Acta Zoologica

Published for The Royal Swedish Academy of Sciences and The Royal Danish Academy of Sciences and Letters

Acta Zoologica (Stockholm) **92**: 101–108 (April 2011) doi: 10.1111/j.1463-6395.2010.00481.x

Spermatogenesis and the structure of the testes in *Isodiametra pulchra* (Isodiametridae, Acoela)

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Keywords:
Acoela, spermatogenesis, ultrastructure, germline, Bilateria

Accepted for publication: 26 November 2009

Abstract

Boone M., Willems M., Claeys M. and Artois, T. 2011. Spermatogenesis and the structure of the testes in *Isodiametra pulchra* (Isodiametridae, Acoela). —*Acta Zoologica* (Stockholm) **92**: 101–108.

Spermatogenesis and the structure of the testes were studied ultrastructurally in *Isodiametra pulchra* (Smith and Bush, *Transactions of the American Microscopical Society* 1991; 110: 12; Hooge and Tyler, *Journal of zoological systematics and evolutionary research* 2005; 43: 100). The testes are paired, compact, non-follicular and lie dorsally and dorso-laterally to the paired ovaries, partially enfolding them. All stages of spermatogenesis, including spermiogenesis, are described at the ultrastructural level and their spatial organization within the testes is discussed. The cells at the early stages of spermatogenesis (spermatogonia and spermatocytes) are located on the dorsal and dorso-lateral sides of the testes, while the late stages (spermatids and filiform spermatozoa with 9+2 axonemes) lie at the ventral and inner periphery of the testes, adjacent to ovaries. All the cell types can be found both at the anterior and the posterior end of the testes. The value of the structure of the testes as a phylogenetic marker is addressed.

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Introduction

According to recent molecular phylogenetic studies, Acoela is the most basal taxon within the Bilateria (Ruiz-Trillo *et al.* 1999, 2002; Jondelius *et al.* 2002; Telford *et al.* 2003; Baguna and Riutort 2004; Philippe *et al.* 2007; Wallberg *et al.* 2007; Baguna *et al.* 2008; but see Dunn *et al.* 2008), which makes it very interesting for evolutionary studies.

Because acoels are soft-bodied worms that show only very few morphological clues that can help to identify them, the phylogeny of the Acoela was rarely studied in the past. Hooge *et al.* (2002) studied the phylogenetic relationships within the Acoela using molecular and morphological markers, but only a limited number of species was included, and a large-scale phylogenetic analysis is still lacking.

The ultrastructural morphology of spermatozoa could provide important characters to infer phylogenetic relationships within acoels (Raikova and Justine 1999; Raikova *et al.* 2001; Hooge *et al.* 2002; Petrov *et al.* 2004), as do the morphology of the penis (Hooge and Tyler 2005; Raikova *et al.* 2006), the body musculature (Hooge *et al.* 2002) and the presence and morphology of the bursal nozzles (Petrov *et al.* 2006). Mature spermatozoa have already been studied in detail in different taxa of acoels (Hendelberg 1969, 1977; Tyler *et al.* 1986; Justine *et al.* 1998; Raikova *et al.* 1998, 2001; Raikova and Justine 1999; Petrov *et al.* 2004; Tekle *et al.* 2007), and these studies have confirmed that all acoel spermatozoa are filiform and have two incorporated axonemes (Hendelberg 1969, 1983, 1986). These axonemes consist of microtubules that can be arranged in either a 9+2, a 9+0 or a 9+1 pattern, a feature considered an important phylogenetic marker (Raikova *et al.* 2001). The mature sperm of *Isodiametra pulchra* has been described by Petrov *et al.* 2004.

The process of forming mature spermatozoa out of primordial germ cells or spermatogonia is called spermatogenesis. The final stages of spermatogenesis, during which spermatids mature into spermatozoa, together constitute spermiogenesis. Whereas spermiogenesis has been described in several species of acoels (Raikova and Justine 1994, 1999; Raikova *et al.* 1997), the early stages of sperm formation have never been studied. These early stages of spermatogenesis (spermatogonia or germ cells) are especially interesting, given that acoels

have pluripotent stem cells (neoblasts), which are responsible for forming all types of differentiated somatic cells and the germ cells. Recently, there has been growing interest in the study of these neoblasts in acoels (Gschwentner *et al.* 2001; Egger *et al.* 2007; Gaerber *et al.* 2007). Given the exceptional fact that neoblasts are the sole source for every cell type, it is important to know whether neoblasts and primordial germ cells can be distinguished at the ultrastructural level.

Rieger *et al.* (1991) described the basic organization of the testes in Acoela. The known diversity in the structure of the testes in acoels seems to be large; testes can be asaccular or saccular, compact or diffuse, paired or unpaired, follicular or non-follicular, mixed or separated from the female gonad (Rieger *et al.* 1991). Some acoels have follicular testes, which means that the testes consist of packages of clusters of cells that develop together and that are spatially separated from other follicles. In other species, no follicles can be found. The testes can be compact or diffuse, depending on the degree of density to which the cells are packed together. It is still unclear how the testes are organized ultrastructurally in different species and how the different stages of spermatogenesis are organized within the testes or follicles. Nevertheless, it is essential to describe and understand the spatial organization of the different cell types in the testes of Acoela, to be able to interpret the results of further studies on the function of germ cells and neoblasts, as for example immunohistochemical stainings and *in situ* hybridization (De Mulder *et al.* 2009). Moreover, a detailed study of the testes in acoels will increase the knowledge on the morphology of this challenging taxon, which will enable us to infer the evolution of the testes in this group and to examine the possibility to use this characteristic in phylogenetic studies.

In this contribution, we present a detailed ultrastructural study of spermatogenesis and the spatial cellular organization within the testis of the acoel *Isodiametra pulchra* (Smith and Bush 1991) Hooge and Tyler 2005; a species that is recently used in studies on regeneration and stem cell dynamics (De Mulder *et al.* 2009), and for which a number of EST's have been sequenced (Philippe *et al.* 2007). It is the first time the complete spermatogenesis of an acoel is described.

Materials and Methods

Cultures

Isodiametra pulchra (Isodiametridae, Acoela) is a small (365 μm long; Smith and Bush 1991), unpigmented, tear-shaped acoel with an isodiametric penis located within the seminal vesicle. Specimens of *I. pulchra* are kept in Petri dishes filled with artificial sea water, which is enriched with nutrients (Guillard's f/2 medium) (see Rieger *et al.* 1988), and are fed with the diatom *Nitzschia curvilineata*. To maintain constant conditions, they are held in an incubator at 20 °C on a 14 : 10 day–night cycle. This culture of *I. pulchra* originated from a culture set up by Prof. Dr. Seth Tyler and Dr. Matthew Hooge of the University of Maine, the type location can be found in the species description (Smith and Bush 1991).

Transmission electron microscopy and sectioning

Mature specimens (16 days post-hatching) were relaxed with 7.14% $MgCl_2$. Immediately after that, the animals were fixed in a cocktail of glutaraldehyde fixative and osmium fixative (Eisenmann and Alfert 1982) for approximately 10 min at 4 °C. The glutaraldehyde fixative was 4% glutaraldehyde in the first buffer (100 mL: 0.2 M sodium cacodylate with 0.58 g NaCl and 11.97 g sucrose; pH 7.2), and the osmium fixative was 1% osmium tetroxide in the second buffer (100 mL: 0.2 M sodium cacodylate with 3.48 g NaCl; pH 7.2). After fixation with the cocktail, the specimens were fixed in glutaraldehyde fixative for 1 h at 4 °C and then post-fixed with osmium fixative for 1 h at 4 °C. After rinsing in the second buffer for 10 min at 4 °C and rinsing for 5 min in double distilled water, they were dehydrated, using acetone series of increasing concentrations. The specimens were subsequently infiltrated with a low-viscosity embedding medium (Spurr 1969), and polymerisation was performed at 70 °C for 8 h. Semi-thin sections of 1 and 2 μm and ultra-thin sections of 60 nm were made on a Reichert-Jung Ultracut E or a Leica-Reichert Ultracut S (Leica, Vienna, Austria). The specimens of *I. pulchra* were sectioned either transversely or longitudinally. Specimens were sectioned semi-thin until the region of interest was reached, after which ultra-thin sections were made. The testes were completely sectioned from the anterior to the posterior tip. Semi-thin sections were studied using an Olympus BX 51 microscope equipped with an Olympus C5060 digital camera. Ultra-thin sections were studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV and pictures were digitized using a Ditabis system (Pforzheim, Germany). A 3D-reconstruction of the reproductive structures of *I. pulchra* was made based on a series of serial sections of 2 μm, using Amira 3.1.1 software (TGS Europe, Bordeaux, France).

Results

Structure of the testes

Sixteen days after hatching, the post-embryonic development in *I. pulchra* has been completed and the worm is able to reproduce. A 3-dimensional reconstruction of the reproductive structures in an adult specimen of *I. pulchra* is presented in Fig. 1A–C. In the adult worms, the testes lie dorso-laterally to the ovaries, with the anterior part of the testes slightly enfolding the ovaries. The bursa and the vesicula seminalis, including the penis, are situated centrally in the caudal part of the body. The testes as a whole are well-defined, but there is no proper delimitation of follicles within the testes. Therefore, the testes are clearly non-follicular. The cells are so densely packed that they build up a compact testes.

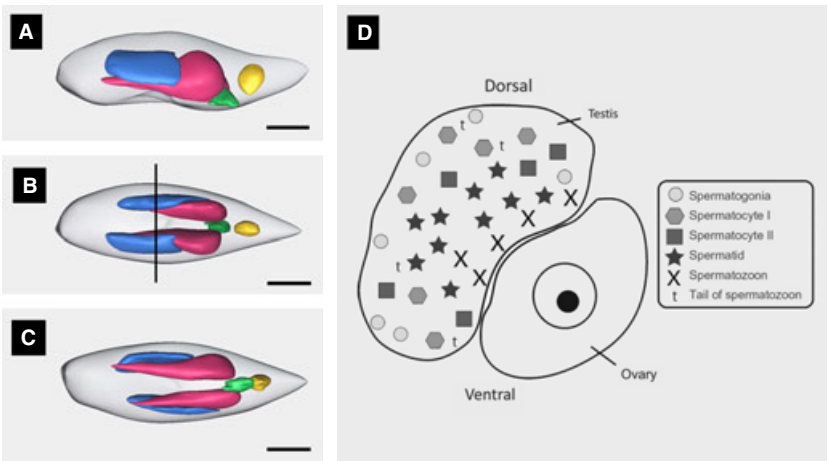

Fig. 1—*Isodiametra pulchra.* —**A**–**C**. 3D reconstruction based on serial semi-thin sections of the reproductive organs in the acoel *I. pulchra*. On each side, sperm ducts connect the testes and the vesicula seminalis (not in visible on 3D reconstruction). **A**. lateral view; **B**. dorsal view with the indication of the level of the cross section presented in 1D; **C**. ventral view. Colour codes: blue, testes; pink, ovaries; green, bursa; yellow, vesicula seminalis. Left is anterior, right is posterior. Scale bar: 100 μm. —**D**. Diagram of a cross section of *I. pulchra* at 130 μm from the anterior tip, where the organization of the different cell types in the testes is shown. The early stages, spermatogonia, spermatocytes I and II, are lying at the outer periphery of each testis (dorso-lateral side), while spermatids are in the centre of each testis and mature spermatozoa are lying at the inner periphery of each testis, on the side adjacent to the ovary. Note that the 'tails' (opposite end of the nucleus) of the spermatozoa are scattered in between all the cell types. This scheme is representative of the organization of each testis along its entire length. Left is the side of the body wall; right is the side of the central parenchyma.

In what follows we will describe the structure of the testes and each successive stage of spermatogenesis, from spermatogonium to mature spermatozoon.

All the stages of spermatogenesis can be found all along the entire length of the testes, anteriorly as well as posteriorly, but they are organized in a way that the early stages (spermatogonia, primary and secondary spermatocytes) are lying on the outside of the testes (meaning at the side of the epidermis), while spermatids and mature sperm can be found at the centre and inner side of the testes (meaning at the side of the ovaries). This organization is presented in Fig. 1D. From our observations, we can conclude that the male germinative zone is found at the side of the testes adjacent to the epidermis. The spermatids and spermatozoa are found in fours, as they originate from a single spermatocyte. The 'tails', the end of the spermatozoon which is opposite to the nucleus and contains the two axonemes, can be found in every region of the testes in between the other cells, even though the shafts of the spermatozoa are found at the inner side of the testes.

Spermatogenesis

The spermatogonium. The nucleus of a spermatogonium (Fig. 2A) contains small clumps of heterochromatin centrally, which are unconnected to each other. The nucleo-cytoplasmic ratio is high; the nucleus fills the larger part of the spermatogonium. The undifferentiated cytoplasm contains a few mitochondria and ribosomes. This ultrastructure is remarkably similar to that of a neoblast (somatic stem cell). Figure 2B shows a neoblast that is situated near the body wall. This cell has a high nucleo-cytoplasmic ratio, comparable to the one in the spermatogonia. Both the cytoplasm and the nucleus of the neoblast resemble those of the spermatogonium: the cytoplasm contains a few mitochondria and ribosomes, while clumps of heterochromatin are found in the nucleus.

The primary spermatocyte. The nucleus of the primary spermatocyte has a round to ovoid shape and is characterized by the presence of synaptonemal complexes, which are clearly visible (Fig. 3, arrowheads), and scattered clumps of heterochromatin. The nucleo-cytoplasmic ratio is still high compared to what is found in somatic cells, but lower than in spermatogonia.

The cytoplasm contains some mitochondria and ribosomes, and sometimes rough endoplasmatic reticulum can be found. Intercellular bridges connect the cytoplasm of adjacent primary spermatocytes (Fig. 3B, arrow).

The secondary spermatocyte. The nucleus of the secondary spermatocyte is round and contains more heterochromatin that is also more condensed than that of the primary spermatocytes. Apart from the mitochondria and ribosomes, also centrioles (Fig. 4, arrowheads), swollen endoplasmatic reticulum and electron-dense granules (Fig. 4, black arrows) can be noticed in the cytoplasm. The granules are not membrane-bound. As in primary spermatocytes, intercellular bridges connect the cytoplasm of adjacent secondary spermatocytes.

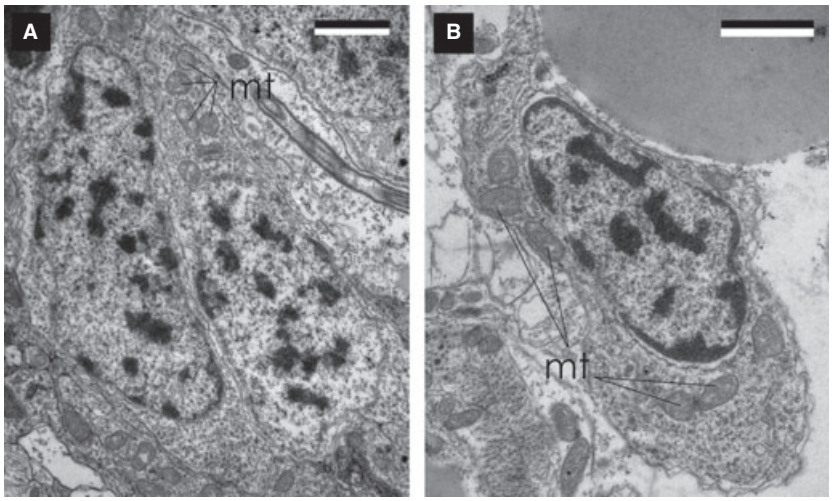

Fig. 2—Spermatogonia and neoblast in *I. pulchra*. —**A**. Spermatogonium with scattered clumps of heterochromatin in the nucleus. The undifferentiated cytoplasm contains mitochondria and ribosomes. —**B**. Neoblast that is located near the body wall (under the muscle layer). Note the undifferentiated cytoplasm with mitochondria and ribosomes, and the scattered clumps of heterochromatin in the nucleus. mt, mitochondria. Scale bars: 1 μm.

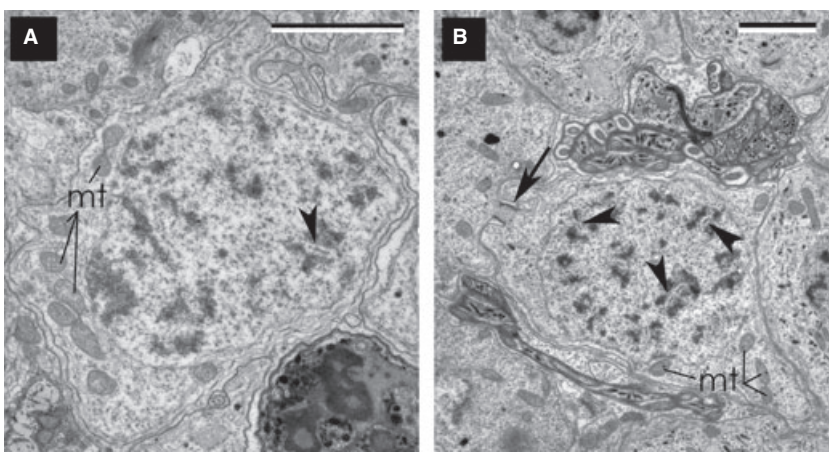

Fig. 3—Primary spermatocytes in *I. pulchra*. —**A**. Primary spermatocyte with scattered chromatin and a synaptonemal complex (arrowhead) in the nucleus. Note the undifferentiated cytoplasm that contains only a few mitochondria and ribosomes. —**B**. Two primary spermatocytes with several synaptonemal complexes (arrowheads) in the nucleus. Note the intercellular bridge between the two cells (arrow). mt, mitochondria. Scale bars: 2 μm.

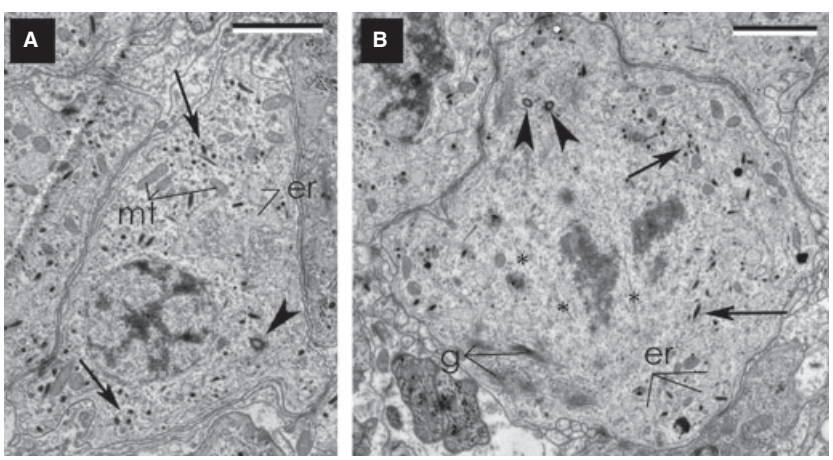

Fig. 4—Secondary spermatocytes in *I. pulchra*. —**A**. Secondary spermatocyte with a centriole (arrowhead), ribosomes, mitochondria, swollen endoplasmatic reticulum and granules in the cytoplasm (black arrows). The nucleus contains scattered chromatin, which is more abundant than in the primary spermatocytes. —**B**. Secondary spermatocyte with a meiotic spindle figure. The two centrioles (arrowheads) of one of the two centrosomes are visible, as well as the spindle fibres (microtubules; asterisks) that are pulling the sister chromatids apart. Two different types of granules in the cytoplasm (black arrows) are present. g, Golgi complex; mt, mitochondria; er, endoplasmatic reticulum. Scale bars: 2 μm.

Every secondary spermatocyte gives rise to two haploid spermatids after the second meiotic division, marked by the presence of centrioles (see Fig. 4B, arrowheads indicate the centrioles).

The spermatid. During the spermatid stage, the round to ovoid cell starts to elongate (Fig. 5). Spermatids are always found in clusters of four cells; each of these clusters originates from one primary spermatocyte.

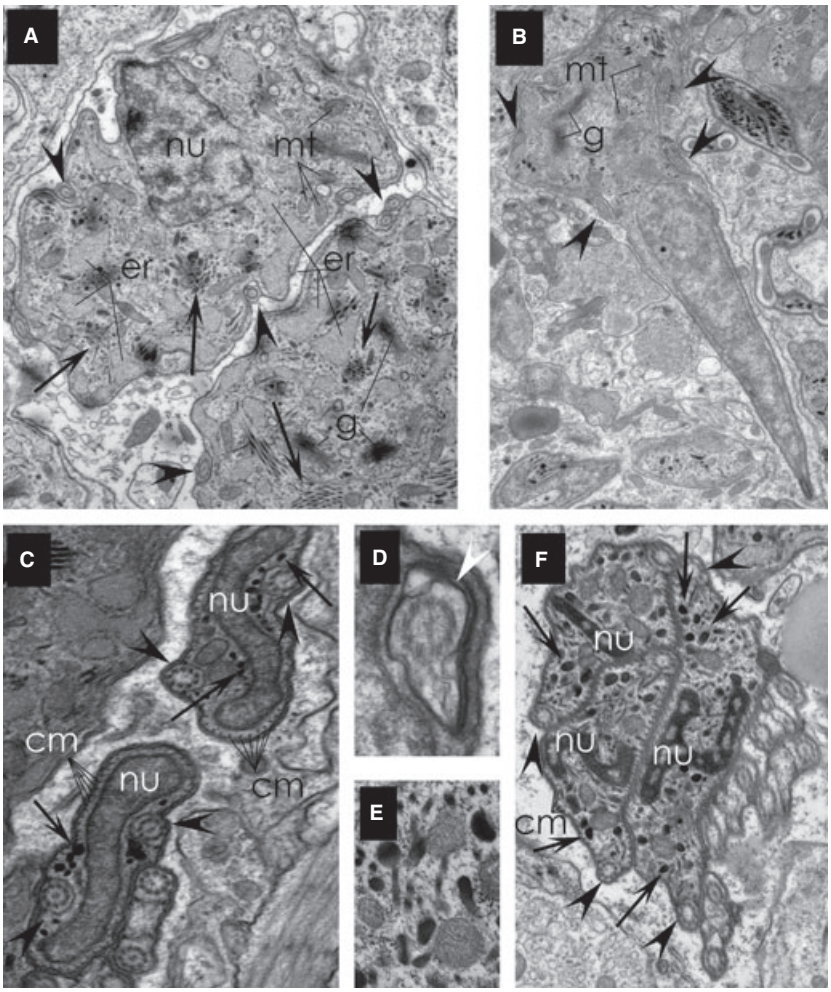

Fig. 5—Spermatids in *I. pulchra*. —**A**. Early spermatids sectioned transversally. The flagella that will later be incorporated as axonemes are indicated with arrowheads; note the granules in the cytoplasm (arrows). —**B**. Early spermatid sectioned longitudinally. The nucleus is elongating but the flagella are not incorporated as axonemes yet (arrowheads). The cytoplasm contains swollen ER, mitochondria, Golgi, polyribosomes and granules. —**C**. Incorporation of flagella as axonemes (arrowheads). The nucleus acquires an S-shape in cross sections. The cytoplasmic granules are indicated with black arrows. Note the cortical microtubules. —**D**. Detail of the incorporation of flagella as axonemes. The flagella are surrounded by two membranes, forming one canal (arrowhead). —**E**. Detail of the granules in the cytoplasm of spermatids, the granules being not membrane-bound. —**F**. Transverse section of late spermatids, sectioned at the nuclear level. The spermatids have elongated into a filiform shape. Note the cortical microtubules. The axonemes are already incorporated, but their membrane is still visible (arrowheads); the arrows point to the granules in the cytoplasm. cm, cortical microtubules; g, Golgi; mt, mitochondria; nu, nucleus; er, endoplasmatic reticulum. Scale-bars: **A**, **B**, **F**: 2 μm; **C**: 1 μm; **D**: 200 nm; **E**: 300 nm.

An early spermatid is somewhat square shaped, not yet fully elongated, but not ovoid anymore either. Cytoplasmic organelles are abundant: mitochondria, Golgi complexes, ribosomes and swollen endoplasmatic reticulum can be found in high numbers. The cytoplasm of the spermatids contains electron-dense granules, which may appear either in groups (arrowhead) or individually. These granules are not membrane-bound (Fig. 5D). The nucleus is elongating, while the shape in cross section is changing from a round-ovoid shape to an S-shape.

Figure 5A,B clearly shows the flagella (arrowheads), which are still free and lie along the cell body at the beginning of spermiogenesis. They will be incorporated in the shaft of the spermatid during the elongation phase. From our observations of transverse sections, the flagella are surrounded by a single canal formed by the two membranes surrounding the axoneme (Fig. 5E). Numerous cortical microtubules are found near the cell membrane (Fig. 5C,F).

The mature spermatozoon. Mature spermatozoa can best be studied in the seminal vesicle (Fig. 6). The mature, elongated sperm cells have a nuclear region on one end and a cytoplasmic region on the other end (Hendelberg 1969; Petrov *et al.* 2004). The nucleus is elongated, electron-dense and not fragmented. The cytoplasmic region is characterized by the presence of 9+2 axonemes (arrowheads in Fig. 6), mitochondria, granules and cortical microtubules. At the posterior (proximal) end of the axonemes, the two central microtubules are absent. The axonemes extend into the nuclear region. Mitochondria are abundant, as well as the electron-dense granules described earlier (black arrows in Fig. 6).

Discussion

Spermatogenesis

Spermiogenesis has been studied in detail in several species of acoels (Raikova and Justine 1994, 1999; Raikova *et al.* 1997), but the complete spermatogenesis and the detailed structure of the testes have never been published.

The spermatogonium is an undifferentiated cell, which divides mitotically to produce two cells: a new

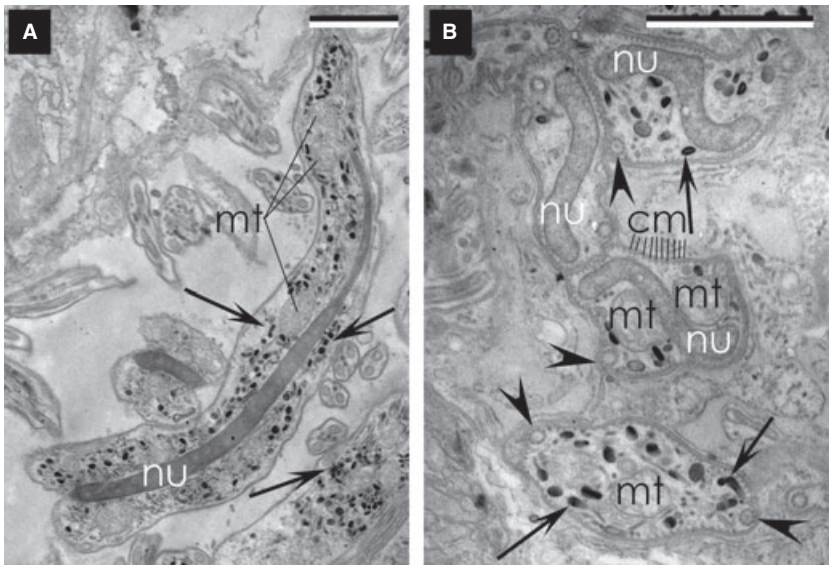

Fig. 6—Mature spermatozoa as they appear in the seminal vesicle of *I. pulchra*. —**A**. Longitudinal section of a mature spermatozoon with an elongated nucleus and granules (arrows) and a few mitochondria. —**B**. Transverse sections of mature spermatozoa. The upper three spermatozoa are sectioned at the nuclear level, while the bottom one is sectioned in the cytoplasmic region. The nucleus is S-shaped when sectioned transversally. The 9+2 structure of the two axonemes (arrowheads) and the cortical microtubules can be clearly seen in this section. Note the granules (arrows) and the mitochondria in the cytoplasmic region. cm, cortical microtubules; mt, mitochondria; nu, nucleus. Scale bars: 2 μm.

spermatogonium and a cell that will differentiate into a primary spermatocyte. In *I. pulchra*, spermatogonia can only be distinguished from somatic neoblasts based on their location (they are in the testes); as to their ultrastructural morphology, they are identical to neoblasts. The function of spermatogonia and somatic stem cells is very similar: both divide to form a new spermatogonium or a stem cell, respectively, and a daughter cell that will differentiate into a germ cell or a somatic cell, respectively.

A primary spermatocyte can easily be identified by the presence of the synaptonemal complexes. The synaptonemal complex is a protein structure, which mediates synapsis and crossing-over during the zygotene phase of the prophase of the first meiotic division (Alberts *et al.* 1994), and because of this function, it cannot be found in any other cell (except primary oocytes). Consequently, this feature is diagnostic of the primary spermatocytes. The fact that only few organelles are present in the cytoplasm is also indicative of this stage, but not exclusive, as it is also found in other stages. After the first meiotic division, each of the primary spermatocytes gives rise to two secondary spermatocytes.

The secondary spermatocyte is the stage at which the second meiotic division starts. Consequently, it is more differentiated than the primary spermatocyte. It already contains the two types of granules that are seen in spermatozoa. The cells have not started to elongate (cf. spermatids), and their nucleus is still round-ovoid in shape, which makes them easily distinguishable from spermatids.

Spermatids in *I. pulchra* are not connected to each other trough a cytophore, as is the case in other acoels (see Raikova and Justine 1994). During their incorporation in the spermatids, the axonemes are bordered by a single canal, formed by the two membranes surrounding the axoneme. This mode of incorporation can also be found in other Euacoela, as e.g. *Actinoposthia beklemischevi* (Raikova *et al.* 2001), and is in contrast with the very complex flagellar incorporation involving complicated cytoplasmic canals in *Paratomella rubra* (Raikova *et al.* 1997, 2001). Incorporation of flagella is also found in other taxa e.g. the Neodermata (Watson 1999) and Kalyptorhynchia (Watson 2001), although the mode of incorporation differs.

As described by Petrov *et al.* (2004), mature spermatozoa in *I. pulchra* have 9+2 axonemes and cortical microtubules. As the other 'small-bodied convolutids', *C. pulchra* was transferred into the new family Isodiametridae, by Hooge and Tyler (2005) because of the presence of an isodiametric penis, with the 9+2 sperm axonemes as an additional character for that family. The electron-dense granules in mature spermatozoa can also be found in other Acoela (Raikova and Justine 1994) but it is difficult to identify homologies between them. Their function is also unclear; an acrosome function was proposed (Hendelberg 1983) but this cannot be assessed in a morphological study alone.

Implications of the structure of the testes

The known diversity in the structure of the testes in acoels seems to be large; testes can differ in position in the body, lining, relation to ovaries, internal spatial organization, direction of maturation of the germ cells, density (Rieger *et al.* 1991), etc.

Testes can be paired or unpaired, and mixed or separated from the female gonad. In *I. pulchra*, the testes are paired but separated from the ovaries, while in *Diopisthoporus longitubus* (Diopisthoporidae) and *Oligofilomorpha karlingi* (Solenofilomorphidae), the testes are mixed with the ovaries and unpaired (Dörjes 1971).

A diffuse testis is a testis in which the germ cells are divided into groups by processes from parenchymal or gut cells, e.g. the

testis in *Paratomella rubra* (Rieger *et al.* 1991). When the germ cells are densely packed, the testes are called compact, as is the case in *I. pulchra*. Follicular testes are composed of follicles of certain stages of spermatogenesis differentiated from a single germ cell, the follicles being spatially separated from each other. This is seen in Paratomellidae and some members of the Proporidae (*Proporus venenosus* and *P. brochi*; Dörjes 1971), but most testes in acoela are non-follicular, e.g. *I. pulchra*.

Although the testes of *I. pulchra* as a whole are well-defined, it is difficult to detect separate regions within the testes. Even though early stages of spermatogenesis are found at the outside of the testes (germinative zone) and spermatids and mature sperm are found at the inside of the testes, a strict layered organization as in some acoels (e.g. *Oxyposthia praedator*; Rieger *et al.* 1991) and Macrostomorpha (*M. lignano*; Willems M., pers. comm.) is lacking. Moreover, the 'tails' (end of the spermatozoon opposite to the nucleus) of the spermatozoa are lying in between the different cell types because of their elongated nature, which makes it even harder to define layers in the testes. It could be that the side of the testis neighbouring the ovarium, where the mature spermatozoa are lying, functions as a sperm duct, with the sperm moving in the direction of the seminal vesicle.

Some of the characters of the testes provided by our analysis of the structure of the testes in *I. pulchra* provide very useful additional information that is impossible to get from the species description or deduce from literature. We believe that the same accounts for other species as well, hence further research is required. Examples of this additional information are the direction of maturation of male sex cells, the presence or absence of layering and the exact position of testes and ovaries. It would be very interesting to create an extended study on the testes of species of many putative unrelated taxa, to detect a pattern in the phylogenetic distribution of the various types of gonads and to be able to construct a datamatrix to infer the evolution of the testes within the Acoela. As illustrated above, the morphology of the male gonads can vary substantially between taxa. Because acoels do not have many other clear morphological characteristics, the structure of the testes surely could provide many additional features that could be important in our study of acoel phylogeny.

Acknowledgements

Specimens of *I. pulchra* were kindly provided by Prof. Dr Seth Tyler and Dr Matthew Hooge from the University of Maine and by Prof. Dr Peter Ladurner from the University of Innsbruck. We thank Natascha Steffanie for the help with the sectioning and Willi Salvenmoser and Johannes Achatz for the discussions. We also thank Prof. Dr. Dominique Adriaens for the use of the Amira software. We greatly acknowledge two anonymous reviewers for their very valuable comments on the previous versions of this manuscript. MB is supported by the FWO Vlaanderen.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. 1994. Germ cells and fertilization. In: Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (Eds.): *Molecular Biology of the Cell*, pp. 1011–1036. Garland Publishing, New York.

Baguna, J. and Riutort, M. 2004. The dawn of bilaterian animals: the case of acoelomorph flatworms. – *Bioessays* **26**: 1046–1057.

Baguna, J., Martinez, P., Paps, J. and Riutort, M. 2008. Back in time: a new systematic proposal for the Bilateria. – *Philosophical Transactions of the Royal Society B* **363**: 1481–1491.

De Mulder, K., Kuales, G., Pfister, D., Willems, M., Egger, B., Salvenmoser, W., *et al.* 2009. Characterization of the stem cell system of the acoel *Isodiametra pulchra*. – *BMC Developmental Biology* **9**: 69.

Dörjes, J. 1971. Monographie der Proporidae und Solenofilomorphidae (Turbellaria, Acoela). – *Senkenbergiana biologica* **52**: 113–137.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., *et al.* 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. – *Nature* **452**: 745–750.

Egger, B., Gschwenter, R. and Rieger, R. M. 2007. Free-living flatworms under the knife: past and present. – *Development, Genes and Evolution* **217**: 89–104.

Eisenmann, E. A. and Alfert, M. 1982. A new fixation procedure for preserving the ultrastructure of marine invertebrate tissues. – *Journal of Microscopy* **125**: 117–120.

Gaerber, C. W., Salvenmoser, W., Rieger, R. M. and Gschwenter, R. 2007. The nervous system of Convolutriloba (Acoela) and its patterning during regeneration after asexual reproduction. – *Zoomorphology* **126**: 73–87.

Gschwentner, R., Ladurner, P., Nimeth, K. and Rieger, R. M. 2001. Stem cells in a basal bilaterian. S-phase and mitotic cells in Convolutriloba longifissura (Acoela, Platyhelminthes). – *Cell and Tissue Research* **304**: 401–408.

Hendelberg, J. 1969. On the development of different types of spermatozoa from spermatids with two flagella in the Turbellaria with remarks on the ultrastructure of the flagella. – *Zoologiska bidrag fran Uppsala* **38**: 1–52.

Hendelberg, J. 1977. Comparative morphology of turbellarian spermatozoa studied by electron microscopy. – *Acta Zoologica Fennica* **154**: 149–152.

Hendelberg, J. 1983. Trends in the evolution of flatworm spermatozoa. In: André, J. (Ed.): *The Sperm Cell*, pp. 450–453. Martinus Nijhoff publishers, The Hague.

Hendelberg, J. 1986. The phylogenetic significance of sperm morphology in the platyhelminthes. – *Hydrobiologia* **132**: 53–58.

Hooge, M. D. and Tyler, S. 2005. New tools for resolving phylogenies: a systematic revision of the Convolutidae (Acoelomorpha, Acoela). – *Journal of zoological systematics and evolutionary research* **43**: 100–113.

Hooge, M. D., Haye, P. A., Tyler, S., Litvaitis, M. K. and Kornfield, I. 2002. Molecular systematics of the Acoela (Acoelomorpha, Platyhelminthes) and its concordance with morphology. – *Molecular Phylogenetics and Evolution* **24**: 333–342.

Jondelius, U., Ruiz-Trillo, I., Baguna, J. and Riutort, M. 2002. The Nemertodermatida are basal bilaterians and not members of the Platyhelminthes. – *Zoologica Scripta* **31**: 201–215.

Justine, J.-L., Iomini, C., Raikova, O. and Mollaret, I. 1998. The homology of cortical microtubules in platyhelminth spermatozoa: a comparative immunocytochemical study of acetylated tubulin. – *Acta Zoologica* **79**: 235–241.

Petrov, A., Hooge, M. and Tyler, S. 2004. Ultrastructure of sperms in Acoela (Acoelomorpha) and its concordance with molecular systematics. – *Invertebrate Biology* **123**: 183–197.

Petrov, A., Hooge, M. and Tyler, S. 2006. Comparative morphology of the bursal nozzles in Acoels (Acoela, Acoelomorpha). – *Journal of Morphology* **267**: 634–648.

Philippe, H., Brinkmann, H., Martinez, P., Riutort, M. and Baguna, J. 2007. Acoel flatworms are not Platyhelminthes: evidence from phylogenomics. – *Plos One* **8**: e717.

Raikova, O. I. and Justine, J. L. 1994. Ultrastructure of spermiogenesis and spermatozoa in 3 Acoels (Platyhelminthes). – *Annales des Sciences Naturelles-Zoologie et Biologie Animale* **15**: 63–75.

Raikova, O. I. and Justine, J. L. 1999. Microtubular system during spermiogenesis and in the spermatozoon of *Convoluta saliens* (Platyhelminthes, Acoela): Tubulin immunocytochemistry and electron microscopy. – *Molecular Reproduction and Development* **52**: 74–85.

Raikova, O. I., Falleni, A. and Justine, J. L. 1997. Spermiogenesis in *Paratomella rubra* (Platyhelminthes, Acoela): Ultrastructural, immunocytochemical, cytochemical studies and phylogenetic implications. – *Acta Zoologica* **78**: 295–307.

Raikova, O. I., Flyatchinskaya, L. P. and Justine, J. L. 1998. Acoel spermatozoa: ultrastructure and immunocytochemistry of tubulin. – *Hydrobiologia* **383**: 207–214.

Raikova, O. I., Reuter, M. and Justine, J. L. 2001. Contributions to the phylogeny and systematics of the Acoelomorpha. In: Littlewood, D. T. J. and Bray, R. A. (Eds.): *Interrelationships of the Platyhelminthes*, pp. 13–23. Taylor and Francis, London.

Raikova, O. I., Tekle, Y. I., Reuter, M. and Jondelius, U. 2006. Copulatory organ musculature in *Childia* (Acoela) as revealed by phalloidin fluorescence and confocal microscopy. – *Tissue and Cell* **38**: 219–232.

Rieger, R. M., Gehlen, M., Hazpruhnar, G., Homlund, M., Legniti, A., Salvenmoser, W. and Tyler, S. 1988. Laboratory cultures of marine Macrostomida (Turbellaria). – *Forts Zool* **36**: 525.

Rieger, R. M., Tyler, S., Smith, J. P. S. and Rieger, G. E. 1991. Platyhelminthes and Nemertinea. In: Harrison, F. W. and Bogitsh, B. J. (Eds.): *Microscopic Anatomy of Invertebrates*, vol 3, pp. 7–140. Wiley-Liss, New York.

Ruiz-Trillo, I., Riutort, M., Littlewood, T. J., Herniou, E. A. and Baguna, J. 1999. Acoel flatworms: earliest extant Bilaterian Metazoans, not members of Platyhelminthes. – *Science* **283**: 1919–1923.

Ruiz-Trillo, I., Paps, J., Loukota, M., Ribera, C., Jondelius, U., Baguna, J. and Riutort, M. 2002. A phylogenetic analysis of myosin heavy chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians. – *Proceedings of the National Academy of Sciences of the United States of America* **99**: 11246–11251.

Smith, J. P. S. and Bush, L. 1991. *Convoluta-pulchra* N-Sp (Turbellaria, Acoela) from the East-Coast of North-America. – *Transactions of the American Microscopical Society* **110**: 12–26.

Spurr, A. R. 1969. A low viscosity epoxy resin-embedding medium for electron microscopy. – *Journal of Ultrastructural Research* **26**: 31–43.

Tekle, Y. I., Raikova, O. I., Justine, J. L., Hendelberg, J. and Jondelius, U. 2007. Ultrastructural and immunocytochemical investigation of acoel sperms with 9+1 axoneme structure: new sperm characters for unravelling phylogeny in Acoela. – *Zoomorphology* **126**: 1–16.

Telford, M. J., Lockyer, A. E., Cartwright-Finch, C. and Littlewood, D. T. J. 2003. Combined large and small subunit ribosomal RNA phylogenies support a basal position of the acoelomorph flatworms. – *Proceedings of the Royal Society of London Series B-Biological Sciences* **270**: 1077–1083.

Tyler, S., Smith, J. P. S., Rieger, R. M., Ehlers, U. and Gremigni, V. 1986. Electron microscopy of turbellarian platyhelminths: a bibliography. – *Hydrobiologia* **132**: 323–343.

Wallberg, A., Curini-Galletti, M., Ahmadzadeh, A. and Jondelius, U. 2007. Dismissal of Acoelomorpha: Acoela and Nemertodermatida are separate early bilaterian clades. – *Zoologica Scripta* **36**: 509–523.

Watson, N. A. 1999. Platyhelminthes. In: Adiyodi, K. G., Adiyodi, R. G. and Jamieson, B. G. M. (Eds.): *Reproductive Biology of Invertebrates: Progress in Male Gamete Ultrastructure and Phylogeny*, vol 9A, pp. 97–142. Osford and IGH Publishing, New Delhi, India.

Watson, N. A. 2001. Insights from comparative spermatology in the 'turbellarian' Rhabdocoela. In: Littlewood, D. T. J. and Bray, R. A. (Eds.): *Interrelationships of the Platyhelminthes*, pp. 217–230. Taylor and Francis, London.