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# Spermiogenesis in *Bythonomus lemani* and the phylogenetic position of the Lumbriculidae (Oligochaeta, Annelida)

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## Abstract

Spermiohistogenesis and spermatozoal morphology of the lumbriculid *Bythonomus lemani* have been investigated by means of electron microscopy. Though spermiohistogenetic events follow the general microdrile pattern, some features are peculiar: chromatin is clumped in the first stages and its condensation is very irregular, as in *Eisenia*. Manchette geometry is similar to that of hirudineans, as the microtubules are helically arranged from the very early stages. A number of mitochondria are always present in the collar region.

The mature sperm also departs from the microdrile model and is more similar to the megadrile one in (1) the strong withdrawal of the base of the acrosome vesicle into the acrosome tube; (2) the apparent development of rudimentary connectives. On the other hand, some features seem to be unique among oligochaetes, including the inclined longitudinal axis of the axial rod and tilted anterior end of the nucleus.

Spermatologically, lumbriculids may be interpreted as advanced microdriles which may be descended from a stock which also gave rise to Haplotaxidae, Moniligastridae, and the true Opisthopores.

## Introduction

The spermatozoon of the Oligochaeta is a long, filiform cell with the primary acrosome vesicle carried on an acrosome tube. It contains an axial rod (perforatorium) and a basal invagination (secondary acrosome invagination), while from the base of the primary acrosome vesicle (PAV) and around the axial rod, there extends a delicate secondary tube. An elongate, highly condensed cylindrical, sometimes spiral nucleus is followed by a cylindrical midpiece which consists of two to eight radially adpressed mitochondria which are not penetrated by the axoneme. A single, distal centriole persists, though modified, at maturity. The axoneme has nine doublets, each with two outer longitudinal rows of glycogen granules, and centrally two singlets accompanied by two solid fibers in a tetragon arrangement (Jamieson, 1978b, 1981b; Ferraguti, 1983; Jamieson *et al.*, 1987).

Collectively these features are unique for male gametes and some individual characters are unique or vitually so for all groups or are limited to euclitellates (Jamieson, 1983a). Thus the acrosome tube is restricted to euclitellates, though a structure somewhat resembling it is seen in nematomorphs (Lora Lamia Donin & Cotelli, 1977). Location of the midpiece mitochondria between the nucleus and axoneme and the presence of a proximal basal cylinder on the axoneme are synapomorphies and possibly autapomorphies, of the euclitellate-onychophoran assemblage (Jamieson, 1986). A prominent central sheath, which forms an extension of the basal cylinder around the central singlets or core of the axoneme, is a further autapomorphy for euclitellates (Ferraguti, 1984). Within the Euclitellata,

oligochaete sperms are distinguished, inter alia, from those of leeches and branchiobdellids by persistence of the central axonemal tetragon into the mature sperm, and by retention of two central singlets; from leeches, at least, by the presence of the secondary acrosome tube (a structure resembling a weakly developed secondary tube is apparent in some published monographs on branchiobdellids). Oligochaete sperm differ further from branchiobdellid sperm in the elongate rather than cork-shaped axial rod and the relatively simple acrosome vesicle lacking internal microvillus-like extensions; from leech sperm in the absence of an anterior prevesicular extension of the acrosome tube (Jamieson, 1981b; Ferraguti, 1983).

In recent years our knowledge of oligochaete spermatology has greatly increased. To date representatives of nine oligochaete families have been studied for sperm ultrastructure (see Jamieson, 1983b, 1984; Jamieson *et al.*, 1987). Interest in oligochaete sperm has been stimulated by two features, among others, both linked to the peculiar morphology of oligochaete spermatozoa: (a) since the spermiohistogenesis occurs within morulae free in the coelomic derivatives, the system appears suitable for the study of endogenous morphogenetic factors; (b) since all oligochaete sperm studied to date are built on a common plan, with variations suitable for numerical computer analysis involving clustering procudures, they provide an interesting model for comparison between the deduced phylogeny of a specialized cell type and more "classical" phylogenies derived from non-spermatozoal characters.

Sperm ultrastructure in oligochaetes was the subject of a study by Jamieson (1983b) in which congruence with a pre-existing holomorphological phylogenetic analysis was demonstrated. Later (Jamieson, 1984), it was used to construct phylogenies of the Oligochaeta using numerical procedures. In these papers it was pointed out that knowledge of spermatozoal ultrastructure in the Lumbriculidae was insufficient for inclusion of this family in the studies beyond some preliminary assessment of its position. Transmission electron microscope data on spermiohistogenesis of lumbriculids are also incomplete, since investigation of this process has been largely limited to early stages (Ferraguti & Lanzavecchia, 1977). These deficiencies in our knowledge have been especially significant in view of the importance accorded to the Lumbriculidae in phylogenetic studies, lumbriculids having been regarded as the most plesiosmorph living oligochaetes in some studies (e.g. Michaelsen, 1928; Brinkhurst & Jamieson, 1971; Jamieson, 1978a, 1981a, b) while Brinkhurst (1984), on the other hand, has argued for derivation of Lumbriculidae from haplotaxid-like ancestors. We will return to these points in the Discussion.

## Materials and methods

Mature specimens of *Bythonomus lemani* have been collected in ponds around Milano, dissected and immediately fixed in cacodylate-buffered Karnowsky's mixture. Following an overnight washing in 0.1 M cacodylate buffer, the specimens were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer and pre-stained in 2% aqueous uranyl acetate for two hours. The dehydration was performed in a graded ethanol series and the embedding was in Spurr's resin. The sections, cut with LKB Ultrotomes III and V, were observed under a Hitachi HU 11 ES electron microscope, after staining in lead citrate.

## Observations

The spermiohistogenetic process is very complicated and involves different transformations of the various portions of the early spermatid. Unfortunately, T.E.M. observations are based on thin sections which give only partial information on the developmental stages of the different organelles. For this reason we have prepared a schematic drawing (Fig. 1) summarizing the main events, whereas we will give a separate description of the spermiohistogenetic events modifying the shape and position of the single organelles.

### *Early spermaids*

It seems that spermatid polarization starts from the very beginning of spermiogenesis: at this stage the

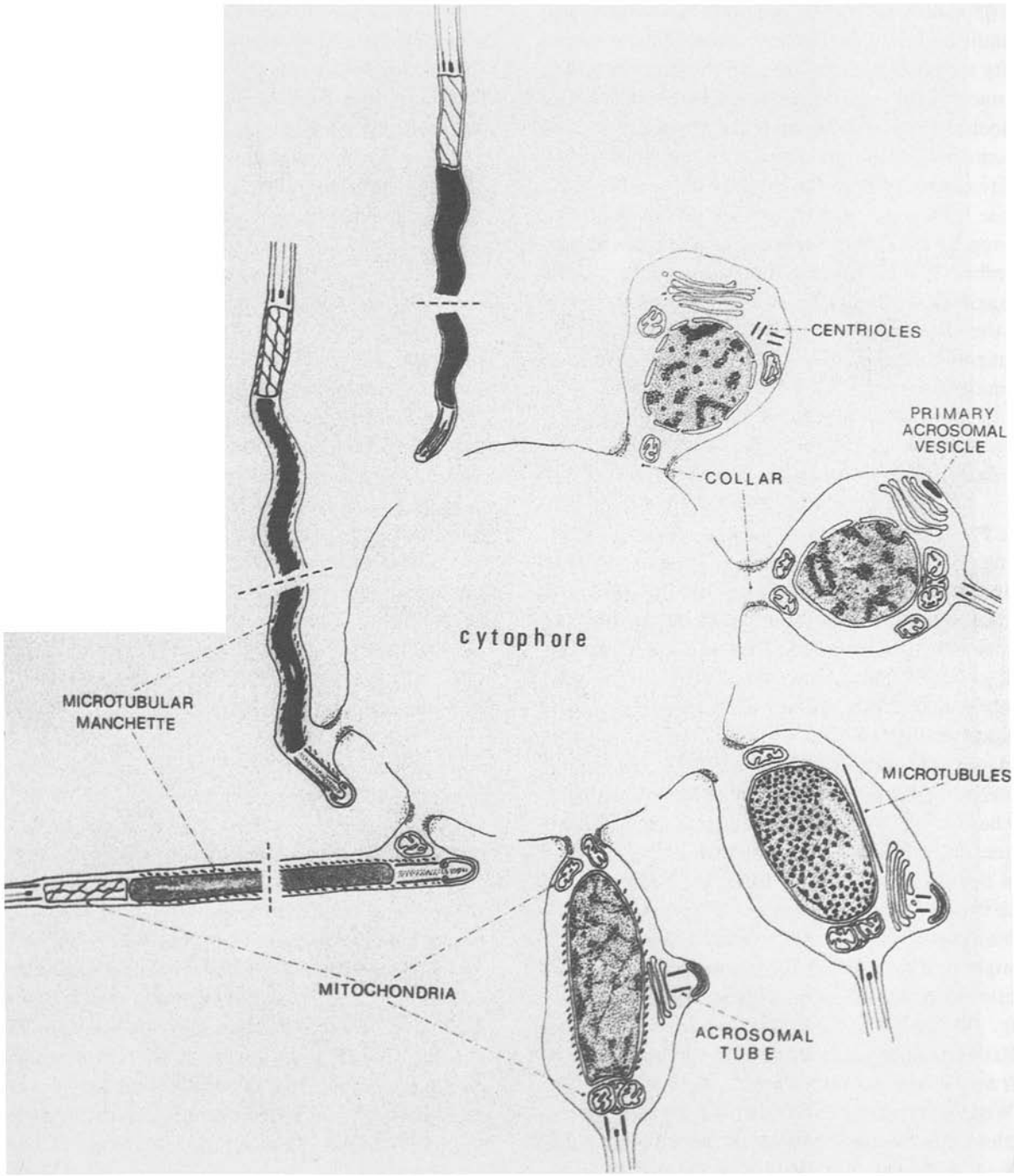



*Fig. 1.* Schematic drawing to illustrate on a single hypothetical morula the main spermiohistogenetic stages in *Bythonomous*.

spermatid is a roundish cell attached by a collar to an anucleate mass called the cytophore (Fig. 2). At the opposite pole with respect to the collar the basal granule and the flagellum are visible. In close proximity six mitochondria are already grouped and in contact with the nuclear membrane. Other mitochondria are to be found in the collar area. A prominent Golgi apparatus lies in the same region: in favourable sections it is possible to see an electron dense vesicle, the primary acrosomal vesicle (PAV), on top of the Golgi cisternae, close to the plasma membrane. This is the first appearance of the acrosomal vesicle. Scattered microtubules are visible in the centriolar area. Chromatin is irregularly clumped in evident masses. Cytoplasm is reduced to a small amount.

*Acrosome morphogenesis*

The PAV soon becomes cup-shaped (Fig. 3) and its convex side maintains contact with the plasma membrane. The contents of the vesicle are divided into a clear matrix which surrounds a darker phase. The acrosomal tube appears at first as a short cylinder (0.2 $\mu$m $\times$ 0.16 $\mu$m) under the PAV which progressively assumes a more convex shape, resembling a telephone receiver (Fig. 4).

As the acrosomal tube elongates (Fig. 5) the dense material inside the PAV breaks into a ring occupying the base of the PAV, whereas centrally the two membranes of the vesicle are pressed against each other and the plasma membrane (Fig. 6). The acrosomal tube finally abuts on the nucleus (Figs. 6 and 17) and subsequently, when the nucleus is completely straight and cylindrical, is ensheathed by the manchette and aligned with the nuclear major axis (Fig. 7). The acrosome is, at this stage, close to the collar and many mitochondria are always present in this area (Figs. 6 and 17).

When the nuclear chromatin is compact and the nucleus has become corkscrew-shaped, the acrosome follows the nuclear bending. At this stage the subacrosomal material becomes apparent as a rod and shortly after the acrosomal vesicle withdraws inside the tube to reach its final position (Figs. 7 and 8). These last events are deduced to be very rapid, since we have never observed intermediate stages.

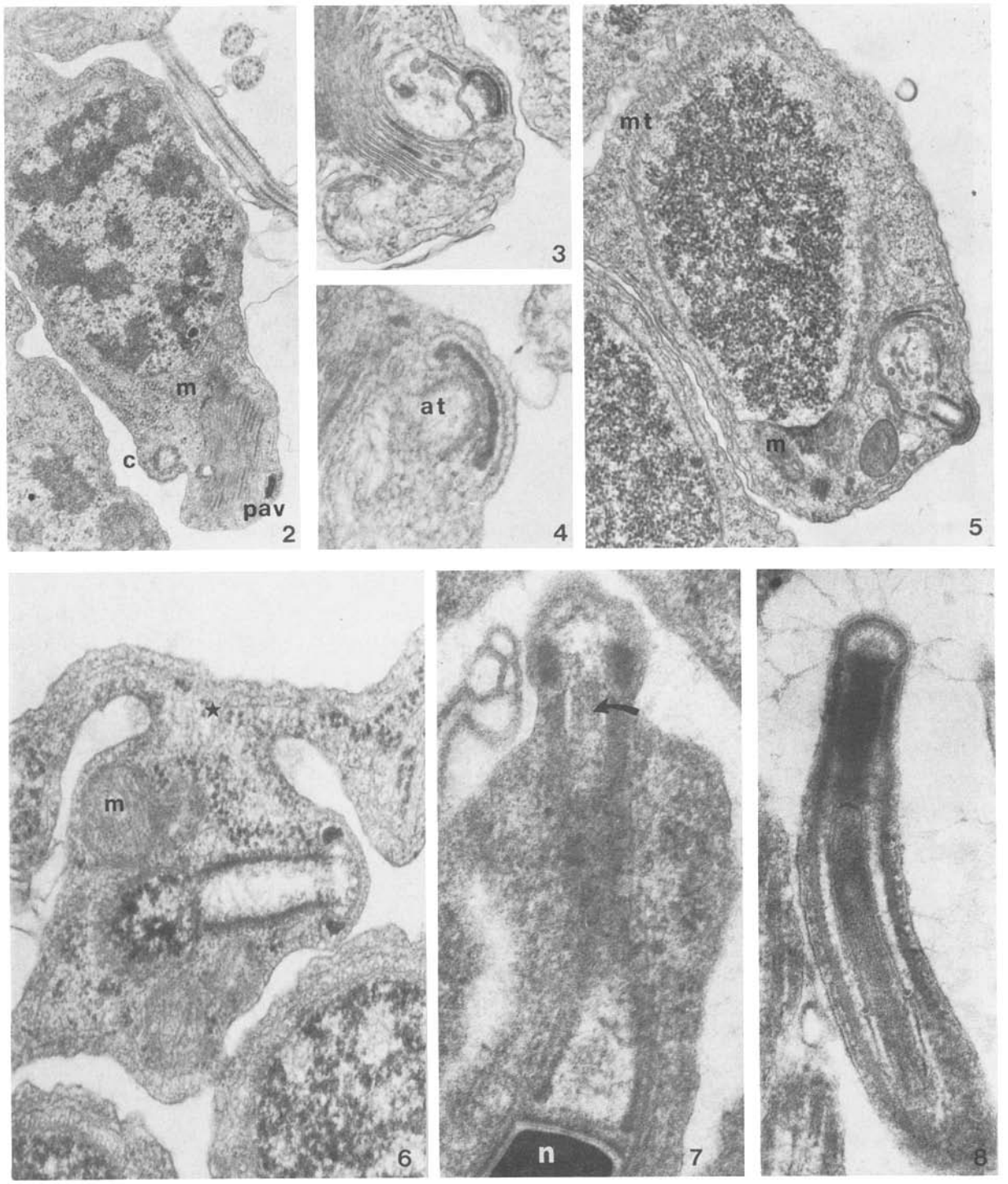
*Nuclear morphogenesis*

The appearance of the chromatin and the nuclear shape vary continuously during spermiogenesis. The chromatin, in large clumps in early spermatids (Fig. 2), assumes at first a finely granular aspect, then progressively condenses to a continuous ring at the periphery of the nucleus surrounding an electron-transparent core. Finally, the core progressively reduces and disappears completely (Figs. 10, 12 and 13).

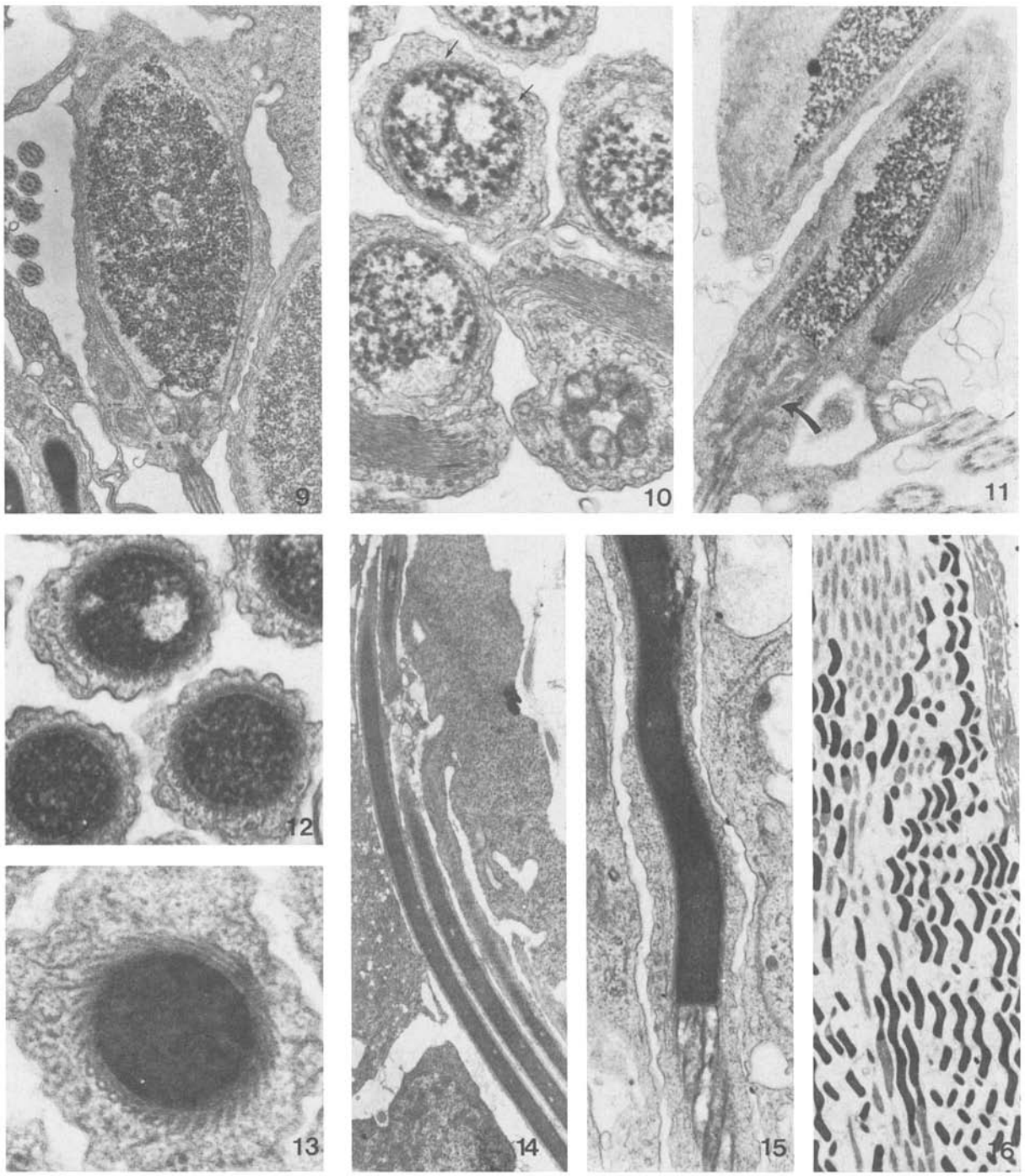
The shape of the nucleus is rounded at first (Fig. 2), then ovoidal (Fig. 9), cylindrical (Fig. 14), and finally corkscrewed (Figs. 15 and 16). Changes in shape are accompanied by the presence of a microtubular manchette which forms from scattered microtubules in the very early stages of spermiogenesis. When the nucleus is ovoidal, the manchette is already complete and, apparently, twisted (Fig. 10). A further twisting of the manchette is visible in the latest stages, when the corkscrew shape of the nucleus appears more visible (Fig. 13). At maturity, the manchette and the residual cytoplasm are eliminated.

*Mitochondrial morphogenesis*

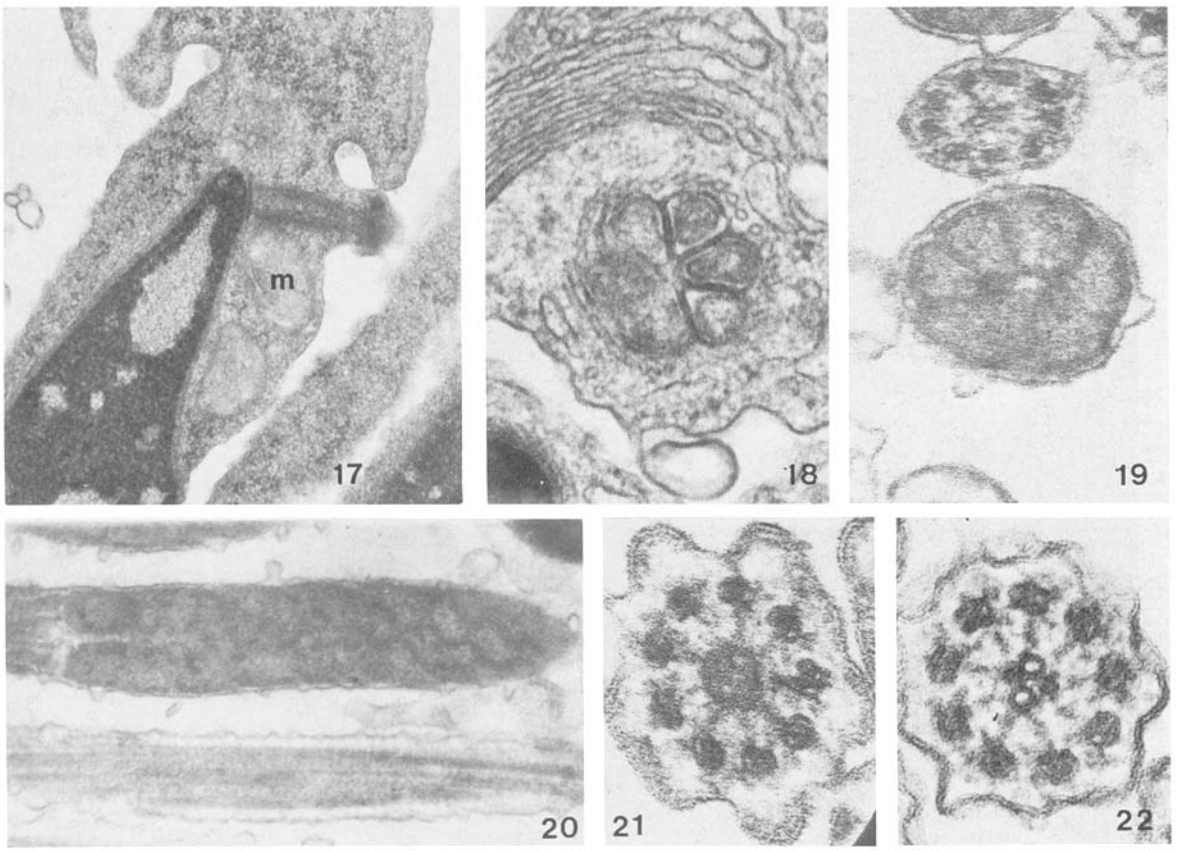
As stated before, grouping of six mitochondria is one of the more precocious events of spermatid polarization (Figs. 9 and 10). The factors controlling this event have not been clarified. Mitochondria seem to be held together by a dense material present between the outer membranes. The mitochondria not forming the midpiece migrate to the collar area, where they persist to the end of spermiogenesis (Figs. 6 and 17), whereas midpiece-forming ones are soon ensheathed by manchette microtubules. With the continuation of spermiogenesis the mitochondria lose their conventional appearance, becoming more and more elongate and similar to cylindrical sectors (Figs. 10, 11, and 18). The process of lengthening is accompanied by a progressive torsion giving rise to the characteristic twisted appearance of the midpiece and the assembled mitochondria of the mature sperm (Figs. 15, 19 and 20). This transformation of mitochondrial shape occurs without any variation in the volume of the organelles.




*Figs. 2–8.* (2) An early spermatid showing a large nucleus with clumped chromatin. Basal granule (c), mitochondria (m) and primary acrosomal vesicle (pav) are visible. (× 38000); (3) A detail showing the PAV with its dark content in close proximity of the plasma membrane. (× 38000); (4) The acrosome tube (at) appears now under the PAV. (× 50000); (5) At a subsequent maturation stage, the chromatin appears finely granular, whereas the acrosome tube is longer and mitochondria (m) are grouped at the base of the nucleus. Some microtubules start forming the manchette (mt). (× 21000); (6) The acrosome lies on top of the nucleus and is close to the collar (*). Mitochondria (m) are present in this area. (× 50000); (7) An advanced spermatid shows the acrosome in line with the nucleus (n), which is completely condensed, and the acrosome rod now apparent (arrow) (× 78000); (8) The acrosome in a mature spermatozoon. For a better understanding of the details, please refer to Fig. 23. (× 75000).




*Figs. 9–16.* (9) An early spermatid showing the granular chromatin and polarization of the cell. (× 14000); (10) Cross section of some elongating spermatids showing the aspect of chromatin, the twisted microtubular manchette (arrows) and the six grouped mitochondria. (× 75000); (11) A spermatid in the same stage as the ones of the former figure, cut in longitudinal section. Note the areas of uncondensed chromatin and the twisted mitochondria (arrow) (× 25000); (12) Cross section of a spermaid at a subsequent maturation stage. Chromatin is nearly condensed and manchette is helically arranged. (× 30000); (13) Cross section of a completely condensed spermatid nucleus. (× 60000); (14) Longitudinal section of spermatids at the stage of Fig. 12. Whereas the manchette is twisted, the nucleus remains straight. (× 8000); (15) Longitudinal section of a spermatid at the stage of Fig. 13. The nucleus is twisted, as is the middle piece mitochondria. (× 20000); (16) Longitudinal section of a group of mature spermatozoa, at the periphery of the seminal vesicle. The cytoplasm is completely absent and the nuclei are highly twisted. (× 4000).




*Figs. 17–22.* (17) Longitudinal section of an advanced spermatid. The acrosome is already on top of the nucleus and some mitochondria (m) are present in the collar area. (× 32000); (18) Cross section of a mid-spermatid to show the helically arranged microtubular manchette around the mitochondria. (× 60000); (19) Cross section of the middle piece of a mature sperm. The six mitochondria are helically arranged. (× 90000); (20) Longitudinal section of the middle piece of a mature spermatozoon. (× 45000); (21) Cross section of the first portion of the flagellum. A prominent central sheath is evident in which the two singlets appear "embedded". (× 130000); (22) Cross section of the main portion of the flagellum, to show the tetragon fibers. (× 130000).

*Mature sperm*

Mature spermatozoa have been found and examined in large amounts within the seminal vesicles of *Bythonomus* (details of spermatozoal dimensions are given in Jamieson *et al.*, 1987; a schematic drawing is given in Fig. 23). The acrosomal complex, at the top of the nucleus, is 1.2 μm long and 0.3 μm wide maximally (at base). The acrosomal tube is 1.1 μm long and 0.24 μm wide at the base, with a wall thickness of approximately 0.02 μm and encloses the acrosomal vesicle for about 0.8 μm. A thin secondary tube, 0.3 μm long is pendant from the base of the acrosomal vesicle and is characteristically biased towards one wall of the acrosomal tube (for a more precise understanding of the acrosomal shape, see Fig. 23). Subacrosomal material is present in the form of a rod 0.8 μm long surrounded by the base of the vesicle and anteriorly lying in an invagination of this (the secondary invagination) which is 0.5 μm long. The acrosomal membrane appears incomplete against the acrosomal tube. The secondary invagination comprises about half of the total length of the PAV. The contents of the portion of the acrosomal vesicle anterior to the axial rod and its investing secondary invagination chiefly consists of an electron dense, finely granular approximately cylindroid core, the dense acrosomal core. A small por-

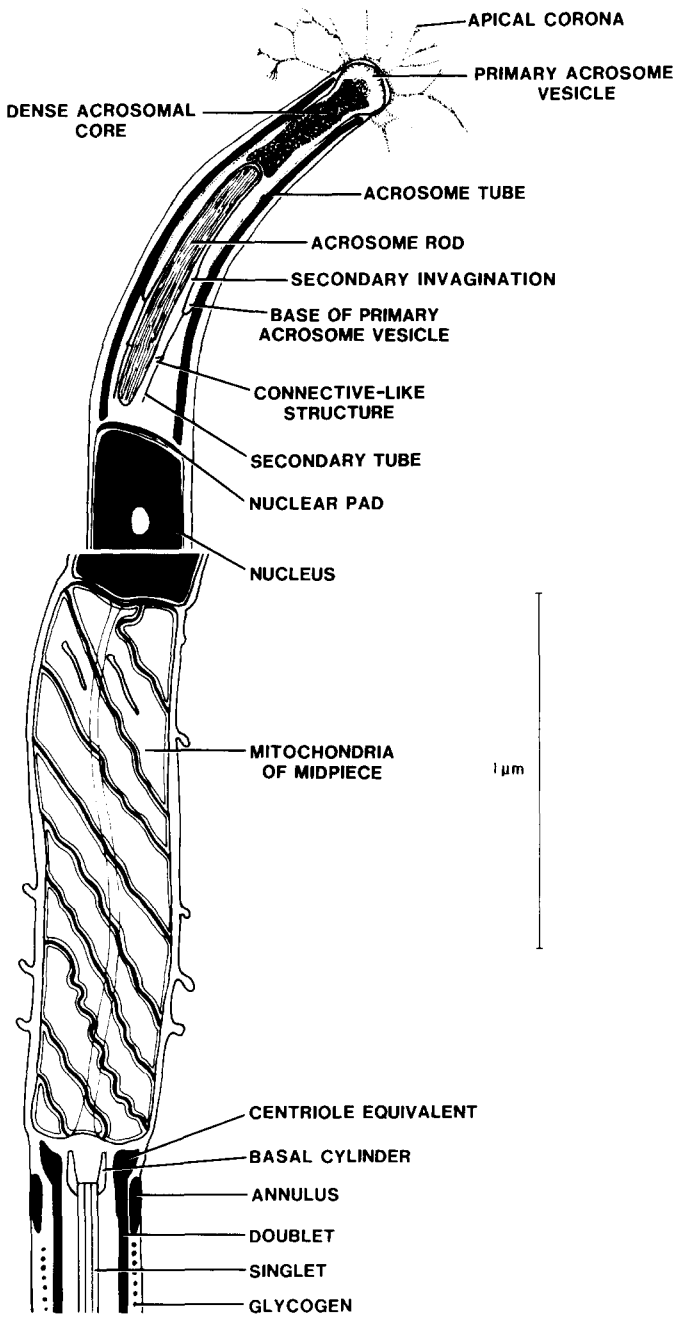



*Fig. 23.* Schematic drawing of a mature sperm of *Bythonomus.*

tion (0.14 of its length) of the acrosome vesicle projects anteriorly beyond the acrosome tube. The plasma membrane covering this rounded protrusion bears long branched filaments which are here termed the apical corona (see Discussion). The acrosome surmounts the tip of the nucleus (about 23 $\mu$m long and 0.3 $\mu$m wide) which is corkscrew-shaped with a pitch of 1.4 $\mu$m. The midpiece is composed by six twisted mitochondria with approximately ten gyres and is 1.6 $\mu$m long by 0.4 $\mu$m wide. The centriole is

here termed the centriole equivalent, since it is highly modified as in all Clitellate by the presence of a central basal cylinder which abuts on the mitochondria, and centriolar triplets have again not been demonstrated. Around is a dense subplasmalemmal collar-like structure, the annulus (or annuloid, Jamieson, 1982). The flagellum shows successively in proximal-distal sequence a portion with prominent central sheath (Fig. 21), a portion with tetragon fibers (Fig. 22), and a portion with the conventional aspect (Ferraguti, 1984). Glycogen granules are present with the usual disposition peripheral to doublets.

## Discussion

Spermatogenesis in *Bythonomus* follows the common pattern for Oligochaeta, with some variations which deserve attention and will be treated in the same order as in the Observations section.

### *Chromatin in the early spermatids*

In all "microdriles" so far studied the chromatin at the beginning of spermiohistogenesis is finely granular, whereas in *Bythonomus* it is in large masses. Whether this situation is to be considered homologous to the one described in the lumbricid "megadrile" *Eisenia foetida* (Martinucci & Felluga, 1979) remains to be established. As spermiogenesis progresses and the manchette is completed, the appearance of the chromatin becomes similar to that of other microdriles. However, large electron transparent areas of incondensed chromatin are irregularly distributed within the nucleus. Such areas have been shown up to now only in the nuclei of advanced megadrile spermatids.

### Acrosome morphogenesis and movement

The first appearance of the PAV occurs very early in spermiogenesis, and the growth of the acrosomal tube is not apparently accompanied by the formation of subacrosomal material which becomes evident only when the acrosome has reached its final position on top of the nucleus. The distance migrated by the acrosome seems to be short. Unlike what happens, for instance, in *Lumbricus* (Troyer & Cameron, 1980), it seems that the collar is, throughout the spermiogenesis, in the acrosomal area. Since the acrosomal complex reaches its final position on top of the nucleus when this starts elongating, the migration has to occur very early, when the cell is still isodiametric. It is not clear to what extent this mechanism is common to all microdriles, but the drawing by Jaana (1983) presents a similar situation in *Tubifex hattai*. The apical corona, composed of branching filaments, described above for *Bythonomus*, has previously been noted only for the megascolecid *Fletcherodrilus* in which it was stated that they may represent extruded secretion (perhaps as a response to fixation) or structural elements of the acrosome concerned with establishing adhesion to the egg (Jamieson, 1978b). They may correspond with external papillation of the plasma membrane of the vesicle described for *Lumbricus terrestris* by Cameron & Fogal (1963), possibly as a type of reaction product.

### *Gross nuclear morphogenesis*

Transitions of shape and the general model of chromatin condensation are very similar to the pattern described in other microdriles. However, the relationships between manchette microtubules, chromatin condensation and nuclear morphogenesis are less clear in *Bythonomus* than in Tubificidae and Enchytraeidae. In particular, although the perinuclear cisterna collapses in correspondence with the microtubules, as observed in other euclitellate groups, no deposition of condensed chromatin is observed in the first stages of spermiogenesis on the inner side of the cisterna. Chromatin seems to start condensation with an irregular pattern, only later following the conventional model. Manchette geometry also differs from that of other microdriles. Whereas in enchytraeids (Webster & Richards, 1977) and in tubificids (Ferraguti & Lanzavecchia, 1971; Jamieson & Daddow, 1979) the manchette is helical in late spermatids, but is initially longitudinal, in *Bythonomus*, it appears from its inception to be

oblique with respect to the major axis of the nucleus, thus resembling the situation in leeches (Ferraguti, 1983).

*Mitochondria*

The precocious aggregation of midpiece-forming mitochondria is already known for other oligochaetes. However, *Bythonomus* is again peculiar in having a number of mitochondria always present in the collar area, close to the acrosome, persisting up to the late stages of spermatogenesis. The significance of this observation is not clear.

*Mature sperm*

The mature spermatozoon of *Bythonomus* agrees with the general pattern for oligochaete outlined in the Introduction. Features attributable to a specifically microdrile model are the short acrosome, in the vicinity of 1 $\mu$m long; the long, approximately cylindrical secondary tube; the relatively short and stout axial rod, and the strongly helical midpiece. Helical coiling of the mitochondria, seen also in the spermatologically very plesiomorphic Enchytraeidae (*Lumbricillus*), in *Phreodrilus* and, weakly developed, in *Haplotaxis* (see Jamieson, 1982), but unknown in "megadriles" is probably also to be regarded as basically a microdrile feature.

There are, however, departures from the microdrile model which approach that of the opisthoporous oligochaetes (oligochaetes in which the male pores are separated by one or more segments from the last testis segment and which are usually larger, being loosely termed "megadriles"). The readily recognizable, qualitative, megadrile tendencies of the lumbriculid sperm are (1) the strong withdrawal of the base of the primary acrosomal vesicle and of the secondary invagination, with its enclosed axial rod, into the acrosome tube, a withdrawal which exceeds that in, for instance, the highly opisthoporous Sparganophilidae, and (2) the apparent development of at least rudimentary connectives from the secondary acrosomal tube to the axial rod, though these are poorly discernible because of difficulties in obtaining adequate fixation. Connectives are also present, however, in the Haplotaxidae (Jamieson, 1982, 1983b) and apparently in *Phreodrilus* (Jamieson, 1981c, 1983b) which is aberrant from other microdriles in sperm morphology, in putative internal fertilization, and in its epizoic way of life.

*Phylogenetic relationships*

Spermatologically, therefore, lumbriculids may be interpreted as advanced microdriles which may be descendent from a stock which also gave rise to the Haplotaxidae, the Moniligastridae (the sperm of which is unknown) and the true opisthophores. The long cylindrical secondary tube precludes their derivation from haplotaxids, but we may perhaps envisage origin of lumbriculids and haplotaxids from a common microdrile stock which in lumbriculids retain the long secondary tube but specialized in the deep withdrawal of the acrosome vesicle and appurtenances and in haplotaxids developed a short divergent secondary tube with true connectives but remained plesiomorph, among other respects, in minimal withdrawal of the acrosome vesicle, of the secondary invagination and of the axial rod.

These inferences can at most be considered preliminary, on the strength of one investigated species, and it is perhaps premature to suggest autapomorphies for the Lumbriculidae but these may prove to be the inclined longitudinal axis of the axial rod relative to the axis of the acrosome tube, and the tilted anterior end of the nucleus, features at present known only in the *Bythonomus* sperm. In other microdriles the nuclear tip is domed, as in most megadriles (the presumed plesiomorph condition), whereas it is apomorphically flattened in the Lumbricidae (Jamieson, 1983b, 1984).

That lumbriculids may be apomorphic microdriles finds support in other, non-spermatozoal, anatomical features. Thus the excretory system is highly apomorphic in *Lumbriculus variegatus*, in having the nephridia interconnected from segment to segment by a longitudinal system of tubules (Boveri-Bonner, 1920). Also it has long been argued that passage of the male duct through the posterior septum of the testicular segment only to loop back

and discharge at a male pore in the testicular segment (the prosopore condition with the male pore in the testicular segment) is a secondary development from a previous plesioporous condition (male pores in the segment behind the corresponding testis) (see, for instance, Brinkhurst, 1984). Furthermore, the ultrastructure of lumbriculid muscles shows some apomorphic features known nowhere else (de Equileor *et al.*, this volume). The similarity of manchette geometry to that of leeches possibly has implications for the view that leeches are descended from lumbriculid-like forms espoused by Michaelsen (1928). This view of the relatively apomorphic status accorded lumbriculids would further imply that the Hirudinea is a specialized group within the Oligochaeta s. lat. but such considerations must await additional evidence.

Lastly, it should be noted that the supposedly apomorphic status of prosopory in the lumbriculids does not logically exclude the possibility of a prosoporous proto-annelid.

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