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The ultrastructure of the tardigrade spermatozoon: a comparison between *Paramacrobiotus* and *Macrobiotus* species (Eutardigrada)

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ABSTRACT: The spermatozoan ultrastructure was investigated in three semiterrestrial eutardigrade species belonging to two different genera of Macrobiotidae (*Paramacrobiotus* and *Macrobiotus*). The spermatozoa of *P. areolatus* and *P. richtersi* are very similar and are made by three regions, namely a long head, a short kidney-shaped middle piece and a short tail with its terminal tuft. In both species the spermatozoa are particularly long (up to 100 μm) and very thin. The peculiar length is due to the remarkably developed head consisting of a cylindrical acrosome and a weakly coiled nucleus increasing in width caudally. The presence of a long nucleus, an electron-dense core of fibrils running parallel to the nucleus, as well as nine outer electron-dense fibers around the proximal part of the axoneme represents novelties in the ultrastructure of the tardigrade spermatozoa. These structures, never described before for a tardigrade spermatozoon, could be related to the movement of those extraordinary long male gametes of *Paramacrobiotus.* The spermatozoon of *M. harmsworthi* too is made up of three regions: the head, including the acrosome and nuclear region, the middle piece and the terminally tufted tail. Nevertheless it is only 46–50 μm in length and the head, including a slightly tight helical nuclear region, is similar in length to the tail. In all macrobiotid species here examined, including those from literature, the spermatozoa within the was deferens always appear folded, with the hinge located between the end of the head and the beginning of the middle piece, thus resembling a long nutcracker. The use of spermatozoan characters as phylogenetic information in tardigrades is also discussed.

KEY WORDS: Tardigrada, Macrobiotidae, *Paramacrobiotus*, *Macrobiotus*, Spermatozoon, Ultrastructure, Phylogeny.

Ультраструктура сперматозоидов тардиград: сравнительный анализ *Paramacrobiotus* и *Macrobiotus* (Eutardigrada)

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РЕЗЮМЕ: Ультраструктура сперматозоидов была исследована у трех видов эутардиград, из двух разных родов *Paramacrobiotus* и *Macrobiotus* (Macrobiotidae). Сперматозоиды *P. areolatus* и *P. richtersi* очень похожи и состоят из трех частей: сильно

вытянутая головка, короткий бобовидный срединный участок и короткий хвост с терминальным пучком. Сперматозоиды этих видов необычайно длинные (до 100 мкм) и очень тонкие. Это связано с необыкновенно развитой длинной головкой, включающей цилиндрическую акросому и слабо закрученное ядро, ширина которого увеличивается каудально. Наличие у изученных представителей длинного ядра, электронноплотной сердцевины из фибрилл, проходящих параллельно ядру, а так же девяти наружных электронноплотных волокон вокруг проксимальной части аксонемы – новые факты в ультраструктуре сперматозоидов тардиград. Эти структуры ранее не были описаны и, вероятно, связаны с движением этих экстраординарно длинных гамет самцов *Paramacrobiotus*. Сперматозоиды *M. harmsworthi* так же представлены тремя частями: головка, включающая акросому и ядерный регион, средняя часть и хвост с терминальным пучком. Тем не менее, их длина составляет всего 46–50 мкм, и головка, вместе с плотно закрученным ядерным регионом, сходна по длине с хвостом. У всех изученных макробиотид, включая данные литературы, сперматозоиды внутри was deferens всегда имеют складчатость, с петлями расположенными между концом головки и началом средней части, и напоминающими длинные щипцы для орехов. Использование особенностей строения сперматозоидов для филогении тардиград обсуждается.

КЛЮЧЕВЫЕ СЛОВА: Тихоходки, Tardigrada, Macrobiotidae, *Paramacrobiotus*, *Macrobiotus*, сперматозоиды, ультраструктура, филогения.

Introduction

Tardigrades are hydrophilous micrometazoans most of which belong to the desiccation-tolerant multicellular organisms. Although all active individuals require water, the environments in which tardigrades live are generally divided into marine and brackish water, freshwater, and terrestrial and limnoterrestrial habitats. The highest number of species has been described from terrestrial habitats, where they are inactive unless surrounded by a film of water (Bertolani et al., 2009).

The phylum Tardigrada has been included in the clade Ecdysozoa (Aguinaldo et al., 1997) and are considered related to Arthropoda and Onychophora comprising the monophyletic taxon Panarthropoda (Nielsen, 1995; Dunn et al., 2008; Budd, Telford, 2009; Rota-Stabelli et al., 2010). On morphological basis, it is subdivided into 2 extant classes (Heterotardigrada and Eutardigrada), 4 orders, 21 families, 106 genera and 1047 species (Guidetti, Bertolani, 2005; Pilato, Binda, 2010). A third class (Mesotardigrada) is dubious. Heterotardigrada and Eutardigrada have been confirmed by molecular studies (Jørgensen, Kristensen, 2004; Guidetti et al., 2005, Nichols et al., 2006; Jørgensen et al., 2009). Within the Eutardigrada, there are two orders: Apochela (with the only family Milnesiidae) and Parachela (made up by 8 families). The parachelan families most rich in species are Macrobiotidae, Hypsibiidae, Eohypsibiidae and Calohypsibiidae. The relationships within and among tardigrade families are mostly unresolved. Recent molecular studies based on 18S rDNA sequences have proposed to upgrade Macrobiotidae and Hypsibiidae to superfamily level (including Calohypsibiidae in Hypsibioidea), to erect the new family Isohypsibiidae (separating several genera from Hypsibiidae) and to erect the new family Ramazzottiidae (Sands et al. 2008), The only family Ramazzottiidae is supported by morphological data. In this paper only taxa supported by morphological data are considered, i.e. the systematics proposed by Pilato and Binda (2010) plus the family Ramazzottiidae.

The ultrastructure of the male gamete is widely used for investigations of phylogeny and adaptations in several animal groups (Franzén, 1970; Jamieson et al., 1999), including tardigrades (for review see Rebecchi et al., 2000). With regard to this phylum, all tardigrade spermatozoa are flagellate, with a "9+2" axoneme. The spermatozoa of Heterotardigrada, considered of primitive type, are short (14–20 μm in length), have a globose or slightly elongated head with a nucleus containing homogeneous, and sometime electron-dense chromatin. They lack a well-defined midpiece and the head is followed by two elongated mitochondria extending from the main axis of the cell and by a flagellum tapering out of its termination (Kristensen, 1979, 1984; Kristensen, Hallas, 1980; Jørgensen et al., 1999; Greven, Kristensen, 2001; Rebecchi et al., 2000, 2003). On the contrary, the spermatozoa of Eutardigrada can be attributed to a derivative type and display a remarkable morphological heterogeneity. They are longer (30–100 μm) than those of Heterotardigrada and in many case are filiform. They elongated head always bears a helical region that corresponds to the acrosome or, more frequently, to the nucleus. The nucleus is made up by homogeneous electron-dense chromatin and it is surrounded by scanty cytoplasm. A neck or midpiece can be present or absent. It is small cup-shaped with large mitochondria, or elongated with many ovoidal elements with a dense core around a mitochondrial sleeve. The flagellum always ends with a tuft of 9–11 fine filaments (Rebecchi et al., 2000; Rebecchi, 2001; Bertolani et al., 2009; Nelson et al., 2010). Only a few taxonomic studies have been conducted to date, but spermatozoan differences should be considered more in depth for identifying phylogenetic relationships. Consequently, in this paper the spermatozoan ultrastructure of three gonochoristic species belonging to two genera (*Paramacrobiotus* and *Macrobiotus*) of Macrobiotidae (Eutardigrada, Parachela) is considered and compared with reference data on the spermatozoan ultrastructure of other macrobiotid species.

Material and methods

Three semiterrestrial species were studied, namely *Paramacrobiotus richtersi* (Murray, 1911), *Paramacrobiotus areolatus* (Murray, 1907) and *Macrobiotus harmsworthi* Murray, 1907, all belonging to the family Macrobiotidae. We should remember that, before the erection of the genus *Paramacrobiotus* (Guidetti et al., 2009), the first two species were also attributed to *Macrobiotus* (Guidetti, Bertolani, 2005).

For each species a bisexual and amphimictic population was examined. Specimens of *P. richtersi* came from hazel leaf litter collected at Formigine (Modena, Northern Italy; N 44° 34.253', E 10°50.892'), at 80 m above sea level. Specimens of *P. areolatus* came from moss on rock collected at Monte Calvario (Reggio Emilia, Northern Italy; N 44°18.764', E 10°36.686'), at 774 m above sea level. Specimens of *M. harmsworthi* came from beech leaf litter collected at Monte Rondinaio (Modena, Northern Italy; N 44°07.378, E 010°55.166), at 1670 m above sea level.

To extract tardigrades from their substrate, samples of leaf litter or moss were sprinkled with tap water, and after 15 min submerged in water for 15 min at room temperature. Tardigrades were extracted from the substrate by sieves (250 and 37 μm mesh size) under running water; then animals were picked up with a glass pipette under a stereomicroscope.

Smears of *in vivo* spermatozoa of all species were examined using light microscopy (LM). The male gametes were mechanically extracted from the testis by dissecting each animal with thin entomologist needles. The slides were viewed with a Leitz Dialux 20 microscope under differential interference contrast (DIC) or phase contrast optics.

For scanning electron microscopy (SEM) analysis, the spermatozoa were extracted from the gonad and prepared according to a technique perfected by Rebecchi and Guidi (1991). For transmission electron microscopy (TEM) analyses of spermatozoa, *in toto* males were prepared according to conventional technique

(Rebecchi, Guidi, 1995). The observations were carried out with a Philips SEM XL 40 or a Philips TEM 400 at the "Centro Interdipartimentale Grandi Strumenti (C.I.G.S.)" of the University of Modena and Reggio Emilia, Modena, Italy.

Results

With SEM, the testicular spermatozoa of *Paramacrobiotus richtersi* and *Paramacrobiotus areolatus* appear composed of three regions: the head, the middle piece and the tail (with its terminal tuft). In both species the spermatozoa are particularly long (up to 100 μm) and very thin. The peculiar length is due to the remarkably developed head.

The spermatozoan head of *P. areolatus* is about 65 μm in length and exhibits two morphologically very different parts. The first one corresponds to a long (36 μm) cylindrical acrosome with a smooth surface, constant diameter and rounded tip (Fig. 1A–C). With TEM, the acrosome exhibits a central electron-lucent core surrounded by a sheath of electron-dense material (Fig. 2A, B). In longitudinal section, the central core appears as a long electron-lucent perforatorium (Fig. 2A). The posterior part of the head corresponds to an elongated and weakly helical nuclear region, whose diameter increases in width caudally, towards the middle piece; its coils increase in tightness caudally (Fig. 2A, B). The transition between the acrosome and nuclear region is clearly evident (Figs. 1C, D; 2A). The nuclear region contains a nucleus made up of very condensed chromatin (Figs. 2C, D). In the anterior part, the nucleus is surrounded by three concentric layers, namely an inner layer made up of moderate electron-dense material, an intermediate layer of electron-lucent material and an outer layer of moderate electron-dense material (Fig. 2C, E). The caudal part of the nucleus is surrounded only by a layer of moderate electron-dense material that can appear organized in thin lamellae (fibrils) that run parallel to the major axis of the nucleus (Fig. 2D). The most caudal coil of the nucleus has an evident V-shaped hollow into which the longitudinal centriole is inserted (Fig. 2H, I). These structures are within the middle piece. In fact, the middle piece is short (about 4.5 μm in length) and kidney-shaped, and contains several structures (Figs. 1A, B; 2F–I). In particular, it contains the last coils of the nucleus, the centriole and the beginning of the axonemal complex surrounded by a long and narrow mitochondrial sheath exhibiting transverse cristae (Figs. 2H, I; 8). In addition, the middle piece contains a large number of ovoid or spherical elements (from 0.5 μm to 1.1 μm in diameter) in tightly packed arrays (Fig. 2F–H). Each of these elements, delimited by a cytomembrane, has an irregularly electron-dense granular core surrounded by a translucent zone with concentric lamellae (Fig. 2F–H). The ovoid or spherical elements are located at one side of the middle piece, between the mitochondrial sheath and the cell membrane. The tail is about 32 μm in length and is always clearly shorter than the head. It is constant in diameter and splits terminally into a tuft of 8–11 elements each about 10 μm in length (Fig. 1A). The tail has the typical "9+2" axonemal complex (Fig. 8). Its proximal part is surrounded by nine outer accessory electron-dense structures (fibers; Fig. 2J–K). The most caudal part of the axoneme loses the accessory fibers and the "9+2" organization.

Figure 3 shows a schematic reconstruction of *P. richtersi* spermatozoon based on SEM and TEM observations. The head (about 70 μm in length) is composed of an acrosome and nucleus (Figs. 4; 5A, B). The acrosome is a long, thin and sinuous cylinder with a smooth surface and rounded tip (Figs. 4; 5A, B). It has a constant diameter (about 0.2 μm). In cross section, the acrosome exhibits a central electron-lucent core surrounded by a sheath of electron-dense material (Fig. 6A, D, E). This sheath is separated from the plasma-membrane by a thin electron-lucent ring. The acrosome is separated from the nucleus by a thin lamina and fits over the apical part of the nucleus like a cap. The nuclear region is weakly coiled and its coils increase in width towards the beginning of the middle piece (Figs. 4; 5A, C). With TEM, the nucleus exhibits homogeneous and electron-dense chromatin and

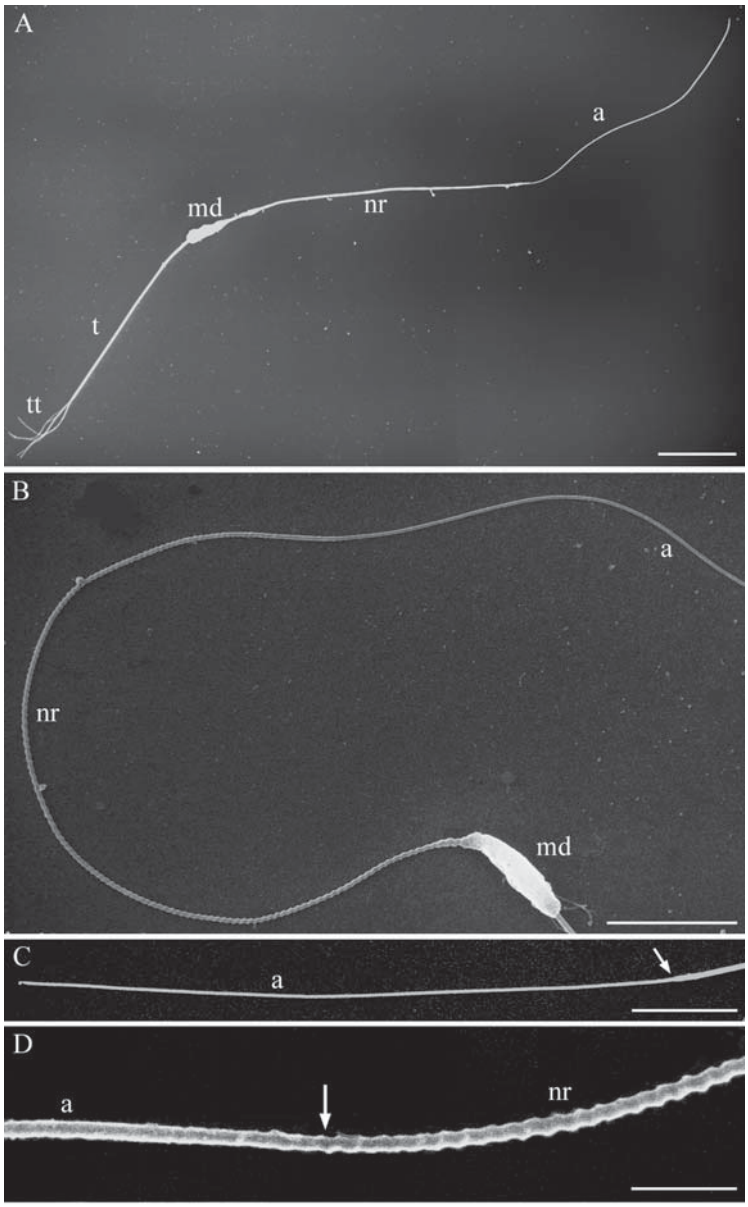


Fig. 1. Testicular spermatozoon of *Paramacrobiotus areolatus* (SEM).
A — *in toto* male gamete; B — acrosome, nuclear region and middle piece; C — cylindrical acrosome with rounded tip and the beginning of the nuclear region (arrow); D — transition area (arrow) between acrosome and proximal part of the nuclear region.
Abbreviations: a — acrosome; md — middle piece; nr — nuclear region; t — tail; tt — terminal tuft. Scale bars: A — 10 μm; B, C — 5 μm; D — 1 μm.

Рис.1. Тестикулярные сперматозоиды *Paramacrobiotus areolatus* (СЭМ).
A — тотальный препарат мужской гаметы; B — акросома, область ядра и средняя часть; C — цилиндрическая акросома с шаровидным кончиком и началом ядерного региона (стрелка); D — промежуточная область (стрелка) между акросомой и проксимальной частью ядерного региона.
Обозначения: a — акросома; md — средний кусочек; nr — ядерный регион; t — хвост; tt — терминальный пучок.
Масштаб: A — 10 мкм; B — 5 мкм; C — 5 мкм; D — 1 мкм.

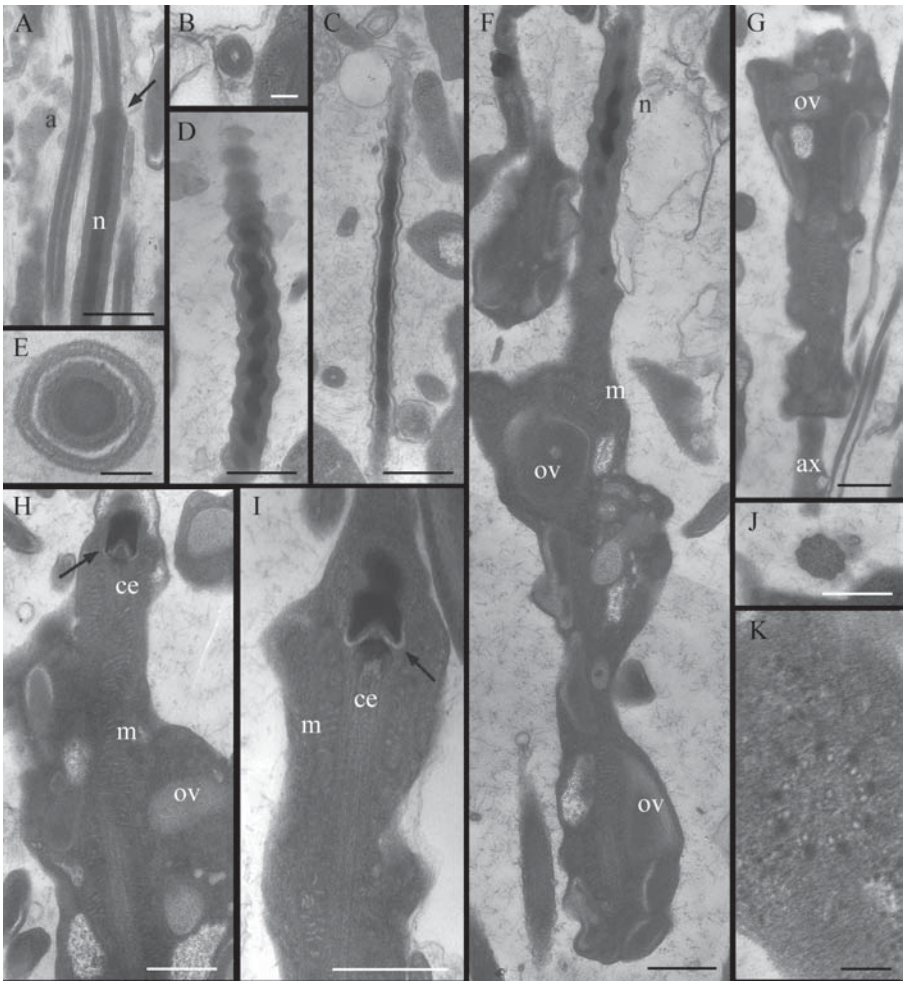


Fig. 2. Ultrastructure of the testicular spermatozoon of *Paramacrobiotus areolatus* (TEM).
A — longitudinal section of the acrosome and of transition area (arrow) between acrosome and nucleus; B — cross section of the acrosome; the internal electron-lucent core is visible; C — Longitudinal section of the anterior part of the nucleus; D — longitudinal section of the posterior part of the nucleus; E — cross section of the anterior region of the nucleus; F — longitudinal section of the distal part of the nucleus and of the middle piece. Ovoid elements and mitochondria are visible; G — middle piece with ovoid elements; H, I — longitudinal sections of the middle piece showing the last V-shaped coil of the nucleus, the longitudinal centriole inserted in it (arrow), the mitochondrial sheath and the ovoid elements; J, K — axonemes with "9+2" organization surrounded by nine outer electron-dense accessory fibers.
Abbreviations: a — acrosome; ax — axoneme; ce — centriole; m — mitochondrial sleeve; n — nucleus; ov — ovoid elements. Scale bars: A, C, D, F–J — 0.5 μm; B, E, K — 0.1 μm.

Рис. 2. Ультраструктура тестикулярных сперматозоидов *Paramacrobiotus areolatus* (ТЭМ).
A — продольный срез акросомы и переходной области (стрелка) между акросомой и ядром; B — поперечный срез акросомы; показана внутренняя электронносветлая сердцевина; C — продольный срез передней части ядра; Д — продольный срез задней части ядра; E — поперечный срез передней области ядра. F — продольный срез дистальной части ядра и среднего кусочка. Показаны овальные элементы и митохондрии; G — средняя часть с овоидными элементами; H, I — продольные срезы средней части показывающий последний V- образный изгиб ядра, включающий продольную центриоль (стрелка), митохондриальную оболочку и овоидные элементы; J, K — аксонемы с организацией микротрубочек «9+2», окруженная девятью наружными электронноплотными дополнительными волокнами.
Обозначения: a — акросома; ax — аксонема; ce — центриоль; m — митохондриальный рукав; n — ядро; ov — овоидные элементы. Масштаб: A, C, D, F–J — 0,5 мкм; B, E, K — 0,1 мкм

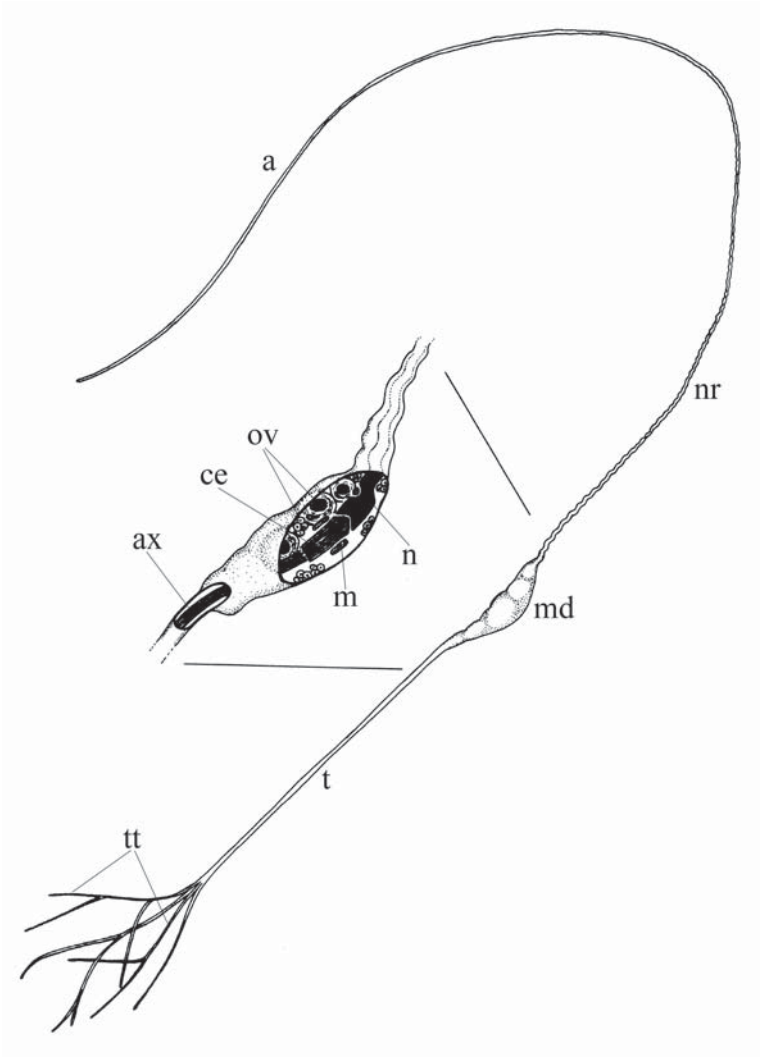


Fig. 3. Drawing of the testicular spermatozoon of *Paramacrobiotus richtersi*.
Abbreviations: a — acrosome; ax — axoneme; ce — centriole; m — mitochondria; md — middle piece; n — nucleus; nr — nuclear region; ov — ovoid elements; t — tail; tt — terminal tuft.
Рис. 3. Схема строения тестикулярного сперматозоида *Paramacrobiotus richtersi*.
Обозначения: a — акросома; ax — аксонема; ce — центриоль; m — митохондрия; md — средняя часть; n — ядро; nr — ядерная область; ov — овоидные элементы; t — хвост; tt — терминальный пучок.

it is surrounded by a small amount of moderate electron-dense material organized in thin lamellae (fibrils) that run parallel to the major axis of the nucleus (Fig. 6A–E). The most caudal coil of the nucleus has a V-shaped hollow into which the longitudinal centriole is inserted (Fig. 6C). Caudally to the head there is a short (3–4 μm in length) and elongated kidney-shaped mid-piece, sometimes exhibiting very small hemispherical protuberances on its surface (Figs. 4, 5A–C). The mid-piece contains the caudal part of the nucleus, the longitudinal centriole and the proximal part of the axonemal complex which is surrounded by 9 accessory electron-dense fibers (Fig. 6F), and small rod-shaped mitochondria (or a mitochondrial sheath). Around all

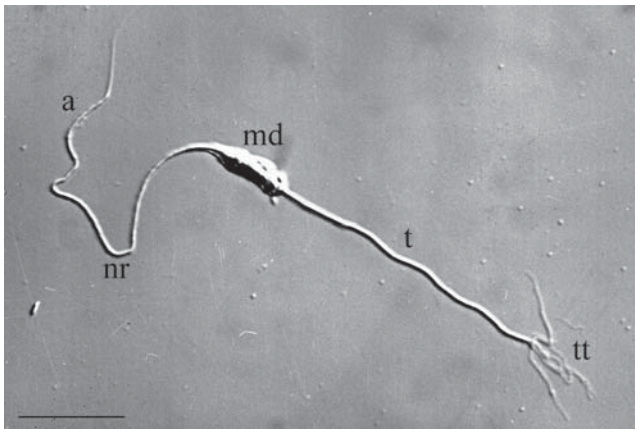


Fig. 4. *In vivo* spermatozoon of *Paramacrobiotus richtersi* extracted from the testis (DIC).
Abbreviations: a — acrosome; md — middle piece; nr — nuclear region; t — tail; tt — terminal tuft. Scale bar 10 μm.
Рис. 4. Сперматозоид *Paramacrobiotus richtersi* выделенный из семенника *in vivo* (DIC).
Обозначения: a — акросома; md — средняя часть; nr — ядерная область; t — хвост; tt — терминальный пучок. Масштаб 10 мкм.

these structures it is present a large number of ovoid or spherical elements morphologically similar to those observed in the middle piece of the *P. areolatus* spermatozoon. In *P. richtersi*, the tail is 25–30 μm in length, constant in diameter and splits terminally into a tuft of 8–11 elements (Figs. 4; 5D). The axonemal complex and the nine outer dense accessory fibers have the same organization as those described in *P. areolatus*. The most caudal part of the axoneme loses the accessory fibers and the "9+2" organization. Groups of microtubules can be observed, comprising two to ten singlets, all inside a single cytomembrane, in addition to numerous individual microtubules surrounded by their own cytomemebrane (Fig. 6E).

The testicular spermatozoon of *Macrobiotus harmsworthi* is 46–50 μm in length. It is made up of three regions: the head, including acrosome and nuclear region, the middle piece and the terminally tufted tail (Fig. 7A). The head is about 18.0 μm in length. The acrosome is about 8.5 μm in length; it has a constant diameter (0.2 μm), smooth surface and rounded tip (Fig. 7A, B). The nuclear region (about 10.5 μm in length) is helical and tightly coiled (Fig. 7A-C). Its coils increase in width (from 0.4 μm to 0.6 μm) caudally, towards the middle piece. The midpiece (about 4.0 μm in length) is kidney-shaped; nevertheless its anterior region is particularly large, whereas its terminal region is elongated and thin (Fig 7A, C). On its surface hemispherical protuberances (ovoid elements) can be present (Fig. 7C). Sometimes the transition between middle piece and tail is indicated by an evident constriction. The tail (about 19.0 μm in length) has a constant diameter and splits terminally into a tuft (Fig. 7A).

In *P. richtersi*, *P. areolatus* and *M. harmsworthi* the spermatozoa within testis and was deferens appear folded, with the hinge located between the end of the head and the beginning of the middle piece, thus resembling a long nutcracker. *In vivo*, spermatozoa inside testis and was deferens are always motile and with the hinge oriented towards the cloaca.

Discussion

Paramacrobiotus areolatus and *P. richtersi* have a very similar male gamete. The spermatozoa of both species are particularly long and thin, and exhibit a peculiar very long head with a weakly coiled nucleus increasing in width caudally, followed by a short midpiece, and a relatively short tail. The presence of a long nucleus as well as of an electron-dense core of fibrils running parallel to the nucleus represent a novelty in the ultrastructure of the tardigrade spermatozoon. The fibrils could have two different functions that are not mutually exclusive. First, the fibrils could support such a long, thin and weakly coiled nucleus. Second, the fibrils could facilitate spermatozoan movement. Another novelty is the presence of nine outer electron-dense structures around the proximal part

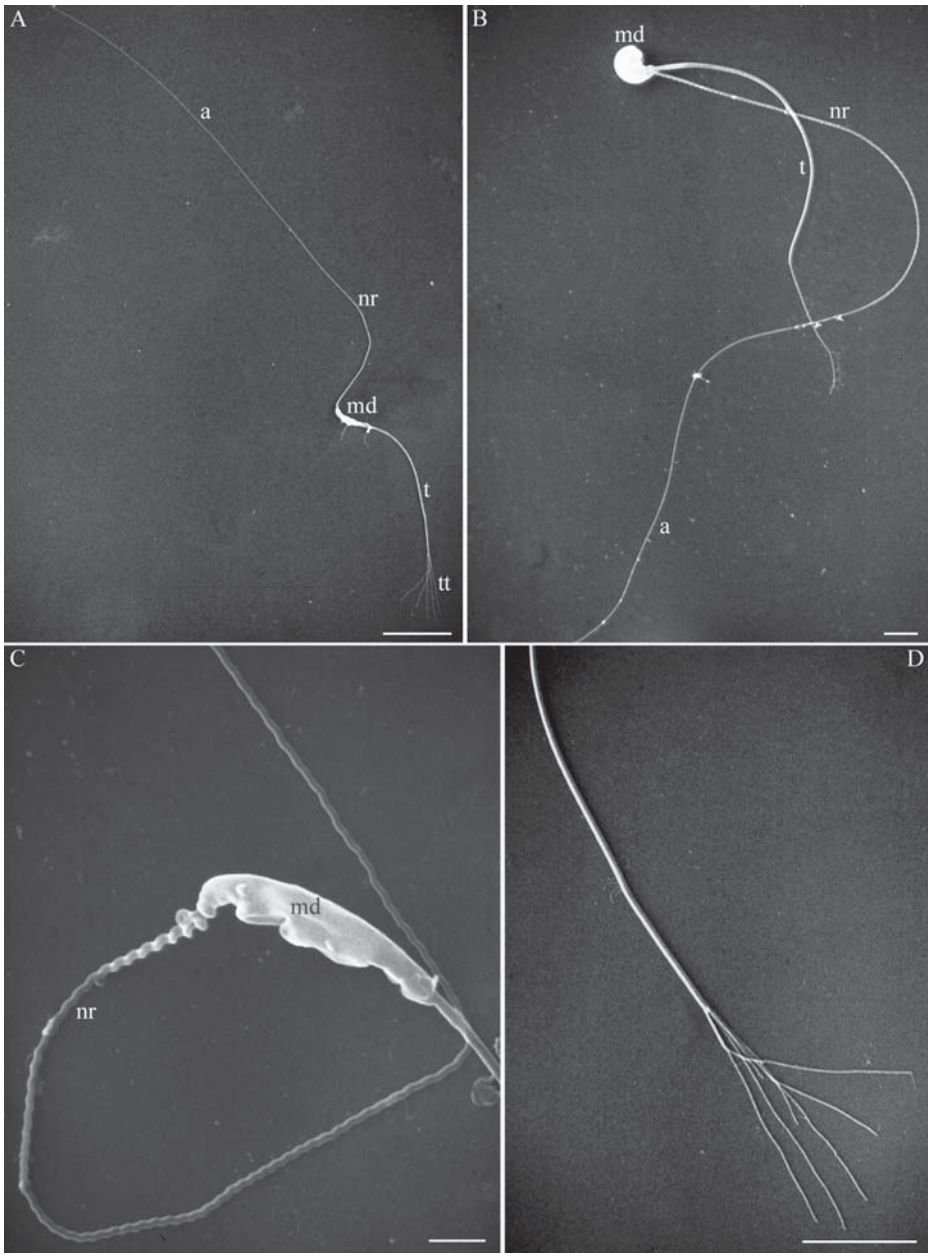
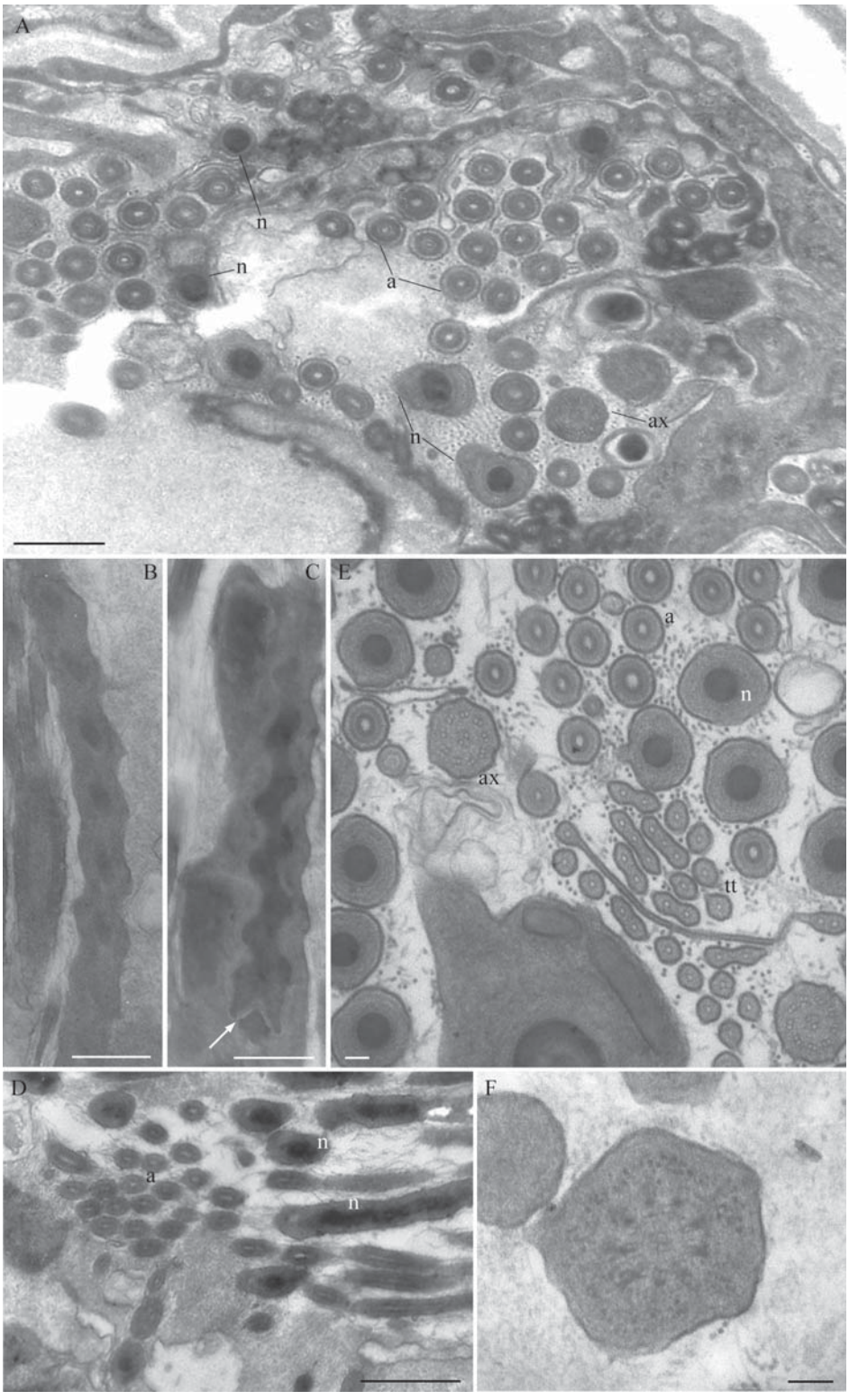


Fig. 5. Testicular spermatozoon of *Paramacrobiotus richtersi* (SEM).
A, B — *in toto* male gametes. In B the sperm cell exhibits a large middle piece; C — kidney-shaped middle piece and weakly coiled nuclear region; D — tail with terminal tuft.
Abbreviations: a — acrosome; md — middle piece; nr — nuclear region; t — tail; tt — terminal tuft. Scale bars: A, B — 10 μm; C, D — 5 μm.
Рис. 5. Тестикулярные сперматозоиды *Paramacrobiotus richtersi* (СЭМ).
A, B — целые мужские гаметы. На рис. B клетка спермы демонстрирует большую среднюю часть; C — средняя часть в виде почки (бобовидная) и слабо закрученный ядерный регион; D — чвост с терминальным пучком.
Обозначения: a — акросома; md — средняя часть; nr — ядерная область; t — хвост; tt — терминальный пучок.
Масштаб: A, B — 10 мкм; C, D — 5 мкм.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of the axonemal complex that could correspond to the outer dense accessory fibers already described for the spermatozoa of some metazoans (Fawcett, Philips, 1970). Once again, this organization, never described before for tardigrades, could be related to the extraordinary length of male gamete by enhancing its movement. In insects, it has widely been assumed that accessory fibers or tubules represent additional motor elements developed in relationship to the acquisition of internal fertilization with its attendant requirement for progression in a more viscous fluid environment (Fawcett, Philips, 1970). Therefore, the spermatozoan morphology of *Paramacrobiotus* species seems to represent an adaptation to internal fertilization. This type of fertilization could be the rule in eutardigrades with spermatozoa of the derived type (Rebecchi et al., 2000), but it is documented only for the limnic species *P. megalonyx* (Rebecchi, Bertolani, 1999) and for the few species in which females bear an internal spermatheca (*Xerobiotus pseudohufelandi*, *Ramazzottius tribulosus* and some *Macrobiotus* species; Rebecchi, Bertolani 1988; Rebecchi, 1997). Spermatheca has never been observed in *Paramacrobiotus* species. In addition, the nine outer dense accessory fibers described only for *Paramacrobiotus* could represent an apomorphy and, by being shared by the two species of the genus, can be used as a phylogenetic signal.

The spermatozoon of *M. harmsworthi* differs from that of *Paramacrobiotus* because the length of its head is similar to that of its tail and the nuclear helical region is slightly coiled compared to that of *Paramacrobiotus* species. However, the midpiece and the tail of *M. harmsworthi* have a similar size and shape as that of *Paramacrobiotus* species. The spermatozoon of *M. harmsworthi* bears some resemblance to the male gamete of *M. islandicus* (see Rebecchi, Bertolani, 1999) but it differs from those of the other *Macrobiotus* studied to date (Baccetti et al., 1971; Rebecchi, Guidi, 1991; Guidi, Rebecchi, 1996; Bertolani, Rebecchi, 1999; Rebecchi, 2001; Rebecchi et al., 2000). The similarities between the male gamete of *M. harmsworthi* and that of *M. islandicus* should be studied more in depth to understand if the similarities are due to a phylogenetic relationship. We should note that the morphology of the sclerified structures (e.g. claws, buccal-pharyngeal apparatuses) of the animals and eggs of the two species is quite different. The testicular spermatozoa of *Paramacrobiotus* and *M. harmsworthi* differ from those of *Macrobiotus* belonging to the "*hufelandi* group" and of *Xerobiotus* having a shorter head with a rod-shaped acrosome and a tightly coiled nucleus, a wider kidney-shaped midpiece always bearing ovoid elements surrounding an incomplete mitochondrial sleeve, and an axonemal complex without the nine outer accessory fibers (Baccetti et al., 1971; Rebecchi, Guidi, 1991; Guidi, Rebecchi, 1996; Rebecchi, 1997, 2001). We can note that this type of spermatozoon exhibits some similarities

Fig. 6. Ultrastructure of the testicular spermatozoon of *Paramacrobiotus richtersi* (TEM).
A — cross section of the testis showing different regions of the male gametes; B, C — longitudinal sections of nuclei. In C, the last V-shaped coil of the nucleus into which the longitudinal centriole is inserted (arrow) is visible; D — nuclei with electron-dense chromatin and acrosomes; E — cross section of acrosomes, nuclei, axonemes and single filament of the tail tuft; F — cross section of the axoneme with "9+2" organization surrounded by nine outer electron-dense accessory fibers.
Abbreviations: a — acrosome; ax — axoneme; n — nucleus; tt — terminal tuft elements. Scale bars: A — 1 μm; B–D — 0.5 μm; E, F — 0.1 μm.

Рис. 6. Ультраструктура тестикулярных сперматозооидов *Paramacrobiotus richtersi* (ТЭМ).
A — на поперечном срезе семенника показаны разные области мужских гамет; B, C — продольный срез ядра. На рис. C показан последний V- образный виток ядра внутри которого видна продольная центриоль (стрелка); D — ядро с электронноплотным хроматином и акросомой; E — поперечный срез акросомы, ядра, ахонемы и одинарные филаменты хвостового пучка; F — поперечный срез аксонемы «9+2», окруженной девятью наружными электронноплотными дополнительными волокнами.
Обозначения: a — акросома; ax — аксонема; n — ядро; tt — элементы терминального пучка. Масштаб: A — 1мкм; B–D — 0,5 мкм; E, F — 0,1 мкм.

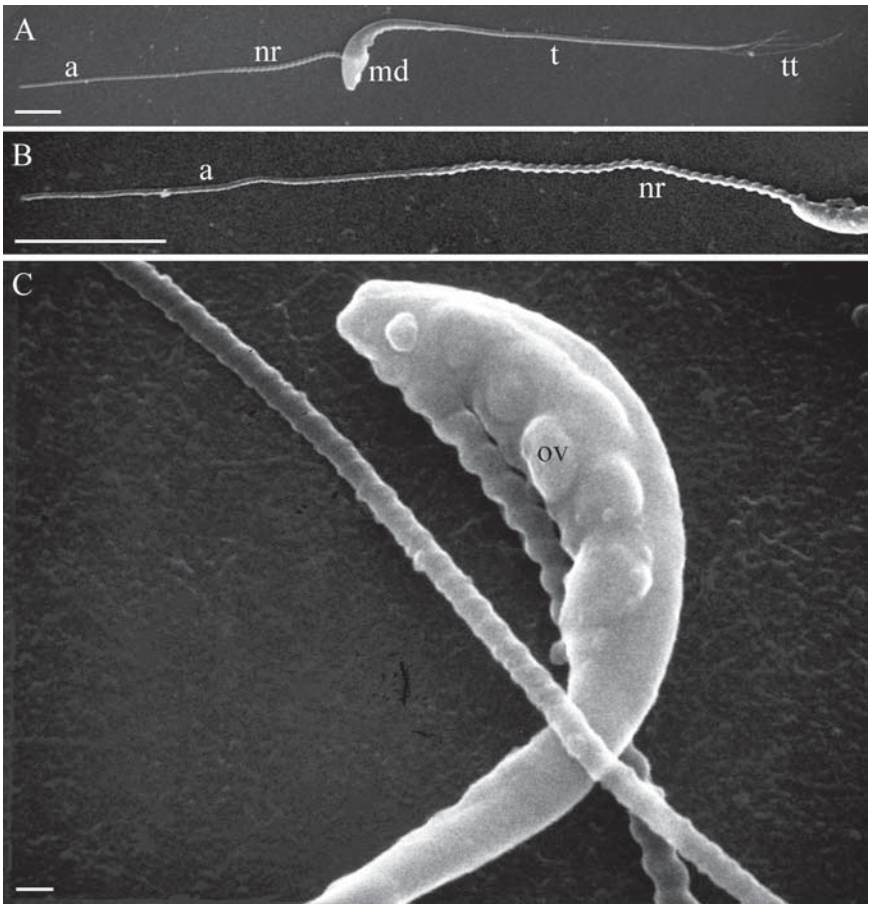

Fig. 7. Testicular spermatozoon of *Macrobiotus harmsworthi* (SEM).
A — *in toto* male gamete; B — cylindrical acrosome and tightly helical nuclear region; C — middle piece with small superficial swellings (ovoid elements) and a portion of the helical nuclear region.
Abbreviations: a — acrosome; md — middle piece; nr — nuclear region; ov — hemispherical protuberances (ovoid elements); t — tail; tt — terminal tuft. Scale bars: A — 10 μm; B, C — 5 μm.
Рис. 7. Тестикулярные сперматозоиды *Macrobiotus harmsworthi* (СЭМ).
A — целая мужская гамета; B — цилиндрическая акросома и слабо спиральный ядерный регион.
Обозначения: a — акросома; md — срединная часть; nr — ядерный регион; ov — полусферические протуберанцы (овоидные элементы); t — хвост; tt — терминальный пучок. Масштаб: A — 10 мкм; B, C — 5 мкм.

to the male gametes of *Hypsibius* (Hypsibiidae) and *Ramazzottius* (Ramazzottidae) (Rebecchi, Bertolani, 1999; Rebecchi, 2001). Nevertheless, further studies are necessary to understand if the similarities among *Ramazzottius*, *Macrobiotus* and *Xerobiotus* are due to a phylogenetic relationship. However, we should note that the hypsibiids *Ramazzottius* lay free ornamented eggs as well as all *Macrobiotus* and *Xerobiotus* species (Guidetti, Bertolani, 2005).

Within the Macrobiotidae family we can identify at least three types of spermatozoa that identify three separated evolutionary lines. Even though within macrobiotids three types of spermatozoa exist, macrobiotids spermatozoa are characterized by a common structure, represented by an evident and cylindrical acrosome with a perforatorium, a coiled nucleus with coils increasing in width caudally, and a kidney-shaped midpiece with ovoid elements and a

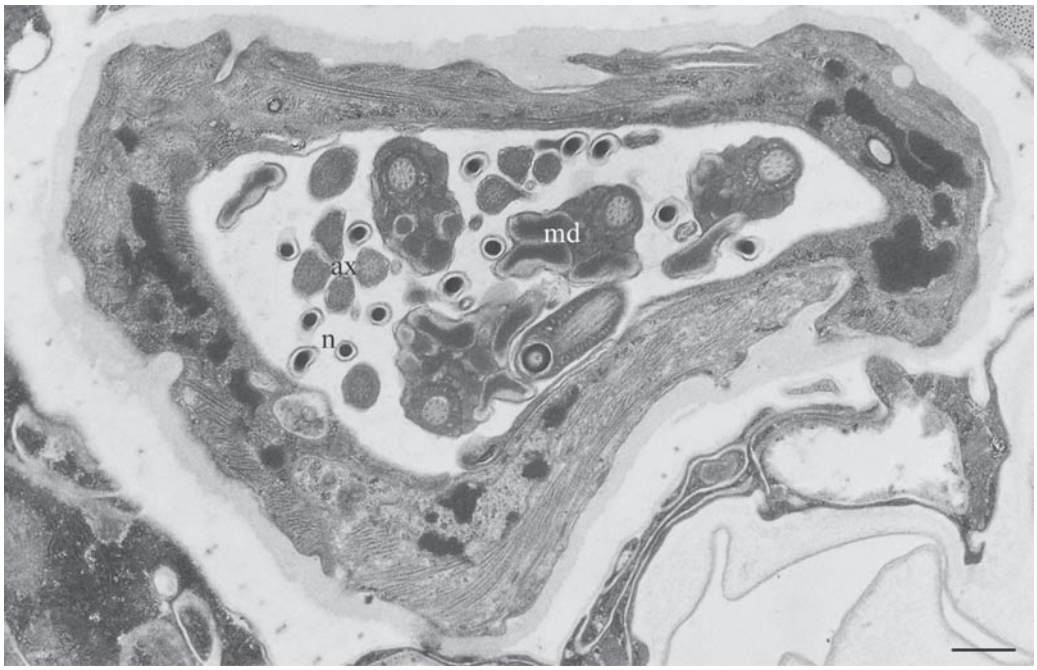

Fig. 8. Cross section of a was deferens of *Paramacrobiotus areolatus* showing nuclei with electron-dense chromatin and middle piece with axonemes, mitochondrial sheaths and ovoid elements (TEM).
Abbreviations: ax — axoneme; n — nucleus; md — middle piece. Scale bar 0.5 μm.
Рис. 8. Поперечный срез выносящего протока у *Paramacrobiotus areolatus*, показано: ядро с электронноплотным хроматином и средняя часть с аксонемой; митохондриальная оболочка и овоидные элементы (ТЭМ).
Обозначения: ax — аксонема; n — ядро; md — средняя часть. Масштаб 0,5 мкм.

mitochondrial sheath. The male gametes of macrobiotids are clearly distinguishable from the spermatozoa of the other two eutardigrade families thus far studied, namely Hypsibiidae and Eohypsibiidae. Similarly, the male gametes of the species belonging to the Hypsibiidae family are morphologically quite different and at least three types of spermatozoa can be identified (Wolburg-Buchholz, Greven, 1979; Baccetti, 1987; Bertolani, Rebecchi, 1999; Rebecchi, Bertolani, 1999; Rebecchi, 2001; Nelson et al., 2010). Instead, the spermatozoa of the species belonging to the Eohypsibiidae family are morphologically quite similar (Rebecchi, Guidi, 1995). This study demonstrates that sperm ultrastructure provides additional phylogenetic signal and can be used for identifying phylogenetic relationships among eutardigrades, especially at lower taxonomic levels (genera and species).

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