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Spermatogenesis in a species of *Paralinhomoeus* (Nematoda, Monhysterida, Linhomoeidae)

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Summary. Spermatogenesis of the free-living marine nematode, *Paralinhomoeus* sp. (Monhysterida, Linhomoeina, Linhomoeidae), was studied with transmission electron microscopy (TEM). The spermatocyte cytoplasm is filled with ribosomes, mitochondria, cisternae of RER and Golgi bodies. The spermatids are subdivided into the residual body, which includes the whole synthetic apparatus of the cell, and the main cell body (MCB) with mitochondria, fibrous bodies (FB) and the eccentrically located nucleus. The MCB surface bears numerous filopodia. The immature spermatozoa from the testes are unpolarized cells with a structure closely resembling one of the spermatid MCB. Membranous organelles (MO), a special component of many nematode spermatozoa, have not been found in either spermatids or spermatozoa of *Paralinhomoeus* sp. The pattern of spermatogenesis found in *Paralinhomoeus* sp. is not unique for nematodes, but is known for several other orders and families within the class Chromadorea (Chromadorida, Desmodorida, Rhabditida). However, it differs distinctly from the pattern described for the suborder Monhysterina whose spermatozoa have typical MO. This difference may be a distinct cytological synapomorphy to consider in phylogenetic analyses. A special component of the cytoskeleton comprises 16-18 nm thick microtubule-like fibres (MLF) that were found in the cytoplasm and in the submembrane position in both the spermatids and the immature spermatozoa of *Paralinhomoeus* sp. Analogous fibres were found in the spermatozoa of many nematodes but have never been detected in other Metazoa, outside the Nematoda.

Key words: Fibrous bodies, membranous organelles, microtubules, MSP, *Paralinhomoeus,* phylogeny, spermatozoa, ultrastructure.

Nematode spermatozoa represent an aberrant type of male gametes in that they are characterized by the absence of an axoneme and acrosome (Justine & Jamieson 1999; Justine 2002). The basic type of nematode spermatozoon is an amoeboid bipolar cell with an anterior pseudopod and posterior main cell body (MCB), which includes a condensed nucleus without a nuclear envelope, mitochondria and so-called 'membranous organelles' (MO) (Wolf *et al.*, 1978). MO are unique aberrant organelles characteristic of developing and mature sperm of many nematodes. Usually MO look like vesicles with dense content and a system of internal finger-like projections of the outer membrane. The MO appear as part of the complexes with paracrystalline fibrous bodies (FB) that are additional unusual components of developing nematode sperm. The prism-shaped FB are composed from densely packed parallel filaments consisting of the unique cytoskeleton protein, called 'major sperm protein' or MSP (Justine & Jamieson, 1999).

The complexes of MO and FB ('FB-MO complexes') dissociate into separate FB and MO during late stages of spermatogenesis. After sperm activation inside the female gonoduct, MO join with the plasmalemma of the sperm MCB resulting in transformation into membranous sacs continuous with the sperm plasmalemma. Sperm activation also is accompanied by transformation of FB into the MSP-based cytoskeleton of a newly-formed pseudopod.

Spermatogenesis in many taxa of soil and parasitic nematodes from the order Rhabditida (class Chromadorea) proceeds by the above described development resulting in sperm similar in structure (Justine & Jamieson, 1999; Justine, 2002; Yushin & Malakhov, 2004). However, the fate of the unusual organelles differs significantly

in several taxa of Chromadorea. These cytological differences may be used for comparative analysis, but such analysis across taxa is limited by lack of ultrastructural data on spermatozoa of diverse taxa (Yushin & Malakhov, 2004).

According to De Ley & Blaxter (2002) the order Monhysterida is subdivided into the suborders Monhysterina and Linhomoeina. All currently available information on monhysterid sperm structure and development was based on several species of Monhysterina (Noury-Sraïri *et al.*, 1993; Nicholas & Stewart, 1997; Justine & Jamieson, 1999; Justine, 2002; Yushin & Malakhov, 2004). In these cases spermatozoa and spermatogenesis closely resemble those of rhabditids (Justine & Jamieson, 1999; Justine, 2002; Yushin & Malakhov, 2004).

We conducted the first TEM study of spermatogenesis in a member of the suborder Linhomoeina, belonging to the genus *Paralinhomoeus* de Man 1907 (Linhomoeidae). This work widens our knowledge on the nematode spermatozoa and also includes preliminary observations concerning male gametes as a character assessing phylogeny of monhysterids.

MATERIAL AND METHODS

A new species of *Paralinhomoeus* was collected at the Vostok Marine Biological Station of the Institute of Marine Biology (Vostok Bay, Sea of Japan) where it is a typical component of the meiobenthos of silty sand at 10 m depth. The holotype and paratype specimens are stored in the Museum of the Far East State University (FESU); the description of the species will be published soon by Dr. N.P. Fadeeva (FESU).

Live males were cut to obtain a piece containing both testes. The specimens were fixed for TEM at 4°C in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 21 mg ml^{-1} NaCl and then postfixed in 2% osmium tetroxide in the same buffer containing 23 mg ml^{-1} NaCl. Postfixation was followed by *en bloc* staining for 2 h in 1% solution of uranyl acetate in distilled water and then specimens were dehydrated in ethanol followed by dehydration in an isopropanol series and finally embedded in Spurr resin. Thin sections were cut with a Reichert Ultracut E and Leica UC6 ultratomes, stained with lead citrate and then examined with a JEOL JEM 100B or JEOL JEM 100S TEM. The testes of four males were observed and the structure of spermatocytes, spermatids, and immature spermatozoa (following the terminology of Shepherd, 1981) were studied.

For light microscopy a suspension of live spermatozoa was extracted from the testes into a drop of filtered sea water and then observed with a Reichert Polyvar microscope using interference contrast.

RESULTS

The male reproductive system of *Paralinhomoeus* sp. comprises two opposite testes each containing successive stages of sperm development: spermatogonia (at each distal tip), diploid spermatocytes, haploid spermatids, and immature spermatozoa. All stages may be easily observed simultaneously in a single longitudinal section.

The spermatocytes are arranged into a successive chain of growing cells. The late spermatocytes are 20 μm diameter with a 9 μm diameter nucleus (Fig. 1A). The nucleus includes several small nucleoli scattered throughout the pale nucleoplasm. The cytoplasm contains ribosomes, mitochondria, cysternae of rough endoplasmic reticulum (RER), and Golgi bodies. The abundance of these organelles is evidence of high synthetic activity which, however, does not result in formation of components such as FB and MO.

Between the distal chain of spermatocytes and the seminal vesicle, a short zone of spermatids has been detected. Each spermatid has a residual body which contains components of a synthetic apparatus: ribosomes, RER and Golgi bodies (Fig. 1B). A cytoplasmic bridge joins the residual body to the MCB, which measures 10 μm in diameter and contains a nucleus that is 3x2 μm in size and is shifted to the cell periphery opposite the residual body. The nucleus is devoid of a nuclear envelope and has an indistinct boundary; its chromatin is granular (Fig. 1B, 2A). A group of FB (1.5 μm long and 0.5 μm thick) occupies the centre of the MCB. The spindle-shaped FB are composed of closely packed parallel fibres about 10 nm thick. The cluster of FB is surrounded by a monolayer of mitochondria that are characterized by an electron-dense matrix.

The MCB bear 0.5 μm thick filopodia enclosing a framework of longitudinally orientated fibres 16-18 nm in diameter (Fig. 2). Similar fibres fill the ectoplasm of the MCB and underlie its plasmalemma as parallel arrays.

The proximal half of both testes is filled with numerous immature spermatozoa of uniform size and structure. Spermatozoa extracted from the testes, when studied with an interference contrast microscopy, are unpolarized opaque cells about 20 μm in diameter (Fig. 3A, insert). Their surface includes numerous long filopodia.

The outlines of spermatozoa observed in longitudinal sections through the seminal vesicles vary from spherical to stretched (Fig. 3A). The sperm nucleus retains an eccentric position, being in close vicinity to the plasmalemma (Fig. 3A, B). The remaining volume of the cell is filled with randomly distributed FB, mitochondria and a loose net of fibrous matter. The worm-like mitochondria (2 μm long and 0.25 μm thick) have electron-dense content. The FB retain a spindle shape but enlarge up to 4 μm x 2 μm and have less tightly packed parallel fibres.

As was observed for the MCB of the spermatids, the surface of each spermatozoon bears numerous 0.5 μm thick filopodia which are squeezed between the cells (Fig 4A, B). The filopodia as well as the rest of plasmalemma enclose an inner monolayer of 16-18 nm thick fibres with the appearance of microtubules due to their characteristically transparent core (Fig. 4A, insert). These microtubule-like fibres (MLF) run longitudinally inside the filopodia and are arranged as parallel arrays beneath the plasmalemma (Fig. 4B).

The MO and resembling structures have not been found both in spermatids and spermatozoa.

DISCUSSION

Sperm development of the monhysterid *Sphaerolaimus hirsutus* (Sphaerolaimidae) has been studied most thoroughly and, in general, its spermatogenesis resembles that of rhabditids (Noury-Sraïri *et al.*, 1993; Justine & Jamieson 1999; Justine, 2002). Typical FB-MO complexes appear first in spermatocytes; the complexes mature and then dissociate into separate FB and MO in the spermatids. The spermatozoa assume a concentric internal structure. The central nucleus lacks a nuclear envelope and is surrounded by FB which now transform into bundles of fibres. A layer of MO and mitochondria covers the central part of the cell. Finally the FB disappears to form thick filamentous layer underlying the plasmalemma. An electron-transparent halo surrounds the nucleus.

The structure of mature (activated) spermatozoa of *S. hirsutus* has not been studied. However, brief information is available on the mature spermatozoa of *Daptonema* spp., species of Monhysterina belonging to the family Xyalidae (Justine & Jamieson, 1999; Justine, 2002; Yushin & Malakhov, 2004). In *Daptonema* the activation of the spermatozoa in the uterus results in formation of the pseudopod and the MCB which contains the nucleus and mitochondria as well as empty MO joined to the sperm plasmalemma and opening to the exterior via pores. Data on the xyalid, *Gonionchus australis*, also confirm the presence of MO in the spermatids and spermatozoa of Monhysterina (Nicholas & Stewart, 1997).

Our study of a member of the family Linhomoeidae, which represents the monhysterid suborder Linhomoeina, results in unexpected data. We assumed that the sperm development and structure in *Paralinhomoeus* sp. would be reasonable like that of the basic pattern of monhysterids and rhabditids. However, the linhomoeid spermatozoa lack MO; the FB appear only in the spermatids as the separate organelles free of MO. The mature spermatozoa from the seminal vesicle contain an eccentrically located nucleus, mitochondria and many FB composed from parallel fibres.

The absence of MO at all the stages of sperm development is well known for several taxa of Chromadorea. In plant-parasitic nematodes from the superfamily Tylenchoidea the FB appear as separate organelles in spermatocytes and sometimes persist even in the spermatozoa from the uterus (Cares & Baldwin, 1995; Justine, 2002). Formation of FB without assistance or presence of MO has also been described for representatives of the orders Chromadorida and Desmodorida (Yushin & Zograf, 2002; Yushin, 2003; Zograf *et al.*, 2004; Zograf & Yushin, 2004; Yushin & Coomans, 2005). Thus, the pattern of spermatogenesis found in the linhomoeid *Paralinhomoeus* sp. is not unique. However, it differs distinctly from the pattern described for Monhysterina and may be used as important differential character when the phylogeny of the order is further revolved.

Unusual components of the cytoskeleton were found in the spermatids and immature spermatozoa of *Paralinhomoeus* sp. These are the MLF which resemble microtubules but are distinctly thinner (16-18 nm) than the tubulin-containing microtubules (24-25 nm). Very similar fibres were observed in the spermatozoa of many other nematodes from a variety of orders: Enoplida, Mononchida, Mermithida, Trichinellida, Chromadorida, Rhabditida (Beams & Sekhon, 1972; Shepherd *et al.*, 1973; Ugwunna & Foor, 1982; Shepherd & Clark, 1983; Hess & Poinar, 1989; Poinar & Hess-Poinar, 1993; Cares & Baldwin, 1994, 1995; Takahashi *et al.*, 1994; Endo *et al.*, 1998; Turpeenniemi, 1998; Yushin & Malakhov 1998; Yushin, 2004; Yushin & Zograf, 2004; Zograf *et al.*, 2004). The difference in thickness between MLF and microtubules was reasonably interpreted by Turpeenniemi (1998), in his study of the free-living marine nematode *Halalaimus dimorphus* (Enoplida, Oxystominidae), as evidence for a difference in their proteinaceous

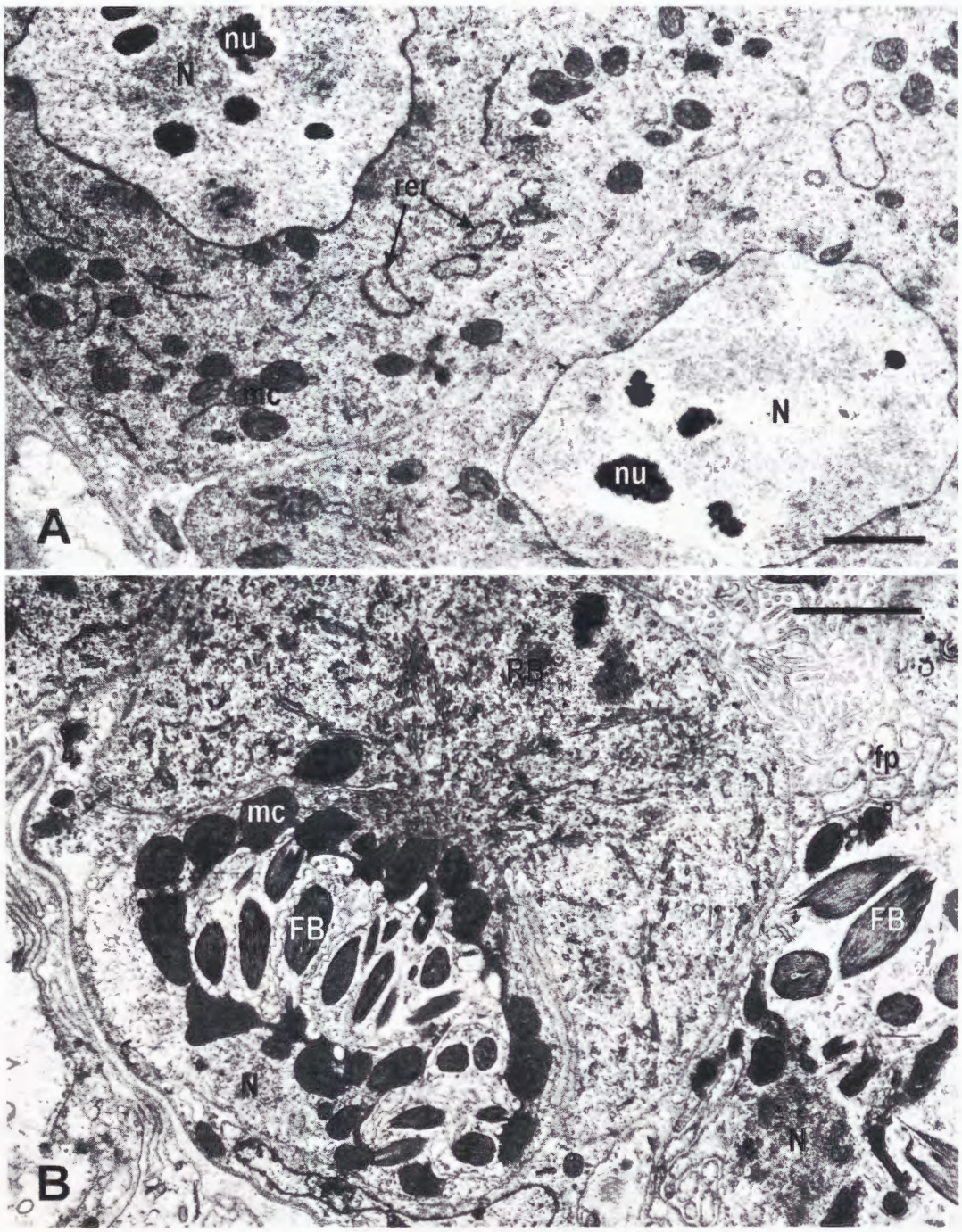

Fig. 1. *Paralinhomoeus* sp., spermatogenesis. A: Spermatocytes; B: Spermatid with the residual body. Scale bars: 2 μm. Abbreviations: FB, fibrous body; fp, filopodia; mc, mitochondria; N, nucleus; nu, nucleoli; RB, residual body; rer, rough endoplasmic reticulum.

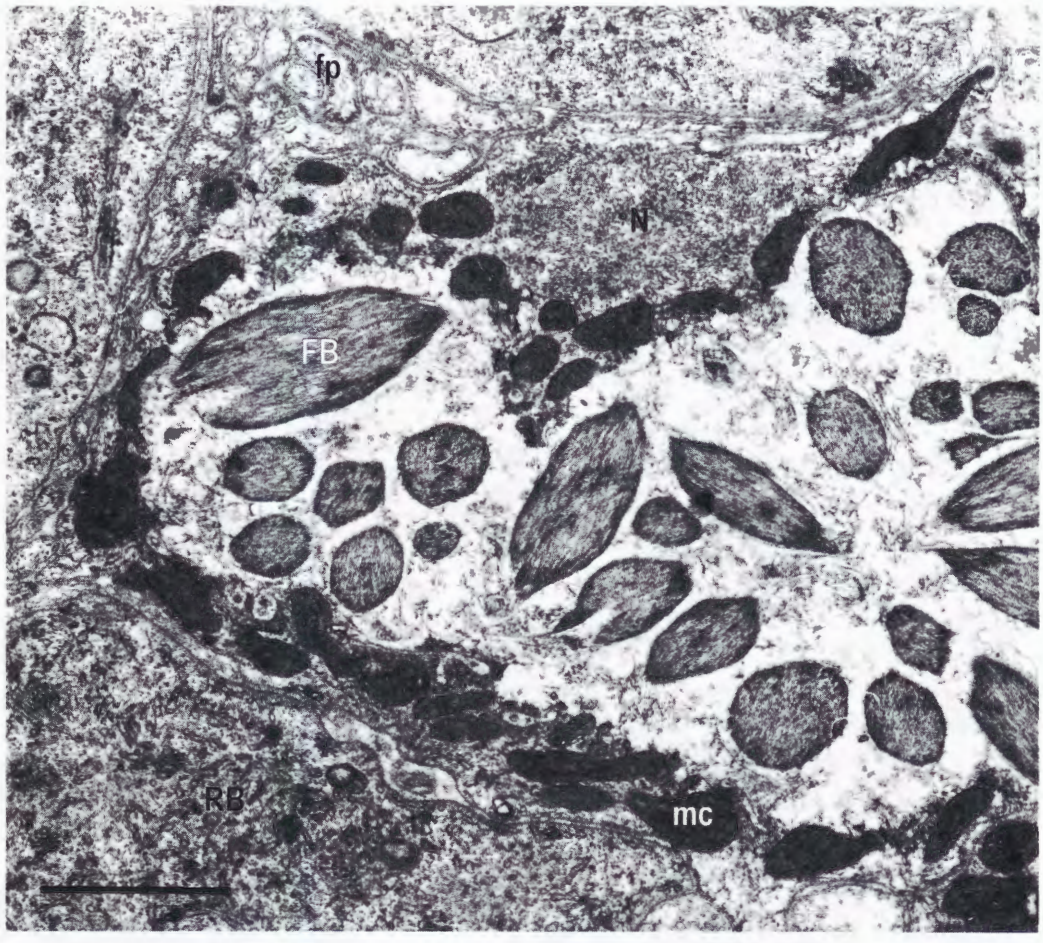

Fig. 2. *Paralinhomoeus* sp. Main cell body of the spermatid. Scale bar: 2 μm. For abbreviations see Fig. 1.

nature. It is very likely that the MLF of nematode spermatozoa are assembled from MSP-based filaments because the MSP is the basic cytoskeleton protein involving in pseudopodium formation and movement (Scott, 1996); while tubulins are rare or absent in nematode spermatozoa (Justine & Jamieson, 1999; Justine, 2002).

Electron microscope immunocytochemistry shows that the MSP in spermatids and immature spermatozoa of nematodes is localized in FB (Hess & Poinar, 1989; Justine, 2002). In *Paralinhomoeus* sp. the MLF and the FB were found simultaneously. This implies that two different forms of MSP fibres (*i.e.* free tubules 16-18 nm thick and packed parallel fibres 10 nm thick) coexist in the spermatogenous cells.

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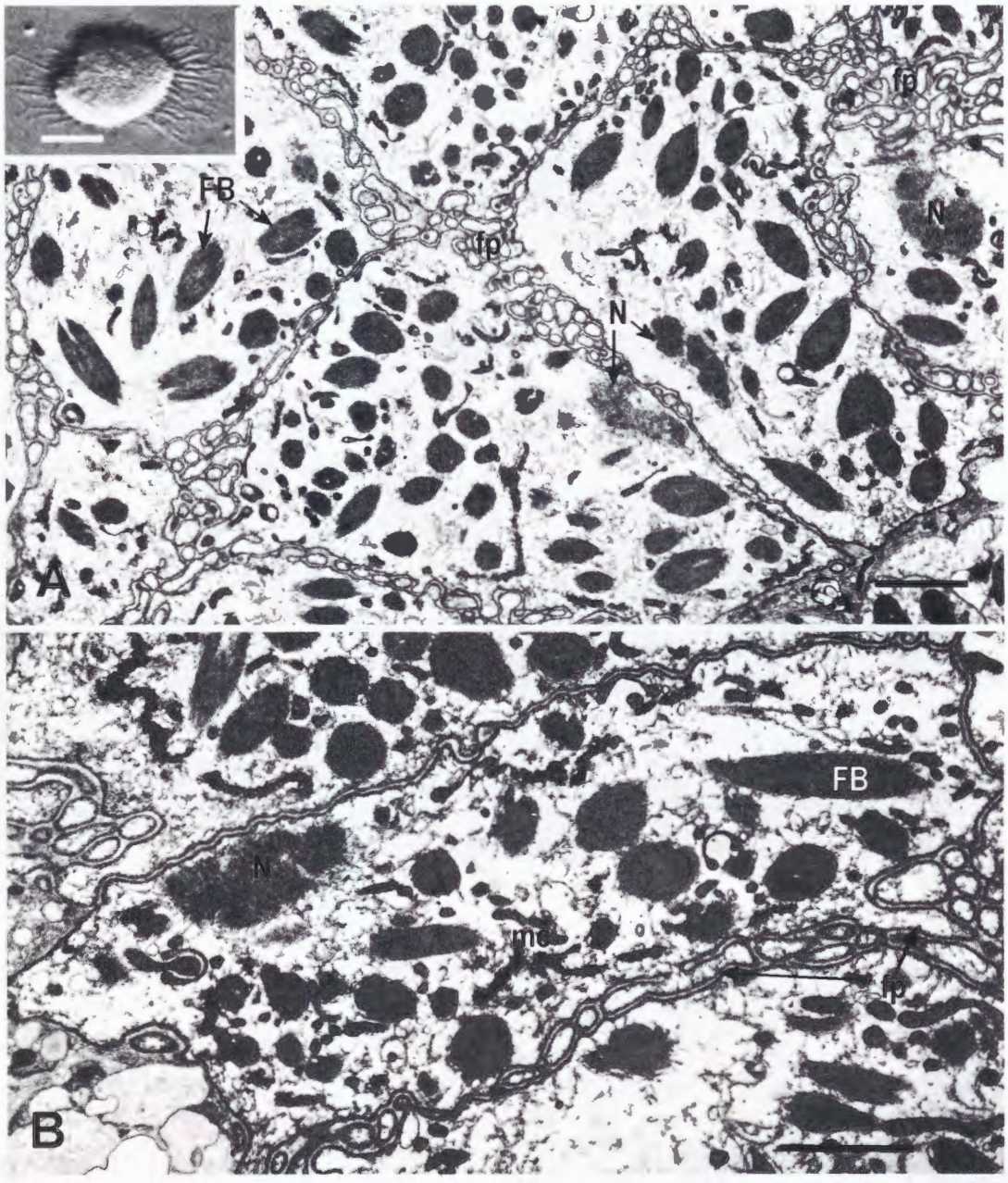

Fig. 3. *Paralinhomoeus* sp., immature spermatozoa (A and B – TEM; insert to A – interference contrast). A: Cluster of spermatozoa in the seminal vesicle. Insert: spermatozoon extracted from the testis; note numerous long filopodia on the cell surface; B: Spermatozoon at a higher magnification. Scale bars: A = 4 μm (insert = 10 μm); B = 3 μm. For abbreviations see Fig. 1.

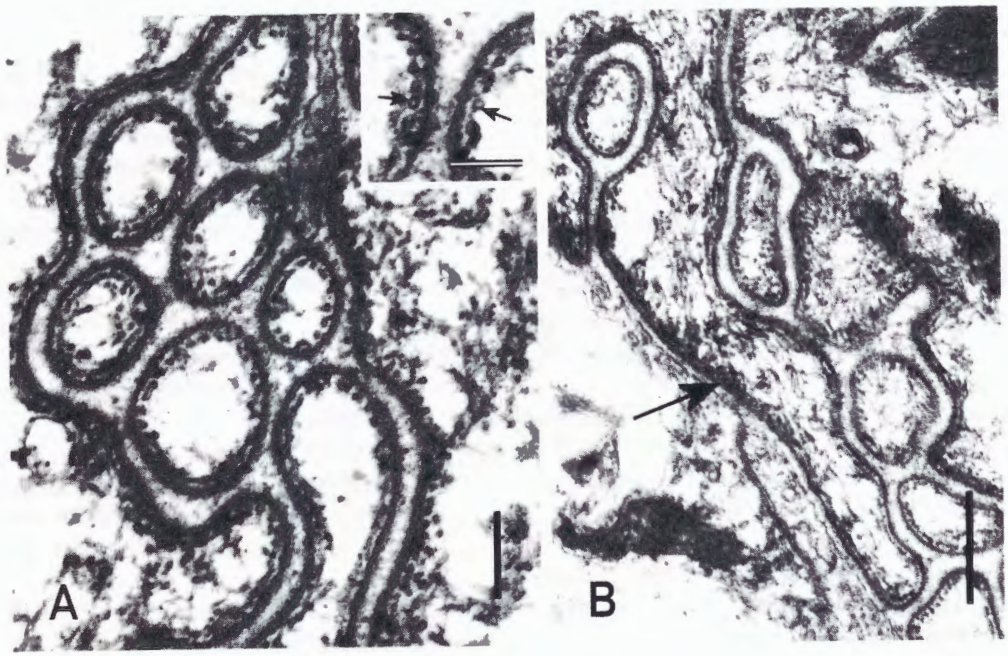

Fig. 4. *Paralinhomoeus* sp., immature spermatozoa. A: Section through the filopodia and plasmalemmata of two neighboring spermatozoa; the membranes are strengthened by MLF. Insert: high magnification of transverse section through filopodia; arrows indicate the submembranous MLF with a transparent core. B: Longitudinal section of the filopodium (arrow) showing longitudinal orientation of the MLF. Scale bars: A = 0.25 μm (insert = 0.1 μm); B = 0.5 μm.

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Yushin V.V. Сперматогенез у свободноживущей морской нематоды из семейства Linhomoeidae (Nematoda, Monhysterida).

Резюме. С помощью электронной микроскопии исследован сперматогенез у свободноживущей морской нематоды *Paralinhomoeus* sp. (Monhysterida, Linhomoeina, Linhomoeidae). Цитоплазма сперматоцитов заполнена рибосомами, цистернами ЭПР и аппарата Гольджи. Сперматида разделена на резидуальное тело, включающее в себя весь синтетический аппарат клетки, и главное тело клетки (ГТК), содержащее эксцентрично расположенное ядро, митохондрии и волокнистые тела (ВТ). Поверхность ГТК покрыта многочисленными филоподиями. Незрелые сперматозоиды из семенников – неполяризованные клетки с эксцентричным ядром, митохондриями и ВТ; поверхность клетки покрыта многочисленными филоподиями. Ни в сперматидах, ни в сперматозоидах не обнаружены мембранные органеллы (МО), характерные для спермиев других нематод. Тип сперматогенеза, обнаруженный у *Paralinhomoeus* sp., вида, принадлежащего подотряду Linhomoeina отряда Monhysterida, известен для ряда таксонов класса Chromadorea (Chromadorida, Desmodorida, Rhabditida). Однако он несомненно отличается от типа сперматогенеза, известного для монхистерид из подотряда Monhysterina, у которых развиваются типичные МО. Это различие может быть использовано как ясный цитологический признак для анализа филогении отряда. В сперматидах и сперматозоидах *Paralinhomoeus* sp. обнаружены специфические компоненты цитоплазмы, микротрубочкоподобные волокна (МПЛ) толщиной 16-18 нм, располагающиеся свободно цитоплазме или образующие субмембранный слой. Аналогичные волокна обнаружены в сперматозоидах многих нематод, но неизвестны для других многоклеточных животных.