Ultrastructure of the spermatozoon of *Macvicaria obovata* (Digenea, Opecoelidae), a parasite of *Sparus aurata* (Pisces, Teleostei) from the Gulf of Gabès, Mediterranean Sea

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Abstract

The ultrastructural organization of the spermatozoon of the digenean *Macvicaria obovata* (Opecoelidae) is described by transmission electron microscopy. Alive digeneans were collected from the digestive tract of *Sparus aurata* (Teleostei, Sparidae), caught from the Gulf of Gabès in Chebba, Tunisia (Eastern Mediterranean Sea). The male gamete of *M. obovata* is a filiform cell, tapered at both extremities and exhibits typical characters such as two axonemes of different lengths showing the 9+'1' trepaxonematan pattern, a nucleus, mitochondria, two bundles of parallel cortical microtubules, external ornamentation of the plasma membrane, spine-like bodies and granules of glycogen. Cortical microtubules are absent in the anterior spermatozoon extremity and appear after the disappearance of the electron-dense material. The first mitochondrion is of moniliform type and it is associated with the external ornamentation of the plasma membrane and spine-like bodies. The second mitochondrion is more posteriorly located, reaching the nuclear region. The present study provides new data on the mature male gamete of *M. obovata* that may be useful for the understanding of digenean relationships and phylogenetic studies.

Keywords: *Macvicaria obovata*, Opecoelidae, Digenea, ultrastructure, spermatozoon.

Introduction

The Opecoelidae is a large, cosmopolitan family of digeneans, with over 90 genera and nearly 900 species, almost solely found in marine and freshwater teleost fishes (Bray *et al.* 2016). According to Cribb (2005) this family was divided into four subfamilies, namely the Opecoelinae, Opecoelininae, Plagioporinae and Stenakrinae. Recently, a phylogenetic study of the Opecoelidae carried on the molecular analysis of 41 species of this family revealed that the Opecoelidae is monophyletic (Bray *et al.* 2016). Moreover, based on this analysis and in the characters of eggs and uterus the new subfamily Helicometrinae was erected for the genera *Helicometra*, *Helicometrina* and *Neohelicometra* (Bray *et al.* 2016).

In this context, the ultrastructural studies of species belonging to the family Opecoelidae are of great importance to bring additional information that complements the molecular results. In this sense, it has been demonstrated that ultrastructural study of the mature spermatozoon provides numerous characters, which are useful for phylogenetic inference in parasitic Platyhelminthes (Justine 1991, 1995, 1998, 2001; Levron *et al.* 2010; Quilichini *et al.* 2010, 2011a).

Recently, a promising increase in spermatological data available for ultrastructural and phylogenetic purposes in the Digenea has been noted. In this sense, eight species from the Opecoelidae were spermatologically investigated. The latter belong to three of the five subfamilies: *Helicometra epinepheli* and *Helicometra fasciata* (Helicometrinae), *Opecoeloides furcatus* and *Poracanthium furcatum* (Opecoelinae), *Allopodocotyle pedicellata*, *Nicolla testiobliquum*, *Nicolla wisniewskii* and *Podocotyloides magnatestis* (Plagioporinae) (Miquel *et al.* 2000; Levron *et al.* 2003, 2004; Quilichini *et al.* 2007a, 2007b, 2011b; Diagne *et al.* 2016; Bakhoum *et al.* 2017).

The aim of the present study is to produce the first description of the ultrastructure of the spermatozoon of the plagioporine *Macvicaria obovata*, contributing to the increase of the

ultrastructural database of the Digenea. Our results were also compared with the available data on digenean spermatology, in particular, with those species belonging to the family Opecoelidae in order to highlight the possible criteria useful for phylogeny.

Materials and methods

Live specimens of *Macvicaria obovata* (Molin, 1859) were collected from the digestive tract of *Sparus aurata* (Teleostei, Sparidae) caught from the Gulf of Gabès in Chebba (34°14′N, 11°06'E) (Tunisia).

After their extraction, adult worms were immediately 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 7.4 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4. They were then postfixed in cold (4 °C) 1% osmium tetroxide (OsO_4) with 0.9% potassium ferricyanide [$K_3Fe(CN)_6$] in the same buffer for 1 h, rinsed in Milli-Q water (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in Spurr resin and finally polymerized at 60 °C for 72 h. Ultrathin sections were obtained using a Reichert-Jung Ultracut-E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate according to Reynolds (1963) methodology. Finally, all stained grids were studied with a JEOL 1010 transmission electron microscope operated at 80 kV, in the 'Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)'.

The Thiéry (1967) technique was used to locate glycogen. Gold grids were treated in periodic acid, thiocarbohydrazide and silver proteinate (PA-TCH-SP) as follows: 30 min in 10% PA, rinsed in Milli-Q water, 24 h in TCH, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark, and rinsed in Milli-Q water.

Results

The interpretation of numerous cross- and longitudinal sections of the mature spermatozoon of *M. obovata* allows us to establish three distinctive regions (I, II and III) from the anterior to the posterior extremities of the male gamete exhibiting different ultrastructural characteristics. The mature spermatozoon of *M. obovata* exhibits the usual structures found in most of digeneans. Indeed, it contains two axonemes of the 9+'1' trepaxonematan pattern, external ornamentation of the plasma membrane, spine-like bodies, nucleus, two mitochondria, two bundles of parallel cortical microtubules and granules of glycogen.

Region I (Figs. 1A-I and 4I) corresponds to the anterior extremity of the spermatozoon. The anterior spermatozoon extremity of *M. obovata* forms a sharp point (Fig. 1A). Cross- and longitudinal sections in the anterior tip are characterized by the presence of an anterior electron-dense material (Figs. 1A, B, 4I), that persists in the anterior extremity of the spermatozoon until the appearance of the second axoneme (Fig. 1 C-F). Both axonemes, longitudinally displaced to each other, are of the 9+'1' trepaxonematan pattern (Fig. 1D, F). Another peculiarity observed in the anterior extremity of the male gamete of *M. obovata* is the absence of cortical microtubules in their anterior tip (Fig. 1B-E). In the middle part of this region, it is worthy to mention that an external ornamentation of the plasma membrane and spine-like bodies are observed in association with cortical microtubules (Figs. 1F-I, 4I) and this area exhibits the first moniliform mitochondrion composed of a mitochondrial cord with joined mitochondrial bulges (Fig. 1G, H). In the distal part of region I, the disappearance of the first mitochondrion (Fig. 1H, I) and the external ornamentation (Fig. 1I, J) marks the transition between regions I and II.

Region II (Figs. 1J, K; 2A and 4II) is the intermediate zone, which is mainly characterized by the lack of external ornamentation of the plasma membrane and the simultaneous presence of two axonemes, two bundles of cortical microtubules and granules of glycogen in its anterior part of this region (Fig. 1J, K). The same characters are present

towards the posterior portion of region II together with the appearance of the second mitochondrion (Fig. 2A).

Region III (Figs. 2B-H, 3 and 4III) corresponds to the nuclear region and posterior spermatozoon extremity. It begins with the simultaneous presence of posterior part of the second mitochondrion and the anterior extremity of the nucleus (Fig. 2B, C). The sequence of characters toward the posterior tip is characterized by: (i) the disorganization and disappearance of the first axoneme (Fig. 2D-F), (ii) the disappearance of the second axoneme (Fig. 2F, G), and (iii) the posterior end of the nucleus (Fig. 2G, H). Thus, only cortical microtubules and glycogen granules are still present in the posterior spermatozoon tip (Fig. 2H).

The granular material observed in all the regions of the mature spermatozoon is evidenced as glycogen using the test of Thiéry (1967) (Fig. 3).

Discussion

The mature spermatozoon of *M. obovata* exhibits the usual ultrastructural elements as most species of digeneans described so far: two axonemes, nucleus, mitochondrion, two sets of parallel cortical microtubules, external ornamentation of the plasma membrane and granules of glycogen. Additional aspects include the presence of spine-like bodies in the ornamented area of the sperm cell and the electron-dense material in the anterior extremity of the spermatozoon. Some of these characters as well as the morphology of both extremities could be potential candidates for phylogenetic analysis.

Anterior spermatozoon extremity

The anterior extremity of the mature spermatozoon of *M. obovata* presents two slightly longitudinally displaced centrioles corresponding to both axonemes of the 9+‘1’

trepaxonematan pattern (Ehlers 1984). This is the typical structure of axonemes observed in all digeneans except for the species of the genus *Schistosoma* with a special 9+‘1’ pattern with a poorly contrasted central element (Justine *et al.* 1993) and species of *Didymozoon* with a 9 + 0 pattern (Justine and Mattei 1983).

The anterior spermatozoon tip of *M. obovata* forms a sharp point. This is a frequently found morphology described in digenean species (Ndiaye *et al.* 2002; Agostini *et al.* 2005; Quilichini *et al.* 2011b; Bakhoum *et al.* 2013, 2015a; Zhukova *et al.* 2014; Kacem *et al.* 2015).

The presence of a submembranous electron-dense material in the anterior extremity of the mature spermatozoon of *M. obovata* was also observed in several digeneans such as *Holorchis micracanthum* (Bâ *et al.* 2011), *Gyliauchen* sp. (Quilichini *et al.* 2011a), *Robphildollfusium fractum* (Bakhoum *et al.* 2012), *Hypocreadium caputvadum* (Kacem *et al.* 2012), *Opechona bacillaris* (Ndiaye *et al.* 2015) or *Neomultitestis aspidogastriformis* (Bakhoum *et al.* 2015a). Concerning the Opecoelidae, an electron-dense material is present in the anterior extremity of the sperm cell of six of the nine studied species, namely *A. pedicellata*, *H. epinepheli*, *H. fasciata*, *M. ovobata*, *P. magnatestis* and *P. furcatum* (Levron *et al.* 2003, 2004; Quilichini *et al.* 2011b; Diagne *et al.* 2016; Bakhoum *et al.* 2017; present study –see Table I). For three of these species, authors do not mention its presence, but they are clearly visible in the published TEM micrographs. This is the case of *H. fasciata* and *P. furcatum* (Levron *et al.* 2003, 2004). Also, in *H. epinepheli*, Quilichini *et al.* (2011b) consider the anterior electron-dense material as a thicker membrane. Finally, the anterior electron-dense material is absent in the mature spermatozoon of *N. testiobliquum*, *N. wisniewskii* and *O. furcatus* (Miquel *et al.* 2000; Quilichini et al. 2007a, 2007b).

Another peculiarity observed in the anterior spermatozoon extremity of *M. obovata* is the absence of cortical microtubules. In fact, the appearance of cortical microtubules is noted only when the anterior electron-dense material disappears.

Spine-like bodies

Spine-like bodies consist of a submembranous electron-dense elements usually present in the ornamented area of the spermatozoon and described for the first time in *O. furcatus* by Miquel *et al.* (2000). In *M. obovata*, these structures were also observed in the ornamented area as most opecoelids. In this family, only *H. fasciata* lacks spine-like bodies (Levron *et al.* 2003) (see Table I). The presence of this character are always associated with the external ornamentation and only the exception of the apocreadiid *Neoapocreadium chabaudi*, in which the spine-like bodies appear posteriorly to the ornamented area, has been reported (Kacem *et al.* 2010).

Another interesting aspect concerning to the spine-like bodies is the presence or absence of periodicity between these elements. Besides, their number is not always evident because of the difficulty in observing longitudinal sections containing successive spine-like bodies. Some digeneans exhibit a clear periodicity and other show an irregular location (see Miquel *et al.* 2013). In fact, it was not possible to evaluate the periodicity of spine-like bodies in *M. obovata* as always mentioned for other digeneans species.

External ornamentation of the plasma membrane

A literature review show that the spermatozoon of *M. obovata* exhibit external ornamentation of the plasma membrane as most opecoelids, namely *A. pedicellata*, *H. epinepheli*, *N. testiobliquum*, *N. wisniewskii*, *O. furcatus*, *P. magnatestis* and *P. furcatum*, (Miquel *et al.*

2000; Levron *et al.* 2004; Quilichini *et al.* 2007a, 2007b, 2011b; Diagne *et al.* 2016; Bakhoum *et al.* 2017). However it has not been described in *H. fasciata* (Levron *et al.* 2003).

Quilichini *et al.* (2011a) distinguished three types of digenean anterior spermatozoon regions according to the localization of the external ornamentation: (i) type 1 presents external ornamentation in the anterior extremity of the spermatozoon; (ii) type 2 presents external ornamentation at a more posterior level, and (iii) type 3 lacks external ornamentation. This attempt of classification could be important at different taxonomic levels. Indeed, according to this criterion most species belonging to the Opecoelidae studied up to now have external ornamentation (group 2) with the exception of *H. fasciata* (Levron *et al.* 2003) (group 3) (see Table I). Thus, the presence or absence of external ornamentation and their location in the spermatozoon may provide additional characters useful for a phylogenetic analysis of the Digenea.

Number of mitochondria

Mitochondrial number and location are spermatological characters with a potential in phylogeny of the Digenea at the family or genus level (Bakhoum *et al.* 2015a). Most digeneans present one or two mitochondria (for a review see Bakhoum 2012). However, three digeneans, namely *Sandonia sudanensis* (Ashour *et al.* 2007), *Anisocoelium capitellatum* (Ternengo *et al.* 2009) and *Euryhelmis squamula* (Bakhoum *et al.* 2009) present three mitochondria. In what refers the Opecoelidae, the mature spermatozoa of *M. obovata* possess two mitochondria like *A. pedicellata* (Bakhoum *et al.* 2017), *H. epinepheli* (Quilichini *et al.* 2011b), *N. testiobliquum* (Quilichini *et al.* 2007a), *N. wisniewskii* (Quilichini *et al.* 2007b) and *P. furcatum* (Levron *et al.* 2004). Other opecoelids such as *H. fasciata* (Levron *et al.* 2003) and *O. furcatus* (Miquel *et al.* 2000) present only one mitochondrion. In *M. obovata* we describe for the second time in the Opecoelidae the presence of a moniliform mitochondria

composed of a mitochondrial cord with joined mitochondrial bulges. This type of mitochondrion was also reported in other digeneans such as *H. micracanthum* (Bâ *et al.* 2011), *Aphallus tubarium* (Foata *et al.* 2012), *O. bacillaris* (Ndiaye *et al.* 2015), *Stephanostomoides tenuis* (Bakhoum *et al.* 2015b) and *A. pedicellata* (Bakhoum *et al.* 2017).

Posterior spermatozoon extremity

The posterior spermatozoon extremity is morphologically variable within digeneans. Quilichini *et al.* (2010) postulate three types of posterior parts of the spermatozoon (opecoelidean type, fasciolidean type and cryptogonimidean type) according to the sequence of the principal characters (cortical microtubules, nucleus and second axoneme) toward the posterior tip of the sperm cell. Thus, the posterior spermatozoon extremity of *M. obovata* corresponds to the opecoelidean type of Quilichini *et al.* (2010) characterized by the following sequence: posterior extremity of the second axoneme, posterior extremity of the nucleus and cortical microtubules. The interest of the posterior spermatozoon extremity for understanding the relationships within the digeneans is evidenced in the present study and also in other reported ultrastructural studies. Yet, all the opecoelids studied to date exhibit a posterior spermatozoon extremity with the sequence characteristic of the opecoelidean type, except *P. magnatestis* that exhibits the nucleus as posterior character (Diagne *et al.* 2016) (see Table I). Other particularities concern *H. epinepheli* (Quilichini *et al.* 2011b) and *P. magnatestis* (Diagne *et al.* 2016) and these two species present a second mitochondrion that reaches the level of the disorganization of the second axoneme.

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Legends

Fig. 1. Spermatozoon of *Macvicaria obovata* (regions I and II). **A** – Longitudinal section of the anterior spermatozoon extremity; **B** – Cross-section showing the anterior spermatozoon tip; **C–E** – Consecutive cross-sections of region I showing the progressive appearance of the two axonemes. Note the location of the anterior electron-dense material; **F–I** – Cross-sections of the ornamented area of the sperm cell showing also the first mitochondrion and spine-like bodies; **J, K** – cross-sections of the anterior areas of region II showing the increase in the number of dorsal cortical microtubules. Scale bars: A = 500 nm, B–K = 300 nm.

Abbreviations for all Figs: ADM – anterior electron-dense material; ASE – anterior spermatozoon extremity; Ax1 – first axoneme; Ax2 – second axoneme; C1 – centriole of the first axoneme; C2 – centriole of the second axoneme; CC – central core; CM – cortical microtubules; D – doublets; EO – external ornamentation of the plasma membrane; G – granules of glycogen; M1 – first mitochondrion; M2 – second mitochondrion; Mb – mitochondrial bulge; Mc – mitochondrial cord; N – nucleus; PM – plasma membrane; PSE – posterior spermatozoon extremity; S – singlets; SB – spine-like bodies.

Fig. 2. Spermatozoon of *Macvicaria obovata* (regions II and III). **A** – Cross-section of the posterior areas of region II showing the second mitochondrion; **B–H** – Cross-sections of region III from the appearance of nucleus to the posterior spermatozoon extremity. Scale bars = 300 nm.

Fig. 3. Transmission electron micrograph showing the positive test of Thiéry. Scale bar = 300 nm.

Fig. 4. Schematic reconstruction of the mature spermatozoon of *Macvicaria obovata*. The sperm cell is organized in three different regions: region I or anterior part, region II or middle part and region III or posterior part. In order to make the diagram clearer, granules of glycogen are not shown in longitudinal sections.

Figure

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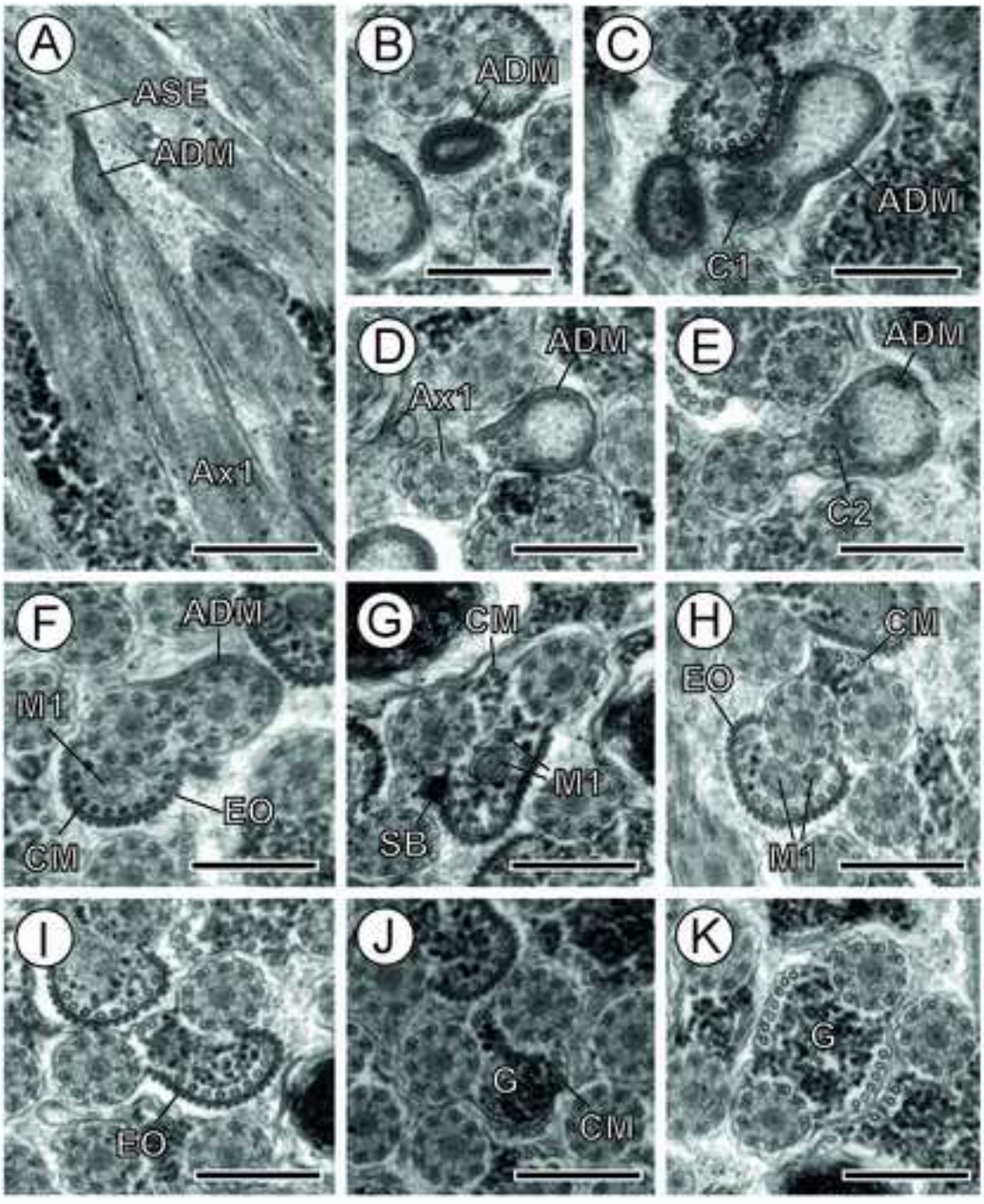

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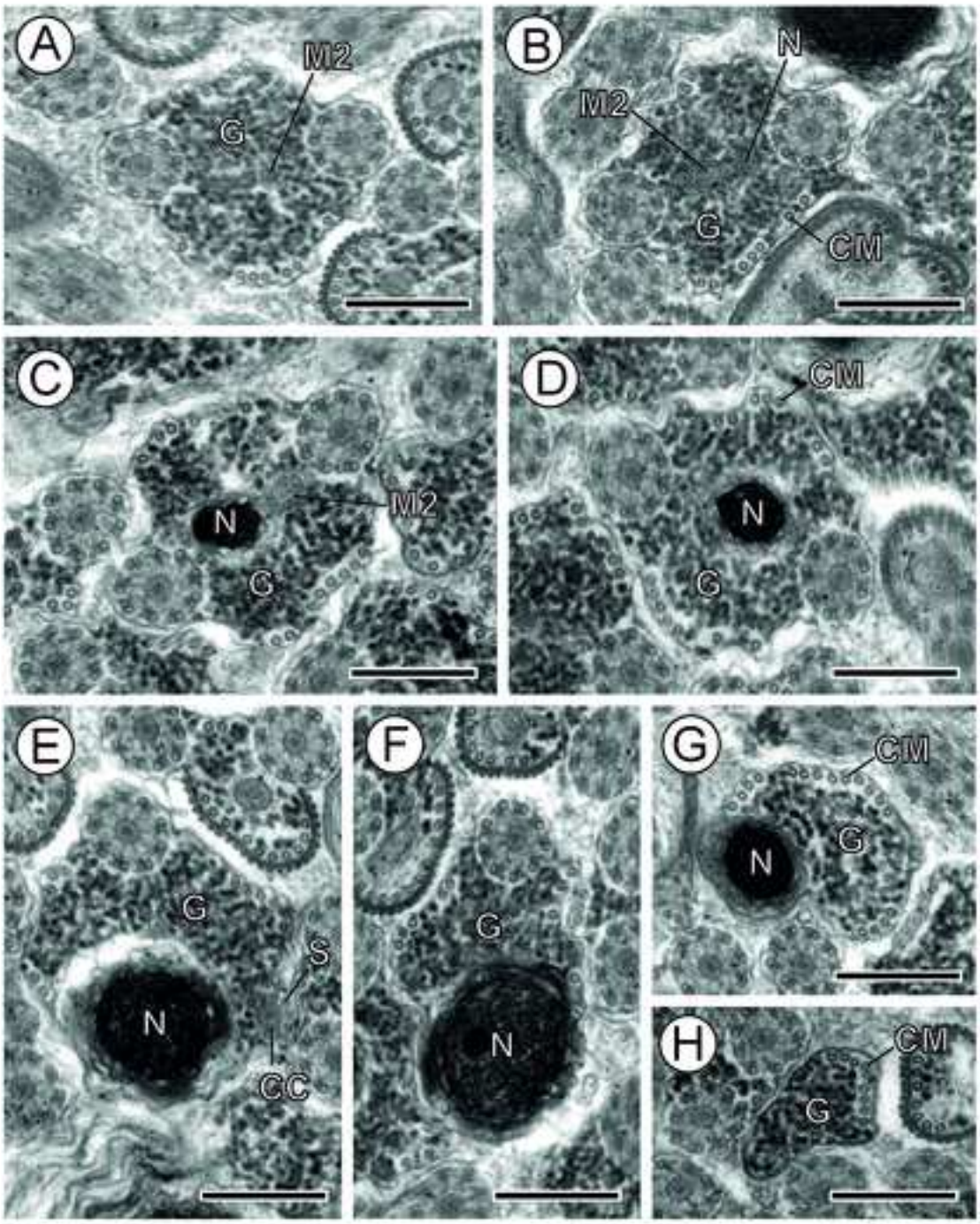

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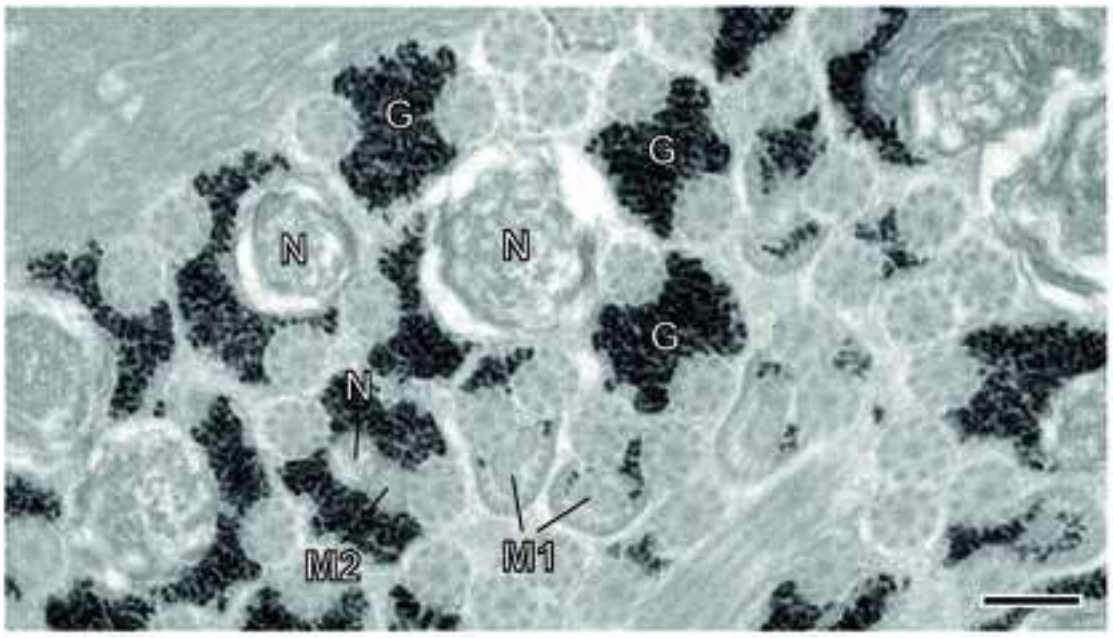

Figure

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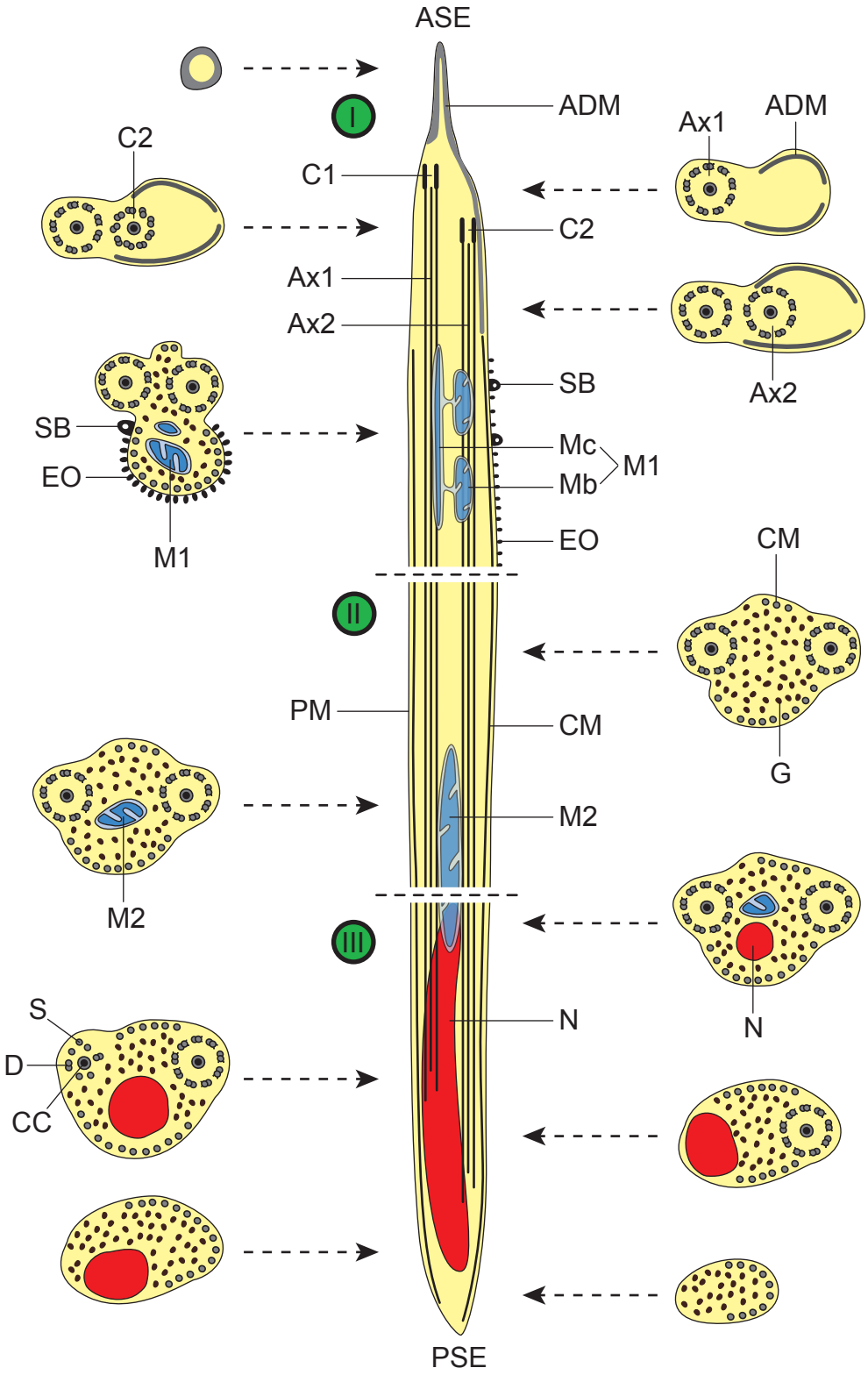

Table I. Spermatological characters in the Opecoelidae.

Subfamilies and species	ADM	EO	EO+CM	LEO	SB	M	PSC	References
Helicometrinae								
Helicometra epinepheli	+	+	+	Post	+	2	CM	Quilichini *et al.* (2011a)
Helicometra fasciata	+	-	-	NA	-	1	CM	Levron *et al.* (2003)
Opecoelinae								
Opecoeloides furcatus	-	+	+	Post	+	1	CM	Miquel *et al.* (2000)
Poracanthium furcatum	+	+	+	Post	+	2	CM	Levron *et al.* (2004)
Plagioporinae								
Allopodocotyle pedicellata	+	+	+	Post	+	2	CM	Bakhoum *et al.* (2017)
Macvicaria obovata	+	+	+	Post	+	2	CM	Present study
Nicolla testiobliquum	-	+	+	Post	+	2	CM	Quilichini *et al.* (2007a)
Nicolla wisniewskii	-	+	+	Post	+	2	CM	Quilichini *et al.* (2007b)
Podocotyloides magnatestis	+	+	+	Post	+	2	N	Diagne *et al.* (2016)

ADM– anterior electron-dense material; CM–cortical microtubules; EO– external ornamentation of the plasma membrane; EO+CM– association external ornamentation-cortical microtubules; LEO– location of the external ornamentation; M– number of mitochondria; N– nucleus; NA– not applicable; Post– posterior; PSC– posterior spermatozoon character; SB– spine-like bodies; +/-– presence/absence of considered character.