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

3

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21 **Abstract**

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

44 **Keywords:** *Aphanurus stossichii*; *Ectenurus lepidus*; Hemiuridae; Digenea;
45 ultrastructure; sperm characters.

46

47 **Introduction**

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

54 The molecular phylogenetic analysis of the family Hemiuridae carried out by
55 Atopkin et al. (2017) indicates that the presence or absence of an ecsoma was not
56 associated with molecular data for hemiurid subfamilies differentiation. Moreover, on the
57 basis of the molecular differentiation found in this study, authors considered that the
58 taxonomic status of Aphanurinae, Dinurinae, Elytrophallinae, and Hemiurinae should be
59 reconsidered with the inclusion of these digeneans in the same subfamily Hemiurinae.

60 Due to several inconsistencies and the shortage of robustness in the existing
61 classification of the Hemiuroidea, numerous changes were made related to the description
62 of new genera and subfamilies (Bray and Nahhas 2002; Pankov et al. 2006; Bursey et al.
63 2008; Bilqees et al. 2009, 2010; Justo and Kohn 2012; Urabe and Shimazu 2013) as well
64 as a change in the taxonomic rank of previously described subfamilies (Sokolov et al.
65 2018).

66 Ultrastructural studies of species belonging to the Hemiuridae, as in other
67 digeneans, are of great importance for a better knowledge of relationships within this
68 family of digeneans since they bring additional information that could complement
69 molecular analyses and morphological data (Justine 1991, 1995, 2001; Quilichini et al.
70 2010a, 2011; Bakhoum et al. 2017). To date, there are ultrastructural studies on the

71 spermatozoon of five species within this family, namely, *Lecithocladium excisum*
72 (Rudolphi, 1819) (Elytrophallinae), *Hemiurus appendiculatus* (Rudolphi, 1802) and
73 *Parahemiurus merus* (Linton, 1910) (Hemiurinae), and *Lecithochirium microstomum*
74 Chandler, 1935 and *Lecithochirium musculus* (Looss, 1907) (Lecithochiriinae). (Ndiaye
75 et al. 2012, 2013a, 2014; Dione et al. 2016). The present study describes for the first time
76 the mature spermatozoa of *Aphanurus stossichii* (Monticelli, 1891) and *Ectenurus lepidus*
77 Looss, 1907 belonging to the subfamilies Aphanurinae and Dinurinae, respectively. Our
78 results provide useful criteria for the elucidation of phylogenetic relationships within the
79 family Hemiuridae.

80

81 **Materials and methods**

82 Adult specimens of *A. stossichii* (Monticelli, 1891) and *Ectenurus E. lepidus* Looss, 1907
83 were gathered live from the digestive tract of the bogue *Boops boops* (Linnaeus, 1758)
84 (Pisces, Teleostei, Sparidae) and the atlantic horse mackerel *Trachurus trachurus*
85 (Linnaeus, 1758) (Pisces, Teleostei, Carangidae), respectively. Hosts were caught in
86 ~~December–March~~ 2019 in the Mediterranean Sea, off La Chebba (34°14'N, 11°06'E)
87 (Tunisia).

88 After their extraction, live worms were immediately rinsed with a 0.9% NaCl
89 solution and fixed in cold (4 °C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer
90 at pH 7.4 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4.
91 They were then postfixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium
92 ferricyanide in the same buffer for 1 h, rinsed in MilliQ water (Millipore Gradient A10),
93 dehydrated in an ethanol series and propylene oxide, embedded in Spurr resin, and finally
94 polymerized at 60 °C for 72 h. Ultrathin sections were obtained using a Reichert-Jung
95 Ultracut-E ultramicrotome, placed on copper grids, and double-stained with uranyl

96 acetate and lead citrate according to Reynolds (1963). Finally, all stained grids were
97 studied with a JEOL 1010 transmission electron microscope operated at 80 kV in the
98 ‘Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)’.

99 The Thiéry (1967) technique was used for cytochemical detection of glycogen.
100 Gold grids were treated 30 min in 10% periodic acid and rinsed in MilliQ water; 24 h in
101 thiocarbohydrazide and rinsed in acetic solutions and MilliQ water; then 30 min in 1%
102 silver proteinate in the dark, and rinsed in MilliQ water.

103

104 **Results**

105 The interpretation of numerous ultrathin sections of the mature spermatozoa of *A.*
106 *stossichii* and *E. lepidus* allow us to distinguish three different regions (I–III) from the
107 anterior to the posterior spermatozoon extremities (see Figs. 1 to 54).

108 Region I (Figs. 1a-i, 32a-d and 54I) corresponds to the anterior part or
109 premitochondrial area of the spermatozoon. Cross-sections through the anterior tip of the
110 spermatozoon of both studied hemiurid species show relatively small differences.

111 The anterior tip of the *A. stossichii* spermatozoon is entirely covered by a
112 filamentous ornamentation associated with short cortical microtubules and also exhibits
113 a few doublets of the first axoneme (Figs. 1a and 54I). At a slightly distal level, the sperm
114 cell is partly covered with typical external ornamentation (Fig. 1b). More posteriorly,
115 cross-sections show the gradual appearance of doublets of the second axoneme and later
116 the complete formation of both axonemes (Fig. 1c-f). In this area a single cortical
117 microtubule appears between both axonemes and the external ornamentation partially
118 surrounds just the second axoneme (Figs. 1d-f and 54I). Finally, the posterior part of
119 region I lacks external ornamentation. It is also characterized by the increase of the

120 number of cortical microtubules reaching seven elements and the appearance of a small
121 amount of glycogen granules (Fig. 1g-i).

122 The *E. lepidus* male gametes anterior tip exhibit ~~classical~~-typical external
123 ornamentation of the plasma membrane surrounding centriole of the first axoneme (Fig.
124 32a and 54I). At a slightly distal level, the first axoneme appears accompanied by the
125 anterior extremity of the second axoneme. Then the sperm cell is partly covered by the
126 external ornamentation of the plasma membrane (Fig. 32b). As previously reported in *A.*
127 *stossichii*, when the second axoneme appears, there is a single cortical microtubule (Fig.
128 32c) and the external ornamentation is partially surrounding both axonemes (Figs. 32c
129 and 54I). The posterior part of region I is characterized by the disappearance of external
130 ornamentation of the plasma membrane and the presence of up to five cortical
131 microtubules disposed only on one side of the spermatozoon as well as some glycogen
132 granules (Figs. 32d and 54I).

133 Region II (Figs. 1j, k, 2, 32e and 54II) is the middle region or mitochondrial area
134 of the spermatozoon. In both hemiurids the sperm region II is characterized by the same
135 features: the appearance of a mitochondrion and a higher number of cortical microtubules
136 (eleven in *A. stossichii* and six in *E. lepidus*) (Figs. 1j, k, 32e and 54II). For *A. stossichii*,
137 this is the maximum number of cortical microtubules. However, an interesting difference
138 is observed in *A. stossichii* spermatozoon: the mitochondrion contains dense granules
139 deposited in the matrix with granule diameter of about 45 nm (Figs. 1j, 2 and 54II).

140 Region III (Figs. 1l-u, 2, 32f-o and 54III) corresponds to the nuclear and posterior
141 spermatozoon extremity. It begins with the simultaneous presence of posterior
142 mitochondrion and the nucleus (Figs. 1l, 2, 32f, g and 54III). In the middle part, the
143 mitochondrion disappears. The sperm cell contains two axonemes, a nucleus, granules of
144 glycogen, and cortical microtubules whose number progressively decreases and

145 disappears (Figs. 1n-p, [32h-k](#) and [54III](#)). However, in the spermatozoon of *E. lepidus* the
146 maximum number of cortical microtubules (8 elements) is observed in the nuclear region
147 (Fig. [32h](#)). The transition of characters toward the posterior tip of the spermatozoon of
148 both studied hemiurid species shows relatively slight differences. In *A. stossichii* the
149 transition of characters is as follows: (i) disappearance of the nucleus, (ii) disorganization
150 of the first axoneme, (iii) and later disorganization of the second axoneme (Fig. 1q-u).
151 Nevertheless, singlets of the first axoneme remain present until the posterior
152 spermatozoon tip (Fig. 1u). In *E. lepidus* spermatozoon the transition is as follows: (i)
153 disorganization of the first axoneme, (ii) disappearance of the nucleus, and (iii)
154 disorganization of the second axoneme (Fig. [32l-o](#)).

155 The glycogenic nature of the electron-dense granules observed along the sperm cell
156 of both studied hemiurids was evidenced by applying Thiéry's test (Fig. [43a, b](#)).

158 Discussion

159 The mature spermatozoa of *A. stossichii* and *E. lepidus* share the general ultrastructural
160 characteristics found in most digeneans described so far: two axonemes with the 9+1'
161 trepaxonematan pattern (Ehlers 1984), a nucleus, a mitochondrion, glycogen granules,
162 ~~external ornamentation of the plasma membrane~~, and parallel cortical microtubules. Thus,
163 they exhibit the characteristics of the type II digeneans sperm cells (Bakhoun et al. 2017),
164 namely, two 9+1' axonemes, external ornamentation not associated with cortical
165 microtubules and located in the anterior part of the sperm cell, a single bundle of cortical
166 microtubules, the maximum number of cortical microtubules located in a middle part of
167 the spermatozoon and the presence of one mitochondrion. The type II of spermatozoon
168 has been described in the faustulid *Pronoprymna ventricosa* and in all the hemiuroideans
169 except species belonging to the Didimozoidae (Justine and Mattei 1982, 1983; Pamplona-

170 [Basilio et al. 2001; Quilichini et al. 2007](#)):- However, there are several specific features
171 mainly located in both anterior and posterior extremities of the spermatozoon. Now, the
172 great variability of the morphology and organization of these extremities in digenean
173 spermatozoa might be a suitable criterion for taxonomy and/or phylogenetic
174 considerations ([Quilichini et al. 2010a, 2011; Bakhoum et al. 2017](#)):-

175 The anterior extremity of the male gamete of both hemiurids is filiform. It is
176 covered by an external ornamentation of the plasma membrane. The presence or absence
177 of this last structure as well as its type, location and association or not with cortical
178 microtubules are considered as interesting ultrastructural criteria used for phylogenetic
179 inference (see [Quilichini et al. 2007, 2011; Bakhoum et al. 2017](#)). The mature
180 spermatozoon of *A. stossichii* displays two types of external ornamentation. The first one
181 is filamentous and appears in the anterior extremity as a discontinuous layer. Each
182 filament of the ornamentation is associated with a cortical microtubule. As for the second
183 one, it is also discontinuous, but not associated with cortical microtubules. It is placed
184 more posteriorly in the anterior part of the spermatozoon. Both types of external
185 ornamentation have been reported in other digeneans belonging to several families of the
186 superfamily Hemiuroidea such as the Didymozoidae *Gonapodasmius* sp. ([Justine and
187 Mattei 1982, 1984](#)), the Hemiuridae *Lecithochirium microstomum*, *Lecithochirium
188 musculus* and *Hemiurus appendiculatus* ([Ndiaye et al. 2014; Dione et al. 2016](#)), and the
189 Sclerodistomidae *Prosorchis palinurichthi* and *Sclerodistomum italicum* ([Ndiaye et al.
190 2013b; Ndiaye et al., 2016](#)). However, the mature spermatozoa of *E. lepidus* like other
191 species belonging of to the Hemiuroidea and most digeneans are characterized by the
192 presence of only one type of external ornamentation. These are the Hemiuridae family,
193 namely: *Lecithocladium excisum* and *Parahemiurus merus* ([Ndiaye et al. 2012; Ndiaye
194 et al., 2013a](#)), the *Lecithasteridae Aponurus laguncula* ([Quilichini et al. 2010b](#)), and the

195 Sclerodistomoididae *Sclerodistomoides pacificus* (Bâ et al. 2020) (see Table 1).
196 ~~is~~
196 ~~characterized by the presence of only one type of external ornamentation that is usually~~
197 ~~described in most digeneans.~~

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

204 Another character that could depict the male gamete is the cortical microtubules.
205 These structures are present in the sperm cells of most digeneans studied so far (see
206 Bakhoum et al. 2017). Three aspects of these tubular structures are considered as
207 interesting ultrastructural criteria used for phylogenetic inference: their presence or
208 absence, the location of their maximum number, and the number of bundles. The cortical
209 microtubules of the mature spermatozoon of *A. stossichii* and *E. lepidus* are arranged only
210 in a ventral bundle. Among digeneans this characteristic is only known in the
211 Hemiuroidea. A low number of these cortical microtubules is present in the mature
212 spermatozoon of *A. stossichii* and *E. lepidus*: 11 and 8, respectively. The reported number
213 of cortical microtubules in the hemiuroids is usually low, varying from 5 to 10. However,
214 they are ranged from 25 to 28 for Didymozoidae (Justine and Mattei 1982) and
215 Sclerodistomidae (Ndiaye et al. 2013b; ~~Ndiaye et al., 2017~~) and Sclerodistomoididae
216 (Bâ et al. 2020). The location of the maximum number of these tubular structures along
217 the sperm cell is also variable depending on the species. Quilichini et al. (2007) proposed
218 that the spermatozoon of digeneans could be divided into two groups according to the
219 location of the maximum number of cortical microtubules along the spermatozoon. The

220 first group has the maximum number of these elements in the anterior part. However, for
221 the second group, the maximum number occurs in the middle or in the more posterior
222 part of the spermatozoon. In the male gamete of both studied hemiurids, the maximum
223 number of cortical microtubules is located in the middle or posterior part of the
224 spermatozoon. Thus, they belong to the second type, as mentioned in the remaining
225 studied hemiuroids except for the didymozoids (see Table 1).

226 The presence of mitochondrion/a in digenean spermatozoa is considered as a
227 plesiomorphic character and, to date, there are descriptions of digenean sperm
228 organization containing one, two, or three mitochondria (see Bakhoun et al. 2017 for a
229 review). In the sperm cells of all the hemiuroids described so far, the presence of one
230 mitochondrion has been observed (see Table 1 for details and references). Both mature
231 spermatozoa of *A. stossichii* and *E. lepidus* follow the hemiuroidean pattern with only
232 one mitochondrion in their spermatozoa. It is worthy to note the presence of large
233 granules of different sizes in the mitochondrial matrix of *A. stossichii*. This is the second
234 time that this character has been reported in the sperm mitochondria of a parasitic
235 flatworm. Recently, Kacem et al. (2019) have observed mitochondrial matrix granules in
236 the male gamete of *Haplospalchnus caudatus*.

237 The posterior spermatozoon extremity presents a great variability in the digenean
238 male gametes as reported with the anterior spermatozoon tip. Three types of posterior
239 spermatozoon extremities in digenean spermatozoa were proposed by Quilichini et al.
240 (2010a) considering the sequence of characters' disappearance towards the posterior tip.
241 These are: type 1 (also called opecoelid type) characterized by the sequence 'axoneme,
242 nucleus, and cortical microtubules'; type 2 (also called fasciolid type) with the sequence
243 'cortical microtubules, axoneme, and nucleus'; and type 3 (also called cryptogonimid
244 type) with the sequence 'cortical microtubules, nucleus, and axoneme'. The two studied

245 hemiurids present a variability. In *A. stossichii* sperm the sequence is: ‘mitochondria,
246 cortical microtubules, nucleus, first and second axonemes’. As far as *E. lepidus* is
247 concerned, the sequence is: ‘mitochondria, cortical microtubules, first axoneme, nucleus
248 and second axoneme’. Other sequences of characters’ disappearance towards the
249 posterior tip have been observed in the Hemiuridae: ‘first axoneme, mitochondria,
250 cortical microtubules, nucleus and second axoneme’ in *L. microstomum*, *L. musculus* and
251 *H. appendiculatus* (Ndiaye et al. 2014; Dione et al. 2016); and ‘mitochondria, first
252 axoneme, cortical microtubules, nucleus, and second axoneme’ in *L. excisum* and *P.*
253 *merus* (Ndiaye et al. 2012, 2013a). Thus, the posterior spermatozoon extremity of
254 digeneans exhibits numerous variations leading to unfitness with the three previous
255 models. This is the principal reason argued by Bakhoum et al. (2017) to scrutinize the last
256 spermatozoon character instead of the sequence of characters. The present study proves
257 that *A. stossichii* and *E. lepidus* show the axoneme as the terminal character of the male
258 gamete as in all previously studied hemiurids.

259
260 The available data on the sperm ultrastructure show a great similarity between the
261 hemiurids studied to date, with the exception of didymozoids. The type II spermatozoon
262 characterizes hemiurids, lecithasterids, sclerodistomids and sclerodistomoidids (see
263 Table 1). The monophyly of the Hemiuridae demonstrated by some authors (Gibson and
264 Bray 1979; Olson et al. 2003; Sokolov et al. 2018; Pérez-Ponce de León and Hernández-
265 Mena 2019) could be supported by the spermatological ultrastructural database.
266 Moreover, two features such as the reduced number of cortical microtubules and their
267 arrangement into a single bundle could also confirm results of the above-mentioned
268 molecular analysis indicating the close relationships between Hemiuridae and
269 Lecithasteridae. Unfortunately, there is no molecular data for representatives of the

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270 [Sclerodistomoididae, the third family of hemiuroids with species presenting only a](#)
271 [ventral bundle of cortical microtubules in their spermatozoa.](#)

272

273 **Compliance with ethical standards**

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

275

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280

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411

412 **Figure captions**

413 **Fig. 1.** Mature spermatozoon of *Aphanurus stossichii*. **a-i** Correlative cross-sections of
414 the anterior spermatozoon extremity (region I). Note the anterior tip with a filamentous
415 ornamentation (fEO) (Fig. 1a). **j-l** Cross-sections of the middle or mitochondrial region

416 (region II). Note the presence of mitochondrial matrix granules (white arrow) (Fig. 1j).
417 **m-u** Consecutive cross-sections of the posterior part or nuclear region (region III). Ax1
418 first axoneme, C2 centriole of the second axoneme, CM cortical microtubules, D1
419 doublets of the first axoneme, D2 doublets of the second axoneme, EO external
420 ornamentation of the plasma membrane, G granules of glycogen, M mitochondrion, N
421 nucleus, S1 singlets of the first axoneme, S2 singlets of the second axoneme. Scale bars
422 = 200 nm.

423 Fig. 2. Mature spermatozoon of *Aphanurus stossichii*. Detail of mitochondrial matrix
424 granules (white arrow) in regions II and III. CM cortical microtubules, G granules of
425 glycogen, M mitochondrion, N nucleus. Scale bar = 200 nm.

426 **Fig. 32.** Mature spermatozoon of *Ectenurus lepidus*. **a-d** Consecutive cross-sections of
427 the anterior spermatozoon extremity (region I). Note the presence of classical external
428 ornamentation in the anterior tip (Fig. 2a) in contrast with *A. stossichii*. **e** Cross-section
429 of the middle or mitochondrial region (region II). **f-o** Correlative cross-sections of the
430 posterior extremity or nuclear region (region III). Ax1 first axoneme, C1 centriole of the
431 first axoneme, CC2 central core of the second axoneme, CM cortical microtubules, EO
432 external ornamentation of the plasma membrane, G granules of glycogen, M
433 mitochondrion, N nucleus, S1 singlets of the first axoneme, S2 singlets of the second
434 axoneme. Scale bars = 200 nm.

435 **Fig. 43.** Evidence of the glycogenic nature of the electron-dense granules by means of
436 the test of Thiéry in the mature spermatozoon *Aphanurus stossichii* (Fig. 3a) and
437 *Ectenurus lepidus* (Fig. 3b). G granules of glycogen, M mitochondrion, N nucleus. Scale
438 bars = 300 nm.

439 **Fig. 54.** Schematic reconstruction of the mature spermatozoon of both studied hemiurids,
440 on the right *Aphanurus stossichii*, on the left *Ectenurus lepidus*. The sperm cell is

441 organized in three different regions: region I or anterior part, region II or middle part and
442 region III or posterior part. In order to make the diagram clearer, granules of glycogen
443 and mitochondrial matrix granules are not shown in longitudinal sections. ASE anterior
444 spermatozoon extremity, Ax1 and Ax2 first and second axoneme, C1 and C2 centriole of
445 the first and the second axoneme, CC2 central core of the second axoneme, CM cortical
446 microtubules, D2 doublets of the second axoneme, EO external ornamentation of the
447 plasma membrane, fEO filamentous ornamentation, G granules of glycogen, M
448 mitochondrion, MMG mitochondrial matrix granules, N nucleus, PM plasma membrane,
449 PSE posterior spermatozoon extremity, S1 and S2 singlets of the first and second
450 axoneme.

Table I. Spermatological characteristics in the superfamily Hemiuroidea

Family, Subfamily and Species	Spermatological characters													References
	Tax	ASC	TAntR	EO	EO+CM	LEO	BCM	MCM	LMCM	M	TPostR	PSC	TS	
DIDYMOZOIDAE														
<i>Didymocystis wedli</i>	9+1'	?	3?	-?	NA	NA	0	0	NA	1?	?	?	?	Pamplona-Basilio et al. (2001)
<i>Didymozoon</i> sp.	9+0	2Ax?	3?	-?	NA	NA	0	0	NA	1?	?	?	?	Justine and Mattei (1983)
<i>Gonapodasmius</i> sp.	9+1'	2Ax-EO	1	+	+	AntA	2	39	AntS	1?	3?	Ax	IV?	Justine and Mattei (1982)
HEMIURIDAE														
Aphanurinae														
<i>Aphanurus stossichii</i>	9+1'	1Ax-EO	1	+ (2 types)	+/-	AntA	1	11	MedS	1	3?	Ax	II	Present study
Dinurinae														
<i>Ectenurus lepidus</i>	9+1'	1Ax-EO	1	+	-	AntA	1	8	PostS	1	3?	Ax	II	Present study
Elytrophallinae														
<i>Lecithocladium excisum</i>	9+1'	1Ax-EO	1	+	-	AntA	1	8	MedS	1	3	Ax	II	Ndiaye et al. (2012)
Hemiurinae														
<i>Hemiurus appendiculatus</i>	9+1'	1Ax-EO	1	+ (2 types)	+/-	AntA	1	7	MedS	1	3	Ax	II	Dione et al. (2016)
<i>Parahemiurus merus</i>	9+1'	1Ax-EO	1	+	-	AntA	1	5	MedS	1	3?	Ax	II	Ndiaye et al. (2013a)
Lecithochiriinae														
<i>Lecithochirium microstomum</i>	9+1'	1Ax-EO	1	+ (2 types)	+/-	AntA	1	8	MedS	1	3	Ax	II	Ndiaye et al. (2014)
<i>Lecithochirium musculus</i>	9+1'	1Ax-EO	1	+ (2 types)?	+/-?	AntA	1	6	MedS	1	3	Ax	II	Ndiaye et al. (2014)
LECITHASTERIDAE														
<i>Aponurus laguncula</i>	9+1'	1Ax-EO	1	+	-	AntA	1	10	MedS	1	3?	Ax	II	Quilichini et al. (2010b)
SCLERODISTOMIDAE														
<i>Prosorhis palinurichthi</i>	9+1'	1Ax-CM	1	+ (2 types)	+/-	AntA	2	30	MedS	1	3	Ax	II	Ndiaye et al. (2013b)
<i>Sclerodistomum italicum</i>	9+1'	1Ax-CM	1	+ (2 types)	+/-	AntA	2	28	MedS	1	3?	Ax	II	Ndiaye et al. (2017)
<u>SCLERODISTOMOIDIDAE</u>														
<i>Sclerodistomoides pacificus</i>	9+1'	2Ax-EO-CM	1	+	-	AntA	1	24	PostS	1*	3?	Ax	II	Bâ et al. (2020)

AntA, anterior part of the anterior region; AntS, anterior part 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.

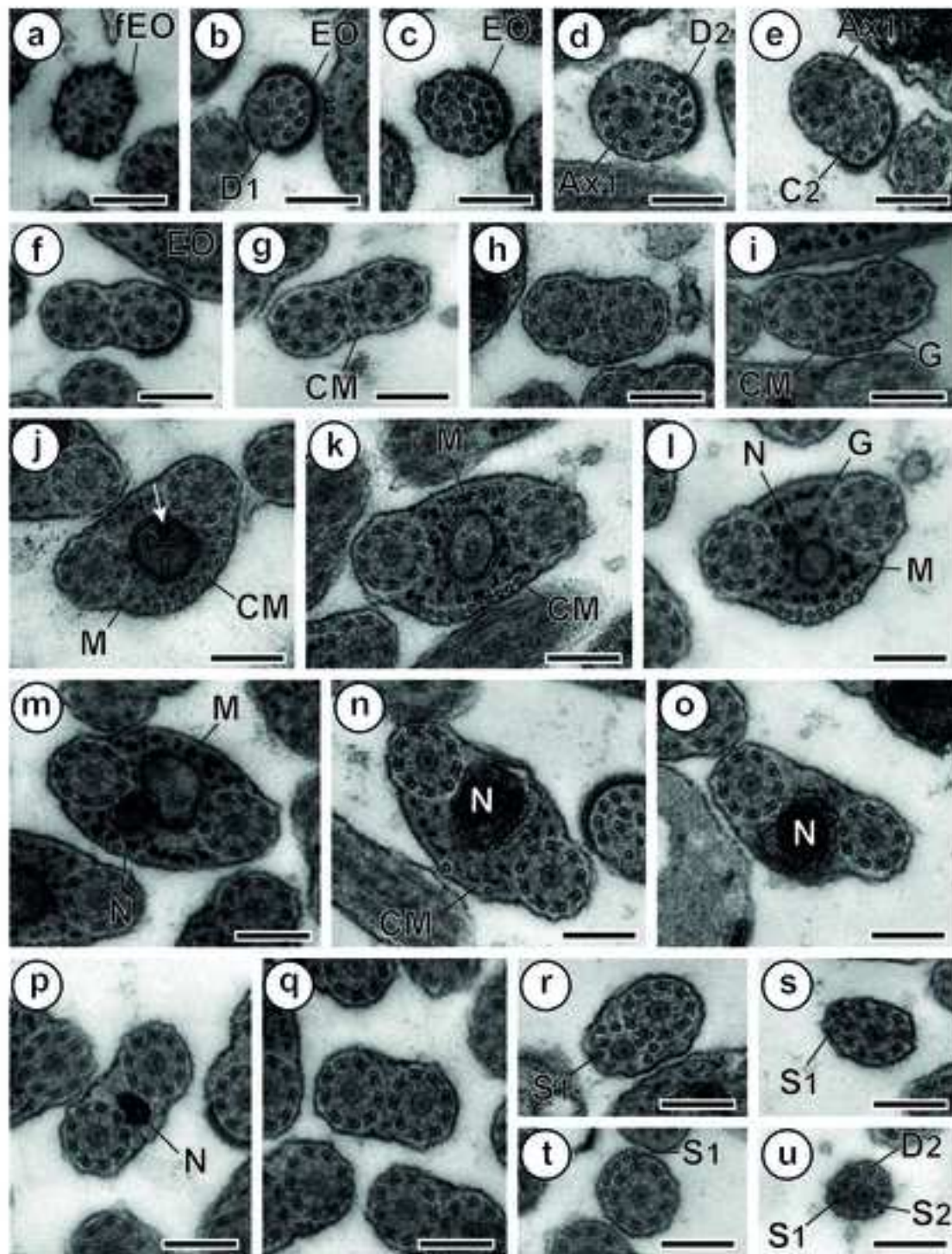
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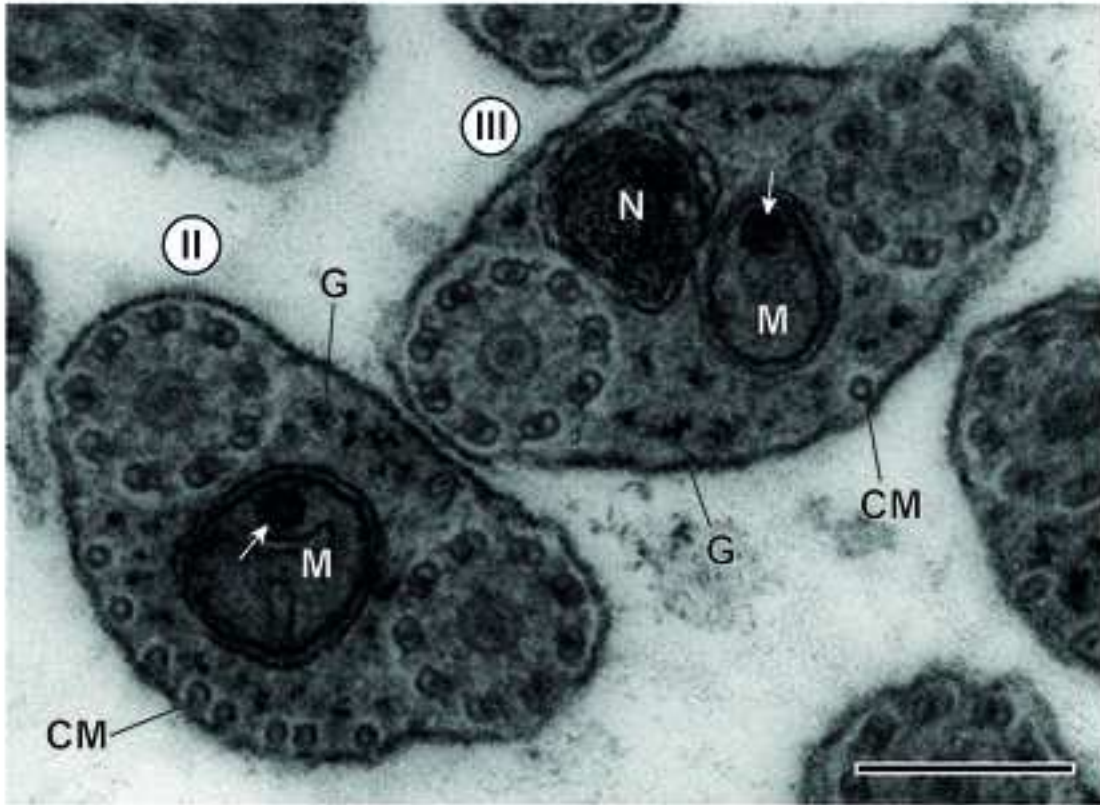
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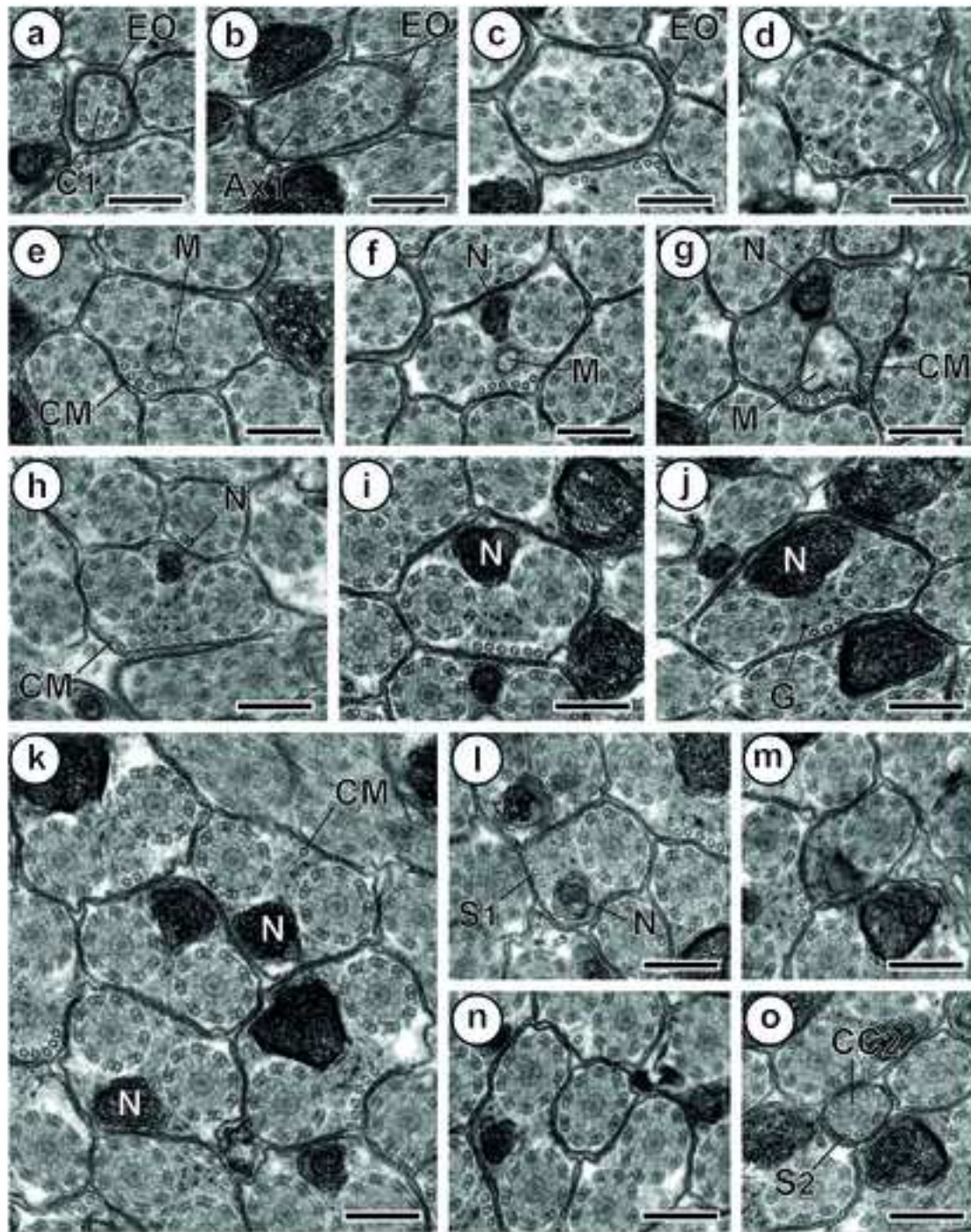
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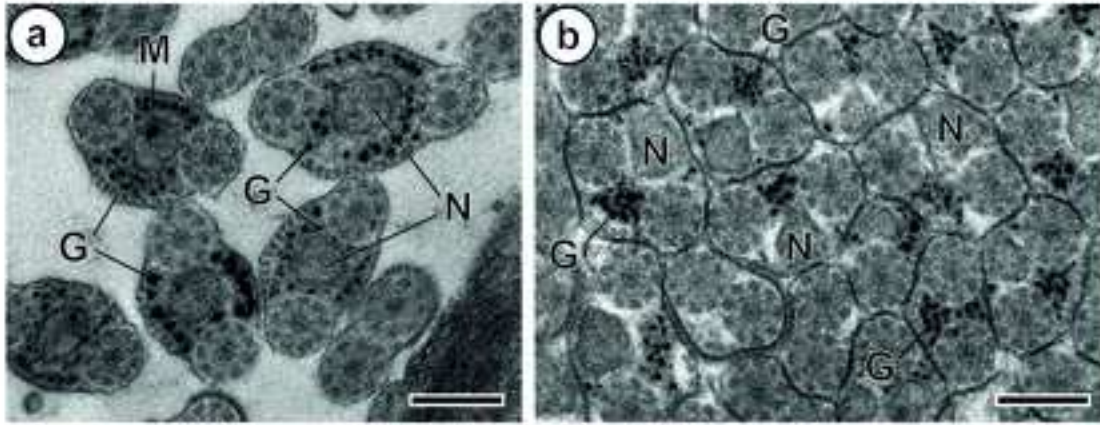
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<i>Didymozoon</i> sp.	9+0	2Ax?	3?	-?	NA	NA	0	0	NA	1?	?	?	?	Justine and Mattei (1983)
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Elytrophallinae														
<i>Lecithocladium excisum</i>	9+1'	1Ax-EO	1	+	-	AntA	1	8	MedS	1	3	Ax	II	Ndiaye et al. (2012)
Hemiurinae														
<i>Hemiurus appendiculatus</i>	9+1'	1Ax-EO	1	+ (2 types)	+/-	AntA	1	7	MedS	1	3	Ax	II	Dione et al. (2016)
<i>Parahemiurus merus</i>	9+1'	1Ax-EO	1	+	-	AntA	1	5	MedS	1	3?	Ax	II	Ndiaye et al. (2013a)
Lecithochiriinae														
<i>Lecithochirium microstomum</i>	9+1'	1Ax-EO	1	+ (2 types)	+/-	AntA	1	8	MedS	1	3	Ax	II	Ndiaye et al. (2014)
<i>Lecithochirium musculus</i>	9+1'	1Ax-EO	1	+ (2 types)?	+/-?	AntA	1	6	MedS	1	3	Ax	II	Ndiaye et al. (2014)
LECITHASTERIDAE														
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<i>Sclerodistomum italicum</i>	9+1'	1Ax-CM	1	+ (2 types)	+/-	AntA	2	28	MedS	1	3?	Ax	II	Ndiaye et al. (2017)
SCLERODISTOMOIDIDAE														
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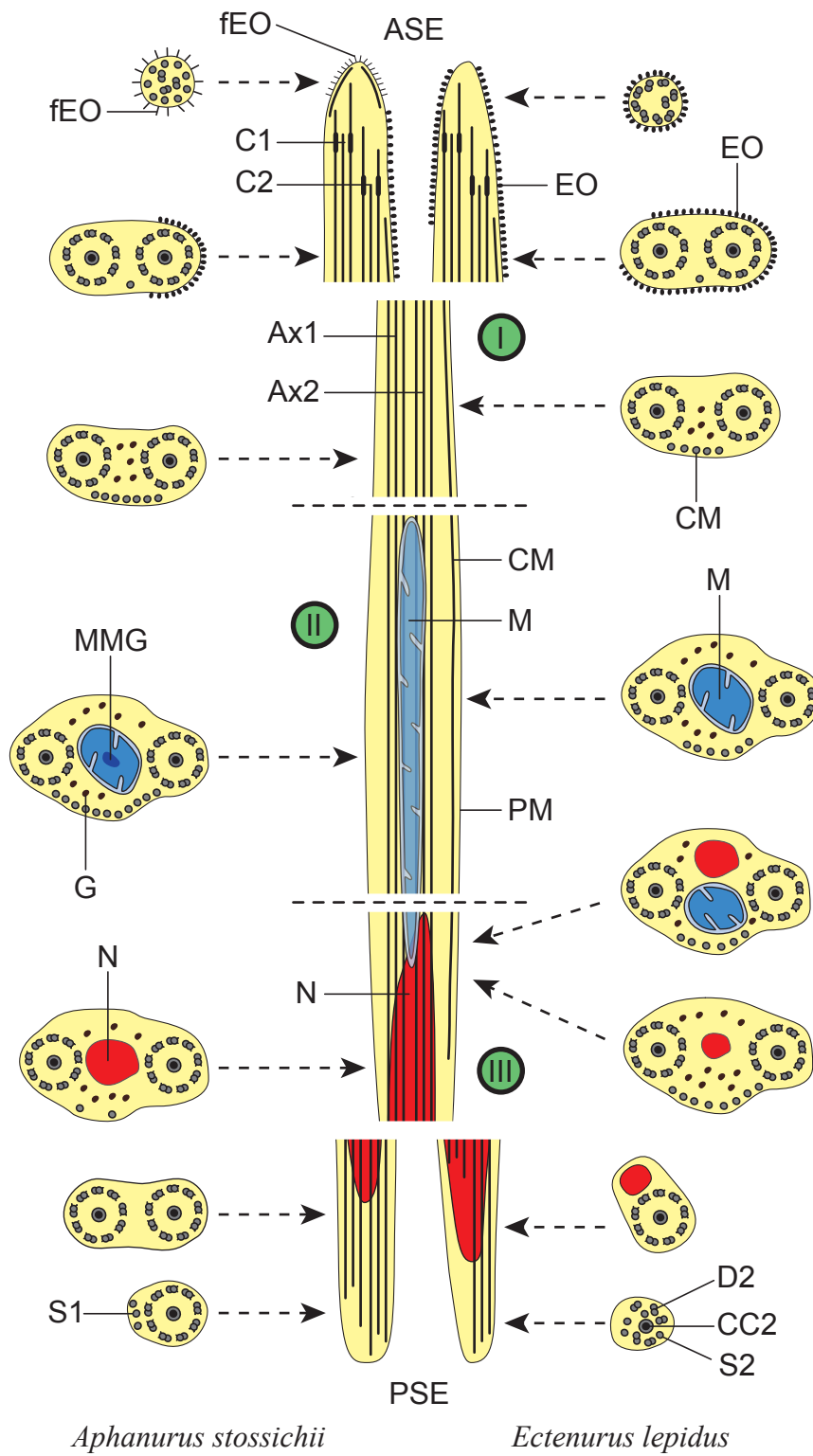
AntA, anterior part of the anterior region; AntS, anterior part of the spermatozoon; ASC, anterior spermatozoon character Ax, axoneme; BCM, number of bundles of cortical microtubules; EO, external ornamentation of the plasma membrane; EO+CM, association 'external ornamentation-cortical microtubules'; 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.











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

3

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21 **Abstract**

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

43 **Keywords:** *Aphanurus stossichii*; *Ectenurus lepidus*; Hemiuridae; Digenea;
44 ultrastructure; sperm characters.

45

46 **Introduction**

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

53 The molecular phylogenetic analysis of the family Hemiuridae carried out by
54 Atopkin et al. (2017) indicates that the presence or absence of an ecsoma was not
55 associated with molecular data for hemiurid subfamilies differentiation. Moreover, on the
56 basis of the molecular differentiation found in this study, authors considered that the
57 taxonomic status of Aphanurinae, Dinurinae, Elytrophallinae, and Hemiurinae should be
58 reconsidered with the inclusion of these digeneans in the same subfamily Hemiurinae.

59 Due to several inconsistencies and the shortage of robustness in the existing
60 classification of the Hemiuroidea, numerous changes were made related to the description
61 of new genera and subfamilies (Bray and Nahhas 2002; Pankov et al. 2006; Bursey et al.
62 2008; Bilqees et al. 2009, 2010; Justo and Kohn 2012; Urabe and Shimazu 2013) as well
63 as a change in the taxonomic rank of previously described subfamilies (Sokolov et al.
64 2018).

65 Ultrastructural studies of species belonging to the Hemiuridae, as in other
66 digeneans, are of great importance for a better knowledge of relationships within this
67 family of digeneans since they bring additional information that could complement
68 molecular analyses and morphological data (Justine 1991, 1995, 2001; Quilichini et al.
69 2010a, 2011; Bakhoun et al. 2017). To date, there are ultrastructural studies on the
70 spermatozoon of five species within this family, namely, *Lecithocladium excisum*

71 (Rudolphi, 1819) (Elytrophallinae), *Hemiurus appendiculatus* (Rudolphi, 1802) and
72 *Parahemiurus merus* (Linton, 1910) (Hemiurinae), and *Lecithochirium microstomum*
73 Chandler, 1935 and *Lecithochirium musculus* (Looss, 1907) (Lecithochiriinae) (Ndiaye
74 et al. 2012, 2013a, 2014; Dione et al. 2016). The present study describes for the first time
75 the mature spermatozoa of *Aphanurus stossichii* (Monticelli, 1891) and *Ectenurus lepidus*
76 Looss, 1907 belonging to the subfamilies Aphanurinae and Dinurinae, respectively. Our
77 results provide useful criteria for the elucidation of phylogenetic relationships within the
78 family Hemiuridae.

79

80 **Materials and methods**

81 Adult specimens of *A. stossichii* and *E. lepidus* were gathered live from the digestive tract
82 of the bogue *Boops boops* (Linnaeus, 1758) (Pisces, Teleostei, Sparidae) and the atlantic
83 horse mackerel *Trachurus trachurus* (Linnaeus, 1758) (Pisces, Teleostei, Carangidae),
84 respectively. Hosts were caught in March 2019 in the Mediterranean Sea, off La Chebba
85 (34°14'N, 11°06'E) (Tunisia).

86 After their extraction, live worms were immediately rinsed with a 0.9% NaCl
87 solution and fixed in cold (4 °C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer
88 at pH 7.4 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4.
89 They were then postfixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium
90 ferricyanide in the same buffer for 1 h, rinsed in MilliQ water (Millipore Gradient A10),
91 dehydrated in an ethanol series and propylene oxide, embedded in Spurr resin, and finally
92 polymerized at 60 °C for 72 h. Ultrathin sections were obtained using a Reichert-Jung
93 Ultracut-E ultramicrotome, placed on copper grids, and double-stained with uranyl
94 acetate and lead citrate according to Reynolds (1963). Finally, all stained grids were

95 studied with a JEOL 1010 transmission electron microscope operated at 80 kV in the
96 ‘Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)’.

97 The Thiéry (1967) technique was used for cytochemical detection of glycogen.
98 Gold grids were treated 30 min in 10% periodic acid and rinsed in MilliQ water; 24 h in
99 thiocarbohydrazide and rinsed in acetic solutions and MilliQ water; then 30 min in 1%
100 silver proteinate in the dark, and rinsed in MilliQ water.

101

102 **Results**

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

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

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

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

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

137 Region III (Figs. 1l-u, 2, 3f-o and 5III) corresponds to the nuclear and posterior
138 spermatozoon extremity. It begins with the simultaneous presence of posterior
139 mitochondrion and the nucleus (Figs. 1l, 2, 3f, g and 5III). In the middle part, the
140 mitochondrion disappears. The sperm cell contains two axonemes, a nucleus, granules of
141 glycogen, and cortical microtubules whose number progressively decreases and
142 disappears (Figs. 1n-p, 3h-k and 5III). However, in the spermatozoon of *E. lepidus* the
143 maximum number of cortical microtubules (8 elements) is observed in the nuclear region
144 (Fig. 3h). The transition of characters toward the posterior tip of the spermatozoon of both

145 studied hemiurid species shows relatively slight differences. In *A. stossichii* the transition
146 of characters is as follows: (i) disappearance of the nucleus, (ii) disorganization of the
147 first axoneme, (iii) and later disorganization of the second axoneme (Fig. 1q-u).
148 Nevertheless, singlets of the first axoneme remain present until the posterior
149 spermatozoon tip (Fig. 1u). In *E. lepidus* spermatozoon the transition is as follows: (i)
150 disorganization of the first axoneme, (ii) disappearance of the nucleus, and (iii)
151 disorganization of the second axoneme (Fig. 3l-o).

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

154

155 **Discussion**

156 The mature spermatozoa of *A. stossichii* and *E. lepidus* share the general ultrastructural
157 characteristics found in most digeneans described so far: two axonemes with the 9+'1'
158 trepaxonematan pattern (Ehlers 1984), a nucleus, a mitochondrion, glycogen granules,
159 and parallel cortical microtubules. Thus, they exhibit the characteristics of the type II
160 digeneans sperm cells (Bakhoun et al. 2017), namely, two 9+'1' axonemes, external
161 ornamentation not associated with cortical microtubules and located in the anterior part
162 of the sperm cell, a single bundle of cortical microtubules, the maximum number of
163 cortical microtubules located in a middle part of the spermatozoon and the presence of
164 one mitochondrion. The type II of spermatozoon has been described in the faustulid
165 *Pronoprymna ventricosa* and in all the hemiuroideans except species belonging to the
166 Didimozoidae (Justine and Mattei 1982, 1983; Pamplona-Basilio et al. 2001; Quilichini
167 et al. 2007). However, there are several specific features mainly located in both anterior
168 and posterior extremities of the spermatozoon. Now, the great variability of the
169 morphology and organization of these extremities in digenean spermatozoa might be a

170 suitable criterion for taxonomy and/or phylogenetic considerations (Quilichini et al.
171 2010a, 2011; Bakhoun et al. 2017).

172 The anterior extremity of the male gamete of both hemiurids is filiform. It is
173 covered by an external ornamentation of the plasma membrane. The presence or absence
174 of this last structure as well as its type, location and association or not with cortical
175 microtubules are considered as interesting ultrastructural criteria used for phylogenetic
176 inference (see Quilichini et al. 2007, 2011; Bakhoun et al. 2017). The mature
177 spermatozoon of *A. stossichii* displays two types of external ornamentation. The first one
178 is filamentous and appears in the anterior extremity as a discontinuous layer. Each
179 filament of the ornamentation is associated with a cortical microtubule. As for the second
180 one, it is also discontinuous, but not associated with cortical microtubules. It is placed
181 more posteriorly in the anterior part of the spermatozoon. Both types of external
182 ornamentation have been reported in other digeneans belonging to several families of the
183 superfamily Hemiuroidea such as the Didymozoidae *Gonapodasmius* sp. (Justine and
184 Mattei 1982, 1984), the Hemiuridae *Lecithochirium microstomum*, *Lecithochirium*
185 *musculus* and *Hemiurus appendiculatus* (Ndiaye et al. 2014; Dione et al. 2016), and the
186 Sclerodistomidae *Prosorchis palinurichthi* and *Sclerodistomum italicum* (Ndiaye et al.
187 2013b, 2017). However, the mature spermatozoa of *E. lepidus* like other species of the
188 Hemiuroidea and most digeneans are characterized by the presence of only one type of
189 external ornamentation. These are the Hemiuridae *Lecithocladium excisum* and
190 *Parahemiurus merus* (Ndiaye et al. 2012, 2013a), the Lecithasteridae *Aponurus*
191 *laguncula* (Quilichini et al. 2010b), and the Sclerodistomoididae *Sclerodistomoides*
192 *pacificus* (Bâ et al. 2020) (see Table 1).

193 Regarding the location of the external ornamentation along the spermatozoon,
194 Quilichini et al. (2011) suggested that digenean spermatozoa could be divided into three

195 types: (i) type 1 presents an external ornamentation in the anterior extremity of the
196 spermatozoon; (ii) type 2 presents an external ornamentation at a more posterior level;
197 and (iii) type 3 lacks external ornamentation. In our case, both spermatozoa of *A.*
198 *stossichii* and *E. lepidus* follow the Quilichini et al.'s type 1 spermatozoon.

199 Another character that could depict the male gamete is the cortical microtubules.
200 These structures are present in the sperm cells of most digeneans studied so far (see
201 Bakhoum et al. 2017). Three aspects of these tubular structures are considered as
202 interesting ultrastructural criteria used for phylogenetic inference: their presence or
203 absence, the location of their maximum number, and the number of bundles. The cortical
204 microtubules of the mature spermatozoon of *A. stossichii* and *E. lepidus* are arranged only
205 in a ventral bundle. Among digeneans this characteristic is only known in the
206 Hemiuroidea. A low number of these cortical microtubules is present in the mature
207 spermatozoon of *A. stossichii* and *E. lepidus*: 11 and 8, respectively. The reported number
208 of cortical microtubules in the hemiuroids is usually low, varying from 5 to 10. However,
209 they are ranged from 24 to 28 for Didymozoidae (Justine and Mattei 1982),
210 Sclerodistomidae (Ndiaye et al. 2013b, 2017) and Sclerodistomoididae (Bâ et al. 2020).
211 The location of the maximum number of these tubular structures along the sperm cell is
212 also variable depending on the species. Quilichini et al. (2007) proposed that the
213 spermatozoon of digeneans could be divided into two groups according to the location of
214 the maximum number of cortical microtubules along the spermatozoon. The first group
215 has the maximum number of these elements in the anterior part. However, for the second
216 group, the maximum number occurs in the middle or in the more posterior part of the
217 spermatozoon. In the male gamete of both studied hemiurids, the maximum number of
218 cortical microtubules is located in the middle or posterior part of the spermatozoon. Thus,

219 they belong to the second type, as mentioned in the remaining studied hemiuroids except
220 for the didymozoids (see Table 1).

221 The presence of mitochondrion/a in digenean spermatozoa is considered as a
222 plesiomorphic character and, to date, there are descriptions of digenean sperm
223 organization containing one, two, or three mitochondria (see Bakhoum et al. 2017 for a
224 review). In the sperm cells of all the hemiuroids described so far, the presence of one
225 mitochondrion has been observed (see Table 1 for details and references). Both mature
226 spermatozoa of *A. stossichii* and *E. lepidus* follow the hemiuroidean pattern with only
227 one mitochondrion in their spermatozoa. It is worthy to note the presence of large
228 granules of different sizes in the mitochondrial matrix of *A. stossichii*. This is the second
229 time that this character has been reported in the sperm mitochondria of a parasitic
230 flatworm. Recently, Kacem et al. (2019) have observed mitochondrial matrix granules in
231 the male gamete of *Haploplanchnus caudatus*.

232 The posterior spermatozoon extremity presents a great variability in the digenean
233 male gametes as reported with the anterior spermatozoon tip. Three types of posterior
234 spermatozoon extremities in digenean spermatozoa were proposed by Quilichini et al.
235 (2010a) considering the sequence of characters' disappearance towards the posterior tip.
236 These are: type 1 (also called opecoelid type) characterized by the sequence 'axoneme,
237 nucleus, and cortical microtubules'; type 2 (also called fasciolid type) with the sequence
238 'cortical microtubules, axoneme, and nucleus'; and type 3 (also called cryptogonimid
239 type) with the sequence 'cortical microtubules, nucleus, and axoneme'. The two studied
240 hemiurids present a variability. In *A. stossichii* sperm the sequence is: 'mitochondria,
241 cortical microtubules, nucleus, first and second axonemes'. As far as *E. lepidus* is
242 concerned, the sequence is: 'mitochondria, cortical microtubules, first axoneme, nucleus
243 and second axoneme'. Other sequences of characters' disappearance towards the

244 posterior tip have been observed in the Hemiuridae: ‘first axoneme, mitochondria,
245 cortical microtubules, nucleus and second axoneme’ in *L. microstomum*, *L. musculus* and
246 *H. appendiculatus* (Ndiaye et al. 2014; Dione et al. 2016); and ‘mitochondria, first
247 axoneme, cortical microtubules, nucleus, and second axoneme’ in *L. excisum* and *P.*
248 *merus* (Ndiaye et al. 2012, 2013a). Thus, the posterior spermatozoon extremity of
249 digeneans exhibits numerous variations leading to unfitness with the three previous
250 models. This is the principal reason argued by Bakhoun et al. (2017) to scrutinize the last
251 spermatozoon character instead of the sequence of characters. The present study proves
252 that *A. stossichii* and *E. lepidus* show the axoneme as the terminal character of the male
253 gamete as in all previously studied hemiurids.

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

266

267 **Compliance with ethical standards**

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

269

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274

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404

405 **Figure captions**

406 **Fig. 1.** Mature spermatozoon of *Aphanurus stossichii*. **a-i** Correlative cross-sections of
407 the anterior spermatozoon extremity (region I). Note the anterior tip with a filamentous
408 ornamentation (fEO) (Fig. 1a). **j-l** Cross-sections of the middle or mitochondrial region
409 (region II). Note the presence of mitochondrial matrix granules (white arrow) (Fig. 1j).
410 **m-u** Consecutive cross-sections of the posterior part or nuclear region (region III). Ax1
411 first axoneme, C2 centriole of the second axoneme, CM cortical microtubules, D1
412 doublets of the first axoneme, D2 doublets of the second axoneme, EO external

413 ornamentation of the plasma membrane, G granules of glycogen, M mitochondrion, N
414 nucleus, S1 singlets of the first axoneme, S2 singlets of the second axoneme. Scale bars
415 = 200 nm.

416 **Fig. 2.** Mature spermatozoon of *Aphanurus stossichii*. Detail of mitochondrial matrix
417 granules (white arrow) in regions II and III. CM cortical microtubules, G granules of
418 glycogen, M mitochondrion, N nucleus. Scale bar = 200 nm.

419 **Fig. 3.** Mature spermatozoon of *Ectenurus lepidus*. **a-d** Consecutive cross-sections of the
420 anterior spermatozoon extremity (region I). Note the presence of classical external
421 ornamentation in the anterior tip (Fig. 2a) in contrast with *A. stossichii*. **e** Cross-section
422 of the middle or mitochondrial region (region II). **f-o** Correlative cross-sections of the
423 posterior extremity or nuclear region (region III). Ax1 first axoneme, C1 centriole of the
424 first axoneme, CC2 central core of the second axoneme, CM cortical microtubules, EO
425 external ornamentation of the plasma membrane, G granules of glycogen, M
426 mitochondrion, N nucleus, S1 singlets of the first axoneme, S2 singlets of the second
427 axoneme. Scale bars = 200 nm.

428 **Fig. 4.** Evidence of the glycogenic nature of the electron-dense granules by means of the
429 test of Thiéry in the mature spermatozoon *Aphanurus stossichii* (Fig. 3a) and *Ectenurus*
430 *lepidus* (Fig. 3b). G granules of glycogen, M mitochondrion, N nucleus. Scale bars = 300
431 nm.

432 **Fig. 5.** Schematic reconstruction of the mature spermatozoon of both studied hemiurids,
433 on the right *Aphanurus stossichii*, on the left *Ectenurus lepidus*. The sperm cell is
434 organized in three different regions: region I or anterior part, region II or middle part and
435 region III or posterior part. In order to make the diagram clearer, granules of glycogen
436 and mitochondrial matrix granules are not shown in longitudinal sections. ASE anterior
437 spermatozoon extremity, Ax1 and Ax2 first and second axoneme, C1 and C2 centriole of

438 the first and the second axoneme, CC2 central core of the second axoneme, CM cortical
439 microtubules, D2 doublets of the second axoneme, EO external ornamentation of the
440 plasma membrane, fEO filamentous ornamentation, G granules of glycogen, M
441 mitochondrion, MMG mitochondrial matrix granules, N nucleus, PM plasma membrane,
442 PSE posterior spermatozoon extremity, S1 and S2 singlets of the first and second
443 axoneme.