Zoomorphology (1992) 112:57–66

Zoomorphology

Spermatogenesis in the earthworm *Microchaetus pentheri* (Oligochaeta, Microchaetidae)

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Received July 9, 1991

Summary. Spermatogenesis in *Microchaetus pentheri* (Microchaetidae) follows the familiar pattern known for other oligochaetes. Spermatogenic stages develop around an anucleate cytophore from which they separate as mature spermatozoa. During spermiogenesis the nucleus elongates and becomes surmounted by a complex, elongate acrosome; the flagellar axoneme develops from the distal centriole. The centriole is positioned posterior to the mid-piece, which consists of six mitochondria radially adpressed to form a cylinder about 2 μm long. *Microchaetus* shows many plesiomorphic features in the structure of its acrosome, which are also seen in two other taxa of the Diplotesticulata, *Haplotaxis* (Haplotaxidae) and *Sparganophilus* (Sparganophilidae, Aquamegadrili), each of which has the greatest number of plesiomorphies in spermatozoal characters in its group. The Aquamegadrili constitute the sister-group of the Terrimegadrili which contain the earthworm families including the Microchaetidae. The numerous symplesiomorphies in spermatozoal characters do not, however, establish monophyly of microchaetids with haplotaxids and sparganophilids. An apomorphy in the acrosome of *Microchaetus* is its greater length (3.8 μm vs less than 1 μm in *Haplotaxis* and 1.5 μm in *Sparganophilus*), in this respect resembling other investigated terrimegadriles, the lumbricids, hormogastrids and megascolecids. The axial rod of the acrosome of *Microchaetus* appears apomorphic relative to that of *Haplotaxis*, *Sparganophilus*, lumbricids and megascolecids in lacking an anterior expansion, the capitulum. It ends posteriorly in a cylindrical body, somewhat resembling the node diagnostic of the axial rod of megascolecid earthworms.

A. Introduction

Spermiogenesis and spermatozoal ultrastructure have been studied in three of the four subclasses of the Oligochaeta: the Tubificata, Lumbriculata and Diplotesticulata (see Ferraguti 1983, 1984; Ferraguti and Jamieson 1984; Jamieson 1981, for literature reviews; Jamieson 1988, for the current classification of the Oligochaeta). The studies on spermiogenesis have made a significant contribution to the appreciation of some cytological events during cell differentiation (Ferraguti 1984). Studies on sperm structure have been valuable in elucidating phylogenetic relationships between oligochaete taxa (Jamieson 1987). To date spermatozoa from 11 of the 26 oligochaete families have been described (see Jamieson et al. 1987) for literature). However, if relationships between all the families of oligochaete are to be resolved, further descriptive studies are required on the outstanding taxa.

The phylogenetic relationships of the exclusively South African family Microchaetidae have been the subject of controversy (Jamieson 1971; Pickford 1975; Simms 1978). As this question is unresolved, we have studied the spermatogenesis and sperm ultrastructure in one species of microchaetid, *Microchaetus pentheri* (for geographical distribution of see Pickford 1975; Simms 1978), and draw comparisons with other oligochaete taxa with a view to elucidating its relationships.

The family Microchaetidae was restricted to the South African genera *Microchaetus* and *Tritogenia* by Jamieson (1971), with the removal of the Ethiopian genus *Alma* and its other African and South American relatives. At the same time, the Microchaetidae were reduced to a tribe Microchaetini in the family Glossoscolecidae, subfamily Glossoscolecinae. The other tribe of the Glossoscolecinae was the large South and Central American tribe Glossoscolecini. Although relationships of microchaetins with glossoscolecins continued to be recognized, the tribe Microchaetini was restored to familial rank, as the Microchaetidae, by Jamieson (1978). Unfortunately, for comparative purposes, the sperm of the Glossoscolecinae are incompletely described, the only reference to them being made by Ferraguti (1983) who described the structure of the sperm mid-piece of a single species.

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B. Materials and methods

Specimens of *Microchaetus pentheri* Rosa, 1898, were collected in Grahamstown in the eastern Cape Province of South Africa after heavy rains in June 1990. After dissection, small pieces of the seminal vesicles were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Tissues were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series and embedded via propylene oxide in an Araldite CY212/Taab resin mixture. Thin sections (silver/gold interface) were stained in 5% aqueous uranyl acetate and Reynolds lead citrate and examined on a Jeol 100CXII transmission electron microscope at 80 kv.

C. Results

I. Pre-spermiogenesis

The earliest stages of spermatogenesis observed in the seminal vesicles were spermatocytes. The spermatocytes are attached to a central anucleate cytophore by fuzzy-coated bridges, each bridge consituting the zonula collaris (Fig. 1a). The cytophore is irregular in shape and contains numerous small mitochondria (about 0.2 μm diameter) and structures which resemble smooth endoplasmic reticulum (Fig. 1a). The nucleus of the primary spermatocyte (Fig. 1b) is oval in shape (about 10×6 μm) and in the plane of section exhibits numerous synaptonemal complexes and one nucleolus (about 0.9 μm diameter).

Division of the primary spermatocyte gives the secondary spermatocyte, the nucleus of which is almost spherical and about 6 μm diameter (Fig. 1a). At this stage most of the prominent organelles (Golgi body; mitochondria, which are elongate and about 1.5×0.2 μm; centrioles) occupy the cytoplasm between the nucleus and cytophore (Fig. 1a). Within the seminal vesicle many spermatocytes were undergoing division. Metaphase or early anaphase in these is illustrated in Fig. 1c.

II. Spermiogenesis

The cytological changes that occur during spermiogenesis in *M. pentheri* are similar to those described for other oligochaetes. For brevity, only the salient features will be described in this paper. In addition, the structural changes which occur in the cytophore are identical to those described in depth by Jamieson (1981) for other species. These will therefore not receive further consideration here.

Ferraguti and Lanzavecchia (1971) divided oligochaete spermiogenesis into six stages (A–F). Although these six stages are artificial divisions of a continuous process, for convenience and comparison they will be referred to in this paper.

The nucleus of the early spermatid (Stage A) occupies the entire cell and is isodiametric with a diameter of about 5 μm (Figs. 2a, 3a). The chromatin is distributed as small granules within the nucleus and the nucleolus has disappeared. In some early spermatids a large portion of the nucleus extended through the zonula collaris into the cytophore (Fig. 2b). Within the cytoplasm several of the mitochondria (about 0.3 μm diameter) become sited at the developing posterior end of the cell (Fig. 3a) in close proximity to the centrioles. Here we call the posterior end of the cell as being that part furthest from the zonula collaris, as the later-formed definitive acrosome (conventionally "anterior" in the sperm) will lie near the zonula. The maximum number of mitochondria revealed in any profile of an early spermatid was 12. In addition a Golgi body (7–10 cisternae) lies posteriorly in the cell (Figs. 2, 3a). In very early spermatids a small proacrosomal vesicle (about 1 μm diameter) was observed lying in close proximity to the Golgi stack. This vesicle gradually elongates and becomes concave (Fig. 3). In some profiles the acrosomal vesicle appeared to be formed within an extension of the Golgi body (Fig. 3d). Beneath the acrosome the rudiments of the acrosomal tube are beginning to develop (Fig. 3c).

As the spermatid matures (Stage B) the oval nucleus begins to elongate (Fig. 4a) and portions of the anterior nuclear envelope become more electron dense due to the aggregation of chromatin on the inner nuclear envelope (Fig. 4b). This process is accompanied by the development, external to the nuclear envelope, of microtubules (Fig. 6; mean number in transverse section $= 171 \pm$ SD 15; values of all microtubule numbers obtained from counts from 20 transverse sections), which are associated only with the electron-dense areas of the envelope where the perinuclear cisterna has collapsed (Fig. 4b). The general chromatin is still granular in appearance. At this stage, the acrosome lies posterolaterally to the nucleus (Fig. 4c, d) near the point of emergence of the axoneme (Fig. 4d). The acrosome consists of the acrosomal vesicle (primary vesicle in a superseded terminology) which overlies a subvesicular space filled with amorphous material. As the base of the acrosomal vesicle becomes concave, a dense ring (*sensu* Jamieson 1981) appears within its rim, on the inner side of the acrosomal membrane; smaller densities are also visible around the inner surface of the membrane (Fig. 4c). The acrosomal tube has increased in size and projects into the cytoplasm towards the Golgi body (Fig. 4c). Within the anterior section of the acrosomal tube a small, stout rod-like structure, the precursor of the acrosomal rod, is observed (Fig. 4c).

The mid-piece of the Stage B spermatid has also begun to develop. The mitochondria destined to form the mid-piece are aggregated to form the nebenkern at the base of the nucleus and begin to elongate (Fig. 4d). Superfluous mitochondria are most probably eliminated by passage through the zonula collaris into the cytophore (Martinucci et al. 1977) as shown in Figs. 3a, 4a. The proximal centriole which was visible in early spermatids (Fig. 2a) has disappeared and the distal, from which the axoneme develops, is positioned posterior to the elongating mitochondria (Fig. 4d).

As maturation proceeds to Stage C, the nucleus undergoes pronounced elongation and further peripheral chromatin condensation occurs (Fig. 5a, c). The microtubular manchette comprises some 142 (SD $\pm$ 20) microtubules (Fig. 6), completely encircles the nucleus

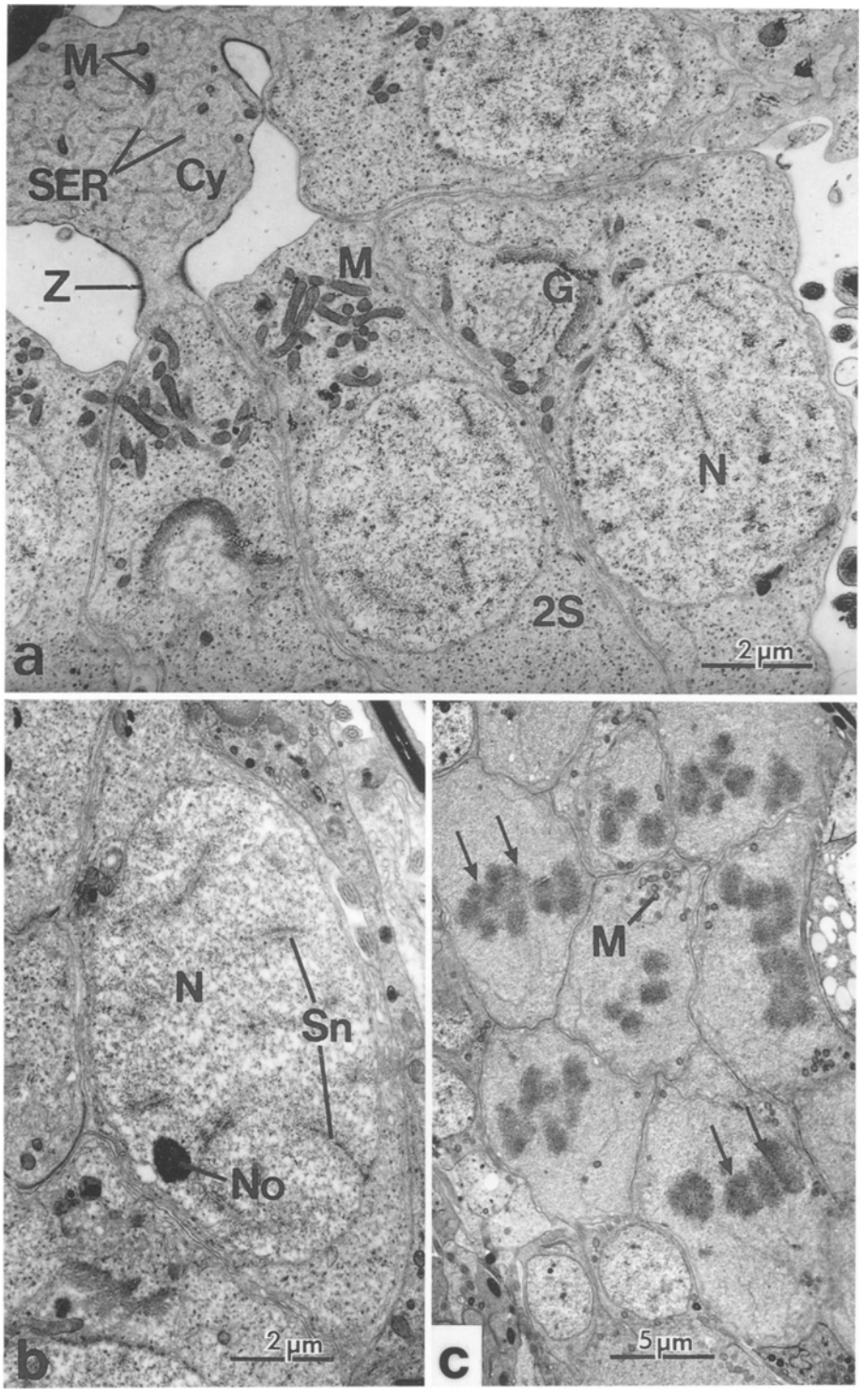

Fig. 1a–c. *Microchaetus pentheri.* **a** Secondary spermatocytes (*2S*) attached to a cytophore (*Cy*) by zonula collaris (*Z*). Note the ordering of the nucleus (*N*), Golgi body (*G*) and mitochondria (*M*) in the spermatocyte. *SER* smooth endoplasmic reticulum. **b** Primary spermatocyte showing nucleus (*N*) with nucleolus (*No*) and synaptonemal complexes (*Sn*). **c** Dividing spermatocytes with chromosomes (*arrowed*) aligned at the metaphase plate. *M* mitochondria

(Fig. 5c) and extends for the length of the developing head and mid-piece to the distal centriole (Fig. 5b–d). The mid-piece now consists of six mitochondria which are radially adpressed and about 1.2 µm long (Fig. 5a, b, d). The Golgi body has been relocated to occupy a lateral position at the base of the nucleus (Fig. 5a).

Spermatid stages D–F are characterised by further chromatin condensation in the nucleus and a reduction in the nuclear diameter to about 0.4 µm (Fig. 7a–c). The number of microtubules of the manchette also changes (Figs. 6, 7a–c). The mid-piece is becoming circular in cross-section (total mid-piece mitochondrial diameter of 0.3–0.4 µm) (Fig. 7f, g) and the mitochondria are now about 2 µm long (Fig. 7d), the length in fully developed sperm (Fig. 7e). The flagellum is well developed. The anterior section of the axoneme is surrounded by glycogen rosettes, two to each doublet of microtubules (Fig. 7f), a constant feature of oligochaetes.

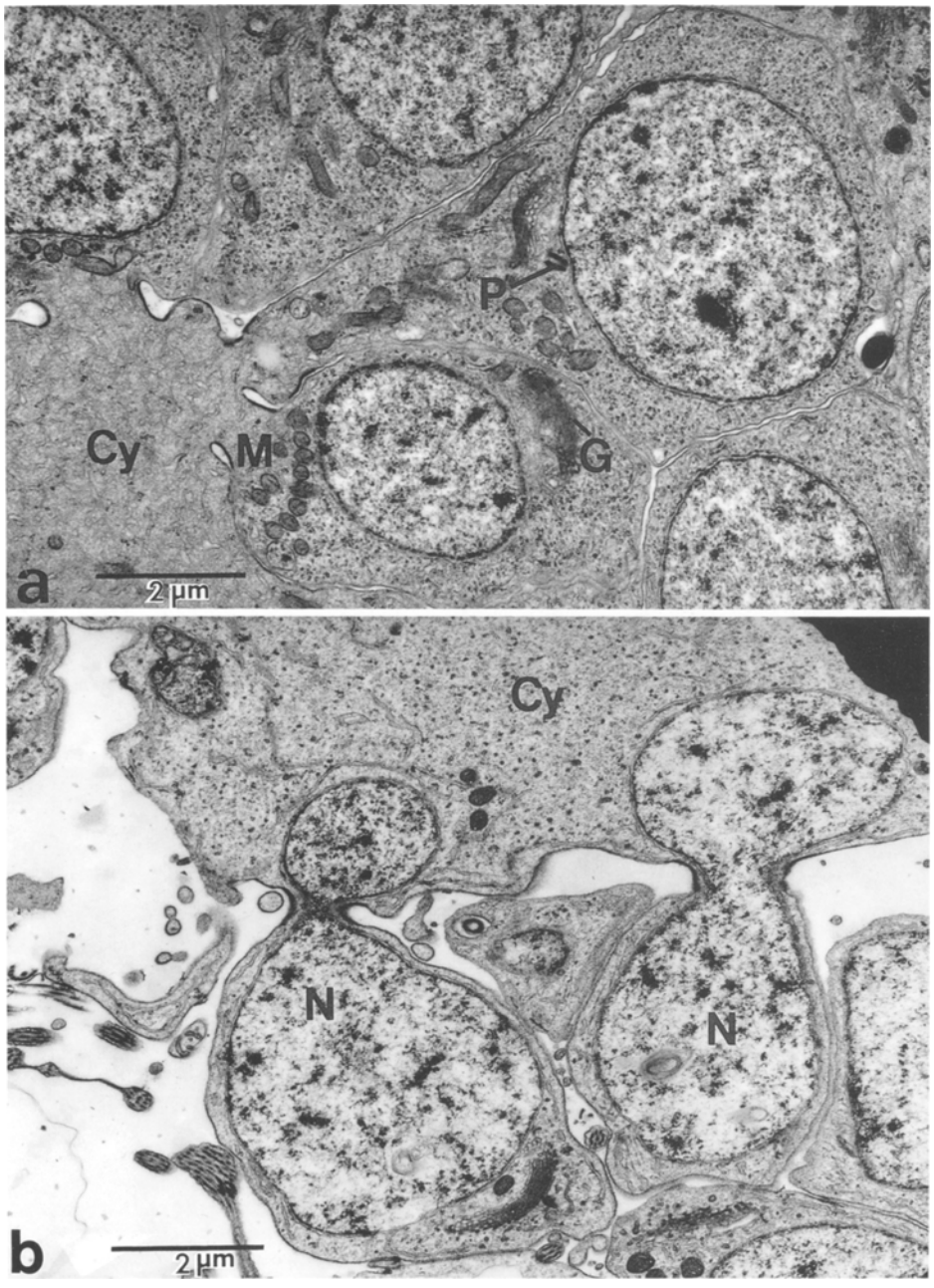

Fig. 2. a Example of early spermatids. In some the Golgi body (*G*) has migrated around the nucleus to the presumptive posterior of the cell. *Cy* cytophore; *M* mitochondrion; *P* proximal centriole. **b** Spermatids in which the nucleus (*N*) extends through the zonula collaris into the cytophore (*Cy*)

In the maturing stage F, as in the remainder of the spermatid (Figs. 6, 7), the excess cytoplasm and all microtubules are greatly reduced in number and finally eliminated (Fig. 7e, g), excepting those of the axoneme and the single, persistent, distal centriole. The acrosome undergoes further elongation. During stages involving elongation of the spermatid, the acrosome migrates from its position at the base of the nucleus to the tip of the spermatid so that by stage F it is located anteriorly on the nucleus in the vicinity of the still persistent zonula collaris (Fig. 8a). The acrosome is still wholly external to, and anterior to, the acrosomal tube. It forms a bulbous structure penetrated by the axial rod. The rod, lying in what is in fact the subacrosomal space, causes a narrow projection of the anterior end of the vesicle and extends backwards into the acrosomal tube (Fig. 8). The acrosome (vesicle and tube) is about about 3.2 µm long. In the last phase of stage F the posterior rim of the acrosomal vesicle, and with it the axial rod, is drawn into the acrosomal tube.

III. Mature acrosome

Transition from spermatid to spermatozoa is marked by severance of the zonula collaris. The head of the mature sperm is about 28 µm long, the majority of which consists of an elongate nucleus (about 24 µm × 0.2 µm, the maximum length observed). The structure of the mature acrosome is illustrated in Fig. 8. The maximum di-

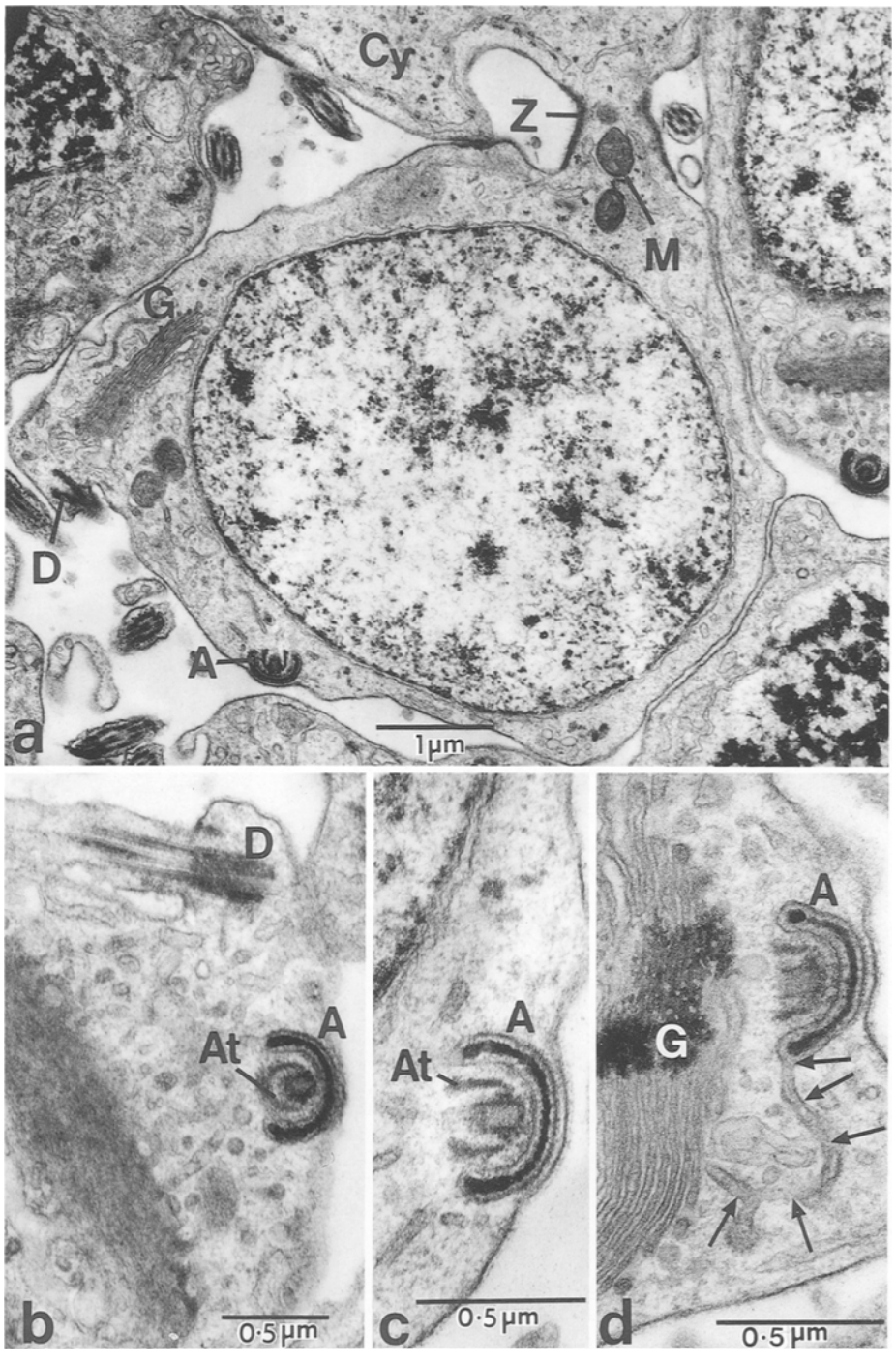

Fig. 3a–d. *M. pentheri.* **a** Stage A spermatid attached to a central cytophore (*Cy*). Note the mitochondrion (*M*) migrating through the zonula collaris (*Z*) and the developing acrosome (*A*). **D** distal centriole; *G* Golgi body. **b–d** Oblique (**b**) and longitudinal (**c**, **d**) sections through developing acrosomes. The acrosome (*A*) is cup-shaped beneath which the acrosomal tube (*At*) has begun to form. In **d** the acrosome is developing within an extension (*arrowed*) of the Golgi body (*G*)

mensions observed for the acrosome are: total length (vesicle + tube), 3.8 μm; length of the basal chamber (from the posterior extremity of the acrosome rod to the posterior end of the acrosome tube), 2.1 μm; greatest diameter at the base, 0.26 μm. The acrosome consists of an acrosomal tube which tapers slightly from its base to the anterior extremity. At its base the acrosomal tube is expanded inwards as a small flange, the limen. Between the limen and the tip of the nucleus is a small convex pad. The lumen of the acrosomal tube is filled with an amorphous material. The part of the acrosomal vesicle emergent from the acrosomal tube forms a prominent, subspherical terminal bulb. The centre of this bulb is occupied by the subvesicular (subacrosomal) space. The wall of this bulb therefore consists of, from the exterior outwards, the plasma membrane, the outer acrosomal membrane, the narrow zone which consists of the lumen of the vesicle, the inner acrosomal membrane and the large subspherical subvesicular space. The axial rod no longer occupies the emergent portion of the acrosomal vesicle but has retreated into the acrosomal tube. The posterior rim of the acrosomal vesicle extends distally as a narrow peripheral tube into the acrosomal tube. This posterior extension of the vesicle encloses the con-

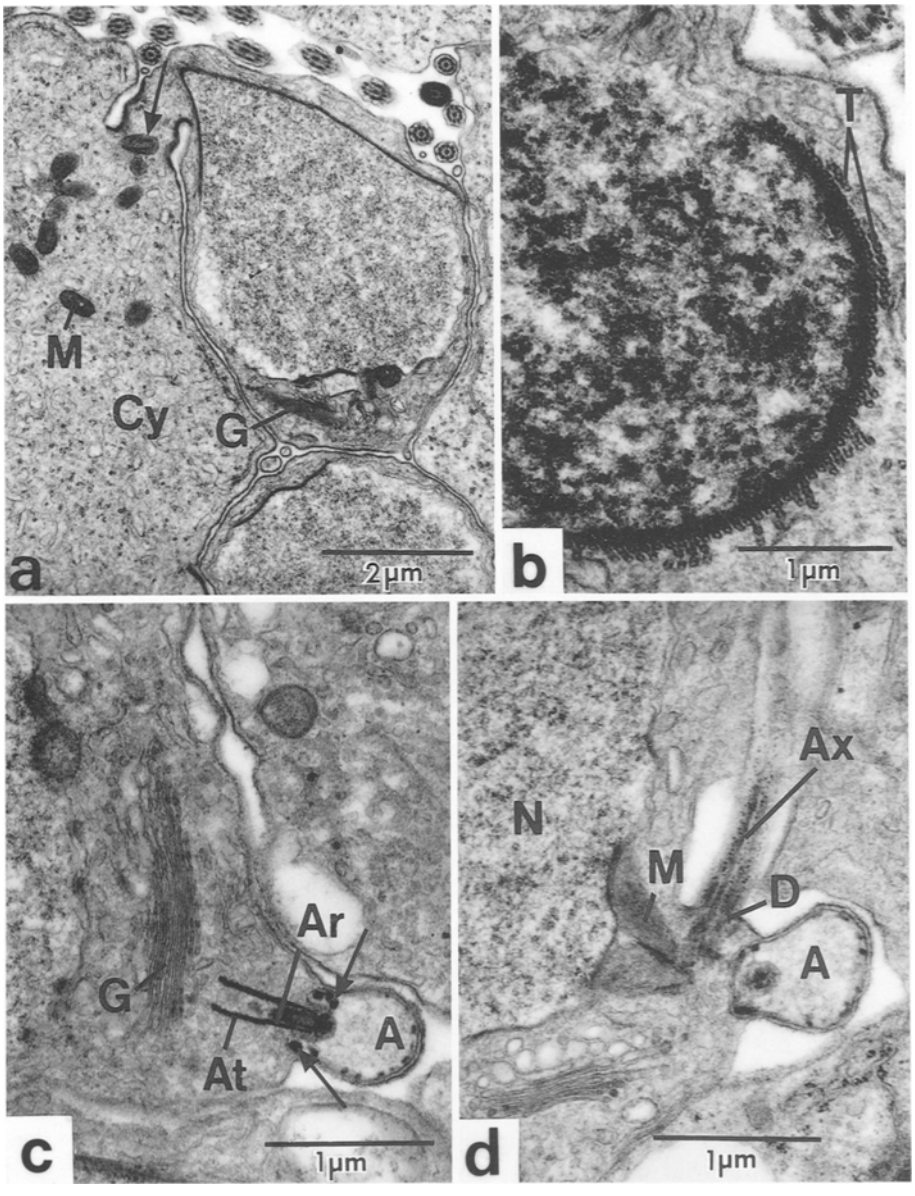

Fig. 4a–d. *M. pentheri.* **a** Longitudinal section through a Stage B spermatid with the nucleus beginning to elongate. The anterior portion of the nuclear envelope is more electron dense due to deposition of chromatin. *Cy* cytophore; *G* Golgi body; *M* mitochondrion; *arrow* indicates mitochondrion undergoing passage through zonula collaris. **b** Transverse section through a portion of the nucleus of a Stage B spermatid showing incomplete ring of microtubules (*T*). **c** Longitudinal section through a developing acrosome. *A* acrosome; *arrows* indicate dense ring structures within basal rim of acrosomal vesicle; *Ar* acrosomal rod; *At* acrosomal tube; *G* Golgi body. **d** Longitudinal section through developing mid-piece showing arrangement of mitochondria (*M*), nucleus (*N*), distal centriole (*D*) from which the axoneme (*Ax*) is developing and acrosome (*A*)

tinuation of the subacrosomal or subvesicular space and the axial rod enclosed in the latter. The rod is electron dense and is about 1 μm long.

The acrosomal rod ends posteriorly in a wider, cylindrical body, about twice as long as wide, somewhat resembling the node diagnostic of the sperm of megascolecid earthworms. A membrane connecting the posterior end of the tubular backward extension of the acrosomal vesicle with this node is clearly homologous with the so-called connectives known for all oligochaete sperm. The node apparently has a developmental origin independent of that of the acrosomal rod. What is here interpreted as a precursor of the node is present in the acrosome tube of the late spermatid (Fig. 8a) before the acrosomal rod has extended into the tube.

D. Discussion

Spermiogenesis in *Microchaetus pentheri* is similar to that described for other families of oligochaetes. Spermatogenic stages develop around an anucleate cytophore from which they separate as mature spermatozoa. During spermiogenesis the nucleus undergoes elongation and becomes surmounted by a complex acrosome, the axoneme develops from the distal centriole (basal body), the proximal centriole disappears and the mitochondria form a cylindrical mid-piece between the nucleus and distal centriole. Interpolation of the midpiece of the mitochondria between the nucleus and distal centriole is universal for the Euclitellata (oligochaetes, branchiobdellids and leeches) but is a rare condition elsewhere. It occurs in the Onychophora, where it is considered to indicate relationship with euclitellates, and independently in, for instance, Chondrichthyean fish (Jamieson 1991).

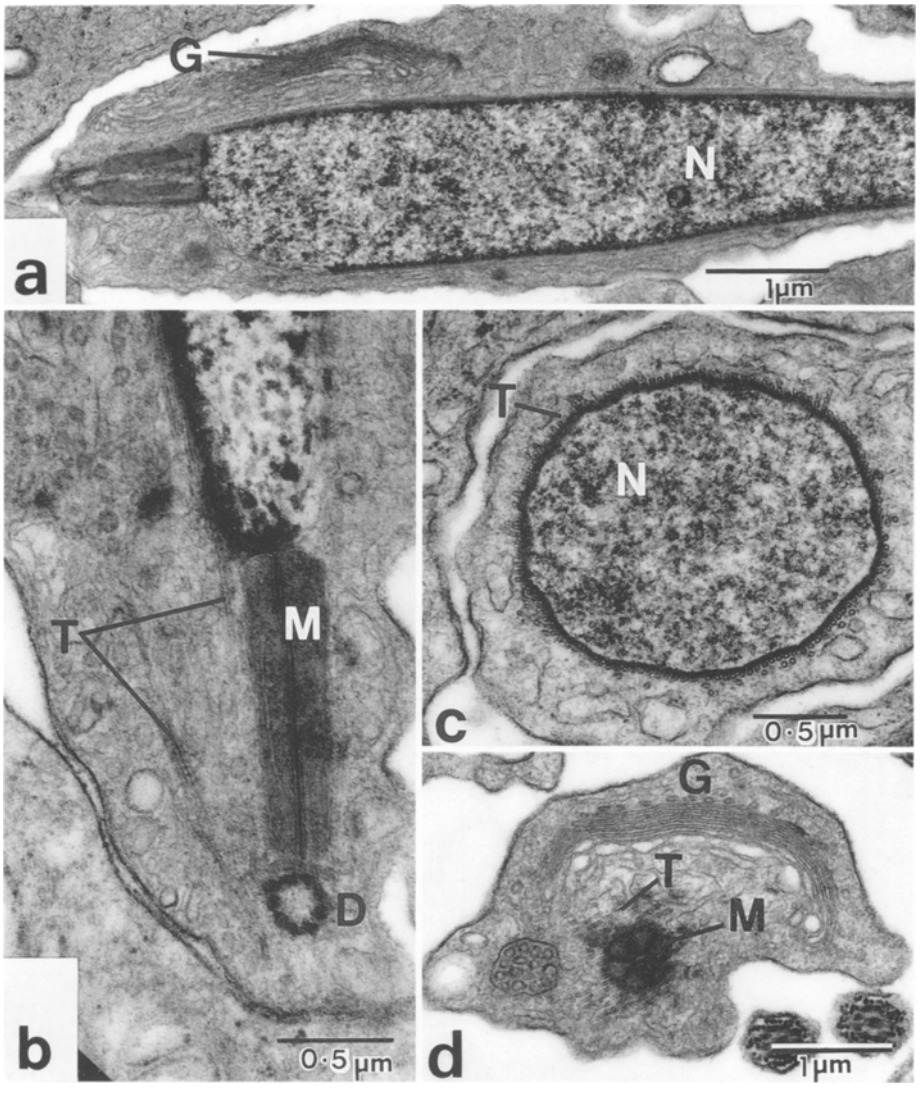

Fig. 5a–d. *M. pentheri.* Longitudinal (**a**, **b**) and transverse (**c**, **d**) sections through Stage C spermatids. The nucleus (*N*) is now elongate and condensation of the chromatin is progressing. Microtubules (*T*) completely surround the nucleus and mid-piece, the Golgi body (*G*) is displaced laterally and the mitochondria (*M*) have become reduced to six in number and are radially adpressed. *D* distal centriole

Nuclear elongation coincides with the development of a microtubular manchette. The location of the microtubule organizing centre has still to be satisfactorily identified in *Microchaetus* and some other oligochaetes (Jamieson 1981). In some species it has been suggested that the microtubules originate from the distal centriole (Anderson et al. 1967; Reger 1967; Troyer and Cameron 1980). However, in the present and other studies the microtubules first appear only at the anterior of the nucleus and not in close proximity to the posteriorly located distal centriole. This association is only apparent in stage C spermatids. The pattern of development of microtubule distribution and numbers during spermiogenesis in *M. pentheri* is, for the most part similar to that reported for tubificids by Jamieson and Daddow (1979), except that in *Microchaetus* the total number of microtubules is greater and there is a rapid decrease in the number during Stage F (Fig. 6).

The mature acrosome of *Microchaetus* has many of the features of the acrosome of *Haplotaxis* (Haplotaxidae) and of *Sparganophilus* (Sparganophilidae). The Haplotaxidae has the greatest number of plesiomorphic character states than any other family of the Subclass Diplotesticulata, to which *Microchaetus* belongs. The same is true of *Sparganophilus* in relation to diplotesticulate Aquamegadrili (aquatic, earthworm-like oligochaetes in the families Sparganophilidae, Biwadrilidae, Almidae, Lutodrilidae and possibly Kynotidae) which constitute the sister-group of the Terrimegadrili (the earthworm families Ocnerodrilidae, Microchaetidae,

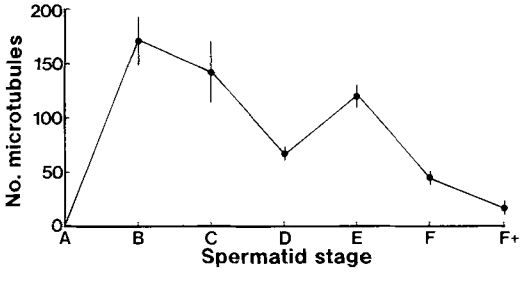

Fig. 6. *M. pentheri.* Total microtubule numbers ($\bar{x} \pm SD$) during spermiogenesis. Mean microtubule numbers was obtained from 20 spermatids from each stage

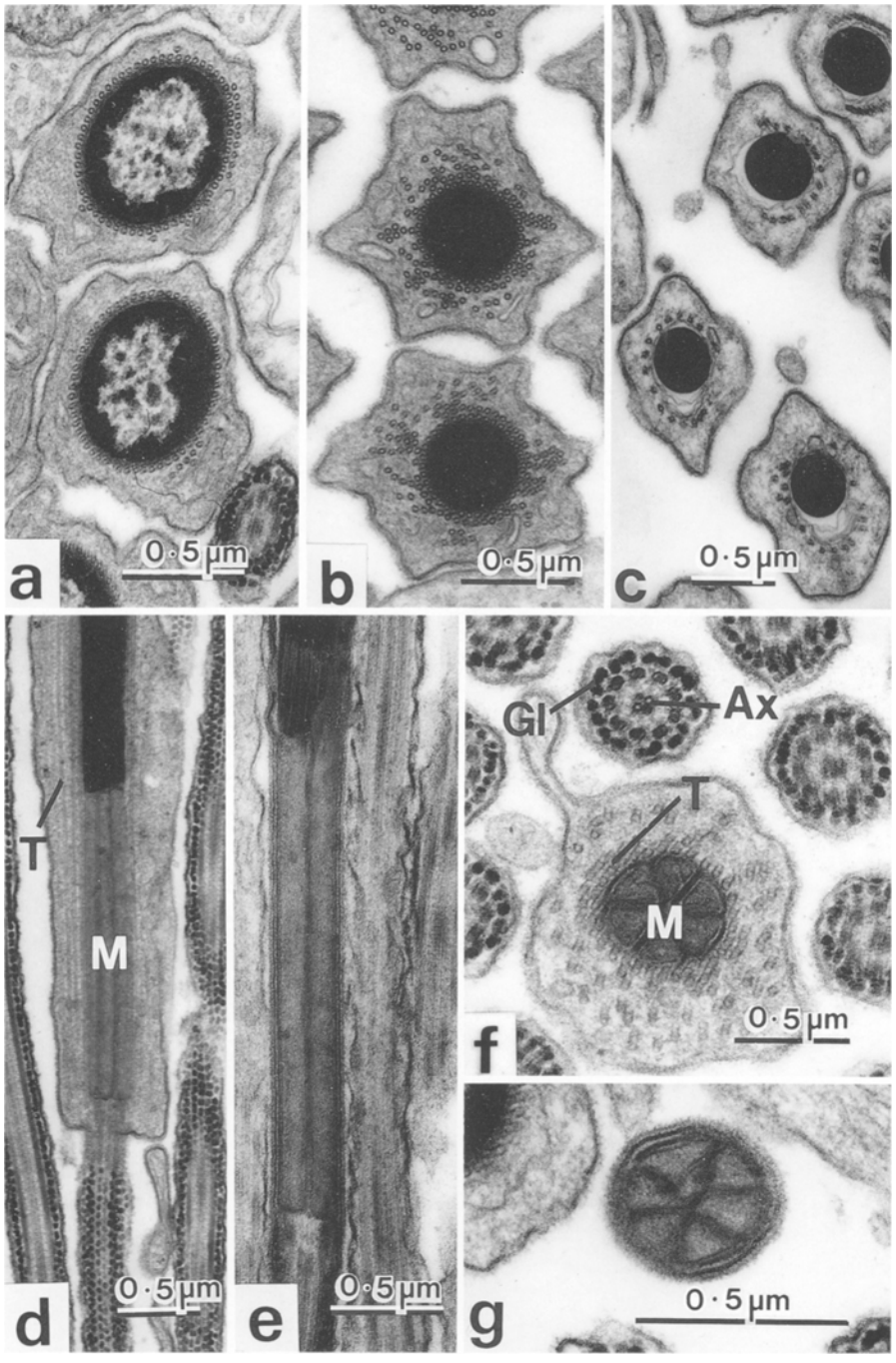

Fig. 7a–g. *M. pentheri.* **a–c** Transverse sections through the mid-nuclear region of spermatid Stages D, E and F respectively. **d–g** Longitudinal and transverse sections through a developing (**d**, **f**) and mature (**e**, **g**) mid-pieces. In the developing mid-piece the mitochondria (*M*) are surrounded by microtubules (*T*) and the axoneme (*Ax*) is surrounded by glycogen rosettes (*Gl*)

Hormogastridae, Glossoscolecidae, Lumbricidae and Megascolecidae). Of these, sperm ultrastructure is known only from the Sparganophilidae, Hormogastridae, Glossoscolecidae, Lumbricidae, Megascolecidae and, in the present work, Microchaetidae.

The chief shared features of the haplotaxid, sparganophilid and microchaetid sperm are, however, symplesiomorphic character states in the acrosome. Although *Microchaetus* has more plesiomorphic states in the acrosome than do other taxa of the Terrimegadrili by virtue of possessing these aquamegadrile features, these plesiomorphies do not establish monophyly of microchaetids with haplotaxids and sparganophilids. These symplesiomorphies include: (1) the bulbous form of the emergent region of the acrosomal vesicle, (2) the fact that the core of this bulb is occupied by an anterior extension of the subacrosomal (subvesicular) space, a feature which is seen only developmentally in lumbricid, hormogastrid and megascolecid sperm, (3) protrusion of the acrosomal rod anteriorly into the bulb, also only a developmental feature of the other three families, and (4) when compared to these three families, the fact that the rim of the primary vesicle and acrosomal rod in *Microchaetus*, like *Haplotaxis* and *Sparganophilus*, penetrates less than half way into the acrosomal tube, leaving a large basal chamber (intrusion of the rod into the tube

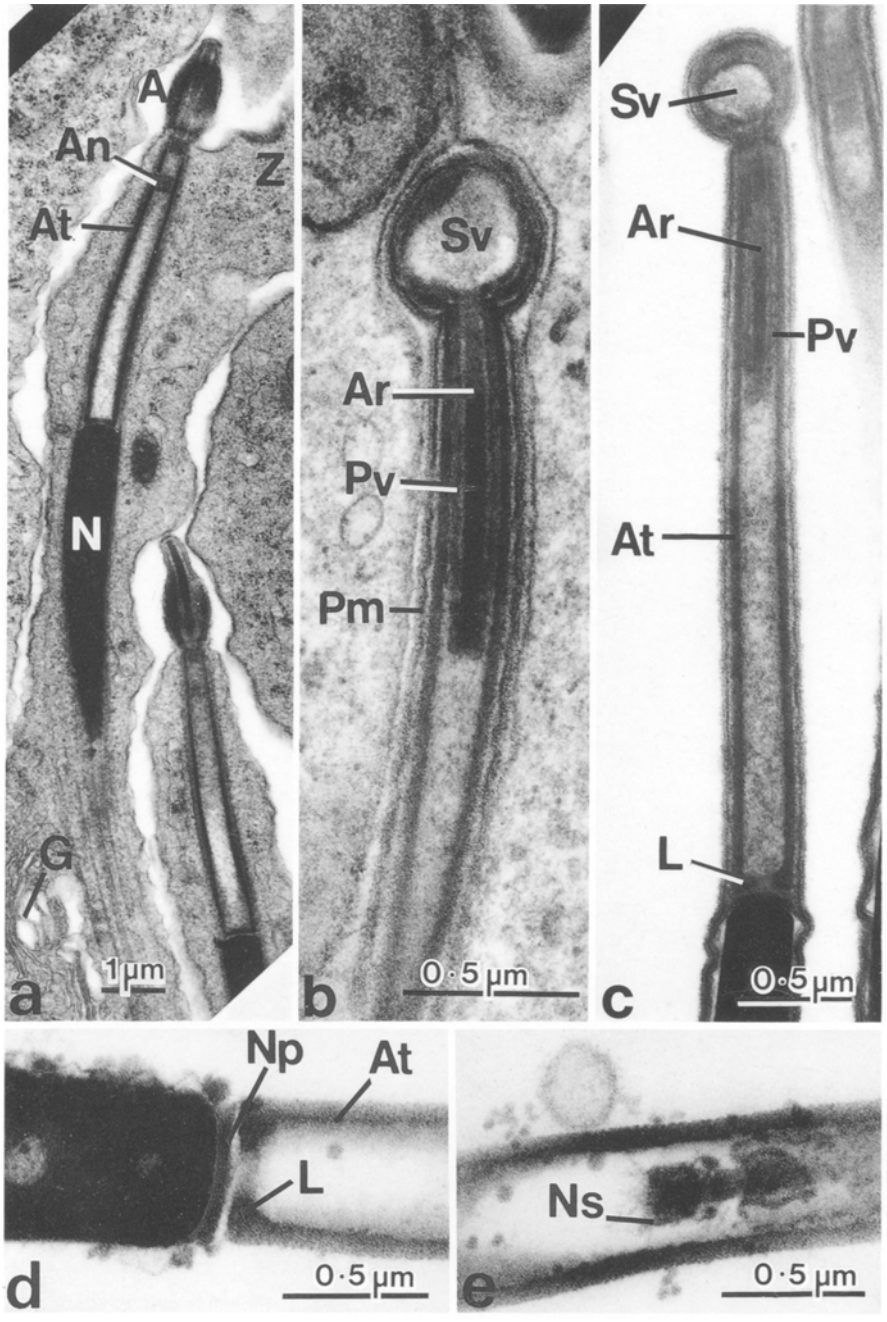

Fig. 8. a Longitudinal section of Stage E or F spermatid with the acrosome (*A*) positioned anterior to the nucleus (*N*) near the zonula collaris (*Z*). A precursor of the acrosomal node (*An*) is sited within the acrosomal tube (*At*) and the Golgi body (*G*) is now sited close to the anterior extremity of the spermatid. **b–e** Longitudinal sections of fully developed acrosomes. *Ar* acrosomal rod; *At* acrosomal tube; *L* limen; *Np* nuclear pad; *Ns* node sheath; *Pm* plasmamembrane; *Pv* primary acrosomal vesicle; *Sv* acrosomal vesicle

is large and the basal chamber small in lumbricids, hormogastrids and megascolecids). A difference between the acrosome of *Microchaetus* on the one hand and *Haplotaxis* and *Sparganophilus* on the other is the larger size in *Microchaetus* (length 3.8 µm vs less than 1 µm and 1.5 µm, respectively). In this respect the acrosome of *Microchaetus* resembles that of the other terrimegadriles, the lumbricids, hormogastrids and megascolecids (Jamieson et al. 1987). The axial rod of *Microchaetus* differs from that of *Haplotaxis*, *Sparganophilus*, lumbricids and megascolecids in lacking an anterior expansion, the capitulum. This is most parsimoniously to be regarded as an apomorphic loss of the capitulum.

Study of a single species makes it impossible to establish that the sperm of *M. pentheri* is representative of the Microchaetidae. However, in other families, intrafamilial variation in sperm ultrastructure has been found to be small where several species of a family have been examined (Jamieson 1981; Jamieson et al. 1987).

Acknowledgements. We wish to thank Miss C. Bojarski, Mr. Robin Cross and Mr. Neil Cannon (Rhodes University) for technical assistance. This project was funded by a grant from the FRD and Rhodes University (ANH) and by an Australian Research Council grant (BJMJ). We are grateful to Rhodes University for providing the opportunity and facilities for our collaboration.

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