Comparative Spermatology of Selected Polyclad Flatworms (Platyhelminthes)

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ABSTRACT Sperm ultrastructure of four acotylean (Idioplana atlantica, Armatoplana leptalea, Styloplanocea fasciata, Melloplana ferruginea) and three cotylean polyclads (Pseudoceros bicolor, Phrikoceros mopsus, Enchiridium evelinae) was investigated. All spermatozoa are biflagellate, exhibiting a 9+1 axoneme pattern. All acotylean axonemes originate and extend within the sperm shaft, and once exiting the shaft, remain attached to it. The flagella of all acotylean spermatozoa exit the shaft immediately and remain free. Structures shared by all species include: an elongated nucleus, in acotyleans located only in the posterior part of the shaft, whereas in cotyleans it extends along the entire sperm body; mitochondria along with small and large dense bodies arranged in a specific pattern; and a ring of microtubules that extends along the entire sperm shaft just beneath the cell membrane. A unique spermatozoon has been found in E. evelinae, where round vesicle-like structures fill the anterior part of the nucleus, and a different type of large dense bodies is present. The spermatozoa of all studied species exhibit numerous characters (axoneme/flagella position, distribution and position of large and small dense bodies, of mitochondria, presence of nuclear vesicles) that may be of phylogenetic value at the family and higher taxonomic levels. J. Morphol. 268:891–897, 2007.

KEY WORDS: Platyhelminthes; Polycladida; comparative spermatology; phylogeny

Phylogenetic reconstructions rely on characters derived from many different fields of study, including morphology, cytology, physiology, behaviour, and molecular. In recent years, sperm structure has been successfully applied to taxonomic considerations for many animal groups, e.g., annelids (Jamieson and Rouse, 1989; Ferraguti and Erséus, 1999; Ferraguti et al., 1999), insects, and other arthropods (Alberti and Weinmann, 1985; Jamieson, 1987, 1991b; Liana and Witalinski, 2005), fish and tetrapods (Jamieson, 1991a, 1995). Studies of the ultrastructure of sperm and spermatogenesis in Platyhelminthes showed that in this group, spermatozoa also carry strong phylogenetic information and can be useful in establishing a taxonomic system (Hendelberg, 1965, 1969, 1974, 1977, 1983, 1986; Ehlers, 1985; Justine, 1995; Båå and Marchand, 1995; Watson and Rohde, 1995; Watson, 1999). Although light microscopic studies exist on sperm morphology of several polyclads (Hendelberg, 1965, 1969), only two studies describe the spermatozoa of the acotyleans Notoplana japonica (Kubo-Irie and Ishikawa, 1983) and Pleioplana atomata (Liana and Litvaitis, personal observation) at the ultrastructural level. Generally, polyclad spermatozoa are filamentiform, biflagellate, possess elongated nuclei, mitochondria or mitochondrial derivatives, and one or more types of refractile bodies (for review see Watson, 1999). Turbellarian sperm morphology has been successfully used for phylogenetic reconstructions (Hendelberg, 1969). According to Hendelberg (1969) the biflagellate spermatozoa of Acocela (recently excluded from Platyhelminthes, see Littlewood et al., 1999; Ruiz-Trillo et al., 1999) Polycladida, Proseriata, and Tricladida represent the ancestral character state in Turbellaria, and all other spermatozoan types (monoflagellate and aflagellate) are derived from them. Moreover, sperm morphology of Polycladida shows distinct differences between the two suborders, Acotylea and Cotylea. Sperm of both taxa possess two flagella, but in Cotylea they are free, whereas in Acotylea they are incorporated or located very close to the sperm shaft (Hendelberg, 1965, 1969).

The aims of our study were to investigate the spermatozoa of representatives of Acotylea and Cotylea at the ultrastructural level and to evaluate the phylogenetic usefulness of their characteristics.

MATERIALS AND METHODS

Four species of Acotylea, Idioplana atlantica Bock, 1913 (Pseudostylochidae), Armatoplana leptalea (Marcus, 1947) (Stylochoplanidae), Styloplanocea fasciata (Schmarda, 1859) (Gnesioceridae), and Melloplana ferruginea (Schmarda, 1859) (Pleioplanidae), and three species of Cotylea, Pseudoceros bicolor Verrili, 1902 (Pseudocerotidae), Phrikoceros mopsus Quiroga et al. 2004 (Pseudocerotidae), and Enchiridium evelinae Marcus, 1949 (Pros...
thiostomidae) were examined. The specimens of *A. leptalea* were collected in Florida in May 2005. Specimens of *E. evelinae* were collected in Panama in June 2005. The remaining species were collected on St. John, US Virgin Islands during May and June 2006.

Animals were fixed in 2.5% glutaraldehyde in PBS for 24 h at 4°C and postfixed for 12 h in 1% OsO₄. After routine dehydration in a graded ethanol series followed by a final dehydration step in propylene oxide, fragments of specimens containing the reproductive systems were embedded in EMbed 812 resin. To assure the presence of mature spermatozoa in our sections, we selected epoxy blocks containing the vasa deferentia, in which the spermatozoa are stored before mating. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a LEO 922 Omega transmission electron microscope.

**RESULTS**

The spermatozoa of all species examined are filiform and biflagellate with elongated nuclei located in the posterior part of the sperm shaft in acotyleans and extending along the entire shaft in cotyleans. Additional common features include numerous electron-dense bodies and mitochondria in the anterior part of the sperm, and complete rings of microtubules located just beneath the cell membrane and extending along the entire sperm body. The axoneme exhibits a microtubular pattern of 9+"1", which is characteristic for all Platyhelminthes. The 9 peripheral doublets have dynein arms and connect to a central cylinder by fine spokes. The central cylinder is about 55 nm in diameter and has three different regions of electron density: a dense central core, a less dense intermediate zone and an outermost zone covered by a thin cortical sheath (Fig. 1B).

Paired flagella are anchored in the very anterior part of the sperm. Intercentriolar bodies (ICB) are present between the two basal bodies in all species (Figs. 1A, 2A,B, 3A, 4A, 5A, and 6F). In all examined cotyleans, the axonemes exit the sperm shaft from its very anterior part and extend as free flagella (Figs. 5A and 6F) (Table 1). In acotyleans, on the other hand, the proximal (with respect to the basal bodies) portions of the axonemes extend inside the sperm shaft for some distance (Figs. 1A,B, 2B–D, and 4B,C) and exit the shaft (Fig. 1C), either at the same level (*Styloplanocera fasciata*) (Fig. 3A) or individually at two different levels (*Idioplana atlantica*, *Armatoplana leptalea*, and *Melloplana ferruginea*) (Figs. 1A, 2A, and 4A). Once outside the sperm shaft, acotylean flagella remain very close or even adhere to the main sperm body (Figs. 2D,E, 3B–E, and 4D–F) (Table 1). The paired flagella are probably of the same length but certainly shorter than the sperm body; thus, they end at the level of the middle part of the nucleus.

Numerous dense bodies fill the anterior part of the sperm in *Idioplana atlantica*. They are round in shape (~0.2 μm) and densely packed (Fig. 1A,C). The middle part of the shaft contains round (~0.2 μm) mitochondrial derivatives along with smaller dense bodies (~0.1 μm) (Fig. 1A,B). The posterior

**Fig. 1.** Spermatozoon of *Idioplana atlantica*. A: Diagrammatic view of the spermatozoon. B: Cross section through the anterior part of the sperm. C: Longitudinal section of the spermatozoon showing axoneme exiting the sperm shaft. D: Cross section through the posterior part of the sperm showing the nucleus and ring of microtubules.

a, axoneme inside the spermatozoon; f, flagellum; lb, large dense bodies; m, mitochondria; n, nucleus; sb, small dense bodies; arrowhead, microtubules; short arrow, basal body.
Fig. 2. Spermatozoon of *Armatoplana leptalea*. A: Diagrammatic view of the spermatozoon. B–F: Cross sections through different levels of the sperm shaft, from anterior to posterior. a, axoneme inside the spermatozoon; f, flagellum; lb, large dense bodies; m, mitochondria; n, nucleus; sb, small dense bodies; arrowhead, microtubules; short arrow, basal body; star, ICB.

Fig. 3. Spermatozoon of *Styloplanocera fasciata*. A: Diagrammatic view of the spermatozoon. B: Cross section through the middle part of the sperm shaft. C, D: Longitudinal sections through the middle (C) and the posterior (D) part of the shaft. E: Cross section through the posterior part of the sperm shaft. f, flagellum; lb, large dense bodies; m, mitochondria; n, nucleus; sb, small dense bodies; arrowhead, microtubules; short arrow, basal body.

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segment of the shaft is filled by the nucleus (0.3 μm in diameter) surrounded by microtubules (Fig. 1D) (Table 1).

The anterior part of the sperm of Armatoplana leptalea also is filled by numerous and round (~0.3 μm) large dense bodies (Fig. 2A, D). The middle part of the shaft possesses large (~0.3 μm) mitochondrial derivatives surrounded by smaller dense bodies (>0.1 μm) (Fig. 2E, F) (Table 1). These structures also surround the anterior, tempered segment of the elongated nucleus (0.3 μm).

The anterior portions of the sperm of Melloplana ferruginea and Styloplanocera fasciata contain numerous large (0.3 μm) dense bodies interspersed with round mitochondrial derivatives (0.6 μm) (Figs. 3A and 4B–E) (Table 1). The middle portion of the sperm shaft is filled by small dense bodies (0.1 μm).
that also surround the anterior part of the elongated nucleus (Figs. 3A–D and 4F). The posterior segment of the spermatozoon possesses a nucleus surrounded by microtubules only (Fig. 3E).

In *Pseudoceros bicolor*, the anterior segment of the sperm shaft is filled by large (0.3 μm) dense bodies along with mitochondrial derivatives (0.4 μm) that are located closer to the nucleus, and a layer of dense bodies (0.2 μm) that separate the mitochondrial derivatives from a ring of microtubules (Fig. 5A, D). In the posterior part of the sperm shaft, no intranuclear vesicles are apparent, although a second type of the dense bodies surrounds the nucleus. These are larger (0.4 μm) than the dense bodies of the anterior sperm portion, elliptical and densely packed. They have regions of different electron opacity. Usually one pole region is more electron dense than the opposite end (Fig. 6A, D, E).

**DISCUSSION**

Sperm structure in high-rank taxa (families to orders) usually shares features allowing for the recognition of a general “structural pattern” characteristic for a given taxon. At lower taxonomic levels, a general pattern still may exist, but many details may be variable. In the spermatozoa of Platyhelminthes, we also can distinguish characters that can be very useful for phylogenetic considerations at different taxonomic levels. In recent morphological and molecular phylogeny analyses of Platyhelminthes (Zamparo et al., 2001; Zrzawy, 2001; Bagunà and Riutort, 2004; Petrov et al., 2004; Willems et al., 2006), sperm morphology always is considered a valuable character. According to Watson and Rohde (1995), homologous characters of sperm that are phylogenetically useful include the number of axonemes, their state (free or incorporated) and placement, split tips of flagella, the location and form of mitochondria, the nature of the nucleus, the presence, number, and types of dense bodies, the presence of peripheral microtubules, and the presence of small (25 nm in diameter) granules in the sperm shaft. Although Watson and Rohde (1995) use these characters at the phylum level, some of...
them also may be useful when considering lower taxonomic categories.

In Polycladida, we found that the position at which flagella exit the sperm shaft and if they remain attached to the sperm shaft or become free varies between the two suborders. In Cotylea, both axonemes exit the sperm shaft at the very anterior end and remain free flagella thereafter, whereas in Acotylea one axoneme emerges first from the anterior part of the sperm shaft, and the second axoneme extends for some distance inside the shaft, exiting further from the anterior end. Furthermore, in acotyleans both flagella remain in close contact to the sperm shaft, probably even adhering to it.

It appears that Styloplanocera fasciata is an exception within the Acotylea because both axonemes exit the sperm shaft at the same level, although not at the very anterior most portion of the sperm body as in the cotyleans. Furthermore, the flagella remain attached to the sperm body. According to Hendelberg (1965), flagella are free in Acotylea at the beginning of sperm maturation but as spermatids elongate and mature, the anterior parts of the flagella become incorporated and the posterior parts adhere to the sperm shaft. We examined mature spermatozoa stored in the vasa deferentia; thus, we assume that incorporation of the axonemes had been completed.

Other characters shown to be of phylogenetic value are the dense bodies (Watson and Rohde, 1995). Watson and Rohde (1995) noted the presence and types of dense bodies. However, we showed that their distribution also should be considered. In the acotyleans Idioplana atlantica and Armatomoplana leptalea, the anterior ends of spermatozoa are filled by large dense bodies only. In Melloplana ferruginea and Styloplanocera fasciata on the other hand, the dense bodies in the anterior part of the sperm are interspersed with mitochondria. Small dense bodies can be either interspersed with mitochondria (A. leptalea and I. atlantica) or be found anterior to the nucleus and surrounding its anterior part (M. ferruginea and S. fasciata). The distribution of these structures in another acotylean species, Pleioplana atomata, is similar to that in M. ferruginea and S. fasciata (Liana and Litvaitis, personal observation).

In the cotyleans Pseudoceros bicolor and Prhiko- ceros mopsus, the nucleus extends almost throughout the entire sperm shaft, restricting the distribution of dense bodies and mitochondria to the anterior most and peripheral areas. Additionally, a different type of dense body has been described in Enchiridium evelinæ. These larger, elliptical dense bodies are restricted to the posterior part of the shaft and are distinct from other dense bodies by exhibiting different electron opacities. However, it is highly likely that they originate in the same way as smaller dense bodies. We found that in Pleioplana atomata, dense bodies are formed during spermatogenesis from vesicles originating from the Golgi apparatus (Liana and Litvaitis, personal observation). Assuming that all dense bodies in polyclad sperm form in this fashion, they can be considered homologous structures, making them useful for phylogeny.

Finally, the length of the nucleus within the sperm shaft may be used to distinguish the two suborders of polyclads. All acotyleans examined have their nucleus located in the posterior part of the sperm shaft. This is in contrast to the cotylean species examined in this study, where the nucleus extends through most of the spermatozoon. Although we did find one species-specific character in Enchiridium evelinæ, namely numerous round vesicle-like structures within the anterior part of the nucleus, sperm ultrastructure at the species level may be too uniform to be of phylogenetic signifi-

<table>
<thead>
<tr>
<th>Species</th>
<th>Level of axoneme exit from sperm shaft</th>
<th>State of axoneme</th>
<th>Position of nucleus</th>
<th>Distribution of large dense bodies</th>
<th>Distribution of small dense bodies</th>
<th>Nuclear vesicles</th>
<th>Distribution of mitochondria</th>
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<tr>
<td>Idioplana atlantica</td>
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<td>Anterior</td>
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<td>Absent</td>
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<td>Armatomoplana leptalea</td>
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<td>Posterior</td>
<td>Anterior</td>
<td>Posterior to large bodies</td>
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<td>Interspersed with small dense bodies</td>
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<tr>
<td>Styloplanocera fasciata</td>
<td>Middle, same level</td>
<td>Attached</td>
<td>Posterior</td>
<td>Anterior</td>
<td>Posterior to large bodies</td>
<td>Absent</td>
<td>Interspersed with large dense bodies</td>
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<tr>
<td>Melloplana ferruginea</td>
<td>Middle, different levels</td>
<td>Attached</td>
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<td>Posterior to large bodies</td>
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<td>Interspersed with large dense bodies</td>
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<td>Entire shaft</td>
<td>Anterior</td>
<td>Posterior to large bodies</td>
<td>Absent</td>
<td>Interspersed with large dense bodies</td>
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<tr>
<td>Phrikoceros mopsus</td>
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<td>Free</td>
<td>Entire shaft</td>
<td>Anterior</td>
<td>Present</td>
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<td>As a layer between nucleus and small dense bodies</td>
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<tr>
<td>Enchiridium evelinæ</td>
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<td>Free</td>
<td>Entire shaft</td>
<td>Posterior</td>
<td>Anterior to large bodies</td>
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cance. Furthermore, without an understanding of spermatogenesis in this species, the origin as well as presumptive function of these intranuclear structures remains unknown. Considering our findings and those of other ultrastructural studies (Kubo-Irie and Ishikawa, 1983; Watson and Rohde, 1995; Watson, 1999) we conclude that the spermatozoa of polyclad flatworms contain many characteristics useful for phylogenetic inferences, especially at the family and higher taxonomic levels.

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LITERATURE CITED


