RESEARCH ARTICLE

Neomultitestis aspidogastriformis Bray and Cribb, 2003 (Digenea, Lepocreadiidae): mature spermatozoon and sperm morphologies in the Lepocreadioidea

Abdoulaye J. S. Bakhoum1,2, Yann Quilichini1*, Jean-Lou Justine3, Rodney A. Bray4, Cheikh T. Bâ2 and Bernard Marchand1

1 CNRS—University of Corsica, UMR 6134-SPE, SERME Service d’Étude et de Recherche en Microscopie Électronique, Corte 20250, Corsica, France
2 Laboratory of Evolutionary Biology, Ecology and Management of Ecosystems, Cheikh Anta Diop University of Dakar, BP 5055, Dakar, Senegal
3 ISYEB, Institut de Systématique, Évolution, Biodiversité (UMR7205 CNRS, EPHE, MNHN, UPMC), Muséum National d’Histoire Naturelle, CP 51, 55 rue Buffon 75231, Paris cedex 05, France
4 Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK

Abstract

Spermatological characteristics are described for the first time in the genus Neomultitestis Machida, 1982, belonging to the family Lepocreadiidae Odhner, 1905. Adult Neomultitestis aspidogastriformis were collected from the digestive tract of Platax teira, caught off New Caledonia. The mature spermatozoon of N. aspidogastriformis is described by transmission electron microscopy (TEM). It includes two axonemes with 9 + "1" pattern of Trepaxonemata, an antero-lateral electron-dense material, attachment zones, two mitochondria, a nucleus, two bundles of parallel cortical microtubules, external ornamentation of the plasma membrane, spine-like bodies, and granules of glycogen. Certain ultrastructural characteristics of the spermatozoon, especially the antero-lateral electron-dense material, are shared by N. aspidogastriformis and all species of the superfamily Lepocreadioidea studied so far. The antero-lateral electron-dense material, present in different families of lepocreadioids, supports the monophyletic status of the superfamily. We also list several ultrastructural characteristics which characterize the mature spermatozoon of the Lepocreadioidea.

Keywords: Neomultitestis aspidogastriformis; phylogeny; Platyhelminthes; spermatozoon; TEM; ultrastructure

Introduction

The most recent classifications of the Lepocreadiidae emphasise the monophyletic status of this family (Bray et al., 2009; Bray and Cribb, 2012). This family represents one of the largest digenean families with over 75 genera and over 200 species (Bray and Justine, 2012). It is the major group within the Lepocreadioidea despite the transfer of several genera to other families, namely the Aephnidiogena and Lepidapedidae. The systematic and phylogenetic relationships of the Lepocreadiidae and the superfamily Lepocreadioidea remain controversial.

In order to better clarify relationships in the Platyhelminthes, several authors have promoted the use of ultrastructural characteristics of the spermatozoa especially in Monogenes and Cestodes (Brooks et al., 1985; Bâ and Marchand, 1995; Justine, 1995, 1998, 2001; Levron et al., 2010).

Recently, there has been a noticeable increase in spermatological data available for ultrastructural and phylogenetic purposes in the Digenea (Miquel et al., 2000, 2006, 2013; Levron et al., 2003, 2004a; Bakhoum et al., 2009, 2011, 2013a,b; Ternengo et al., 2009; Kacem et al., 2010, 2012; Quilichini et al., 2010a,b, 2011a,b; Ndiaye et al., 2011, 2013a). However, despite the increase of ultrastructural studies of the spermatozoon, several families and genera remain unexplored.

The present study is the first for a member of the genus Neomultitestis. A comparative study is made between Neomultitestis aspidogastriformis and other digenean species. The presence and phylogenetic interest of the antero-lateral electron-dense material, a character that might support the monophyletic status of the epocreadioidea sensu Bray and Cribb (2012), is discussed.

Materials and methods

A very fresh specimen of Platax teira (Ephippidae) was purchased on 12 March 2009 from the fishmarket in...
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Nouméa (New Caledonia). Numerous digeneans were collected from the digestive tract by a “wash” method (Cribb and Bray, 2010; Justine et al., 2012). Among these, ten adult live specimens of N. aspidogastriformis Bray and Cribb, 2003 were found: four were immediately fixed for electron microscopy, and the others were processed for morphological study and mounted on slides; voucher specimens are kept in the collection of the Muséum National d’Histoire Naturelle, Paris, France, under registration number MNHN JNC2872B. Worms were rinsed with a 0.9% NaCl solution and fixed in cold (4°C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2, rinsed in 0.1 M sodium cacodylate buffer at pH 7.2, post-fixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.2, dehydrated in ethanol and propylene oxide series, embedded in Spurr’s resin, and polymerized at 60°C for 24 h.

Ultrathin sections (60–90 nm thick) were obtained using an Ultramicrotome (Power tome PC, RMC Boeckeler Instruments, Tuscon, AZ, USA) with diamond knife. Some sections placed on copper grids were double-stained with uranyl acetate and lead citrate (Reynolds, 1963). Other sections placed on gold grids were also made in order to reveal the presence of glycogen according to Thiéry’s (1967) technique. They were treated in periodic acid, thio-carbohydrazide, and silver proteinate (PA-TCH-SP) as follows: 20 min in 1% PA, rinsed in distilled water, 45 min TCH, rinsed in acetic solutions and distilled water, 30 min in 1% SP in the dark, and rinsed in distilled water.

All grids were examined in a Hitachi H-7650 transmission electron microscope (High Technologies Corporation, Tokyo, Japan) operated at 80 kV, in the “Service d’Etude et de Recherche en Microscopie Électronique” of the University of Corsica Pasquale Paoli (Corte, France).

Results

The mature spermatozoon of N. aspidogastriformis is described from several cross- and longitudinal sections which allows the interpretation of four distinctive regions from the anterior to the posterior spermatozoon extremity: Region I or anterior region of the spermatozoon; Region II, ornamented region with the first mitochondrion; Region III, median region of the spermatozoon without mitochondrion or nucleus, and Region IV, posterior region of the spermatozoon with second mitochondrion and nucleus (Figures 1–3).

Region I

Region I shows an anterior spermatozoon extremity with a sharp tip, characterized by presence of one axoneme of the 9 + “1” pattern of Trepaxonemata and antero-lateral electron-dense material (Figures 1a, 1b and 3I). The second axoneme appears in the area containing the electron-dense material and when both axonemes are completely formed, cross-sections lack cortical microtubules (Figures 1c, 1d, and 3I). In the more posterior area of region I, cortical microtubules appear and their numbers increase from two to five. Moreover, in this area a residual section of the anterolateral electron-dense material is noted (Figures 1e, 1f and 3I). Importantly, four attachment zones are visible when the centriole of the second axoneme appears and are still present in consecutive cross-sections at the posterior area of Region I (Figure 1c–1f).

Region II

Region II corresponds to the ornamented area, showing in cross-sections the presence of external ornamentation of the plasma membrane, first mitochondrion, four attachment zones, and cortical microtubules (Figures 1g–1j, 3II). The presence of spine-like bodies in the ornamented area (Figure 1h) is remarkable. The external ornamentation is observed on the ventral side (side containing mitochondrion), associated with cortical microtubules, of which the maximum number increases from the anterior to the posterior part of this Region II: 14 cortical microtubules (Figure 1g), 15 cortical microtubules (Figure 1h and 1i), and 20–22 cortical microtubules (Figure 1j).

Region III

Region III is a transitional region characterized in cross-sections by the presence of only the two axonemes, parallel cortical microtubules distributed into two submembranous bundles and granules of glycogen (Figures 2a–2c, and 3III). The maximum number of cortical microtubules in the sections decreases from 19 (Figure 2a), 15 (Figure 2b) to about 4–5 (Figure 2c). Note that the four attachment zones (arrowheads) are still visible in this region.

Region IV

Region IV represents the posterior spermatozoon extremity (Figures 2d–g, and 3IV). In its proximal area, cross-sections show the presence of the second mitochondrion, two axonemes, four attachment zones, granules of glycogen, and cortical microtubules, of which the maximum number is about 4 (Figures 2d and 3IV). When the nucleus appears, the second mitochondrion is still present. However, in the same area cross-sections show disorganization of the second axoneme exhibiting six doublets of microtubules (Figure 2e). After the disappearance of the second mitochondrion, only the nucleus, one axoneme, microtubules, and granules of glycogen are observed in cross-sections (Figures 2e and 2f,
Figure 1 Mature spermatozoon of Neomultitistis aspidogastriformis. (a) Longitudinal section of Region I showing the anterior spermatozoon extremity with a sharp tip; (b–f) consecutive cross-sections showing the anterior spermatozoon with one axoneme and antero-lateral electron-dense material (b), the formation of the second axoneme surrounded by antero-lateral electron-dense material (c and e), the appearance and increase of cortical microtubules (from 2 to 5) with residual electron-dense material (e and f); (g–j) Region II or external ornamentation areas containing the first mitochondrion and granules of glycogen. Note the increase in the number of cortical microtubules associated with external ornamentations from 14 in (g) to 15 in (i). Note also the presence of spine-like body in this ornamented region (h). In posterior part of Region II, when the first mitochondrion disappears the external ornamentations are still present and associated with about 17 cortical microtubules (j). Aldm, antero-lateral electron-dense material; Arrowheads, attachment zones; Ase, anterior spermatozoon extremity; Ax1, first axoneme; Ce2, centriole of second axoneme; Cm, cortical microtubules; EO, external ornamentation of the plasma membrane; M1, first mitochondrion; Sb, spine-like body.

and 3IV). In distal area of region IV, the second mitochondrion disappears and the disorganization of the doublets of the second axoneme is observed (Figures 2f and 3IV). Hence, the posterior spermatozoon tip exhibits only the nucleus (Figures 2g and 3IV) and granules of glycogen evidenced by the test of Thiéry (Figure 2h).

Discussion

Spermatological characteristics observed in the mature spermatozoon of N. aspidogastriformis emphasize the presence of two axonemes of the 9 + “1” trepanomatan pattern (Ehlers, 1984), a nucleus, two mitochondria, parallel cortical microtubules, and granules of glycogen. These structures are common to all digenean species studied until now, except those belonging to the schistosomatids and some didymozoids (Justine and Mattei, 1983; Justine et al., 1993; Yang et al., 2003). In addition to these classical structures, several other characters vary according to digenean genera, families, or superfamilies. Those are the antero-lateral electron-dense material, external ornamentation of the plasma membrane, spine-like bodies, and the morphologies of the anterior and posterior spermatozoon extremities. All these characters are present in the spermatozoon of N. aspidogastriformis.

This variability of the digenean spermatozoon, highlighting the phylogenetic significances of ultrastructural characters, is discussed below for the superfamily Lepocreadioidea.

Anterior spermatozoon extremity and antero-lateral electron-dense material

The anterior spermatozoon extremity of N. aspidogastriformis forms a sharp point. This is the most frequent
maturity described in digenean species (Ndiaye et al., 2002; Agostini et al., 2005; Quilichini et al., 2011a; Bakhoum et al., 2013b; Zhukova et al., 2014). In cross-section, the anterior extremity of the mature spermatozoon of *N. aspidogastriformis* exhibits one axoneme associated with antero-lateral electron-dense material. This latter appears as a submembranous layer in the anterior spermatozoon tip and is located around the second axoneme, when it appears. This is also the case in all lepocreadioidean species studied, namely the Aephnidiiogenidae *Holorchis micracanthum* (Bâ et al., 2011), the Gyliauchenidae *Gyliauchen* sp. and *Robphildolfusium fratum* (Quilichini et al., 2011b; Bakhoum et al., 2012), and the Lepocreadiidae *Hypocreadium caputvadum* and *Opechona bacillaris* (Kacem et al., 2012; Ndiaye et al., 2015).

Another peculiarity observed in the anterior spermatozoon of *N. aspidogastriformis* and all lepocreadioidean species is the absence of cortical microtubules in their anterior tip. In fact, the appearance of cortical microtubules is noted only when both axonemes are completely formed. The antero-lateral electron-dense material could be a characteristic of the Lepocreadiidea and could be a synapomorphy supporting the monophyletic status of this superfamily, the classification of which is discussed by Bray (2005), Bray et al. (2009), and Bray and Cribb (2012). However, more ultrastructural studies are needed in other

**Figure 2** Mature spermatozoon of *Neomultitestis aspidogastriformis*. (a–c) Consecutive cross-sections in Region III showing the cortical microtubules distributed into two fields and their maximum number decreasing from 19 in (a), 15 in (b), to 5 in (c). (d) Proximal part of the region IV showing both axonemes, cortical microtubules (about 4), and the appearance of the second mitochondrion. (e) Middle part of region IV exhibiting simultaneous presence of the nucleus and the second mitochondrion (e). Note the progressive disorganization of the first axoneme and section with only nucleus, one axoneme, second mitochondrion, microtubules, and granules of glycogen. (f and g) Posterior spermatozoon where second mitochondrion disappearance and second axoneme disorganizes. Hence the posterior spermatozoon tip contains only the nucleus and granules of glycogen (g). (h) Cross-section in which granules of glycogen are evidenced by Thiéry (1967)'s test. Arrowsheads, attachment zones; Cm, cortical microtubules; D, doublets of microtubules; G, granules of glycogen; M2, second mitochondrion; N, nucleus.
Figure 3 Schematic reconstruction of the mature spermatozoon of *Neomultitessis aspidogastriformis*. To simplify the diagram, the granules of glycogen are not drawn. Aae1, anterior extremity of first axoneme; Aae2, anterior extremity of second axoneme; Aldm, antero-lateral electron-dense material; Ase, anterior spermatozoon extremity; Ax1, first axoneme; Ax2, second axoneme; Az, attachment zones; Ce2: centriole of second axoneme; Cm, cortical microtubule; D, doublet of microtubules; Eo, external ornamentation; M1, first mitochondrion; M2, second mitochondrion; N, nucleus; Pae1, posterior extremity of axoneme 1; Pae2, posterior extremity of axoneme 2; Pm, plasma membrane; Pse, posterior spermatozoon extremity; Sb, spine-like body.
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leperoidean families or genera in order to better highlight the importance of antero-lateral electron-dense material.

Ornamented area of the spermatozoon and cortical microtubules

The mature spermatozoon of N. aspidogastriformis presents, just after the anterior extremity, an ornamented zone characterized by external ornamentation of the plasma membrane, cortical microtubules, and the first mitochondrion. In N. aspidogastriformis, the external ornamentations are associated with about 14–17 cortical microtubules (located on the ornamented side), whereas in the other leperoidean species they were associated with about seven cortical microtubules in H. micracanthum (Bâ et al., 2011), 12 in Gyliauchen sp. (Quilichini et al., 2011b), four in Robphilidolfesium fratum (Bakhoum et al., 2012), 14–15 in Hypocreadium caputvadum (Kacem et al., 2012), and 13 in Opechona bacillaris (Ndiaye et al., 2015).

The association “external ornamentation + cortical microtubules” has been reported in most digenean species (Quilichini et al., 2011b; Bakhoum, 2012). Moreover, Quilichini et al. (2011b) have established three types of digeneans according to the location of external ornamentation in the spermatozoon. The mature spermatozoon of N. aspidogastriformis presents external ornamentation of type 2, i.e., located at a distal part of the anterior spermatozoon region and associated with the anterior mitochondrion (see Quilichini et al., 2011b). This type 2 of external ornamentation was also reported in all leperoidean species described so far.

It is also interesting to remark that external ornamentation not associated with cortical microtubules was described in certain digenean species, especially belonging to the families Lecithasteridae (Quilichini et al., 2010a), Hemiuridae (Ndiaye et al., 2012, 2013a), and Sclerocestomidae (Ndiaye et al., 2013b). Thus, the association, or absence of external ornamentation with cortical microtubules and their location in the spermatozoon might provide additional characters useful for a phylogenetic analysis of the Digenea.

Concerning the role of the external ornamentation, Justine and Mattei (1984) reported that during the fertilization process, the anterior spermatozoon penetrates the oocyte after a lateral fusion. Thus, considering the anterior location of external ornamentation in the spermatozoon, they postulated their possible participation in the fertilization process. However, the real role of external ornamentation remains unknown and requires more detailed studies.

Spine-like bodies

Spine-like bodies appear in the ornamented area in the mature spermatozoon of N. aspidogastriformis. In most digeneans in which these structures were observed, they are present in anterior areas of the spermatozoon and are associated with external ornamentation of the plasma membrane, usually in the mitochondrial region, as observed in N. aspidogastriformis and other digenean species (Levron et al., 2004b; Quilichini et al., 2007a, 2010b; Bakhoum et al., 2011; Foata et al., 2012; Miquel et al., 2013). Moreover, in N. aspidogastriformis the spine-like bodies resemble little vesicles interrupting the external ornamentation of the plasma membrane and are irregularly distributed. However, an exception has been reported in the apocreadiid Neopocreadium chabaudi (Kacem et al., 2010) in which the spine-like bodies appear posteriorly to the ornamented area, i.e., they are not associated to external ornamentation.

In the leperoidean species studied until now, spine-like bodies were observed in three species, namely Gyliauchen sp., Raphidolopsis fratum, and O. bacillaris (Quilichini et al., 2011b; Bakhoum et al., 2012; Ndiaye et al., 2015). In the remaining leperoidean species, such as the aepnidiogenid H. micracanthum (Bâ et al., 2011) and leperoideid H. caputvadum (Kacem et al., 2012), spine-like bodies are absent.

It is likely that the spine-like bodies participate in the fertilization process, as postulated previously for the external ornamentation of the plasma membrane.

Number of mitochondria

Taking into account that it is very difficult, if not nearly impossible, to observe and determine the number of mitochondria using longitudinal sections, several authors have determined the presence of more than one mitochondrion by logical interpretations of numerous cross-sections. Thus, one, two, or three mitochondria have been reported in digeneans according to species (Miquel et al., 2006; Bakhoum et al., 2011; Bakhoum, 2012; Zhukova et al., 2014).

The mature spermatozoon of N. aspidogastriformis presents two mitochondria with a first one in the ornamented area, and a second one more posteriorly located, i.e., in the nuclear region. Similar disposition of mitochondria had been reported in other digeneans such as the opecoelids Porocanthium furcatum, Nicolla testiobliquum, and N. wisniewskii (Levron et al., 2004b; Quilichini et al., 2007b, c), the dicrocoeliid Dicrocoelium hospes (Agostini et al., 2005), the triclotrematid Troglotrema acutum (Miquel et al., 2006), the ophalometrid Rubenstroma exasperatum (Bakhoum et al., 2011), or the opisthorchiid Opisthorchis felineus (Zhukova et al., 2014). Within the Lepocreadioidea, two mitochondria have been described in R. fratum, H. caputvadum, and O. bacillaris (Bakhoum et al., 2012; Kacem et al., 2012; Ndiaye et al., 2015), whereas in the remaining species only one mitochondrion has been reported (Bâ et al., 2011; Quilichini et al., 2011b). In addition to the variability in
the number of mitochondria, authors have described the particular morphology of some mitochondria, which show a succession of bulges and cords; these “moniliform” mitochondria were reported in *H. micrancanthum* and *O. bacillaris* (Bà et al., 2011; Ndiaye et al., 2015).

Thus, the variability in the number of mitochondria and their morphologies could be additional characters to compare digenean species at the family or genus level.

Considering the absence of mitochondrion as a synapomorphy in Eucestoda (Justine, 1995), we agree with previous studies that the presence of mitochondrion is a plesiomorphic character in Digenea. However, no evolutionary aspect is observed concerning the variability in the number of mitochondrion within digenean species.

**Posterior spermatozoon extremity**

The morphology of the posterior spermatozoon extremity is variable in digenean species and has received particular attention in recent years. Quilichini et al. (2010b) proposed three types of posterior spermatozoon extremities according to the sequence or succession of the terminations of ultrastructural characteristics moving towards the posterior spermatozoon extremity. Those are:

- type 1 or opecoelidean type, characterized by the posterior extremity of the second axoneme, the nucleus, and cortical microtubules;
- type 2 or fasciolidean type, with the disappearance of cortical microtubules, the posterior extremity of the second axoneme, and the posterior extremity of the nucleus;
- and type 3 or cryptogonimidean type, characterized by disappearance of the cortical microtubules, the posterior extremity of the nucleus, and the posterior extremity of second axoneme.

The mature spermatozoon of *N. aspidogastriformis* appears to exhibit the type 2 posterior spermatozoon extremity. In fact, in the posterior part of the spermatozoon of *N. aspidogastriformis*, cortical microtubules, and the second axoneme disappear at the same level. Hence, we could not say which of these two structures disappears first. However, the single structure in the posterior spermatozoon tip is the nucleus.

Among the family Lepocreadiidae, only type 3 or cryptogonimidean type had been described (Kacem et al., 2012; Ndiaye et al., 2013a). Thus, this study describes the type 2 posterior spermatozoon extremity in the Lepocreadiidae for the first time. However, a type 2 posterior spermatozoon has been reported in lepocreadioid species, particularly in the gyliauchenid *Robphildolfusium fractum* by Bakhoum et al. (2012).

It is interesting to remark that *N. aspidogastriformis* was included in the molecular phylogenetic estimate presented by Bray et al. (2009) which utilised *lsrDNA + nad1* sequences. In that study, *N. aspidogastriformis* was found to be embedded within the Lepocreadiidae sensu stricto along with *Opechona* and *Hypocreadium* species.

In our ultrastructural findings the existence of two types of posterior spermatozoon extremities (which would correspond to two spermatozoon models) within the Lepocreadiidae is an ultrastructural argument not supporting the monophyletic status of the family. However, it is too soon to confirm the mono- or polyphyletic status of the Lepocreadiidae from sperm characteristics considering that this family is the largest within the Lepocreadioidea. Moreover, more ultrastructural studies are required in the unexplored taxa that compose the Lepocreadioidea in general and the Lepocreadiidae in particular.

**Conclusions**

The present contribution presents, for the first time, spermatological characteristics in the genus *Neomultitestis*, a member of the family Lepocreadiidae. The morphology of the mature spermatozoon of *N. aspidogastriformis* and those reported from other lepocreadioidean species reveal some common characteristics: (1) the presence of two axonemes with the 9 + 1” pattern characteristic of the Trepaxonematida, (2) antero-lateral electron-dense material, (3) an association “external ornamentation + cortical microtubules,” (4) two bundles of parallel cortical microtubules, (5) one or two mitochondria, (6) spine-like bodies, and (7) the posterior extremity of the spermatozoon containing the axoneme or nucleus. These seven characteristics may define a “lepocreadioidean model” of spermatozoon, which could differentiate the superfamiliy from other digeneans.

The description of the antero-lateral electron-dense material confirms the particularity of this character for the lepocreadioidean species. Moreover, this structure may be an ultrastructural argument supporting the monophyletic status of the superfamiliy Lepocreadioidea.

Another interesting aspect is the morphology of the posterior spermatozoon extremity exhibiting only the nucleus and, therefore, corresponding to type 2. This morphology is described here for the first time in the family Lepocreadiidae, whereas type 3 has been reported in previous studies of lepocreadiid species. An understanding of the phyllogenetic significance of these differences, which may not be reflected in the molecular studies (e.g. Bray et al., 2009), awaits further study.

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