

Ultrastructure of spermiogenesis and spermatozoa of *Notocotylus neyrai* González Castro, 1945 (Digenea, Notocotylidae), intestinal parasite of *Microtus agrestis* (Rodentia: Arvicolidae) in Spain

PAPA IBNOU NDIAYE¹, JORDI MIQUEL^{1*}, CARLOS FELIU¹ and BERNARD MARCHAND²

¹Laboratori de Parasitologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028 Barcelona, Spain
Tel. +34 (93) 402-4500; Fax: +34 (93) 402-4504; email: jmiquel@farmacia.far.ub.es

²Laboratoire Parasites et Écosystèmes Méditerranéens, Faculté des Sciences et Techniques, Université de Corse, F-20250 Corte, France

Received 24 May 2002; Accepted 18 November 2002

Summary

To our knowledge, this is the first ultrastructural study on spermiogenesis and the spermatozoon of a trematode belonging to the family Notocotylidae, *Notocotylus neyrai*. Spermiogenesis begins with the formation of the zone of differentiation which comprises striated rootlets associated with the two centrioles and an intercentriolar body in-between. It is characterised by an asynchronous flagellar rotation and subsequent proximodistal fusion with a median cytoplasmic process. The migration of the nucleus toward the median cytoplasmic process before its fusion with the free flagella is also described. The mature spermatozoon of *N. neyrai* is filiform and tapered at both ends and presents all the features found in the Digenea gamete: two axonemes, mitochondrion, nucleus and two bundles of parallel cortical microtubules. Nevertheless, several characters allow us to distinguish *N. neyrai* from other digenetic trematodes.

Key words: Ultrastructure, spermiogenesis, spermatozoon, *Notocotylus neyrai*, Trematoda, Digenea, Notocotylidae

Introduction

Several authors have carried out ultrastructural studies on Platyhelminthes for phylogenetic purposes (Euzet et al., 1981; Brooks, 1989; Hoberg et al., 1997; Justine, 1991, 1995, 1997, 1998, 2001; Justine et al., 1985; Rohde, 1990; Bâ and Marchand, 1994, 1995). Numerous reports on the ultrastructure of the spermatozoon and the process of spermiogenesis are thus available: 150 papers on parasitic Platyhelminthes, of which more than 80 deal with digenetic trematodes,

although spermatozoa have been examined in only 47 genera of Digenea (Chen et al., 1996; Gracenea et al., 1997; Iomini, 1998; Tang et al., 1998; Miquel et al., 2000; Baptista-Farias et al., 2001; Justine 2001). The spermatozoa described in Digenea share a few characteristics: nucleus in posterior areas of the sperm, one or more mitochondria, parallel cortical microtubules and two axonemes of the 9+“1” pattern of trepaxonematan Platyhelminthes (Ehlers, 1985). However, many other particularities have been described, but they lack infor-

*Corresponding author.

mation about the pattern of spermiogenesis and spermatozoon particularities for determine phylogenetic purposes.

After the review of Simón-Vicente et al. (1985), three species of the genus *Notocotylus* are accepted as parasites of rodents in Europe: *Notocotylus noyeri* Joyeux, 1922, *N. neyrai* González Castro, 1945 and *N. gonzalezi* Simón Vicente, Mas Coma, López Román, Tenora et Gállego, 1985. There are limited ultrastructural data available on gametogenesis in the Notocotylidae family. Here, we present the first ultrastructural study on the spermiogenesis and spermatozoon of one species of this family, *N. neyrai*.

Materials and Methods

Live *N. neyrai* specimens were collected from *Microtus agrestis* from Vall d'Arties (Lleida, Spain). Adults were kept in 0.9% NaCl solution. Several portions of these specimens were dissected and fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 h, rinsed in 0.1 M sodium cacodylate buffer at pH 7.2, postfixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 h, rinsed in 0.1 M sodium cacodylate buffer at pH 7.2, dehydrated in ethanol solutions and propylene oxide, embedded in Spurr and polymerised at 60°C for 48 h. Ultrathin sections (60–90 nm) of testes and seminal ducts were obtained using a Reichert-Jung Ultracut E ultramicrotome, placed on 200 mesh copper grids and double-stained with uranyl acetate and lead citrate following Reynolds (1963).

Copper grids were examined under a Hitachi H-600 EM at 75 kV in the "Serveis Científicotècnics" of the University of Barcelona (Spain) and in the University of Corsica (Corte, France).

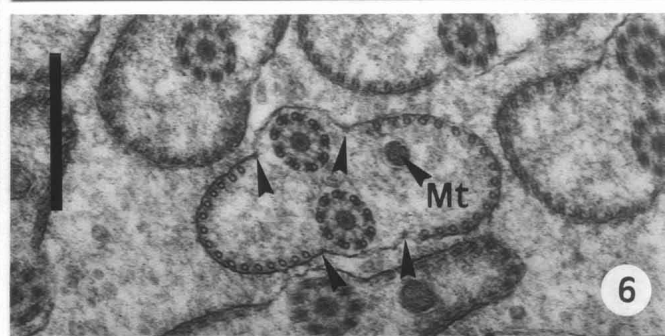
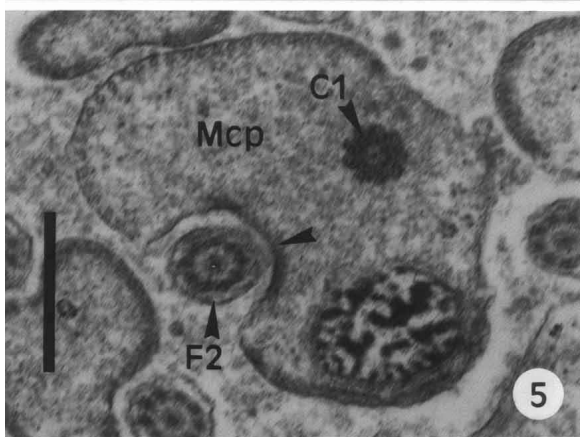
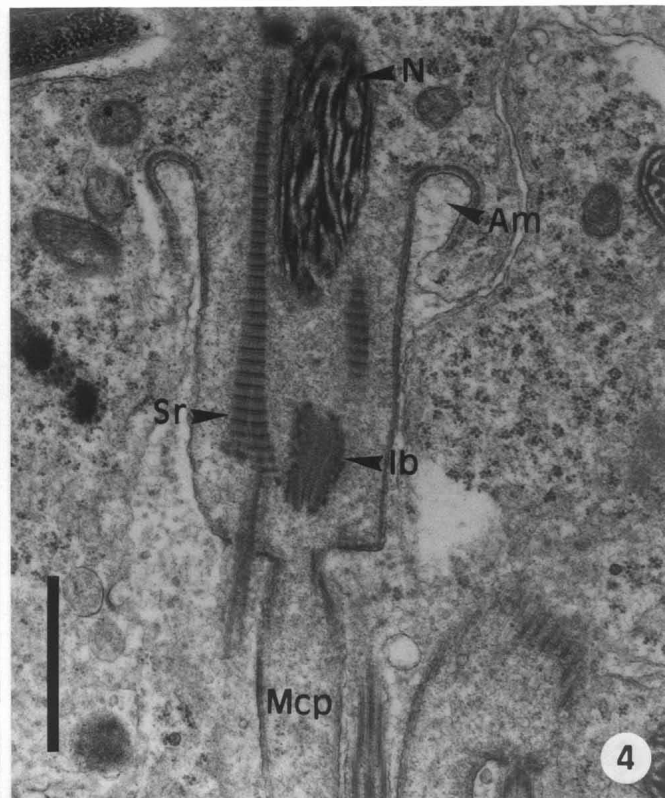
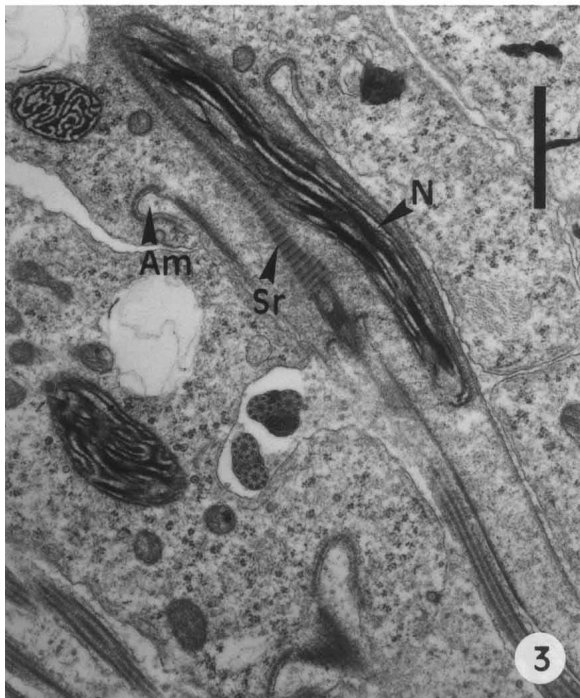
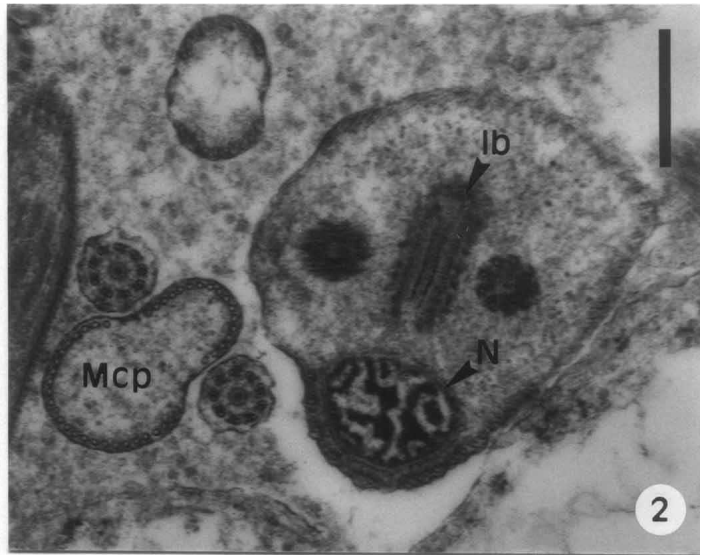
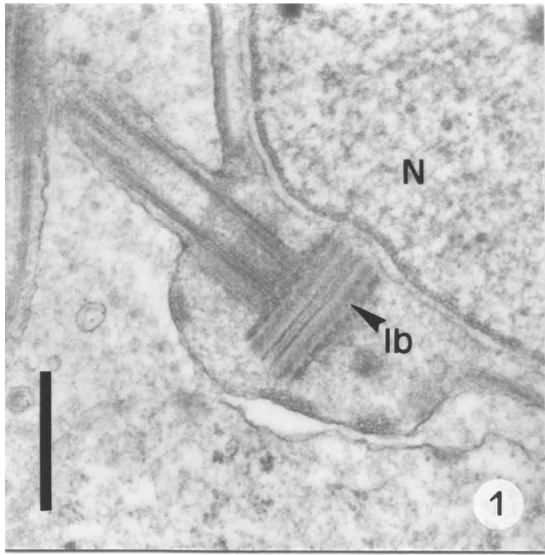
Results

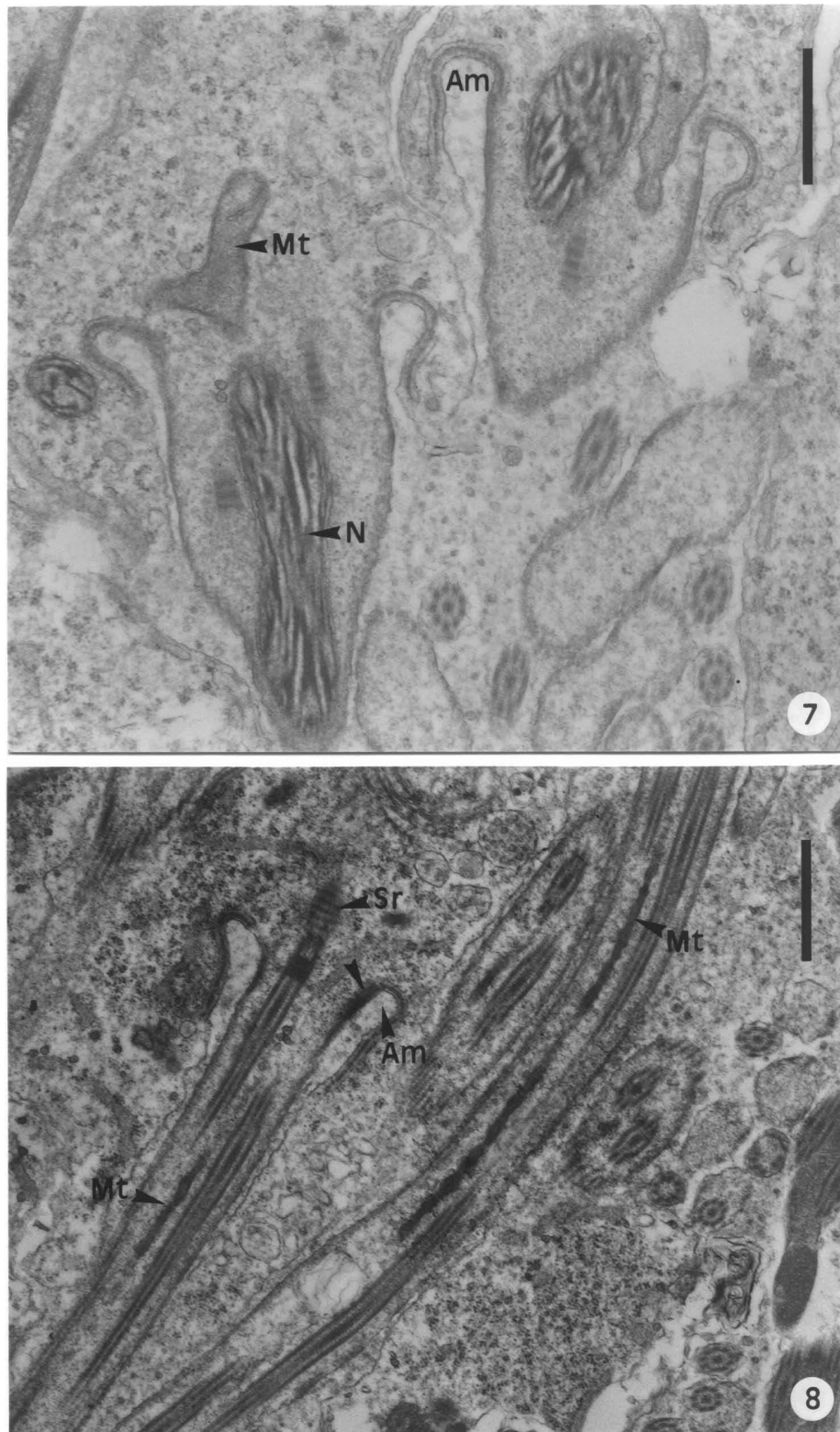
Spermiogenesis in *N. neyrai* (Figs. 1–8, 27A–F) begins with the formation of a differentiation zone in the spermatid. This is a conical area, characterised by the presence of arched membranes and bordered by a layer of cortical microtubules (Fig. 1). It also contains two centrioles separated by an intercentriolar body, which show an associated striated rootlet (Figs. 1–4) and develop a flagellum that grows externally, and an emerging median cytoplasmic process (Figs. 1, 3, 4). The cortical microtubules initiate their migration along this median process (Fig. 2). At the beginning of their development, the two flagella grow orthogonal to the median cytoplasmic process (Fig. 1). Thereafter, they

undergo a rotation, become parallel to the cytoplasmic extension and fuse with it (Figs. 2–4). The rotation and proximodistal fusion of the free flagella are asynchronous: one flagellum fuses before the other (Fig. 5). Electron-dense areas between the dorsal and ventral cortical microtubules are observed in the median cytoplasmic process before the proximodistal fusion of the free flagella (Fig. 5). They are the origin of the future attachment zones (Fig. 6). The fusion of the flagella with the median cytoplasmic process determines the appearance of two sets of cortical microtubules (Figs. 5, 6). The nucleus migrates toward the median cytoplasmic process before the proximodistal fusion (Figs. 3, 4). However, a longitudinal section of the differentiation zone revealed that nucleus migration takes place after flagellar rotation (Figs. 3–5). The mitochondria migrate along the spermatid body after the complete fusion of the two flagella and the median cytoplasmic process (Figs. 6–8). Finally, the ring of arched membranes is strangled and the young spermatozoon detaches from the residual cytoplasm.

The mature spermatozoon of *N. neyrai* (Figs. 9–26, 28I–VI) is characterised by the presence of two axonemes, at least two mitochondria, nucleus and two sets of parallel cortical microtubules. Other ultrastructural features and their location are described below. The observation of a large number of longitudinal and transverse sections allowed us to establish six regions with distinct ultrastructural features:

Figs. 1–6. Spermiogenesis of *Notocotylus neyrai*. Fig. 1. Zone of differentiation showing the growth of the first flagellum. Ib, intercentriolar body; N, nucleus. Bar = 0.5 µm. Fig. 2. Two cross sections of zones of differentiation. One of them shows the two centrioles, the intercentriolar body (Ib) and the nucleus (N). The other shows the median cytoplasmic process (Mcp) and the two free flagella before the proximodistal fusion. Bar = 0.5 µm. Fig. 3. Longitudinal section of a zone of differentiation showing the nuclear migration. Am, arched membranes; N, nucleus; Sr, striated roots. Bar = 1 µm. Fig. 4. Longitudinal section of a zone of differentiation after the flagellar rotation of the two flagella. Am, arched membranes; Ib, intercentriolar body; Mcp, median cytoplasmic process; N, nucleus; Sr, striated roots. Bar = 1 µm. Fig. 5. Cross section of a zone of differentiation after the proximodistal fusion of the first flagellum. Note the appearance of dense material in the zone of fusion (arrow heads). C1, centriole of the first flagellum; F2, second flagellum; Mcp, median cytoplasmic process. Bar = 0.5 µm. Fig. 6. Cross section of spermatid showing the attachment zones (arrow heads) and the mitochondrion (Mt). Bar = 0.5 µm.





Figs. 7, 8. Spermiogenesis of *Notocotylus neyrai*. Fig. 7. Longitudinal sections of two zones of differentiation. The mitochondrion (Mt) starts its migration. Am, arched membranes; N, nucleus. Bar = 0.5 μm . Fig. 8. Longitudinal sections of spermatids showing the mitochondrial migration in the final stages of spermiogenesis. Striated roots (Sr) remain at the base of the spermatid and dense material (arrow head) appears before the stragulation of the ring of arched membranes (Am). Mt, mitochondrion. Bar = 1 μm .

Region I (Figs. 9–17, 28I). This region encompasses the anterior end of the spermatozoon. It is characterised by the presence of two axonemes of the 9+“1” pattern of Trepaxonemata, one mitochondrion and external ornamentation of the plasma membrane. At the beginning of the process, it is sharp (Fig. 9), shows a single axoneme (Figs. 9, 10, 13) and lacks cortical microtubules (Figs. 9, 10). A second axoneme, cortical microtubules and external ornamentation soon appear (Figs. 11–17). Cortical microtubules are numerous (30–40), parallel to the long axis of the spermatozoon, and they appear as a continuous submembranous layer. The plasma membrane is devoid of submembranous cortical microtubules only in a small area where two attach points are observed (Figs. 12, 16). Most transverse sections of region I reveal a mitochondrion (Figs. 16, 17) and some of them show spine-like bodies (Figs. 12, 15, 17). These structures consist of triangular prominences containing a submembranous and electron-dense spherical vesicle.

Region II (Figs. 18–20, 28II). This region is characterised by the disappearance of membranous ornamentation, the absence of mitochondria and a clear bilateral symmetry (Figs. 18, 20). Cross sections of the spermatozoon in this region show only two axonemes and two bundles of cortical microtubules (Figs. 18, 20). Granules of glycogen appear progressively between the axonemes (Figs. 18–20).

Region III (Figs. 20, 21, 24, 28III). In addition to the structures observed in Region II, this region presents a mitochondrion between the two axonemes (Figs. 20, 21, 24), which may differ from that observed in Region I. Therefore, we show a schematic drawing of sperm containing two mitochondria (Fig. 28I–VI).

Region IV (Figs. 20, 26, 28IV). This region is characterised by the simultaneous presence of two axonemes, mitochondrion, nucleus and abundant glycogen granules (Figs. 20, 26). In a transversal section, the cortical microtubules form two bundles (Figs. 20, 26).

Region V (Figs. 20, 22, 23, 28V). This region presents a single axoneme, mitochondrion, nucleus and numerous glycogen granules (Figs. 20, 22). The cross sections of the nucleus are larger than in region IV.

Region VI (Figs. 22, 24–26, 28VI). This region includes the posterior part of the mature spermatozoon. The cross sections reveal a single axoneme, nucleus and glycogen granules (Figs. 22, 24, 25). The disruption of the axoneme occasionally begins at the nucleus (Fig. 25). Cortical microtubules, glycogen granules and nucleus disappear before the axonemal doublets and singlets. Therefore, the posterior extremity of the

spermatozoon exhibits only several axonemal microtubules (Figs. 22, 26).

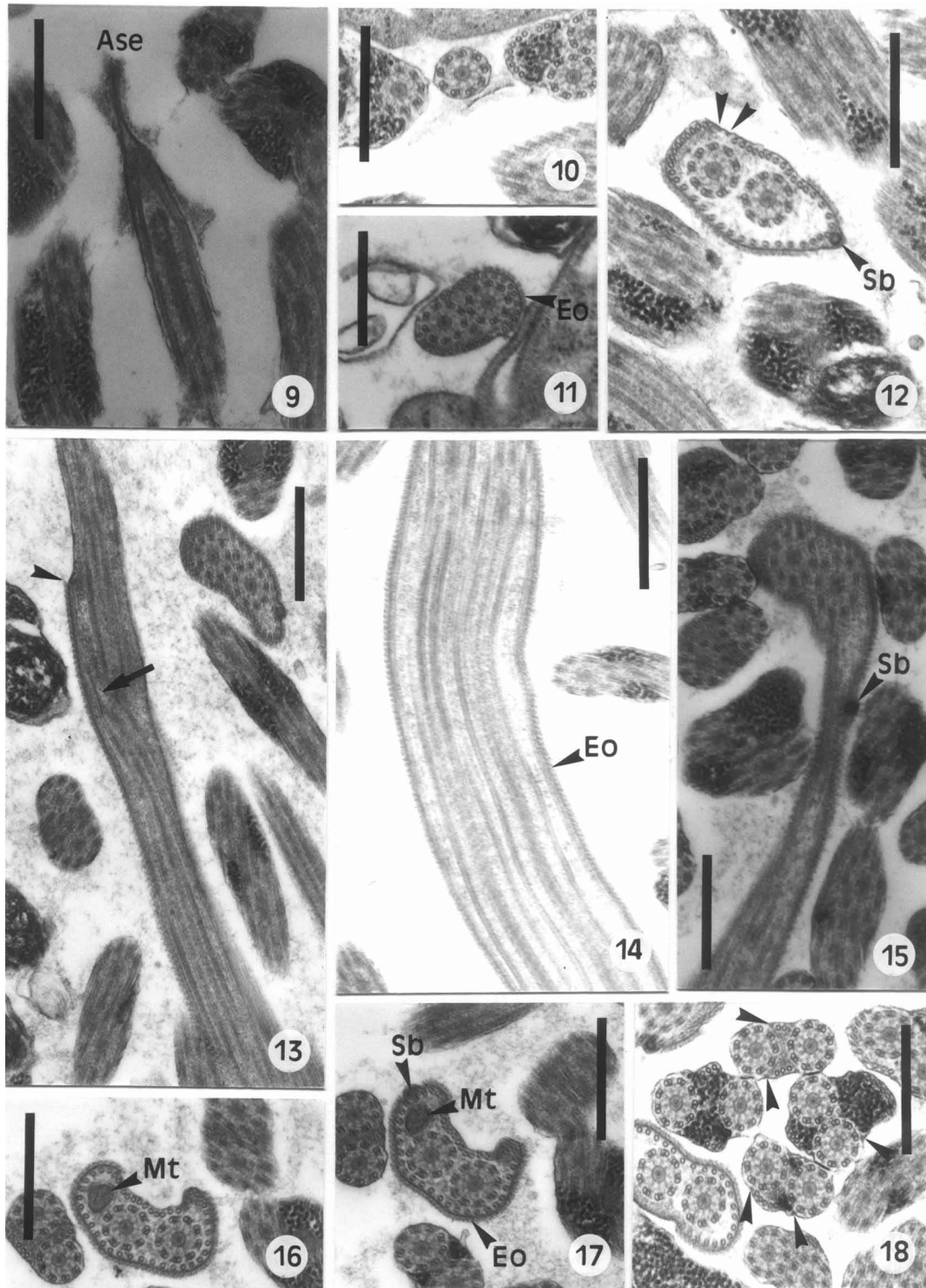
Discussion

Spermiogenesis in *N. neyrai* follows the general pattern found in all the digenetic trematodes studied to date (Burton, 1972; Erwin and Halton, 1983; Gracenea et al., 1997; Miquel et al., 2000; Baptista-Farias et al., 2001, etc.). In *Opecoeloides furcatus*, Miquel et al. (2000) described an asynchronous process of proximo-distal fusion and the first fused flagellum migrates to distal areas of the spermatid after its fusion with the median cytoplasmic process but before the complete proximodistal fusion of the second flagellum. This may account for the origin of the anterior extremity of the future spermatozoa with a single axoneme. In the anterior extremity of the *N. neyrai* spermatozoon, we also observed a single axoneme both in cross and longitudinal sections as a result of the relative displacement of one of the axonemes with respect to the other.

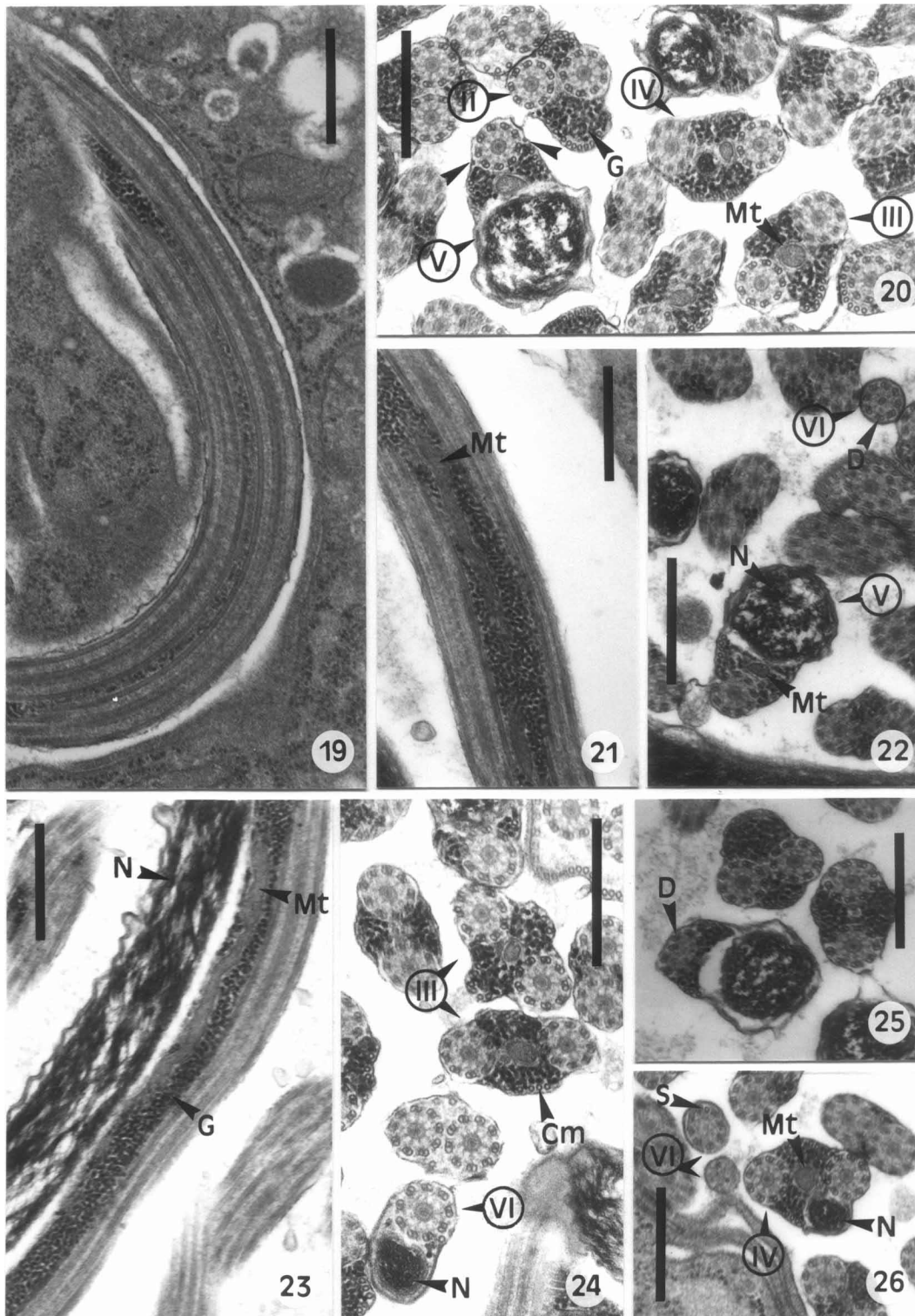
The present study and most published reports (Burton, 1972; Erwin and Halton, 1983; Hendow and James, 1988; Iomini and Justine, 1997; Miquel et al., 2000; Baptista-Farias et al., 2001) describe the nucleus migration toward the median cytoplasmic process before the migration of the mitochondrion, in contrast with the results obtained by Gracenea et al. (1997) in *Postorchigenes gymnesicus*. Only these authors have described mitochondrion migration before the nucleus migration in Digenea.

The basal bodies, striated rootlets and intercentriolar body have never been described in the mature spermatozoon. Both in trematodes and cestodes, it is thus assumed that these structures remain in the residual cytoplasm and degenerate (Burton, 1972; Mokhtar-Maamouri and Swiderski, 1975; Rees, 1979; Erwin and Halton, 1983; Brunanska et al., 2001). In late *N. neyrai* spermiogenesis, a crescent proximity of striated roots to the arched membranes is noted, and this fact support this hypothesis.

The presence of more than one mitochondrion has been described in most digeneans, namely *Haematoloechus medioplexus* (Burton, 1972), *Pharyngostomoides procyonis* (Grant et al., 1976), *Cryptocotyle lingua* (Rees, 1979), *Paragonimus ohirai* (Orido, 1988), *P. gymnesicus* (Gracenea et al., 1997) and *N. neyrai* (present study). However, the presence of a single mitochondrion has been reported in other species of digeneans such as *Bucephaloides gracilescens* (Erwin and Halton, 1983), *Echinostoma caproni* (Iomini and Justine, 1997) and *O. furcatus*



Figs. 9–18. Spermatozoon of *Notocotylus neyrai*. Fig. 9. Longitudinal section of the anterior spermatozoon extremity (Ase). Bar = 0.5 µm. Fig. 10. Cross section of the anterior area of Region I characterised by the presence of a single axoneme. Bar = 0.5 µm. Fig. 11. Cross section of Region I showing the external membranar ornamentation (Eo) covering only one of the two axonemes. Bar = 0.5 µm. Fig. 12. Cross section of Region I showing the spine-like body (Sb) and the presence of only two attachment zones (arrow heads). Bar = 0.5 µm. Fig. 13. Longitudinal section of Region I at the level of the beginning of the extramembranar ornamentation (arrow head) and showing the appearance of the second axoneme (arrow). Bar = 0.5 µm. Fig. 14. Longitudinal section of Region I showing the external ornamentation of the plasma membrane (Eo). Bar = 0.5 µm. Fig. 15. Tangent section of Region I showing the spine-like body (Sb). Bar = 0.5 µm. Fig. 16. Cross section of Region I showing the mitochondrion (Mt). Bar = 0.5 µm. Fig. 17. Another cross section of Region I in area with simultaneous presence of mitochondrion (Mt) and spine-like body (Sb). Eo, extramembranar ornamentation. Bar = 0.5 µm. Fig. 18. Several cross sections of Region II. Note the presence of four attachment zones (arrow heads) in each section. Bar = 0.5 µm.



Figs. 19–26. Spermatozoan of *Notocotylus neyrai*. All bars = 0.5 μm. Fig. 19. Longitudinal section of Region II. Fig. 20. Cross sections of Regions II, III, IV and V. In Region V note the two attachment zones (arrow heads) G, granules of glycogen; Mt, mitochondrion. Fig. 21. Longitudinal section of Region III. Mt, mitochondrion. Fig. 22. Cross sections of Regions V and VI. D, doublet; Mt, mitochondrion; N, nucleus. Fig. 23. Longitudinal section of Region V. G, granules of glycogen; Mt, mitochondrion; N, nucleus. Fig. 24. Cross sections of Regions III and VI. Cm, cortical microtubules; N, nucleus. Fig. 25. Cross section of Region VI showing the axonemal disorganization at the level of nucleus. D, doublets. Fig. 26. Cross sections of Regions IV and VI. Mt, mitochondrion; N, nucleus; S, singlets.

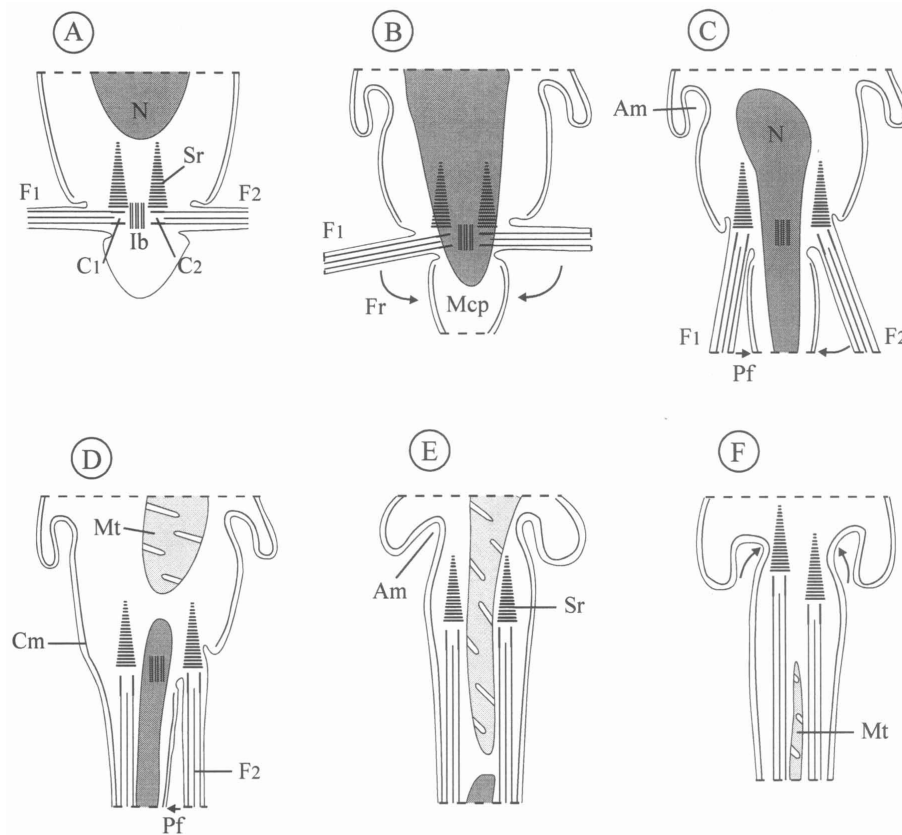


Fig. 27. (A–F). Diagram showing the main stages of spermiogenesis of *Notocotylus neyrai*.

(Miquel et al., 2000). Burton (1972) has suggested that numerous mitochondria of the spermatid accompany the nucleus into the median cytoplasmic process where they apparently fuse together to form the long mitochondrion of the mature sperm. Regarding parasitic Platyhelminthes, a single mitochondrion has also been described in the spermatozoon of monogeneans but, to our knowledge, not in cestodes (Justine, 1995, 1998, 2001).

Most studies reveal the presence of an external ornamentation of the plasma membrane in the spermatozoon of digeneans (Jamieson and Daddow, 1982; Justine and Mattei, 1982a, 1982b; Gracenea et al., 1997; Iomini and Justine, 1997; Miquel et al., 2000), but the localisation of this ornamentation along the spermatozoon can vary in these species. Structures similar to external ornamentation of the plasma membrane have also been described in the anterior part of the spermatozoon of a few monogeneans: *Microtyle* sp. (Microcotylidae) and *Pseudomazocraes cf. monsi-vaisae* (Chauhaneidae) (Justine and Mattei, 1985). According to Justine (1991, 1995), this external ornamentation characterises the region of the spermatozoon originating from the zone of differentiation, which corresponds with the anterior areas in the mature spermatozoon, whereas the rest of the spermatozoon

originates from fusion of the three processes and has no ornamentation.

Until today, spine-like bodies have previously been described only in *Opecoeloides furcatus* (Miquel et al., 2000), and in this species they are located at the level of mitochondrial areas. These structures probably may be formed in late spermiogenesis.

Attachment zones in mature spermatozoa indicate the area of fusion of flagella with the median cytoplasmic process (Burton, 1972). In anterior and posterior areas of mature spermatozoon of *N. neyrai*, only two attachment zones are detected, pointing to the origin of the anterior areas of the first axoneme present in the anterior extremity of the spermatozoon from the zone of differentiation. This flagellum rotates, but proximodistal fusion does not occur in its anterior extremity. In contrast, the second axoneme fuses totally with the median cytoplasmic process during the proximodistal fusion and determines the appearance of two attachment zones throughout its length. These events are confirmed by the presence of a continuous layer of submembranous cortical microtubules not organised in ventral and dorsal bundles. In *Echinostoma caproni*, similar sections (containing two axonemes, extramembranar ornamentation and mitochondrion) but with four attachment points, as a result of fusion of the two free

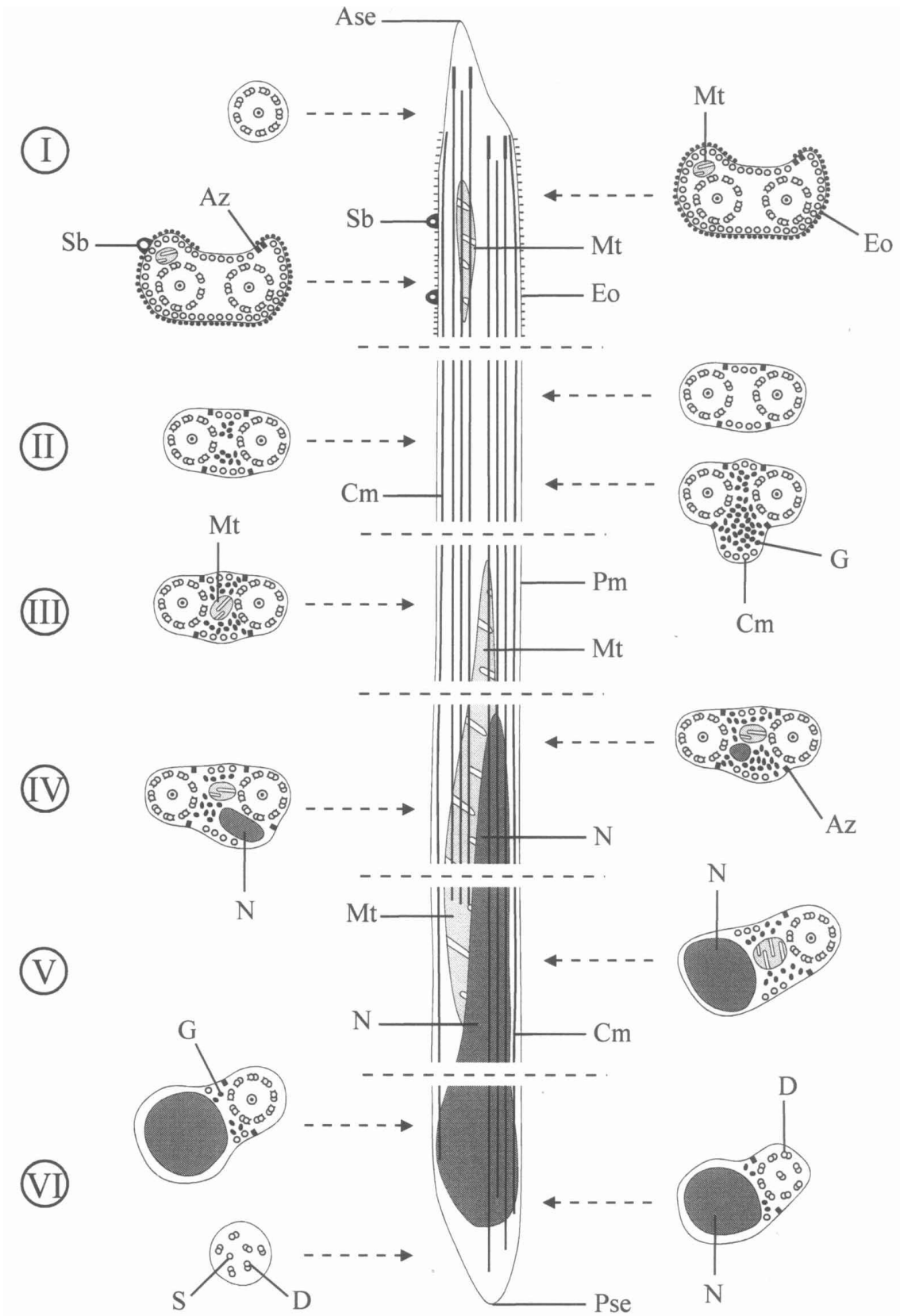


Fig. 28. (I–VI). Diagram showing the ultrastructural organization of mature sperm of *Notocotylus neyrai*. To make the diagram clearer, the granules of glycogen are not included in the longitudinal sections of the drawing.

flagella with the median cytoplasmic process during spermiogenesis, have been described (Iomini and Justine, 1997). On the other hand, the posterior spermatozoon areas of *N. neyrai* with a single axoneme also show only two attachment zones as a result of the fusion of the second flagellum with the median cytoplasmic process and reveal the different growth in length of the free flagella before their proximodistal fusion.

These posterior areas of the mature sperm are characterised by the great development of the nucleus, as in other digeneans like *Mesocoelium monas* (Iomini et al., 1997), *E. caproni* (Iomini and Justine, 1997), *P. gymnesicus* (Gracenea et al., 1997) and *O. furcatus* (Miquel et al., 2000). However, there are differences in the posterior extremity. That of the spermatozoon of *N. neyrai* differs from that of *O. furcatus* in some respects (Miquel et al., 2000). In the latter, the second axoneme disorganises and disappears before the posterior end of the nucleus. Thus, the posterior extremity of the *O. furcatus* sperm contains the nucleus and several cortical microtubules that may reach the posterior tip of the spermatozoon. A similar situation occurs in *E. caproni* (Iomini and Justine, 1997), whereas in *P. gymnesicus* (Gracenea et al., 1997), the posterior axoneme disappears after the nucleus and cortical microtubules do not reach this area, but end their parallel course along the sperm at the biflagellate area containing mitochondrion and nucleus. In *N. neyrai*, the peripheral microtubules stop at posterior-most areas of sperm, containing nucleus and axoneme.

In our opinion, further extensive and complete ultrastructural studies on the spermatology of digeneans are needed to evaluate the variability in the pattern of spermiogenesis, e.g., the synchronicity of flagellar rotation and proximodistal fusion, the movement of striated roots toward the base of the differentiation zone, among others. On the other hand, related to mature sperm, the spine-like bodies and the extramembranar ornamentation were probably the most useful characters for phylogenetic purposes.

Acknowledgements

We thank the Serveis Científicotècnics of the University of Barcelona for their help in the preparation of material. The study was partially supported by the Comissionat per a Universitats i Recerca de la Generalitat de Catalunya (2001-SGR-00088) and project BOS2000-0570-CO2-01 of the Ministerio de Ciencia y Tecnología of Spain. Papa Ibnou Ndiaye is the recipient of a grant from the Agencia Española de

Cooperación Internacional –AECI of the Ministerio de Asuntos Exteriores.

References

- Bâ, C.T. and Marchand, B., Similitude ultrastructurale des spermatozoïdes de quelques Cyclophyllidea. *Parasite*, 1 (1994) 51–55.
- Bâ, C.T. and Marchand, B., Spermiogenesis, spermatozoa and phyletic affinities in the Cestoda. *Mém. Mus. nat. Hist. nat.*, 166 (1995) 87–95.
- Baptista-Farias, M.F.D., Kohn, A. and Cohen, S.C., Ultrastructure of Spermatogenesis and sperm development in *Saccocoeloides godoyi* Kohn & Froes, 1986 (Digenea, Haploporidae). *Mem. Inst. Osw. Cruz*, 96 (2001) 61–70.
- Brooks, D.R., A summary of the data base pertaining to the phylogeny of the major groups of parasitic platyhelminths, with a revised classification. *Can. J. Zool.*, 67 (1989) 714–720.
- Brunanská, M., Nebesárová, J. and Scholz, T., Spermiogenesis in the pseudophyllid cestode *Eubothrium crassum* (Bloch, 1779). *Parasitol. Res.*, 87 (2001) 579–588.
- Burton, P.R., Fine structure of the reproductive system of a frog lung fluke. III. The spermatozoon and its differentiation. *J. Parasitol.*, 58 (1972) 68–83.
- Chen, K., Huang, H., Lu, W. and Dai, W., Ultrastructure of sperm and spermatogenesis of *Hypoderaeum conoideum*, Bloch 1872 (Trematoda: Digenea: Echinostomatidae). *J. Shanghai Agric. Coll.*, 14 (1996) 186–195.
- Ehlers, U., Phylogenetic relationships within the Platyhelminthes. In: *The Origins and Relationships of Lower Invertebrates*, S. Conway Morris, J.D. George, R. Gibson and H.M. Platt (eds.), Oxford University Press, Oxford, 1985, pp. 143–158.
- Erwin, B.E. and Halton, D.W., Fine structural observations on spermatogenesis in a progenetic Trematode, *Bucephaloides gracilescens*. *Int. J. Parasitol.*, 13 (1983) 413–426.
- Euzet, L., Swiderski, Z. and Mokhtar-Maamouri, F., Ultrastructure comparée du spermatozoïde des cestodes. Relations avec la phylogénèse. *Ann. Parasitol.*, 56 (1981) 247–259.
- Gracenea, M., Ferrer, J.R., González-Moreno, O. and Trullols, M., Ultrastructural study of spermatogenesis and the spermatozoon in *Postorchigenes gymnesicus* (Trematoda, Lecithodendriidae). *J. Morphol.*, 234 (1997) 223–232.
- Grant, W.C., Harkema, R. and Muse, K.E., Ultrastructure of *Pharyngostomoides procyonis* Harkema 1942 (Diplostomatidae). I. Observations on the male reproductive system. *J. Parasitol.*, 62 (1976) 39–49.
- Hendow, H.T. and James, B.L., Ultrastructure of spermatozoon and spermatogenesis in *Maritrema linguilla* (Digenea, Microphallidae). *Int. J. Parasitol.*, 18 (1988) 53–63.
- Hoberg, E.P., Mariaux, J., Justine, J-L, Brooks, D.R. and Weekes, P.J., Phylogeny of the orders of the Eucestoda (Cercomeromorphae) based on comparative morphology:

- Historical perspectives and a new working hypothesis. *J. Parasitol.*, 83 (1997) 1128–1147.
- Iomini, C., Le cytosquelette des spermatozoïdes des Plathelminthes parasites: la tubuline et ses modifications post-traductionnelles. Thèse de doctorat, Mus. natn. Hist. nat., Paris, 1998.
- Iomini, C. and Justine, J.-L., Spermiogenesis and spermatozoon of *Echinostoma caproni* (Platyhelminthes, Digenea): transmission and scanning electron microscopy, and tubulin immunocytochemistry. *Tissue Cell*, 29 (1997) 107–118.
- Iomini, C., Mollaret, I., Albaret, J.-L. and Justine, J.-L., Spermatozoon and spermiogenesis in *Mesocoelium momas* (Platyhelminthes: Digenea): ultrastructure and epifluorescence microscopy of labelling of tubulin and nucleus. *Folia Parasitol.*, 44 (1997) 26–32.
- Jamieson, B.G.M. and Daddow, L.M., The ultrastructure of the spermatozoon of *Neochasmus* sp. (Cryptogonimidae, Digenea, Trematoda) and its phylogenetic significance. *Int. J. Parasitol.*, 12 (1982) 547–559.
- Justine, J.-L., Phylogeny of parasitic Platyhelminthes: A critical study of synapomorphies proposed from the ultrastructure of spermiogenesis and spermatozoa. *Can. J. Zool.*, 69 (1991) 1421–1440.
- Justine, J.-L., Spermatozoal ultrastructure and phylogeny in the parasitic Platyhelminthes. *Mém. Mus. natn. Hist. nat.*, 166 (1995) 55–86.
- Justine, J.-L., La classification générale des Plathelminthes parasites: changements récents et utilisation des caractères ultrastructuraux, en particulier des spermatozoïdes. *Bull. Soc. zool. Fr.*, 122 (1997) 269–277.
- Justine, J.-L., Spermatozoa as phylogenetic characters for the Eucestoda. *J. Parasitol.*, 84 (1998) 385–408.
- Justine, J.-L., Spermatozoa as phylogenetic characters for the Platyhelminthes. In: *Interrelationships of the Platyhelminthes*, D.T.J. Littlewood and R.A. Bray (eds.), Taylor & Francis, New York, 2001, pp. 231–238.
- Justine, J.-L. and Mattei, X., Étude ultrastructurale de la spermiogénèse et du spermatozoïde d'un Plathelminthe: *Gonapodasmius* (Trematoda: Didymozoidae). *J. Ultrastruct. Res.*, 79 (1982a) 350–365.
- Justine, J.-L. and Mattei, X., Réinvestigation de l'ultrastructure du spermatozoïde d'*Haematoloechus* (Trematoda: Haematoloechidae). *J. Ultrastruct. Res.*, 81 (1982b) 322–332.
- Justine, J.-L. and Mattei, X., Particularités ultrastructurales des spermatozoïdes de quelques Monogènes Polyopisthocotylea. *Ann. Sc. Nat., Zool., Paris*, 7 (1985) 143–152.
- Justine, J.-L., Lambert, A. and Mattei, X., Spermatozoon ultrastructure and phylogenetic relationships in the monogeneans (Platyhelminthes). *Int. J. Parasitol.*, 15 (1985) 601–608.
- Miquel, J., Nourrisson, C. and Marchand, B., Ultrastructure of spermiogenesis and the spermatozoon of *Opecoeloides furcatus* (Trematoda, Digenea, Opecoelidae), a parasite of *Mullus barbatus* (Pisces, Teleostei). *Parasitol. Res.*, 86 (2000) 301–310.
- Mokhtar Maamouri, F. and Swiderski, Z., Etude en microscopie électronique de la spermatogénèse de deux cestodes *Acanthobothrium filicollis benedenii* Loennberg, 1889 et *Onchobothrium uncinatum* (Rud., 1819) (Tetraphyllidea, Onchobothriidae). *Z. Parasitenkd.*, 47 (1975) 269–281.
- Orido, Y., Ultrastructure of spermatozoa of the lung fluke, *Paragonimus ohirai* (Trematoda: Troglotrematidae), in the seminal receptacle. *J. Morphol.*, 196 (1988) 333–343.
- Rees, F.G., The ultrastructure of the spermatozoon and spermiogenesis in *Cryptocotyle lingua* (Digenea: Heterophyidae). *Int. J. Parasitol.*, 9 (1979) 405–419.
- Reynolds, E.S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, 17 (1963) 208–212.
- Rohde, K., Phylogeny of Platyhelminthes, with special reference to parasitic groups. *Int. J. Parasitol.*, 20 (1990) 979–1007.
- Simón-Vicente, F., Mas-Coma, S., López-Román, R., Tenora, F. and Gállego, J., Review of *Notocotylus* species (Trematoda: Notocotylidae) parasitizing rodents in Europe. *Folia Parasitol.*, 32 (1985) 21–33.
- Tang, J., Wang, W. and Wang, G., Studies on ultrastructure of spermatogenesis and sperm in *Pseudorhipidocotyle elpichthys*. *Acta Hydrobiol. Sin.*, 22 (1998) 168–173.