First ultrastructural observation of spermatozoa in an araeolaimid nematode (Nematoda: Araeolaimida: Axonolaimidae)

Vladimir V. Yushin and Lyubov A. Gliznutsa

A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Paltchevsky Street 17, 690041, Vladivostok, Russia
e-mail: vvyushin@yandex.ru

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Summary. The mature spermatozoa from the uterus of the free-living marine nematode, *Axonolaimus arcuatus* (Araeolaimida: Axonolaimidae), were studied. These are clearly polarised cells 8-10 µm in size each subdivided into a pseudopod and a main cell body; the latter includes a central nucleus without a nuclear envelope surrounded by transparent halo and peripheral layers of organelles, *i.e.* mitochondria and large membranous cisterns interpreted as membranous organelles (MO) characteristic of nematode sperm. The MO in *A. arcuatus* look like large serpentine or ring-like cisterns opening to the exterior *via* multiple pores. The pseudopods with irregular contours contain no organelles and are filled with fibrous material. The spermatozoa of *A. arcuatus* show similarity with spermatozoa in Monhysterida-Sphaerolaimoidea and many taxa of the order Rhabditida. However, development of specific extensive MO in *A. arcuatus* spermatozoa is a marked deviation from the 'rhabditid' sperm pattern and may be proposed as an apomorphy of Araeolaimida.

Key words: *Axonolaimus arcuatus*, membranous organelles, nematode sperm pattern, pseudopod, spermatogenesis.

The basic features of structure and development of nematode sperm have been studied in details in representatives of the diverse order Rhabditida belonging to the class Chromadorea (*sensu* De Ley & Blaxter (2002); this classification will be used in the present paper). Many species studied within this order produce similar spermatozoa of the 'rhabditid' pattern, described as an amoeboid bipolar cell with an anterior pseudopod and posterior main cell body, which includes a nucleus (without nuclear envelope), mitochondria and unique 'membranous organelles' (MO) (Justine & Jamieson, 1999; Justine, 2002; Yushin & Malakhov, 2014). The MO develop as part of complexes with paracrystalline fibrous bodies (FB) composed of a unique cytoskeleton protein MSP ('major sperm protein') (Justine, 2002; Chu & Shakes, 2013; Yushin *et al.*, 2016). During the late stages of spermatogenesis the complexes of FB and MO ('FB-MO complexes') dissociate into separate FB and MO. After sperm activation inside the female gonoduct, MO join to the plasmalemma of the spermatozoon main cell body and release their content into the uterus lumen. The empty MO, appearing as numerous membranous pouches continuous with the sperm plasmalemma, are retained as a stable feature of the mature sperm. Sperm activation is also accompanied by the transformation of FB into the MSP-based cytoskeleton of a newly formed pseudopod (Justine, 2002; Chu & Shakes 2013; Yushin *et al.*, 2016).

The order Rhabditida includes many taxa that have deviations of sperm structure and development from the 'rhabditid' pattern (Justine & Jamieson, 1999; Yushin & Spiridonov, 2001; Justine, 2002; Yushin & Malakhov, 2004, 2014; Yushin *et al.*, 2016). At the same time, in at least one more nematode order (Monhysterida), spermatozoa very reminiscent of the 'rhabditid' pattern were described (Noury-Sraïry *et al.*, 1993; Justine & Jamieson, 1999; Justine, 2002; Yushin *et al.*, 2018). These confusing data on male gametes need to be interpreted from a phylogenetic point of view.

Spermatozoa usually display a large number of informative morphological characters that can be analysed in the framework of metazoan taxonomy and phylogeny (Baccetti, 1985; Jamieson *et al.*, 1995, Liana & Witalinski, 2005; Pitnick *et al.*, 2008; Levron *et al.*, 2010; Dallai *et al.*, 2016; Bakhoum *et al.*, 2017). Unlike many other Metazoa, data on the

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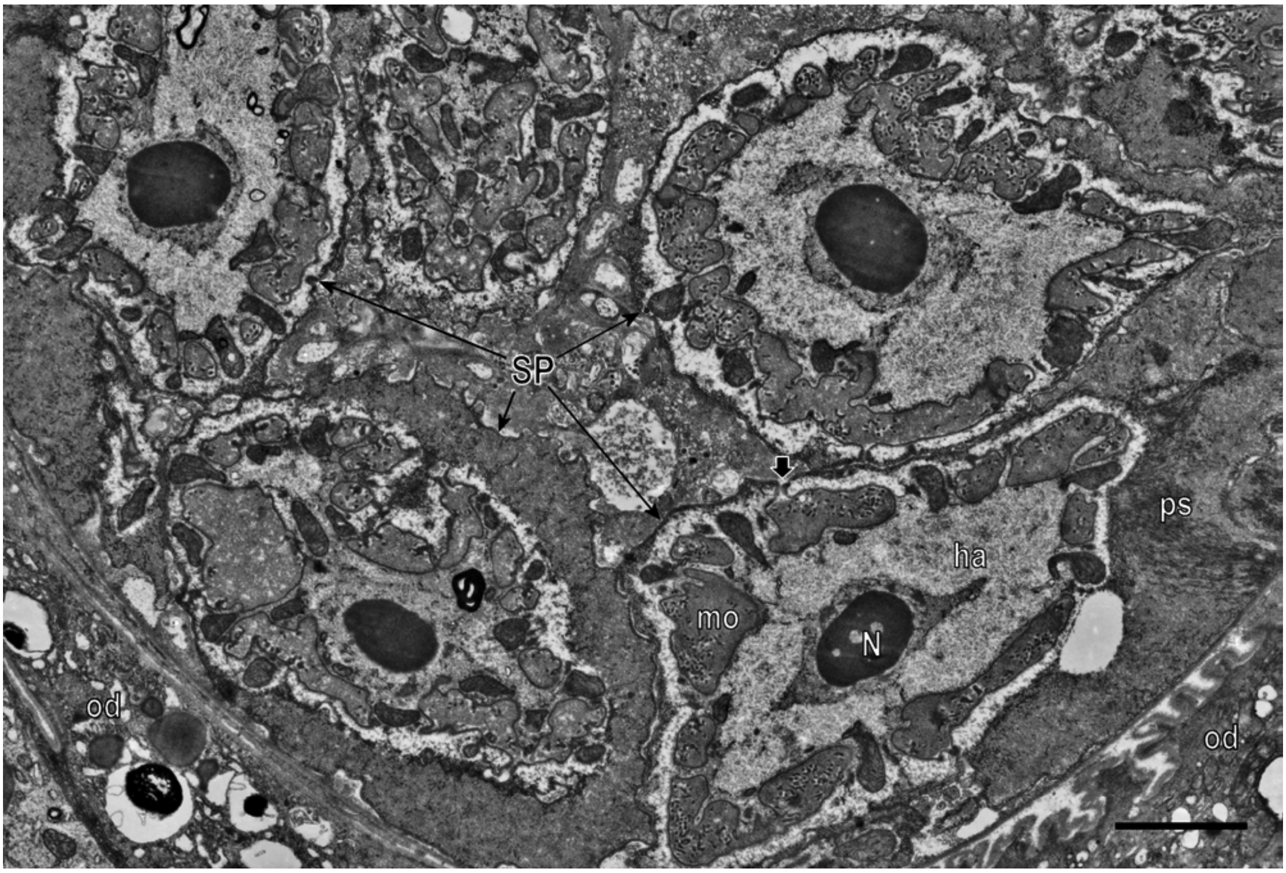

Fig. 1. *Axonolaimus arcuatus*, mature spermatozoa from the uterus, TEM. Cluster of spermatozoa at the joining point of the uterus and oviduct. Abbreviations: ha – halo; mo – membranous organelles; N – nucleus; od – oviduct wall; ps – pseudopod; SP – spermatozoa. Thick arrow shows opening of membranous organelle to exterior. Scale bar: 2 μm.

sperm structure and spermatogenesis are rarely discussed in the analyses of phylogenetic relationships in nematodes, probably due to lack of studies on the relatively primitive free-living nematodes from several important orders of the class Chromadorea. For example, the nematodes of the order Araeolaimida, which includes mainly free-living marine nematodes, were presented only by brief outlines of sperm development in two species in the mini-review that contains no photographs (Yushin & Malakhov, 2004).

To improve understanding of the basic patterns and diversity of male gametes in Chromadorea the detailed ultrastructural observations of the spermatozoa in the araeolaimids are warranted. The free-living marine nematode *Axonolaimus arcuatus* Schuurmans-Stekhoven, 1950 (Areolaimida: Axonolaimidae) was chosen for the first ultrastructural description of the araeolaimid spermatozoon; the ultrastructure of mature spermatozoa from the uterus has been studied in details. These new data will enable brief discussion of sperm patterns in Chromadorea.

MATERIAL AND METHODS

Gravid females of *A. arcuatus* were extracted from samples of sand collected on August 2001 at a depth of 3 m in the Priboynaya Cove of the Vostok Bay, the Sea of Japan (42°53'13.6" N; 132°43'35.2" E). Before fixation for transmission electron microscopy (TEM), the head and tail of each female were removed to facilitate the subsequent tissue fixation and embedding processes. The specimens were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.05 M cacodylate buffer with 21 mg ml^{-1} NaCl, 0.5 mg ml^{-1} $MgCl_2$, 1% dimethylsulphoxide (DMSO) and then postfixed in 2% osmium tetroxide in the same buffer containing 23 mg ml^{-1} NaCl. The specimens were dehydrated in ethanol followed by isopropanol series and embedded in Spurr resin. Thin longitudinal sections were made with a diamond knife using Reichert Ultracut E and Leica UC6 ultramicrotomes, stained with uranyl acetate and lead citrate, and examined with JEOL JEM 100B and Zeiss Sigma 300 VP electron microscopes.

The structure of mature spermatozoa of two females was examined for the present paper, in which the terminology of Shepherd (1981) for stages of spermatogenesis is employed.

RESULTS

The female reproductive system of *A. arcuatus* has two branches; each includes an outstretched ovary, oviduct and uterus. The uterus lumen of the females observed was filled with fertilised eggs with egg-shells; the small cluster of spermatozoa was identified on semithin sections at the joining point of uterus and oviduct. On the thin sections observed with TEM the spermatozoa were found as a cluster of densely packed cells in the distal tip of the uterus adjoining the oviduct (Fig. 1). These are pseudopod bearing amoeboid cells, which may be identified as the mature (activated) spermatozoa capable for egg fertilisation. These spermatozoa have irregular outlines and may be estimated only approximately as 8-10 μm in size.

The spermatozoa are clearly polarised cells each subdivided into a pseudopod and a main cell body that contains a central nucleus inside the wide halo surrounded by layers of peripheral organelles, *i.e.* mitochondria and large membranous cisterns (Figs 1, 2 & 4). As a result, the arrangement of cell components provides a remarkable concentric structure to the main cell body. The oval nucleus, about 2 μm in size, has no nuclear envelope; the highly condensed nuclear chromatin has a clear-cut boundary. The nucleus is surrounded by filamentous coat and voluminous halo without organelles (Figs 1, 2 & 4).

At the cell periphery the large membranous cisterns are regularly interspersed by mitochondria with dense matrix (Figs 1 & 2). The cisterns contain a system of internal tubes formed by finger-like invaginations of their outer membrane (Figs 2 & 3A). The cisterns open to the exterior *via* pores through 0.3 μm long ducts (Figs 1 & 2). This remarkable structure of the cisterns suggests that they are homologous to the MO of other nematode spermatozoa. The MO in *A. arcuatus* are variable in size and shape; their thickness is about 0.5 μm but actually they extend under the plasma membrane as a long serpentine or even ring-like cistern (Fig. 3C). Analysis of sections shows that each cistern has more than one opening to the exterior (Fig. 3B). Despite openings the internal space of MO is filled with matter of moderate density.

Each spermatozoon bears a prominent (2-3 μm long) pseudopod, which provide distinct polarity to the cell (Figs 1, 2 & 4). The pseudopods have irregular contours, contain no organelles and are filled with diverse fibrous material (Figs 1, 2 & 3D). The layer of 20 nm thick parallel fibres with tubular profiles was detected under the plasma membrane of the main cell body (Fig. 3D, E). These fibres are arranged as a submembranous net, clearly visible on the tangential sections (Fig. 3F). The surface of spermatozoa is smooth and bears no filopodia.

DISCUSSION

The mature spermatozoa of the araeolaimid *A. arcuatus* in general follow the well known pattern of nematode sperm described in many taxa of the extensive order Rhabditida belonging to the same class Chromadorea. This 'rhabditid' pattern outlined in the introduction, was described for a variety of representatives of the order Rhabditida (Spiruromorpha, Ascaridomorpha, Panagrolaimomorpha, Tylenchomorpha, Diplogasteromorpha, Rhabditomorpha and Myolaimina), as well as for the aquatic nematodes of the orders Monhysterida (Monhysteroidea) (Justine & Jamieson, 1999; Justine, 2002; Giblin-Davis *et al.*, 2010; Yushin & Malakhov, 2004, 2014; Zograf, 2014; Slos *et al.*, 2015; Yushin *et al.*, 2016, 2018). In *A. arcuatus* the mature spermatozoa are clearly polarised cells each subdivided into a pseudopod and a main cell body that contains a central nucleus without nuclear envelope surrounded by a layer of peripheral organelles, *i.e.* mitochondria and MO (Fig. 4). However, this formal structural description of spermatozoa does not exclude distinct morphological differences. The spermatozoa of free-living marine nematodes are generally larger than the regular rhabditid spermatozoa, which usually are about 5 μm (Yushin *et al.*, 2011, 2016). In the monhysterids *Sphaerolaimus hirsutus* and *Daptonema* sp. immature spermatozoa are approximately 15 and 10 μm in size, respectively (Justine & Jamieson, 1999; Yushin *et al.*, 2018). In *Daptonema* sp. mature spermatozoa, the main cell body is 8 μm in diameter with pseudopods up to 5 μm long. The mature spermatozoa of *A. arcuatus* have comparable size, 8-10 μm. However, diversity of the rhabditid spermatozoa also includes extremely large spermatozoa (Bird & Bird, 1991; Justine, 2002; Yushin *et al.*, 2007).

Specific to the monhysterids and *A. arcuatus* is a clearly visible concentric arrangement of organelles

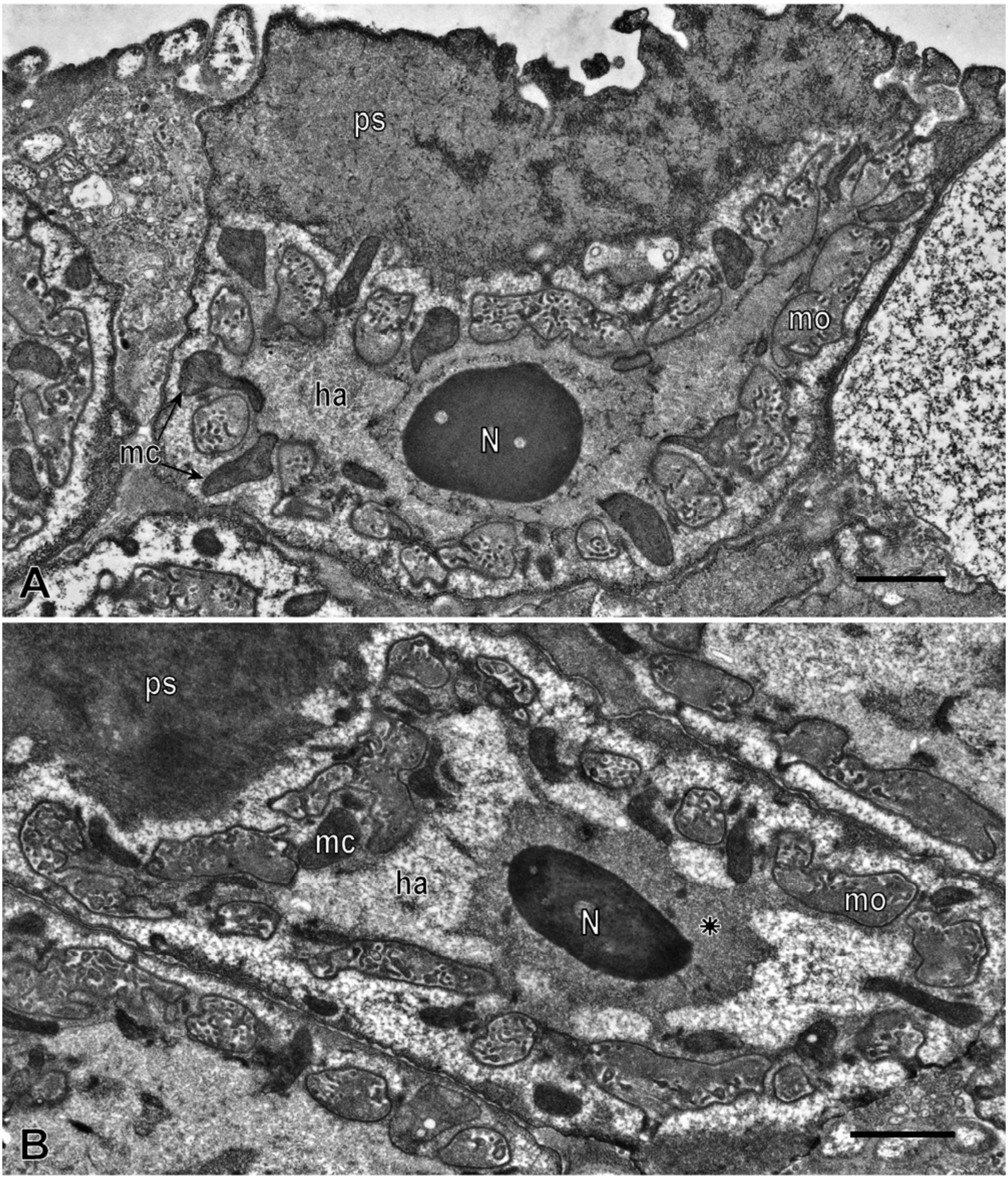

Fig. 2. *Axonolaimus arcuatus*, mature spermatozoa from the uterus, TEM. A: General view, the spermatozoon is clearly subdivided into the pseudopod and the main cell body. B: Concentric arrangement of cell components in the main cell body of spermatozoon; asterisk marks the filamentous matter surrounding the nucleus. Abbreviations: ha – halo; mc – mitochondria; mo – membranous organelles; N – nucleus; ps – pseudopod. Scale bars: 1 μm.

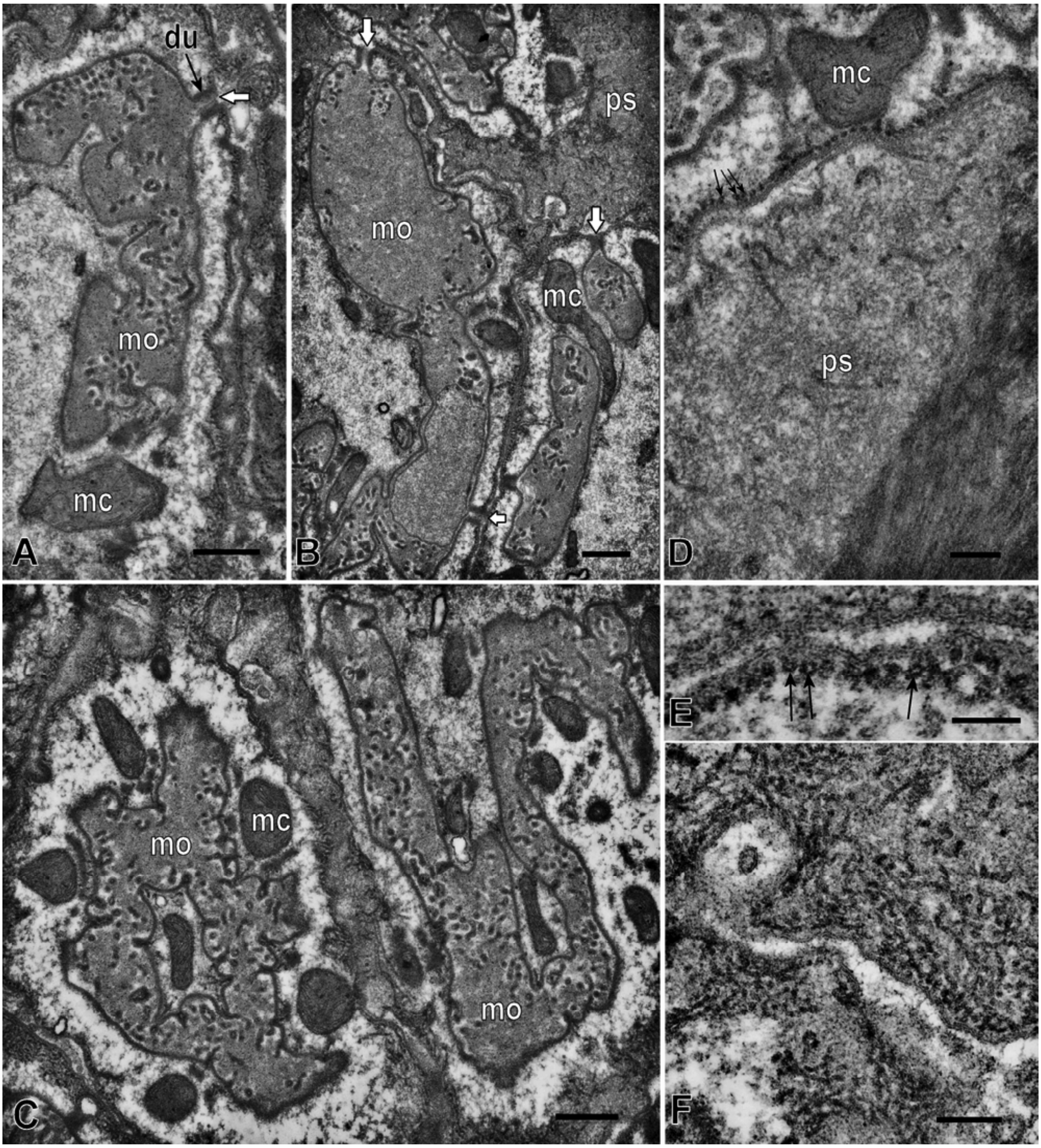

Fig. 3. *Axonolaimus arcuatus*, mature spermatozoa from the uterus, TEM. Higher magnification of the spermatozoa showing ultrastructural details. A: The membranous organelle opens to exterior *via* duct and pore. B: One membranous organelle opens to exterior *via* two ducts and pores. C: Tangential section of periphery of two neighbouring spermatozoa showing extensive membranous organelles of ring-like and serpentine shape. D: The pseudopod with diverse fibrous components and neighbour spermatozoon (upper) with fibres under plasma membrane; arrows point to submembranous fibres. E: The submembranous layer of fibres at higher magnification; arrows point to cross profiles of fibres. F: Tangential section of periphery of two neighbouring spermatozoa showing net of submembranous fibres. Abbreviations: du – duct of membranous organelle; mc – mitochondria; mo – membranous organelles; ps – pseudopod. Thick arrows on A and B show opening of membranous organelle to exterior. Scale bars: A-C = 0.5 µm; D, F = 0.2 µm; E = 0.1 µm.

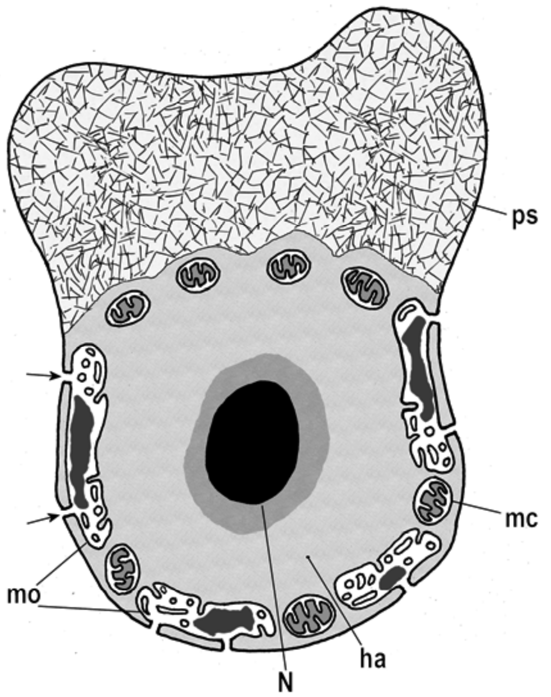

Fig. 4. *Axonolaimus arcuatus*, schematic drawing of the mature spermatozoon from the uterus; not to scale. The bipolar cell bears the anterior pseudopod (ps); the condensed nucleus (N) is surrounded by the filamentous coat and transparent halo (ha); at the cell periphery large memabranous organelles (mo) are interspersed with mitochondria (mc). The membranous organelles contain the matter of moderate density and are open to exterior *via* pores (arrows).

in spermatozoa where the nucleus is separated from peripheral organelles and pseudopods by a wide transparent halo free of organelles but containing fibrous matter (Noury-Sraïri *at al.*, 1993; Yushin *et al.*, 2018). This halo around the nucleus seems not to be characterisric of the rhabditid spermatozoa but was described in the representative of the chromadorean order Desmodorida (Yushin & Coomans, 2005).

Despite general similarity with the 'rhabditid' pattern, the spermatozoa of *A. arcuatus* show strong individual character in the structure of MO. Usually in the rhabditids and also in the monhysterids (Sphaerolaimoidea) MO are numerous vesicles that in the mature spermatozoa form an equivalent number of pouches each opening to exterior *via* a pore (Justine, 2002; Yushin & Malakhov, 2014; Yushin *et al.*, 2018). In *A. arcuatus* MO are large ramified membranous cisterns extended over the sperm membrane. These cisterns may be interpreted as a result of fusion of individual MO during sperm development. Two and possibly more ducts that open the internal space of MO to the exterior are indirect evidence of this fusion.

It is reported that in the araeolaimid nematode *Sabatieria palmaris* from the family Comesomatidae, typical paracrystalline FB appear in the complex with the sole giant MO formed in the spermatocytes by fusion of numerous small MO of usual structure (Yushin & Malakhov, 2004). This hypertrophied FB-MO complex appears in immature spermatozoa and is retained in the sperm found in uteri. Data on two araeolaimid nematodes from different families suggest that the hypertrophied membranous organelles may characterise the specific pattern for spermatozoa of the order Araeolaimida. The diversity of the sperm patterns in Rhabditida and other orders of Chomadorea includes also reduction of MO and even of all specific organelles (Justine, 2002; Yushin & Malakhov, 2004, 2014).

The spermatozoa of *A. arcuatus* are polarised and have well developed pseudopod filled with a cytoskeleton fibres. Specific submembranous tubule-like fibres spread under the membrane of spermatozoa of *A. arcuatus* are also known from ultrastructural descriptions of many other nematodes from a variety of diverse orders representing very distant taxa from both classes Enoplea and Chromadorea (Turpeenniemi, 1998; Justine & Jamieson, 1999; Yushin & Zograf, 2004; Yushin *et al.*, 2018), and may be considered as one of the general subcellular characters of the nematode sperm.

CONSLUSION

Our first observation of the araeolaimid nematode *A. arcuatus* shows similarity of its mature spermatozoa with spermatozoa in Monhysterida-Sphaerolaimoidea and in many taxa of the order Rhabditida. However, specific extensive MO of *A. arcuatus*, which hypothetically are fusions of 'unit' MO of regular structure, is a marked deviation from the 'rhabditid' pattern and may be considered a well defined apomorphy of Araeolaimida. The study of spermatogenesis in Axonolaimidae and, especially, Comesomatidae may elucidate a new pattern of sperm development for Araeolaimida.

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В.В. Юшин и Л.А. Глизнуца. Первое ультраструктурное исследование сперматозоидов у нематоды ареолаймиды (Nematoda: Araeolaimida: Axonolaimidae).

Резюме. Изучены зрелые сперматозоиды из матки свободноживущей морской нематоды *Axonolaimus arcuatus* (Araeolaimida: Axonolaimidae). Это поляризованные клетки размером 8-10 мкм, каждая из которых подразделяется на псевдоподию и основное тело клетки; последнее включает центральное ядро без ядерной оболочки, окруженное прозрачным гало, и периферический слой органелл, т.е. митохондрий и крупных мембранных цистерн, которые интерпретируются как результат слияния мембранных органелл (МО), характерных для сперматозоидов нематод. МО у *A. arcuatus* представлены большими змеевидными или кольцевыми цистернами, открывающимися наружу через множество пор. Псевдоподии не содержат органелл и наполнены волокнистым материалом различной плотности. Сперматозоиды *A. arcuatus* демонстрируют сходство со сперматозоидами у Monhysterida-Sphaerolaimoidea и многих таксонов отряда Rhabditida. В то же время развитие гипертрофированных МО у *A. arcuatus* – это значительное отклонение от «рабдитидного» паттерна строения сперматозоидов и может быть предложено в качестве апоморфии отряда Araeolaimida.