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Published in:
Zoologischer Anzeiger

DOI:
10.1016/j.jcz.2014.07.003

Publication date:
2014

Document version
Publisher's PDF, also known as Version of record

Zoologischer Anzeiger 253 (2014) 497–511

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz

Zoologischer Anzeiger

Spermatozoa in the reproductive system of a hermaphroditic marine tardigrade, *Orzeliscus belopus* (Tardigrada: Arthrotardigrada)

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 28 June 2014
Accepted 10 July 2014
Available online 18 July 2014
Corresponding Editor: P. Michalik.

Keywords:
Ovotestis
Hermaphrodites
Ultrastructure
Sperm
Bermuda
Tardigrada

ABSTRACT

Orzeliscus belopus has long been regarded as the only hermaphroditic marine tardigrade, yet there has been no published detailed information on its internal anatomy. Our study elucidates the ultrastructure of the ovotestis and the spermatozoa of *Orzeliscus* cf. *belopus* from Bermuda. The ovotestis had no septum to separate male and female germ cells and, while early stages of spermatogenesis were not observed, many spermatozoa were found at the periphery in both anterior and posterior areas of the gonad. The nucleus and the mitochondria of the spermatozoa in the ovotestis extend backward from the centriole region, forming a half-headed arrow-shape with the nucleus on the outer side of the 'arrowhead'. The cross section of a long vesicular body (the paranuclear vesicle) is dumbbell- or horseshoe-shaped and attached to almost the entire length of the nucleus. In the seminal receptacle, we found both a complete spermatozoon and some which started to degrade. There is an indication that sperm is further modified after discharge as within the receptacle duct the sperm is no longer half-headed arrow-shaped but has a straight nucleus. This modification might be correlated with the degeneration of the paranuclear vesicle. Our observations clearly show that *O. belopus* is a simultaneous hermaphrodite, and suggests that the reproductive mode includes copulation and cross-fertilization.

1. Introduction

Bisexual reproduction has been observed in almost all marine tardigrades. The only exception so far is *Orzeliscus belopus* Du Bois-Reymond Marcus, 1952. Some hermaphrodite specimens of this species were found by one of the authors (RMK) and this observation was referred to as a 'personal communication' in a paper by Bertolani (1987). This reference has frequently been cited in articles on tardigrade reproduction (e.g. Bertolani and Rebecchi, 1999; Rebecchi et al., 2000) but detailed information about the ovotestis structure had never been published.

O. belopus was at first described from São Sabastião, Brazil (Du Bois-Reymond Marcus, 1952), and many other reports revealed its wide distribution including, in the Atlantic Ocean, Bermuda (Renaud-Mornant, 1970; Kristensen and Sterrer, 1986; Kristensen and Neuhaus, 1999), Antilles (Pollock, 1982), Scotland (Pollock, 1971), France (Renaud-Debyser, 1963), and Spain (Veiga et al., 2009); the Mediterranean (De Zio Grimaldi et al., 2001); and, in the Pacific Ocean, New Caledonia (Renaud-Mornant, 1967), and Galapagos (McKirdy et al., 1976). Furthermore, we had access to the University of Copenhagen tardigrade collection which included unpublished *Orzeliscus* data of specimens from Egypt, Florida, Japan and Australia (Heiner and Kristensen, 2003). Another species, *O. septentrionalis* (see Schulz, 1953) described from the North Sea, was synonymized with *O. belopus* by Pollock (1982). Hence the monotypic genus has been recognized as a cosmopolitan, though several undescribed species are held in the Copenhagen collection and some of the above-mentioned records may also include new species.

In the literature there is limited information regarding the reproductive mode of this tardigrade and no reports of males. The aim of this paper is to demonstrate the coexistence of spermatozoa and oocytes in the gonad, and to describe the ultrastructure of *O. belopus* spermatozoa from Bermudan specimens, with light microscopy images from an Egyptian specimen.

2. Materials and methods

2.1. Materials

The Egyptian specimens of *O. belopus* were collected by H. Ramløv on 29 August 1981, at El Hurghada, 4 km south of the Marine Biological Station in the Red Sea, at 1.5 m depth. The clean, coarse coralline sand samples were treated by fresh water shock

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http://dx.doi.org/10.1016/j.jcz.2014.07.003

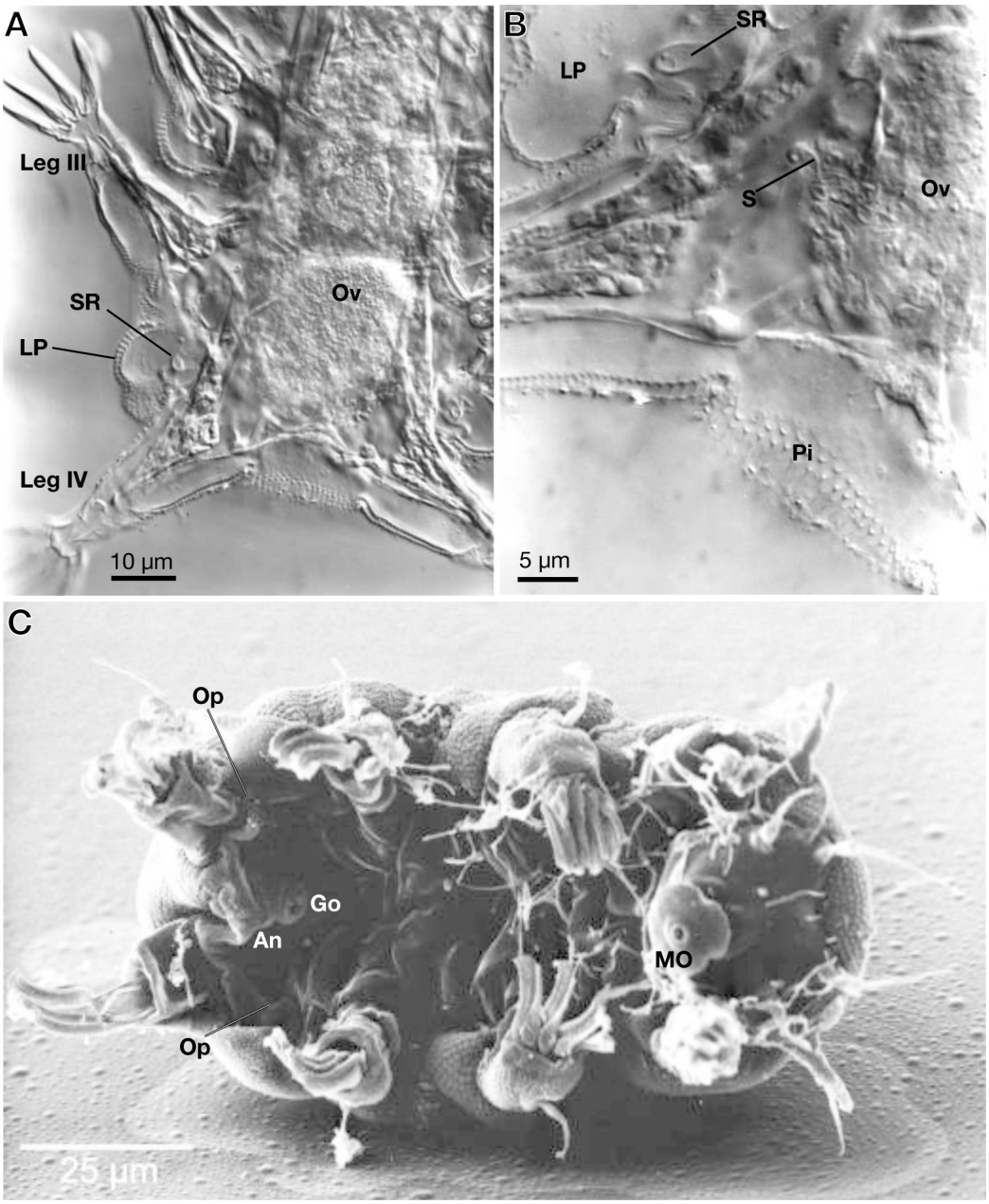

Fig. 1. *Orzeliscus* cf. *belopus*. Light microscopy images of a specimen from Egypt (A and B) and a SEM image of a specimen from Bermuda (C). (A) Posterior region between legs III and IV. (B) Higher magnification of gonad showing seminal receptacle and sperm. (C) Ventral habitus. An, anus; Go, gonopore; LP, lateral projection; MO, mouth opening; Op, opening of seminal receptacle; Ov, ovary; Pi, pillars of epicuticle; S, spermatozoa; SR, seminal receptacle.

and the supernatant was decanted through a 32 µm mesh net, and the content on the mesh was fixed with 4% buffered formaldehyde in seawater. Several additional specimens were collected by R.M. Kristensen on 20 May 1983, at Castle Harbour near Castle Island, Bermuda, from fine coralline sand at 8 m depth (see Kristensen and Neuhaus, 1999). The samples were processed as above. The specimens for transmission electron microscopy were collected by R. M. Kristensen on 17 May 1983, at Saint David's Head, Bermuda from coarse coralline sand at 1.5 m depth. After the short fresh water shock the content on the mesh was fixed in trialdehyde (Lake, 1973).

2.2. Preparations

The animals for light microscopy were fixed with 4% formaldehyde in seawater and stored for several months before the tardigrades were sorted out. Then each specimen was mounted on slide glass with cover slip in the fixative and micrographs were taken with a Zeiss Ultraphot 3 with DIC-optic (Nomarski-technique) before the specimens were transferred to glycerol. Each specimen was finally embedded in glycerol and the cover was sealed with Glyceel. The specimens for transmission electron microscopy (TEM) were fixed in trialdehyde in diluted seawater for 2 h (after Lake, 1973 and Jørgensen et al., 1999 modification), rinsed and stored in 0.1 M sucrose/sodium cacodylate buffer (pH 7.4). Tardigrades were sorted under stereomicroscope and postfixed in 1% osmium tetroxide for 1 h. The animals were dehydrated in an ascending ethanol series, transferred to propylene oxide, and embedded in EPON 812. The ultrathin sections were cut on a Reichert OM U3 ultramicrotome and stained with uranyl acetate and lead citrate. TEM observation was done with JEM-1010 (JOEL) microscope at 80 kV. Furthermore, semi-ultrathin overview

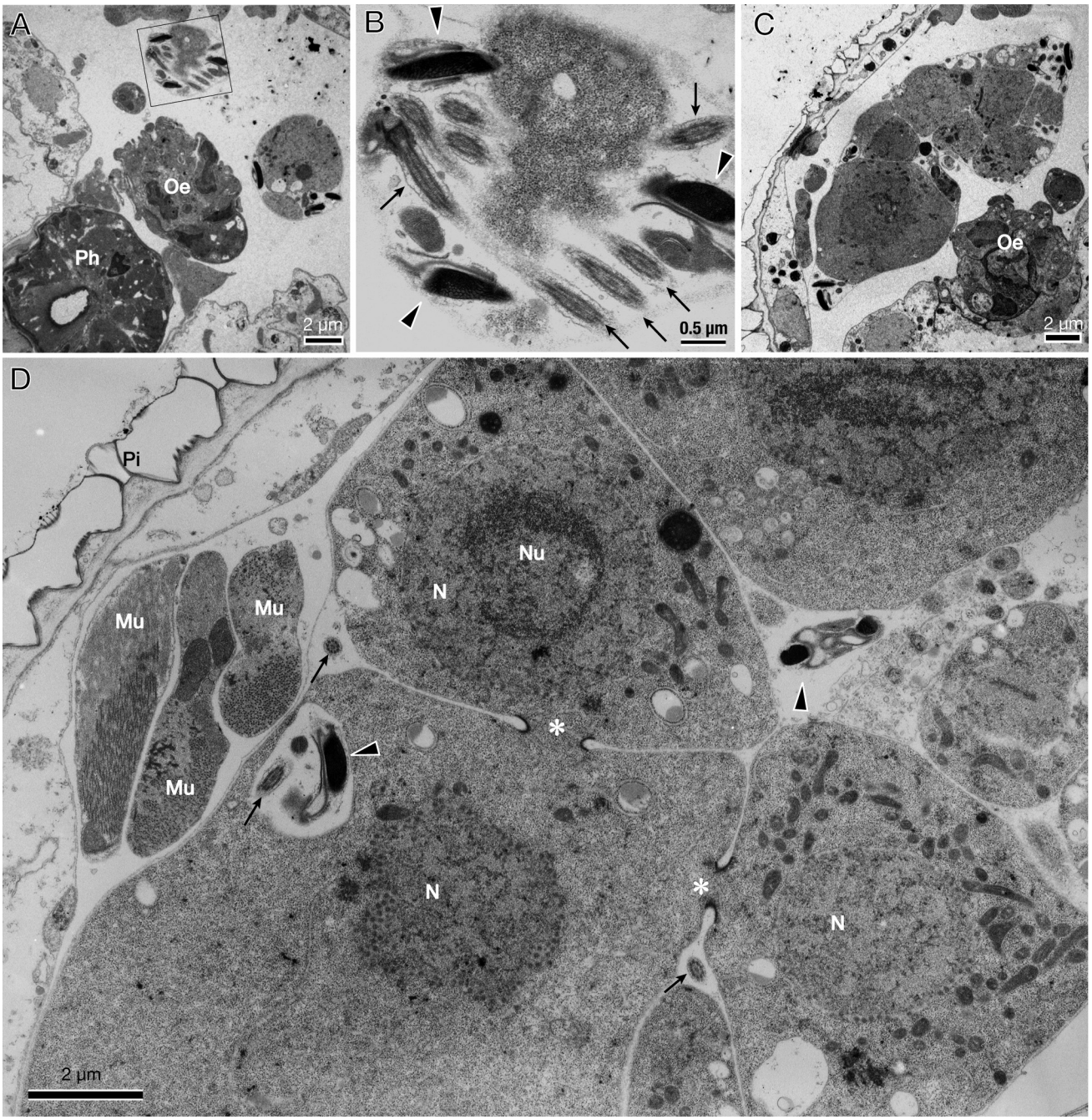

Fig. 2. *Orzeliscus* cf. *belopus* (from Bermuda). Anterior structures of the ovotestis – TEM. (A) Cross section of the most anterior region of the trunk. (B) Enlargement of region marked in A showing sperm heads (arrowheads) and flagella (arrows) around a germ cell. (C) Young germ cells and spermatozoa near the oesophagus (Oe). (D) Young germ cells connected by intercellular bridges (asterisks). Sperm heads (arrowheads) and flagella (arrows) among the cells. Mu, muscle; N, nucleus; Nu, nucleolus; Ph, pharynx; Pi, pillar of epicuticle.

sections (1 µm) were cut for every 5 grids. These sections on microslides were stained with toluidine blue, and later embedded in Entellan. Specimens for scanning electron microscopy (SEM) were dehydrated through an ascending ethanol/acetone series prior to critical point drying. The critical point dried animals were mounted on aluminium stubs and sputter coated with gold (Kristensen and Neuhaus, 1999) for observation in a JEOL JSM-840 scanning electron microscope.

2.3. Three dimensional reconstruction

The outlines of seminal receptacle and spermatozoa serial sections were traced, coloured and their orientation adjusted using Adobe Illustrator CS5, with the individual images saved as jpeg files in Adobe Photoshop CS5. Three-dimensional representations of these structures were reconstructed from these stacked files in the freeware programme, DeltaViewer (http://delta.math.sci.osaka-u.ac.jp/DeltaViewer/).

2.4. Directional terms for the spermatozoa

We used the terms “proximal/distal” instead of ‘anterior/posterior’ when we describe the nucleus and nuclear associated parts of the gonadal spermatozoa because they are bent backward from the centriole region.

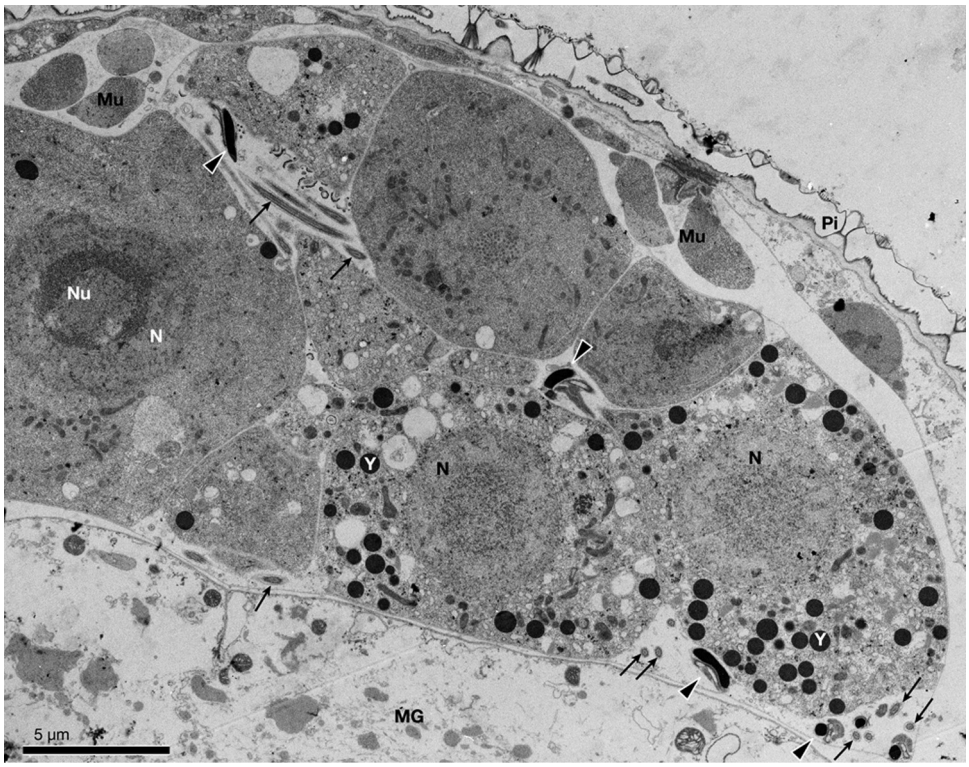

Fig. 3. *Orzeliscus* cf. *belopus*. Spermatozoa among young germ cells and vitellogenic oocytes. TEM. Arrowheads and arrows indicate the sperm heads and flagella, respectively. MG, mid gut; Mu, muscle; N, nucleus; Nu, nucleolus; Pi, pillar; Y, yolk granules.

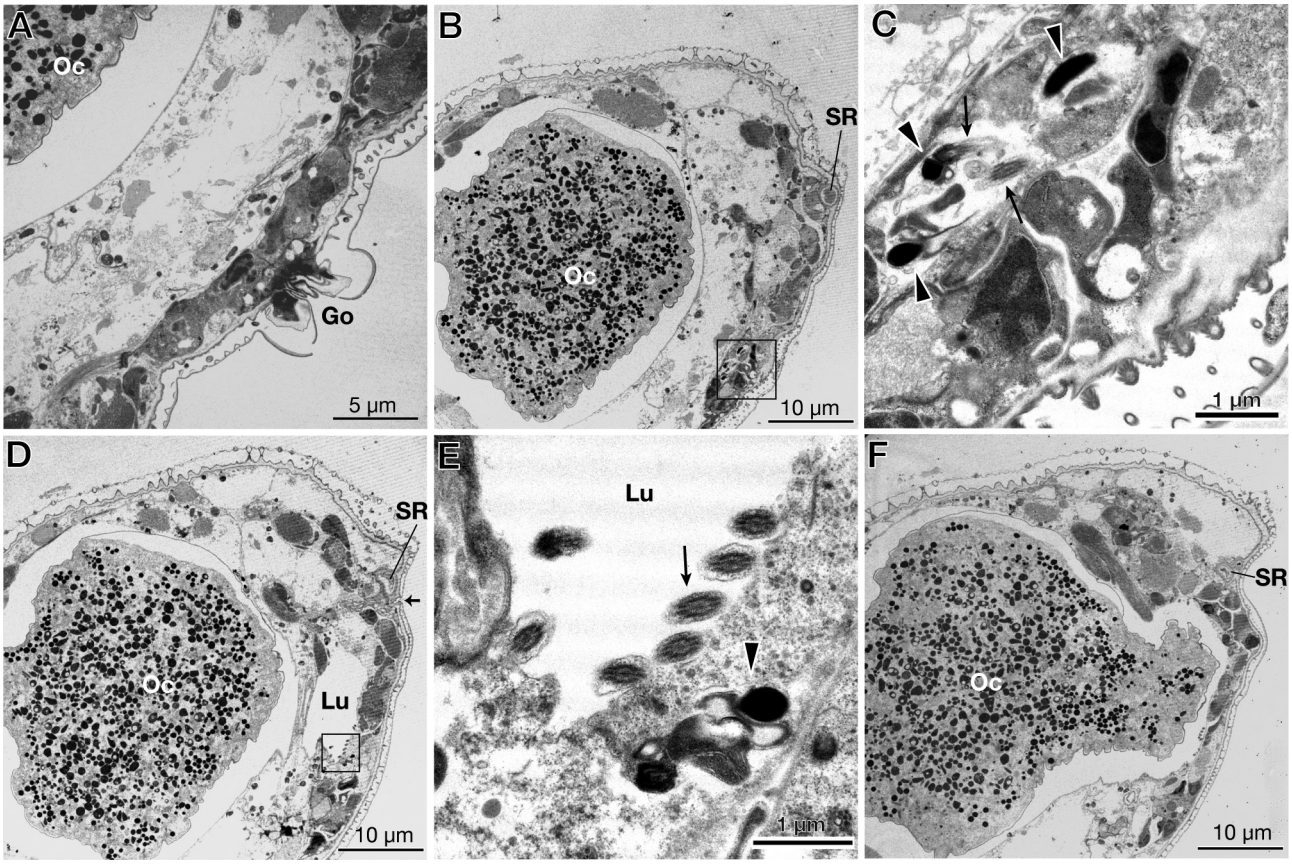

Fig. 4. *Orzeliscus* cf. *belopus*. Ovotestis and reproductive system. TEM. (A) Section showing the gonopore (Go), which opens mid-ventrally in front of the 4th legs. (B) Section slightly dorso-posterior to the gonopore with sperm observed in the area delineated by square. Seminal receptacle (SR) located near the lateral projection. (C) Enlargement of region marked in B, showing sperm head region (arrowheads) and flagellum (arrows). (D) Section showing gonoduct (oviduct) lumen (Lu) and the opening of the seminal receptacle (SR) indicated by a small arrow. (E) Enlargement of region marked in D, showing sperm in the oviduct lumen (Lu). (F) Section showing part of the large oocyte (Oc), which protrudes into the entrance of the oviduct.

3. Results

3.1. Ovotestis and germ cells

Fig. 1A and B (light microscopy) shows the hermaphroditic structure of *O.* cf. *belopus* from the Red Sea, which was the individual originally recognized as a hermaphrodite marine tardigrade (Bertolani, 1987). The two micrographs were taken in the formaldehyde fixative before the animal was transferred to the glycerol preparation. In the glycerol preparation the spermatozoan are difficult to observe. A large egg is visible in the posterior half of the gonad (Fig. 1A) with spermatozoa around the egg (Fig. 1B). Two lateral seminal receptacles (Fig. 1A), not previously reported for this genus, are situated near the base of lateral projections between the 3rd and 4th legs. The ventral habitus of a Bermudan specimen is shown in Fig. 1C (SEM). We observed two specimens by TEM and found that one was hermaphrodite while the second was a female with an ordinary ovary. Here we describe the gonad of the former

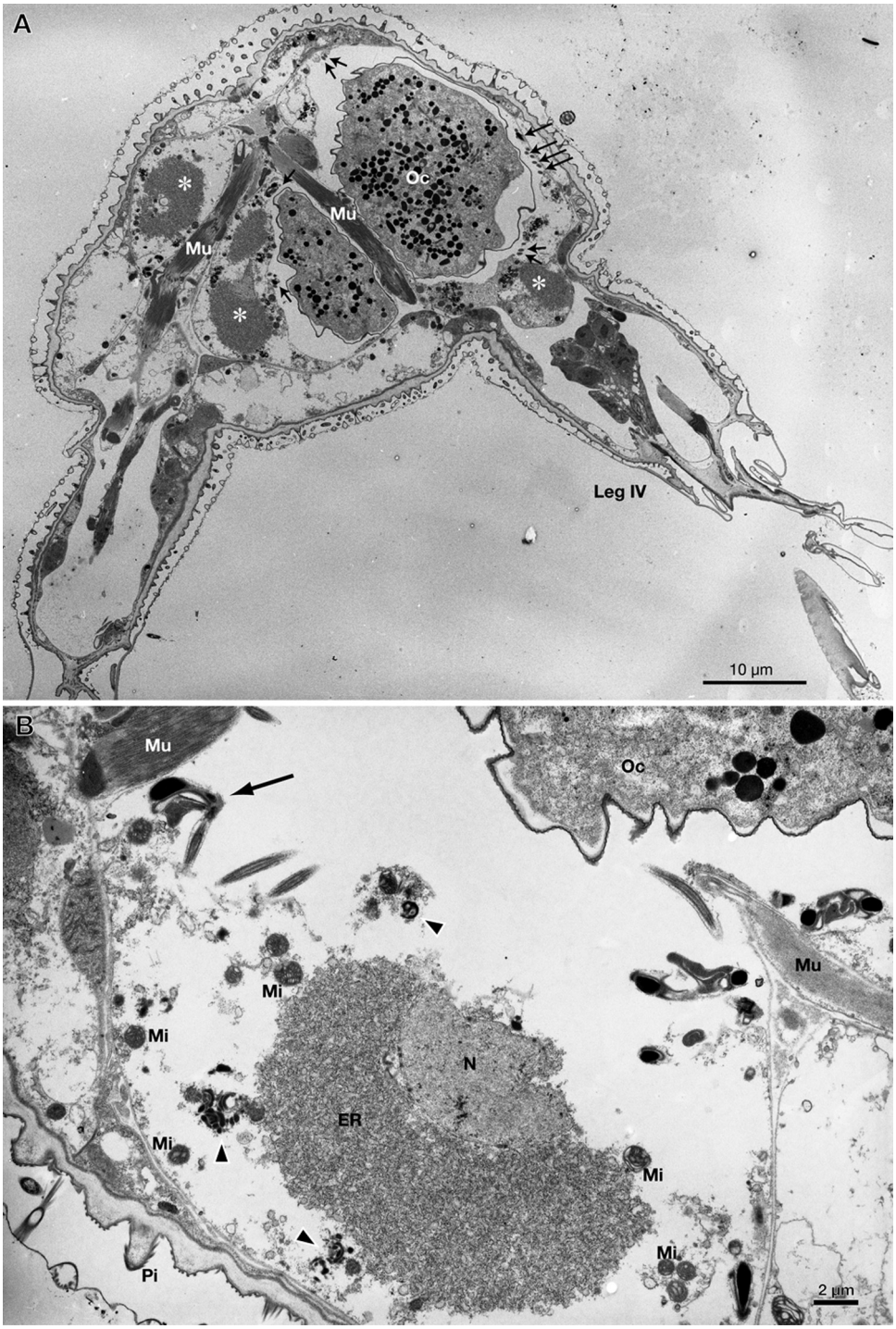

Fig. 5. *Orzeliscus* cf. *belopus*. Ovotestis between the 4th pair of legs. TEM. (A) Posterior region of the large oocyte (Oc), which appears to be divided by muscle bundles (Mu). Within the gonad, around the periphery, large cells (asterisks) with very rich membranes of endoplasmic reticulum. Spermatozoa are marked by small arrows. (B) Higher magnification of section showing peripheral ER-rich cell and spermatozoa. A longitudinal section of a spermatozoal neck region is marked by an arrow. Conspicuous black bodies (arrow heads) and spherical mitochondria (Mi) are present and the prominent membranous structures (ER) partially encircling a nucleus (N). The cuticle with internal pillar (Pi) structure is present in the lower left.

specimen. Males were not found in either the Bermuda or in the Red Sea populations.

The ovotestis forms an unpaired sac that runs dorsal to the alimentary tract for most of its length, with the most anterior part lying close to the pharyngeal bulb (Fig. 2 A–C). Both, male and female germ cells, co-exist in the same ovotestis chamber without separating septa. Young germ cells, which may be either spermatocytes or oocytes, were observed in the anterior section, followed by vitellogenic oocytes. A large egg occupied the posterior half of the gonad. Fully formed, mature sperm were found around the periphery of both anterior and posterior regions of the gonad but earlier spermatogenesis stages were not observed in this specimen.

3.2. Sperm in the reproductive system – from ovotestis to the gonopore

Young germ cells in the most anterior region of the gonad were connected by intercellular bridges (Fig. 2D) and surrounded

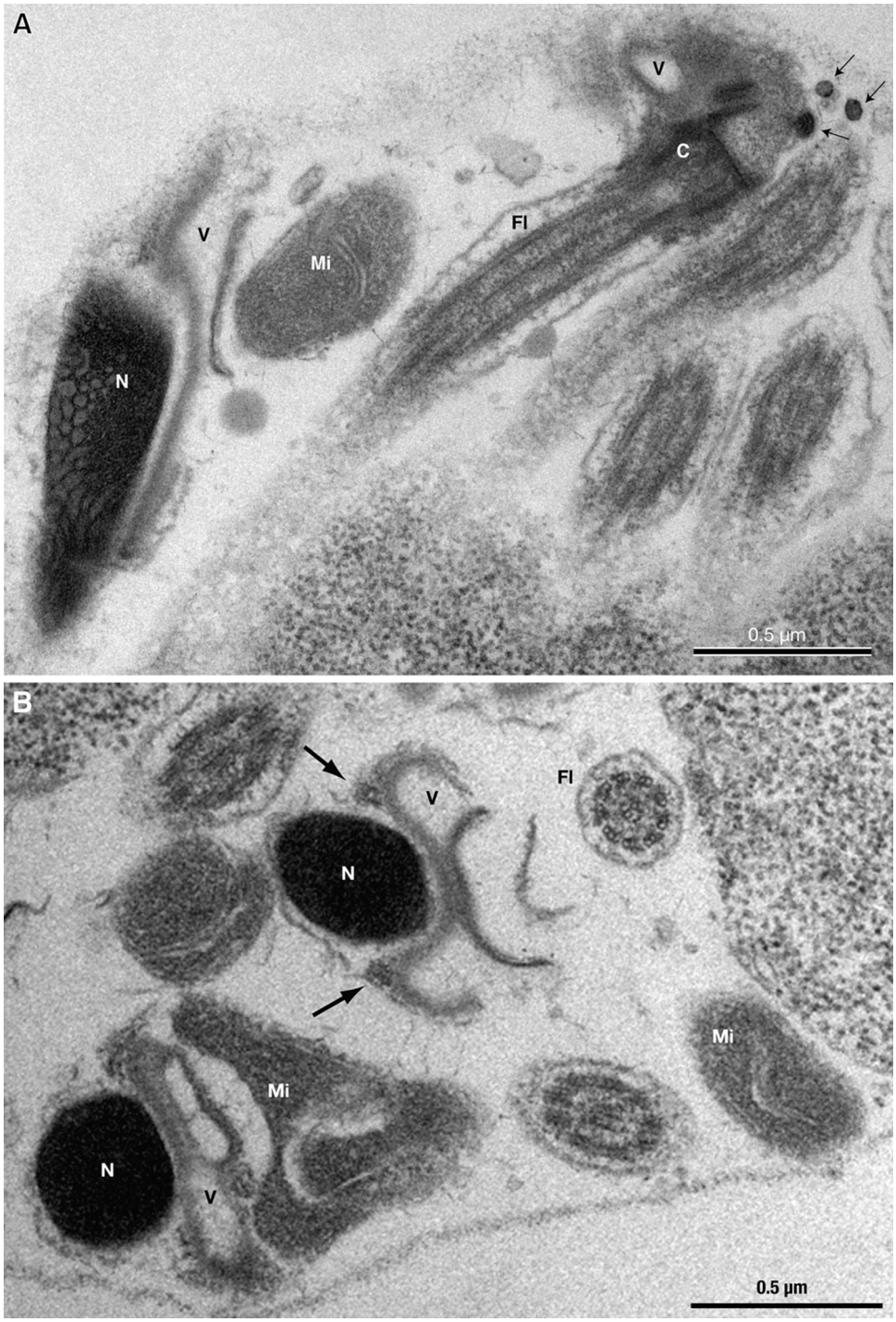

Fig. 6. *Orzeliscus* cf. *belopus*. Fine structure of spermatozoa. TEM. (A) Longitudinal section (enlargement from Fig. 2B) comprising centriole (C), flagellum (Fl), mitochondria (Mi), paranuclear vesicle (V) and small dense bodies (arrows) attached anteriorly. (B) Cross section of main sperm organelles. Electron dense material (arrows) is present inside the cell membrane between the paranuclear vesicle (V) and nucleus (N). The mitochondria of the upper spermatozoon (Mi at far right) protrude from the cell body and is 'free'. The second spermatozoon (lower left) shows a more proximal cross section where the mitochondria are located, with paranuclear vesicle and nucleus, inside the cell body.

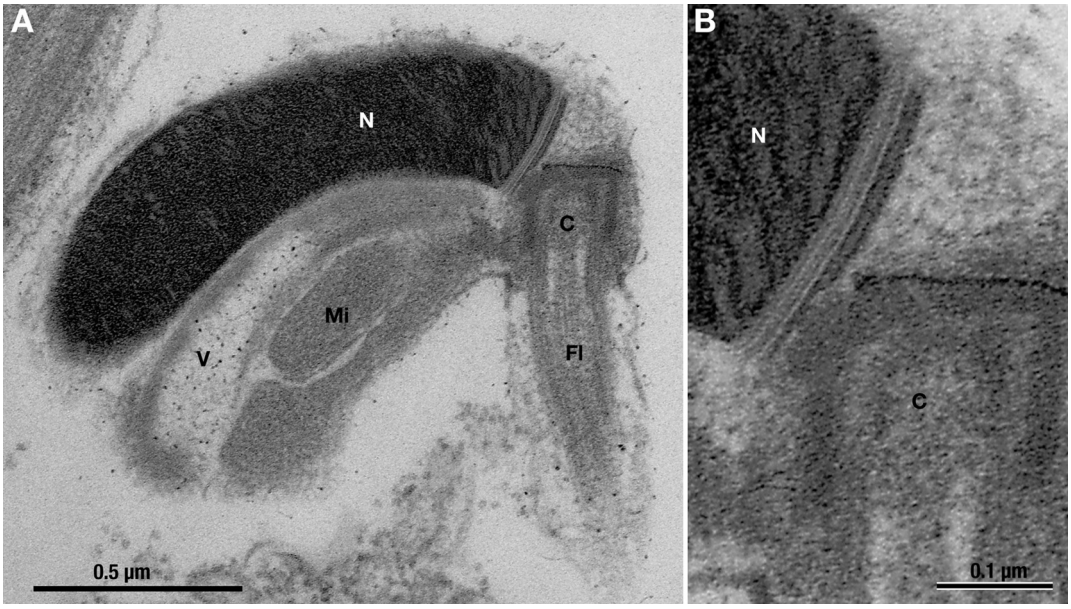

Fig. 7. *Orzeliscus* cf. *belopus.* Fine structure of spermatozoa. TEM. (A) Longitudinal section showing the connecting region of the sperm head. (B) Enlargement of the connecting region between the nucleus and the centriole. C, centriole; Fl, flagellum; Mi, mitochondria; N, nucleus; V, paranuclear vesicle.

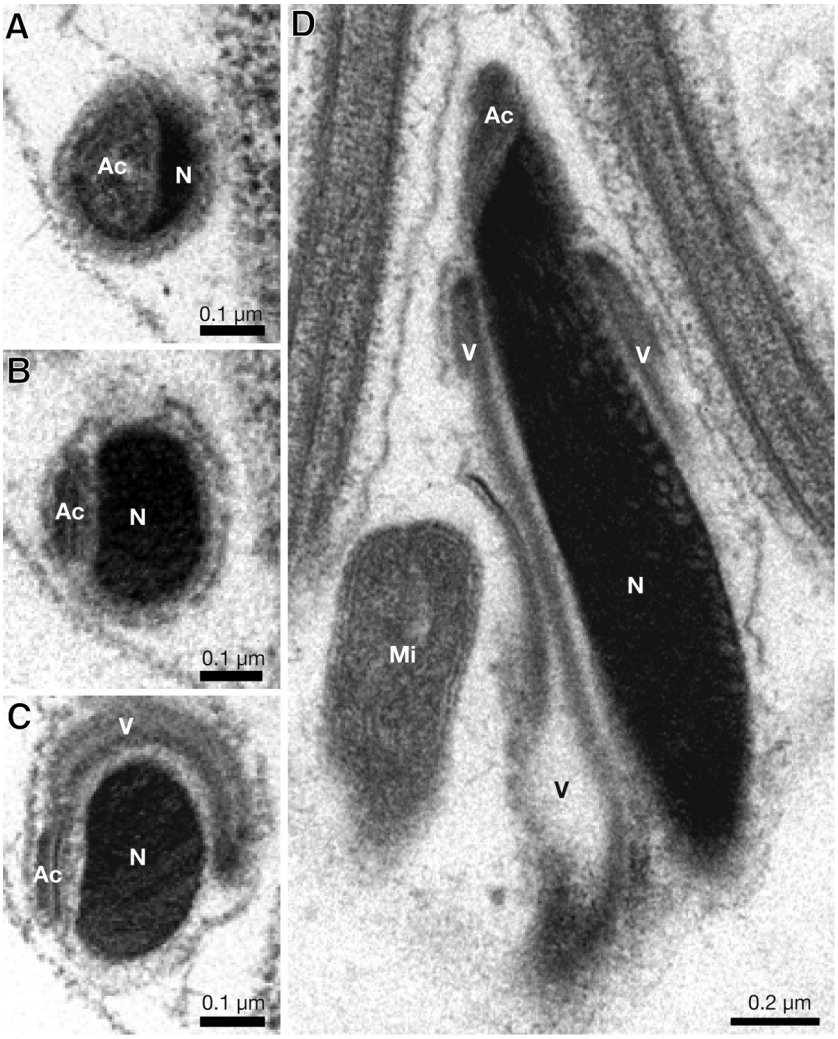

Fig. 8. *Orzeliscus* cf. *belopus.* Fine structure of spermatozoa – acrosomal region. TEM. (A–C) Tip of the nucleus showing the acrosome in three cross sections from distal to proximal region. (D) Longitudinal section of the acrosomal region. Ac, acrosome; Mi, mitochondria; N, nucleus; V, paranuclear vesicle.

by mature sperm (Fig. 2A–D). Some of the sperm appeared to be invaginated into a young germ cell (Fig. 2D), but no connection was found between the two. Several sperm are also observed between young oocytes and vitellogenic oocytes (Fig. 3).

The female rosette gonopore is located mid ventrally in front of the fourth pair of legs, as seen in Figs. 1C and 4A. In TEM sections, slightly dorso-posterior to the gonopore, a number of sperm were observed in the unpaired gonoduct that connects the gonad to the gonopore (Fig. 4B–F).

In a section of the posterior region between the 4th pair of legs a number of sperm were observed (Fig. 5A, small arrows), and a large egg protrudes into the posterior area behind the leg muscle bundles. Inside the posterior gonad, around the periphery, were several large cells having prominent endoplasmic reticulum (ER) membranes, spherical mitochondria and peculiar electron-dense bodies (Fig. 5B). Although a number of spermatozoa were observed near these cellular masses there was no clear relationship.

3.3. Morphology of the spermatozoa in the ovotestis

The organelles of the gonadal spermatozoa consist of a nucleus, acrosome, paranuclear vesicle, the two mitochondria, centriole, and a flagellum (Fig. 6). The nucleus, an extremely electron dense body (Figs. 6–9), also exhibited compacted fibrous material (Figs. 6A, 7, 8C and D). The paranuclear vesicle always accompanied the nucleus (Figs. 6–9), partially encircling almost the entire length. In cross section the paranuclear vesicle appeared dumbbell-shaped (Figs. 6B and 9D) and separated the nucleus from the mitochondria. Most of the area inside the vesicle was electron-lucent, though there were certain regions of lightly osmiophilic materials beneath the vesicle membrane and, on the mitochondrial side, electron-dense deposition on the membrane was observed (Figs. 6A and B and 9D). Moreover, electron dense material was also observed just inside the cell membrane between the paranuclear vesicle and nucleus (Fig. 6B, arrow).

The anterior end of the spermatozoal head, incorporating the nucleus, paranuclear vesicle and mitochondria, were attached at an angle to the centriole forming a half-headed arrow shape (Figs. 5B (arrow), 6A and 7A). In section the nuclear base was covered by two unit membranes, or nuclear envelope, and a thick, electron-dense trilaminar zone that formed an acute angle with a layer of dense material at the anterior of the centriole (Fig. 7A and B). In some sections certain dense material appeared as centriolar adjunct (Fig. 10). The mitochondrion was attached to the lateral wall of the centriole (Fig. 7A), and separated by a layer of electron-dense material. In the cross section the mitochondria have two intertwined components that were shaped like the Yin-Yang symbol (Fig. 9D). At the anterior end of the spermatozoal head the two mitochondria were in tight association with the nucleus and the paranuclear vesicle (Figs. 6B and 7A), but more posteriorly the two mitochondria were partially 'free' from the cell body (Fig. 6B). The acrosome forms the distal tip of the nucleus, and was not covered by the paranuclear vesicle, which was slightly shorter than the nucleus (Fig. 8). The centriole,

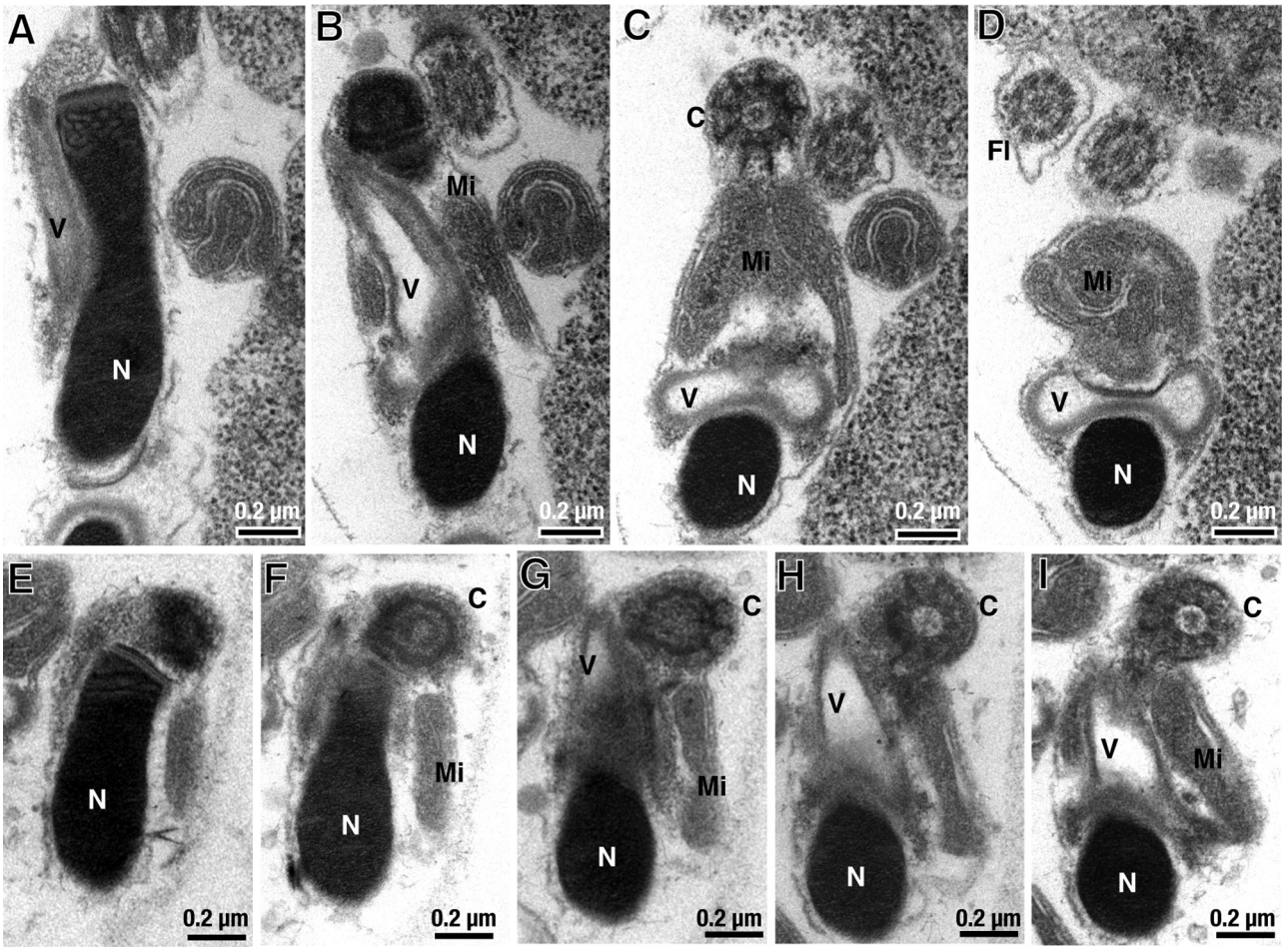

Fig. 9. *Orzeliscus* cf. *belopus.* Fine structure of spermatozoa – centriole region. TEM. (A–D) Sperm head region in four serial cross sections. (E–I) Five serial sections through a sperm head. C, centriole; Fl, flagellum; Mi, mitochondria; N, nucleus; V, paranuclear vesicle.

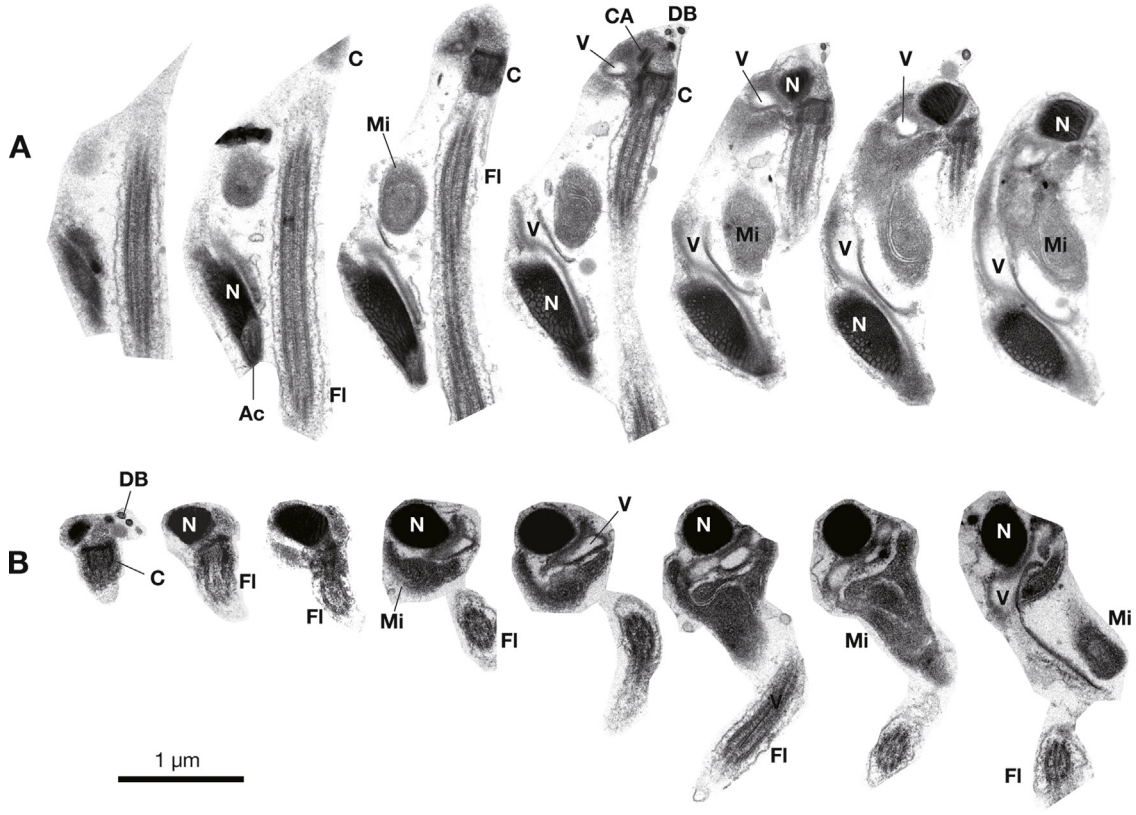

Fig. 10. *Orzeliscus* cf. *belopus.* Fine structure of spermatozoa. TEM. Composite images of serial sections through head regions of two spermatozoa. (A) Longitudinal sections. (B) Cross sections arranged anterior–posterior from the left. C, centriole; CA, centriolar adjunct; DB, small dense bodies; Fl, flagellum; Mi, mitochondria; N, nucleus; V, paranuclear vesicle.

at the anterior of the flagellum (Figs. 6A, 7A and 9B, C, E–I), is attached by peripheral satellite rays (Fig. 9C and G), which in turn probably attached to the cell membrane. A group of small dense bodies were observed at the junction of the nucleus and centriole (Fig. 6A), and were always present in the serial sections (Figs. 10 and 11).

From a series of 29 cross section TEM images (Fig. 11A) (from a total 43 sections, 14 of which were damaged or hidden by grid mesh) we verified the morphology of the sperm head region by 3-D reconstruction (Fig. 11B). In the reconstruction (Fig. 11B), it is possible to see that the long nucleus curved backwards in an arc starting from the side of the centriole. The paranuclear vesicle encircled the inner curve of the nucleus and was dumbbell-shaped in the proximal region of the nucleus but horseshoe-shaped more distally. The mitochondria, initially sandwiched between the paranuclear vesicle and the flagellum, extended free of all other spermatozoal head structures. At the most anterior of the spermatozoa were the small dense bodies.

3.4. Seminal receptacle and spermatozoa

A pair of seminal receptacles is present about 25 µm laterally and slightly posterior to the gonopore (Fig. 4B, D and F). From a nearly continuous series of 107 TEM sections the entire seminal receptacle structure was observed, and 59 of these sections (55%) were appropriate to retrieve photographs (part of the series is shown in Figs. 12 and 13). Using these images we were able to reconstruct a 3D model of the seminal receptacle (Fig. 14A–C) and a spermatozoon located within (Fig. 14D–F). The seminal receptacle consisted of a gourd-shaped chamber (cuticle covered vesicle) and winding cuticular duct (Figs. 12 and 14). The external opening to the seminal receptacle duct was situated 1 µm medially to the receptacle chamber (Fig. 12C), invaginating medially from the trunk cuticle, turning and elongating laterally, before turning medially to connect with the anterior portion of the receptacle chamber. An epicuticular honeycomb layer (similar to that which lines the epidermis but thicker; ca. 150 nm and 60 nm, respectively), lined the internal surface of the seminal receptacle, with the hexagons of ca. 13 nm diameter (Fig. 12A). This implies that the spermatozoa in the receptacle are situated outside the epicuticle.

A spermatozoon was observed in the seminal receptacle (Figs. 12–14) with its head located in the duct and the flagellum extended into the receptacle chamber. The endpiece of the flagellum (Fig. 12B) reached the innermost part of the chamber, suggesting a total length of 13.4 µm. Based on our serial sections (Figs. 12 and 13) two electron dense nuclei were identified, one of which (4.4 µm in length) was situated anteriorly to the centriole and extended towards the receptacle duct opening. The connection between this nucleus (Fig. 13A) and the centriole region (Fig. 13B) was unclear due to the loss of four serial sections. The other nucleus (4.1 µm in length) appeared posteriorly to the centriole and was positioned at the entrance of the receptacle chamber, but there did not appear to be a direct connection of this nucleus to the spermatozoon (Fig. 13D–G). Moreover the periphery of the latter nucleus was made fuzzy by fine fibrous materials (Fig. 13E–G), which were never observed in the gonadal spermatozoa, suggesting its deteriorated condition. Therefore, these serial sections were showing the nucleus of an entire sperm extended anteriorly with, nearby, a degraded nucleus from an older sperm. Within the receptacle chamber other cellular fragments of degraded sperm could be observed (Fig. 12B–D). The bipartite origin of the two mitochondria was present (Fig. 13C–D) further posteriorly. Fig. 13E shows the cross-section of the acutely bent duct where the two

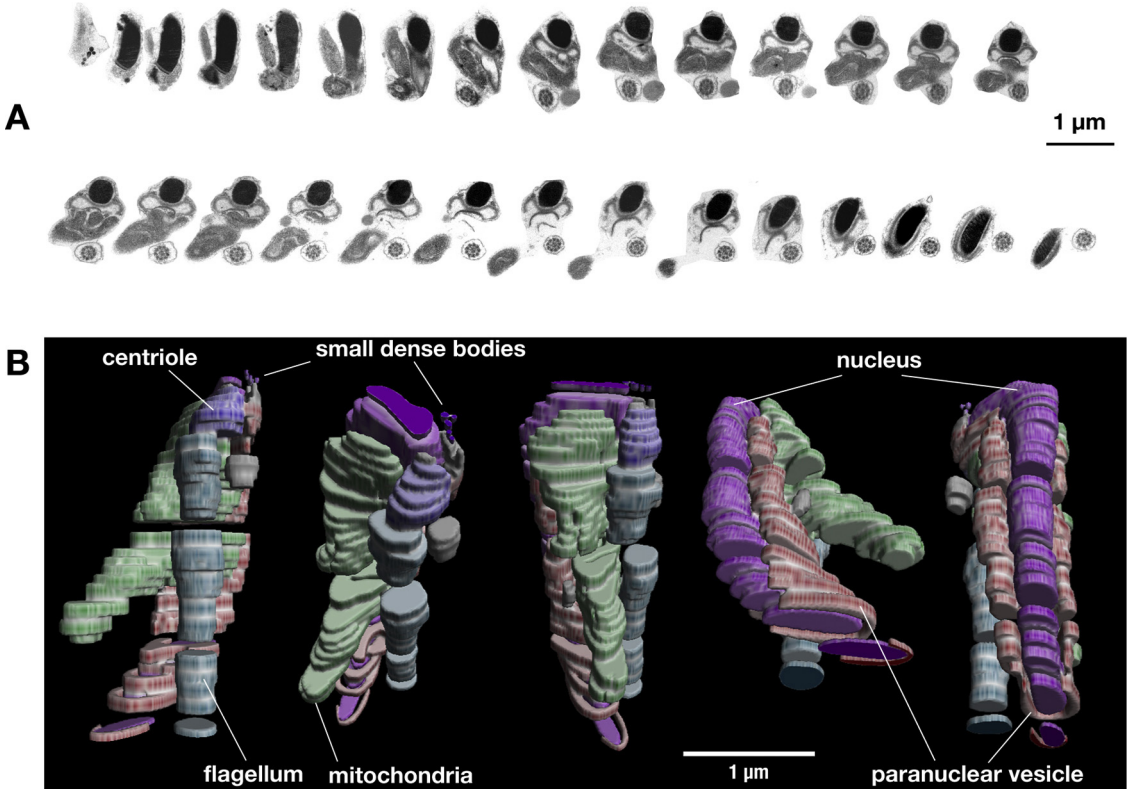

Fig. 11. *Orzeliscus* cf. *belopus.* Fine structure of spermatozoa – head region. (A) Composite image of 29 serial TEM sections, arranged anterior–posterior from top left. (B) 3D reconstruction based on the serial sections depicted in A.

mitochondria are present in the left (proximal) section but no longer visible in the right (distal) section. The 3-D reconstruction of the spermatozoon within the receptacle duct (Fig. 14E and F) clearly shows the modified location of organelles with the anterior nucleus. The mitochondria are now apart from the nucleus and situated along the flagellum. A limited region in the sperm flagellum (Fig. 14E – asterisk, six sections) was slightly thickened with cytosol.

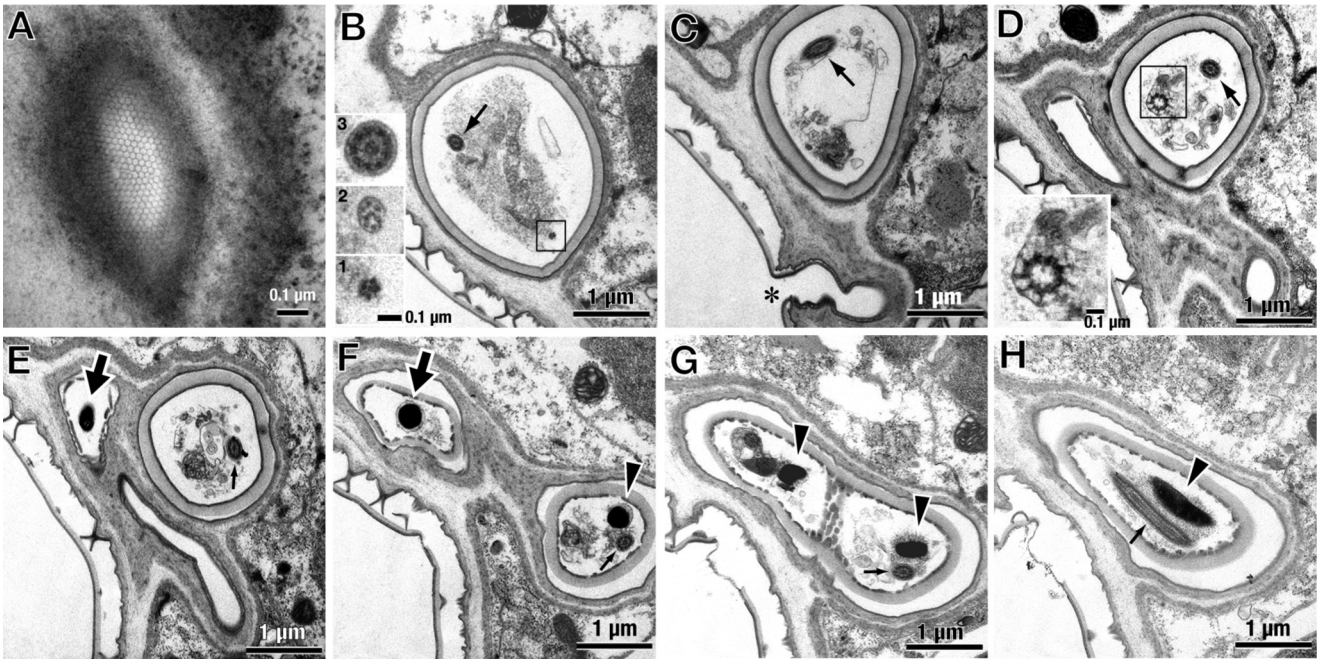

Fig. 12. *Orzeliscus* cf. *belopus.* Seminal receptacle structure. TEM serial sections, from anterior–posterior (A–H). (A) Tangential section showing the honeycomb layering of the receptacle. (B) Cross section of the seminal receptacle. Flagellum (arrow) and its endpiece (in square) of a spermatozoon around a central mass composed of degrading sperm. Insets: 1, enlargement of the endpiece; 2, endpiece from a more proximal serial section; 3, cross section of the flagellum. (C) Duct opening (asterisk) of the seminal receptacle. (D) Cross section of centriole of a degraded sperm (in square) found in the receptacle chamber. Inset: enlargement. (E) The most anterior region of the sperm appears as a dense body in the duct (large arrow). Small arrow indicates the flagellum. (F) Another dense part seen in the bottom of the seminal receptacle (arrow head) next to the flagellum (small arrow). (G–H) Showing part of the curved region of the receptacle duct. Arrowheads, the same dense part as in F; small arrow, sperm flagellum.

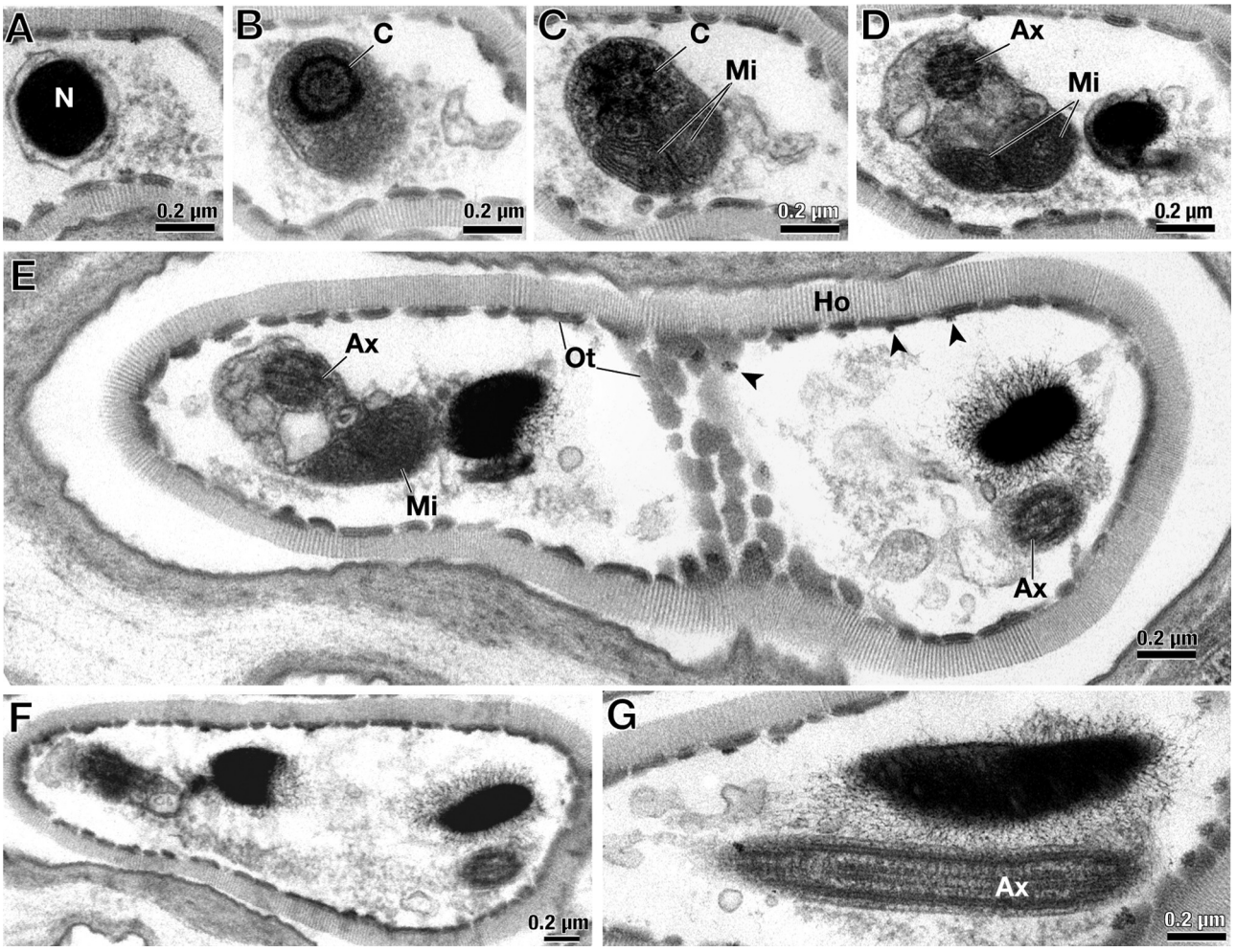

Fig. 13. *Orzeliscus* cf. *belopus*. Spermatozoon in the receptacle duct – with details from a series of TEM sections in the region indicated in Fig. 12F and H. (A) Enlargement of the nucleus in cross-section; from top left of Fig. 12F. (B) The most anterior section of centriole complex (C). (C) Centriole complex with satellite ray and basal section of the two mitochondria (Mi). (D) Cross section of the axoneme (Ax), mitochondria, and to the right the most anterior section of a separate degraded nucleus. (E) Enlargement of Fig. 12G, showing a tangential section of the outer trilaminary layer (OT) as it appears at the point at which the receptacle duct bends acutely; short spine bundles (arrowheads) were found on the trilaminary layer. Honeycomb layer (Ho) formed a lining under the trilaminary layer. (F–G) Longitudinal section of the winding receptacle duct.

4. Discussion

4.1. Sperm morphology

The individual examined in this study did not permit the evaluation of *O. belopus* early stage spermatogenesis as only mature sperm were observed. However, although early stages of spermatids were absent, significant cellular mass with very rich ER membranes (Fig. 5) were observed and suggested the possibility of spermatid nurse cells as in *Actintarctus doryphorus* (see Jørgensen et al., 1999). The fully developed ovotestis allowed us to reveal the ultrastructure of *O. belopus* spermatozoon by serial TEM sectioning. Moreover, the spermatozoon located in the seminal receptacles unravel further interesting details about the final metamorphosis process. These observations were synthesized into schematic drawings showing the overall shape of the spermatozoa in the ovotestis and seminal receptacles of *Orzeliscus belopus* (Fig. 15).

4.1.1. Spermatozoa in ovotestis

Mature sperm in the ovotestis comprise a long nucleus and two mitochondria that are partially 'free' of the nucleus, which together extend backwards from the centriole region, giving an overall shape of a half-headed arrow (Fig. 15A). This sperm head shape is also described in other marine heterotardigrades, i.e. *Batillipes noerrevangi* Kristensen, 1978 (see Kristensen, 1979), *Echiniscoides sigismundi* (Schultze, 1865) (see Kristensen and Hallas, 1980; Greven and Kristensen, 2001), *Wingstrandarctus corallinus* Kristensen, 1984, and *Actinarctus doryphorus* Schulz, 1935; only in the last species the two mitochondria are not 'free' from the cell body (Jørgensen et al., 1999).

The presence of a paranuclear vesicle is an interesting finding. It was attached along the entirely inner-lateral side of, and nearly as long as, the nucleus. In cross section the paranuclear vesicle showed dumbbell-shaped whether in the proximal section it was sandwiched between the mitochondria and the nucleus. The impression was that the paranuclear vesicle partially encased the nucleus and, hypothetically, its function could be to attach the nucleus to the mitochondria, holding the nucleus in the backward pointing position (see below).

At the junction of the flagellum and nucleus, the centriole was accompanied by a structure similar to the 'satellite rays' or the 'anchoring fibre apparatus' of jellyfish (Afzelius and Franzén, 1971; Harrison and Jamieson, 1999) and the 'centriolar apparatus' of priapulids (Storch et al., 2000), which probably fixes the flagellum to the sperm cell body. This structure may be also homologous to what was considered as an apomorphic condition of 'outer dense accessory fibres' (Rebecchi et al., 2011), which were found in two species of eutardigrades, *Paramacrobiotus areolatus* (Murray,

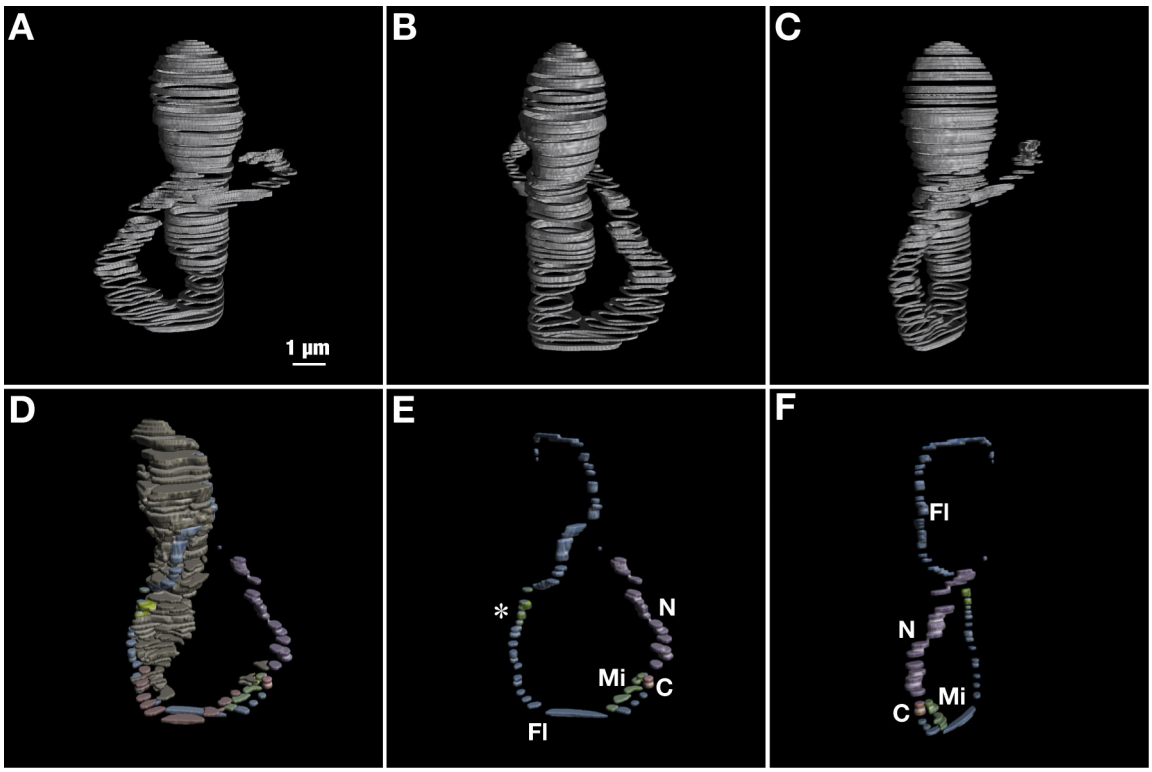

Fig. 14. *Orzeliscus* cf. *belopus*. Seminal receptacle as 3D reconstruction based on TEM serial sections (A–C). The bottom of receptacle chamber (top of the images) directs anteriorly in the animal. (D) 3-D reconstruction of the internal contents of the receptacle. Same orientation as B. (E and F) 3-D reconstruction of a complete spermatozoon (omitting additional receptacle contents) correlated in orientation as B and C, respectively. C, centriole (red); Fl, flagellum (blue); Mi, mitochondria (green); N, nucleus (purple). Contents of the receptacle, mostly comprised of degraded sperm, are expressed as grey except for the degraded nucleus (pink). The greenish regions in the sperm flagellum (E – asterisk, six sections) were slightly thickened with cytosol.

1907) and *P. richtersi* (Murray, 1911). However, the presence of such a structure in heterotardigrades suggests that it represents a plesiomorphic condition. The absence of the proximal centriole in *Orzeliscus* spermatozoa, as well as in all other tardigrades examined so far, is interesting because it might be an apomorphy for Tardigrada, as in Hexapoda, Acantocephala, and Nematoda (Krioutchkova and Onishchenko, 1999). To reveal whether a centriolar duplication at the end of the first meiotic division is lacking studies on early spermatogenesis are needed. At the potential location of a proximal centriole a group of small dense bodies are present. These small dense bodies, of unknown function, were situated at the most anterior part of the gonadal sperm.

4.1.2. Spermatozoa in seminal receptacle

The spermatozoon in the seminal receptacle was different in shape from the gonadal spermatozoa as the nucleus was no longer bent backwards and we found no evidence of the paranuclear vesicle. This relocation of the nucleus might be explained by the breakdown of the paranuclear vesicle, thus, as mentioned above, the paranuclear vesicle played a role in keeping the spermatozoon shape before discharging into the seminal receptacle.

Within the seminal receptacle duct the sperm nucleus, no longer bent backwards, faced outwards towards the duct opening with the flagellum lying in the receptacle chamber (Fig. 14E). This orientation suggests that the spermatozoon either turned its position after entering into the duct or entered backwards (tail first). The most plausible story would be that the fully developed spermatozoon, still half-headed arrow-shaped, enters the receptacle duct where a reaction (which might be like an acrosomal reaction) occurs to breakdown the paranuclear vesicle, liberating the nucleus and mitochondria, and allowing the flagellum to straighten so the spermatozoon is completely stretched. It is not known whether the small dense bodies, initially situated at the most anterior point of the fully formed spermatozoon, might also take a part in this metamorphosis. We believe the spermatozoon observed near the receptacle duct opening was the most recently injected as all the others within the receptacle were more or less degraded. A second, slightly degraded nucleus was found behind the most recent spermatozoon in a position that indicated it also faced towards the exit with the nucleus ahead (Fig. 14D, red). Indeed, all spermatozoa within the seminal receptacle would need to face the opening in order to exit for fertilization. If the receptacle chamber was empty the spermatozoa could use the space to turnaround but the specimen studied had a mass of some materials, which included degraded spermatozoa, occupying the chamber. We therefore hypothesize that the spermatozoon is turned in the receptacle duct as illustrated in Fig. 15C–F. The inner diameter of the duct (ca. 1 µm) would be wide enough to reverse the orientation of the tail, while keeping the nucleus (ca. 4.4 µm in length) in position. An alternative would be that the spermatozoon is straightened before entering the seminal receptacle as speculated for eutardigrade spermatogenesis (Rebecchi, 1997). It is clear that in *O. belopus* the final metamorphosis takes place after ejaculation of the spermatozoa as the hermaphrodite form lacks seminal vesicles (which are present in all male marine heterotardigrades) and all fully developed spermatozoa, from the ovotestis to those near the gonopore, exhibit the half-headed arrow shape.

4.1.3. Paranuclear vesicle and its homologous elements in other tardigrades

There have been several opinions on the marine heterotardigrade 'acrosome' and whether the structure is a true acrosome or the variously described 'vesicles' or 'dense bodies' are analogous structures (Kristensen, 1979; Jørgensen et al., 1999; Greven and

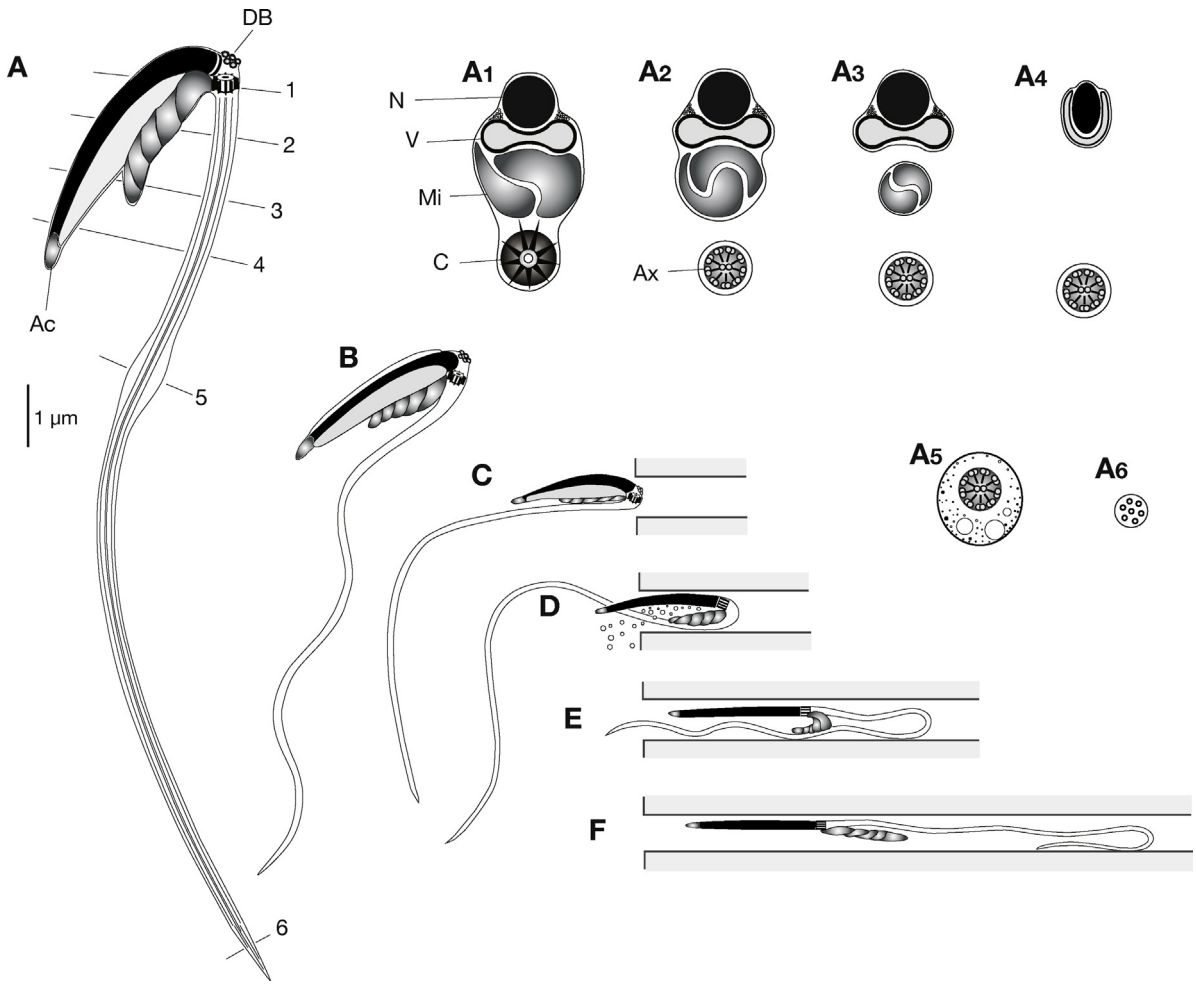

Fig. 15. *Orzeliscus* cf. *belopus*. Schematic representation of the spermatozoa. (A) Spermatozoon in ovotestis and cross-sections through the sperm at different levels (A1–A6). (B) Sperm in front of the gonopore. (C–F) Suggested scenario of the sperm's final metamorphosis in the receptacle duct. Ac, acrosome; Ax, axoneme; C, centriole; DB, small dense bodies; Mi, mitochondria; N, nucleus; V, paranuclear vesicle.

Kristensen, 2001). In the present study, we use the term 'acrosome' for the *Orzeliscus* sperm with respect to its position only, located at the distal tip of the nucleus. Without additional observations that elucidate the origin and biological function of this structure, we cannot provide further clarification. Instead, we try to prove a homology between the *Orzeliscus* 'paranuclear vesicle' and the *Actinarctus* 'vesicle' (Jørgensen et al., 1999).

The large, spherical 'vesicle', produced by the Golgi apparatus, is a major component of *Actinarctus doryphorus* spermatids occupying about 1/3 of the cell and situated near the nucleus (Jørgensen et al., 1999). This vesicle has three definable regions: tubular, light osmiophilic and osmiophobic substructures part, which might correlate to the substances in the *Orzeliscus* paranuclear vesicle. Unfortunately, the complete morphology of the *Actinarctus* vesicle is not known as stage 5, the latest detailed morphological stage of the studied *Actinarctus* spermatid, appeared to still have a nucleus in a rather early stage of condensation and elongation (Jørgensen et al., 1999). Further development of the spermatid was presumed to occur in the seminal vesicles where light microscopy observations revealed mature sperm with a rod-like nucleus bent backward to touch the flagellum (Jørgensen et al., 1999). It is therefore possible that the vesicle also changes its shape, along with the transformation of the nucleus, resembling the shape of the *Orzeliscus* paranuclear vesicle. In the *Actinarctus* seminal receptacle the large vesicle is described as to be, 'exploded in the copulatory act and only debris from this dominant structure is recognizable' (Jørgensen et al., 1999).

A vesicle was also described for *Wingstrandarctus corallinus* Kristensen, 1984. In this species the spermatids appear to be like small grains of wheat with a tail, with a huge dominating vesicular structure. Within the female seminal receptacle the sperm were filliform and the vesicle reduced or lacking (Kristensen, 1984).

Based on the observations from the above mentioned genera, *Actinarctus* and *Wingstrandarctus*, and our data, we hypothesize that the vesicle is involved in straightening the male germ cell from a grain-like cell, in which the acrosome and the nucleus are bent backwards, to a rod-like spermatozoon with the acrosome and the nucleus located anterior to the flagellum (Jørgensen et al., 1999). It is, therefore, quite probable that the 'paranuclear vesicle' of *O. belopus* is homologous to the 'vesicle' of *A. doryphorus* and *W. corallinus*.

Most of the investigated marine heterotardigrades have this vesicle, or 'dense body' (*Batillipes noerrevangi* (see Kristensen, 1979), *Echiniscoides sigismundi* (see Kristensen and Hallas, 1980; Greven and Kristensen, 2001), as well as four terrestrial Echiniscidae species (Rebecchi, 2001; Rebecchi et al., 2003)). Although in these tardigrades the vesicle is not involved in straightening the spermatozoa, they likely have a homologous origin.

Furthermore, the 'condensed body' found in the eutardigrade *Isohypsibius granulifer* Thulin, 1928, extends over the whole length of the long nucleus (Wolburg-Buchholz and Greven, 1979) and

is similar to the organization of the paranuclear vesicle and nucleus in *Orzeliscus*. Indeed, morphological change of the nucleus has been reported in, *Xerobiotus pseudohufelandi* (Iharos, 1966) (Eutardigrada), in which spermatozoa change from a bent shaped to an elongated appearance in the female spermatheca (Rebecchi, 1997). In this case, the 'ovoid elements' (Rebecchi and Guidi, 1995; Rebecchi, 1997), like the 'vesicle', were involved in the straightening process.

4.2. Reproductive mode of O. belopus

The Bermudan *Orzeliscus* specimen was observed to be a simultaneous hermaphrodite, with both a mature egg in the ovotestis and a large number of male mature gamete cells (gonadal spermatozoa), indicating this individual was in a breeding period. Moreover, the mode of spermatogenesis is not continuous in *Orzeliscus*, as described for other marine tardigrades such as *Batillipes* (Pollock, 1970; Kristensen, 1979) and *Wingstrandarctus* (see Kristensen, 1984). Although both mature stage sexual gametes were observed in *Orzeliscus*, self-fertilization seemed unlikely as the presence of spermatozoa in the seminal receptacles indicated a reproductive mode involving copulation. However, marine arthrotardigrades usually mate by the transfer of the spermatozoa to the seminal receptacle by protruding the ovoid male gonopore up to the opening of the female seminal receptacle. As this hermaphroditic individual had a female rosette gonopore and no males were found in the population, it is unclear how the spermatozoa would have been transferred to the seminal receptacle. It is, of course, still possible that males are present in the population. We have only two ultra-sectioned specimens, but we have studied 22 specimens from Bermuda (Kristensen and Sterrer, 1986; Kristensen and Neuhaus, 1999); 19 specimens on microslides, one specimen on a SEM-stub and two on TEM grids. All these specimens have a female gonopore. No males are present in our collections from Bermuda. Our observations of the hermaphroditic individual showed not only spermatozoa in the receptacle duct but also cellular debris from degraded sperm deeper in the receptacle chamber. This fact suggests that copulation with different individuals may occur. If so, a sperm competition could be possible.

Our observation of the ovotestis by TEM has confirmed the existence of hermaphroditism in a population of *Orzeliscus*, but does not imply that all individuals of this population are hermaphrodites; indeed, our second specimen was a female. However, no males were observed in the Bermuda population, thus this population may consist of a mixture of female and hermaphrodite individuals. Probably, a female sometimes carries out spermatogenesis, becoming a hermaphrodite. This hypothesis is derived from the observation that the gonoduct of the hermaphrodite is unpaired as oviduct, which may be the default morphology of the ovotestis. *Opydorscus fonsecae* Renaud-Mornant, 1989 (Orzeliscinae) is known to be gonochoristic (Renaud-Mornant, 1989) and although males are not reported for *Orzeliscus* cf. *belopus*, there probably are several gonochoristic species within this genus; for example, an Australian *Orzeliscus* sp. (Heiner and Kristensen, pers. com.).

Acknowledgements

The first author thanks all members of the Zoological Museum, Natural History Museum of Denmark, for their great hospitality during his stay there at the start of this work. Dr. Sandra McInnes (British Antarctic Survey, Cambridge) is greatly acknowledged for the English revision and comments on the manuscript. We are grateful to Professor Dr. Øjvind Moestrup and Dr. Gert Hansen (Department of Biology, Copenhagen University), for use of the TEM facility, and to Mrs. Else Schiøtt Hansen (former Institute of Comparative Anatomy, Copenhagen University) for making all the ultrasections. Finally, Professor Dr. Hans Ramløv (Roskilde University Centre (RUC), Denmark) and Dr. Wolfgang Sterrer (Former Director of Biological Station at Bermuda) are acknowledged for supporting the collections of *Orzeliscus belopus* in Egypt and Bermuda. This work was financially supported by grants from Keio Gijuku Academic Development Funds, Smithsonian Institution, Washington D.C., Carlsberg Foundation (Grant No. 9231988 and 970345/30-488) and the Danish Research Council (Grant No. FNU 272-08-0576 and 09-066003/FNU).

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