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Spermiogenesis and sperm ultrastructure in *Thylacorhynchus ambronensis* (Schizorhynchia, Kalyptorhynchia, Platyhelminthes)

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Abstract. Comprehensive ultrastructural investigation of spermiogenesis and the mature sperm in *Thylacorhynchus ambronensis* revealed a number of features valuable for cladistic analysis. Two basal bodies lie on either side of an intercentriolar body in the zone of differentiation of the spermatid, but only one develops into a normal flagellum while the other remains as a small bud, eventually disappearing. Structures within and surrounding the two basal bodies differ, and, contrary to observations in another monoaxonemal schizorhynch (*Baltoplana magna*), the two basal bodies become separated and only that of the normal flagellum is carried distally from the cytophore which unites an isogenic group of spermatids. A spiralling ridge develops on a projection of the spermatid that is distal to the flagellar basal body; the normal flagellum becomes incorporated into the shaft of the sperm, paralleling its long axis, in the distal to proximal orientation; and the nucleus and a string of mitochondria become tightly coiled around the axoneme. Cortical microtubules surrounding the nucleus, mitochondria, and axoneme are also helically wound around the shaft, except at the proximal end. Mature sperms are very long and filiform, with a corkscrew structure ~7 μm long at the distal end. The nucleus extends throughout most of the length of the sperm, while mitochondria and the central axoneme terminate some distance from the proximal end. There are no dense bodies in any region of the sperm. Although terminal corkscrew structures and, separately, monoaxonemal sperms have been found in other platyhelminth taxa, evidence suggests that neither of these features is homologous between *T. ambronensis* and those taxa.

Additional key words: phylogeny, Turbellaria

Comparative spermatology is proving to be a useful tool in phylogenetic analyses of invertebrate phyla (see chapters and references in Jamieson et al. 1995). Ultrastructural studies of mature sperms reveal a range of character variables used to examine relationships between and within various conventional taxonomic levels, from phylum to family and even genus and species. Some aspects of spermiogenesis have also been examined although not usually included in such analyses.

In the phylum Platyhelminthes—in which a major taxon, Trepaxonemata, is recognized as monophyletic on the basis of a unique structure in the sperm axoneme (Ehlers 1984)—most sperm studies have concentrated on the parasitic groups (Neodermata), especially monogeneans and cestodes (see reviews by Bâ & Marchand 1995 and Justine 1995). Far fewer studies (see Watson & Rohde 1995a) have treated the remaining platyhelminth taxa, collectively known as the "Turbellaria," a paraphyletic grouping (and possibly polyphyletic, e.g., Smith et al. 1986). The large number of variable characters in platyhelminth sperms makes them useful for phylogenetic studies, especially if the long filiform sperms are examined throughout their length and a full reconstruction presented. Most platyhelminth sperms have two flagella, either free or incorporated to varying degrees in the sperm body. The processes involved during spermiogenesis, especially those associated with the flagella, also provide a number of additional characteristics for phylogenetic analysis.

Several recent detailed investigations of sperm ultrastructure and spermiogenesis (Watson et al. 1993; Watson & Rohde 1994a, 1995b; Watson & Jondelius 1995; Watson et al. 1995) have shown that such studies may be particularly fruitful in the turbellarian rhabdocoels, a large taxon of mainly free-living families classified into Kalyptorhynchia, Typhloplanida, Dalyelliida, and Temnocephalida. Monophyly of this group appears likely based on DNA and protonephridial ultrastructural data (Rohde 1991) but relationships between and within the various sub-taxa are unclear. Kalyptorhynchia, a well-defined taxon within Rhabdocoela, is sub-divided into Schizorhynchia and Eu-

kalyptorhynchia, based on the nature of the proboscis musculature. There have been two transmission electron microscopical studies dealing with only some features of sperm and spermiogenesis in kalyptorhynchs (Rohde et al. 1987; L'Hardy 1988), but only one comprehensive study encompassing all aspects necessary to permit use of the data for phylogenetic resolution (Watson & L'Hardy 1995). That investigation revealed for the first time the manner in which the sperm of *Baltoplana magna* KARLING 1949 becomes uniflagellate.

Here we report an ultrastructural investigation of the uniflagellate sperm and spermiogenesis in a schizorhynch kalyptorhynch from a different family, as part of a plan to build a detailed database of sperm and spermiogenetic characteristics across a wide range of rhabdocoel turbellarians, for ultimate use in cladistic analyses.

Methods

Mature specimens of *Thylacorhynchus ambronensis* SCHILKE 1970 were collected from beach sand, from Bredene on the Belgian coast, and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer made with boiled, filtered seawater (replacing distilled water), pH 7.3, for 13 hours at 4° C. They were then washed in the same buffer, post-fixed in buffered 1% OsO_4, dehydrated in ethanol, and embedded in Spurr resin. Ultrathin serial sections through testes and sperm ducts were stained with uranyl acetate and lead citrate and examined with Philips 400 and JEOL 1200 EX transmission electron microscopes (TEM).

Results

Spermiogenesis

Thylacorhynchus ambronensis has paired testes anterior to the pharynx, and germinal cells are surrounded by a thin epithelium with sparse flattened nuclei. Incomplete cytokineses of the products from divisions of spermatogonia and spermatocytes result in a rosette of early spermatids attached to a central cytophore (Fig. 1). Spermiogenesis begins with the movement of spermatid nuclei to peripheral positions and the formation of a zone of differentiation (ZD) at the apical cell membrane distal to each nucleus.

The ZD contains a series of parallel disc-shaped electron-dense plates known as an intercentriolar body (ICB), flanked on either side by a basal body at 90° to the parallel plates of the ICB. From one of the basal bodies, a flared rootlet extends toward a depression in the nuclear envelope, and a normal flagellum grows out (Figs. 2, 3). The other basal body has an electron-dense intracentriolar granule, a much reduced rootlet, and no adjacent depression in the nuclear envelope; only a small flagellar bud grows out from it (Figs. 2, 3). The long free flagella from different spermatids form conspicuous tight bundles in the testis, many of these groups containing 32 flagella, although several with greater numbers were seen (Fig. 3).

The outermost plates of the ICB begin to thicken, but the one beneath the normal flagellum becomes distinctly thicker than the one beneath the flagellar bud. At this time, microtubules appear in a dense region beneath the cell membrane in the ZD (Fig. 3 shows this dense region), and a projection lined with peripheral microtubules grows distally (Fig. 4). The basal body of the full flagellum then rotates toward the spermatid cell body, remaining in contact with the dense heel formed from the outermost plate of the ICB, the ICB itself, and the smaller dense heel that was nearest to the flagellar bud (Figs. 4–6). During this movement, the basal body of the flagellar bud loses its connection to the ICB, and small vesicles are consistently seen near the ICB (Figs. 5, 6). The bud then remains near

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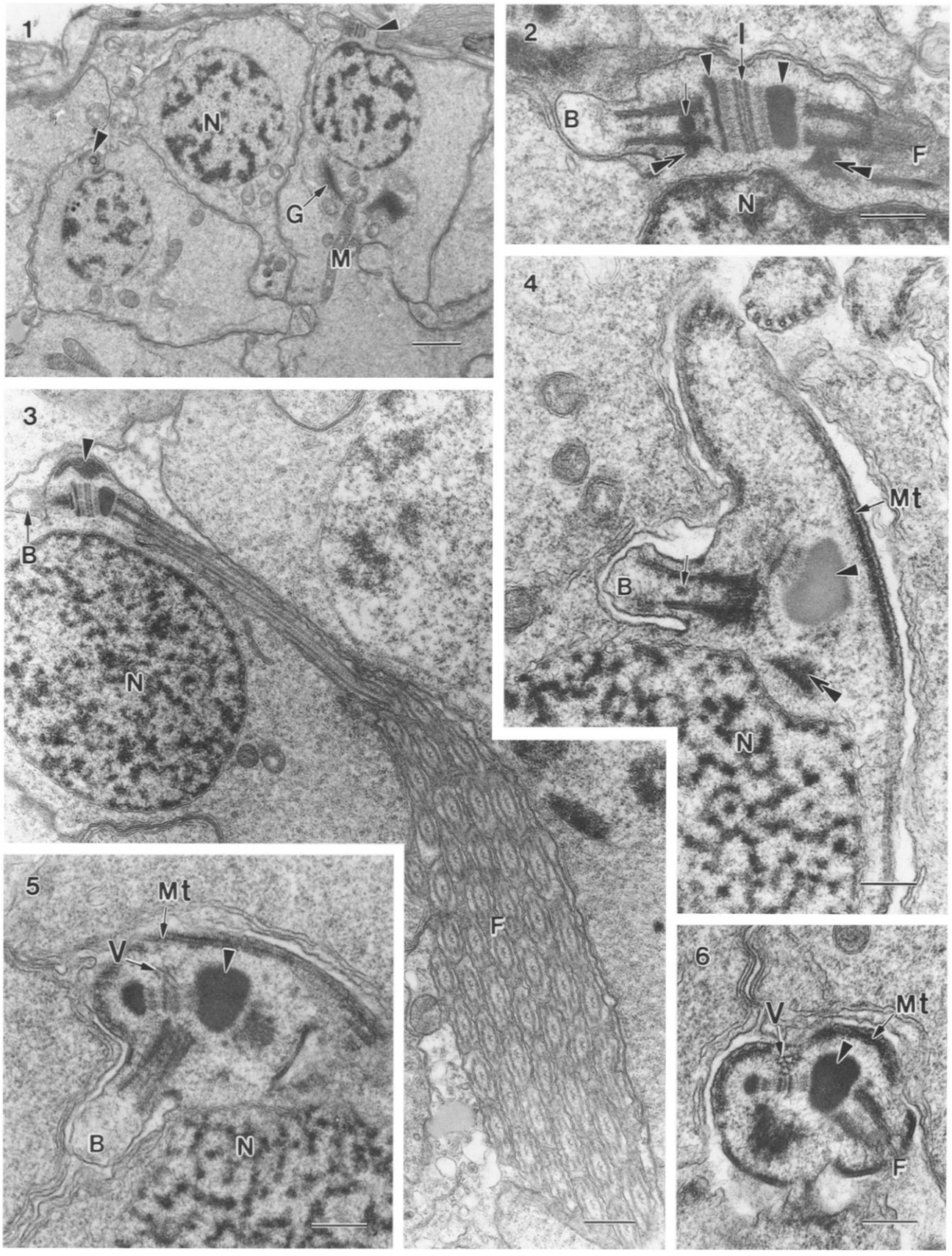
Figs. 1–6. Early stages of spermiogenesis in *Thylacorhynchus ambronensis*. TEM. Scale bars 2 μm in Fig. 1; 1 μm in Fig. 3; 0.5 μm in Figs. 2, 4–6. **Fig. 1.** Early spermatids arranged around a central cytophore. Peripheral nuclei (N), zones of differentiation (arrowheads), Golgi apparatus (G), mitochondria (M). **Fig. 2.** Early zone of differentiation showing intercentriolar body, ICB (I) with unequally thickened outer dense plates (arrowheads). Basal bodies at right angles to the ICB give rise to one flagellar bud (B) and one normal flagellum (F). Note unequal rootlet formation between basal bodies and the nucleus (double arrowheads); a depression in the nuclear outline only beneath the normal flagellum; and intracentriolar granule (arrow) of the flagellar bud. **Fig. 3.** Spermatid in which the flagellum has begun to rotate toward the spermatid body. Microtubules will develop from the dense region in the small distal projection (arrowhead). Note flagellar bud (B), nucleus (N), and bundle of closely associated free flagella (F) from a number of spermatids. **Fig. 4.** Spermatid in which the distal projection with cortical microtubules (Mt) has begun to extend. Nucleus (N), flagellar bud (B) with intracentriolar granule (arrow), dense plate (arrowhead) and rootlet (double arrowhead) of the free flagellum. **Fig. 5.** Spermatid zone of differentiation showing the flagellar bud (B), which has lost its former connection with the plates of the intercentriolar body. Nucleus (N); microtubules (Mt); vesicles (V); large dense plate or heel (arrowhead) associated with the full flagellum. **Fig. 6.** Transverse section through the zone of differentiation during rotation of the free flagellum (F), still associated with its dense plate (arrowhead). Note cortical microtubules (Mt), vesicles (V).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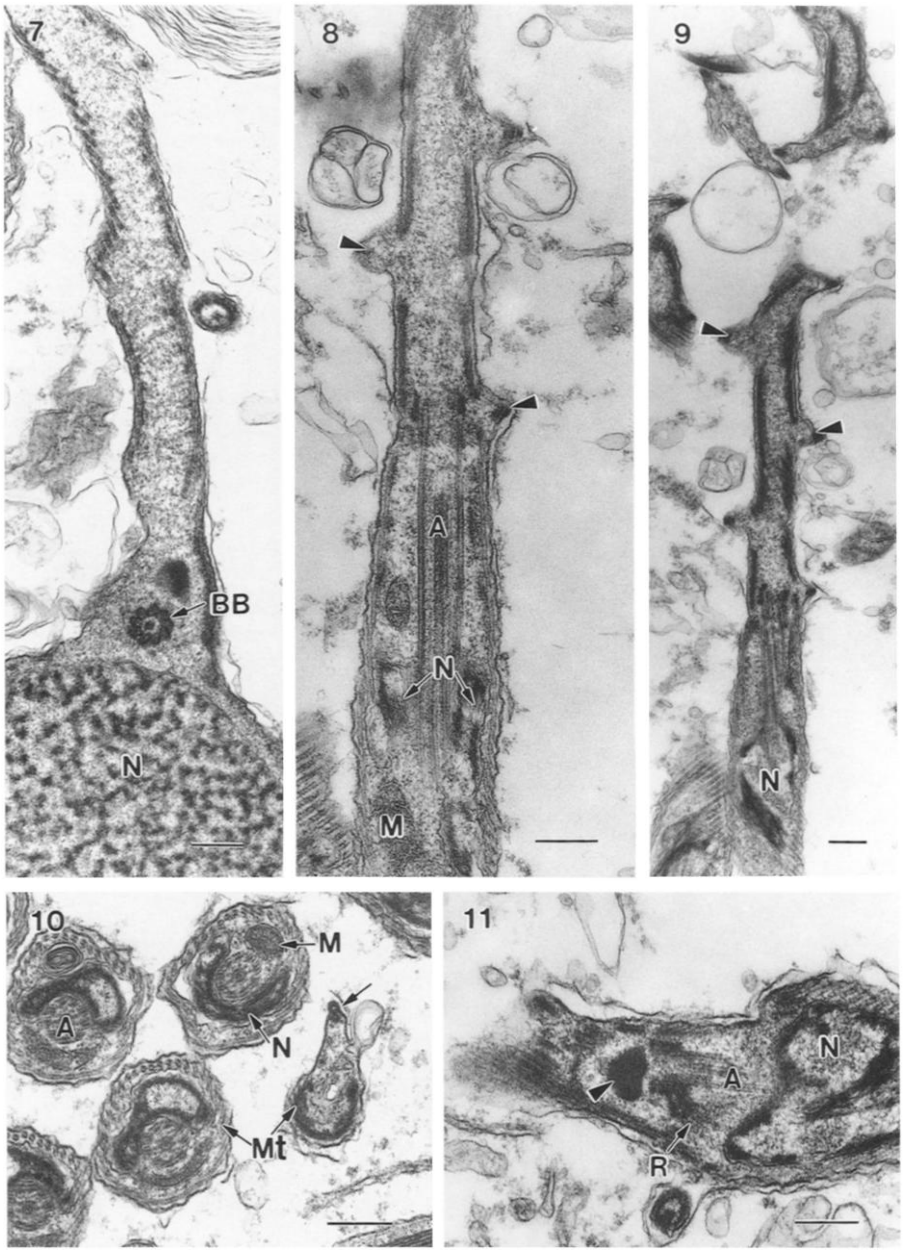


Figs. 7–11. Later stages of spermiogenesis in *Thylacorhynchus ambronensis*. TEM. Scale bars, 0.5 μm. **Figs. 7-9.** Stages in the formation of the corkscrew from the still rounded spermatid stage shown in Fig. 7—note basal body (BB) of the bud, and intracentriolar granule—to incorporation of the flagellar axoneme (A) enveloped by the nucleus (N) (Fig. 8), to further condensation of the corkscrew ridges (arrowheads) and twisting of the shaft (Fig. 9). In Fig. 7, the flagellum at right does not belong to this spermatid. **Fig. 10.** Transverse sections through late spermatids showing twisting cortical microtubules (Mt), nucleus (N), and mitochondrion (M) partially enwrapping the axoneme (A). Note the developing ridge of the corkscrew (arrow). **Fig. 11.** Sub-distal region of a spermatid after spatial separation of the two basal bodies and (at least terminal) incorporation of the flagellum; dense plate (arrowhead) still associated with the basal body of the partially incorporated axoneme (A); rootlet (R) extends from the basal body toward the elongating nucleus (N). The flagellum below does not belong to this spermatid.

to the main spermatid mass (i.e., proximal) (Fig. 7) while the basal body of the full flagellum is carried some distance distally by elongation of the spermatid shaft. The nucleus remains closely associated with the basal body and rootlet of this flagellum (Fig. 11), and thus becomes located in the elongating shaft, moving past the bud, as was seen in numerous cross sections. Mitochondria also move alongside the nucleus, and the free flagellum fuses completely with the shaft (Figs. 8, 9) in a distal to proximal orientation. At this time the accessory structures (ICB, heels, and rootlets) have disappeared.

Concurrently, the distal projection lined with microtubules develops a spiralling ridge or projection that originates as a fold of the cell membrane containing a single microtubule (Figs. 7–10) or occasionally two microtubules. At the same time, the distal projection and the main shaft begin to twist so that all components acquire a spiralling configuration. The flagellar bud remains proximal, and eventually disappears, either by resorption or by being pinched off.

Mature sperms

Mature sperms were found tightly packed in the sperm duct (Figs. 12–21). The wall of the duct is

formed from a thin cellular epithelium that is wider where the perikarya intrude into the canal lumen. There are septate junctions between wall cells, and a thin fibrous basal lamina beneath them; the duct is surrounded by longitudinal or slightly spiralling muscles (Figs. 12, 13, 16).

Sperms have the following configuration, depicted diagrammatically in Fig. 22. Beneath the cell membrane the entire sperm body except for a short region at each end is tightly twisted, so that in cross sections most cortical microtubules appear oblique (Figs. 14, 15, 17, 19). The helical nucleus enwraps the single incorporated axoneme, and both nucleus and a tight string of oblong mitochondria spiral with a relatively short pitch within the shaft (Figs. 13, 15–18). The axoneme also spirals but with a much longer pitch (Fig. 16), such that the other components appear twisted around the axis of the axoneme.

The distal end of the sperm consists of a corkscrew at least 7 μm long with at least 10 turns of the projections (Figs. 12–14). The corkscrew is lined with spiralling cortical microtubules that become crowded and straight for only a short distance at the very end (Fig. 12). Single microtubules from the former basal body of the axoneme also extend some distance into this end-piece (Fig. 14). Proximal to the end-piece, first mitochondria, then the nucleus and a string of mitochondria surround the axoneme over the greater part of the sperm body.

Near the proximal end, mitochondria terminate. The axoneme then tapers and terminates (Fig. 19); microtubular doublets are reduced to singlets and some singlets cease before others. A few sections (e.g., Fig. 18 bottom left) showed only nucleus and mitochondrion, indicating that the axoneme may terminate earlier or mitochondria continue farther in some sperms. The nucleus alone then occupies the entire shaft within the still spiralling cortical microtubules, eventually diminishing in diameter and terminating less than a micron from the proximal end of the shaft (Fig. 20). Cortical microtubules are barely twisted in this short terminal region (Fig. 21).

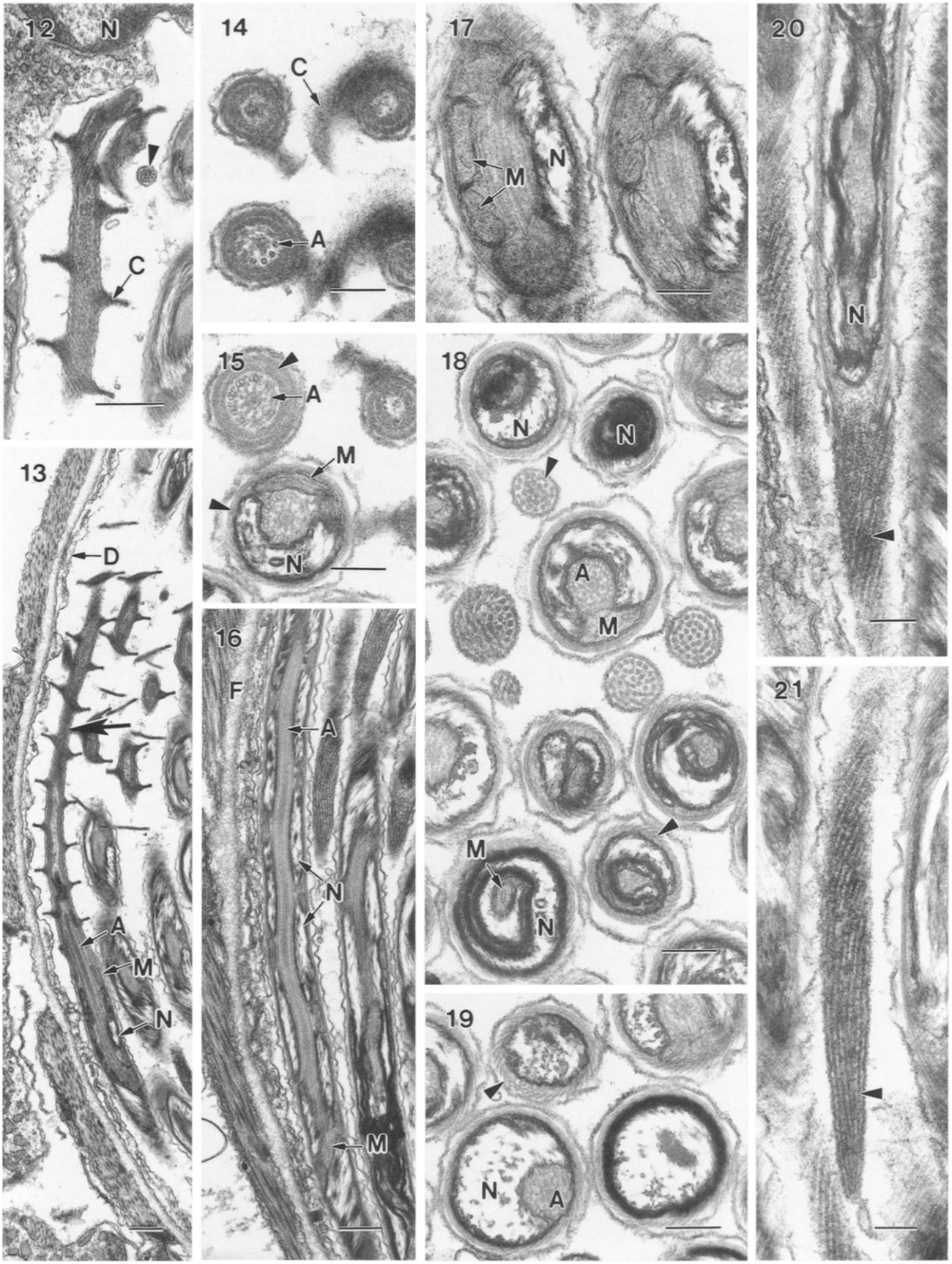
Mitochondria have well-defined cristae (Fig. 17); the axoneme has the 9+'1' configuration (9 doublets and a single complex central element) typical for trepaxonematan platyhelminths (Fig. 15); and the nucleus has dense peripheral chromatin strands, which spiral with the pitch of the nucleus (Fig. 16), and a central open mesh of chromatin. This mesh is much more open where the nucleus alone fills the shaft. There are no dense bodies, granules, or other inclusions within the sperm shaft.

Discussion

Platyhelminths have internal fertilization with introsperm (terminology of Rouse & Jamieson 1987), deposited either into the female ducts or via hypodermic injection. They are highly modified from the basic primitive type of Franzén (1956). Most have two flagella, either free or incorporated along the sperm shaft, deeply embedded or more superficially attached. Kalyptorhynchs previously examined have two axonemes fully incorporated into the sperm body but *Baltoplana magna* (Schizorhynchia, Karkinorhynchidae) was reported to have only one axoneme (L'Hardy 1972; Hendelberg 1975, 1983), the first recorded observation of a single flagellum in turbellarian flatworms apart from the primitive members of Nemertodermatida. This was confirmed in a full study of spermiogenesis and reconstruction of the mature sperm (Watson & L'Hardy 1995).

Now we have shown that another kalyptorhynch from a different family (Schizorhynchidae) also has a monoaxonemal sperm, and that this condition arises similarly through incomplete development and subsequent degeneration of one of the two flagella formed at the beginning of spermiogenesis. In *Thylacorhynchus ambronensis,* the intercentriolar body and its outer swollen dense regions remain in contact and in line with the normal flagellum during its rotational movement and distal movement accompanying the elongation of the shaft. As a result, the flagellar bud on the other side of the shaft loses contact with the ICB; the bud does not move distally but remains proximal and eventually disappears. This distinct separation and loss of contact between the two basal bodies was not seen in *B. magna,* nor was there any noticeable difference between the basal bodies in the thickened regions at their bases. Sperms of both species are completely lacking in dense bodies, although both have prominent Golgi apparatus in developing spermatids.

Other differences include the following: Sperms of *T. ambronensis* do not have dense chromatin rods in the nuclei as do sperms of *B. magna;* they have a tight string of individual mitochondria instead of a single mitochondrial rod; cortical microtubules spiral tightly around most of the shaft instead of running parallel to it; and the nucleus enwraps the axoneme. Most conspicuously, the corkscrew structure at the distal end of *T. ambronensis* is completely lacking in *B. magna,* although in both species the axoneme at its distal end contains only single microtubules. Furthermore, the temporary tight association of free flagella from a number of spermatids, a striking feature in the testis of *T. ambronensis,* was not seen in *B. magna,* nor to our knowledge has it been reported for any other tur-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bellarian, although it is a common occurrence during spermiogenesis in many neodermatans (see, e.g., Justine & Mattei 1986). Similar flagellar bundles occur in another schizorhynch, *Karkinorhynchus bruneti* SCHILKE 1970 (unpubl. obs.).

Schizorhynchia is regarded as a monophyletic taxon on the basis of the split musculature of the proboscis (schizorhynch). The presence of monoaxonemal sperms formed by a similar process in these two species from different families supports that hypothesis. The only other turbellarian taxon known to have a single axoneme in its sperms is Nemertodermatida, but the sperms of *Meara* (Hendelberg 1977) and *Nemertoderma* (Tyler & Rieger 1975, 1977) are very different from those of the kalyptorhynchs although their spermiogenesis has not yet been investigated. Some groups of cestodes (Bâ & Marchand 1995) and monopisthocotylean monogeneans (Justine 1991) also have monoaxonemal sperms, but these represent independently derived conditions. Cestodes and monogeneans belong to the Neodermata, a well-recognized monophyletic taxon in which the plesiomorphic state is biaxonemal sperms. The monoaxonemal condition has therefore arisen by convergent evolution in the schizorhynchs and these neodermatans.

Furthermore, it is generally accepted that the schizorhynch structure is derived from the conorhynch of the Eukalyptorhynchia (Karling 1961; Ehlers 1985; Ax 1987) and therefore that the sister group to Schizorhynchia probably is or lies within the Eukalyptorhynchia, on this basis. Sperms of three species of the latter taxon have been briefly examined (Rohde et al. 1987; L'Hardy 1988) and all have two axonemes incorporated into the shaft, further confirmation that the single axoneme condition in Schizorhynchia and Neodermata is a homoplasy.

Reduction to a single axoneme may result in a more streamlined sperm with improved competitive locomotion. The corkscrew end (which is equivalent to the end found to be anterior in terms of the direction of movement in other platyhelminth studies, e.g., Hendelberg 1983; Ishida et al. 1991) may confer a similar advantage and/or aid in penetration of the egg. In the latter context, no platyhelminth sperms have been shown to possess a true acrosome with the possible exception of those of Nemertodermatida (Tyler & Rieger 1975); however, the dense bodies present along much of the length of many sperms may possibly perform an acrosomal function (Hendelberg 1983; Sopott-Ehlers 1986) and there are dense regions at the "anterior" end in several species (e.g., Webb 1979—*Temnocephala daddowae;* Watson & Rohde 1993—*Kronborgia isopodicola*). *T. ambronensis* and *B. magna* both have conspicuous Golgi apparatus in their developing spermatids, and multivesicular bodies form but no secretion moves distally to form an acrosome.

Terminal corkscrew structures are also known from a number of cestodes (see Bâ & Marchand 1995), and from some monogeneans (e.g., Tappenden & Kearn 1991; Watson & Rohde 1994b). These structures, known as crested bodies in the cestodes, resemble that found in sperms of *T. ambronensis.* Many of the cestodes with terminal crests or corkscrews also have a single axoneme enwrapped by the nucleus, and a tight spiralling of cortical microtubules and of nucleus within the shaft. However, assuming that Neodermata is monophyletic and that within it the cestodes are a derived group, then the combined incidence of these

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Figs. 12–21. Transverse (TS) and longitudinal sections (LS) of mature sperms of *Thylacorhynchus ambronensis,* found in the sperm duct. TEM. Scale bars, 0.5 μm in Figs. 12, 13, 16; 0.2 μm in Figs. 14, 15, 17–21. **Fig. 12.** The distal tip in LS and (arrowhead) TS. Ridge of the sperm corkscrew (C); nucleus of the cellular duct wall (N). **Fig. 13.** Distal regions of sperms including the corkscrew region with tight spiral of microtubules (large arrow without letter), the beginning of the main shaft with central axoneme (A), peripheral nucleus (N), and mitochondria (M). Note cytoplasmic layer of duct wall (D). **Fig. 14.** Distal sperm regions showing the corkscrew (C) in TS and the single microtubules of the tapering axoneme (A). **Fig. 15.** More proximal region (top left) with axoneme (A) surrounded by tightly spiralling microtubules (arrowhead), and principal region (bottom) with central axoneme, spiralling nucleus (N), mitochondria (M), and microtubules (arrowhead). **Fig. 16.** LS of the principal region of a sperm showing a slight spiralling of the axoneme (A), but a tight coiling of the nucleus (N) and mitochondria (M). Note fibrous basal lamina (F) underlying the cellular lining of the sperm duct. **Fig. 17.** Oblique LS of sperm showing that mitochondria (M) are arranged end-to-end. Nucleus (N). **Fig. 18.** Sections through sperms near the proximal end. The axoneme (A) and mitochondria (M) terminate; the nucleus (N) continues for some distance, filling most of the shaft. As the shaft diminishes in width, microtubules become less twisted and finally run parallel with it (arrowheads). Bottom left shows a section with axoneme terminated but small mitochondrion still present. **Fig. 19.** Sperm regions where the nucleus (N) dominates; axoneme and mitochondria have terminated (arrowhead) or the axoneme (A) is still present. **Fig. 20.** LS where the nucleus (N) terminates and microtubules are much less twisted around the shaft (arrowhead). **Fig. 21.** LS of the proximalmost tip where microtubules become parallel with the shaft (arrowhead).

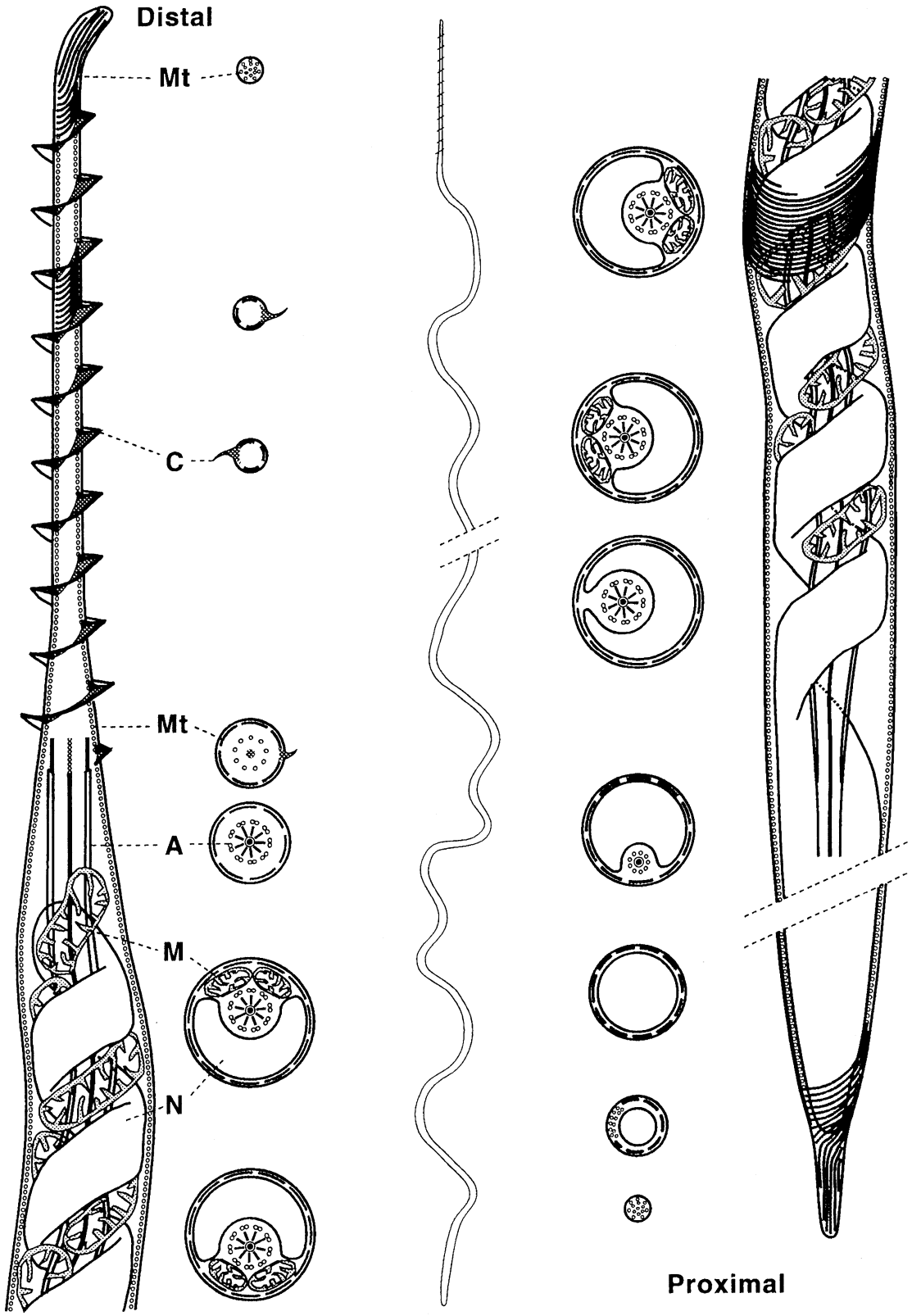

Fig. 22. Representation of the living sperm of *Thylacorhynchus ambronensis* (center) and diagrammatic longitudinal and transverse sections of the sperm at various levels. Axoneme (A); corkscrew region (C); mitochondrion (M); cortical microtubules (Mt); nucleus (N).

characteristics in such cestodes and in *T. ambronensis* must also represent homoplasy. The corkscrew in two species of *Calicotyle* (Monogenea) (Tappenden & Kearn 1991; Watson & Rohde 1994b) does not resemble that in *T. ambronensis* either in its formation or final appearance. A crested structure in another monogenean, *Calceostoma* sp. (see Justine & Mattei 1986), is not terminal, nor associated with spiralling microtubules, and appears to arise from cytoplasmic condensation.

A terminal corkscrew has also been reported in the sperm of one other turbellarian—*Troglocaridicola* sp. (Temnocephalida) (Iomini et al. 1994). Its formation has not been documented but the authors suggested that it may originate from a central electron-dense structure and microtubules lining an apical process. Terminal spiralling structures have also been found sporadically in other invertebrate phyla—e.g., Tardigrada (Rebecchi & Guidi 1991, 1995), Polychaeta (Rouse 1995), Gastrotricha (Fischer 1994; Ferraguti & Balsamo 1995); that they have arisen independently implies a selective advantage during movement or egg penetration. We now intend to trace the development of this and other distinctive characteristics of sperms and spermiogenesis within Schizorhynchia and Eukalyptorhynchia and to use them for phylogenetic analyses.

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