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Ultrastructure of spermiogenesis and spermatozoa in *Diurodrilus subterraneus* (Polychaeta, Diurodrilidae)

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Abstract Spermiogenesis in the polychaete species *Diurodrilus subterraneus* may be divided into six stages. These stages, as well as the ultrastructure of the mature spermatozoa, are described based on TEM studies. The spermatozoa are unusual in having a very large acrosome followed by a region containing the nucleus and several ovoid mitochondria. A secondary acrosomal membrane forms a manchette around the nucleus and mitochondria. In this region, the plasma membrane is modified, with many small, mushroom-shaped cytoplasmic processes, each including filaments. The flagellum may be divided into three sequential regions; the longest, middle one is covered by a helically arranged mucous coat. Spermatozoa of the type described here are unknown among polychaetes but show certain superficial resemblances to those in oligochaetes. The resemblance of the mushroom-shaped bodies to spermatozoal microvilli in certain gnathostomulids is discussed. The phylogenetic relationships of Diurodrilidae are considered on the basis of this new information.

A. Introduction

The so-called Archiannelida are now generally believed to be a collection of polychaete families that are not closely related to one another (Protodrilidae, Protodriloidae, and Saccocirridae being an exception; see Purschke and Jouin 1988; Purschke 1993). Practically all of them are interstitial forms that share a number of general adaptations to that environment. Among the "archiannelids", the genus *Diurodrilus* is one of the most aberrant groups. Remane (1925) placed it within the family Dinophilidae. Kristensen and Niilonen (1982) erected a new family, Diurodrilidae, for this genus based on ultrastructural evidence such as specialization of the locomotory apparatus, ciliophores, cuticle, and pre- and postpharyngeal glands. Westheide (1985) subsequently erected a new order, Diurodrilida, for this family.

Kristensen and Niilonen (1982) also provided a number of preliminary observations on the morphology of spermatozoa in *Diurodrilus subterraneus* Remane, 1934. In the present study, the spermiogenesis of this species is investigated using transmission electron microscopy (TEM). The present investigation provides ultrastructural evidence showing that the spermatozoa of *D. subterraneus* are highly specialized and among the most unusual found in those families previously referred to Archiannelida. The type of spermatozoon cannot be related to any of those previously described for "dinophilid" dorvilleids or other polychaetes.

Theories on the polyphyletic (Orrhage 1974; Franzén 1977; Westheide 1985, 1987) or monophyletic origin of Archiannelida (Hatchek 1893; Heider 1922; Hanström 1928; Dales 1962; Hermans 1969; Nørrevang 1970) are discussed from the viewpoint of comparative spermatology. It is possible that some "archiannelids" could be primarily adapted to interstital life, e.g., Diurodrilidae, whereas others as Protodrilidae, Saccocirridae, Protodriloidae, Nerillidae, and those Dorvilleidae that were previously referred to Dinophilidae are secondarily modified polychaetes. This hypothesis is discussed in the context of groups such as the interstitial annelid family Lobatocerebridae (Rieger 1980, 1991a, b) and the Gnathostomulida (Ax 1956; Riedl 1969).

B. Materials and methods

D. subterraneus was sampled on June 8, 1975 at a water depth of 1 m near Ystad, Sweden. Additional material was collected in the summer of 1979, from June 1 to August 15, at a sediment depth of 10–30 cm on the sandy beach slope at Flakkerhuk, Disko Island, West Greenland. In all, more than 100 specimens were collected and fixed.

The animals from Sweden were fixed for 2 hours at 4° C in 1% OsO_4 in 50% seawater adjusted to pH 7.4 with Na cacodylate buffer. The material from Greenland was fixed in similarly buffered 2% glutaraldehyde in seawater for 2 hours at 4° C, postfixed in 2% OsO_4, and transferred to a 0.1 M Na cacodylate buffer for 1 hour

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at 20° C. The animals were rinsed in the same buffer for a few minutes, after which they were dehydrated in an ethanol gradient, transferred to propylene oxide, and finally embedded in Epon 812. Ultrathin sections were cut on a Reichert OM U2 ultramicrotome, stained in 3.5% uranyl acetate at 60° C and in lead citrate at 20° C (Reynolds 1963). Observations were made using a Zeiss EM 9 S-2 transmission electron microscope (TEM).

All TEM photographs shown in this paper are based on the study of two males from Ystad, Sweden. This is partly because we wished to avoid the mixing of material from two widely separated geographical regions. Furthermore, the fixation of *Diurodrilus* specimens for TEM is notoriously difficult and the spermatozoa were satisfactory only in the two cases mentioned.

Supporting light microscopy was performed on living material with a Zeiss interference-phase-contrast microscope (Nomarski technique) and on semi-ultrathin (1-µm-thick) epon sections stained with toluidine blue-borax. One male was stained with Feulgen light-green before semi-ultrathin sectioning.

C. Results

I. Structure of the male reproductive system

The testis of *Diurodrilus* species has been described by Ax (1967) for *D. ankeli* (Ax, 1967) on the basis of light microscopy. In *D. subterraneus* is consists of two thin-walled sacs, but in mature males the two sacs are not well separated as the wall of the testis, which consists of a single layer of epithelium, disappears or is discontinuous. Consequently, the maturing spermatids lie free in the coelom in contact with the gut, protonephridia, and muscle tissues (Fig. 3). Posteriorly the spermatozoa are in contact with two seminal vesicles, separated from the testis only by a basal lamina. The seminal vesicles open through a funnel-shaped passage into the cloaca. No distinct penis structure is found in *D. subterraneus.*

The cells of the reproductive system are of three different types: phagocytic vegetative cells, nutritive vegetative cells (nurse cells), and germinal cells. The phagocytic vegetative cells are always found attached to the wall of the testis. They contain large, phagocytic vesicles enclosing pieces of the very characteristic spermatozoa. One nutritive vegetative cell surrounds each germinal cell in early spermiogenesis and disappears almost totally in the mature parts of the testis. This vegetative cell contains enormous ergastoplasmic vesicles with many free ribosomes as well as 10–15 small mitochondria lying near the nucleus. The latter has one large nucleole. The germinal cell found during the first stage of spermatogenesis (primary spermatogonium) is unspecialized, with many (more than 20) small mitochondria and without ergastoplasmic vesicles. Spermatogenesis begins peripherally and spreads to the middle of the testis. However, even at an early stage when the testis still contains many immature spermatids, the seminal vesicles are filled with large spermatozoa (Fig. 3). In the mature male, the reproductive system consists almost exclusively of mature spermatozoa lying "free" in the coelom.

Both investigaed specimens of *D. subterraneus* from Ystad contained endosymbiotic bacteria in the gonads and tissues related to the coelom (Figs. 5, 9, 12). In the material from Greenland this symbiosis was not observed.

II. Mature spermatozoon (Figs. 1, 12, 16, 17)

Preliminary light microscopical (Nomarski technique) observations on the morphology of the spermatozoa in *D. subterraneus* demonstrated the presence of a large head (11 µm in length) with a small, apical appendage superficially resembling an acrosome. Near the flagellum a crescent-shaped area, which has earlier been interpreted as the mitochondrial region, is visible.

TEM studies revealed that most of the large head region consists of a giant acrosome, which is divided into three subunits, a small, 2- to 3-µm-long, hook-shaped "snout", an osmiophobic middle part, and a posterior osmiophilic area (Fig. 1). The nucleus is small and lies together with the mitochondria in the middle piece. The nucleus and mitochondria are totally embedded within an extra-acrosomal substance and the whole complex is surrounded by a "secondary acrosomal membrane".

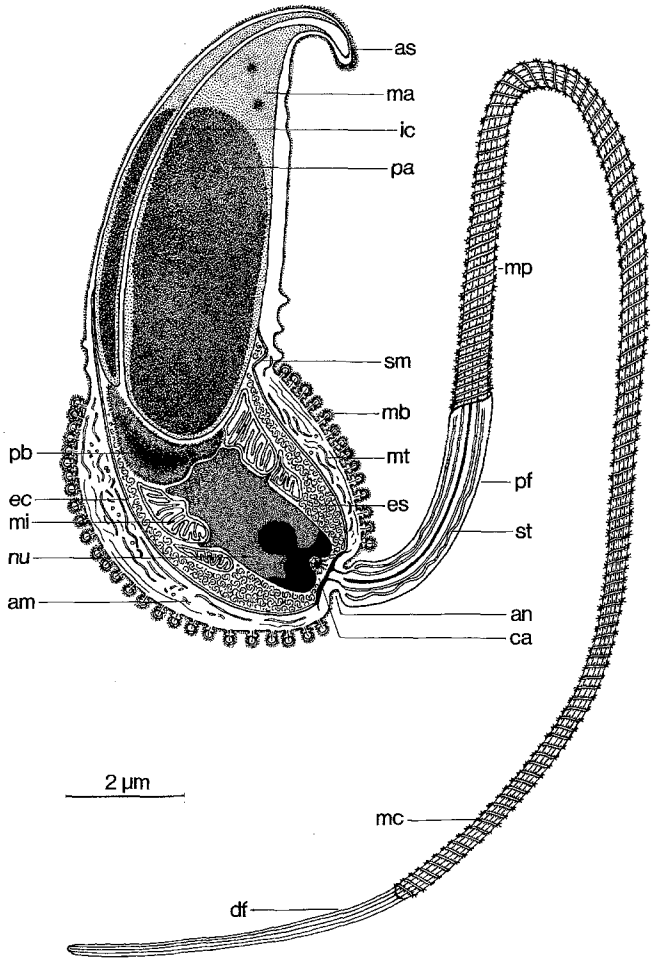


Fig. 1 *Diurodrilus subterraneus.* Semi-schematic drawing of mature spermatozoon

The total length of mature spermatozoa from the seminal vesicles is 40–48 µm, including the tail. The acrosome occupies 7–8 µm and the nucleus only 3.3–3.5 µm. Five to seven large mitochondria, each about 1.3 µm long, are in close contact with the nucleus. Between the acrosome and the nucleus, a strong, 1.8-µm-long osmiophilic postacrosomal body is present. The plasma membrane of the spermatozoon is strongly modified in the region where the extra-acrosomal manchette, the nucleus, and the mitochondria lie. Here, the plasma membrane has many small, mushroom-shaped cytoplasmic extensions, each with a maximal diameter of 0.17 µm (Figs. 13–17). The tail is 32–35 µm long and has a thick proximal part (length 4.5–5.0 µm, diameter 0.7–0.8 µm) with secondary tubuli, a middle part (length 21.0–25.0 µm, diameter 0.5–0.7 µm) with a helically arranged mucous coat, and a thin distal part (length 5.5–6.0 µm, diameter 0.3–0.4 µm) lacking a mucous coat.

III. Spermiogenesis

In the immature testis, germinal cells occur as a developmental series in the following order: spermatogonia, spermatocytes, spermatids, and spermatozoa. The spermatozoa are found only in the seminal vesicles. Spermatogenesis from spermatogonia to early spermatids is of the ordinary type described by Wingstrand (1972).

Spermiogenesis is very complex and, to facilitate its description, the development from spermatid to spermatozoon will be divided into six stages. Spermatid 1 is an early, small spermatid with intercellular bridges and one small Golgi complex. In spermatid 2, formation of the acrosome begins as a thin vesicle bounded by a single membrane and there are two Golgi complexes. Spermatid 3 is characterized by the formation of the mushroom-shaped bodies and the extra-acrosomal manchette. The intercellular bridges disappear in spermatid 4 and the secondary acrosomal membrane surrounds the nucleus and mitochondria. In spermatid 5, the Golgi complexes and other cytoplasmic organelles disappear and the condensation of nuclear chromatin is completed. Stage 6 is the mature spermatozoon, which is characterized by the formation of the acrosomal, hook-shaped "snout".

1. Spermatid 1

The cell is nearly spherical without a clear anterior-posterior polarity. It has a diameter of 2.8 µm and lacks a distinct nucleolus and flagellum; many small rod-shaped mitochondria are spread throughout the cytoplasm. The mitochondria contain a few cristae, but otherwise appear "empty". Very strong intercellular bridges are found between the four daughter spermatids (as in Fig. 3, inset). Two centrioles and a small Golgi complex are present in each. Only a few spermatids of this stage were observed, which may indicate that it is very short in duration. The cytoplasm is very osmiophilic and late in this stage nurse cells begin to surround the spermatids.

2. Spermatid 2 (Fig. 3, inset)

Spermatid 2 is lens shaped and nearly twice the size (10.5 µm long and 4.8 µm wide) of spermatid 1. The cell is divided into two compartments and is no longer spherical. The anterior compartment encloses the developing acrosome, whereas the posterior compartment contains the nucleus and is surrounded by the nurse cell. Laterally a flagellum is present, but it is without contact with the nucleus and the 10–12 mitochondria.

Within the nucleus, a finely granular nucleoplasm and a large nucleolus are found. Patches of condensed chromatin are also present. The nuclear membrane consists of outer and inner membranes with many nuclear pores. Most mitochondria begin to arrange themselves around the nucleus, although a few are still seen near the flagellar pit. The mitochondria grow in length and width and the cristae are much more complex than in stage 1.

The development of the acrosome begins as a very thin, membrane-bound vesicle in close contact with the plasma membrane. In stage 2 it is nearly spherical, 2.2 µm in diameter, and consists of an osmiophilic core and a thin, osmiophobic periphery (Fig. 11, sp_2). The two Golgi complexes are in close contact with the acrosome and form many saccules of two different types, one containing a strongly osmiophilic material, the other with an osmiophobic appearance.

The endoplasmic reticulum forms small sacs near the plasma membrane. In the region between the nucleus and

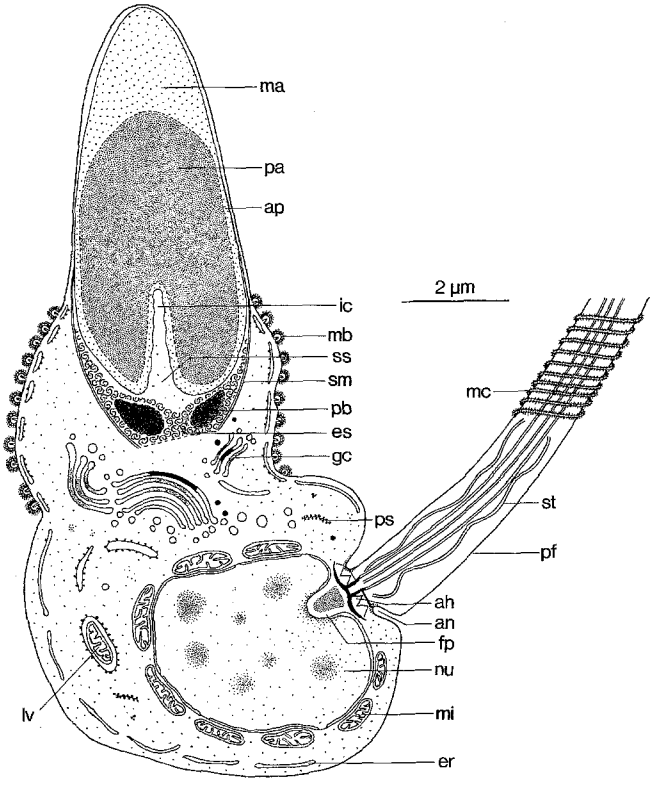
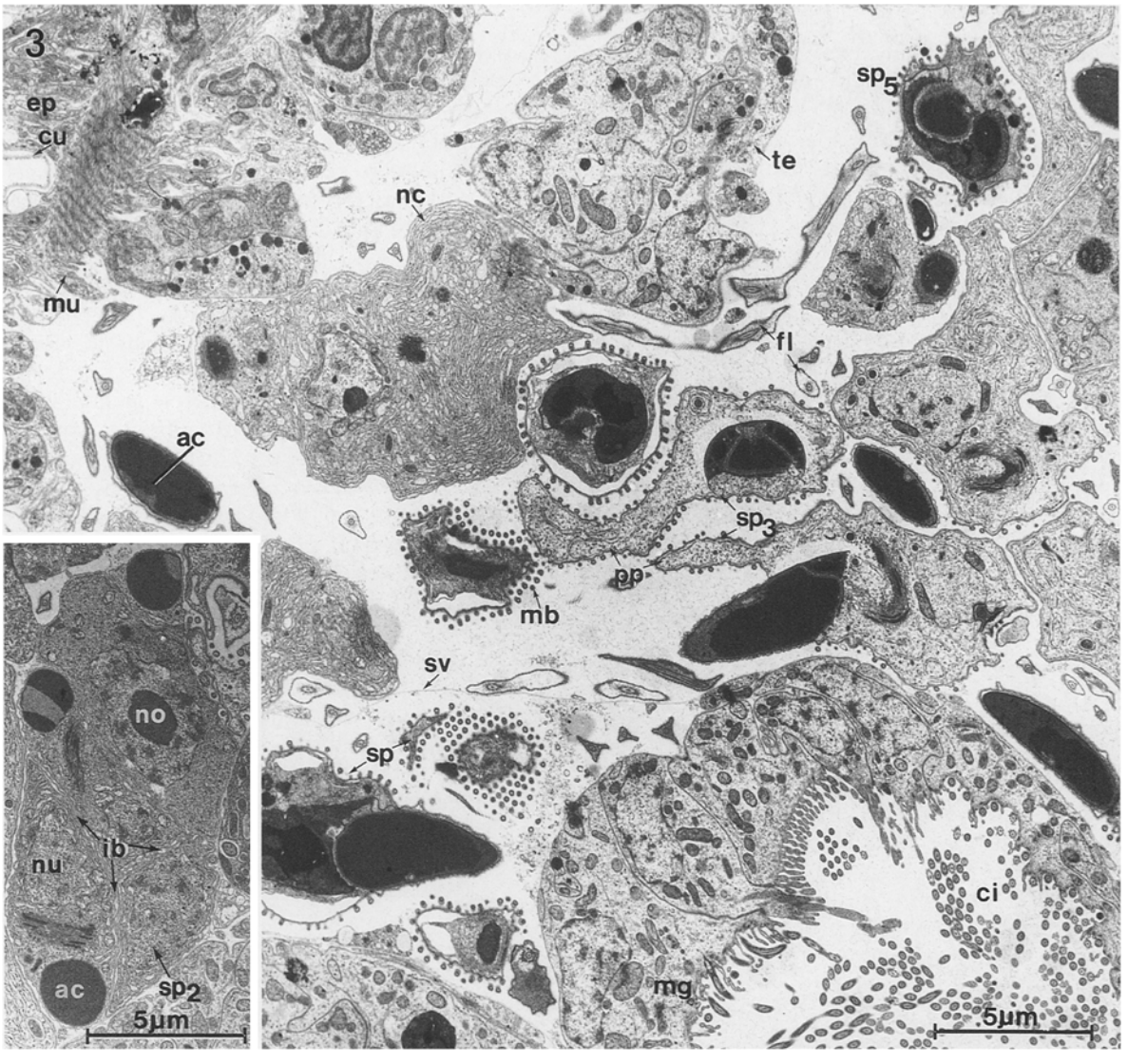


Fig. 2 *D. subterraneus*. Semi-schematic drawing of spermatid 3 (most of flagellum excluded)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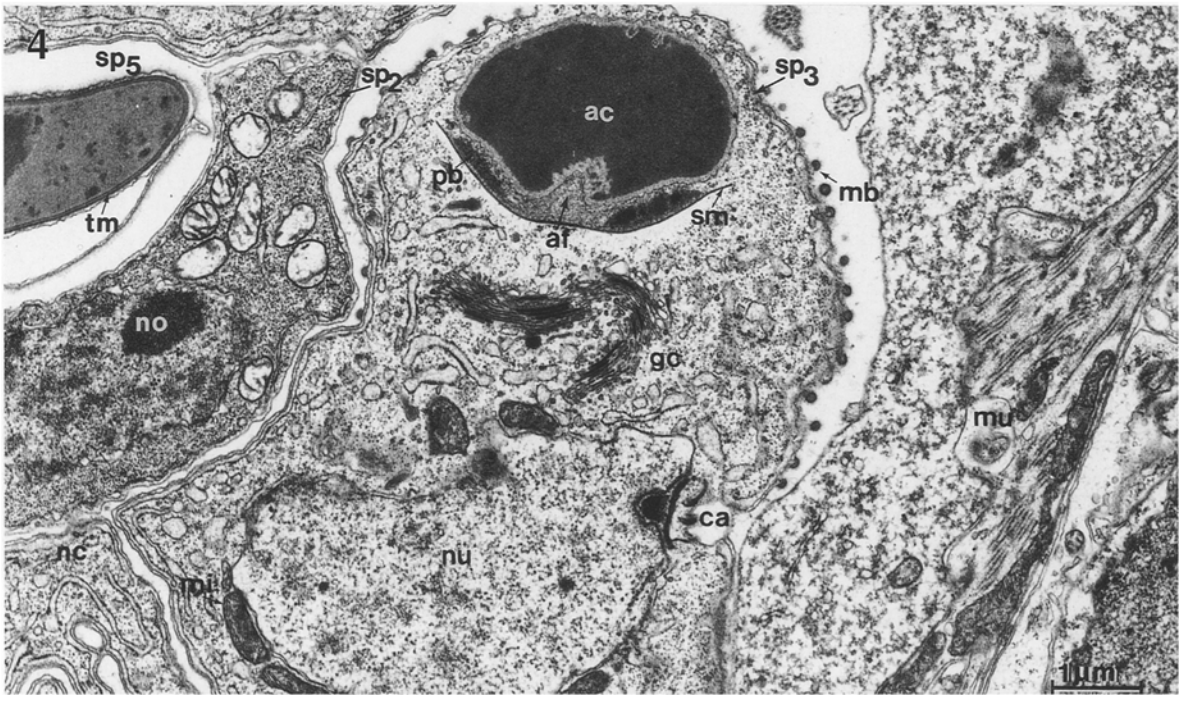
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the acrosome, the plasma membrane begins to undulate, resembling small waves. Many free ribosomes are present in the cytoplasm.

3. Spermatid 3 (Figs. 2, 4–8, 13, 14)

The body of spermatid 3 has attained the maximal width of 7.5 μm and a length of 14.7 μm (Fig. 2). It is very irregular and bears many pseudopodia (Fig. 3). These spermatids may be amoeboid, they lack contact with the nurse cells, and contain many polysomes and a rich ergastoplasm. A characteristic feature is the presence of endoplasmic reticular sacs in regular contact with the plasma membrane, where they form the mushroom-shaped bodies. Late in stage 3 the pseudopodia disappear.

The nucleus is in contact with the flagellar region, the centriolar adjunct. The nucleus is still spherical and the nucleoplasm is pale and finely granular with 3–5 patches containing condensed granular chromatin (Fig. 8). Where the nucleus is in contact with the centriolar adjunct, the outer and inner nuclear membranes are fused and the nucleoplasm is condensed (Fig. 4). All of the five to nine mitochondria are in close contact with the nucleus and have very complex cristae. A few degenerated mitochondria are seen in lysosomic vesicles (Fig. 2).

The neck region, in which the head and flagellum are connected, has been established. At an early point in stage 3, the distal centriole has a peripheral location in the cell body at the base of the flagellum. The proximal centriole has disappeared and the distal centriole forms a large anchoring fiber apparatus consisting of nine radiating fibers (Figs. 4, 5). This complicated apparatus is later invaginated into the cell body and forms a deep flagellar pit or funnel.

Late in stage 3 the cell loses its amoeboid appearance, the centriolar anchoring apparatus comes into contact with the nucleus, and an apical membrane is formed close to the nuclear membrane. From the apical membrane, a granular material forms and is invaginated in the nucleus as the centriolar adjunct (Fig. 4). The axoneme in the tail consists of the normal 9+2 pattern of microtubuli. In the proximal part of the flagellum, nine accessory tubules are present, perhaps developed from the anchoring fiber apparatus (Fig. 10). A large Golgi complex is found in connection with the formation of the centriolar adjunct and the anchoring fiber apparatus.

However, the most significant organelle in spermatid 3 is the giant acrosome. From being a small spherical sac in stage 2, it develops into a long conical structure, twice the size of the nucleus (Figs. 4, 6, 7).

Late in stage 3 the spermatid is curved in the middle and thus, the acrosome is oriented perpendicularly to the nucleus (Figs. 2, 4). The nucleus and the acrosome are still separated by a large amount of cytoplasm containing three enormous Golgi complexes. At the beginning of the elongation of the acrosomal vesicle, an acrosomal filament grows into it from behind (Figs. 2, 4, 7). The acrosomal filament originates from extra-acrosomal material separated from the cytoplasm by a secondary acrosomal membrane (Fig. 4). The extra-acrosomal material is differentiated as a subacrosomal substance, which contains the acrosomal filament with longitudinal subfilaments (Fig. 13) and two postacrosomal bodies consisting of a condensed amorphous material. In a cross-section (Fig. 3, upper sp_3) of the acrosomal complex, a very characteristic configuration may be seen. The acrosome is triangular and formation of the acrosomal filament begins as an invagination from one of its corners (Fig. 13, sp_3). Later in spermatid 3, the filament is completely submerged into the acrosome, creating a peripherally placed intra-acrosomal canal (Figs. 9, 13) that is open at both ends.

The formation of the tertiary acrosomal membrane has begun (Figs. 4, 7 inset, 11, 13; not included in Figs. 1, 2). Its development starts apically and it does not completely envelop the acrosome until the spermatid 5 stage (see below).

At the sides of the acrosome are the two postacrosomal bodies, surrounded by the secondary acrosomal membrane. However, this membrane does not enclose the whole acrosomal complex. It ends blindly and the extra-acrosomal manchette still lies freely in the cytoplasm in connection with the Golgi complex. A very characteristic feature is that all organelles such as ribosomes and endoplasmic reticulum are lacking in the cytoplasm around the secondary acrosomal membrane.

The mushroom-shaped bodies are developing in the region between the acrosome and the nucleus, where the plasma membrane was undulated in stage 2. Each mushroom-shaped body is established as a thickening of the plasma membrane with its glycocalyx. The nearby specialized endoplasmic reticulum sends out small offshoots into the bodies. In stage 3, only the head of each body is formed (Figs. 13, 14; for a full description, see below).

4. Spermatid 4 (Figs. 9, 14 inset)

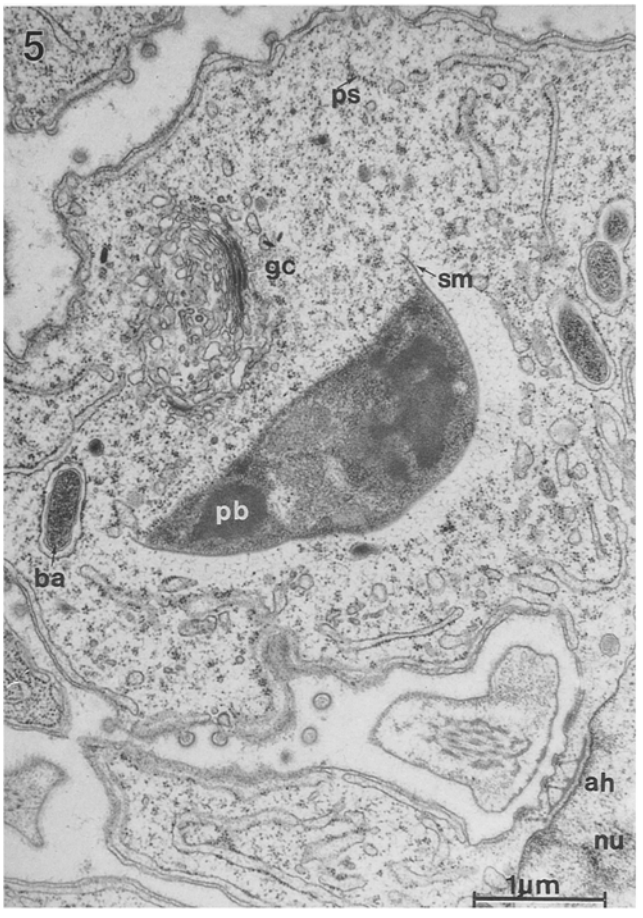
This stage is characterized by the establishement of contact between the nucleus and the mitochondria and by the surrounding of the acrosomal-nuclear complex by the secondary acrosomal membrane. The volume of cytoplasm decreases and spermatid 4 is, therefore, smaller than spermatid 3.

The nucleolus is large, containing helically arranged chromatin (Fig. 8). Several patches of condensed chro-

Figs. 3, 4 *D. subterraneus*

Fig. 3 Low magnification micrograph showing "testis" with nurse cells and spermatids in different stages of development lying free in coelom between intestinal epithelium (lower right) and outer body wall (upper left). *Inset* shows spermatids in stage 2 connected by intercellular bridges

Fig. 4 Spermatid 3, early stage, showing centriolar adjunct apposed to nucleus, well-developed Golgi complexes, developing mushroom-shaped bodies, and developing acrosome with postacrosomal bodies, acrosomal filament, and secondary acrosomal membrane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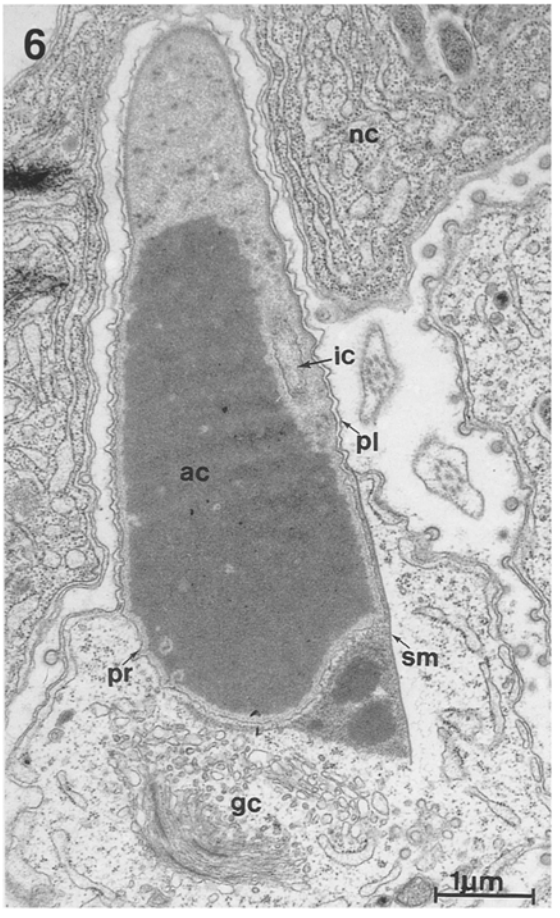
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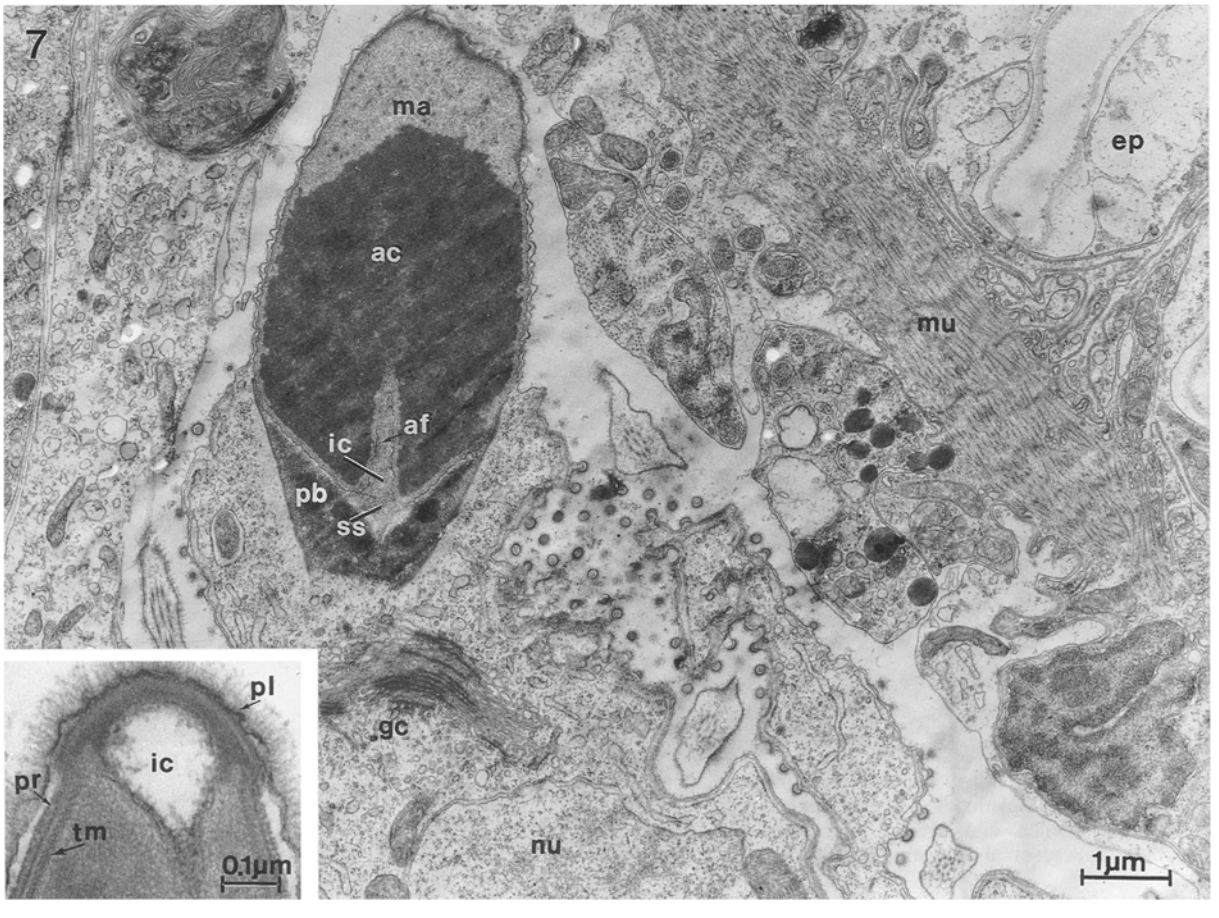
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matin are present in the nucleoplasm as strong osmiophilic spots. The nuclear membrane is fused in those regions where contact is established with the centriolar adjunct and the extra-acrosomal manchette. The number of mitochondria is still between five and nine and they have reached the maximal length of 1.3 μm. The mitochondria are aggregated close to the nucleus and the border between them cannot be distinguished by the light microscope.

The centriolar anchoring apparatus has lost contact with the nucleus. In longitudinal section the apparatus resembles a double hammerhead (Fig. 10; cross-section on Fig. 11 inset). The flagellar pit is reduced and the anchoring apparatus is once again located near the plasma membrane. The centriolar adjunct is totally invaginated into the nucleus as a small sphere. The retraction of cytoplasm from the proximal part of the flagellum begins from the annular thickening of the cell membrane at the bottom of the flagellar pit. The middle of the flagellum is covered by a spiralized glycocalyx.

The formation of the giant acrosome is complete and the extra-acrosomal material forms a protective manchette around the mitochondria and nucleus (Fig. 9). This material consists of two amorphous postacrosomal bodies lateral to the acrosome and a microfilamental material which totally surrounds the nuclear-mitochondrial complex. Late in stage 4, the secondary acrosomal membrane reaches and fuses with the centriolar anchoring apparatus. The nucleus and the mitochondria are now totally separated from the cytoplasm.

The formation of the mushroom-shaped bodies is almost complete. They are densely placed on the plasma membrane from the posterior one-third of the acrosome to the flagellar pit. Each body is developing the characteristic "velum" (Fig. 14, inset; see below). Inside the bodies, microfilaments develop from the endoplasmic reticulum. The head of each body is covered with a thick glycocalyx (Fig. 17).

5. Spermatid 5 (Fig. 10, 11, 15)

The two last Golgi complexes found posteriorly around the flagellar pit disappear. The cytoplasm outside the secondary acrosomal membrane is reduced and the cytoplasm is filled with a new organelle, microtubuli (Figs. 11, 16 inset). Ultimately, when the spermatid is transformed into the mature spermatozoon, the microtubuli will be substituted by accessory membranes.

The chromatin of the nucleus is totally condensed. The posterior part of the nucleus, which is in contact with the centriolar adjunct, is more osmiophilic than the anterior part (Fig. 10).

Inside the membrane of the acrosomal vesicle, the tertiary membrane had completed formation. In cross-section, four different membrane systems may be seen in the middle of the acrosome: 1) the plasma membrane (outermost), 2) the secondary acrosomal membrane, which also surrounds the nuclear-mitochondrial complex, 3) the membrane of the acrosomal vesicle, and 4) a thick tertiary membrane developed inside the acrosome (Fig. 13). Furthermore, the intra-acrosomal canal contains numerous acrosomal filaments composed of actin. This canal is surrounded only by the membrane of the acrosomal vesicle.

The flagellar funnel has disappeared, but the annular thickening of the cell membrane is still present and reinforced (Fig. 10). The cytoplasm at the proximal part of the flagellum is reduced, the latter consisting only of the axoneme and the secondary microtubuli.

The mushroom-shaped bodies are densely packed on the plasma membrane from the postacrosomal bodies to the flagellar pit. The formation of the bodies has been completed and the endoplasmic reticulum has disappered (Figs. 15, 16). Each body is a modified microvillus with a thick glycocalyx, a plasma membrane, and six microfilaments (Figs. 16, 17).

The "middle piece" of spermatid 5 consists of accessory membranes and microvilli, the extra-acrosomal manchette, five to seven mitochondria, and the nucleus. A nucleolus is lacking, but the centriolar adjunct (flagellar pit) superficially resembles one (Fig. 10).

6. Final transformation (Figs. 11, 12)

Spermatid 5 (Fig. 11) continues directly into the mature spermatozoon (Fig. 12). The final transformation takes place in the seminal vesicles. The small, hook-shaped apical part of the acrosome becomes apparent. The acrosomal material is condensed in two parts. The microtubuli in the middle piece disappear and are substituted by irregular accessory membranes.

IV. Mushroom-shaped bodies (Figs. 13–17)

The plasma membrane of the so-called "middle piece" is modified and covered with small dots resembling mushrooms. We have called these unique structures mushroom-shaped bodies (not to be confused with the presumably associative centers dorsally attached to the brains of, e.g., polynoid polychaetes).

During spermiogenesis, the development of the mushroom-shaped bodies could be followed step by step. The first indications of them are seen in spermatid 2. The en-

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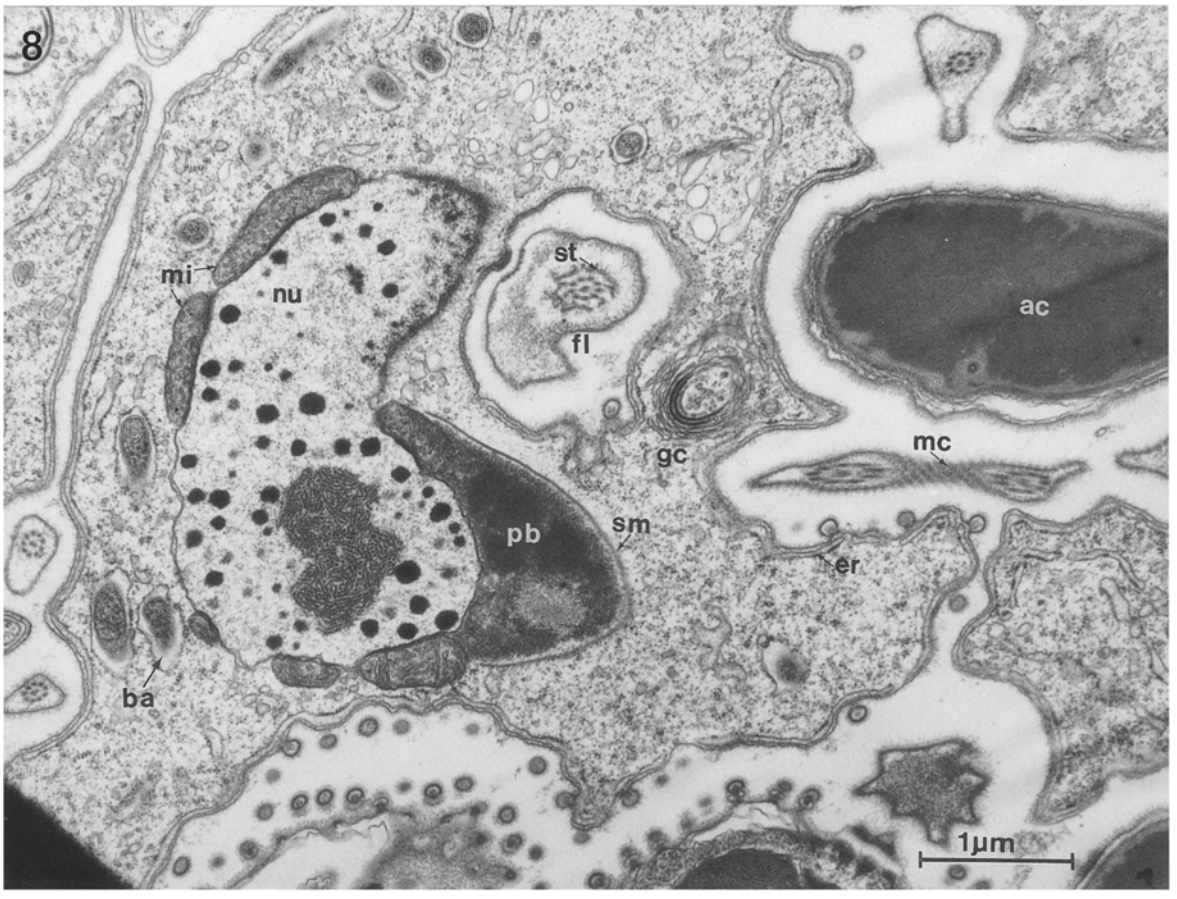
Figs. 5–7 *D. subterraneus,* spermatid 3

Fig. 5 Early stage, centiolar anchoring apparatus of another spermatid may be seen at lower right

Fig. 6 Middle stage, showing elongation of acrosome

Fig. 7 Late stage, showing developing acrosome with acrosomal filament, postacrosomal bodies, secondary acrosomal membrane, and nearby Golgi complexes. *Inset* shows tip of acrosome (future acrosomal snout) with tertiary acrosomal membrane, late stage 3 spermatid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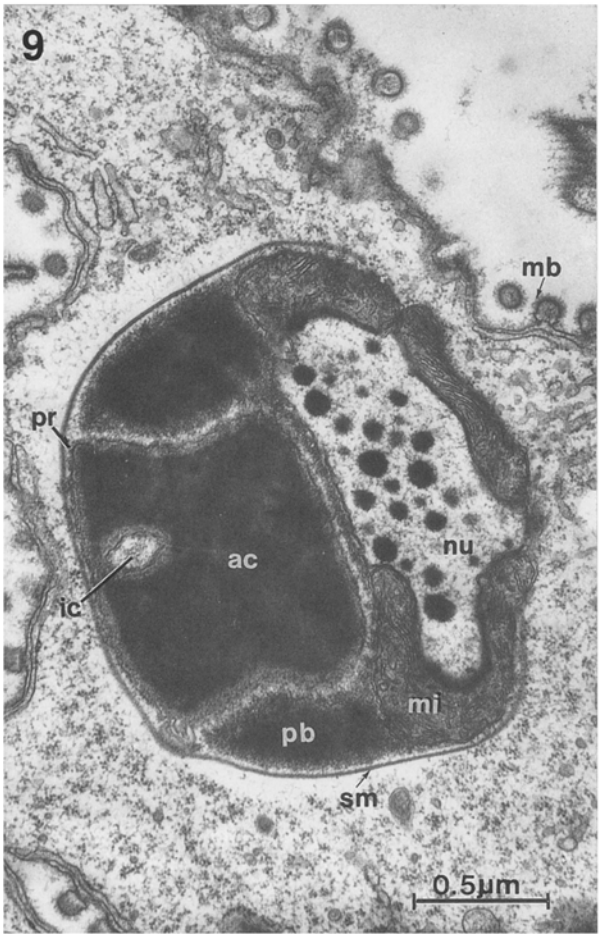
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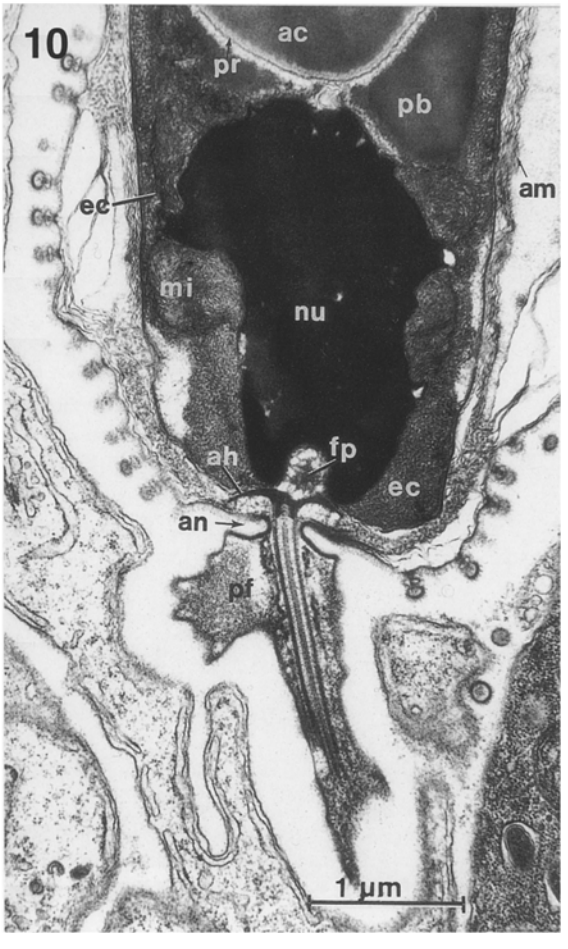
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doplasmic reticulum (ER) forms small sacs near the plasma membrane of the "middle piece". The surface of the ER is covered by a large number of ribosomes (rough ER) and small vesicles produced by exocytosis are seen fusing with the plasma membrane. In the area of fusion, a glycocalyx is beginning to form.

In early spermatid 3, the first indication of the mushroom-shaped head is seen as a lunular thickening of the plasma membrane (Fig. 13). The distance between the centers of adjacent heads is about 0.2 μm and each ER sac forms six to seven bodies in a very regular pattern. In late spermatid 3, the entire head has been formed (Fig. 14).

In a cross-section of the head, four layers can be distinguished (Figs. 16, 17). The surface of the head is covered by 20-nm-long glycocalyx filaments, which are oriented as a halo-layer around the head. Each filament bears one or two osmiophilic dots of globular proteins. The second layer is 10 nm thick, strongly osmiophilic, and contains a grid-like substance. This is followed by a 2-nm-thick osmiophobic layer. Finally, the lumen of the head is covered by a plasma membrane of the normal, unit membrane type.

In early spermatid 4, the "velum" of the mushroom-shaped body is formed. The velum lacks the grid-like second layer mentioned above, but has instead an amorphous layer below the plasma membrane, inside the body. Late in spermatid 4, microtubuli (diameter=20 nm) are formed in the cytoplasm close to the endoplasmic sacs (Fig. 16 inset). Inside the bodies, non-homologous microfilaments of a lesser diameter (10 nm) are developed from the ER.

In spermatid 5, the ER has disintegrated and many irregular, accessory membranes are formed in the adjacent cytoplasm. The formation of the bodies has been completed. Each contains six strongly osmiophilic microfilaments. They may have a supporting function for the mushroom-shaped body. The entire structure may be regarded as a highly aberrant microvillus, completely unknown from the spermatozoa of other annelids.

D. Discussion

I. Comparisons with earlier descriptions of spermatozoa in species of *Diurodrilus*

Previous light microscopical studies of the spermatozoa of *Diurodrilus dohrni* (Gerlach, 1952) and their spermiogenesis in *D. ankeli* indicated that spermatozoa in representatives of this genus have a typical, large, rounded head with a small acrosome and a large nucleus, a middle region with unmodified mitochondria, and a normal flagellum as in primitive spermatozoa (Gerlach 1952; Ax 1967). However, the head is unusually large (about 12 μm long, according to Ax 1967), causing Franzén (1956, citing Jägersten) to interpret thse spermatozoa as aberrant.

Franzén further regarded the sperm as modified because the mature spermatozoon retains a nucleole within the nucleus. However, this interpretation was seen to be problematical when Feulgen-treated spermatozoa were examined in a preliminary analysis. The so-called "nucleus with nucleole" in the large head is Feulgen negative, whereas a small area near the flagellum reacts positively.

According to Mock (1981), the head of the mature spermatozoon in *D. subterraneus* is completely occupied by a "very large, two-partite, ovoid acrosome". The nucleus and mitochondria are not specifically mentioned, but were presumably thought to occupy the "bead-like middle piece" (maximum diameter ca. 2.3 μm, according to his Fig. 4e) between the head and the flagellum. There is some doubt concerning the specific identity of the spermatozoa described by Mock (1981) as belonging to *D. subterraneus*. In *D. ankeli*, the acrosome has a distal "hook" (Ax 1967) or snout, as does that described in this paper. This hook was not observed on the spermatozoa illustrated by Mock. His measurements of the "head" region correspond well to ours (12, 11 μm long, respectively). However, the absence of an acrosomal snout on the cells observed by Mock could be an indication that they were either not completely mature or came from a different species of the genus.

No distinct penis structure was found in *D. subterraneus*, but in *D. westheidei* Kristensen and Niilonen, 1982, clusters of sperm that had been inseminated into the female were found (Kristensen and Niilonen 1982). The anal cone may function as a copulatory apparatus in these two species. In *D. ankeli*, two club-like cuticular structures are present at the point where the opening of the genital duct is seen in *D. subterraneus* (see Ax 1967). They may have a penis function, which would agree well with Franzén's conclusion that fertilization in species of *Diurodrilus* is internal (Franzén 1956).

The spermatozoa of *D. subterraneus* exhibit a number of unusual features. Most noticeably, the acrosomal development is of hitherto unknown proportions among polychaetes (comparisons with information provided by Franzén and Rice 1988; Jamieson and Rouse 1989). Other striking features are the mushroom-shaped bodies, the secondary acrosomal membrane surrounding a nuclear-mitochondrial complex, the presence of a presumably amoeboid stage (in spermatid 3) with pseudopodia, the invagination of the centriolar adjunct within the nucleus, and the presence of post-acrosomal bodies, a tertiary acrosomal membrane, a centriolar anchoring apparatus consisting of nine radiating fibers plus a double hammer-

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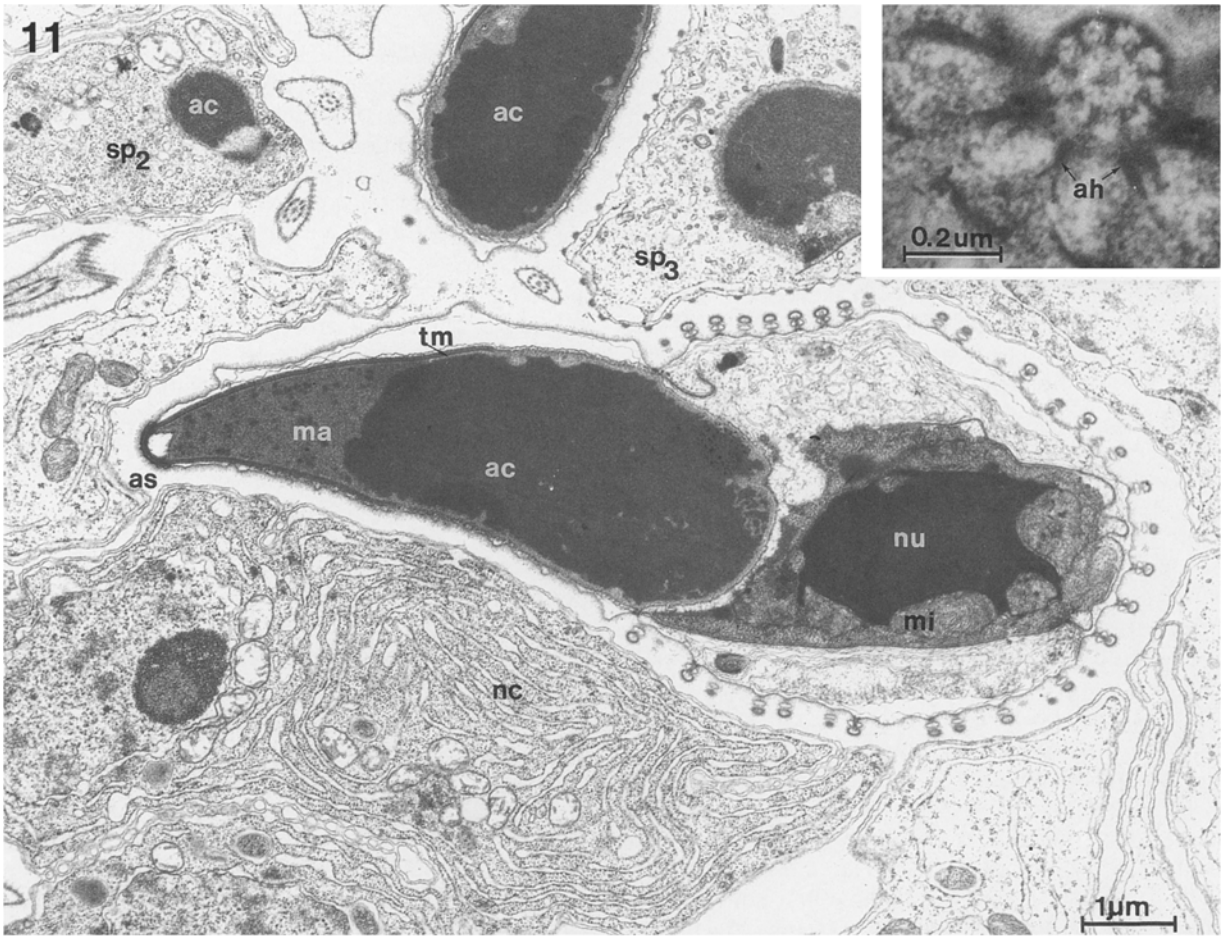
Figs. 8–10 *D. subterraneus*

Fig. 8 Spermatid 3, middle stage, showing chromatin in nucleus beginning to condense

Fig. 9 Spermatid 4, slanted cross-section of nucleus, mitochondria, and acrosomal complex, showing peripheral location of intra-acrosomal canal

Fig. 10 Spermatid 5, middle piece and base of flagellum, showing nucleus with condensed chromatin, centriolar adjunct, and anchoring fiber apparatus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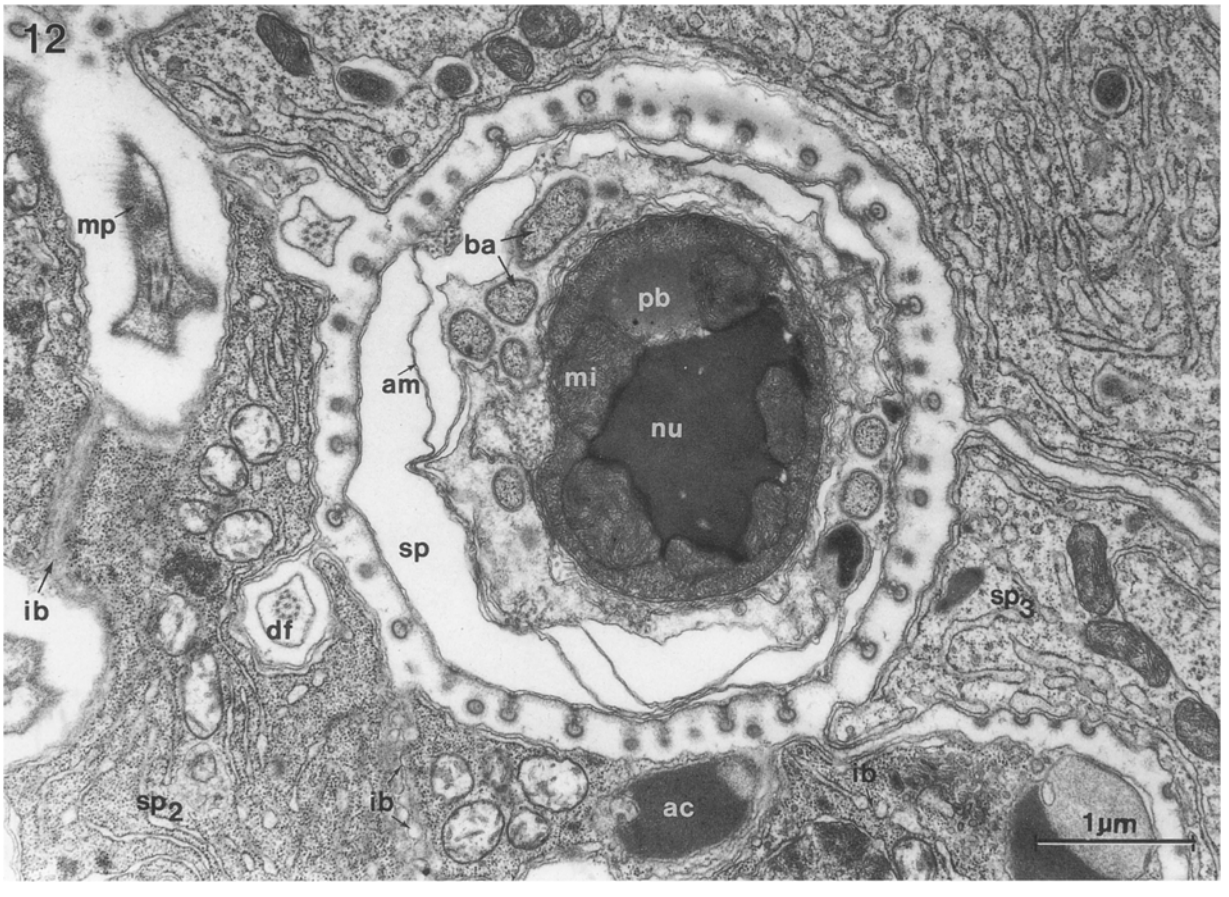
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head-shaped anchoring plate, accessory tubules in the proximal region of the flagellum, and a helical mucous coat on the middle region of the flagellum. Some of these traits are discussed in the following.

II. Comparisons with spermatozoa found among other so-called "archiannelids"

The ultrastructure of spermiogenesis and the spermatozoa of the so-called Archiannelida was first investigated by Franzén (1956, 1977), but only in three genera: *Polygordius, Protodrilus,* and *Dinophilus*. The spermatozoa of *Polygordius lacteus* (Schneider, 1868), are unspecialized with a typical short head consisting of an acrosome and a nucleus, a midpiece containing unmodified mitochondria, and a normal flagellum (Franzén 1977). This type of spermatozoon is very similar to that found in other animals with external fertilization (Afzelius and Franzén 1971; Baccetti and Afzelius 1976). Members of the genera *Protodrilus* and *Dinophilus* have filiform spermatozoa, as is often the case in species with internal fertilization (via spermatophores and hypodermic injection, respectively).

The spermatozoa in species of the dorvilleid genus *Trilobodrilus* are of the same type as those in *Dinophilus*, although more highly specialized (Scharnofske 1986; both genera were previously placed in the family Dinophilidae, but have recently been referred to Dorvilleidae, see Eibye-Jacobsen and Kristensen 1994). Franzén and Sensenbaugh (1984) described the spermatozoa of *Nerilla antennata* (O.F. Schmidt, 1848), which resemble those of representatives of *Protodrilus* and *Dinophilus* in being filiform, but show a number of autapomorphic specializations (e.g., its supporting, ribbon-like structure). An aberrant, aflagellate type of spermatozoon has been described for *Protodriloides symbioticus* (Giard 1904; see Jouin 1978). This morphology may somehow be adapted to external fertilization within a cocoon (a similar correlation is found in species of *Ophryotrocha* (Dorvilleidae); see discussion by Pfannenstiel and Grünig 1990).

Representatives of several other groups, that like *Diurodrilus* have previously been referred to as archiannelids, also have spermatozoa in which the acrosome is strongly elongated, e.g., *Dinophilus* and *Trilobodrilus* (see Scharnofske 1986) and *Protodrilus* (see von Nordheim 1989). However, in none of these cases is the acrosome so much larger than the nucleus as in *Diurodrilus*. There are other important differences in the sperm of species belonging to these three genera, as well as to *Nerilla* (Franzén and Sensenbaugh 1984), in comparison to *Diurodrilus*. Thus, the spermatozoa found in all four genera have a greatly elongated nucleus and large, elongate mitochondria. The mitochondria are highly specialized and reduced in number (four are often present, only one in *N. antennata*). In *D. subterraneus* and presumably also other members of the genus, the nucleus and mitochondria show no such features and differ from those of the "primitive" sperm type (the ectaquasperm of Rouse and Jamieson 1987) only in the location of the mitochondria around the nucleus and the envelopment of this entire complex by a secondary acrosomal membrane.

In most polychaete spermatozoa, the sequence of organelles from anterior to posterior is: acrosome, nucleus, mitochondria, axoneme. Even in highly specialized sperm, this sequence is visible (e.g., species of *Protodrilus*). This is also the case in representatives of *Trilobodrilus, Dinophilus,* and *Nerilla*, although there is a considerable degree of overlap between the regions. In less extreme cases, overlap usually involves the mitochondria, which can be placed around the proximal region of the axoneme (e.g., *Polydora ciliata,* (Johnston, 1838), see Franzén 1974; *Protodrilus purpureus,* (Schneider 1868; see von Nordheim 1987) or around the nucleus (*D. subterraneus*). Considering the function of the mitochondria during spermatozoan movement, overlap may generally tend to make the delivery of energy to the axoneme more efficient. From this point of view, the position of the mitochondria in *D. subterraneus,* around the nucleus and at a considerable distance from the centriolar adjunct, is somewhat puzzling.

Supporting structures, which seem to be characteristic of spermatozoa specialized for internal fertilization, have been described for several different groups of polychaetes. Such structures include more or less elaborate rods in members of *Trilobodrilus* and *Dinophilus* (Scharnofske 1986), vesicles in species of *Protodrilus* (von Nordheim 1987), ribbon structures in *N. antennata* (Franzén and Sensenbaugh 1984), and double supporting walls in *Hesionides arenaria* (Friedrich, 1937; Westheide 1984). It is clear that the structures involved are analogous, probably having the same basic function but being independently derived by convergence (interestingly, although obviously belonging to the family Hesionidae and having never been considered "archiannelids", the genus *Hesionides* consists of interstitial forms, as do all the other genera mentioned above). It is possible that the secondary acrosomal membrane of *Diurodrilus* has the same function. In any case, this membrane appears to be an autapomorphic feature of the genus.

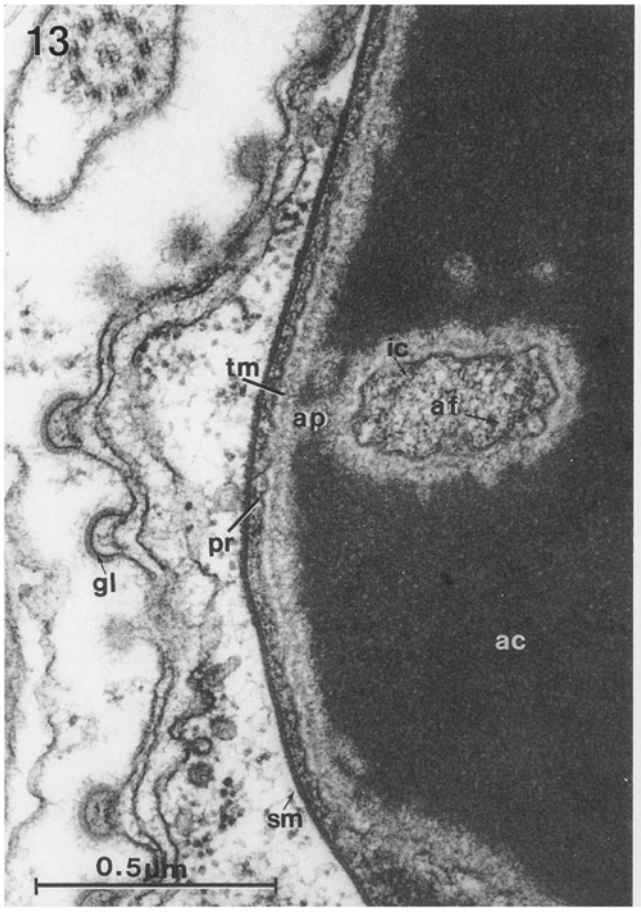
III. Comparisons with spermatozoa among other polychaetes and the clitellates

Baccetti and Afzelius (1976) noted that the flagellum of modified spermatozoa often have nine accessory ele-

Figs. 11, 12 *D. subterraneus*

Fig. 11 Sagittal section of spermatid 5, showing zones of acrosome, nucleus surrounded by mitochondria, fully developed mushroom-shaped bodies, and a small amount of cytoplasm. *Inset* shows transverse section of axoneme and anchor complex

Fig. 12 Transverse section of mature spermatozoon, showing nuclear-mitochondrial complex, chromatin densely condensed; apparently endosymbiotic bacteria can be seen outside the secondary acrosomal membrane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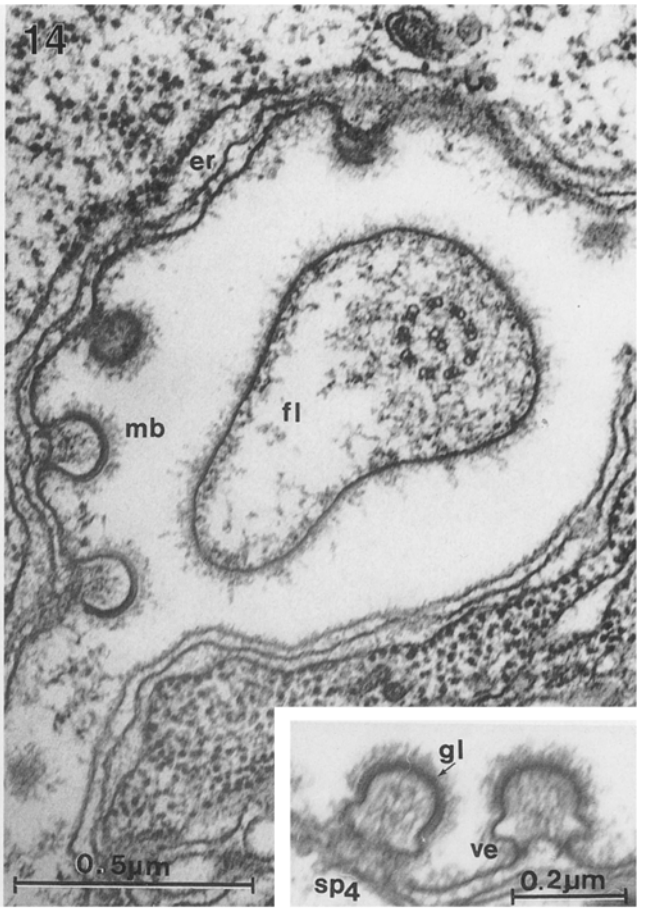
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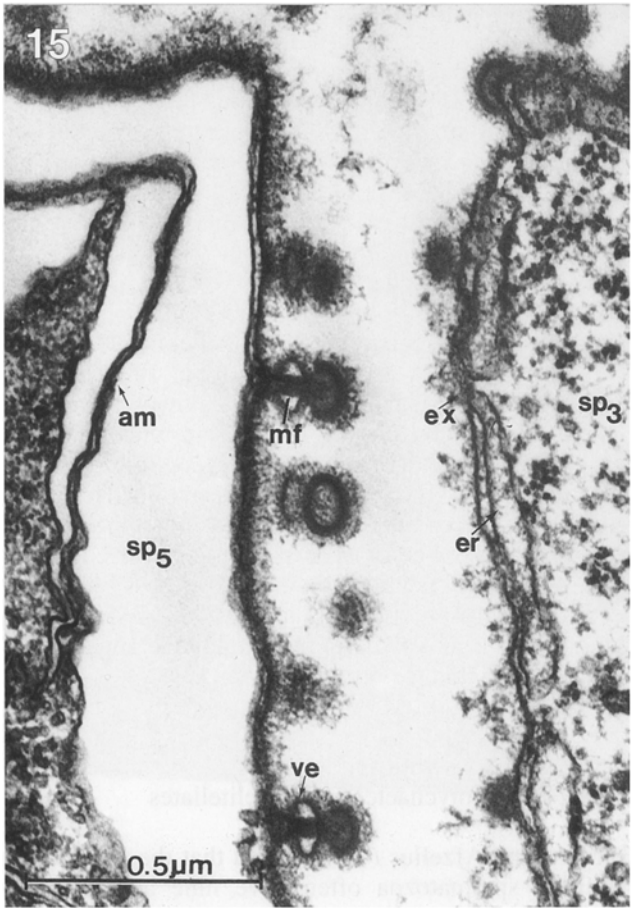
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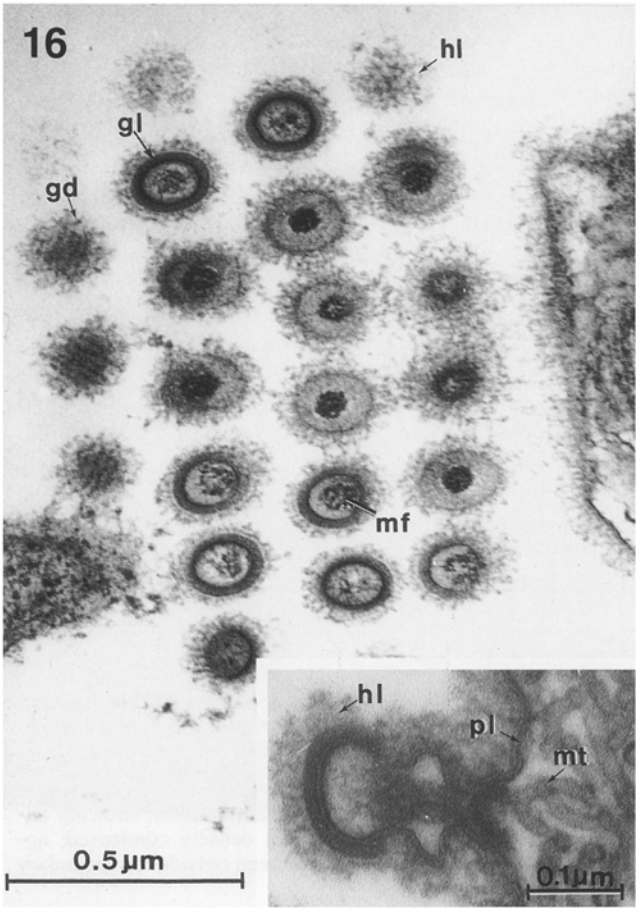
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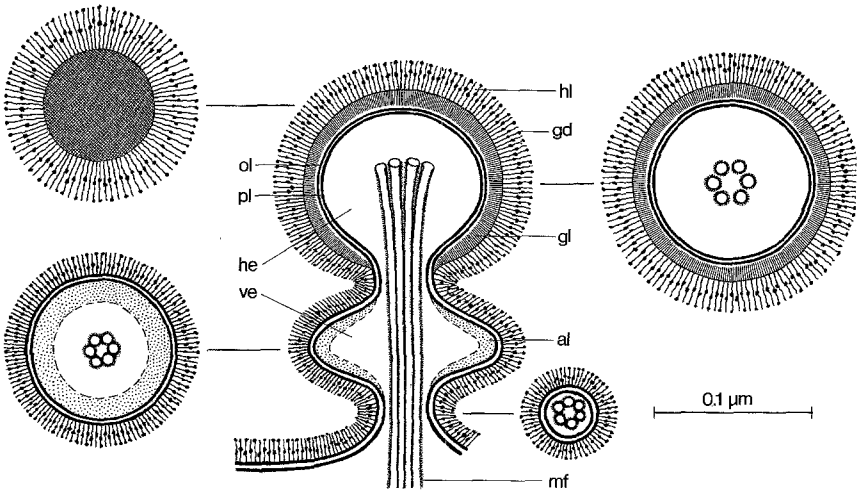

Fig. 17 *D. subterraneus.* Semi-schematic drawing of mushroom-shaped body from mature spermatozoon: longitudinal section and four transverse sections at indicated levels

ments which are peripheral to the 9+2 microtubules. They reported that these elements had thus far been found in Annelida (Oligochaeta), Mollusca, Onychophora, Arthropoda, and Chordata.

In polychaetes, peripheral tubules occur in the spermatozoa of representatives of the oligochaete-like genus *Questa* (Jamieson 1983a, b). There are three extra microtubules outside each of the nine doublets in the proximal part of the flagellum. In the same region, *D. subterraneus* has one microtubule peripheral to each of the nine doublets. The nine accessory elements in *D. subterraneus* are perhaps not true peripheral microtubules like those found in onychophorans and insects, as they describe a spiral around the axoneme. The function of these peripheral microtubules is not known.

In the spermatids described here, the proximal centriole disappears in stage 3 and the distal centriole forms a large anchoring apparatus consisting of nine radiating fibers ("satellite rays", see Rouse 1988a). The rays branch before they join the plasma membrane (Fig. 11, inset). Branching of the rays in the anchoring apparatus is a widespread feature in polychaete spermatozoa (Jamieson and Rouse 1989) and is also found in other so-called archiannelids, such as *Polygordius lacteus.* This character, thus, probably lacks great phylogenetic value among polychaetes. On the other hand, the presence of an anchoring plate resembling a double hammerhead is unique for *Diurodrilus.* In polychaetes, anchoring plates have only been found in *Owenia fusiformis* delle Chiaje, 1844 (Rouse 1988a) and *Prionospio* cf. *queenslandica* (see Rouse 1988b).

A large number of papers have been published describing the spermatozoan ultrastructure of polychaete species belonging to families that have never been considered archiannelids (many of them are reviewed by Jamieson and Rouse 1989). In general, comparisons of the spermatozoa found among species of *Diurodrilus* with these descriptions reveal only coincidental resemblances.

An example is the presence of a central canal in the acrosome of *Micromaldane* sp. (Rouse and Jamieson 1987). Closer study shows important differences, such as the fact that the canal found in this species of *Micromaldane* is centrally placed (not peripheral) and is closed at its anterior end. It is also interesting to note that the acrosomal material of this species is divided into an osmiophobic anterior region and an osmiophilic posterior region, and that the axoneme is deeply embedded in the nucleus, as in *D. subterraneus.* However, the differences in detail are numerous and there is no obvious indication of a close phylogenetic relationship between maldanids and diurodrilids.

The spermatozoa of members of *Diurodrilus* resemble those in representatives of Clitellata in having only one centriole. In Clitellata, this strongly modified centriole is called the centriolar equivalent. However, in *Diurodrilus,* the centriole is implanted in the nucleus, in clitellates at the caudal end of the mitochondrial derivatives of the midpiece. Another superficial resemblance is that of the acrosomal tube found in oligochaetes (Jamieson 1984, 1986; Jamieson et al. 1987) and hirudineans (Garavaglia et al. 1974) to the secondary acrosomal membrane in *D. subterraneus.* However, in clitellates the acrosomal tube surrounds the acrosomal rod and is entirely anterior to the nucleus. The secondary acrosomal membrane of *D. subterraneus* surrounds the nuclear-mitochondrial complex and any structure resembling an acrosomal rod is lacking. The spermatozoa in members of *Diurodrilus* also lack the above-mentioned midpiece consisting of mitochondrial derivatives separating the nucleus from

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Figs. 13–16 *D. subterraneus,* mushroom-shaped bodies

Fig. 13 Early spermatid 3, bodies without stalk or tubules; the acrosomal filament submerged in the acrosome may also be seen

Fig. 14 Late spermatid 3, bodies with fully developed head. *Inset* shows bodies of spermatid 4

Fig. 15 Spermatid 5, bodies in longitudinal section, complete with stalk, velum, and microfilaments

Fig. 16 Spermatozoon, bodies in transverse section, complete. *Inset* shows longitudinal section at high magnification

the axoneme. Also absent is the central flagellar sheath which is found in Branchiobdellida, Hirudinea, and certain oligochaetes (Ferraguti and Lanzavecchia 1977). Ferraguti (1984) concluded that the presence of a central flagellar sheath should be regarded as a plesiomorphic feature among the Clitellata. Thus, the structure of the spermatozoa in representatives of *Diurodrilus* does not indicate any close relationship between this genus and any member of the Clitellata.

IV. Diurodrilidae and Gnathostomulida

It appears that the information presented here does not provide positive evidence for the membership of Diurodrilidae in any particular subgrouping of the Polychaeta and practically precludes any affiliation with the Clitellata. In the following, the spermatozoan structure of *Diurodrilus* will be compared to that of a few groups of psammobiontic "worms" that are generally thought to be associated with Annelida (e.g., Nielsen 1995), primarily the Gnathostomulida (Kristensen and Nørrevang 1977).

The mushroom-shaped bodies found on the surface of spermatozoa in *D. subterreaneus* are certainly the homologues of microvilli, such as those found on the spermatozoa of certain gnathostomulids (Graebner 1969, where the microvilli are called "Füßchen"). Both contain a central core of microfilaments and have an underlying layer of microtubuli. Furthermore, their lengths and minimum diameters are comparable [in *Gnathostomula paradoxa* (Ax, 1956); length shorter in *G. axi* (Kirsteuer, 1964) and much greater in *G. jenneri* (Riedl, 1969)].

However, very important differences are present. At the functional level, it is worth noting that the spermatozoa of these species of *Gnathostomula* are aflagellar, and movement is performed with the aid of the microvilli. In representatives of *Diurodrilus*, the mature spermatozoa are flagellar and the mushroom-shaped bodies are not involved in their movements. Two types of sperm movement were observed in live specimens of *D. subterraneus*. In early spermatid 3, the germ cell has several pseudopodia in the "middle piece" and the spermatid moves as an amoeboid cell. Later, when the mushroom-shaped bodies are fully developed, the pseudopodia disappear and strong flagellar movement is observed.

There are also considerable structural differences between microvilli and mushroom-shaped bodies. For example, the microvilli of *G. paradoxa* apparently contain four microfilaments each (the "Membrankreuz" of Graebner, not observed in other species), which are attached to the nuclear membrane at their proximal end. In mushroom-shaped bodies, six microfilaments are present and they are very probably attached to nearby microtubuli. Also, the microvilli observed by Graebner lack all the specializations that are found in mushroom-shaped bodies: a well-defined head, a velum, the velar amorphous layer, and the grid layer (although some thickening of the plasma membrane was observed in the gnathostomulid microvilli).

Thus, although the determination of homologies in ultrastructural characters is especially difficult (Rieger and Tyler 1979, 1985), we believe that these structures, while fundamentally homologous, occur on the spermatozoa of the above-mentioned species of *Gnathostomula* and *D. subterraneus* as the result of convergent evolution. The similarities between the spermatozoa of *Diurodrilus* and those of the suborder Scleroperalia (e.g., *Gnathostomula*) must be regarded as convergent, especially since the sperm found in representatives of Scleroperalia appear to be apomorphic compared to those of other gnathostomulids (Ax 1985; Sterrer et al. 1985).

In species of *Lobatocerebrum* and *Jennaria*, both of which are regarded as secondarily acoelomate annelids, the spermatozoa are nearly of the same type as that found in species of the gnathostomulid genus *Haplognathia* (not a member of Scleroperalia), but the characters involved are to be regarded as plesiomorphic (Rieger 1991a, b). The spermatozoa of *Lobatocerebrum psammicola* (Rieger, 1980) also show some superficial similarities to those among oligochaetes (i.e., the configuration of the acrosome resembling an acrosomal tube and the presence of a middle piece containing mitochondrial derivatives and separating the nucleus from the axoneme). However, apart from the slight invagination of the axoneme in the nucleus of *Jennaria pulchra* (Rieger, 1991), there are absolutely no advanced similarities between the spermatozoa in species of *Lobatocerebrum* and *Jennaria* on one hand and *D. subterraneus* on the other.

V. Conclusions

The phylogenetic relationships of Diurodrilidae still remain unclear. The search for apomorphic characters shared by this family and other annelid groups is as yet inconclusive. In Clitellata, sperm ultrastructure has been employed as a major tool in the understanding of phylogenetic relationships within the group. The fact that this approach seems to have been so successful is probably in part due to the circumstance that reproductive strategy is more or less constant among representatives of this class (i.e., hermaphroditism, the use of spermatophores, etc.). In Polychaeta, reproductive strategies are much more diverse and many instances of homoplasy have evolved (e.g., in hypodermic impregnation and in specific methods of spermatophore transmission). Most cases where studies of sperm ultrastructure have yielded useful results in the elucidation of polychaete relationships have been concerned with more limited problems. An excellent example of this is the very strong evidence that investigations of spermatozoa have provided demonstrating that *Dinophilus* and *Trilobodrilus* are sister groups, two genera which as adults show profound differences in external morphology.

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Abbreviations *ac* acrosomal core (osmiophilic) · *af* acrosomal filament · *ah* anchor complex · *al* amorphous layer · *am* accessory membranes (irregular) · *an* annulus · *ap* acrosomal periphery (osmiophobic) · *as* acrosomal snout · *ba* endosymbiotic bacteria · *ca* centriolar adjunct · *ci* cilia · *cu* cuticle · *df* distal part of flagellum · *ec* extra-acrosomal manchette · *ep* epidermis · *er* endoplasmic reticular sacs · *es* extra-acrosomal substance · *ex* exocytotic vesicle · *fl* flagellum · *fp* flagellar pit · *gc* Golgi complex · *gd* glycocalyx dot · *gl* grid-like layer · *he* head · *hl* halo layer · *ib* intercellular bridges · *ic* intra-acrosomal canal · *lv* lysosomic vesicle (with degenerated mitochondrion) · *ma* middle part of acrosome (osmiophobic) · *mb* mushroom-shaped bodies · *mc* mucous coat (spirally oriented) · *mf* microfilament · *mg* midgut · *mi* mitochondrion · *mp* middle part of flagellum · *mt* microtubuli · *mu* muscle · *nc* nurse cell · *no* nucleolus · *nu* nucleus · *ol* osmiophobic layer · *pa* posterior part of acrosome (osmiophilic) · *pb* postacrosomal body (osmiophilic) · *pf* proximal part of flagellum · *pl* plasma membrane · *pp* pseudopodium · *pr* primary acrosomal membrane · *ps* polysome · *sm* secondary acrosomal membrane · *sp* spermatozoon · sp_2-sp_5 spermatid 2-5 · *ss* subacrosomal substance · st secondary tubuli · *sv* seminal vesicle · *te* testis · *tm* tertiary acrosomal membrane · *ve* velum

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