Sperm characters in the Hemiuridae (Digenea): first data on *Aphanurus stossichii* (Aphanurinae) and *Ectenurus lepidus* (Dinurinae)

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Abstract

The present work provides the first ultrastructural analysis of spermatozoa of two digeneans (*Aphanurus stossichii* (Monticelli, 1891) and *Ectenurus lepidus* Looss, 1907) belonging to the unexplored subfamilies of the Hemiuridae, namely, the Aphanurinae and the Dinurinae. In March 2019, these hemiurids were collected respectively from the digestive tract of the bogue *Boops boops* (Teleostei, Sparidae) and the Atlantic horse mackerel *Trachurus trachurus* (Teleostei, Carangidae) captured in the coastal zone of the Mediterranean Sea, off La Chebba (Tunisia). The ultrastructural study reveals that both spermatozoa exhibit the Bakhoum et al.’s type II of the digenean sperm cells characterized by the presence of two 9+1’ axonemes, an external ornamentation of the plasma membrane not associated with cortical microtubules and located in the anterior part of the spermatozoon, a single bundle of cortical microtubules, the maximum number of cortical microtubules located in a middle part of the sperm cell, and one mitochondrion. Moreover, they share several ultrastructural features with the studied spermatozoa of Hemiuridae such as the presence of two axonemes with the 9+1’ trepaxonematan pattern, a reduced number of parallel cortical microtubules organized into one field with their maximum number located in the median (*A. stossichii*) or posterior (*E. lepidus*) part of the spermatozoon, an external ornamentation of the plasma membrane in the anterior part of the spermatozoon, one mitochondrion, a nucleus, and a small amount of glycogen granules. However, the two studied hemiurids could be distinguished by the morphology of the anterior and posterior spermatozoon extremities and the presence of mitochondrial matrix granules in *A. stossichii*.

Keywords *Aphanurus stossichii* • *Ectenurus lepidus* • Hemiuridae • Digenea • Ultrastructure • Sperm characters

Introduction

The superfamily Hemiuroidea is a large group of digenetic trematodes that are predominantly parasitic of the digestive tract, especially the stomach of a wide range of marine and freshwater teleosts, elasmobranchs, and occasionally amphibians and reptiles (Gibson 2002). The Hemiuroidea is considered as the most complex superfamily with generic richness comprising fourteen families among them the Hemiuridae with twelve subfamilies (Gibson and Bray 1979).

The molecular phylogenetic analysis of the family Hemiuridae carried out by Atopkin et al. (2017) indicates that the presence or absence of an ecsoma was not associated with molecular data for hemiurid subfamilies differentiation. Moreover, on the basis of the molecular differentiation found in this study, the authors considered that the taxonomic status of Aphanurinae, Dinurinae, Elytrophallinae, and Hemiurinae should be reconsidered with the inclusion of these digeneans in the same subfamily Hemiurinae.
Due to several inconsistencies and the shortage of robustness in the existing classification of the Hemiuroidae, numerous changes were made related to the description of new genera and subfamilies (Bray and Nahhas 2002; Pankov et al. 2006; Bursey et al. 2008; Bilqees et al. 2009, 2010; Justo and Kohn 2012; Urabe and Shimazu 2013) as well as a change in the taxonomic rank of previously described subfamilies (Sokolov et al. 2018).

Ultrastructural studies of species belonging to the Hemiuridae, as in other digeneans, are of great importance for a better knowledge of relationships within this family of digeneans since they bring additional information that could complement molecular analyses and morphological data (Justine 1991, 1995, 2001; Quilichini et al. 2010a, 2011; and Bakhoun et al. 2017). To date, there are ultrastructural studies on the spermatozoon of five species within this family, namely, *Lecithochirium excisum* (Rudolphi, 1819) (Elytrophallinae), *Hemiuris appendiculatus* (Rudolphi, 1802) and *Parahemiuris merus* (Linton, 1910) (Hemiurinae), and *Lecithochirium microstomum* Chandler, 1935 and *Lecithochirium musculus* (Looss, 1907) (Lecithochiriinae) (Ndjaye et al. 2012, 2013a, 2014; Dione et al. 2016). The present study describes for the first time the mature spermatozoa of *Aphanurus stossichii* (Monticelli, 1891) and *Ectenurus lepidus* Looss, 1907 belonging to the subfamilies Aphanurinae and Dinurinae, respectively. Our results provide useful criteria for the elucidation of phylogenetic relationships within the family Hemiuridae.

### Materials and methods

Adult specimens of *A. stossichii* and *E. lepidus* were gathered live from the digestive tract of the bogue *Boops boops* (Linnaeus, 1758) (Pisces, Teleostei, Sparidae) and the Atlantic horse mackerel *Trachurus trachurus* (Linnaeus, 1758) (Pisces, Teleostei, Carangidae), respectively. Hosts were caught in March 2019 in the Mediterranean Sea, off La Chebbâ (34° 14′ N, 11° 06′ E) (Tunisia).

After their extraction, live 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 with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in MilliQ 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). 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 for cytochemical detection of glycogen. Gold grids were treated 30 min in 10% periodic acid and rinsed in MilliQ water; 24 h in thiocarbohydrazide and rinsed in acetic solutions and MilliQ water; then 30 min in 1% silver proteinate in the dark; and rinsed in MilliQ water.

### Results

The interpretation of numerous ultrathin sections of the mature spermatozoa of *A. stossichii* and *E. lepidus* allows us to distinguish three different regions (I–III) from the anterior to the posterior spermatozoon extremities (see Figs. 1, 2, 3, 4, and 5).

Region I (Figs. 1a–i, 3a–d, and 5I) corresponds to the anterior part or premitochondrial area of the spermatozoon. Cross sections through the anterior tip of the spermatozoon of both studied hemiurid species show relatively small differences.

The anterior tip of the *A. stossichii* spermatozoon is entirely covered by a filamentous ornamentation associated with short cortical microtubules and also exhibits a few doublets of the first axoneme (Figs. 1a and 5I). At a slightly distal level, the sperm cell is partly covered with typical external ornamentation (Fig. 1b). More posteriorly, cross sections show the gradual appearance of doublets of the second axoneme and later the complete formation of both axonemes (Figs. 1c–f). In this area, a single cortical microtubule appears between both axonemes and the external ornamentation partially surrounds just the second axoneme (Figs. 1d–f and 5I). Finally, the posterior part of region I lacks external ornamentation. It is also characterized by the increase of the number of cortical microtubules reaching seven elements and the appearance of a small amount of glycogen granules (Fig. 1g–i).

The *E. lepidus* male gamete anterior tip exhibits typical external ornamentation of the plasma membrane surrounding the centriole of the first axoneme (Figs. 3a and 5I). At a slightly distal level, the first axoneme appears accompanied by the anterior extremity of the second axoneme. Then, the sperm cell is partly covered by the external ornamentation of the plasma membrane (Fig. 3b). As previously reported in *A. stossichii*, when the second axoneme appears, there is a single cortical microtubule (Fig. 3c), and the external ornamentation is partially surrounding both axonemes (Figs. 3c and 5I). The posterior part of region I is characterized by the disappearance of the external ornamentation of the plasma membrane and the presence of up to five cortical microtubules disposed only on one side of the spermatozoon as well as some glycogen granules (Figs. 3d and 5I).

Region II (Figs. 1j, k, 2, 3e, and 5II) is the middle region or mitochondrial area of the spermatozoon. In both hemiurids,
the sperm region II is characterized by the same features: the appearance of a mitochondrion and a higher number of cortical microtubules (eleven in A. stossichii and six in E. lepidus) (Figs. 1j, k, 3e, and 5II). For A. stossichii, this is the maximum number of cortical microtubules. However, an interesting difference is observed in A. stossichii spermatozoon: the mitochondrion contains dense granules deposited in the matrix with a granule diameter of about 45 nm (Figs. 1j, 2, and 5II).

Region III (Figs. 1l–u, 2, 3f–o, and 5III) corresponds to the nuclear and posterior spermatozoon extremity. It begins with the simultaneous presence of the posterior part of the mitochondrion and the nucleus (Figs. 1l, 2, 3f, g, and 5III). In the middle part, the mitochondrion disappears. The sperm cell contains two axonemes, a nucleus, granules of glycogen, and cortical microtubules whose number progressively decreases and disappears (Figs. 1n–p, 3h–k, and 5III). However, in the spermatozoon of E. lepidus, the maximum number of cortical microtubules (8 elements) is observed in the nuclear region (Fig. 3h). The transition of characters toward the posterior tip of the spermatozoon of both studied hemiurid species shows relatively slight differences. In A. stossichii, the transition of characters is as follows: (i) disappearance of the nucleus, (ii) disorganization of the first axoneme, and (iii) later disorganization of the second axoneme (Fig. 1q–u). Nevertheless, singlets of the first axoneme remain present until the posterior spermatozoon tip (Fig. 1u). In E.
lepidus spermatozoon, the transition is as follows: (i) disorganization of the first axoneme, (ii) disappearance of the nucleus, and (iii) disorganization of the second axoneme (Fig. 3I–o).

The glycogenic nature of the electron-dense granules observed along the sperm cell of both studied hemiurids was evidenced by applying the Thiéry’s test (Fig. 4a, b).

**Discussion**

The mature spermatozoa of *A. stossichii* and *E. lepidus* share the general ultrastructural characteristics found in most digeneans described so far: two axonemes with the 9+1’ treponematan pattern (Ehlers 1984), a nucleus, a mitochondrion, glycogen granules, and parallel cortical microtubules. Thus, they exhibit the characteristics of the type II digeneans sperm cells (Bakhoum et al. 2017), namely, two 9+1’ axonemes, external ornamentation not associated with cortical microtubules and located in the anterior part of the sperm cell, a single bundle of cortical microtubules, the maximum number of cortical microtubules located in a middle part of the spermatozoon, and the presence of one mitochondrion. The type II of spermatozoon has been described in the faustulid *Sclerodistomoides pacificus* (Bâ et al. 2020) (see Table 1).

Regarding the location of the external ornamentation along the spermatozoon, Quilichini et al. (2011) suggested that digenean spermatozoa could be divided into three types: (i) type 1 presents an external ornamentation in the anterior extremity of the spermatozoon; (ii) type 2 presents an external ornamentation at a more posterior level; and (iii) type 3 lacks external ornamentation. In our case, spermatozoa of both *A. stossichii* and *E. lepidus* follow the Quilichini et al.’s type 1 spermatozoon.

Another character that could depict the male gamete is the cortical microtubules. These structures are present in the sperm cells of most digeneans studied so far (see Bakhoum et al. 2017). Three aspects of these tubular structures are considered as interesting ultrastructural criteria used for phylogenetic inference: their presence or absence, the location of their maximum number, and the number of bundles. The cortical microtubules of the mature spermatozoon of *A. stossichii* and *E. lepidus* are arranged only in a ventral bundle. Among digeneans, this characteristic is only known in the Hemiuroidea. A low number of these cortical microtubules are present in the mature spermatozoon of *A. stossichii* and *E. lepidus*: 11 and 8, respectively. The reported number of cortical microtubules in the hemiurids is usually low, varying
Fig. 3  Mature spermatozoon of *Ectenurus lepidus*. a–d Consecutive cross sections of the anterior spermatozoon extremity (region I). Note the presence of typical external ornamentation in the anterior tip (Fig. 3a) in contrast with *A. stossichii*. e Cross section of the middle or mitochondrial region (region II). f–o Correlative cross sections of the posterior extremity or nuclear region (region III). Ax1, first axoneme; C1, centriole of the first axoneme; CC2, central core of the second axoneme; CM, cortical microtubules; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M, mitochondrion; N, nucleus; S1, singlets of the first axoneme; S2, singlets of the second axoneme. Scale bars = 200 nm

Fig. 4  Evidence of the glycogenic nature of the electron-dense granules by means of the test of Thiéry in the mature spermatozoon *Aphanurus stossichii* (Fig. 4a) and *Ectenurus lepidus* (Fig. 4b). G, granules of glycogen; M, mitochondrion; N, nucleus. Scale bars = 300 nm
from 5 to 10. However, they are ranged from 25 to 28 for Didymozoidae (Justine and Mattei 1982), Sclerodistomidae (Ndiaye et al. 2013b, 2017) and Sclerodistomoididae (Bâ et al. 2020). The location of the maximum number of these tubular structures along the sperm cell is also variable depending on the species. Quilichini et al. (2007) proposed that the spermatozoon of digeneans could be divided into two groups according to the location of the maximum number of cortical microtubules along the spermatozoon. The first group has the maximum number of these elements in the anterior part. However, for the second group, the maximum number occurs in the middle or in the more posterior part of the...
## Table 1  Spermatological characteristics in the superfamily Hemiuroidea

| Family, subfamily, and species | Spermatological characters | References |
|-------------------------------|-----------------------------|------------|
| **Didymozoidae**              |                             |            |
| Didymocystis wedli            | 9+’1’ 2Ax? 3? −? 1Ax-EO 1+ | Pamplona-Basilio et al. (2001) |
| Didymozoont sp.               | 9+0 2Ax? 3? −? 1Ax-EO 1+ | Justine and Mattei (1983) |
| Gonopodasmius sp.             | 9+1’ 2Ax-EO 1+ + | Justine and Mattei (1982) |
| **Hemiuridae**                |                             |            |
| Aphaniurinae                  |                             | Present study |
| Aphanurus stossichii          | 9+1’ 1Ax-EO 1 + (2 types) ± AntA 1 11 MedS 1 3? Ax II | Ndiaye et al. (2012) |
| Dinurinae                     |                             | Present study |
| Ectenurus lepidus             | 9+1’ 1Ax-EO 1 + | Dione et al. (2016) |
| Elytrophallinae               |                             | Ndiaye et al. (2013a) |
| Lecithocladium excisum        | 9+1’ 1Ax-EO 1 + | Ndiaye et al. (2014) |
| Hemiurinae                    |                             | Ndiaye et al. (2014) |
| Hemius appendiculatus         | 9+1’ 1Ax-EO 1 + (2 types) ± AntA 1 7 MedS 1 3? Ax II | Quilichini et al. (2010b) |
| Parahemiurinae                |                             | Ndiaye et al. (2013b) |
| Lectichochirinae              |                             | Ndiaye et al. (2017) |
| Lectichochirium microstomum   |                             | Bă et al. (2020) |
| Lectichochirium musculus      |                             |           |
| Lecithasteridae               |                             |            |
| Apomurus laguncula             | 9+1’ 1Ax-EO 1 + | Quilichini et al. (2010b) |
| Sclerodistomidae              |                             |            |
| Prosorchis palinrichthi       | 9+1’ 1Ax-CM 1 + | Ndiaye et al. (2013b) |
| Sclerodistomum italicum       | 9+1’ 1Ax-CM 1 + | Ndiaye et al. (2017) |
| Sclerodistomoididae           |                             | Bă et al. (2020) |
| Sclerodistomoides pacificus   | 9+1’ 2Ax-EO-CM 1 + | | |

**Note**: AntA, anterior part of the anterior region; AntS, anterior region of the spermatozoon; ASC, anterior spermatozoon character; Ax, axoneme; BCM, number of bundles of cortical microtubules; EO, external ornamentation of the plasma membrane; EO+CM, association “external ornamentation-cortical microtubules”; LEO, location of external ornamentation; LMCM, location of maximum number of cortical microtubules; M, number of mitochondria; MCM, maximum number of cortical microtubules; MedS, median region of the spermatozoon; NA, not applicable; PostS, posterior region of the spermatozoon; PSC, posterior spermatozoon character; TAntR, type of anterior region; TAx, type of axoneme; TPostR, type of posterior region; TS, type of spermatozoon; *, moniliform mitochondrion; ±, presence/absence of considered character; ?, doubtful or unknown data.
spermatozoon. In the male gamete of both studied hemiurids, the maximum number of cortical microtubules is located in the middle or posterior part of the spermatozoon. Thus, they belong to the second type, as mentioned in the remaining studied hemiurids except for the didymozoids (see Table 1). The presence of mitochondrion/a in digenean spermatozoa is considered as a plesiomorphic character, and, to date, there are descriptions of digenean sperm organization containing one, two, or three mitochondria (see Bakhoum et al. 2017 for a review). In the sperm cells of all the hemiurids described so far, the presence of one mitochondrion has been observed (see Table 1 for details and references). Mature spermatozoa of both A. stossichii and E. lepidus follow the hemiuroidean pattern with only one mitochondrion in their spermatozoa. It is worthy to note the presence of large granules of different sizes in the mitochondrial matrix of A. stossichii. This is the second time that this character has been reported in the sperm mitochondria of a parasitic flatworm. Recently, Kacem et al. (2019) have observed mitochondrial matrix granules in the male gamete of Haplosporanchus caudatus.

The posterior spermatozoon extremity presents a great variability in the digenean male gametes as reported with the anterior spermatozoon tip. Three types of posterior spermatozoon extremities in digenean spermatozoa were proposed by Quilichini et al. (2010a) considering the sequence of characters’ disappearance toward the posterior tip. These are type 1 (also called opecoelid type) characterize by the sequence “axoneme, nucleus, and cortical microtubules”; type 2 (also called fasciolid type) with the sequence “cortical microtubules, axoneme, and nucleus”; and type 3 (also called cryptogonimid type) with the sequence “cortical microtubules, nucleus, and axoneme.” The two studied hemiurids present a variability. In A. stossichii sperm, the sequence is “mitochondria, cortical microtubules, nucleus, and first and second axonemes.” As far as E. lepidus is concerned, the sequence is “mitochondria, cortical microtubules, first axoneme, nucleus, and second axoneme.” Other sequences of characters’ disappearance toward the posterior tip have been observed in the Hemiuriidae: “first axoneme, mitochondria, cortical microtubules, nucleus, and second axoneme” in L. microstomum, L. musculus, and H. appendiculatus (Ndiaye et al. 2014; Dione et al. 2016); and “mitochondria, first axoneme, cortical microtubules, nucleus, and second axoneme” in L. excisum and P. merus (Ndiaye et al. 2012, 2013a). Thus, the posterior spermatozoon extremity of digeneans exhibits numerous variations leading to unfitness with the three previous models. This is the principal reason argued by Bakhoum et al. (2017) to scrutinize the last spermatozoon character instead of the sequence of characters. The present study proves that A. stossichii and E. lepidus show the axoneme as the terminal character of the male gamete as in all previously studied hemiurids.

The available data on the sperm ultrastructure show a great similarity between the hemiurids studied to date, with the exception of didymozoids. The type II spermatozoon characterizes hemiurids, lecithasterids, sclerodistomids, and sclerodistomoidids (see Table 1). The monophyly of the Hemiuriidae demonstrated by some authors (Gibson and Bray 1979; Olson et al. 2003; Sokolov et al. 2018; Pérez-Ponce de León and Hernández-Mena 2019) could be supported by the spermatological ultrastructural database. Moreover, two features such as the reduced number of cortical microtubules and their arrangement into a single bundle could also confirm results of the above-mentioned molecular analysis indicating the close relationships between Hemiuriidae and Lecithasteridae. Unfortunately, there is no molecular data for representatives of the Sclerodistomoididae, the third family of hemiuroids with species presenting only a ventral bundle of cortical microtubules in their spermatozoa.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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