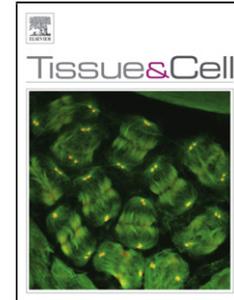


# Journal Pre-proof

Spermatological characters in the Lepocreadioidea, with first data on *Holorchis pycnopus* (Aepnidiogenidae), a parasite of the Striped seabream *Lithognathus mormyrus* (Sparidae) from the Gulf of Gabes (Tunisia)

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**Spermatological characters in the Lepocreadioidea, with first data on *Holorchis pycnopus* (Aepnidiogenidae), a parasite of the Striped seabream *Lithognathus mormyrus* (Sparidae) from the Gulf of Gabes (Tunisia)**

**Running title:** Spermatozoon of *Holorchis pycnopus*

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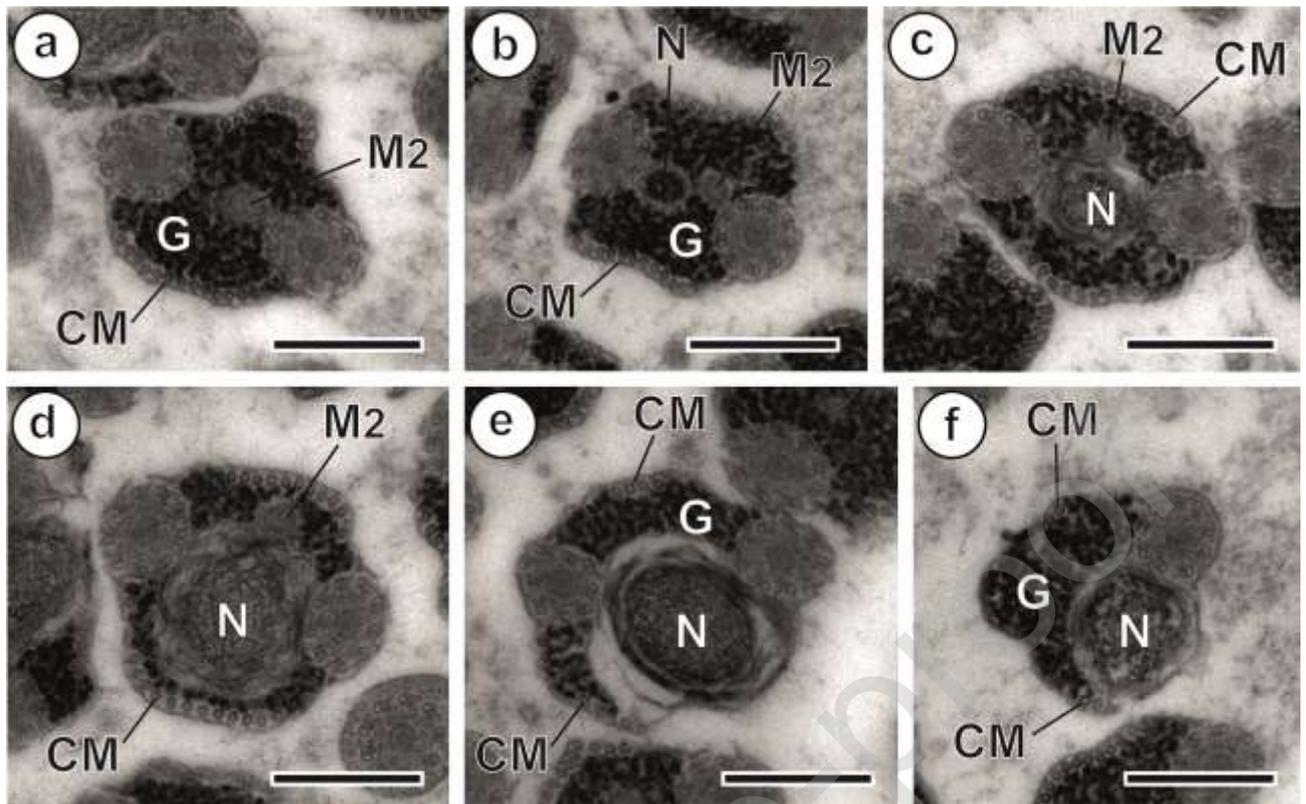
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## Graphical abstract



## Highlights

- Sperm characters of *Holorchis pycnopus* are described by means of TEM.
- *Holorchis pycnopus* has the type III of digenean spermatozoa.
- The Lepocreadioidea have no homogeneous model of the spermatozoon.

## Abstract

The ultrastructural characteristics of the mature spermatozoon of *Holorchis pycnopus* (Digenea, Lepocreadioidea, Aepnidiogenidae) are described by means of transmission electron microscopy (TEM). Live worms were collected from the digestive tract of the Striped seabream *Lithognathus mormyrus* (Teleostei, Sparidae), from off the Gulf of Gabès at La Chebba (Tunisia). The ultrastructural study reveals that the male gamete of *H. pycnopus* is a filiform cell tapered at both extremities and exhibiting the type III of the digenean spermatozoon proposed by Bakhoun et al. (2017a), characterized by the presence of (1) two axonemes with the 9 + '1' pattern of the Trepaxonemata, (2) external ornamentation of the plasma membrane located in a posterior part of the anterior region of the spermatozoon and associated with cortical microtubules, (3) two bundles of parallel cortical microtubules with maximum number located in located in the middle part of the spermatozoon, and (4) generally two mitochondria. Moreover, *H. pycnopus* shares a set of ultrastructural characteristics with the studied Aepnidiogenidae such as: (1) two 9+'1' axonemes of different lengths, (2) an anterior electron-dense material, (3) mitochondrion/a, (4) an external ornamentation of the plasma membrane associated with cortical microtubules, and (5) two bundles of parallel cortical microtubules with their maximum number (around 24 microtubules) located in the middle or posterior part of the spermatozoon. In the Aepnidiogenidae, the mature spermatozoon exhibits a similar ultrastructural pattern. Some differences are observed, particularly the location of maximum number of cortical microtubules and the number of mitochondria. The presence of the anterolateral electron-dense material is the major particularity in species belonging to the Lepocreadioidea. This anterior dense material could be a synapomorphy for the superfamily and an ultrastructural argument supporting the monophyletic status of the Lepocreadioidea (Bray and Cribb, 2012).

**Keywords:** Digenea; spermatozoon; ultrastructure; TEM.

## 1. Introduction

The superfamily Lepocreadioidea Odhner, 1905 comprises several groups of digenetic trematodes of wide range of pelagic and benthic marine teleosts, predominantly in the Indo-West Pacific and the North-East Atlantic. The Lepocreadioidea is considered as one of the complex and problematic digenean superfamilies (Kostadinova and Pérez-del-Olmo, 2014). The systematics and phylogenetic study of this group has been subject to many controversies. According to Bray (2005), the Lepocreadioidea includes 10 families and 137 genera recognized in the "Keys to the Trematoda". Nevertheless, using a molecular phylogenetic approach, Bray et al. (2009) and Bray and Cribb (2012) recovered the Lepocreadioidea as monophyletic and comprising six well-supported clades at the family rank, namely the Aephnidiogenidae Yamaguti, 1934, Enenteridae Yamaguti, 1958, Gorgocephalidae Manter, 1966, Gyliachenidae Ozaki, 1933, Lepidapedidae Yamaguti, 1958 and Lepocreadiidae Odhner, 1905. More recently, Bray et al. (2018), based on phylogenetic analysis, demonstrate that the monotypic genus *Gibsonivermis* Bray, Cribb & Barker, 1997 is isolated from all other lepocreadioids and supports the raise of Gibsonivermidae Bray, Cribb & Cutmore, 2018 as a new family, having specific morphological characteristics.

Due to the complexity and lack of robustness in the classification of the Lepocreadioidea, it would be important to add ultrastructural characters of the spermatozoon to the morphological and molecular datasets for a better knowledge of relationships within this superfamily of digeneans. Such all of ultrastructural study of the mature spermatozoon is worth being a reliable source of characters and as an important tool to elucidate the relationships between the parasitic Platyhelminthes (Justine, 1991, 1998, 2001; Bâ and Marchand, 1995; Levron et al., 2010; Quilichini et al., 2010a, 2011; Bakhroum et al., 2017a).

Spermatological data in the Lepocreadioidea are available in 12 species belonging to five families. These are two Aephnidiogenidae *Aephnidiogenes senegalensis* Dollfus & Capron, 1958 and *Holorchis micracanthum* (Stossich, 1889) (see Bâ et al., 2011, 2018), one Apocreadiidae Skrjabin, 1942, *Neopocreadium chabaudi* Kohn & Fernandes, 1982 (see Kacem et al., 2010); one Deropristidae Cable & Hunninen, 1942 *Deropristis inflata* (Molin, 1859) (see Foata et al., 2007); two Gyliachenidae *Gyliachen* sp. and *Robphildollfusium fractum* (Rudolphi, 1819) (see Quilichini et al., 2011; Bakhoum et al., 2012), and six Lepocreadiidae, namely, *Bianum plicatum* (Linton, 1928), *Bianum arabicum* Sey, 1996, *Hypocreadium caputvadum* Kacem, Derbel & Neifar, 2011, *Neomultitestis aspidogastriformis* Bray & Cribb, 2003, *Opechona bacillaris* (Molin, 1859) and *Prodistomum polonii* (Molin, 1859) (see Kacem et al., 2012; Bakhoum et al., 2015a; Ndiaye et al., 2015; Quilichini et al., 2015; Kacem and Miquel, 2020).

In the present work, we describe the ultrastructural sperm characteristics of *Holorchis pycnopus* Stossich, 1901 and their organization in the mature spermatozoon for the first time, thus contributing to expand the current knowledge of sperm ultrastructure in the superfamily Lepocreadioidea in order to highlight the potential criteria useful for phylogeny.

## 2. Material and methods

### 2.1. Specimens

Live adult specimens of *H. pycnopus* were collected in November 2015 and December 2016 from the digestive tract of the Striped seabream *Lithognathus mormyrus* (Linnaeus, 1758) (Teleostei, Sparidae) captured in the Mediterranean Sea, off the coast of La Chebba (34°14'N, 11°06'E) (Tunisia).

## 2.2. Transmission electron microscopy (TEM)

After their extraction, adult worms were immediately rinsed with a 0.9% NaCl solution, 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, and rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4. They were then postfixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>] 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 at the level of seminal vesicle were obtained using a Reichert-Jung Ultracut-E ultramicrotome, placed on copper grids and double-stained with 2% uranyl acetate (aqueous solution) for 10 min and lead citrate [1.33 g Pb(NO<sub>3</sub>)<sub>2</sub> + 1.76 g Na<sub>3</sub>(C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>).2H<sub>2</sub>O] for 3 min (Reynolds, 1963). 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)'.

## 2.3. Cytochemistry

The Thiéry (1967) technique was applied to locate glycogen in sections placed on gold grids using the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP). Gold grids were treated in PA-TCH-SP as follows: 30 min in 10% PA, rinsed in Milli-Q water, 30-45 min in 0.2% TCH in 20% acetic acid, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark, and rinsed in Milli-Q water.

## 3. Results

The interpretation of numerous ultrathin sections of the mature spermatozoa of *H. pycnopus* from the seminal vesicle by means of TEM has allowed the establishment of three

well defined regions from the anterior to the posterior spermatozoon extremity with characteristic features (I to III) (Figs. 1-3).

Region I (Figs. 1I and 2a-l) corresponds to the anterior region of the spermatozoon. Cross-sections through the anterior tip show doublets of the first axoneme accompanied by an electron-dense material (Fig. 2a). At a slightly distal level, the first axoneme appears with a mass of electron dense material located around the anterior extremity of the second axoneme (Fig. 2b, c). The appearance of the first mitochondrion (of moniliform type) is also observed in this part of region I (Fig. 2c); however only the mitochondrial cord is present in this part. More posteriorly, when the two axonemes are completely formed, small deposits of glycogen granules and cortical microtubules appear (Fig. 2d, e).

In the middle part of region I, an external ornamentation of the plasma membrane associated with cortical microtubules is observed on the ventral side that contains mitochondrion (Fig. 2f-i). The external ornamentation of plasma membrane is formed externally to the membrane surrounding the second axoneme in the area with the anterior dense material, and follows a displacement toward a ventral position (Fig. 2f-h). The particular placement of attachment zones also demonstrates this fact (compare Fig. 2e, i with black and white arrows). Attachment zones are submembranous electron-dense areas that appear in the median cytoplasmic process during spermiogenesis and that are visible in the spermatozoa after the proximodistal fusion of the three involved structures (two free flagella and the median cytoplasmic process). The above-mentioned disposition of attachment zones in Fig. 2i allows to establish a possible relation between external ornamentation and the anterior dense material. The number of cortical microtubules increases from the anterior to the posterior part of region I (Fig. 2e-j, l). Moreover, it is worthy to mention that this area exhibits a mitochondrial cord with joined mitochondrial bulges characterising the first mitochondrion (Figs. 1I and 2j-l).

In the distal part of region I, the disappearance of the external ornamentation and the posterior extremity of the first mitochondrion marks the transition between regions I and II (Fig. 2l).

Region II (Figs. 1II, 2m and 3a) corresponds to the middle region of the mature spermatozoon. It is a transitional area before the nuclear region, which is mainly characterized by the disappearance of the first mitochondrion. The anterior part of this region shows the increase in the number of cortical microtubules to 10+12 microtubules and in the amount of glycogen granules which are tightly packed between both axonemes (Fig. 2m). In the posterior part of this region, we notice the appearance of the second mitochondrion and the presence of the maximum number of cortical microtubules (11+12) in region II (Figs. 1II and 3a).

Region III (Figs. 1III and 3b-i) corresponds to the nuclear and posterior spermatozoon region. In its proximal part, we notice the appearance of the nucleus with the simultaneous presence of the second mitochondrion, two axonemes, granules of glycogen, and cortical microtubules (Figs. 1III and 3b-d). Towards the posterior part of region III, the transition of characters is as follows: (i) disappearance of the second mitochondrion (Fig. 3d, e), (ii) disorganization and disappearance of the first axoneme (Fig. 3e, f), and finally (iii) disappearance of the nucleus during the disorganization of the second axoneme (Fig. 3g, h). A progressive reduction in the number of cortical microtubules (13+11; 4+5; 2+2; 0+0) is observed along this part of region III (Fig. 3d-h). In fact, the maximum number of cortical microtubules in the spermatozoon of *H. pycnopus* is observed in the anterior part of region III containing both the second mitochondrion and the nucleus; this maximum value is around 24 microtubules (Fig. 3d). Therefore, it is interesting to note that the posterior spermatozoon tip exhibits granules of glycogen (Fig. 3i).

The glycogen granules are clearly highlighted with the Thiéry method, and TEM micrographs (Fig. 3j) show their presence in regions II and III of the mature spermatozoon of *H. pycnopus*.

#### 4. Discussion

The ultrastructural study of the mature spermatozoa of *H. pycnopus* reveals a general architecture previously reported in some digeneans. Several specific features –mainly located in anterior and posterior regions of sperm cells– are considered very potential for robustness of phylogenetic inferences and are used by Bakhoun et al. (2017a) to establish five different Digenea spermatozoon models. Our ultrastructural study reveals that the mature spermatozoon of *H. pycnopus* exhibits the characteristics of Bakhoun et al.'s type III of digenean spermatozoa.

##### 4.1. Anterior region of the spermatozoon and associated characters

The anterior extremity of the male gamete of *H. pycnopus* has two axonemes slightly longitudinally displaced from one another. These axonemes exhibit the 9+'1' trepaxonematan pattern constituted by nine peripheral doublets of microtubules disposed around a central core (Ehlers, 1984). This type of axonemes is present in all digeneans except for the species of *Schistosoma* Weinland, 1858 that have a special 9+'1' pattern (see Jamieson and Justine, 2017) and *Didymozoon* Taschenberg, 1879 with a 9+0 pattern (see Justine and Mattei, 1983).

Another feature present in the anterior extremity of the male gamete of *H. pycnopus* is the anterior electron-dense material as occurs in all the Lepocreadioidea studied up to now, except in apocreadiids and deropristids (Foata et al., 2007; Kacem et al., 2010), as well as in numerous species belonging to Atractotrematidae Yamaguti, 1939 (see Bakhoun et al., 2015b), Cryptogonimidae Ward, 1917 (see Kacem and Miquel, 2019), and Opecoelidae

Ozaki, 1925 (see Bâ et al., 2020a). This structure presents distinct spatial distribution patterns according to the species. It appears (i) as a cytoplasmic mass of electron-dense material located around the second axoneme or (ii) as a submembranous layer on the opposite side of the first axoneme beneath the plasma membrane. The first pattern is observed in the anterior spermatozoon extremity of *H. pycnopus* and also reported in other lepecreadioids, namely the Aephnidiogenidae *H. micracanthum* and *A. senegalensis* (Bâ et al., 2011, 2018), the Gyliachenidae *Gyliachen* sp. (Quilichini et al., 2011) and the Lepocreadiidae *B. plicatum* and *B. arabicum* (Quilichini et al., 2015), whereas in the other lepecreadioids, it appears as a submembraneous layer (Bakhoun et al., 2012, 2015a; Kacem et al., 2012, 2020; Ndiaye et al., 2015) –see Table 1.

The external ornamentation of the plasma membrane has been reported in the anterior region of the spermatozoon of most digeneans; their presence or absence and other features as well as its type, its location and its association or not with cortical microtubules are considered as potentially useful criteria for clarifying relationships within digeneans (Quilichini et al., 2007a, 2011; Bakhoun et al., 2017a). Regarding the location of the external ornamentation along the spermatozoon, Quilichini et al. (2011) suggested that digenean spermatozoa can be recognized into three distinct types (type 1, type 2 and type 3). In *H. pycnopus*, the external ornamentation is located at the level of the first mitochondrion and hence follows the Quilichini et al.'s type 2 spermatozoon. Type 2 was also described in all the currently studied Lepocreadoidea, except in *N. chabaudi* (Kacem et al., 2010), presenting the Quilichini et al.'s type 1 characterized by the anterior location of the external ornamentation. Moreover, the male gamete of *H. pycnopus* displays an external ornamentation of the plasma membrane associated with cortical microtubules as occurs in all the Lepocreadoidea studied to date (see Table 1).

Spine-like bodies were firstly described by Miquel et al. (2000) in the opecoelid *Opecoeloides furcatus* (Bremser in Rudolphi, 1819) as submembraneous and prominent electron-dense elements which contain a sort of vesicular structure. Since their original description, spine-like bodies have frequently been observed in the anterior part of digenean mature spermatozoa. There are different aspects related to this structure: presence/absence, their number, size, periodicity or not, and their location in ornamented, non-ornamented or both areas. All of these aspects have been subject to numerous discussions by several authors due to its great variability, even within the same family, and are not retained here as an important ultrastructural criterion for phylogenetic purposes. In the superfamily Lepocreadioidea, there is variability in the presence/absence of spine-like bodies in the spermatozoon. *Holorchis pycnopus* lacks spine-like bodies, and this absence is also the case for the aepnidiogenids *A. senegalensis* and *H. micracanthum*, the deropristid *D. inflata*, and the lepocreadiids *H. caputvadum* and *P. polonii* (Foata et al., 2007; Bâ et al., 2011, 2018; Kacem et al., 2012; Kacem and Miquel, 2020) –see Table 1.

#### 4.2. Cortical microtubules

The presence of cortical microtubules has been described in the spermatozoa of the major parasitic groups of platyhelminths with the exception of the species of *Didymocystis* Ariola, 1902 and *Didymozoon* (see Justine and Mattei, 1983; Pamplona-Basilio et al., 2001). Three important aspects related to this structure (their disposition, the number of bundles, and the location of their maximum number) are considered as remarkable criteria which have led to efficient and robust phylogenetic inference (see Bakhoun et al., 2017a). As occurs in *H. pycnopus* and other lepocreadioideans, and digeneans, these submembraneous ultrastructural elements are present as parallel tubular structures underlying the plasma membrane. However, in most distantly cestodes, such as tetrabothriideans and cyclophyllideans (excluding

mesocestoidids), cortical microtubules have a spiralled disposition (Stoitsova et al., 1995; Miquel et al., 1999; Levron et al., 2010).

Concerning their arrangement along the male gametes, cortical microtubules are normally arranged into two fields in the principal region of the spermatozoon (mitochondrial and nuclear regions), as occurs in *H. pycnopus*, as well as in the previously studied lepecreadioids (see Table 1). However, in several other species belonging to the Hemiuridae Looss, 1899, and the lecithasterid *Aponurus laguncula* Looss, 1907, the sclerodistomid *Sclerodistomum italicum* (Stossich, 1893), the sclerodistomoidid *Sclerodistomoides pacificus* Kamegai, 1971 and the faustudid *Pronoprymna ventricosa* (Rudolphi, 1819) do not follow this pattern and present a single set of cortical microtubules placed on the ventral side (see Quilichini et al., 2007a, 2010b; Ndiaye et al., 2017; Bâ et al., 2020b; Kacem et al., 2020).

The location of the maximum number of cortical microtubules is another crucial characteristic for the establishment of sperm model in digeneans. On the light of this last character, Quilichini et al. (2007b) and posteriorly Bakhoun et al. (2017a) proposed that the spermatozoon of digenean parasites could be divided into two groups: (i) a first one in which the highest number is located in the anterior part of the spermatozoon and (ii) a second one with the maximum number located in the middle or more posterior part of the spermatozoon. In *H. pycnopus* as in all lepecreadioids studied until now, the maximum number of microtubules –which ranges from 15 to 28– is quite homogeneous, located in the middle region of the sperm cell, with the exceptions of the apocreadiid *N. chabaudi*, the deropristid *D. inflata* and the lepecreadiid *H. caputvadum* (Foata et al., 2007; Kacem et al., 2010, 2012) that exhibit the maximum number in the anterior part of the spermatozoon (see Table 1). Therefore, *H. pycnopus* can be classified in the Quilichini et al.'s type 2 of sperm cell according to the location of the maximum number of cortical microtubules, but in this

aephnidiogenid this feature is observed more posteriorly than for the remaining lepoproadioids, in the nuclear region (see Table 1).

#### 4.3. Mitochondria: number, location and morphology

The presence of mitochondria has been described in the spermatozoa of all parasitic groups of flatworms except the Eucestoda in which the loss of mitochondrion is considered as a synapomorphy (Justine, 1991). Various aspects related to this element, namely, the number, the morphology and the location have been considered as potentially informative for phylogenetic inference. Concerning the number of mitochondria in the spermatozoon of digeneans, it is still a matter of controversy considering the difficulty to observe and determine their number using only longitudinal sections. To resolve this problem and to make a logical interpretation of numerous cross-sections, one, two or three mitochondria have been reported in the male gamete of digeneans (see Bakhoun et al., 2017a). In the Lepoproadioidea, all the species currently analysed have two mitochondria except the Aephnidiogenidae *H. micracanthum* and the Gyliuchenidae *Gyliuchen* sp. which exhibit one mitochondrion (Bâ et al., 2011; Quilichini et al., 2011) (Table 1). In general, when the sperm cell contains two mitochondria, the first one is placed in the anterior part and the second one in the posterior part. However, in *Dicrocoelium hospes* Looss, 1907, both mitochondria are located in a parallel disposition in the anterior part of the spermatozoon (Agostini et al., 2005) and in *Haplospalchnus caudatus* (Srivastava, 1937) the first mitochondrion appears in the anterior end of the spermatozoon before the complete formation of both axonemes (see Kacem et al., 2019).

Considering the mitochondrial morphology, it is variable and considered as potential character to discern phylogenetic relationships in Digenea. Different shapes of mitochondria have been described in the sperm cells of digenean species, namely, filiform, moniliform and

U-shaped mitochondrion (Kacem et al., 2019). In fact, *H. pycnopus* presents a moniliform mitochondrion composed of a mitochondrial cord with joined mitochondrial bulges. This shape of mitochondria was firstly described in *H. micracanthum* by Bâ et al. (2011), and posteriorly reported in the male gamete of some digeneans such as the acanthocolpid *Stephanostomoides tenuis* (Manter, 1963) (see Bakhoun et al., 2015c), the cryptogonimids *Aphallus tubarium* (Rudolphi, 1819) and *Timoniella imbutiformis* (Molin, 1859) (see Foata et al., 2012; Kacem et al., 2017a), the opecoelids *Allopodocotyle pedicellata* (Stossich, 1887) and *Macvicaria obovata* (Molin, 1859) (see Bakhoun et al., 2017b; Kacem et al., 2017b), the plagiorchiid *Enodiotrema reductum* Looss, 1901 (see Ndiaye et al., 2012), and the sclerodistomoidid *S. pacificus* (see Bâ et al., 2020b). In the Lepocreadioidea, this type of mitochondrion has been noticed in three species, the above-mentioned *H. micracanthum* and also *O. bacillaris* and *P. polonii* (Bâ et al., 2011; Ndiaye et al., 2015; Kacem and Miquel, 2020).

#### 4.4. Posterior region of the spermatozoon

The posterior spermatozoon extremity of the digenean male gametes shows a great variability of characteristics that emphasise the usefulness of this criterion while establishing spermatozoon models in Digenea. Quilichini et al. (2010a), considering the sequence of disappearance of the following characters nucleus, axoneme, and cortical microtubules, postulated three different posterior spermatozoon extremities (opecoelidean type, fasciolidean type, and cryptogonimidean type). Nevertheless, based on some inconsistencies with these three models, Bakhoun et al. (2017a) recommend to scrutinize just the last spermatozoon character instead of the sequence of characters. Thus, three types of posterior spermatozoon extremities have been established: (i) a posterior spermatozoon extremity containing only cortical microtubules, (ii) a posterior spermatozoon extremity containing

only the nucleus, and (iii) a posterior spermatozoon extremity containing only one axoneme. The present study shows that spermatozoa of *H. pycnopus* present the second axoneme as the terminal character, as occurs in all the studied lepecrearioideans except *B. arabicum*, *B. plicatum*, *N. chabaudi*, *N. aspidogastriformis* and *R. fractum*, which exhibit the nucleus as the posterior sperm character (Kacem et al., 2010; Bakhoun et al., 2012, 2015a; Quilichini et al., 2015).

#### 4.5. Glycogen

The presence of glycogen as a source of energy in the mature spermatozoa of digeneans has been described in the majority of the currently studied species (Bakhoun et al., 2017a). The fixation and staining techniques can affect to the visibility of this granular material and, consequently, the use of specific cytochemical test of Thiéry (1967) is essential to evidence both the presence and nature of granular material present in digenean sperm cells. The majority of digeneans, as occurs in the case of *H. pycnopus* and in the remaining lepecrearioids, exhibit a large amount of glycogen in the cytoplasm in different regions of the spermatozoon, especially in mitochondrial and nuclear regions. In contrast, spermatozoa of hemiurids present a small quantity of granules of glycogen randomly dispersed in the cytoplasm (see Bakhoun et al 2017a; Kacem et al. 2020). A particular pattern in the location of glycogen in digenean sperm cell is described in the bucephalid *Prosorhynchus aculeatus* Odhner, 1905 (see Miquel et al. 2017). In this species the granules of glycogen are placed not only in the cytoplasm but also in the axonemes, located between peripheral doublets and the central core.

## 5. Conclusions

In the Lepocreadioidea, there is no homogeneous model for the organization of ultrastructural characters along the spermatozoon. The analysis of the available data permits us to make some considerations. Most species have type III sperm cell of Bakhoum et al. (2017a). Thus, all the Aepnidiogenidae and Lepocreadiidae exhibit this ultrastructural organization, with the sole exception of the lepocreadiid *H. caputvadum* (Kacem et al., 2012). The gyliachenid *R. fractum* also follows this type (Bakhoum et al., 2012). Type III is basically characterized by two 9+'1' axonemes, external ornamentation associated with cortical microtubules and not being located in the anterior extremity, cortical microtubules organized into two bundles and with their maximum number in the middle part of the spermatozoon and two mitochondria in general. Contrary, the Deropristidae and also the above-mentioned *H. caputvadum* present type IV mainly differentiated by its very anterior location of the maximum number of cortical microtubules. For the remaining lepocreadiids, the sperm model is not clear. Thus, *N. chabaudi* (Kacem et al., 2010) seems to follow a type V with the external ornamentation in the anterior part of the proximal region and maximum number of cortical microtubules in the anterior part of the gamete, but it lacks a lateral expansion characteristic of this type V. Finally, *Gyliachen* sp. (Gyliachenidae) (Quilichini et al., 2011) shares several characteristics of models III and IV, but it is not totally congruent with any of them; the main problem is the doubtful placement of the maximum number of cortical microtubules. In the Aepnidiogenidae, the mature spermatozoon exhibits a similar ultrastructural pattern. Some differences are observed, particularly the location of the maximum number of cortical microtubules and the number of mitochondria. The presence of the anterolateral electron-dense material is the major particularity in species belonging to the Lepocreadioidea. This anterolateral electron-dense material could be a synapomorphy for the superfamily and an ultrastructural argument supporting the monophyletic status of the Lepocreadioidea (Bray and Cribb, 2012).

**AUTHOR STATEMENT**

Hichem Kacem participated in the field work and in the identification of the species, performed the TEM study and wrote the first draft manuscript. Jordi Miquel participated in the field work and in the identification of the species, performed the TEM study and critically revised the final version of manuscript.

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**Declaration of Competing Interest**

The authors declare that they have no conflict of interest.

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**CRedit authorship contribution statement**

HK participated in the field work and in the identification of the species, performed the TEM study and wrote the first draft manuscript. JM participated in the field work and in the identification of the species, performed the TEM study and critically revised the final version of manuscript.

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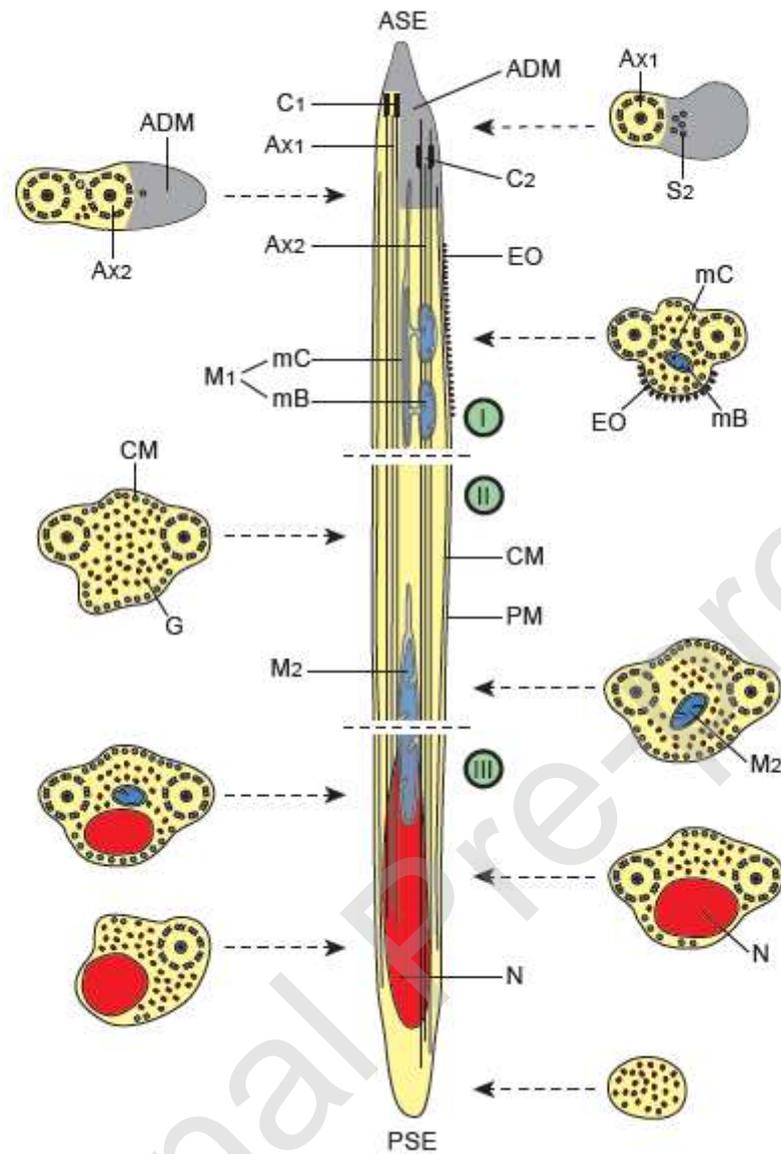
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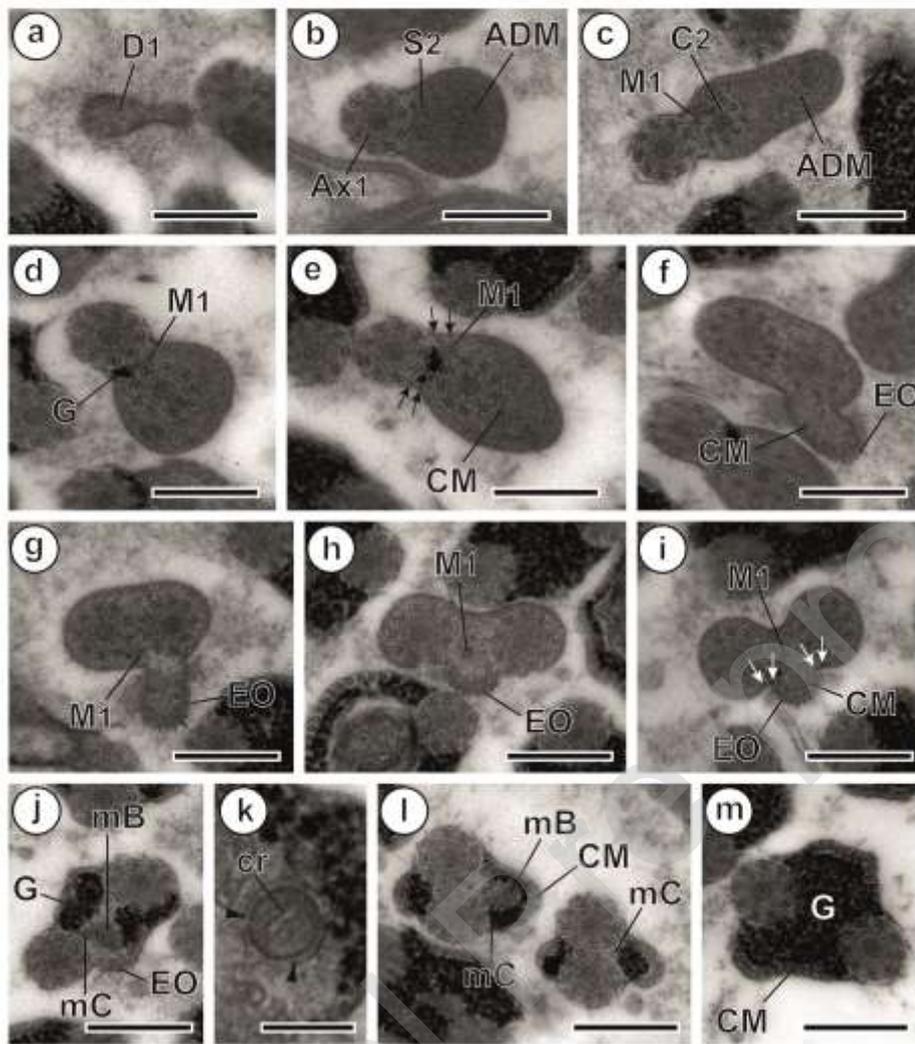
**Figure captions**

**Fig. 1.** Schematic drawing showing the ultrastructural organization of the mature spermatozoon of *Holorchis pycnopus*. 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. ADM, anterior electron-dense material; ASE, anterior spermatozoon extremity; Ax1, Ax2, first and second axoneme; C1, centriole of the first axoneme; C2, centriole of the second axoneme; CM, cortical microtubules; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M1, M2, first and second mitochondrion; mB, mitochondrial bulge; mC, mitochondrial cord; N, nucleus; PM, plasma membrane; PSE, posterior spermatozoon extremity.



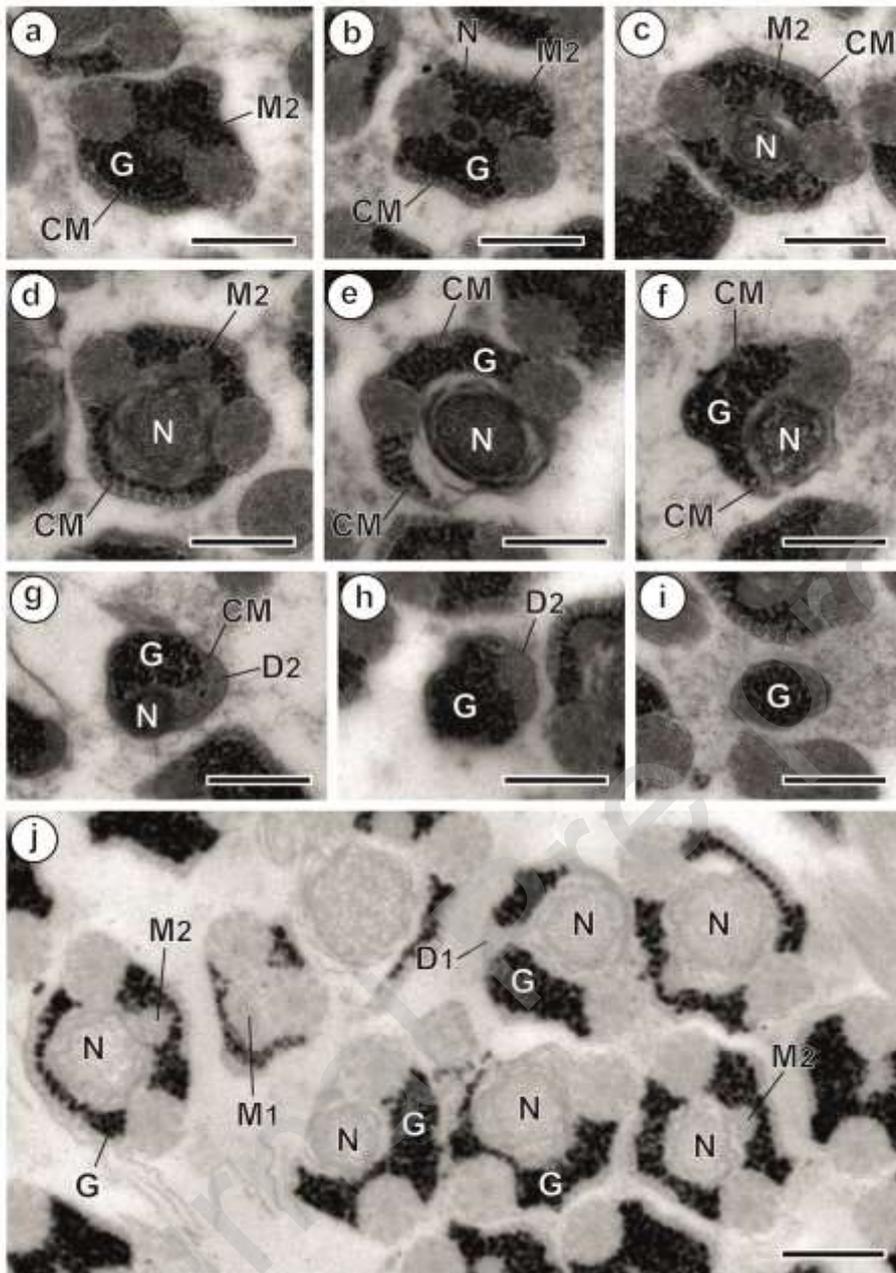
**Fig. 2.** Mature spermatozoon of *Holorchis pycnopus*: (a) anterior spermatozoon extremity illustrating doublets of the first axoneme; (b, c) consecutive sections of region I showing the formation of the second axoneme surrounded by the anterior electron-dense material; (d, e) cross-sections at the level of the appearance of the first mitochondrion and the cortical microtubules; (f-j) ornamented area of region I showing the first mitochondrion of moliliform type, formed by a cord with several bulges; (k) detail of the first mitochondrion (mitochondrial bulge) showing a crest and the double mitochondrial membrane; (l) posterior

part of region I lacking external ornamentation; (m) cross-section of the anterior part of region II. Arrows, attachment zones; Arrowheads, double mitochondrial membrane; ADM, anterior dense material; Ax1, first axoneme; C2, centriole of the second axoneme; CM, cortical microtubules; cr, mitochondrial crest; D1, doublets of the first axoneme; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M1, first mitochondrion; mB, mitochondrial bulge; mC, mitochondrial cord; S2, singlets of the second axoneme. Scale bars (a-j, l, m) = 300 nm; (k) = 100 nm.



**Fig. 3.** Mature spermatozoon of *Holorchis pycnopus*: (a) posterior part of region II showing the second mitochondrion; (b-d) consecutive cross-sections of the anterior part of region III with the simultaneous presence of the second mitochondrion and the nucleus. Note the

progressive reduction in the number of cortical microtubules; (e, f) correlative sections showing the disappearance of the second mitochondrion and the first axoneme; (g, h) consecutive sections showing the disappearance of the nucleus and cortical microtubules during the disorganization of the second axoneme; (i) spermatozoon tip containing only glycogen; (j) cytochemical test of Thiéry evidencing glycogen at ultrastructural level. CM, cortical microtubules; D1, D2, doublets of the first and second axoneme; G, granules of glycogen; M1, M2, first and second mitochondrion; N, nucleus. Scale bars = 300 nm.



**Table 1.** Spermatological characteristics in the superfamily Lepocreadioidea

Families and species	Spermatozoon characteristics											References
	Principal characters								Secondary characters			
	TA x	L E	E O	EO+ CM	LE O	BC M	LM CM	M	AD M	SB	PSC	
<b>Aepnidiogenidae</b>												
<i>Aepnidiogenes senegalensis</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2	Med S	2	+(# )	-	Ax	Bâ et al. (2018)
<i>Holorchis micracanthum</i>	9+ <sup>1</sup> 1'	+?	+	+	Pos tA	2	Med S	1*	+(# )	-	Ax	Bâ et al. (2011)
<i>Holorchis pycnopus</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2	Post S	2(1 *)	+(# )	-	Ax	Present study
<b>Apocreadiidae</b>												
<i>Neopocreadium chabaudi</i>	9+ <sup>1</sup> 1'	-	+	+	Ant A	2	AntS	2	-	+	N?	Kacem et al. (2010)
<b>Deropristidae</b>												
<i>Deropristis inflata</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2	AntS	2	-	-	Ax	Foata et al. (2007)
<b>Gyliauchenidae</b>												
<i>Gyliauchen</i> sp.	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2	Med S?	1	+(# )	+	Ax	Quilichini et al. (2011)
<i>Robphildollfusium fractum</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2	Med S	2	+(† )	+	N	Bakhoum et al. (2012)
<b>Lepocreadiidae</b>												
<i>Bianium arabicum</i>	9+ <sup>1</sup> 1'	+?	+	+	Pos tA	2	Med S	2	+(# )	+	N	Quilichini et al. (2015)
<i>Bianium plicatum</i>	9+ <sup>1</sup> 1'	+?	+	+	Pos tA	2	Med S	2	+(# )	+	N	Quilichini et al. (2015)
<i>Hypocreadium caputvadum</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2	AntS	2	+(† )	-	Ax	Kacem et al. (2012)
<i>Neomultitestis</i>	9+ <sup>1</sup>	-	+	+	Pos	2	Med	2	+(† )	+	N	Bakhoum et al.

<i>aspidogastriformis</i>	1'				tA	S	)					(2015a)
<i>Opechona bacillaris</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2 S	Med S	2(1 *)	+(† )	+	Ax	Ndiaye et al. (2015)
<i>Prodistomum polonii</i>	9+ <sup>1</sup> 1'	-	+	+	Pos tA	2 S	Med S	2(1 *)	+(† )	-	Ax	Kacem and Miquel (2020)

ADM, anterior dense material; AntA, anterior part of the anterior region; AntS, anterior part of the spermatozoon; Ax, axoneme; BCM, number of bundles of cortical microtubules; EO, external ornamentation of the plasma membrane; EO+CM, association 'external ornamentation-cortical microtubules'; LE, lateral expansion; LEO, location of external ornamentation; LMCM, location of maximum number of cortical microtubules; M, number of mitochondria; MedS, median part of the spermatozoon; PostS, posterior part of the spermatozoon; PSC, posterior spermatozoon character; SB, spine-like bodies; Tax, type of axoneme; +/-, presence/absence of considered character; \*, moniliform mitochondrion; #, anterior dense material as a cytoplasmic mass; †, anterior dense material as a submembranous layer; ?, doubtful or unknown data.