

Chapter 8

Reproduction, Development and Life Cycles



Tiziana Altiero, Atsushi C. Suzuki, and Lorena Rebecchi

Abstract In tardigrades reproduction occurs only through eggs, fertilized or unfertilized, and therefore only through gametes. Tardigrades exploit several reproductive modes, amphimixis, self-fertilization and thelytokous parthenogenesis (both apomixis and automixis). These modes are often in close relationship with the colonized environment. As regards sexuality, tardigrades can be gonochoristic (bisexual or unisexual) or hermaphroditic. The anatomy of the reproductive apparatus of males, females and hermaphrodites and the maturative patterns of male and female germinal elements are presented and discussed, as well as the ultrastructure of spermatozoa and eggs, including their phylogenetic implications. In addition, mating and fertilization patterns, embryonic and post-embryonic development, sexual dimorphism and parental care are considered and discussed. Finally, vegetative reproduction does not occur in tardigrades, and their capability to regenerate is limited to a physiological tissue restoration of a few cells.

8.1 Introduction

8.1.1 Sexuality and Reproductive Modes

Tardigrades are unable to propagate via vegetative reproduction. Their reproduction occurs only by means of gametes, in particular through fertilized or unfertilized eggs. Heterochromosomes have never been identified in tardigrades. Both amphimixis due to cross- or self-fertilization and parthenogenesis take place in tardigrades, often in

T. Altiero
Department of Education and Humanities, University of Modena and Reggio Emilia, Reggio Emilia, Italy

A. C. Suzuki
Department of Biology, University of Keio, Yokohama, Japan

L. Rebecchi (✉)
Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
e-mail: lorena.rebecchi@unimore.it

close relationship with the environment colonized (Bertolani et al. 1990; Bertolani 2001).

Tardigrades probably originated in the sea and marine species (in practice all of them heterotardigrades) are gonochoristic (bisexual), with a sex ratio close to 1:1. Only a hermaphroditic species is known in marine environments. Therefore, in marine habitats there is only amphimixis, and parthenogenesis does not occur. The successive colonization of freshwater and terrestrial habitats led tardigrades to change their reproductive modes in order to adapt to less stable environments, with respect to the marine one. Limnic and terrestrial species (both eutardigrades and heterotardigrades) are mainly unisexual (i.e. composed of only females) and reproduce via parthenogenesis that can be automictic or apomictic and often associated with polyploidy. Limnic and terrestrial species can be also bisexual with a sex ratio close to 1:1 and characterized by amphimixis. In addition, even hermaphroditism occurs. It is the less frequent sexual condition in tardigrades and has been found in several families both in limnic and terrestrial eutardigrades (Bertolani 2001).

8.2 Anatomy of the Reproductive Apparatus

An unpaired gonad (ovary, testis or ovotestis), one or two gonoducts (oviduct or vasa deferents, respectively), a gonopore or a cloaca and, eventually, spermatheca/seminal receptacle and seminal vesicles form the reproductive apparatus of tardigrades (Fig. 8.1). The gonad is always a dorso-caudal sack, dorsally overlying to the midgut and anteriorly suspended at the cuticle by one or two ligaments. In heterotardigrades a basal membrane limits the gonad, whereas in eutardigrades a discontinuous epithelium with bundles of contractile-like filaments has been described. Other evident anatomical differences in the reproductive apparatus between the two classes of tardigrades will be described in the next paragraphs.

8.2.1 Female Reproductive Apparatus

The single ovary present in tardigrade females varies in size with animal age and reproductive stage. For instance, the ovary (Figs. 8.1 and 8.2) size increases considerably when oocytes increase in their volume as a consequence of vitellogenesis. In some species the ovary can be extended from the pharynx up to the posterior end of the animal. Anteriorly, it is attached to the dorsal body wall through a single and median ligament in heterotardigrades and two ligaments in eutardigrades (Fig. 8.1a). The oviduct originates from the posterior part of the ovary, extends beyond it and leads directly outside through the gonopore (heterotardigrades) or leads into the rectum and successively in the cloaca (eutardigrades) (Bertolani 1983a). Six cuticular plates surround the female gonopore of heterotardigrades, making it like a

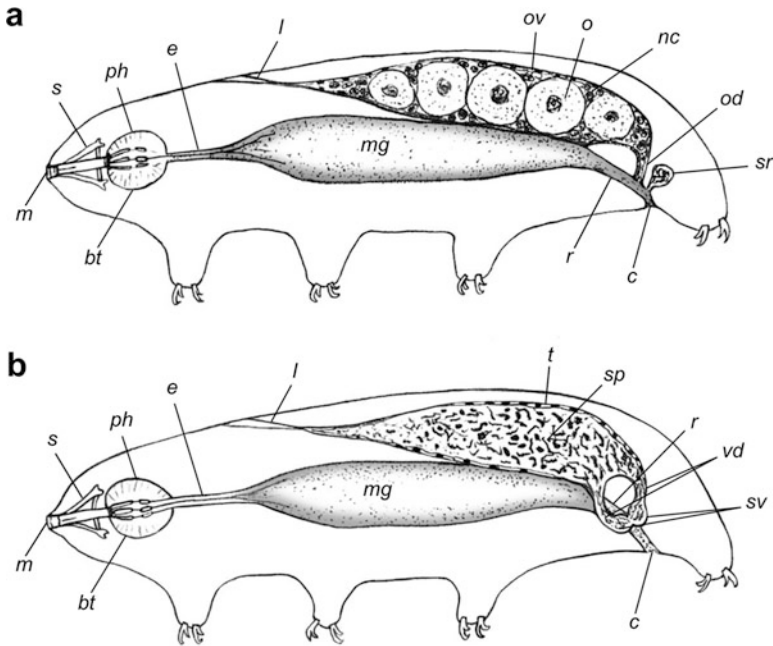


Fig. 8.1 Reproductive and digestive apparatuses in female (a) and male (b) eutardigrades. Abbreviations: bt = Buccal tube; m = mouth; s = stylet; ph = pharynx; e = esophagus; mg = midgut; r = rectum; c = cloaca; l = dorsal ligaments of the gonad; ov = ovary; o = oocyte; nc = nurse cell; od = oviduct; sr = spermatheca; t = testis; sp = sperms; vd = vas deferents; sv = seminal vesicles

rosette with six petals (Fig. 8.3a). Gonopore is located anteriorly to the anus between the third and the hind legs.

In heterotardigrades, females of the most part of marine species and those of the terrestrial family Oreellidae have two external cuticular seminal receptacles located ventrolaterally. Each receptacle has a long, slightly curved genital duct that opens at a distance from the gonopore (Kristensen 1984; Jørgensen et al. 1999). These organs, with their content of spermatozoa, are abandoned with the exuvia during the moulting process. The new cuticular receptacles, without spermatozoa, appear as thin cuticular organs in newly moulted females of the marine species *Actinarctus doryphorus* and again filled of spermatozoa when the ovary contains one mature oocyte (Jørgensen et al. 1999). Cuticular seminal receptacles have never been observed in the intertidal family Batillipidae; an internal epithelial spermatheca has been found only in *Batillipes pennaki* (see Grimaldi de Zio and D'Addabbo Gallo 1975). An internal epithelial spermatheca (Fig. 8.2c) that opens into the rectum was observed also in a few terrestrial eutardigrade species of the genus *Macrobiotus*, *Ramazzottius* and *Xerobiotus* (see Bertolani 1983a; Rebecchi and Bertolani 1988; Rebecchi 1997).

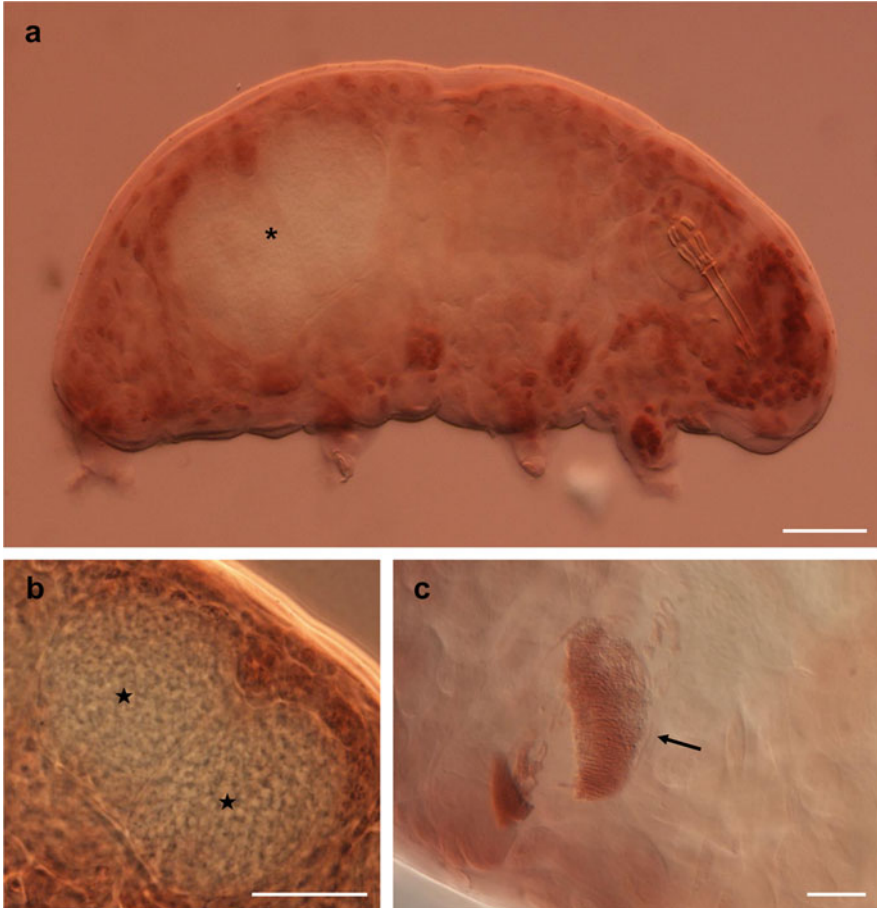
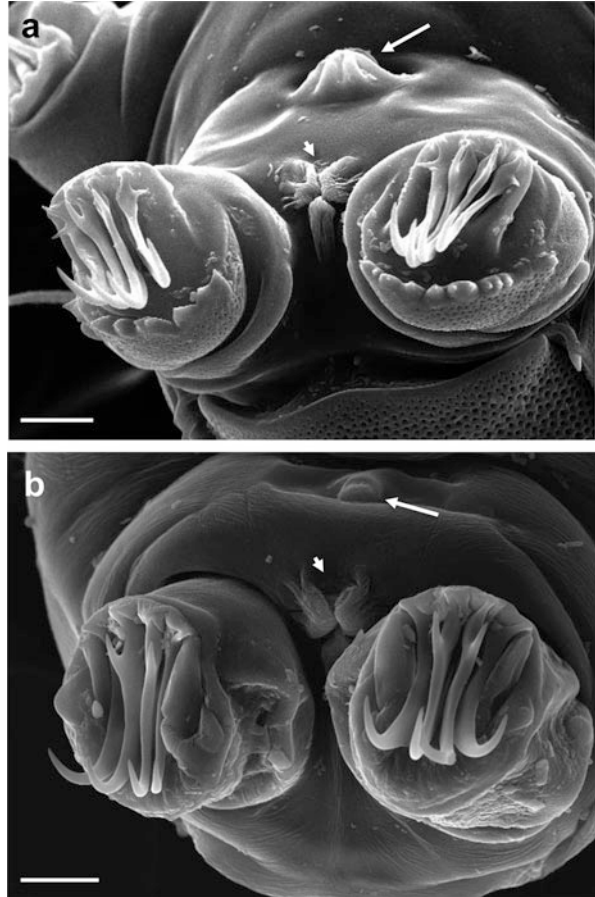


Fig. 8.2 Female reproductive apparatus of the eutardigrade *Macrobiotus* sp. **(a)** *In toto* female with two oocytes into the ovary (asterisk); **(b)** ovary with two oocytes (stars); **(c)** spermatheca (arrow). **(a, c)** Orcein staining and differential interference contrast (DIC), **(b)** orcein and phase contrast. Scale bars = 10 μ m

8.2.2 Male Reproductive Apparatus

The testis of marine heterotardigrades is often small and triangle-shaped with an anteriorly oriented apex (e.g. *Opydorscus*, *Wingstrandarctus*, *Tanarctus*, *Florarctus*; see Rebecchi et al. 2000a). In parachelan eutardigrades, the sac-shaped testis can change in size, similarly to the ovary, depending to the developmental stage of the male germinal cells (Figs. 8.1 and 8.4). The only exception is represented by apochelan eutardigrades (*Milnesium* species), which always have a small testis composed by two fusiform portions united medially to form an inverted V (Rebecchi and Nelson 1988; Suzuki 2006). A basal membrane surrounds the male

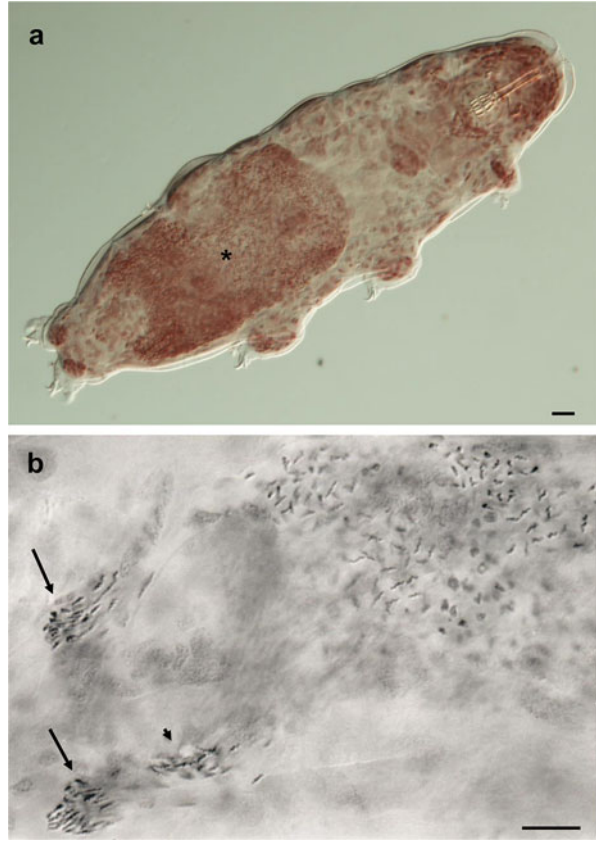
Fig. 8.3 Sexual dimorphism in the heterotardigrade *Echiniscus* sp. (a) Female with anus (arrow head) and rosette-shape gonopore (arrow); (b) male with anus (arrow head) and tubular-shaped gonopore (arrow). (a, b) Scanning electron microscopy. Scale bars = 10 μ m



reproductive apparatus of the heterotardigrades (e.g. *Batillipes*, *Tetrakentron*, *Halechiniscus*; Bertolani 1983b; Rebecchi et al. 2000a), whereas in the terrestrial eutardigrade *Milnesium*, a discontinuous squamous epithelium showing cells with bundles of contractile-like filaments forms the wall of the testis (Dewel et al. 1993).

The two very thin deferents turn ventrally around the midgut, join medially and open into the rectum and successively in the cloaca in eutardigrades. In heterotardigrades the two deferents join to form a short common duct that opens into the gonopore. Differently from the female gonopore, the male gonopore of the heterotardigrades is closer to the anus and generally tubular (Fig. 8.3b). It can show some species-specific peculiar morphologies. For instance, a round or oval-shaped opening, sometimes covered anteriorly by a cuticular fold, is present in *Zioella*, while in other genera, the male gonopore is located on a small ovoid papilla (Bertolani 1983b; Rebecchi et al. 2000a). In some species of both heterotardigrades and eutardigrades, males have two small internal seminal vesicles. In eutardigrades, the seminal vesicles are due to a small swelling of the distal part of each deferent

Fig. 8.4 Male reproductive apparatus of eutardigrades. (a) In toto male of *Macrobotus* gr. *hufelandi* with the large sac-shape testis (asterisk) containing germinal cells; (b) testis of *Hypsibius* cf. *dujardini* with one of the two deferents (arrow head) and seminal vesicles full of spermatozoa (arrows). (a, b) Orcein staining and DIC. Scale bars = 10 μ m

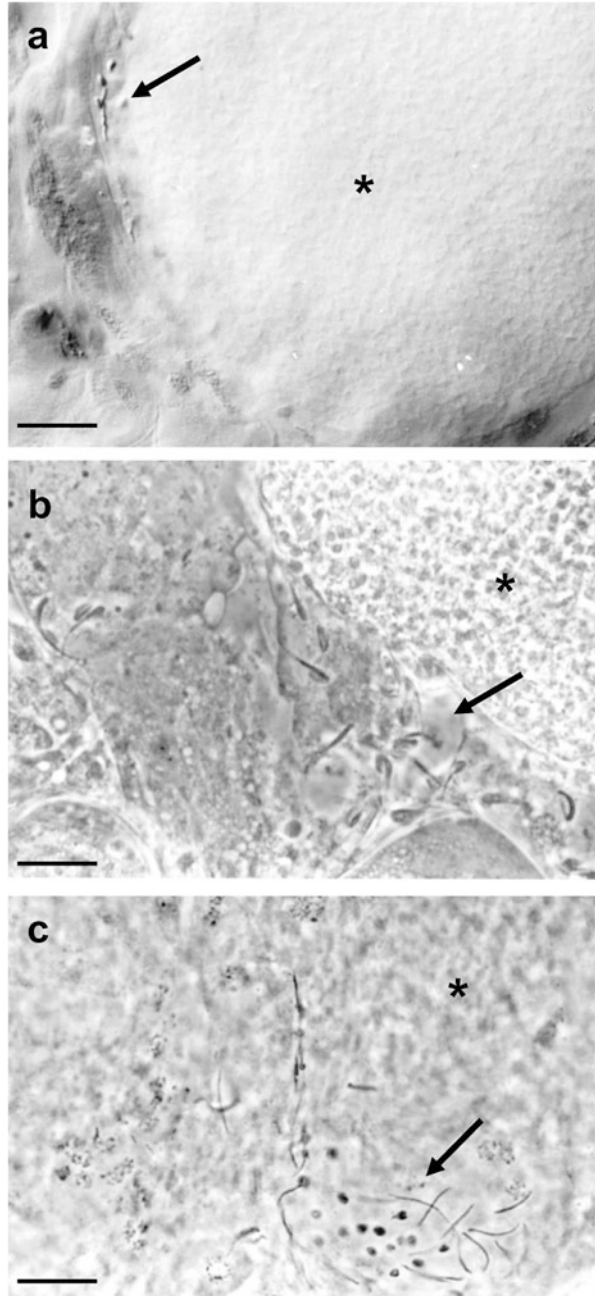


such as in *Diphascion* and *Platicrista*, whereas in the heterotardigrades of the genera *Wingstrandarctus*, *Styraconyx* and *Florarctus*, they look as two caudal lateral bulges of the testis. In male eutardigrades, spermatozoa within the spermiducts and seminal receptacles are motile and clumped in bundle (Rebecchi et al. 2000a).

8.2.3 Hermaphroditic Reproductive Apparatus

In hermaphrodite eutardigrades, a single sac-like ovotestis develops dorsally and posteriorly, overlying the midgut. The ovotestis is followed by a short gonoduct, and it is built of a single layer of cells. Within the ovotestis no septum or other kinds of morphological barriers separating clusters of female and male germinal cells have been observed (Fig. 8.5). In *Macrobotus joannae* and *Bertolanius weglarkae*, the ovotestis varies in size in relationship to the body size, the degree of maturation of the germinal elements and the predominant type (female or male) of gamete. The anterior part of the ovotestis ends with two blind appendices connected with two

Fig. 8.5 Ovotestis of hermaphroditic eutardigrades with an oocyte (asterisk) at the end of the vitellogenesis and male germinal cells (arrow) at different stage of development around it; **(a)**, **(b)** *Macrobiotus joannae*; **(c)** *Isohypsibius monoicus*. **(a)** Orcein and DIC; **(b)** In vivo and phase contrast; **(c)** orcein and phase contrast. Scale bars = 10 μ m



ligaments (protrusions of the gonad wall cells), through which the ovotestis is anchored to the dorsal cuticle (Węglarska 1987; Rebecchi et al. 2000b; Poprawa et al. 2015a). In *M. joannae*, a caudal sack-like diverticulum of the ovotestis containing a large number of spermatozoa and spermatids was evidenced (Rebecchi et al. 2000b). In *Isohypsibius granulifer granulifer*, the apical part of the gonad, germarium, is filled with oogonia; the central part, vitellarium, contains developing oocytes and nurse cells; the last part of the gonad is filled with spermatogonia (Węglarska 1987).

The ovotestis of *Orzeliscus cf. belopus* (the only hermaphroditic marine heterotardigrade) is a sac-like structure without septum to separate male and female germ cells, but fully formed and mature male cells are located only at the periphery in both anterior and posterior regions of the gonad. In addition, a single gonoduct and a pair of seminal receptacles are present laterally and posteriorly to the gonopore. The gonopore has the shape of a rosette-like structure (Suzuki and Kristensen 2014).

8.2.4 Sexual Dimorphism

Secondary sex characters have been observed in many heterotardigrade species and a few eutardigrade ones. In both classes, males are generally smaller than females, even though the body size cannot represent a useful criterion to distinguish sex in tardigrades, as mature males may be larger than immature females (Bertolani 1992; Rebecchi and Nelson 1988). Other than the already mentioned sexual dimorphism in the shape of the heterotardigrade gonopore in heterotardigrades (Fig. 8.3), two kinds of dwarf males have been evidenced in the marine heterotardigrade *Tetrakentron synaptae*: stationary pygmy males having a flattened body and vagile males with longer secondary clavae and unflattened body (Kristensen 1980). Small and large males have been described in another marine heterotardigrade *Tholoarctus natans*; the small ones have no secondary or accessory spurs on the external digits, and the large ones have spurs on the external claws (Kristensen and Renaud-Mornant 1983). Additional sexual dimorphism may be exhibited by some heterotardigrades in the size of the cephalic appendages, especially the clavae, which are often much longer and larger in males than in females, and may play a role in mating (Bertolani 1992).

In a few eutardigrade species, mature males have a basal hook-shaped modified claw with a small spur on the front legs; the appearance of modified claws occurs only with the moulting preceding the mating since they are probably used in attaching the females during copulation (Bertolani 1992; Rebecchi and Nelson 1988; Suzuki 2008). Classical example are represented by the secondary branch of the inner claw of the limnic *Pseudobiotus* species and primary and secondary branch of the front legs of *Milnesium* and *Milnesioides* species (Fig. 8.6). Adult females of the genus *Pseudobiotus* moult producing modified claws on the hind legs before laying eggs; these claws are shorter than normal ones in order do not to destroy or damage eggs laid within the exuvia (Bertolani 1992). A lateral conical papilla is

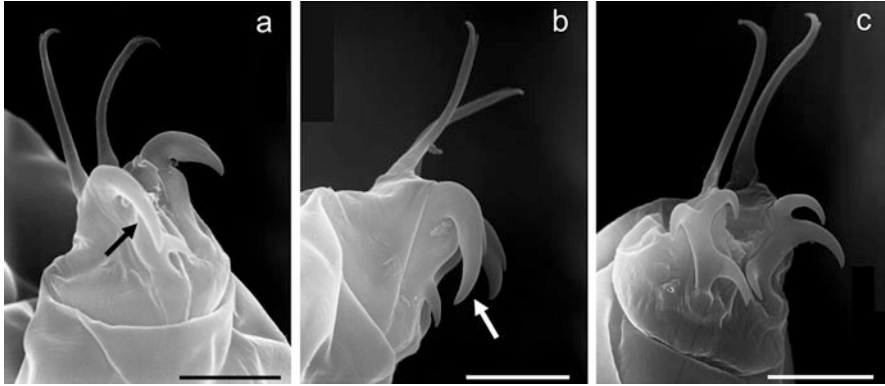


Fig. 8.6 Sexual dimorphism in the claw of *Milnesium* sp. from Yokohama, Japan. (a, b) First claw pair of a mature male with hook (arrow); (c) first claw pair of a female. Scanning electron microscopy. Scale bars = 10 μ m

present on the hind legs of males of *Ramazzottius oberhaeuseri* and, rarely, in *Macrobotus* species (Bertolani 1992). Since some of these sex characters in eutardigrades are related to reproductive cycle, they cannot be used to distinguish sex features and to evaluate sex ratio.

8.3 Maturative Patterns of the Gonad

8.3.1 Maturative Pattern of the Ovary

In tardigrades, oocyte maturation usually begins after the second moulting, rarely even after the first one. Asynchronous maturation of oocytes occurs in most marine heterotardigrades, which lay a single egg at a time with the most mature oocyte located at the posterior part of the ovary (Rebecchi et al. 2000a). Only females of *Batillipes* spp. had showed a different kind of oocyte maturation. In practice, a synchronous maturation of oocytes (up to 8) occurs in spring-summer, while in autumn-winter oocytes mature asynchronously (Pollock 1970). In non-marine heterotardigrades and in limnic and terrestrial eutardigrades, oocytes development is synchronous, and groups of 2 up to more than 40 eggs are laid, as evidenced in the terrestrial eutardigrade *Paramacrobotus richtersi* (currently attributable to *Paramacrobotus fairbanksi*; Altiero et al. 2006, 2015; Guidetti et al. 2019).

Maturation of oocytes and their oviposition occur synchronously with moulting and several times throughout adult female life of both non-marine heterotardigrades (echiniscids) and eutardigrades, which are iteroparous. In particular, four maturation stages of the ovary, corresponding to the oogenesis phases, have been identified in eutardigrades: pre-vitellogenesis, early vitellogenesis, late vitellogenesis and mature oocyte (Węglarska 1975, 1979; Rebecchi and Bertolani 1994; Poprawa 2005a; Hyra et al. 2016; Poprawa et al. 2015a, b, c). Both in young and adult females right after

the oviposition, the small ovary contains undifferentiated cells in pre-vitellogenesis, very similar to each other. Within an ovary at the early vitellogenesis, two kinds of cells are present: oocytes at the beginning of the auxocytosis and nurse cells. The first ones are distinguishable by cytoplasm rich in vacuoles and nucleus with uncoiled chromatin and a large nucleolus. Females in *simplex* stage (i.e. without the sclerified parts of the buccal-pharyngeal apparatus) have gonads at the middle-late vitellogenesis, which have grown in size as a consequence of the synthesis/accumulation of yolk in oocytes. At this stage, within oocytes a large nucleolus is visible, and chromosomes are poorly distinguishable. Moreover, nurse cells with a large nucleus are recognizable among the oocytes. Finally, at the fourth maturative stage, the ovary extends for nearly all the animal's length leaving little space to the midgut and contains mature oocytes, filled with spheres of yolk. At this stage, first metaphase chromosomes can be visible in oocytes.

8.3.2 *Maturative Pattern of the Testis*

The maturation pattern of the testis was poorly analysed, especially that of marine species. The testis of newborns is undifferentiated, and its maturation may occur already after the first moulting. The only exception known has been described in the marine ectoparasite heterotardigrade *Tetrakentron synaptae*, in which dwarf males showed mature germinal cells within the gonad since birth (Kristensen 1980). The arrangement of male germinal elements in *Batillipes* and *Wingstrandarctus* is zonal and uniform, respectively (Kristensen 1979, 1984), while in *Actinarctus* spermatids surround a nurse cell, and males are probably iteroparous (Jørgensen et al. 1999).

In males, conversely to the egg maturation in females, the spermatozoan maturation does not seem correlated with moulting. Males can be iteroparous or semelparous, and their testis displays two pattern of spatial arrangement of the developing germinal elements, i.e. zonal and uniform, and three temporal maturation patterns have been described (Rebecchi and Bertolani 1994).

Firstly, a continuous pattern in which spermatogenesis occurs continuously in time and mature spermatozoa, spermatids and spermatogonia are always present in the testis (e.g. *Paramacrobiotus*, *Minibiotus*, *Xerobiotus*, *Ramazzottius*, *Diphascon*, *Platicrista*).

Secondly, a progressive zonal maturation pattern with gonad completely filled with only spermatozoa, as it takes place in semelparous males of the eutardigrade *Pseudobiotus* and in marine heterotardigrades.

Thirdly, a cyclical maturation pattern in which the testis contains either exclusively spermatozoa or immature germinal cells as observed in the eutardigrade *Bertolanius*; these males are therefore iteroparous as females.

8.3.3 *Maturative Patterns of the Ovotestis*

Hermaphroditic tardigrades are iteroparous and often protandrous. In the eutardigrades *Macrobotus joannae* and *Bertolanius weglarskae*, the maturative pattern of the ovotestis can be subdivided into four stages. The first stage includes three phases (undifferentiated, male and pre-vitellogenic phases), whereas stages 2–4 correspond to the steps of the vitellogenesis during which groups of 3–8 oocytes mature in strict relationship to the moulting cycle. Apart from the first two phases of stage 1, all other stages simultaneously possess male and female germinal elements. The gonad looks as an exclusively “male gonad” in relatively small specimens, indicating that the male phase is present only in the first reproductive cycle (Rebecchi et al. 2000b). Only in the limnic species *Isohypsibius monoicus*, the ovotestis shows an alternation of predominantly male and predominantly female germ cell maturation indicating a cyclic maturation (Węglarska 1987). The contemporary presence of mature oocytes and spermatozoa in close contact with each other evidences that tardigrades are simultaneous hermaphrodites and that self-fertilization can occur even though it may lead to loss of genetic variability. Although the marine *O. cf. belopus* is considered a simultaneous hermaphrodite with both mature eggs and a large number of mature male gametes in the ovotestis, this species should have cross-fertilization elucidated by the presence of spermatozoa in the seminal receptacles, with the modified sperm shape implying the additional maturation process after ejaculation (Suzuki and Kristensen 2014).

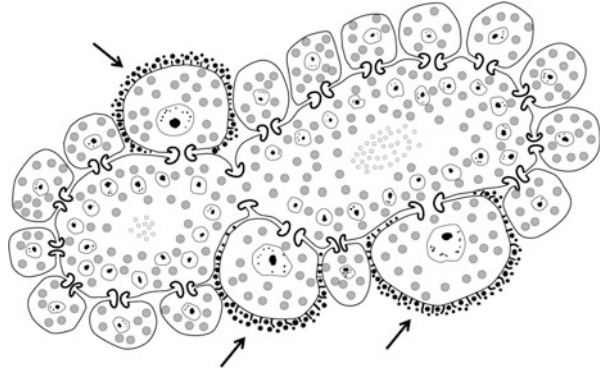
In general, hermaphroditic individuals have a higher energy cost than gonochoristic species, being energy allocated for both male and female gametes. However, self-fertilizing hermaphroditism has the advantage to allow the colonization of a new area by a single specimen (Bertolani 2001).

8.4 Gametogenesis and Gamete Morphology

8.4.1 *Oogenesis*

The ultrastructural process of the oogenesis in tardigrades is still understudied. After Węglarska’s studies (1979, 1982, 1987, 1989) on the details of oogenesis in the eutardigrades *Paramacrobotus cf. richtersi* and *Isohypsibius granulifer*, we have waited for about 25 years before to know more about oogenesis of other eutardigrades species (Suzuki 2006; Poprawa 2005a; Poprawa et al. 2015a, b, c; Hyra et al. 2016). A meroistic oogenesis, with nurse cells (trophocytes) and follicular cells, has been evidenced in tardigrades. Stable cytoplasmic bridges connect cystocytes (both trophocytes and the oocyte) each other forming a cluster. During pre-vitellogenesis, all cystocytes of a cluster synthesize and accumulate macromolecules and organelles, which will eventually transfer by cytoplasmic bridges to the only cell that will differentiate into an oocyte. A mixed vitellogenesis takes place in

Fig. 8.7 Representation of *Milnesium* sp. ovary showing the relationship of the germ cell cluster. Yolk granules (grey circle) are formed in every cell, but only large oocytes (arrows) are growing to be eggs with chorion



tardigrades: a part of the yolk is produced by the different kind of cells within the gonad (both ovary and ovotestis), and another part is produced by cells/organs located outside of the gonad. In particular, within the gonad yolk material is produced by oocytes (autosynthesis) and nurse cells. Outside of the gonad, the yolk is synthesized by storage cells and cells of the midgut epithelium; this yolk is absorbed by oocytes via micropinocytosis (Węglarska 1979; Poprawa 2005a, Hyra et al. 2016; Rost-Roszkowska et al. 2011, Poprawa et al. 2015a, b, c). A different pattern was observed in the apochelan *Milnesium* sp. (formerly shown as *M. tardigradum*) in which four large multinuclear cells, connected each other through cytoplasmic bridges, are surrounded by many mononuclear cells (oocytes; Fig. 8.7). Multinuclear cells and most of mononuclear ones play a role in vitellogenesis (nurse cells), whereas only some of the mononuclear cells develop in eggs (Suzuki 2006).

8.4.2 Eggs Shell Morphology

Laid eggs of tardigrades are often spherical or oval in shape, usually between 50 and 100 μm in diameter and sometimes even up to 235 μm when the size of shell processes are considered (Fig. 8.8). Those of the marine heterotardigrades are surrounded by a sticky smooth shell and are laid freely. The eggs of non-marine heterotardigrades and those of eutardigrades are surrounded by a rigid and sclerified shell that can be smooth when laid in the exuvia (both in eutardigrades and heterotardigrades except for *Oreella*) or ornamented when laid freely (eutardigrades). In particular, in some genera of limnic and terrestrial eutardigrades (e.g. *Macrobiotus*, *Paramacrobiotus*, *Murrayon*, *Minibiotus*, *Dactylobiotus*, *Acutuncus*, *Bertolanius*, *Eohypsibius*, *Ramazottius*, *Hebesuncus* and *Fractonotus*), as well as in the heterotardigrade *Oreella*, the egg shell shows species-specific ornamentation with a variety of shape processes, reticulation and pits (Fig. 8.8a–e). These morphological traits have an essential taxonomical significance, especially in the genera of eutardigrades in which animals are morphologically indistinguishable

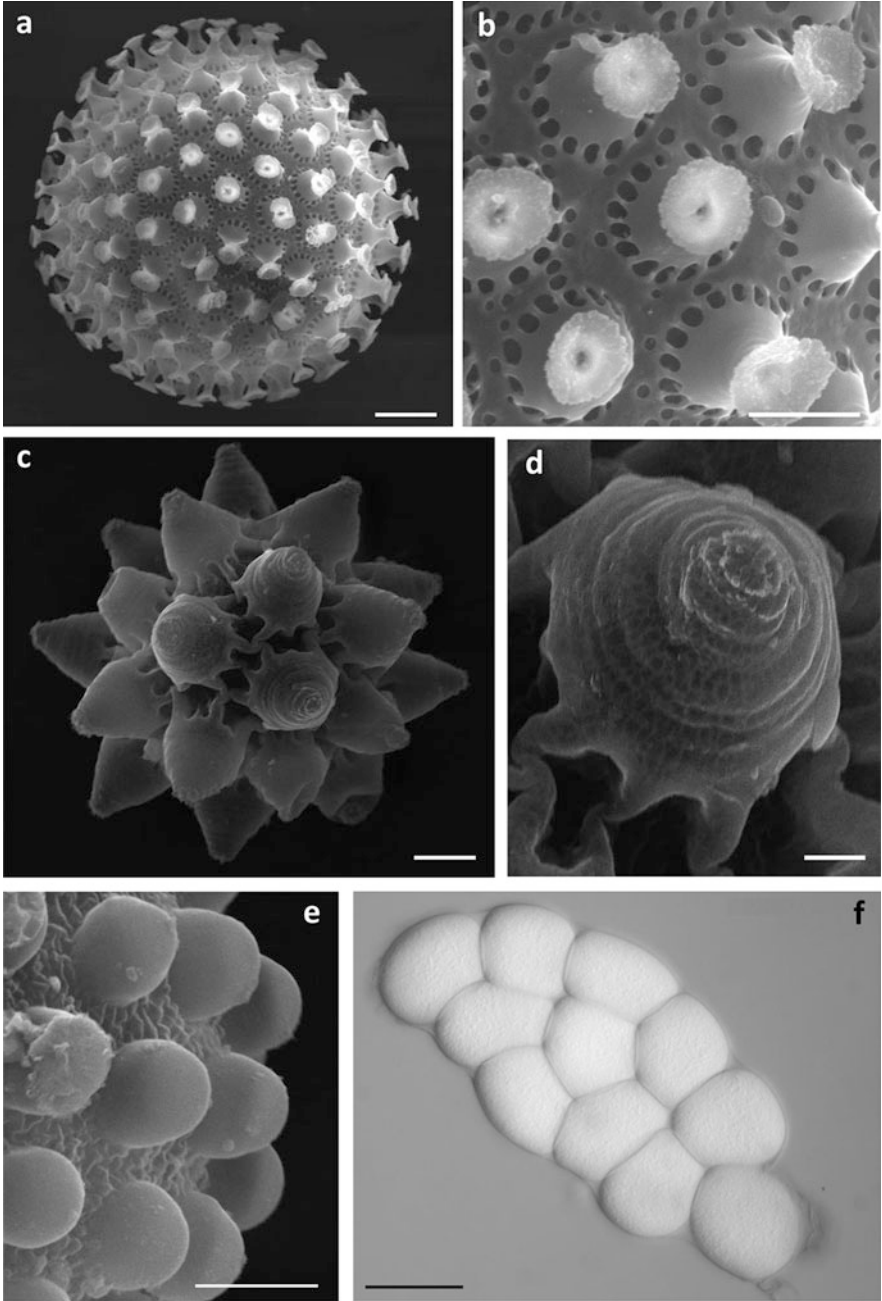


Fig. 8.8 Morphology of the egg shell of eutardigrade species. (a, b) *Macrobiotus macrocalix*; (c, d) *Paramacrobiotus* cf. *richtersi*; (e) *Ramazzottius oberhaeuseri*; (f) exuvium of *Hypsibius dujardini* containing eggs with smooth shell. (a–e) Scanning electron micrographs; (f) orcein staining and DIC. Scale bars: (b, d, e) = 5 μm ; (a, c) = 10 μm ; (f) = 50 μm

to each other (Bertolani and Rebecchi 1993; Bertolani et al. 1996). So far, the egg shell of tardigrades does not exhibit a mycropilar opening. In some eutardigrade species, egg envelope formation starts during middle or late vitellogenesis. The choriogenesis occurs with synthesis and secretion activity of the oocyte and somatic cells of the ovary wall. Afterwards, the oocyte produces precursors of the vitelline envelope which are released in the space between oolemma and chorion. At the end of the oogenesis, the egg capsule, with a protective function, is completely formed, and it is composed of two envelopes: a very thin vitelline envelope, which overlaps the oolemma, and a thick egg shell (chorion). The vitelline envelope consists of polysaccharides, proteins and lipids. The chorion is three-layered: a thin inner layer, the middle labyrinthine layer and an outer layer (Węglarska 1975, 1982; Poprawa 2005b, 2011; Rost-Roszkowska et al. 2011).

8.4.3 Spermatogenesis

Spermatogenesis usually begins after the first or second moulting of the animal. Spermatogonia and spermatocytes can be distinguished each other by the larger size or by the typical star-shaped metaphases of the latter (Bertolani 1971; Altiero and Rebecchi 2003). Data on spermiogenesis at ultrastructural level in tardigrades are still very limited and often too anecdotal to allow reasonable conclusion (Wolburg-Buchholz and Greven 1979; Kristensen 1979; Rebecchi 1997; Jørgensen et al. 1999; Greven and Kristensen 2001). During the spermatogenesis, a spermatogonium undergoes to mitosis leading to two primary spermatocytes, which after two subsequent meiotic divisions give rise to a multinucleate cell. Thereafter, this multinucleate cell turns into a cluster of eight spermatids joined together by cytoplasmic bridges. These cytoplasmic bridges have been evidenced both in eutardigrades and heterotardigrade and both in males and in hermaphrodites. In the eutardigrade *Xerobiotus pseudohufelandi*, spermatids are sphere-shaped (Fig. 8.10a) and have a large nucleus, and their chromatin appears condensed. Afterwards, spermatids elongate, the acrosome originates from the Golgi apparatus, the amount of cytoplasm decreases, and the nucleus shape changes from round/comma to helicoidal. Lastly, spermatids differentiate into spermatozoa: the nucleus moves from the centre of the spermatid cytoplasm outwards and coils; the flagellum develops from the unique centriole present; the number of mitochondria decreases until only two (Rebecchi 1997). In marine species of the genera *Echiniscoides* and *Batillipes*, early spermatids contain two unmodified mitochondria closely attached and an evident acrosome located close to the nucleus. Instead, in *Actinarctus*, five spermatid stages are recognized, and the spermatid stage 5 is only found in the posterior part of the testis (i.e. in the ventral seminal vesicles) and in the gonoducts (Jørgensen et al. 1999).

8.4.4 Ultrastructure of the Spermatozoa

The spermatozoa of tardigrades are always flagellate with a classical axoneme ultrastructure (9 + 2; Figs. 8.9, 8.10, 8.11 and 8.12). A noteworthy morphological heterogeneity in the spermatozoa of eutardigrades has been evidenced with several different structural features that may be associated with colonized habitat or with phylogeny (Figs. 8.9 and 8.10c–e). Nevertheless, the spermatozoa of eutardigrades are thin and filiform with a length that comprises between 25 and 100 μm , according

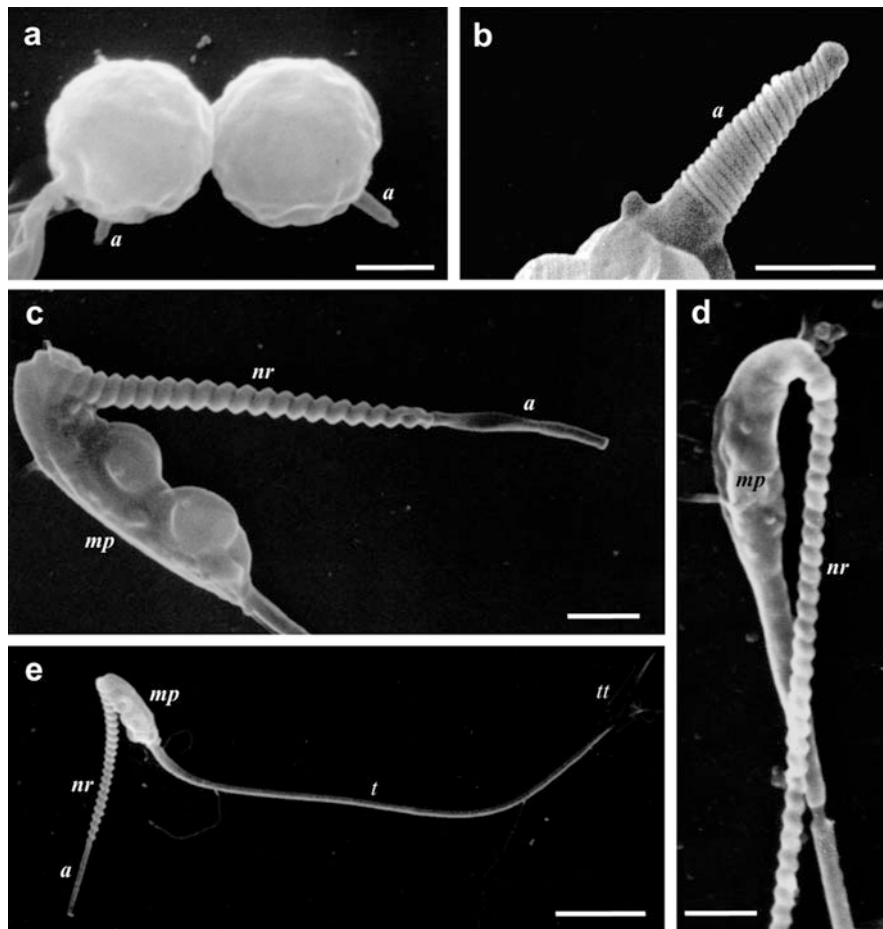


Fig. 8.9 Male germinal cells of eutardigrade species. (a) Roundish spermatids of *Diphyscon nobilei*; (b) acrosome of the spermatozoon of *Bertolanius volubilis*; (c) head (acrosome and nuclear region) and middle piece of the male gamete within the testis of *Xerobiotus pseudohufelandi*; (d) nuclear region and midpiece of the male gamete of *Mesobiotus harmsworthi*; (e) in toto spermatozoon of *Macrobiotus macrocalix*. (a–e) Scanning electron microscopy. Scale bars: (a–d) = 1 μm ; (e) = 5 μm . Abbreviations: a = Acrosome; nr = nuclear region; mp = middle piece; t = tail; tt = terminal tuft

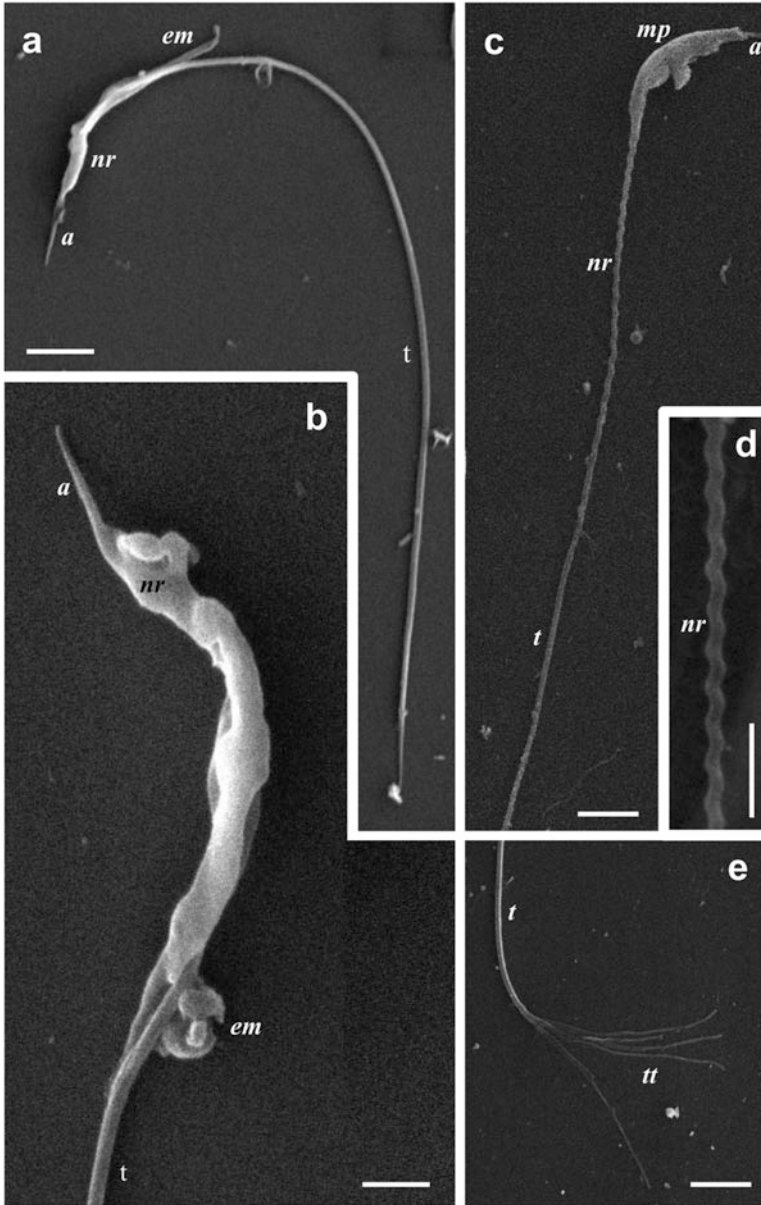


Fig. 8.10 Ultrastructure of tardigrade spermatozoa. (a) *In toto* sperm cell of the moss-dwelling heterotardigrade *Echiniscus duboisi*. (b) Head of the sperm cell of *Echiniscus duboisi*; (c) middle piece and nuclear region of the male gamete of the moss-dwelling eutardigrade *Minibiotus furcatus*; (d) detail of the nuclear region of the male gamete of *Minibiotus furcatus*; (e) terminal tuft of the tail of the male gamete of *Minibiotus furcatus*. (a–e) Scanning electron microscopy. Scale bars: (a, c, e) = 2 μm (b, d) = 1 μm . Abbreviations: a = Acrosome; em = external mitochondria; nr = nuclear region; mp = middle piece; t = tail; tt = terminal tuft

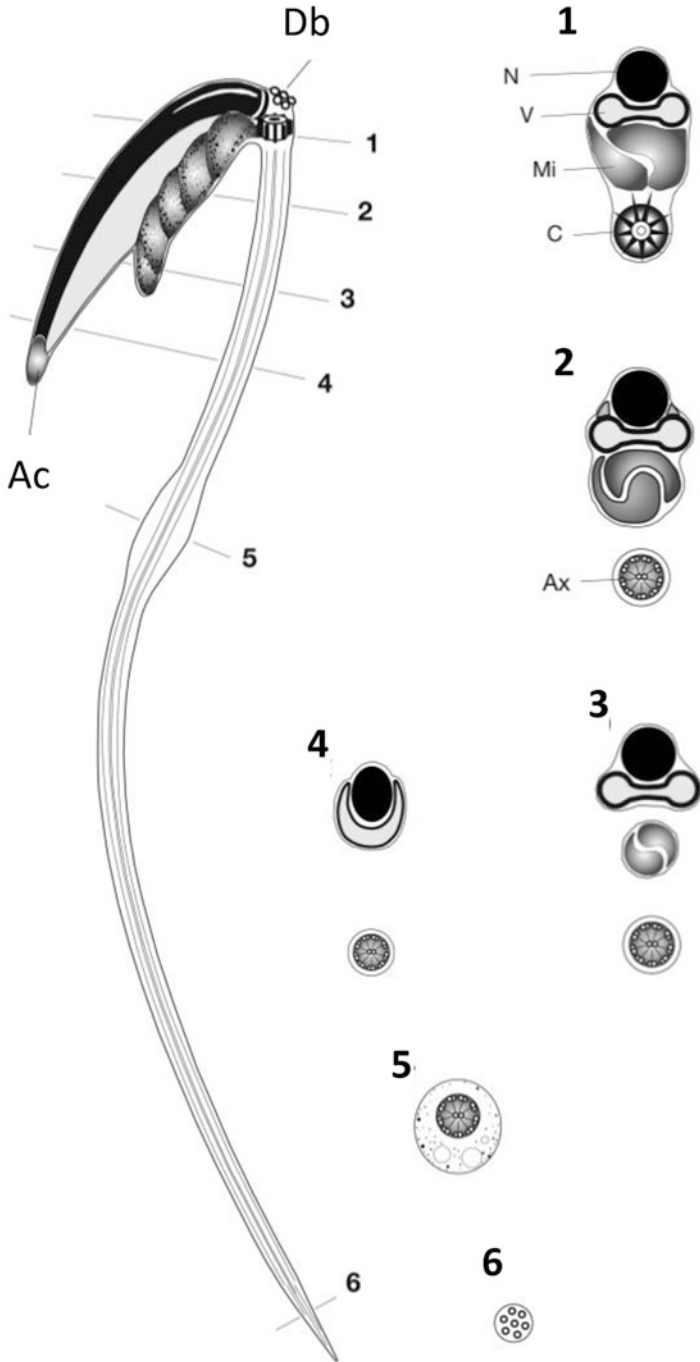


Fig. 8.11 Schematic representation of *in toto* spermatozoon of the hermaphroditic heterotardigrade *Orzeliscus cf. belopus* in the ovotestis and its cross-sections at different levels (1–6). Abbreviations: Ac = Acrosome; Ax = axoneme; C = centriole; Db = small dense bodies; Mi = mitochondria; N = nucleus; V = paranuclear vesicle. Redrawn from Suzuki and Kristensen (2014)

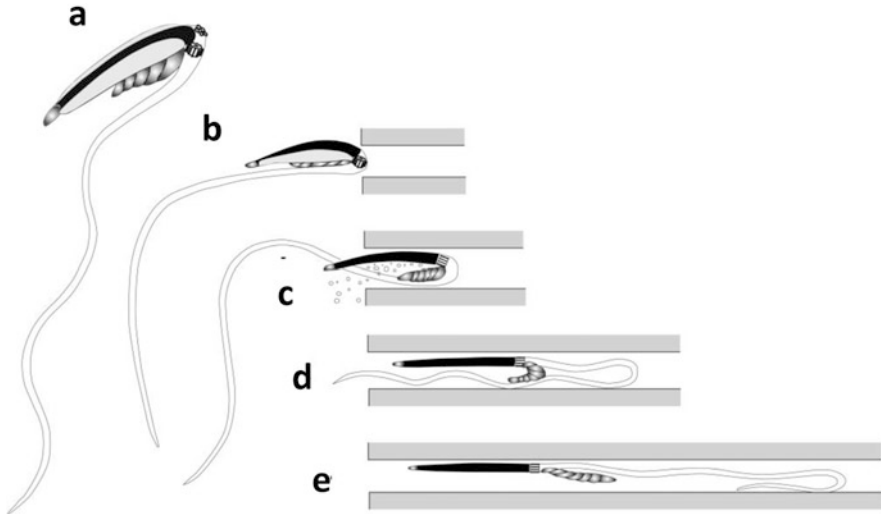


Fig. 8.12 (a) Schematic representation of the spermatozoon of *Orzeliscus* cf. *belopus* in front of the gonopore; (b–e) scenario of the morphological modification of the male gamete in the duct of the female receptacle. Redrawn from Suzuki and Kristensen (2014)

to the species. They are made up by the following regions: an elongated coiled head in which acrosome and nucleus can be easily recognized, a neck or an elongated midpiece (sometimes absent) and a flagellum. The acrosomes can be (1) conical and corkscrew-shaped, with tightly coiled spires (e.g. *Bertolanius*, *Adropion*); (2) -rod-shaped, with smooth surface, and relatively short (e.g. *Macrobiotus*, *Xerobiotus*, *Ramazzottius*); (3) cylindrical and very thin, with smooth surface and longer than the nucleus (e.g. *Paramacrobiotus*, *Mesobiotus*); and (4) very short and comma-shaped (e.g. *Pseudobiotus*, *Isohypsibius*, *Doryphoribius*, *Platicrista*, *Dipascon*, *Isohypsibius*). The acrosome is always bilayered and fits on the anterior part of the nucleus like a cup; in *Bertolanius* species the acrosome shows a narrow longitudinal canal and caudal small vesicles (Rebecchi and Guidi 1995; Rebecchi et al. 2000a).

In all eutardigrade species, the spermatozoon nucleus is always made up by condensed and electron-dense chromatin. In *Bertolanius*, the nucleus is cylindrical and surrounded by cytoplasm organized into ovoidal elements delimited by own cytomembrane. In all other eutardigrade species, the nucleus is always coiled even though with differences in the coil morphology. For instance, in *Platicrista* and *Adropion*, the corkscrew-shaped nucleus is made up by 3–4 coils (Rebecchi et al. 2000a). In *Macrobiotus*, *Xerobiotus* and *Ramazzottius* species, the nucleus exhibit tightly coils increasing in diameter caudally, and it is surrounded by a thin layer of cytoplasm, while in *Paramacrobiotus* the nucleus is longer than that of the previous species, but it is weakly coiled and contains fibrils running parallel to the nucleus (Rebecchi et al. 2011). These fibres could have an important role in facilitating the movement of the spermatozoa. In *Doryphoribius* the drill-shaped long nucleus has a constant diameter pitch (Baccetti 1987; Rebecchi 2001), while in the filiform and

thin nucleus of *Pseudobiotus*, the helical shape is due to a thin helical ridge wrapped around a central axial rod (Rebecchi and Bertolani 1999). In *Isohypsibius*, a “condensed body” with unknown function surrounds on one side the whole length of the helicoidal drill-shaped nucleus from the end of the acrosome to the beginning of the tail (Wolburg-Buchholz and Greven 1979; Rebecchi et al. 2000a).

A short- and cup-shaped neck with large mitochondria around the only centriole, as in *Bertolanius*, *Platicrista* and *Adropion*, is located between nuclear region and tail (Rebecchi and Guidi 1995; Guidi and Rebecchi 1996; Rebecchi et al. 2000a). The male gametes of *Macrobotus*, *Xerobiotus*, *Ramazottius* and *Paramacrobotus* have a rod-shaped elongated midpiece in which ovoidal elements surround a mitochondrial sleeve which envelope the only centriole and the first part of the axoneme (Rebecchi 1997, 2001; Rebecchi and Guidi 1995; Rebecchi and Bertolani 1999; Rebecchi et al. 2000a, 2011). The midpiece (and mitochondria) is absent in limnic species with filiform sperm cells of the genera *Pseudobiotus*, *Doriphoribius* and *Isohypsibius* (Rebecchi and Bertolani 1999; Rebecchi et al. 2000a, 2011; Rebecchi 2001).

The spermatozoan tail has a constant diameter and splits terminally into a tuft of 9–11 fine elements (Rebecchi et al. 2000a). Two exceptions are known. In *Paramacrobotus* the proximal part of the axoneme is surrounded by nine outer accessory fibres (Rebecchi et al. 2011), while in *Doriphoribius* the tail is flanked for the most part of its length by an undulating membrane that is internal supported by a palisade of microtubules (Baccetti 1987; Rebecchi et al. 2000a). In eutardigrades, the spermatozoa within the gonoducts and seminal vesicles are always motile, clumped in bundles and oriented with the head towards the cloaca. In particular, the testicular spermatozoa of *Macrobotus*, *Paramacrobotus*, *Xerobiotus* and *Mesobiotus* are folded back between head and midpiece resembling a nutcracker.

Marine and non-marine heterotardigrade spermatozoa, considered of primitive type, are shorter (14–30 μm in length) than those of eutardigrades. They have a globose or slightly elongated head without coils, “free” mitochondria and a tapering tail without tuft and do not have a clear midpiece (Figs. 8.10a, b, 8.11, 8.12). The spermatozoa of terrestrial Echiniscidae (e.g. *Pseudechiniscus*, *Novechiniscus*, *Echiniscus*, *Antechiniscus*, *Proechiniscus*) are made up by (1) a cylindrical acrosome with smooth surface containing an electron-dense core surrounded by cytomembranes, (2) a cylindrical nucleus with electron-dense chromatin surrounded by a thin layer of cytoplasm, (3) an electron-dense sickle-shaped band which represents the beginning of the centriole located within a tapering tail and (4) a voluminous vesicle, located laterally to the centriole and representing the beginning of a long free mitochondria (Rebecchi 2001; Rebecchi et al. 2003a). The spermatozoa of the marine heterotardigrade *Echiniscoides* have an elongated acrosome, an osmiophilic cylindrical nucleus and two mitochondria shifted parallel to the flagellum and then considered “free” (Greven and Kristensen 2001). A similar pattern was evidenced in the marine *Batillipes* species, even though the acrosome is considered aberrant and the nucleus shows a “snout” of condensed chromatin in contact with three lamellated rings (Kristensen 1979; Rebecchi et al. 2000a). Within the ovotestis, the spermatozoa (Fig. 8.11) of the hermaphrodite species *O. cf. belopus* show acrosome, head,

centriole and flagellum; the head, incorporating a cylindrical nucleus, a paranuclear vesicle and two mitochondria, is attached at the angle to the centriole forming a half-headed arrow shape (Suzuki and Kristensen 2014).

8.4.4.1 Storage of Spermatozoa Within the Female Reproductive Apparatus

The spermatozoa stored in spermatheca of the female eutardigrades or in the seminal receptacles of female heterotardigrades are immobile and appear to have a different shape with respect to those within the testis. The spermatozoa of *Xerobiotus pseudohufelandi* within the spermatheca are straight, have a midpiece thin and without hemispherical swelling, and the tail is reduced to a short stub without terminal tuft (Rebecchi 1997).

In marine heterotardigrades, cuticular and external seminal receptacles and their content in spermatozoa are renewed at each moulting (Kristensen 1984; Jørgensen et al. 1999). In *Actinarctus*, male gametes undergo strong postcopulatory modification as in the seminal receptacle, the acrosome is oriented straight forward, the nucleus becomes rod-shaped and the vesicle in the head region disappear; in addition spermatozoa are immotile and imbedded in an osmiophilous secretion (Jørgensen et al. 1999). In *Orzeliscus* cf. *belopus*, the spermatozoa are located outside the epicuticle of the seminal receptacle and are modified after their discharge since within the duct the cell was no longer bent backwards and the paranuclear vesicle was not visible (Fig. 8.12, Suzuki and Kristensen 2014).

8.5 Mating and Fertilization

Information about mating and fertilization in tardigrades are limited. In the marine and gonochoristic heterotardigrade *Batillipes noerrevangi*, the male stimulates the female with his sense organs, and the female lays her eggs on a sand grain (Kristensen 1979). This species has external fertilization which occurs at the time of oviposition as in *Actinarctus* (Jørgensen et al. 1999). In the limnic eutardigrade *Pseudobiotus megalonyx*, one or more males copulate a single female. In particular, up to nine males of *P. megalonyx* clinch a female with their modified internal claws on the front legs and near-mature oocytes in the ovary not yet completely released by the female in the old cuticle (von Erlanger 1895; von Wenck 1914). According to von Erlanger (1895), the male gametes are inserted in the cloaca of old cuticle, while according to Henneke (1911), the males penetrated the old cuticle with their buccal stylets and then introduce sperms into the old cuticle, and from there spermatozoa rapidly reach the female cloaca. In any case, insemination in *P. megalonyx* takes place in a protected environment and fertilization is internal (Rebecchi et al. 2000a). In another bisexual eutardigrade, *Isohypsibius dastychi*, mating includes a mutual stimulation between male and female which precedes sperm ejaculation and egg

oviposition (Bingemer et al. 2016). For instance, the male holds the female with his first pair of legs, and the female stimulates the male by moving the stylets and contracting the sucking pharynx. Spermatozoa are ejaculated from the male seminal vesicles through the cloaca. During the third moulting, the female develops eggs, and after the cuticle and stylets have been rebuilt, the gravid female remains inside the exuvia, and she is ready for mating; if mating does not occur, egg absorption is observed (Bingemer et al. 2016). Lastly, copulation has not yet been observed in *Milnesium*, even though a sexual behaviour was observed, in which a rare male in thelytokous population approached a female, who had already oviposited in the old cuticle before ecdysis, and the male walked around the female for several minutes, pushing her with his mouth (Suzuki 2008).

As regards the hermaphroditic tardigrades, self-fertilization was demonstrated in the limnic eutardigrade *Isohypsibius monoicus* in which individuals kept isolated from birth to death had been able to reproduce (Altiero and Rebecchi 2001). Marine hermaphrodite *Orzeliscus* cf. *belopus* should have copulation and cross-fertilization as described above, even though it is unclear how spermatozoa could have been transferred to the seminal receptacles (Suzuki and Kristensen 2014).

External fertilization can be presumed for all marine and terrestrial heterotardigrade species on the basis of sperm morphology. In addition, the presence of two external seminal receptacles on the ventral cuticle of the females and far from the female gonopore represents another evidence of external fertilization in heterotardigrades. The more specialized morphology of eutardigrade spermatozoa and the presence of an internal epithelial spermatheca connected to the rectum may represent adaptations to internal fertilization. In line with internal fertilization, the contemporary presence in eutardigrade spermatozoa of a helicoidal head and a tufted tail may help stabilize and direct motion in non-canalized tracts (Rebecchi et al. 2000a). In *Paramacrobotus* species, the fibrils running parallel to the nucleus could have an important role in facilitating the movement of the spermatozoa. Moreover, other structures, as the accessory fibres, may represent additional motor elements, allowing the acquisition of the internal fertilization (Rebecchi et al. 2011).

8.6 Parthenogenesis

Parthenogenesis is unknown in tardigrades colonizing marine habitats, whereas it is very frequent in tardigrades colonizing limno-terrestrial habitats. Among terrestrial heterotardigrades, many species of the genus *Echiniscus* reproduce via parthenogenesis and among eutardigrades the limnic species of the family Murrayidae, some limnic species of *Pseudobiotus*, *Thulinus* and *Hypsibius*, and several limno-terrestrial or terrestrial species of Macrobiotidae. Their parthenogenesis can be automictic or apomictic and in the latter case is often associated with polyploidy (triploidy, the most frequent, and rarely tetraploidy; Bertolani 1982; Rebecchi et al. 2003b). The family Murrayidae represents an example of evolution and persistence of asexual lineages; males have never been found to date indicating that the entire

line made up of three genera and more than 20 described species differentiated without sexual reproduction (Guidetti et al. 2000).

Only thelytokous and continuous parthenogenesis has been found in tardigrades, and heterogony has never been found. Nevertheless, the appearance of males with modified claw in the first pair of legs in a thelytokous strain of *Milnesium* sp. was detected (Suzuki 2008). It is unknown whether these males can actually function in sexual reproduction; however, they might allow some possibility of genetic exchange among clonal populations. No environmental factors that generate males were determined (Suzuki 2008). When animals reproduce by automixis for several generations, the loss of genetic variability, unless of mutations, leads to a complete homozygosity. On the other hand, the heterozygosity level of animals reproduce by apomixis may be kept or increased in time, as a consequence of mutation events (Bertolani 2001).

The success of the parthenogenesis in tardigrades from non-marine habitats may be due to the advantage that this kind of reproduction offers to the colonization of new habitats by means of passive dispersal. A relationship among the evolution of parthenogenesis and cryptobiosis and the colonization of terrestrial environment may be recognized in tardigrades (Bertolani 2001). In limnic and terrestrial habitats, many species of tardigrades can survive drying and freezing, entering anhydrobiosis and cryobiosis, respectively. These adaptive strategies allow tardigrades to be subjected both to a lower environmental selective pressure and to passive dispersal. By means of passive dispersal, even a single tardigrade can colonize a new territory if it is parthenogenetic or has been previously fertilized (Bertolani 2001). Since parthenogenesis is more widespread than cross- and self-fertilization, it is favourably selected, and its permanence across many generations may have been aided by anhydrobiosis. Moreover, parthenogenesis and hermaphroditism have never been noted simultaneously in populations attributable to the same tardigrade species; therefore the widespread occurrence of parthenogenesis in limno-terrestrial habitats has been selected for over hermaphroditism, which only evolves when parthenogenesis is absent (Bertolani 2001).

8.7 Development

8.7.1 Embryonic Development

Literature data on tardigrade development has mainly referred on Marcus' work (1929a, b) for a long time. We waited for nearly 70 years for a reinvestigation on tardigrade embryology by Eibye-Jacobsen (1996/97), then followed by other more recent studies (Hejnol and Schnabel 2005; Gabriel and Goldstein 2007; Gabriel et al. 2007; Gross et al. 2015; Smith et al. 2016).

Embryonic development time in tardigrades lasts from 5 to more than 100 days according to the species and obviously to the rearing/environmental temperature. Tardigrades produce homolecithal eggs. The embryo undergoes an irregular

indeterminate cleavage pattern without early fate determination. In particular, Eibye-Jacobsen (1996/97) found a total and equal first cleavage that becomes asynchronous from the second cleavage onwards in the eutardigrade *Halobiotus crispae* and in the heterotardigrade *Echiniscoides sigismundi*. This is in line with successive studies carried out on other two eutardigrades, namely, *Thulinus stephaniae* and *Hypsibius dujardini* (see Hejnlol and Schnabel 2005; Gabriel and Goldstein 2007; Gabriel et al. 2007; Gross et al. 2015). In the latter species, the authors describe a radial and stereotyped and highly regulative cleavage pattern, including nuclear migrations that can predict the orientation of the embryonic axis, and a stereotyped pattern of stem cell-like asymmetric divisions. The blastula lacks the blastocoel, even though a small blastocoel seems to be present in *H. dujardini* (see Gabriel et al. 2007). In the embryos of *T. stephaniae*, the gastrulation starts after the 64-cell stage with the immigration of single blastomeres at two spatially distinct regions on the ventral surface of the embryo (Hejnlol and Schnabel 2005). Mesodermal precursor cells proliferate and form bands along the right and left side of the prospective pharynx and midgut. Later, the mesodermal bands develop into four pairs of somites without a cavity and disconnected with the archenteron, but more recently endo-mesodermal pouches have been described as the earliest morphological evidence of segmentation in *H. dujardini* embryos (Gabriel and Goldstein 2007; Gabriel et al. 2007; Gross et al. 2015). Primordia of all major organs were present from about 7 days after egg laying in *Halobiotus crispae*, when the embryo orientation is determined, and maturation continues after hatching (Eibye-Jacobsen 1996/97). Expression patterns of key developmental genes, including axis-determining segmentation and Hox genes, are recently evaluated (Gabriel and Goldstein 2007; Smith et al. 2016).

8.7.2 Post-Embryonic Development

As soon as embryonic development is complete, the tardigrade emerges from the egg with the action of the stylets and the pharyngeal pump. By sucking water through the corion into the gut, the embryo increases the inner hydrostatic pressure and breaks the egg shell also with the help of the stylets and the hind legs. Post-embryonic development takes place by growing of the single cells and animal moulting without any evidence of cell number increase. Tardigrades are not considered true eutelic animals, having been observed mitosis in some of their tissues.

In eutardigrades, newborns are very similar to the adults, apart from the smaller size, the immature gonad, slight differences in the claws and buccal apparatus anatomy (Bertolani 1990) and sometimes for the still immature eyespots and Malpighian tubules, as in *Halobiotus crispae* (see Eibye-Jacobsen 1996/97). For instance, in *Richtersius* cuticular pores are present in the newborns and are lost with first moulting, a novelty in the life cycle of eutardigrades (Guidetti et al. 2016). Another example from *Milnesium* shows increasing number of secondary branch with juvenile growth into adult (Morek et al. 2016).

In the heterotardigrade echiniscids, newborns show some anatomical differences from the adults. Anus, gonopore and the two external claws (out of four in the adults) per leg are absent in the juveniles. The juveniles need two moultings before they become adults with anus, gonopore and four claws per leg (Bertolani 1990). Sometimes, the number of cuticular filaments and spines of heterotardigrade specimens can increase with the age, apart from *Mopsechiniscus* in which it decreases. A similar pattern was detected in marine heterotardigrades in which two or four post-embryonic developmental stages (juvenile stages) have been identified (Bertolani 1990). For instance, in a new species of *Florarctus*, two distinct juvenile stages during which the anus is formed and the gonads mature prior to the development of the gonopore have been recognized. These juvenile stages are followed by one adult stage in female and two adult stages in males (Hansen et al. 2016). The morphology of the cuticular structures changes during the post-embryonic development showing an adult and juvenile appearance. Nevertheless, a pedomorphosis phenomenon could be evidenced in males having a mature gonad and juvenile characters of the cuticular appendages (Hansen et al. 2016).

8.8 Parental Care

Limnic and terrestrial tardigrades produce either smooth shelled eggs laid in the moulted cuticle (exuvia) or ornamented shelled eggs normally laid freely (i.e. not within the exuvia); instead marine tardigrades laid smooth shelled eggs freely. When the eggs are laid freely, parental care are not observed. On the contrary, when the eggs are laid within the exuvia, some forms of parental care can occur, even though they are very limited and mainly observed in limnic species of eutardigrades. *Pseudobiotus* females carry their exuvia filled with their eggs, keeping it attached to the caudal part of the body until the eggs hatch. Sometimes two subsequent exuviae filled with eggs of different ages can be found (for review see Bertolani 1990). Specimens of the hermaphrodite *Borealibius zetlandicus* can carry their exuvia filled with eggs, and adults were observed within the old cuticle alongside embryonated eggs and/or newborns (Pilato et al. 2006). Moreover, females of *Isohypsibius annulatus* attach the exuvia containing the eggs to the anterior-dorsal part of the body, and females of *Murrayon* genus lay their eggs within the exuvia of cladocerans (Bertolani et al. 2009).

8.9 Life Cycles

8.9.1 Life History Traits

Although certain useful information might be estimated from field sampling, most of the life history traits from birth to death of a tardigrade would be only available by laboratory culture. The oldest record of tardigrade rearing (Von Wenck 1914) still

provides us important knowledge about mating behaviour and embryology. The necessity of culture for further study of tardigrades has already been emphasized in the earlier monograph “Handbuch der Zoologie” (Richters and Krumbach 1926). However, investigations on life history traits of tardigrades had not increased until around the 1960s, and the second phase of lab-culture works has begun in the new century (Table 8.1). All of these studies have been done on eutardigrades and in particular on parthenogenetic tardigrades, even though a few studies on gonochoristic animals [e.g. *Paramacrobiotus tonollii*, *Macrobiotus sapiens* (see Lemloh et al. 2011) and *Isohypsibius dastychi* (see Bingemer et al. 2016)] are recently available (Table 8.2). On the contrary, in spite of the mention about rearing of *Batillipes* and *Echiniscoides* by Marcus (1929b), there is practically no detailed record of heterotardigrades culture so far.

The reproductive life cycle of a eutardigrade female includes the following steps: embryonic development, hatching, juvenile growth with successive moults, achievement of sexual maturity, egg production, egg laying and adult moulting (Fig. 8.13). From studies under controlled conditions, life history traits such as life span, age at first oviposition, clutch size (number of eggs per oviposition), total number of eggs per life span and hatching phenology can be extracted (Table 8.2). Considerable variations in life history traits may occur within populations of the same species or among different species, as evidenced in Table 8.2. For instance, Dougherty (1964) recorded detailed life history traits of an Antarctic tardigrade, *Hypsibius arcticus*, which is possibly regarded as *Acutuncus antarcticus* (see Dastych 1991), although his culture might not maintained well because of a progressive deterioration, that differs from those recorded by Tsujimoto et al. (2015, 2016a, b) and Altiero et al. (2015) using different populations of the same species, *A. antarcticus*. On the contrary, two populations of *Paramacrobiotus kenianus* did not show any significant differences with respect to their longevity, oviposition and hatching phenology (Schill 2013). A carnivorous species, *Milnesium tardigradum*, was at first reared by Baumann (1964), but their life history was elucidated in detail by a cultured Japanese strain (Suzuki 2003, 2008). However, the latter might not be actually *M. tardigradum* but *Milnesium* sp. with [3-3]-[3-3] claw system, which is not distinguished by DNA analysis from Tübingen strain of *Milnesium* sp. (see Hengherr et al. 2008). Although Tübingen strain was suggested to have longer life span (up to 107 days) than Hiyoshi strain (up to 58 days), the author afterword observed some individuals of Hiyoshi strain lived up to 4 months (at 23 °C), which is similar data with Tübingen’s one. Therefore, more statistical data are necessary for precise comparison between these populations.

The active life span of tardigrades has been estimated from 18 to 30 months (Ramazzotti and Maucci 1983) even though from lab cultures the longest recorded longevity was 518 days (ca. 17 months), from a clonal strain (CDMr01) of the terrestrial and carnivorous *Paramacrobiotus fairbanksi* (see Altiero et al. 2006) and 18 months for the *pseudosimplex 1* stage of the marine eutardigrade *Halobiotus crispae* (see Kristensen 1982). Hengherr et al. (2008) also carried out an experiment on tardigrade longevity concluding that repeated desiccation-rehydration events has no effect on the total active life.

Table 8.1 Records on tardigrade rearing

Author/s (Year)	Species	Habitat	Food	Ref. ^a
Von Wenck (1914)	<i>Macrobiotus lacustris</i> ^b	Freshwater	Green algae	–
Węglarska (1957)	<i>Dactylobiotus dispar</i>	Freshwater	Algae (<i>Chlorosphaera</i> , Diatoms)	–
Baumann (1961)	<i>Hypsibius convergens</i>	Green algae (<i>Blidingia</i>)	Green algae (<i>Chlorella pyrenoidosa</i>)	1
Ammermann (1962, 1967)	<i>Hypsibius dujardini</i>	Freshwater	Green algae (<i>Chlorella</i> sp.?)	2
Baumann (1964)	<i>Milnesium tardigradum</i>	Moss	Bacteria, protozoa, rotifers	3
Dougherty (1964)	<i>Hypsibius arcticus</i> ^c	Freshwater	Green algae	4
Baumann (1966)	<i>Ramazzottius oberhaeuseri</i>	Moss	Green algae (<i>Pseudochlorella</i> sp.)	5
Sayre (1969)	<i>Isohypsibius myrops</i> ^d	Freshwater (moist sphagnum)	Nematode (<i>Panagrellus redivivus</i>)	6
Baumann (1970)	<i>Macrobiotus hufelandii</i>	Moss	Green algae (<i>Pseudochlorella</i> sp.)	7
Bertolani and Buonagurelli (1975)	<i>Dactylobiotus parthenogeneticus</i>	Freshwater	Pond water, algae and Erd-Schreiber solution	8
Altiero and Rebecchi (2001)	<i>Paramacrobiotus fairbanksi</i>	Leaf litter	Nematodes ^e	
	<i>Macrobiotus joannae</i>	Leaf litter	Nematodes ^e	9
	<i>Diphascion</i> cf. <i>scoticum</i>	Freshwater	Green algae (<i>Scenedesmus acutus</i>)	9
	<i>Isohypsibius monoicus</i>	Freshwater	Natural sediment with algae	9
Suzuki (2003)	<i>Milnesium</i> sp. ^e	Moss	Rotifer (<i>Lecane inermis</i>)	10
Hohberg (2006)	<i>Paramacrobiotus</i> cf. <i>richtersi</i>	Soil	Nematodes ^h	11
Gabriel et al. (2007)	<i>Hypsibius dujardini</i>	Freshwater	Green algae (<i>Chlorococcum</i> sp.)	12
Horikawa et al. (2008)	<i>Ramazzottius varieornatus</i>	Moss	Green algae (<i>Chlorella vulgaris</i>)	13
Altiero et al. (2006, 2010)	<i>Paramacrobiotus fairbanksi</i>	Leaf litter	Nematodes ^e	14
Hengherr et al. (2008)	<i>Milnesium</i> sp. ^f	Moss	Rotifer (<i>Philodina citrina</i>)	15
Lemloh et al. (2011)	<i>Paramacrobiotus tonolli</i>	Moss	Green algae (<i>Chlorogonium elongatum</i>) + rotifer (<i>Philodina citrina</i>)	16
	<i>Macrobiotus sapiens</i>	Moss	Green algae (<i>Chlorogonium elongatum</i>)	16
Kagoshima et al. (2013)	<i>Acutuncus antarcticus</i>	Moss	Green algae and/or cyanobacteria	–

(continued)

Table 8.1 (continued)

Author/s (Year)	Species	Habitat	Food	Ref. ^a
Schill (2013)	<i>Paramacrobiotus kenianus</i>	Moss	Rotifer (<i>Philodina citrina</i>)	17
	<i>Paramacrobiotus palaui</i>	Moss	Rotifer (<i>Philodina citrina</i>)	17
Altiero et al. (2015)	<i>Acutuncus antarcticus</i>	Freshwater sediment	Green alga (<i>Chlorococcum</i> sp.)	18
Poprawa et al. (2015b)	<i>Dactylobiotus parthenogeneticus</i>	Freshwater pond	Green algae (<i>Chlorella</i> sp., <i>Chlorococcum</i> sp.)	–
Stec et al. (2015)	<i>Macrobiotus paulinae</i>	Moss	Green algae and rotifers ⁱ	–
Poprawa et al. (2015c)	<i>Macrobiotus polonicus</i>	Moss	Alga (<i>Chlorella</i> sp.), nematode (<i>C. elegans</i>)	–
Tsujimoto et al. (2015)	<i>Acutuncus antarcticus</i>	Moss/freshwater	Green algae (<i>Chlorella vulgaris</i>)	19
Bingemer et al. (2016)	<i>Isohypsibius dastychi</i>	Post-mining site	Green algae (<i>Chlorella vulgaris</i>)	20
Ito et al. (2016)	<i>Isohypsibius myrops</i>	Sewage sludge	Green algae (<i>Parachlorella beyerinckii</i>), rotifers (<i>Lecane inermis</i>)	21
Koszyła et al. (2016)	<i>Diphascion higginsi</i>	Leaves in pond	Green algae and rotifers ⁱ	–
	<i>Hypsibius</i> cf. <i>dujardini</i>	Leaves in pond	Green algae and rotifers ⁱ	–
	<i>Isohypsibius pushkini</i>	Leaves in pond	Green algae and rotifers ⁱ	–
	<i>Milnesium</i> cf. <i>alpigenum</i>	Moss	Rotifers and green algae ⁱ	–
	<i>Paramacrobiotus</i> cf. <i>richtersi</i>	Moss	Rotifers and green algae ⁱ	–
	<i>Thulinus ruffoi</i>	Sewage sludge	Green algae and rotifers ⁱ	–

^aReference number correlate with Table 8.2

^bOriginally described as *Macrobiotus lacustris*; today this name is not valid. On the basis of the original drawing, specimens are attributable to *Pseudobiotus megalonyx*

^c*Acutuncus antarcticus*

^dTentatively identified. Sayre's animal tolerated against dehydration

^eHiyoshi-1 strain, originally described as *M. tardigradum*

^fTubingen strain, originally described as *M. tardigradum*

^g*Pristionchus iheritieri*, *Panagrolaimus rigidus*, *Caenorhabditis elegans*

^h*Pelodera teres*, *Acrobeloides nanus*

ⁱAlgae: *Chlorococcum* sp., *Chlorella* sp. Rotifer: *Lecane inermis*

Sexual maturity is reached with the second or third moulting, rarely even with the first one. Reared females lay from 1 to 44 eggs at each oviposition, depending on the species, age, and nutritional state of the specimens. Ramazzotti and Maucci (1983)

Table 8.2 Life history traits of cultured tardigrades

Species	Life span (days)	Max. longevity (days)	Age at first oviposition (days)	No. of eggs/clutch	No. of ovipositions/animal	No. of eggs/animal	Hatching success (%)	Hatching time (days)	Temp. (°C)	Ref. ^a	Reproductive modes
<i>Acutuncus antarcticus</i>	69.2 ± 36.4	162	9.3 ± 1.1	1–10	7.5	34.4 ± 22.6	97.6	8.5 ± 0.7	15	19	P
<i>Acutuncus antarcticus</i>	88.8 ± 20.0	130	17.1 ± 3.6	1.8 ± 0.8	6.4 ± 1.0	13.0 ± 3.7	67.1 ± 22.7	8.8 ± 2.6	14	18	P
<i>Dactylobiotus dispar</i>	–	–	–	411	–	–	–	–	19	8	P
<i>Diphiascon (Adropion) scoticum</i>	–	263	–	1–11	15	–	–	5–14	14	9	P
<i>Hypsibius arcticus</i>	–	160	21–45	2–13	11	84	69.0	15–39	4–7	4	P
<i>Hypsibius convergens</i>	–	159	–	2–12	–	–	–	5–10	–	1	–
<i>Hypsibius digardini</i>	–	–	14	3–10	–	–	–	5	21–23	2	P
<i>Hypsibius digardini</i>	–	–	13.6 ± 0.8	3.4 ± 1.9	–	–	–	4–4.5	10–18	12	P
<i>Isohypsibius dasychi</i>	–	–	44.9 ± 6.5	–	–	–	80.0	13.1 ± 1.5	12	20	A/G
<i>Isohypsibius dasychi</i>	–	–	28.2 ± 4.6	–	–	–	66.0	7.6 ± 1.1	20	20	A/G
<i>Isohypsibius myrops</i> (strain Im1)	18.8 ± 7.0	~22	10.3 ± 0.7	19.0 ± 7.6	3.5 ± 2.8	67.3 ± 61.3	80.0 ± 29.4	3.6 ± 0.6	22/23	21	P
<i>Isohypsibius monoicus</i>	–	197	–	1–3	3	–	–	12–20	14	9	A/H
<i>Macrobrotius joannae</i>	–	266	–	1–7	–	–	–	14–39	20	9	A/H
<i>Macrobrotius sapiens</i>	83.0 ± 33.5	145	16.5 ± 3.8	5.1 ± 3.1	–	–	78	11.9 ± 2.7	20	16	A/G
<i>Macrobrotius hufelandii</i>	–	84	31	1	3	3	–	26–31	20	7	P
<i>Milnesium tardigradum</i>	–	–	41	1–6	–	–	–	4–12	–	3	P
<i>Milnesium</i> sp. (Hiyoshi)	42.7 ± 11.8	58	15.3 ± 1.6	7.0 ± 2.2	3.3 ± 1.6	22.9 ± 12.9	77.2	11.2 ± 2.4	25	10	P
<i>Milnesium</i> sp. (Tübingen)	82.7 ± 2.7	107	–	–	–	–	–	–	20	15	P
<i>Paramacrobrotius kenianus</i> (population 1)	125 ± 35	204	10	–	–	138 ± 71	33	7	20	17	P
<i>Paramacrobrotius kenianus</i> (population 2)	141 ± 54	212	10	–	–	124 ± 78	51	8	20	17	P
<i>Paramacrobrotius palaui</i>	97 ± 31	187	10	–	–	42 ± 54	54	9	20	17	P
<i>Paramacrobrotius richtersi</i> (Berzdorf)	–	–	64.2 ± 1.7	4.0 ± 0.8	–	–	100	28.1 ± 1.2	16	11	

<i>Paramacrobiotus fairbanksi</i> (CGMr01)	194.9 ± 164.4	518	76.9 ± 16.4	11.0 ± 7.1	3.4 ± 2.1	37.8 ± 29.3	83.1 ± 12.7	41.0 ± 9.5	14	14	P
<i>Paramacrobiotus fairbanksi</i> (CGMr02)	137.3 ± 136.4	457	70.7 ± 19.4	8.0 ± 6.2	2.0 ± 1.2	17.8 ± 16.0	51.0 ± 36.0	60.0 ± 16.7	14	14	P
<i>Paramacrobiotus ionellii</i>	69.0 ± 45.1	237	24.4 ± 4.4	6.5 ± 4.3	–	–	82.2	16.7 ± 6.7	20	16	A/G
<i>Ramazzottius oberhaeuseri</i>	–	70	–	1–11	7	–	–	8–20	–	5	P
<i>Ramazzottius varicornatus</i>	35.0 ± 16.4	87	9	–	–	7.85	82.5	5.7 ± 1.1	25	13	P

P = parthenogenesis; A/G = amphimixis, gonochoric; A/H = amphimixis, hermaphroditic

Data are shown as either average ± SD, the range or simple available data

Minus sign indicates no data

^aCorrelate with reference numbers in Table 8.1

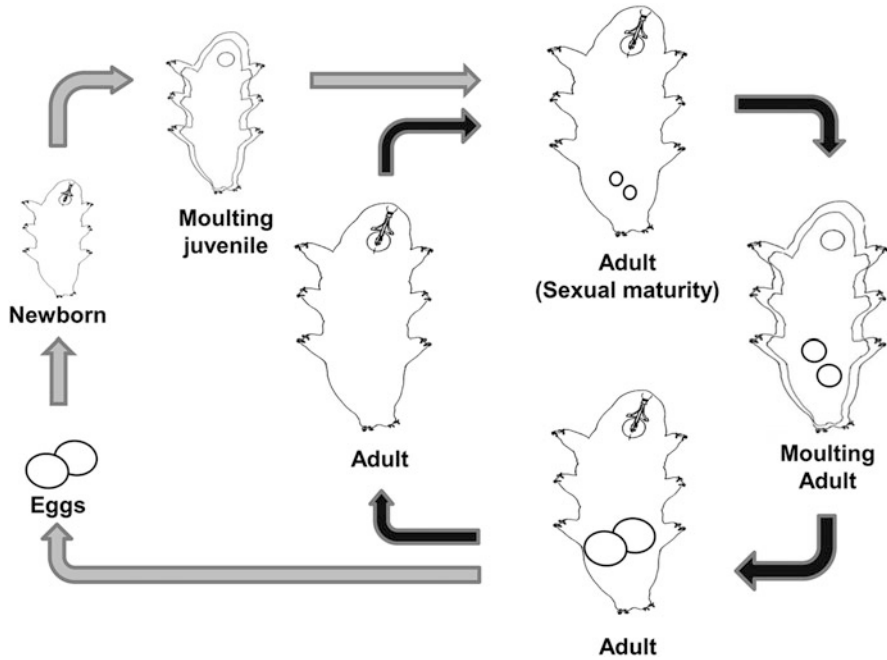


Fig. 8.13 Schematic representation of the steps of the life cycle of a eutardigrade laying smooth eggs within the old cuticle (exuvia)

reported a maximum of 60 eggs at each oviposition, but this data have not been confirmed by laboratory cultured species. Egg production continues throughout adult life, although moulting can occur without egg production, up to 190 eggs (Table 8.2). The development time of eggs varies from 4 to 87 days, depending on rearing conditions (e.g. temperature, oxygen amount) and species/populations, but strong variations have been evidenced even in a same clonal lineage (Table 8.2).

8.9.2 Moulting Process

Tardigrades grow up even after sexual maturity, through periodical moultings, occurring throughout their life. Body length increases at every moulting until maximum size is reached, although a decrease in size may be caused by the lack of food. The moulting process lasts 5–10 days, and it involves the expulsion through the buccal opening of the whole cuticular lining of the foregut together with the buccal tube, placoids, stylets and stylet supports (Guidetti et al. 2012). After that, animals are in the so-called “simplex” stage; they cannot feed because of the closure of the mouth opening and the absence of every sclerified buccal-pharyngeal apparatus. In the meanwhile, the epithelial tissue of the oesophagus starts the

reconstruction of the posterior part of the buccal apparatus, the underlying epidermis of body cuticle, and hindgut lining begins to synthesize these new cuticular structures, and the claw (pedal) glands in each leg regenerate new claws. Finally, animal can release its old cuticle (exuvia, including the lining of the hindgut), which many species of tardigrades use for laying their eggs. For that species, a synchronization between moulting and oviposition is present (Nelson et al. 2015).

8.9.3 Resting Eggs

Experimental cultures of *Paramacrobiotus fairbanksi*, a leaf litter-dwelling species, have allowed to document the presence of resting eggs in tardigrades (Altiero et al. 2010). Resting eggs, needing an environmental cue to hatch (dehydration followed by rehydration), represent only a small portion (<10%) of all laid eggs. The remaining 90% of laid eggs, morphological indistinguishable from resting eggs, involved subitaneous eggs (>58%) that hatch in 30–40 days and delayed-hatching eggs (ca. 30%), which completed their development in 41–62 days, together with a very small fraction of abortive eggs (Altiero et al. 2010). Winter and summer eggs with differently shaped egg processes have been evidenced in *Bertolanius nebulosus* from Greenland: the former type may be interpreted as resting eggs (Hansen and Katholm 2002). Resting eggs, together with the high variability in hatching time of tardigrade eggs, might be considered as a bet-hedging adaptive strategy to cope with unpredictable environments, such as those colonized by tardigrades.

8.9.4 Rearing Tardigrades for Life History Trait Analysis

Cultured tardigrades, mainly belonging to eutardigrades, came from freshwater sediments, leaf litter or moss (Table 8.1). Reared herbivorous tardigrades fed on green algae, e.g. *Chlorella* spp., *Scenedesmus acutus*, *Chlorococcum* sp. and *Chlorogonium elongatum* (see Table 8.1). The actual food preference of four herbivorous tardigrade species (*Macrobiotus sapiens*, *M. persimilis*, *Richtersius coronifer* and *Echiniscus granulatus*) was examined by sequence analysis of a chloroplast gene from mosses and algae, revealing that algae seem to be a more likely food for these moss-dwelling species (Schill et al. 2011). Reared carnivorous tardigrades fed on rotifers or nematodes (see Table 8.1).

Pool cultures of tardigrade species can be kept in a flask with food source, but to collect data on life history traits, eggs must be collected immediately after their deposition and kept individually till hatching, as well as each newborn must be reared singularly from hatch to death. The recent culture system generally consists of appropriate containers (such as plastic Petri dishes) with 1.2–2% agar coat or with the bottom scratched with fine sand to aid tardigrade locomotion, water layer, food according to the rearing species and incubator for temperature and light/dark

conditioning. The medium has to be changed periodically (1–2 weeks), and pool cultures have to be divided in order to prevent overcrowding.

Examples from recent culture protocols are shown as follows.

Paramacrobiotus fairbanksi (from Altiero and Rebecchi 2001, with a slight modification). Animals are reared in small plastic dishes (15 mm diameter and 7 mm height) with bacto agar (Difco, 1.2% w/v in water) and some drops of natural mineral water (San Benedetto). They feed on three species of nematodes, *Pristionchus iheritieri*, *Panagrolaimus rigidus* and *Caenorhabditis elegans*, which are cultured utilizing the bacterium *Escherichia coli*. The diploid strain seemed to prefer 20 °C, whereas the triploid one seemed to prefer 14 °C. Newborns were fed with algae.

Milnesium tardigradum (from Suzuki 2003, with a slight modification). Animals are reared in plastic dishes (35 mm diameter). The bottom of each dish is coated with 2.0% agar (Agar Noble, Difco) in KCM solution (7 mg KCl, 8 mg CaCl₂ and 8 mg MgSO₄·7H₂O in 1000 ml of water), and a layer of water (Milli-Q, Millipore) is added. To simplify, agar solution can be prepared in water, then a layer of natural mineral water (e.g. Volvic) is added. Prey species of rotifer *Lecane inermis* is maintained with rice grain immersed in KCM solution. Bdelloid rotifers can also be used as food. This method is applicable to other milnesiid species.

Ramazzottius varieornatus (from Horikawa et al. 2008, with personal communication from Kunieda). Animals are reared in plastic dishes (35 or 90 mm diameter) with bacto agar (1.5% in either Milli-Q or Volvic water). Solution with *Chlorella vulgaris*, purchased from Chlorella Industry Co., Ltd. (Tokyo, Japan), is diluted ca. 1:100 in Volvic and added to the dish (1–2 mm height). Temperature should be kept at 20–25 °C. Recently, *Acutuncus antarcticus* also proved to be cultured well by this method (Tsujimoto et al. 2015) at 5–15 °C.

References

- Altiero T, Rebecchi L (2001) Rearing tardigrades: results and problems. *Zool Anz* 240:217–221
- Altiero T, Rebecchi L (2003) First evidence of achiasmatic male meiosis in the water bears *Richtersius coronifer* and *Macrobiotus richtersi* (Eutardigrada, Macrobiotidae). *Hereditas* 139:116–120
- Altiero T, Rebecchi L, Bertolani R (2006) Phenotypic variations in the life history of two clones of *Macrobiotus richtersi* (Eutardigrada, Macrobiotidae). *Hydrobiologia* 558:33–40
- Altiero T, Bertolani R, Rebecchi L (2010) Hatching phenology and resting eggs in *Paramacrobiotus richtersi* (Eutardigrada, Macrobiotidae). *J Zool* 280:290–296
- Altiero T, Giovannini I, Guidetti R, Rebecchi L (2015) Life history traits and reproductive mode of the tardigrade *Acutuncus antarcticus* under laboratory conditions: strategies to colonize the Antarctic environment. *Hydrobiologia* 761:277–291
- Ammermann D (1962) Parthenogenese bei dem Tardigraden *Hypsibius dujardini* (Doy.). *Naturwissenschaften* 49:115
- Ammermann D (1967) Die Cytologie der Parthenogenese bei dem Tardigraden *Hypsibius dujardini*. *Chromosoma* 23:203–213

- Baccetti B (1987) The evolution of the sperm cell in the phylum Tardigrada (Electron microscopy of Tardigrades. 5). In: Bertolani R (ed) *Biology of Tardigrades, selected symposia and monographs*, UZI, vol 2. Mucchi, Modena, pp 87–91
- Baumann H (1961) Der Lebensablauf von *Hypsibius (H.) convergens* Urbanowicz (Tardigrada). *Zool Anz* 167:362–381
- Baumann H (1964) Über den Lebenslauf und die Lebensweise von *Milnesium tardigradum* Doyère (Tardigrada). *Veröff Überseemus Bremen* 3:161–171
- Baumann H (1966) Lebenslauf und Lebensweise von *Hypsibius (H.) oberhaeuseri* Doyère (Tardigrada). *Veröff Überseemus Bremen* 3:245–258
- Baumann H (1970) Lebenslauf und Lebensweise von *Macrobiotus hufelandii* Schultze (Tardigrada). *Veröff Überseemus Bremen* 4:29–43
- Bertolani R (1971) Contributo alla carilogia dei Tardigradi. Osservazioni su *Macrobiotus hufelandii*. *Atti Accad Naz Lincei Rend Ser* 8^a(50):772–775
- Bertolani R (1982) Cytology and reproductive mechanisms in tardigrades. In: Nelson DR (ed) *Proc. Third International Symposium on the Tardigrada*. East Tennessee State Univ. Press, Johnson City, Tennessee, pp 93–114
- Bertolani R (1983a) Tardigrada. In: Adiyodi KG, Adiyodi RG (eds) *Reproductive biology of invertebrates. Oogenesis, oviposition, and oosorption*, vol 1. Wiley, Chichester, pp 431–441
- Bertolani R (1983b) Tardigrada. In: Adiyodi KG, Adiyodi RG (eds) *Reproductive biology of invertebrates. Spermatogenesis and sperm function*, vol 2. Wiley, Chichester, pp 387–396
- Bertolani R (1990) Tardigrada. In: Adiyodi KG, Adiyodi RG (eds) *Reproductive biology of invertebrates. Fertilization, development and parental care*, vol 4, Part B. Wiley, Chichester, pp 49–60
- Bertolani R (1992) Tardigrada. In: Adiyodi KG, Adiyodi RG (eds) *Reproductive biology of invertebrates. Sexual differentiation and behaviour*, vol 5. Wiley, Chichester, pp 255–266
- Bertolani R (2001) Evolution of the reproductive mechanisms in tardigrades. A review. *Zool Anz* 240:247–252
- Bertolani R, Buonagurelli GP (1975) Osservazioni carilogiche sulla partenogenesi meiotica di *Macrobiotus dispar* (Tardigrada). *Atti Accad Naz Lincei Rend Ser* 8^a(58):782–786
- Bertolani R, Rebecchi L (1993) A revision of the *Macrobiotus hufelandii* group (Tardigrada, Macrobiotidae), with some observations on the taxonomic characters of eutardigrades. *Zool Scr* 22:127–152
- Bertolani R, Rebecchi L, Beccacchioli G (1990) Dispersal of *Ramazzottius* and other tardigrades in relation to type of reproduction. *Invertebr Reprod Dev* 18:153–157
- Bertolani R, Rebecchi L, Claxton SC (1996) Phylogenetic significance of egg shell variation in tardigrades. *Zool J Linnean Soc* 116:139–148
- Bertolani R, Altiero T, Nelson DR (2009) Tardigrada (Water Bears). In: Likens GE (ed) *Encyclopedia of Inland Waters*, vol 2. Elsevier, Oxford, pp 443–455
- Bingemer J, Hihberg K, Schill RO (2016) First detailed observations on tardigrade mating behaviour and some aspects of the life history of *Isohypsibius dastychi* Pilato, Bertolani & Binda 1982 (Tardigrada, Isohypsibiidae). *Zool J Linnean Soc* 1787:856–862
- Dastych H (1991) Redescription of *Hypsibius antarcticus* (Richters, 1904), with some notes on *Hypsibius arcticus* (Murray, 1907) (Tardigrada). *Mitt Hamb Zool Mus Inst* 88:141–159
- Dewel RA, Nelson DR, Dewel WC (1993) Tardigrada. In: Harrison FW, Rice ME (eds) *Microscopic anatomy of invertebrates*, vol 12. Wiley, New York, pp 143–183
- Dougherty EC (1964) Cultivation and nutrition of micrometazoa. II. An Antarctic strain of the tardigrade *Hypsibius arcticus* (Murray, 1907) Marcus, 1928. *Trans Am Microsc Soc* 83:7–11
- Eibye-Jacobsen J (1996/97) New observation on the embryology of the Tardigrada. *Zool Anz* 235:201–216
- Gabriel WN, Goldstein B (2007) Segmental expression of Pax3/7 and Engrailed homologs in tardigrade development. *Dev Genes Evol* 217:421–433
- Gabriel WN, McNuff R, Patel SK et al (2007) The tardigrade *Hypsibius dujardini*, a new model for studying the evolution of development. *Dev Biol* 312:545–559

- Greven H, Kristensen RM (2001) Some aspects of spermiogenesis and spermatozoa ultrastructure in *Echiniscoidea sigismundi* (Heterotardigrada: Echiniscidae). *Zool Anz* 240:331–339
- Grimaldi de Zio S, D'Addabbo Gallo M (1975) Reproductive cycle of *Batillipes pennaki* Marcus (Heterotardigrada) and observation on the morphology of the female genital apparatus. *Stn Zool Napoli* 39:212–225
- Gross V, Treffkorn S, Mayer G (2015) Tardigrada. In: Wanninger A (ed) *Evolutionary biology of invertebrates Ecdysozoa 1: Non Tetraconata*, vol 1. Springer, Wien, pp 36–52
- Guidetti R, Rebecchi L, Bertolani R (2000) Cuticle structure and systematics of the Macrobiotidae (Tardigrada, Eutardigrada). *Acta Zool* 81:27–36
- Guidetti R, Altiero T, Marchioro T, Sarzi-Amadè L, Avdonina AM, Rebecchi L (2012) Form and function of the feeding apparatus in Eutardigrada (Tardigrada). *Zoomorphology* 131:127–148
- Guidetti R, Rebecchi L, Bertolani R, Jönsson KI, Kristensen RM, Cesari M (2016) Morphological and molecular analyses on *Richtersius* (Eutardigrada) diversity reveal its new systematic position and lead to the establishment of a new genus and a new family within Macrobiotidea. *Zool J Linnean Soc* 178:834–845
- Guidetti R, Cesari M, Bertolani R, Altiero T, Rebecchi L (2019) High diversity in species, reproductive modes and distribution within the *Paramacrobotus richtersi* complex (Eutardigrada, Macrobiotidae). *Zool Lett* 5:1. <https://doi.org/10.1186/s40851-018-0113-z>
- Guidi A, Rebecchi L (1996) Spermatozoan morphology as a character for tardigrade systematics: comparison with sclerified parts of animals and eggs in eutardigrades. *Zool J Linnean Soc* 116:101–113
- Hansen JG, Katholm AK (2002) A study of the genus *Amphibolus* from Disko Island with special attention on the life cycle of *Amphibolus nebulosus* (Eutardigrada: Eohypsibiidae). In: Hansen JG (ed) *Arctic biology field course*, Godhavn, Zoological Museum, University of Copenhagen. H.C.Ø. TRYK, Copenhagen, pp 129–163
- Hansen GJ, Kristensen RM, Jørgensen A, Accogli G, D'Addabbo R, Gallo M (2016) Postembryonic development, paedomorphosis, secondary sexual dimorphism and population structure of a new *Florarctus* species (Tardigrada, Heterotardigrada). *Zool J Linnean Soc* 178:871–877
- Hejnol A, Schnabel R (2005) The eutardigrade *Thulinia stephaniae* has an indeterminate development and the potential to regulate early blastomere ablations. *Development* 132:1349–1361
- Hengherr S, Brümmer F, Schill RO (2008) Anhydrobiosis in tardigrades and its effects on longevity traits. *J Zool* 275:216–220
- Henneke J (1911) Beiträge zur Kenntnis der Biologie und Anatomie der Tardigraden (*Macrobotus macronyx* Duj.). *Z Wiss Zool* 97:721–752
- Hohberg K (2006) Tardigrade species composition in young soils and some aspects on life history of *Macrobotus richtersi* J. Murray, 2011. *Pedobiologia* 50:267–274
- Horikawa DD, Kunieda T, Abe W, Watanabe M, Nakahara Y, Yukuhiro F, Sakashita T, Hamada N, Wada S, Funayama T, Katagiri C, Kobayashi Y, Higashi S, Okuda T (2008) Establishment of a rearing system of the extremotolerant tardigrade *Ramazzottius varieornatus*: a new model animal for astrobiology. *Astrobiology* 8:549–556
- Hyra M, Poprawa I, Włodarczyk A, Student S, Sonakowska L, Kszuk-Jendrysik M, Rost-Roszkowska MM (2016) Ultrastructural changes in the midgut epithelium of *Hypsibius dujardini* (Doyère, 1840) (Tardigrada, Eutardigrada, Hypsibiidae) in relation to oogenesis. *Zool J Linnean Soc* 178:897–906
- Ito M, Saigo T, Abe W, Kubo T, Kunieda T (2016) Establishment of an isogenic strain of the desiccation sensitive tardigrade *Isohypsibius myrops* (Parachela, Eutardigrada) and its life history. *Zool J Linnean Soc* 178:863–870
- Jørgensen A, Møbjerg N, Kristensen RM (1999) Ultrastructural studies on spermiogenesis and postcopulatory modifications of spermatozoa of *Actinarctus doryphorus* Sculz, 1935 (Arthrotardigrada: Halechiniscidae). *Zool Anz* 238:235–257
- Kagoshima H, Imura S, Suzuki AC (2013) Molecular and morphological analysis of Antarctic tardigrade, *Acutuncus antarcticus*. *J Limnol* 72(s1):15–23

- Kosztyła P, Stec D, Morek W, Gąsiorek P, Zawierucha K, Michno K, Ufir K, Małek D, Hlebowicz K, Laska A, Dudziak M, Frohme M, Prokop ZM, Kaczmarek Ł, Michalczyk Ł (2016) Experimental taxonomy confirms the environmental stability of morphometric traits in a taxonomically challenging group of microinvertebrates. *Zool J Linnean Soc* 178:765–775
- Kristensen RM (1979) On the fine structure of *Batillipes noerrevangi* Kristensen, 1978 (Heterotardigrada). 3. Spermiogenesis. *Zesz Nauk Univ Jagiellon Pr Zool* 25:97–105
- Kristensen RM (1980) Zur Biologie des marinen Heterotardigraden *Tetrakentron synaptae*. *Helgol Meer* 34:165–177
- Kristensen RM (1982) The first record of cyclomorphosis in Tardigrada based on a new genus and species from Arctic meiobenthos. *Z Zool Syst Evol – forsh* 20:249–270
- Kristensen RM (1984) On the biology of *Wingstrandarctus corallinus* nov. gen. et spec., with notes on the symbiotic bacteria in the subfamily Florarctinae (Arthrotardigrada). *Vidensk Meddr Dansk Naturhist Foren* 145:201–218
- Kristensen RM, Renaud-Mornant J (1983) Existence d'Arthrotardigrades semi-benthiques de genres nouveaux de la sous-famille des Styraconyxinae, subfam. nov. *Cah Biol Mar* 24:337–354
- Lemloh M-L, Brümmer F, Schill RO (2011) Life-history traits of the bisexual tardigrades *Paramacrobotus tonollii* and *Macrobotus sapiens*. *J Zool Syst Evol Res* 49(s1):58–61
- Marcus E (1929a) Zur embryologie der tardigrade. *Zool Jahrb* 50:333–384
- Marcus E (1929b) Tardigrada. In: Bronn HG (ed) *Klassen und Ordnungen des Tier-Reichs*. Akademische Verlagsgesellschaft, Bd 5, IV-3, Leipzig, pp 1–608
- Morek W, Stec D, Gąsiorek P, Schill RO, Kaczmarek Ł, Michalczyk Ł (2016) An experimental text of eutardigrade preparative methods for light microscopy. *Zool J Linnean Soc* 178:785–793
- Nelson DR, Guidetti R, Rebecchi L (2015) Phylum Tardigrada. In: Thorp J, Rogers DC (eds) *Ecology and general biology, IV edition: Thorp and Covich's freshwater invertebrates, Chapter 17*. Academic Press, New York, pp 347–380
- Pilato G, Guidetti R, Rebecchi L, Lisi O, Hansen JG, Bertolani R (2006) Geonemy, ecology, reproductive biology and morphology of the tardigrade *Hypsibius zetlandicus* (Eutardigrada: Hypsibiidae) with erection of *Borealibius* gen. n. *Polar Biol* 29:595–603
- Pollock LW (1970) Reproductive anatomy of some marine Heterotardigrada. *Trans Am Microsc Soc* 89:308–316
- Poprawa I (2005a) The ovary structure, previtellogenic and vitellogenic stages in parthenogenetic species *Dactylobiotus dispar* (Murray, 1907) (Tardigrada: Eutardigrada). *Tissue Cell* 37:385–392
- Poprawa I (2005b) The structure and the formation of egg shells in the parthenogenetic species *Dactylobiotus dispar* Murray, 1907 (Tardigrada: Eutardigrada). *Folia Biol* 53:173–177
- Poprawa I (2011) Ultrastructural studies of the formation of the egg capsule in the hermaphroditic species, *Isohypsibius granulifer granulifer* Thulin, 1928 (Eutardigrada: Hypsibiidae). *Zool Sci* 28(1):37–40
- Poprawa I, Hyra M, Kszuk-Jendrysik M, Rost-Roszkowska MM (2015a) Ultrastructural changes and programmed cell death of trophocytes in the gonad of *Isohypsibius granulifer granulifer* Thulin, 1928 (Tardigrada, Eutardigrada, Isohypsibiidae). *Micron* 70:26–33
- Poprawa I, Hyra M, Rost-Roszkowska MM (2015b) Germ cell cluster organization and oogenesis in the tardigrade *Dactylobiotus parthenogeneticus* Bertolani, 1982 (Eutardigrada, Murrayidae). *Protoplasma* 252:1019–1029
- Poprawa I, Schlechte-Welnicz W, Hyra M (2015c) Ovary organization and oogenesis in the tardigrade *Macrobotus polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003 (Eutardigrada, Mrobotiidae): ultrastructural and histochemical analysis. *Protoplasma* 252:857–865
- Ramazzotti G, Maucci W (1983) Il Phylum Tardigrada. III Edizione riveduta e aggiornata. *Mem Ist Ital Idrobiol* 41:1–1012
- Rebecchi L (1997) Ultrastructural study of spermiogenesis and the testicular and spermathecal spermatozoon of the gonochoristic tardigrade *Xerobiotus pseudohufelandi* (Eutardigrada, Macrobotiidae). *J Morphol* 234:11–24

- Rebecchi L (2001) The spermatozoon in tardigrades: evolution and relationships with the environment. *Zool Anz* 240:525–533
- Rebecchi L, Bertolani R (1988) New cases of parthenogenesis and polyploidy in the genus *Ramazottius* (Tardigrada, Hypsibiidae) and a hypothesis concerning their origin. *Invertebr Reprod Dev* 14:187–196
- Rebecchi L, Bertolani R (1994) Maturative pattern of ovary and testis in eutardigrades of freshwater and terrestrial habitats. *Invertebr Reprod Dev* 26:107–118
- Rebecchi L, Bertolani R (1999) Spermatozoon morphology of three species of Hypsibiidae (Tardigrada, Eutardigrada) and phylogenetic evaluation. *Zool Anz* 238:319–328
- Rebecchi L, Guidi A (1995) Spermatozoon ultrastructure in two species of *Amphibolus* (Eutardigrada, Eohypsibiidae). *Acta Zool* 76:171–176
- Rebecchi L, Nelson DR (1988) Evaluation of a secondary sex character in eutardigrades. *Invertebr Biol* 117:194–198
- Rebecchi L, Guidi A, Bertolani R (2000a) Tardigrada. In: Jamieson BGM (ed), *Progress in male gamete ultrastructure and phylogeny. Reproductive biology of invertebrates*, Adiyodi KG, Adiyodi RG series, vol IX, Part B, Chapter 6, pp 267–291
- Rebecchi L, Guidi A, Bertolani R (2000b) Maturative pattern of the ovotestis in two hermaphroditic species of eutardigrades. *Invertebr Reprod Dev* 37:25–34
- Rebecchi L, Guidi A, Bertolani R (2003a) The spermatozoon of the Echiniscidae (Tardigrada, Heterotardigrada) and its phylogenetic significance. *Zoomorphology* 122:3–9
- Rebecchi L, Rossi V, Altiero T, Bertolani R, Menozzi P (2003b) Reproductive modes and genetic polymorphism in the *Richtersius coronifer* (Eutardigrada, Macrobiotidae). *Invertebr Biol* 122:19–27
- Rebecchi L, Altiero T, Guidi A (2011) The ultrastructure of the tardigrade spermatozoon: a comparison between *Paramacrobrotus* and *Macrobrotus* species (Eutardigrada). *Invertebr Zool* 8:63–77
- Richters F, Krumbach T (1926) Tardigrada. In: Kükenthal W, Krumbach T (eds) *Handbuch der Zoologie*, vol 3. Walter de Gruyter & Co, Berlin, pp 1–68
- Rost-Roszkowska MM, Poprawa I, Wójtowicz M, Kaczmarek Ł (2011) Ultrastructural changes of the midgut epithelium in *Isohypsibius granulifer granulifer* Thulin, 1928 (Tardigrada: Eutardigrada) during oogenesis. *Protoplasma* 248:405–414
- Sayre RM (1969) A method for culturing a predaceous tardigrade on the nematode *Panagrellus redivivus*. *Trans Am Microsc Soc* 88:266–274
- Schill RO (2013) Life-history traits in the tardigrade species *Paramacrobrotus kenianus* and *Paramacrobrotus palaui*. *J Limnol* 72(s1):160–165
- Schill RO, Jönsson KI, Pfannkuchen M, Brümmer F (2011) Food of tardigrades: a case study to understand food choice, intake and digestion. *J Zool Syst Evol Res* 49(s1):66–70
- Smith FW, Boothby TC, Giovannini I, Rebecchi L, Jockusch E, Goldstein B (2016) The compact body plan of tardigrades evolved by the loss of a large body region. *Curr Biol* 26:224–229
- Stec D, Smolak R, Kaczmarek Ł, Michalczy Ł (2015) An integrative description of *Macrobrotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. *Zootaxa* 4052(2):501–526
- Suzuki AC (2003) Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment. *Zool Sci* 20:49–57
- Suzuki AC (2006) Ovarian structure in *Milnesium tardigradum* (Tardigrada, Milnesiidae) during early vitellogenesis. *Hydrobiologia* 558:61–66
- Suzuki AC (2008) Appearance of males in a thelytokous strain of *Milnesium cf. tardigradum* (Tardigrada). *Zool Sci* 25:849–853
- Suzuki AC, Kristensen RM (2014) Spermatozoa in the reproductive system of a hermaphroditic marine tardigrade, *Orzeliscus belopus* (Tardigrada: Arthrotardigrada). *Zool Anz* 253:497–511
- Tsujimoto M, Suzuki AC, Imura S (2015) Life history of the Antarctic tardigrade, *Acutuncus antarcticus*, under a constant laboratory environment. *Polar Biol* 38:1575–1581

- Tsujimoto M, Komori O, Imura S (2016a) Effect of lifespan and age on reproductive performance of the tardigrade *Acutuncus antarcticus*: minimal reproductive senescence. *Hydrobiologia* 772 (1):93–102
- Tsujimoto M, Imura S, Kanda H (2016b) Recovery and reproduction of an Antarctic tardigrade retrieved from a moss sample frozen for over 30 years. *Cryobiology* 72(1):78–81
- von Erlanger R (1895) Beiträge zur Morphologie der Tardigraden. I. Zur Embryologie eines Tardigrade *Macrobiotus macronyx* Dujardin. *Morphol Jahrb* 22:491–513
- Von Wenck W (1914) Entwicklungsgeschichtliche Untersuchungen an Tardigraden (*Macrobiotus lacustris* Duj.). *Zool Jahrb Abt Anat Ontog Tiere* 37:465–514
- Węglarska B (1957) On the encystation in Tardigrada. *Zool Pol* 8:315–323
- Węglarska B (1975) Studies on the morphology of *Macrobiotus richtersi* Murray, 1911. *Mem Ist Ital Idrobiol* 32:445–464
- Węglarska B (1979) Electron microscope study on previtellogenesis and vitellogenesis in *Macrobiotus richtersi* J. Murr. (Eutardigrada). *Zesz Nauk Uniw Jagiellon Prace Zool* 25:169–190
- Węglarska B (1982) Ultrastructural study of the formation of egg envelops in *Macrobiotus richtersi* (Eutardigrada). In: Nelson DR (ed), *Proceedings third international symposium Tardigrada*, East Tennessee State Univ Press, Johnson City, Tennessee, pp 115–128
- Węglarska B (1987) Yolk formation in *Isohypsibius* (Eutardigrada). *Zoomorphology* 107:287–292
- Wolburg-Buchholz K, Greven H (1979) On the fine structure of the spermatozoon of *Isohypsibius granulifer* Thulin 1928 (Eutardigrada) with reference to its differentiation. *Zesz Nauk Uniw Jagiellon Prace Zool* 25:191–197