counter-intuitive to expect clinically useful information to be obtained from species as diverse as fishes and insects, but because fundamental biological mechanisms share evolutionary origins they transcend species barriers. Epigenetic mechanisms fulfil this expectation admirably as more and more is discovered about the basic operational rules of inheritance, which are much more elaborate than formerly thought. Only a few decades ago, although the complex interplay between genes, inheritance and the environment was recognized, it was difficult to explain. Recent discoveries about the controlling influences of gene silencing through DNA and histone methylation, the roles of so-called non-coding DNA and microRNA, and the way in which these factors respond to environmental conditions have started to shed light on these basic mechanisms. Diverse model species allow epigenetic mechanisms to be studied from different perspectives; for example, some are better suited to studies of sex determination while others may be more convenient for studying the earliest stages of organ development, growth and the influence of nutrition on future wellbeing. The rationale for including this chapter in a book that is focused on uncovering relationships between periconception nutrition in humans is to highlight the opportunities and insights that may be gained by focusing attention on studies in non-mammalian model species.

Keywords Development • Sex determination • Environment • Inheritance • Aquatic organisms • Fishes • Reptiles

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1 Introduction

Model organisms have been studied extensively in biology and have provided a wealth of information on basic physiological and developmental processes. In principle, because species from multiple genera share common, albeit often very ancient, evolutionary origins with humans, studies of these organisms can produce valuable information that is directly useful in understanding human diseases. The choice of model species is usually related to the topic under investigation, the facilities available to researchers, rapidity of growth and reproduction and indeed the expertise of the researchers. Model organisms can also range across orders of scale, from bacteria, yeasts, nematodes and protozoa to large farm animals; all have their place in biological research. In fact, a systematic study of animal model organisms used in studies of development and covering the years 1965, 1975, 1985, 1995 and 2005 (Davies 2007) showed that model species came from 287 genera. There was, however, a strong bias towards the mouse and fruit fly, which together accounted for 40% of the 4615 papers examined.

Recent developments in high throughput genomics and proteomics have, however, called into question the value of working with animal models when research into human diseases can frequently be conducted directly upon human subjects (Fields and Johnston 2005). Indeed, the increasing emphasis on replacing, reducing and refining the use of research animals (known as the 3Rs; Graham and Prescott 2015) is positively encouraging scientists to explore methods that avoid the use of whole animals. Developmental biology is, however, such a conserved process that model organisms will undoubtedly continue to provide a wealth of information that is relevant to humans. Advances in our understanding of reproductive sciences and developmental biology have recently converged in ways that blur the boundaries between traditionally recognized biological disciplines. It is also apparent that understanding the extent to which developmental processes are affected by various aspects of an organism's environment is increasingly important. Organismal responses to the environment appear to share common mechanisms across species and genera, and model organisms are able to provide basic information that illuminate the entire scope of the subject.

Organisms of all types tend to adapt and evolve in order to make the best of their situation and the adaptive responses often involve subtle, or even major, phenotypic changes that can occur surprisingly quickly, even from one generation to the next (Gluckman et al. 2009; Bateson et al. 2014). Such short-term processes, considered to be forms of developmental plasticity, do not necessitate changes in DNA sequence, but instead rely on the controlled modulation of gene expression pathways using epigenetic mechanisms (Burggren and Crews 2014) such as DNA methylation and histone modification (Schaefer and Nadeau 2015) that were not even recognized until relatively recently (Daxinger and Whitelaw 2010). Understanding developmental plasticity as a conserved process is therefore highly relevant to unravelling mechanisms that affect human health, and the objective of this review is to highlight the way in which a few key non-mammalian model species are well suited when it comes to exploring such complexities.

2 Environmental Effects on Reproduction and Development

Developmental programmes and embryonic growth involve some degree of forecasting the state of the future environment so that embryo development can be matched against expectations (Nichelmann 2004). In fact, this relationship is at the heart of reproductive seasonality, where species have developed physiological strategies that typically synchronize breeding and parturition with climatic conditions for the optimal survival of their offspring (Bronson 2009). If the forecast turns out to be inaccurate because the environment changes and defies expectations, the mismatch can have unfortunate consequences for adult health. If the expectation does not match the reality, the outcome often results in disease; such relationships are now well recognized in humans, owing mainly to a series of studies linking early life nutrition to the onset of diseases in adulthood (see, e.g. Barker et al. 2010; Kwong et al. 2000). The realization that there are systematic relationships between pre- and/or periconception nutritional conditions and chronic disease conditions in adults has stimulated a great deal of research in humans and other species. In fact, the subject is now widely known as "DoHad" or the "Developmental Origins of Health and Disease", and there is even a specific journal devoted to the topic. The scope of the DoHad concept is ever widening, as exemplified by a recent article (Ferraro et al. 2016) in that journal (*Journal of Developmental Origins of Health and Disease*) which links air pollution, caesarean section and domestic violence during pregnancy into the DoHad paradigm. Transgenerational effects on body condition in males caused by father's and grandfather's smoking habits have also been described (Pembrey et al. 2006), as well as, in rats, the transgenerational impacts of experimentally administered agricultural chemicals (Anway et al. 2006a, 2006b). Our current understanding of DoHad therefore encompasses the effects of anthropogenic environmental chemicals on humans and animals (Haugen et al. 2015), and a discussion of model organisms would be incomplete if these aspects were omitted.

The word "environment" covers a multiplicity of factors in which organisms survive; it is apparent that the developmental plasticity exhibited by various species may be responses to changes in nutrition, food availability, population density, the prevalence of infectious diseases, presence of competitors and many other stresses. Uncovering epigenetic mechanisms across a wide range of species is therefore highly instructive for understanding details about the ways in which evolution progresses. Knowledge of transcriptomics was not available to Darwin or Lamarck when they formulated their concepts of evolution and selection, and it is therefore very valuable to keep evolutionary biology in mind as novel epigenetic mechanisms come to light (Van Soom et al. 2014).

3 Fishes as Model Organisms

Nutritional support is provided to mammalian embryos via their complex interactions with the placenta with the result that, with the exception of monotremes, live offspring are born; this process is known as viviparity. Analogous but diverse

embryonic support mechanisms leading to the birth of live offspring are believed to have evolved about 150 times in different vertebrate clades (Blackburn 2015), including fishes and amphibians. There are more than 25,000 species of fish, of which about 500 are viviparous (Wourms et al. 1988). Their reproductive adaptations are highly variable and do not always involve the provisioning of embryos via placenta-like structures. However, in the context of DoHad and the investigation of periconception nutrition, it is worth mentioning two groups of fishes, the Poeciliidae and the Syngnathidae, as potential research models.

The Poeciliidae are a family of around 200 small tropical fish species (Pires et al. 2010), some of which have evolved internal fertilization and placental modes of embryo support. These are already used extensively as valuable model species in reproductive studies and in studies on the way in which their development is affected by environmental pollution. It is of interest here that insulin-like growth factor 2 (IGF2), which is involved in the regulation of mammalian embryonic growth, has been found to be expressed in two species of Poeciliidae, *Heterandria formosa* and *Poeciliopsis prolifica* (Lawton et al. 2005; O'Neill et al. 2007). Placental morphology in the Poeciliidae has been studied in some detail (Kwan et al. 2015; Schindler 2015), and the impact upon ovarian function and fecundity of feeding diets that vary in protein content (Dahlgren 1980) was investigated over 30 years ago. However, because these fishes are considered to be well suited for laboratory research (Petrescu-Mag et al. 2014), they offer an excellent opportunity for novel investigations.

A series of experiments aimed at investigating relationships between reproductive outcomes and the quality of food supplied experimentally to female sailfin mollies (*Poecilia latipinna*) prior to ovulation (Trexler 1997), showed that if large females received low quality diet, they responded to their circumstance by providing a greater degree of placental support to their embryos than females receiving high quality diet. One member of the Poeciliidae (*Phalloptychus januarius*) that shows a particularly high level of matrotrophy (i.e. post-fertilization embryo provisioning) has also been used as a model species to investigate relationships between maternal diet, embryo survival and growth (Pollux and Reznick 2011). In this particular experiment, there was no evidence for embryo abortion under low food conditions, but developing offspring were small at birth. The value of these experiments lies not only in the insights they provide about the Poeciliidae themselves but also the insights into the underlying fundamental biology that transcends the phylogenetic divisions. As noted elsewhere in this book, the immediate and future developmental potential of embryos is significantly determined by maternal nutritional status, both before and during pregnancy, and being able to explore these interactions using a small and rapidly reproducing viviparous species provides an efficient means of investigating the principles involved.

The Poeciliidae are unusual in that they have also evolved the phenomenon of superfetation, whereby females can carry several independently fertilized broods of embryos at the same time (for review, see Pollux et al. 2009). Although this ability

has been investigated for its evolutionary and ecological benefits, few if any developmental studies have explored the complex nutritional experiments that may be possible.

Seahorses and pipefishes are another group of teleostian candidate model organisms, the *Syngnathidae*, that hold considerable promise for exploring the effects of periconceptional nutrition on embryonic growth and survival. Seahorse and pipefish embryos, like those of most fishes, possess a yolk sac as a source of nutrients. However, unlike most fishes, these embryos develop within a brood pouch, which is a specialized body cavity only found in males (Schmid and Senn 2002; Stölting and Wilson 2007). Recent radioisotope studies have demonstrated that male pipefishes contribute towards embryonic nutrition via the brood pouch (Ripley and Foran 2009; Kvarnemo et al. 2011; Kornienko 2001); the embryos become embedded within well-vascularized (Ripley et al. 2010) depressions of the interior lining of the brood pouch (Carcupino et al. 2002), and the fertilized embryos develop to term within a closed environment (Fig. 1). Thus, the brood pouch of the male is becoming more widely regarded as functionally equivalent to a mammalian uterus. Indeed, recent genomic studies have shown that the seahorse/pipefish pouch expresses genes more commonly associated with the mammalian uterus and oviduct (Whittington et al. 2015). Despite the intensive degree of husbandry needed for successful seahorse and pipefish breeding in the laboratory, these species hold considerable promise as model organisms suitable for the study of the periconception environment in relation to offspring health and survival.

As seahorses are widely exhibited in zoos and aquaria, there is a substantial body of research on the merits of diets and feeding regimes (Wong and Benzie 2003;

Fig. 1 Photograph of a male seahorse (*Hippocampus hippocampus*) in its natural environment off the coast of Gran Canaria, Spain. The prominent brood pouch indicates that this individual is pregnant. Scale bar = 1 cm (Photograph courtesy of Elodie Turpin)

Woods 2000b, a, 2002, 2003); however, relatively little attention has been paid to the experimental study of pre- and periconception diet in relation to embryonic growth and survival. One study (Otero-Ferrer et al. 2014) in which male and female *Hippocampus reidi* were fed separately with different qualities of diet ("wild caught" or "commercially available") showed, however, that preconception dietary effects could be distinguished on the basis of gender. When females that had only received commercial diet prior to mating were mated with males that had received the natural, wild-caught, diet, the resultant embryos showed poor survival after birth, coupled with distortion of relative head and snout size. When, conversely, males had been provided only with commercial diet prior to mating with females that had received wild-caught diet, the resultant offspring were abnormally large and also showed poor survival. The main difference between diets concerned the content of polyunsaturated fatty acids, with the commercial feed being somewhat, but not enormously, less enriched. In principle these results showed that seahorses could potentially be used as model organisms, where, because of their biology, it is possible to distinguish between dietary effects induced via oocyte quality (thereby mediated solely by females) and those affected by the quality of pouch (placental) support, which are mediated via males. This contrasts with the difficulty of separating oocyte-specific and placenta-specific effects of periconception diets in mammals and other viviparous vertebrates, because any diet fed to females would normally affect both ovarian and uterine function. The possibility that male-mediated dietary effects are influenced by sperm quality as well as pouch function is still something to respect; however, ingenious experimental designs could overcome this problem and allow researchers more flexibility than can be achieved using mammals.

Environmental influences on fish development have long been a topic of intense interest to researchers (reviewed by (Jonsson and Jonsson 2014)). Multiple aspects of the life history of fishes, including growth and adult body size, sex ratio, egg size, lifespan and tendency to migrate, are affected by early influences and although the mechanisms responsible are poorly understood, it is becoming ever more apparent that epigenetics is a central theme. As an example, using sea bass (*Dicentrarchus labrax)* as a model species, Navarro-Martin et al. (2011) demonstrated that sex ratio skewing in this species, where higher temperatures during a critical period in early development induce higher proportions of male offspring, are associated with increased DNA methylation of the aromatase promoter. The temperature-dependent sex ratio skew was therefore attributed to the prevention of aromatase gene expression via an epigenetic mechanism. Recent studies have demonstrated that epigenetic mechanisms are also involved in the temperature-dependency of embryonic growth in fishes via modulation of DNA methylation and gene expression. For example, thermal stress in Atlantic cod embryos resulted in stage-specific changes in the expression profiles of 5-methyltransferase enzymes (Skjaerven et al. 2014), while temperature changes altered the expression of microRNAs during embryonic development in Senegalese sole (*Solea senegalensis*) (Campos et al. 2014); these changes were associated with variations in growth rate, muscle development and lipid metabolism.

The zebrafish (*Danio rerio*) is widely used as a model organism for developmental and molecular studies, partly because it is relatively easy to manage and breed in the laboratory, and partly because of the ease with which the early stages of development can be viewed microscopically. Despite the huge number of published studies that have focused on zebrafish development, the mechanisms that drive sex determination remain stubbornly unclear. Elements of both genetic and temperature-dependent sex determination have been identified (Lopez-Olmeda and Sanchez-Vazquez 2011; Abozaid et al. 2012; Liew et al. 2012; Takatsu et al. 2013; Liew and Orban 2014; Shen and Wang 2014), with a bias towards the development of males at higher temperatures. Development of gender itself is unexpectedly complex, whereby the initial formation of immature ovaries in all individuals is followed by masculinization and testicular development in the case of males. This level of complexity is almost certainly driven epigenetically, but at present the mechanisms are unknown. Sex determination in marine species such as the bluehead wrasse (Fig. 2) (*Thalassoma bifasciatum*) is also a complex issue, where individuals initially develop as females but under specific circumstances can turn into males after reaching adulthood; the socially controlled mechanism is known as protogyny. To make the situation more complicated there are two male phenotypes, distinguished by both body size and colour markings. Recent transcriptomic studies comparing male and female gonads and brains have shown that sex differences are correlated with major changes in gonadal gene expression (Liu et al. 2015). The authors believed that the sex determining pathways in this species were not notably

Fig. 2 Photograph of a male *bluehead* wrasse (*Thalassoma bifasciatum*), a species found on coral reefs in the Caribbean sea. These fishes reach a maximum length of 25 cm, with a maximum reported age of 3 years, but individuals can reach sexual maturity when they are less than 4 cm in length. Males develop the striking *blue* head once they are sexually mature, but a small proportion of adult females also develop this coloration when they undergo a sex change induced by their social environment (Photograph courtesy of Jeff Whitlock; http://www.theonlinezoo.com)

different from those found in other vertebrates, which presumably means that signals emanating from the social environment are sufficient to cause the sexual plasticity.

4 Amphibians and Epigenetics

The amphibians are a group of species that includes the frogs, toads, newts, salamanders and caecilians. One of the most remarkable characteristics of the urodele amphibians, i.e. the newts and salamanders, is the ability to regenerate parts of the body such as the limbs, tail, jaw, retina and even brain, via a process known as epimorphic regeneration (Grigoryan 2016). Regenerative abilities are not completely absent in other species (Godwin 2014), even deer regenerate their antlers annually prior to the breeding season, but some amphibians have evolved this ability to an exceptional degree. One of the most fascinating but puzzling aspects of tissue regeneration in certain amphibians is their ability to replicate an entire limb, with all of the appropriate cell and tissue types in their correct places. Such an ability implies that cells possess an intrinsic "positional memory" which directs cell division and differentiation. While such a hypothesis has been around for many years, it is only now that the likely role of epigenetic mechanisms is being identified. For example, studies of *Xenopus tropicalis* (Hayashi et al. 2015) have identified histone acetylation and deacetylation as important epigenetic control mechanisms in the determination of positional memory. One of the longest established animal models in biology is the axolotl (Fig. 3) (*Ambystoma mexicanum*), which is a species of salamander from the lakes around Mexico City (Reiss et al. 2015). All amphibians pass through a larval stage, where they use gills for respiration, before metamorphosing into adults, but the axolotl is exceptional in that it rarely goes through this process and spends its life in the larval stage even after reaching sexual maturity. Axolotls also possess exceptional abilities when it comes to limb regeneration and can regenerate an entire limb in 100–200 days, depending on species. Positional memory is clearly essential for the regeneration of entire limbs, given that nerves, cartilage,

Fig. 3 Photograph of an Axolotl (*Ambystoma mexicanum),* a salamander from the lakes around Mexico City. Individuals of this species typically reach about 30 cm in length and remain in the larval stage throughout their adult life (Photograph courtesy of Jeff Whitlock; http://www.theonlinezoo.com)

muscle and blood vessels have to be correctly assembled. A recent study by Kragl et al. (2009) showed that the cells at the site of injury rapidly organize themselves into a new site for growth. The various cell types retain their original identity and are not pluripotent, and grow synchronously in order to form the new limb or other organ. Such observations show that the axolotl is a perfect model species for a wide range of epigenetic studies. Nevertheless, there are still relatively few publications dealing with epigenetic processes and tissue regeneration in axolotls. Data on the expression of microRNA during tail regeneration in the axolotl with directly valuable implications for vertebrate development were published recently by Gearhart et al. (2015). These authors identified 4564 microRNA families known to be widely conserved among vertebrates, as well as 59,811 reads of putative novel microRNAs. The authors considered that their data supported the hypothesis that microRNAs play key roles in managing the precise spatial and temporal patterns of gene expression during tissue repair and development. Other authors (Aguilar and Gardiner 2015) have also confirmed that tissue repair requires subtle modification of the DNA methylation status, which changes quickly after injury, following changes in the expression of DNA methyltransferases. Amphibians exhibit many other usual aspects of developmental plasticity that are almost certainly epigenetically controlled; one recent report has shown that the capacity of *Xenopus laevis* to survive prolonged periods without food is brought about by an ability to reduce gene transcription in the intestine and thus to depress metabolic rate (Tamaoki et al. 2016).

Xenopus tropicalis, as well as its larger cousin *Xenopus laevis*, are widely used as a model species in biological research and toxicology, and now rival the rat, mouse and zebrafish in their usage. Breeding facilities such as the European Xenopus Resource Centre (EXRC) in Portsmouth, UK, supply animals to other researchers in biomedical research, and even provide them with genetic resources such as frozen spermatozoa and unfrozen oocytes.

5 Aquatic Organisms as Model Species in Reproductive Toxicology

Some species of the fish group Poeciliidae have been used as bio-indicators of pollution in aquatic environments, especially with respect to the detection and effects of endocrine disrupting chemicals (EDCs). A study of Eastern mosquitofish (*Gambusia holbrooki*) in the St Johns River, Florida (Bortone and Cody 1999), found that females showed significant elongation of the anal fin and the gonopodium, an anal fin that is modified into an intromittent organ in male Poeciliidae. A similar study in China that focused on another mosquitofish (*Gambusia affinis*) (Hou et al. 2011) found evidence of masculinizing effects on the anal fin but also detected increased testis mass. Interestingly, these study sites both received effluents from a local paper mill. Paper mill effluents have since been implicated as causing female masculinization in other sites (Deaton and Cureton 2011). Multiple other effects of endocrine disrupting chemicals have been detected using Poeciliidae,

including changes in female mating behaviour, altered offspring sex ratios, diminished body size in masculinized females and lower fecundity (Tian et al. 2012; Sharbidre and Sopanrao Patode 2012; Vigario and Saboia-Morais 2014; da Silva et al. 2014; Shenoy 2014). One study has even reported changes in risk taking behaviour (Heintz et al. 2015) induced in male and female *Poecilia reticulata* individuals by the estrogenic compound, 17-ethinylestradiol and the androgenic compound, 17-trenbolone.

The Poeciliidae are not, of course, the only group of aquatic organisms that exhibit sensitivity to endocrine disrupting chemicals. Many environmental studies of wild fish have demonstrated male feminization and female masculinization mediated via the presence of anthropogenic compounds, including heavy metals, plasticisers, flame retardants, polychlorinated biphenyls, that typically show some ability to bind, albeit weakly, to hormone receptors (for reviews, see Kumar and Holt 2014; Bhandari et al. 2015; Wilkinson et al. 2016).

Apart from fishes, it is also noteworthy that many aquatic invertebrates are used, or can be used, as model organisms because they also respond to EDCs in the environment (European Environment Agency 2012; Kumar and Holt 2014). Molluscs appear to possess oestrogen-like receptors, but these are apparently not activated by the vertebrate oestrogen, estradiol, or by other known vertebrate EDCs. Nevertheless, mud snails responded to 12.5% and 25% sewage by increased embryo production, while higher sewage concentration (50%) reduced it (Jobling et al. 2004). Later studies (Sternberg et al. 2008) suggested that mud snails do, in fact, respond to androgens and oestrogens, but through an alternative signalling route, possibly the retinoid system. Similar studies on sewage exposure have detected increased vitellogenin-like proteins in males, feminized sex ratios and low gonadosomatic indices (Chesman and Langston 2006; Gomes et al. 2009). Potent androgen receptor agonists and aromatase inhibitors, as well as the marine anti-fouling paint component tributyltin (TBT), induce "imposex" in female gastropod molluscs at concentrations as low as parts per billion. This is where the penis "imposes" on the normal female anatomy, blocking the oviduct and inducing sterility (Pascoal et al. 2013). As knowledge of epigenetics in marine organisms is growing fast, the use of next-generation sequencing combined with studies of epigenetic mechanisms in marine invertebrates has recently been proposed as a source of rapid and powerful biomonitoring tools for tracking the quality of the marine environment (Suarez-Ulloa et al. 2015).

6 Sex Determination and Epigenetics

Environmentally controlled sex determination occurs in both vertebrates (Valenzuela and Lance 2004) and invertebrates (Adams et al. 1987), being affected by photoperiod, ambient temperature, availability of resources, populations density and even, in the nematode *Mermis nigrescens*, the size of the grasshopper that hosts it (Craig and Webster 1982). Temperature-dependent sex determination (TSD) in reptiles is

well documented and has been studied for many years, but until recently it has been difficult to explain mechanistically. Recent advances in genomics have begun to implicate epigenetic mechanisms, however, and therefore although it would be impractical to regard crocodiles, alligators and turtles as model species, it is worth mentioning them here. A recent study of temperature effects (Yatsu et al. 2016) on the gonadal transcriptome in American alligator (*Alligator mississippiensis*) embryos incubated at male and female producing temperatures of 33.5 and 30 °C, respectively, revealed sex-dependent expression of 230 genes, 25 of which were involved in transcriptional regulation. The authors also reported the sexually dimorphic expression of non-coding RNA. In keeping with observations in mammals that environmental factors can impact subsequent generations, similarly long-lasting effects have been induced experimentally in an Australian reptile, the jacky dragon *Amphibolurus muricatus*, whereby the offspring sex ratio is affected by the incubation temperature experienced by the father several years earlier (Warner et al. 2013). In this study the eggs were incubated at female producing temperatures (23 and 33 °C) and an intermediate temperature (27 °C) that produces both sexes. Applying an aromatase inhibitor to 50% of the eggs in each treatment resulted in the production of males, even at female producing temperatures; the other 50% were used as controls. The essential outcome of this study was that the males produced forcibly by the combination of low temperature and aromatase went on to produce predominantly male offspring when they bred about 3 years later. Interestingly, the offspring sex ratio produced by fathers forcibly bred at the higher temperature was skewed in the opposite direction, i.e. towards the production of females.

Zebrafish (*Danio rerio*), although not viviparous, are widely used for laboratory research into the influence of endocrine disrupting chemicals (EDCs) on development. A simple search for "zebrafish" + "endocrine*" + "disrupt*" on "Web of Science" detected more than 1200 publications describing various aspects of such research. A major advantage of the zebrafish over other research models stems from the wealth of background information about development and the role of epigenetics already available for this species (see, e.g. Saxena et al. 2016; Liu et al. 2016). Although there is still relatively little known about the way in which EDCs impact on epigenetic processes during zebrafish development, this is a growing field. In a recent review, Kamstra et al. (2015) discussed DNA methylation at length, reporting that zebrafish possess eight highly conserved DNA methyltransferase genes. As DNA methylation and demethylation occurs during zebrafish development, and these processes are affected by a range of toxicants, it is clear that the impacts of environmental contaminants must be mediated via epigenetic mechanisms, and that endocrine effects are likely to be downstream of these initial impacts.

Water fleas, such as *Daphnia*, which is a genus of freshwater crustacean, occur naturally within the zooplankton assemblages found in bodies of freshwater. They have long been a favourite model species for biologists interested in developmental plasticity because of their ability to modify their life history traits in response to environmental changes. Many species respond to the presence of predators by developing head and tail spines, and can accelerate their developmental rate, switch between sexual and asexual reproductive modes and change the sex ratios of their

offspring as appropriate. *Daphnia* have therefore become an important group of model species for studies in aquatic toxicology (Shaw et al. 2008). However, *Daphnia* are also a valuable organism for research in epigenetics because they parthenogenetically produce individuals, which are essentially clones of the parent, and display phenotypic variation that depends on environmental conditions (for review, see (Bonasio 2015)). Given these attributes, it is not surprising that the *Daphnia* genome has now been sequenced.

Sexual differentiation in *Daphnia* is controlled by the environment (LeBlanc and Medlock 2015) and environmental cues such as season (and therefore ambient temperature), availability of food resources, population density and loss of habitat are known to be involved. The mechanisms of sex determination are known to involve neuropeptides, ecdysone and methyl farnesoate (Toyota et al. 2015; La et al. 2014) and a genetic component also seems likely. However, despite the extensive body of work the sex determination mechanism remains stubbornly unclear. Nevertheless, recent studies have documented the existence of both histone modifications and DNA methylation in *Daphnia*, and therefore it seems likely that this group of species will become an excellent model for future epigenetic studies (Robichaud et al. 2012; Harris et al. 2012).

7 Epigenetics and Social Insects

Social insects provide an unusual opportunity to examine the putative involvement of epigenetics in development because despite fertilized eggs often being produced by a single female, the queen, in a given colony, the variation in eventual phenotypes is astonishing. Not only do the offspring differentiate as males or females, usually depending on the season, but the adults develop highly varied behaviours and phenotypes (Yan et al. 2015). As an example, the workers in a honey bee (*Apis mellifera*) colony are all female and their role is in foraging for food and caring for the developing larvae. However, each year a group of males is produced whose sole duty is to emerge from the colony and mate with a queen, thereby providing the queen with spermatozoa that are then stored in the female reproductive tract for a prolonged period and used for fertilization. While the queen is capable of mating and producing fertilized eggs, the female workers are sterile and their ovaries do not develop. This form of social organization, with a caste system, has contributed to the global success of social insects, leading some authors to draw parallels between insect and human societies in terms of the division of labour (Middleton and Latty 2016). Over the last decade there has been considerable interest in the possibility that the various phenotypes within the insect caste systems are controlled through epigenetic mechanisms (Maleszka 2008; Yan et al. 2015; Yan et al. 2014). The early development of Honey bees (*Apis mellifera*) seems to be determined very early in life, starting from the time when the larvae receive different quality diets from workers (worker jelly) or from the queen (royal jelly). Royal jelly contains factors that have a direct impact on DNA methylation status; for example, knockdown of an

Apis mellifera DNA methyltransferase (*Dnmt3*) by RNA interference results in the development of a queen phenotype from larvae destined to become workers.

Investigators studying the composition of worker jelly have shown that it is highly enriched in microRNA species compared to royal jelly (Guo et al. 2013) and, moreover, that the nature of the microRNA changes on a daily basis during development. Such observations, coupled with data showing that royal jelly is also rich in 10-hydroxy-2-decenoic acid (Spannhoff et al. 2011; Wang et al. 2014) (sometimes known as queen bee acid), which comprises up to 5% of royal jelly and can inhibit histone deacetylation, is suggestive of an epigenetic basis for the development of caste-specific phenotypes. Although these observations relate specifically to honey bees, the hypothesis that morphology and behaviour in social insect societies are controlled via epigenetic mechanisms, and especially through DNA methylation (Standage et al. 2016), is receiving ever more support (Sumner et al. 2006; Patalano et al. 2012; Maleszka 2008; Weiner et al. 2013).

8 Conclusions

Embryonic development always begins with a single cell that contains a large, but arguably finite, number of "genes", and the estimated number of genes in a human embryo is believed to be somewhere between 20 and 25,000. Cell division ensues shortly after fertilization and eventually a multitude of different tissue and cell types are produced by growth and differentiation. Even the cells within a single organ are demonstrably different from each other in terms of their morphology, function and transcriptome; the gonads provide a good example, with morphologically distinct germ cells, specialized somatic cells with and without endocrine functionality and a diversity of biochemical functions and responses. How can this impressively enormous amount of variability be squeezed out of a finite number of genes? In the early days of genomic research the definition of a gene was fairly straightforward; each protein was believed to be encoded by a single DNA sequence and gene expression was controlled via regulatory feedback mechanisms (Beckwith 2011). The number of genes needed for producing phenotypic variability was therefore believed to be correspondingly high, possibly more than 100,000 for a mammal. However, more recently "the gene" has become a much more complex concept as it now incorporates the many permutations by which parts of DNA sequences can be expressed. The timing of gene expression is also crucially important in development and day to day living; gene expression is capable of dynamic control, being modulated by extrinsic and intrinsic factors such as day length and circadian rhythm. It is therefore apparent from the foregoing discussion that many, or indeed all, species have evolved regulatory mechanisms to control the way in which DNA sequences are transcribed and translated. These mechanisms, which, because they are often modulated by small RNA molecules, are themselves encoded within the organism's own genome, thus suggesting that the evolution of species involves selective forces that act upon the epigenetic regulatory mechanisms. Such musings lead towards the

hypothesis that epigenetic mechanisms must be continuously evolving in tandem with environmental influences, always striving to predict and adapt to future conditions in which a new generation will develop, and promoting the selection and survival of well adapted species. While this is not an original hypothesis, it is one that has so far not received much attention (Smith et al. 2016); however, it will without doubt provide a rich and novel topic for future investigations. It will probably also provide novel insights into ways that the environment controls many aspects of evolution and integrate more molecular thinking into future ecological and evolutionary research.

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