1 **Sperm characters of the aspidogastrean *Rohdella amazonica* ~~Giese et al., 2014~~**

2 **(Aspidogastridae, Rohdellinae), a parasite of the banded puffer fish *Colomesus psittacus***

3 ~~**(Bloch & Schneider, 1801)**~~

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Abstract

~~Ultrastructural~~ The ultrastructural characteristics of the mature spermatozoon of the aspidogastrean *Rohdella amazonica* (Aspidogastridae, Rohdellinae) w~~as~~ere studied by means of transmission electron microscopy. The sperm cell ~~has~~ shows two axonemes of the 9+'1' trepaxonematan pattern of Platyhelminthes, parallel cortical microtubules, a well-developed lateral expansion, external ornamentation of the plasma membrane, one mitochondrion, an electron-dense ring, a nucleus and granules of glycogen. The p~~P~~resent results ~~are~~ were compared with those observed in the ~~remaining studied~~ aspidogastreans studied to date and in other Platyhelminthes. ~~Two characters are typical for aspidogastreans, the~~ The lateral expansion and the electron-dense ring are typical characters for aspidogastreans. A~~lthough a~~ lateral expansion ~~that longitudinally describes an undulating membrane~~ has been described in other Platyhelminthes such as monogeneans and digeneans, the Aspidogastrea show a ~~but its higher~~ much ~~larger~~ larger-sized lateral expansion ~~and the presence of~~ with ~~numerous~~ both peripheral and internal microtubules ~~are characteristic of the Aspidogastrea~~. The dense ring is observed as a cylinder in a longitudinal view and shows a ~~different aspect~~ more granular appearance in sperm cells from the seminal vesicle ~~and~~ in comparison to a more electron-dense appearance in sperm cells from the seminal uterine receptacle.

Keywords: *Rohdella amazonica*; Aspidogastrea; Aspidogastridae; Rohdellinae; sperm characters; ultrastructure

Introduction

The Aspidogastrea is a small ~~and archaic~~ group of Neodermata that occupies a basal position within the class Trematoda. It comprises species that infect freshwater and marine molluscs, chondrichthyans, teleosts and freshwater turtles as final hosts. The~~ir~~ known life-cycles include

2

49 some molluscs as intermediate hosts. Aspidogastreans are composed of four families, the

50 Aspidogastridae, Multicalycidae, Rugogastridae and Stichocotylidae. ~~The,~~With respect to the

51 Aspidogastridae, this family ~~and the first of them~~ includes three subfamilies:~~, the~~ Aspidogastrinae,

52 Cotylaspidinae and Rohdellinae (Rohde 1994, 2002).

53 Morphological and molecular studies have shown that the Aspidogastrea are the sister group

54 of the Digenea (Littlewood et al. 1999; Litvaitis and Rohde 1999; Rohde 2001). Moreover,

55 phylogenetic studies do not support the ~~actual~~ currently accepted classification of the family

56 Aspidogastridae, revealing that the subfamilies Aspidogastrinae and Cotylaspinae are

57 monophyletic groups ~~but, in contrast,~~whereas the subfamily Rohdellinae is a paraphyletic group

58 (Zamparo and Brooks 2003). *Rohdella amazonica* Giese et al., 2014 belongs to the subfamily

59 Rohdellinae and is the ~~unique~~ only ~~sole~~ aspidogastrean described to date in fish from the Amazon

60 biome (Alves et al. 2015).

61 Currently, the usefulness of the ultrastructural analysis of the spermatozoon as a source of

62 characters and as an important tool to elucidate relationships between the Platyhelminthes is well

63 known and accepted (Justine 1991a, b, 1998, 2001; Levron et al. 2010; Quilichini et al. 2010, 2011;

64 Bakhoum et al. 2017; Justine and Poddubnaya 2018). In this sense, the ultrastructural study of the

65 spermatozoon in aspidogastreans is of great interest because of their phylogenetic position as a key

66 taxon linking parasitic and free-living Platyhelminthes (Schludermann et al. 2005). To date, there

67 are only a few studies on spermatogenesis and the sperm ultrastructure in the Aspidogastrea. Thus,

68 ~~the~~ some data is available on the ~~ultrastructure of~~ sperm cell~~s~~ ultrastructure ~~is more or less known~~

69 ~~in~~of the Aspidogastridae *Aspidogaster conchicola*, *A. limacoides*, *Lobatostoma manteri* and

70 *Multicotyle purvisi* (Bakker and Diegenbach 1973; Rohde et al. 1991; Watson and Rohde 1991;

71 Levron et al. 2009), all of them included in the subfamily Aspidogastrinae~~,~~.~~:~~ Data is also available

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on the sperm cell ultrastructure of ~~and in~~ the Rugogastridae *Rugogaster hydrolagi* (Watson and Rohde 1992).

In the present work, we describe~~d~~ for the first time the ultrastructural sperm characteristics and their organisation in the mature spermatozoon in an aspidogastrid of the subfamily Rohdellinae, *R. amazonica*, thus contributing to expanding the current knowledge of sperm ultrastructure in the Aspidogastrea.

Materials and methods

Specimens

Live adult specimens of *Rohdella amazonica* Giese et al., 2014 were collected from the intestinal tract of a naturally infected banded puffer fish *Colomesus psittacus* (Bloch & Schneider, 1801) captured in Bay of Marajó, at the mouth of the Paracauari river, Marajó (Pará, Brazil) in February 2019.

Transmission electron microscopy (TEM)

For TEM, several live adult worms were immediately rinsed with a 0.9% NaCl solution and fixed in cold (4 °C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h. Worms were then~~,~~ rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in Milli-Q water (Millipore Gradient A10) and~~,~~ dehydrated in an ethanol series and propylene oxide. After dehydration, worms were~~,~~ embedded in Spurr's resin and polymerised at 60 °C for 72 h. Ultrathin sections (60–90 nm thick) at the level of the seminal vesicle and the uterine seminal receptacle were obtained using a Reichert-Jung Ultracut E ultramicrotome. Sections were placed

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96 on 200 μm -mesh copper and gold grids. Sections placed on copper grids were double-stained with

97 uranyl acetate and lead citrate according to the Reynolds (1963) procedure. Copper grids were

98 examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of

99 80 kV, in the 'Centres Científics i Tecnològics' of the University of Barcelona (CCiTUB).

100

101 *Cytochemistry*

102 Sections placed on gold grids were treated according to the Thiéry (1967) test to reveal the presence

103 of glycogen at an ultrastructural level. Thus, they were treated in periodic acid (PA),

104 thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-

105 Q water, 24 h in TCH, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark

106 and rinsed in Milli-Q water. Sections were examined in a JEOL 1010 transmission electron

107 microscope in the CCiTUB.

108

109 **Results**

110 The observation of mature spermatozoa of *Rohdella amazonica* from the seminal vesicle and the

111 uterine seminal receptacle by means of TEM ~~allows~~ allowed ~~to~~ establish~~ing~~ four well-defined

112 regions with characteristic features (I to IV). The detailed description of the ultrastructural

113 arrangement of the spermatic cell components is illustrated in Figures 1 to 4. T~~hus, t~~he male gamete

114 has two trepaxonematan axonemes, parallel cortical microtubules, a well-developed lateral

115 expansion, external ornamentation of the plasma membrane, one mitochondrion, an electron-dense

116 ring, a nucleus and granules of glycogen.

117   Region I (Figs. 1a-g and 4I) is the anterior extremity of the spermatozoon. It is characterised

118 by the appearance of both axonemes, which are slightly longitudinally ~~shifted~~ displaced (Fig. 1a,

119 b). ~~S~~ ~~The presence of s~~ubmembraneous ~~and~~ parallel cortical microtubules are ~~present~~ observed

5

120 when ~~the two~~both axonemes are ~~already~~ fully formed (Fig. 1c, d). The posterior part of region I

121 exhibits a well-developed lateral expansion ~~describing a sort of undulating membrane along this~~

122 ~~part of the sperm cell~~ (Fig. 1e-g). In areas at maximum development, the lateral expansion measures

123 more than 2.5 μm (Fig. 1g). In this expansion, ~~the~~ cortical microtubules are ~~not only~~ arranged as a

124 submembraneous layer and ~~but~~ ~~there are~~ also some cortical microtubules randomly distributed in

125 the cytoplasm (Fig. 1e-g). It is interesting to note the presence of nuclear membrane as remnants

126 of the nuclear migration during spermiogenesis (Fig. 1e, g) and also the external membrane of the

127 mitochondrion (Fig. 1e).

128   Region II (Figs. 1h-l and 4II) is mainly characterised by the presence of the external

129 ornamentation of the plasma membrane (Fig. 1h-j, l), the well-developed mitochondrion (Fig 1h,

130 j, l) and an electron-dense material (Fig. 1h, j-l). This dense material shows the shape of a ring in

131 cross-sections (Fig. 1h, j, k) and describes a cylinder in a longitudinal view (Fig 1l). ~~It~~ This

132 character ~~has been observed~~presents ~~with~~ a granular ~~aspect~~appearance in spermatozoa from the

133 seminal vesicle~~,~~ ~~while~~and a more electron-dense appearance and more well-defined margins in

134 spermatozoa ~~those~~at from the uterine seminal receptacle ~~presents a dense ring with more well-~~

135 ~~defined margins and is more electron-dense~~ (compare Fig. 1h and Fig. 1j, k). ~~The~~ In this region,

136 the external ornamentation surrounds all the gamete and it is associated with cortical microtubules,

137 which ~~and surrounds all the gamete externally (Fig. 1h, j). Then, cortical microtubules also~~

138 describe a continuous layer under the plasma membrane (Fig. 1h, j). Tangential sections at the level

139 of the external ornamentation show its particular aspect, with alternating electron-dense and

140 electron lucent transversal rows. There are two types of electron-dense rows, a thin band and a

141 thicker one. The latter is formed by regularly arranged electron-dense patches (Fig. 1i).

6

142   Region III (Figs. 2a-d, 3 and 4III) is characterised by the absence of the electron-dense ring

143   and by the discontinuous arrangement of the external ornamentation and cortical microtubules in

144   ventral and dorsal sides of the spermatozoon (Fig. 2a-d). The external ornamentation disappears

145   progressively, first in the ventral side and later in the dorsal side of the sperm cell (Fig. 2a-c). ~~The~~

146   ~~presence of a~~A large amount of granular material is also observed throughout this region, which is

147   also observed in-between cortical microtubules (Fig. 2a,b-d). This material was identified as

148   glycogen by means of the cytochemical test of Thiéry (Fig. 3). ~~These granules of glycogen are~~

149   ~~abundant and even present between cortical microtubules (Fig. 2b, d).~~

150   Region IV (Figs. 2e-j, 3 and 4IV) is the posterior and nuclear region of the spermatozoon. In

151   the anterior part of this region, the sperm cell presents ~~both~~ nucleus, ~~and~~ mitochondrion and

152   granules of glycogen (Fig. 2e-h). The nucleus is the only character present in the posterior

153   spermatozoon extremity ~~is characterised by the unique   presence of the nucleus~~ (Fig. 2i-j).

154   ~~Moreover, numerous granules of glycogen are present with the exception of the posterior end (Fig.~~

155   ~~2e-j).~~ Additionally, the number of cortical microtubules decreases progressively throughout this

156   region. It is interesting to remark that the disorganisation of ~~both~~ the axonemes and the posterior

157   extremity of the mitochondrion were not observed. Considering ~~the working effort and~~ the high

158   number of observed specimens and sections, we ~~presume~~ assume that the area where they occur is

159   extremely reduced.

160

161   **Discussion**

162   *Anterior and posterior spermatozoon extremities*

163   The anterior extremity of the mature spermatozoon of *Rohdella amazonica* is characterised by the

164   appearance of two axonemes that are slightly longitudinally displaced from one ~~to~~ another. These

165   axonemes present the 9+'1' trepaxonematan pattern established by Ehlers (1984). Thus, they have

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the typical nine peripheral doublets arranged around a central element or central core that is formed

by twisted fibres clearly visible in a longitudinal view. This particular central core is the most

characteristic feature ~~that exhibit~~in both axonemes and flagella in the sperm cells of

trepaxonematan Platyhelminthes. The sole exceptions are schistosomes (with a 9+'1' special

pattern) and some didymozoids (with 9+0 axonemes) (see Justine and Mattei 1982; Jamieson and

Justine 2017).

In the male gamete of *R. amazonica* the posterior axonemal extremities have not been

observed. However, they occur before the posterior nuclear extremity. ~~We assume that~~ The area

presenting the disorganisation of both axonemes ~~should be~~is presumably~~appears to be very reduced

in relation to the total length of the mature spermatozoon, as ~~we~~ explained in the results section.

Several descriptions were provided ~~In~~in the remaining studied aspidogastreans ~~there are different

situations in this matter. However,~~ although details ~~of the disruption~~ of axonemal disorganisation~~es

has~~were never ~~been~~ clearly shown in any species. ~~Levron et al. (2009) described the most similar

results to our study. Thus,~~ ~~I~~in *Aspidogaster limacoides* (Levron et al. 2009)~~these authors have not

observed the ending of axonemes but, as in~~the ending of axonemes ~~*R. amazonica*Levron et al.

(2009) also~~ ~~, it~~occurs before the disappearance of the nucleus. However, similarly to the present

study on *R. amazonica*, the axonemes disorganization itself was never observed in *Aspidogaster

limacoides* (Levron et al. 2009). In *Multicotyle purvisi* the disruption of axonemes seems to occur

simultaneously and posteriorly to the disappearance of both nucleus and mitochondrion (Watson

and Rohde 1991). A year later, the same authors showed a great variability in the posterior

axonemal extremities in the case of *Rugogaster hydrolagi* spermatozoa: (i) simultaneous disruption

of both axonemes before and (ii) after the disappearance of the nucleus~~ and (ii) also after the

nucleus stopping~~, (iii) asynchronous/unmatched ~~not simultaneous~~ disruption of axonemes or (iv)

axonemal disorganisation in the mitochondrial region (see Watson and Rohde 1992). Finally, the

190   axonemes' posterior extremity was not described neither in *Aspidogaster conchicola* nor in

191   *Lobatostoma manteri* ~~has the posterior extremity of axonemes been described~~ (see Bakker and

192   Diegenbach 1973 and Rohde et al. 1991, respectively).

193   Considering all these studies, the posterior spermatozoon extremity is only well illustrated in

194   *A. limacoides* and *R. amazonica* (Levron et al. 2009; present study). In ~~these two~~both

195   aspidogastreans the nucleus is the ~~sole~~ only character present in the posterior tip of the sperm cell.

196

197   *Lateral expansion ~~or undulating membrane~~*

198   The presence of a well-developed lateral expansion ~~describing an undulating membrane~~ is a

199   common characteristic of the mature sperm of most aspidogastreans (Rohde et al. 1991; Watson

200   and Rohde 1992, 1995; Levron et al. 2009; present study). Only *A. conchi~~x~~cola* apparently lacks

201   this structure (see Bakker and Diegenbach 1973~~.~~). A lateral expansion is clearly visible in *R.*

202   *hydrolagi* illustrations although there is no mention of the structure in the publication (~~In *R.*~~

203   ~~hydrolagi,~~ Watson and Rohde (1992) ~~do not mention this structure, but it is clearly visible in the~~

204   ~~published illustrations. In *M. purvisi* Watson and Rohde (1995) remark on the formation of a~~ A

205   lateral flange formation is described during spermiogenesis ~~I~~in *M. purvisi* (Watson and Rohde

206   (1995). However, ~~while it~~such structure ~~is~~was not mentioned in ~~its~~the previous ~~ly published~~ work

207   on *M. purvisi* ~~the~~ spermatozo~~on~~a (Watson and Rohde 1991).

208   A lateral expansion presenting peripheral microtubules under the plasma membrane has also

209   been described in other Platyhelminthes such as monogeneans and digeneans. Nevertheless,

210   monogeneans or digeneans do not present internal microtubules in this ultrastructural character~~both~~

211   ~~monogeneans and digeneans lack internal microtubules in the lateral expansions of their~~

212   ~~spermatozoa; they just have peripheral microtubules under the plasma membrane~~. Concerning the

213   Monogenea, several authors reported ~~an undulating membrane~~this ~~structure~~character in some

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species of the Polyopisthocotylea, e.g. *Concinnocotyla australensis*, *Discocotyle sagittata*, *Gonoplasius* sp. and *Gotocotyla acanthura* (Justine and Mattei 1985; Rohde and Watson 1994; Watson et al. 1995; Cable and Tinsley 2001). The lateral expansion was named "undulating membrane" Althoughby some authors refer this expansion as a(????refs????)"undulating membrane",although only Justine and Mattei (1985) described "in vivo" the undulating high-frequency movement of the lateral expansion present in the spermatozoon of *G. acanthura*. So, if there is no a clear evidence of the functional role of these structures, it is preferable to name them as lateral expansions rather than "undulating membranes". In reference toWithin the Digenea, some species possess a lateral expansion in their spermatozoa particularly those belonging to the families Bucephalidae, Diplodiscidae, Echinostomatidae, Fasciolidae and Paramphistomidae possess a lateral expansion in their spermatozoa (Ndiaye et al. 2003, 2018; Seck et al. 2008; Bakhoum et al. 2011; Miquel et al. 2018). However, in the majority of these species the lateral expansions are smaller than in aspidogastreans. Only spermatozoa of *Diplodiscus subclavatus* (Bakhoum et al. 2011) exhibit a well-developed lateral expansion, which but is morphologically different to from that of aspidogastreans in some aspects. ThusIn fact, in the *D. subclavatus* sperm cell the a lateral expansion has with cortical microtubules associated with an external ornamentation of the plasma membrane is present in the *D. subclavatus* sperm cell, while the lateral expansion in in the aspidogastreans there ispresents no ornamentation in the expansion. Moreover, the lateral expansion of aspidogastreans has internal microtubules and also a higher number of internal microtubules additional to the peripheral microtubules. In digeneans having sperm cells with lateral expansion, as well as in aspidogastreans, the maximum number of cortical microtubules is always observed in the lateral expansion region presenting this structure. This is also true for aspidogastreans. Around 75 cortical microtubules were present in this region in *D. subclavatus* (Bakhoum et al. 2011)s, which represents the highest number of cortical microtubules in digenean

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238 sperm cells. In the present study, the lateral expansion region of *R. amazonica* presented ~~is the~~

239 ~~digenean In the present study, we have observed more than~~over 150 peripheral microtubules, ~~in~~

240 ~~the lateral expansion of *R. amazonica*, and this number is~~a much higher number than that present

241 in digenean spermatozoa.~~; e.g. *D. subclavatus* is the digenean presenting the highest number of~~

242 ~~cortical microtubules in their sperm cells and their number is around 75 (Bakhoum et al. 2011).~~,

243

244 *External ornamentation*

245 The external ornamentation of the plasma membrane has been observed in the sperm cell of several

246 groups of Platyhelminthes (see Bakhoum et al. 2017; Justine and Poddubnaya 2018). This

247 ornamentation is usually associated with cortical microtubules, but in some digeneans another type

248 of external ornamentation not associated with peripheral microtubules has been described (see

249 Bakhoum et al. 2017). In the aspidogastreans, an external ornamentation associated with cortical

250 microtubules occurs in *A. conchicola*, *A. limacoides*, *L. manteri*, *R. amazonica* and *R. hydrolagi*

251 ~~and in all these species it is associated with cortical microtubules~~ (Bakker and Diegenbach 1973;

252 Rohde et al. 1991; Watson and Rohde 1992; Levron et al. 2009; present study). Rohde et al. (1991)

253 describe external rod-like structures and an internal granular rod (analogue to the dense ring, see

254 below) in *L. manteri* ~~a specialised~~ particular region of the ~~*L. manteri* sperm cell~~spermatozoon

255 ~~constituted by external rod-like structures and an internal granular rod (analogue to the dense ring,~~

256 ~~see below)~~. The ~~presented~~ respective TEM micrographs ~~of this area of *L. manteri* spermatozoon~~

257 (see Rohde et al. 1991, Figs. 5-7 and 9) ~~are~~ show similarities to the structures ~~similar to our~~

258 observ~~ations~~ed in the present study, indicating that ~~and we think that~~ they probably are ~~really are~~

259 tangential sections of the ornamented area of the male gamete.

260

261 *Electron-dense ring*

262 An electron-dense ~~material~~ ring is present in the mature spermatozoon of *R. amazonica* as in most

263 of the studied aspidogastreans (Bakker and Diegenbach 1973; Rohde et al. 1991; Watson and

264 Rohde 1991; Levron et al. 2009). Justine (1995) emphasizes the presence of electron-dense rings

265 as a possible synapomorphy for the Aspidogastrea~~,~~. ~~and within its congeners,~~ Among

266 aspidogastreans, only the sperm cell of *R. hydrolagi* lacks this structure ~~and diverges from this~~

267 ~~pattern~~ (Watson and Rohde, 1992). The dense ring is present in the spermatozoon's mitochondrial

268 region ~~of the spermatozoon~~, which ~~having~~ also shows an external ornamentation ~~that covers all the~~

269 ~~periphery of gamete~~. ~~It~~ The dense ring has been described under different names such as dark rods

270 (Watson and Rohde 1991), granular rods (Rohde et al. 1991) or electron-dense zone (Levron et al.

271 2009). In *A. conchi~~i~~cola*, Bakker and Diegenbach (1973) misinterpreted the electron-dense ring as

272 a cylindrical part of the nucleus. However, it is clear that this dense ring lacks any membrane.

273 Levron et al. (2009) described ~~the presence of~~a dense ring with three layers of different electron

274 density in *A. limacoides*, ~~but~~ although this was not observed in the remaining species ~~this was not~~

275 ~~observed~~.

276 ~~An interesting aspect that we have observed in *R. amazonica* concerns the different~~

277 ~~morphological aspect that the electron~~It is noteworthy that electron-dense rings in *R. amazonica*

278 show different morphological appearances depending on the observation area. ~~So, s~~Spermatozoa

279 in the seminal vesicle have electron-dense rings ~~with~~ with a~~a~~ granular ~~aspect~~appearance, while

280 those ~~from~~ in the uterine seminal receptacle show this structure to be more compact, more electron-

281 dense and with more well-defined margins. ~~It~~These differences could be related to the capacitation

282 process of mature sperm cells. In this respect, Ishida et al. (1991) described diverse ultrastructural

283 changes in spermatozoa of several species of tricladid turbellarians. These authors observed

284 ultrastructural differences in spermatozoa from testis, ovovitelline duct and seminiferous tubule~~,~~

285 ~~and they have been related~~ associated to spermatozoa movement ~~behavioural~~pattern changes ~~in~~

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spermatozoa observed "in vivo" ~~that concern their differential movement~~ in ~~these ducts and~~ ~~thus~~each area, which were attributed to the ~~a~~capacitation of sperm cells (see Ishida et al. 1991).

Compliance with ethical standards

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

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416

417 **Figure captions**

418 **Fig. 1** Mature spermatozoon of *Rohdella amazonica*: regions I (**a-g**) and II (**h-l**). **a-c** Progressive

419 appearance of the two axonemes. **d** Cross-section showing a complete row of submembraneous

420 cortical microtubules. **e-g** Progressive development of the lateral expansion. **h-l** Sections of region

421 II. Note the morphology of the external ornamentation in a tangential section (i) and the electron-

422 dense ring both in cross- and longitudinal sections (j-l). Ax axoneme, Ax1 and Ax2 first and second

423 axoneme, C1 and C2 centrioles of the first and second axoneme, CM cortical microtubules, DR

424 electron-dense ring, EO external ornamentation of the plasma membrane, LE lateral expansion, M

425 mitochondrion, MM mitochondrial membrane, NM nuclear membrane, tEO tangential section of

426 the external ornamentation, Scale bars (a-h, j, l) 300 nm; (i) 200 nm; (k) 100 nm.

18

427 **Fig. 2** Mature spermatozoon of *Rohdella amazonica*: regions III (**a-d**) and IV (**e-j**). **a-d** Cross-

428 sections showing the arrangement of the external ornamentation in two bundles and its progressive

429 disappearance. Note the presence of a large amount of granules of glycogen. **e-i** Sections showing

430 the transition of characters along the nuclear region (region IV) towards the posterior spermatozoon

431 extremity. **j** Longitudinal section of the posterior spermatozoon tip. CM cortical microtubules, EO

432 external ornamentation of the plasma membrane, G granules of glycogen, M mitochondrion, N

433 nucleus, NM nuclear membrane, PSE posterior spermatozoon extremity, Scale bars 300 nm.

434 **Fig. 3** Mature spermatozoon of *Rohdella amazonica*: cytochemical test of Thiéry evidencing

435 glycogen at ultrastructural level. G granules of glycogen, M mitochondrion, N nucleus, Scale bar

436 300 nm.

437 **Fig. 4** Schematic diagram illustrating the ultrastructural organisation of the mature spermatozoon

438 of *Rohdella amazonica*. In order to make the diagram clearer, granules of glycogen and remnants

439 of nuclear and mitochondrial migration are not shown in longitudinal sections. ASE anterior

440 spermatozoon extremity, Ax1 and Ax2 first and second axoneme, C2 centriole of the second

441 axoneme, CM cortical microtubules, DR electron-dense ring, EO external ornamentation of the

442 plasma membrane, G granules of glycogen, LE lateral expansion, M mitochondrion, MM

443 mitochondrial membrane, N nucleus, NM nuclear membrane, PM plasma membrane, PSE

444 posterior spermatozoon extremity.
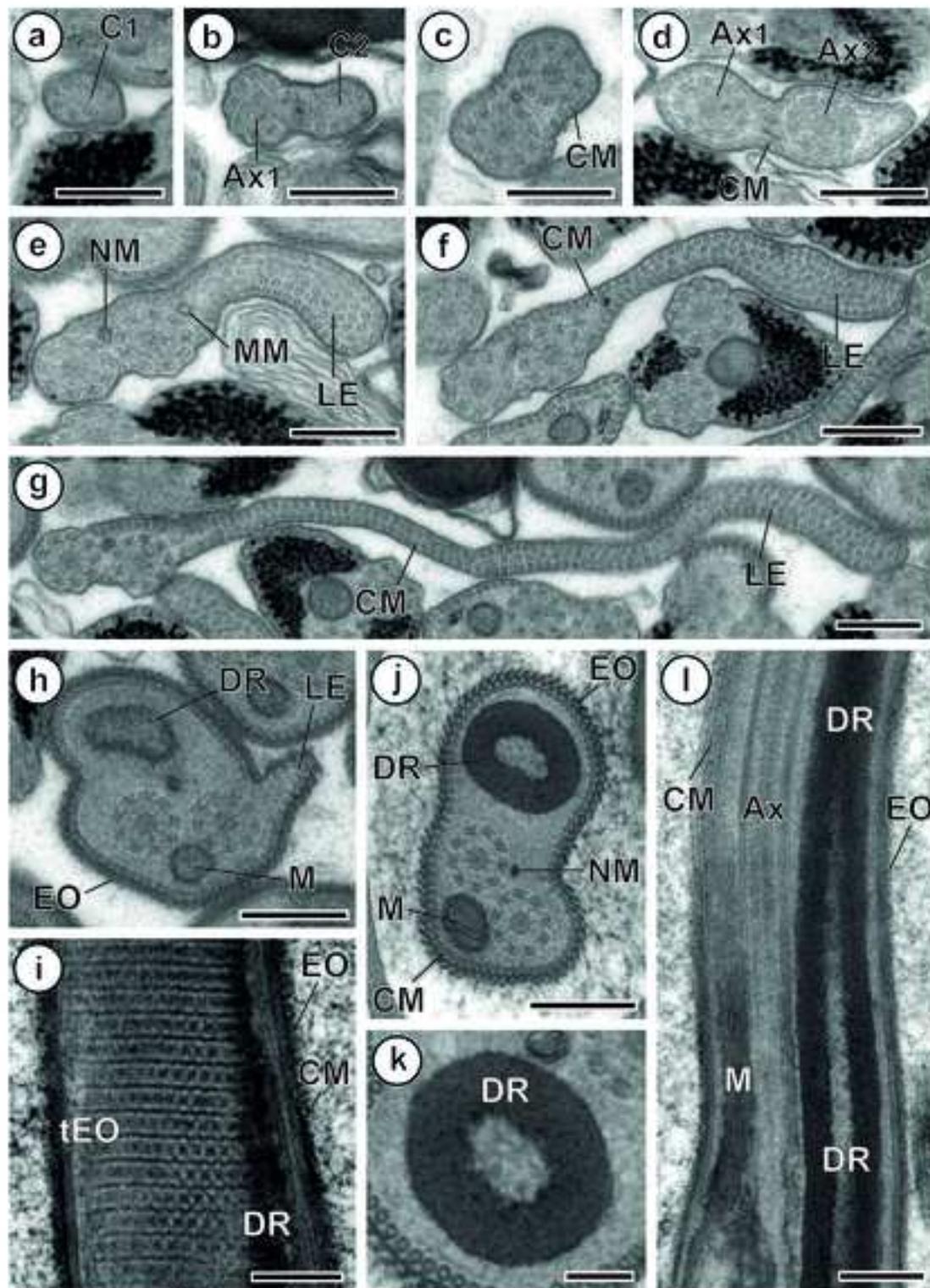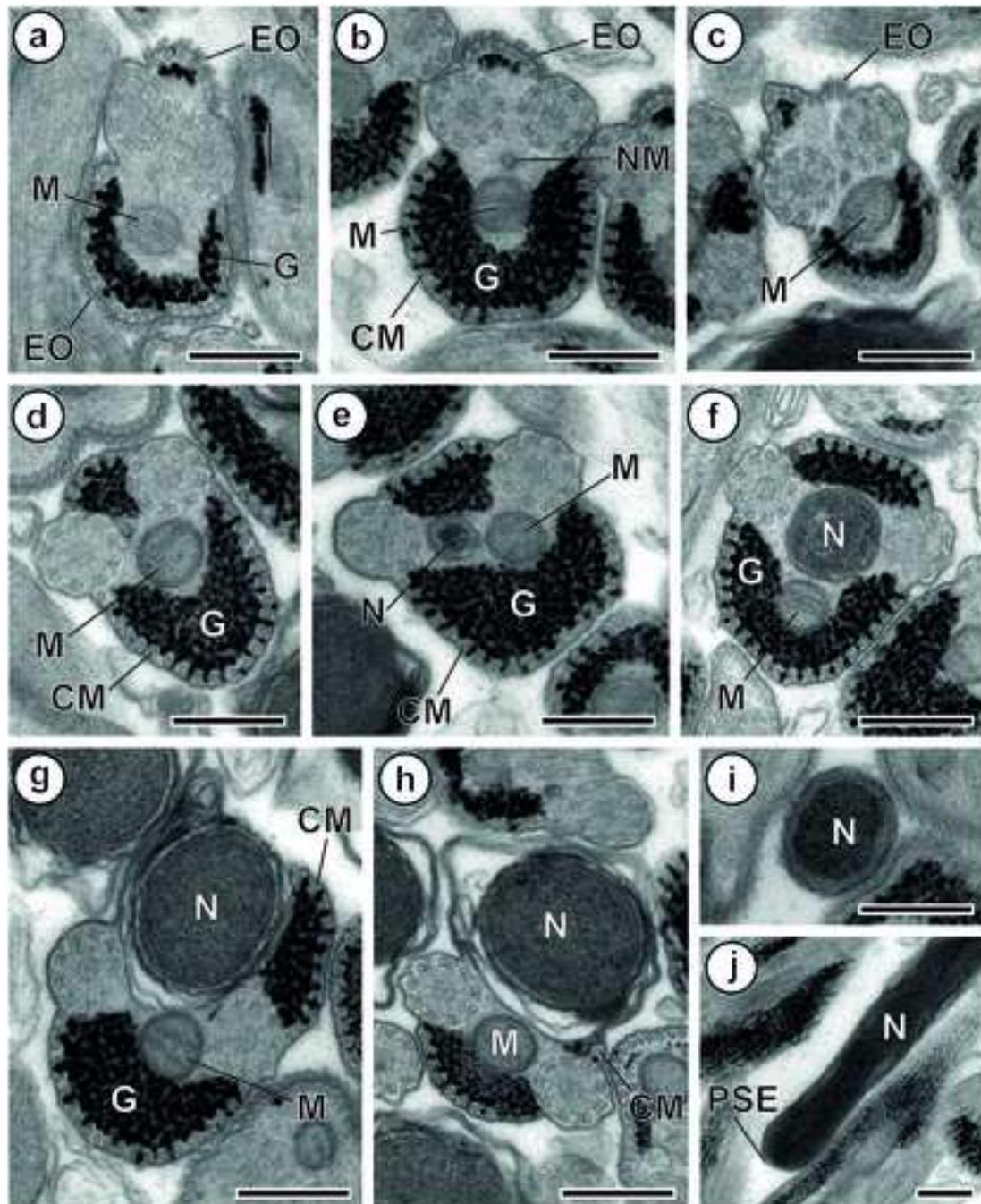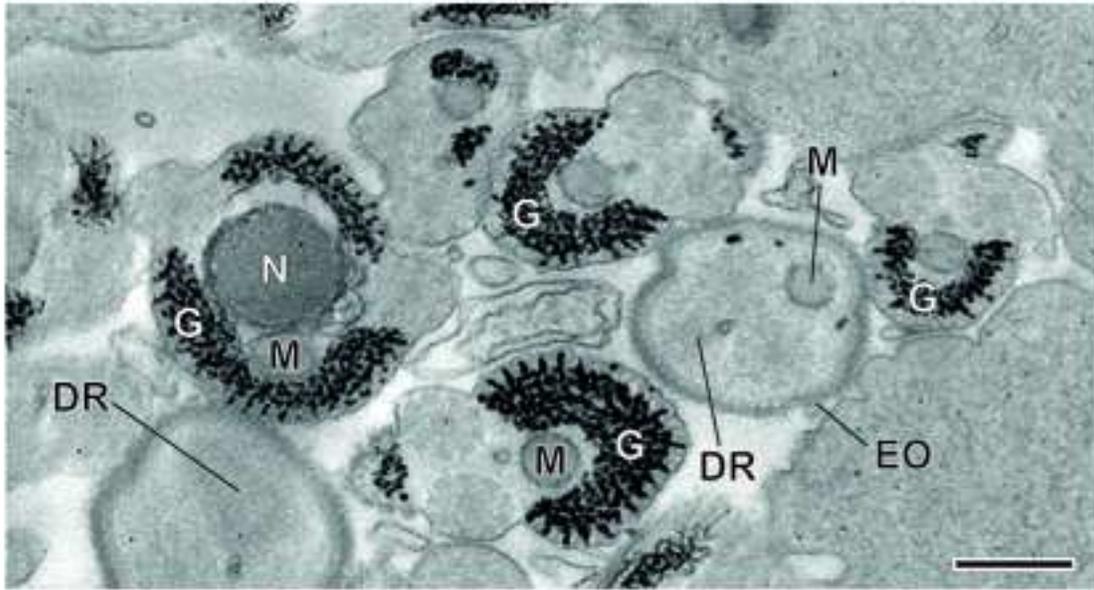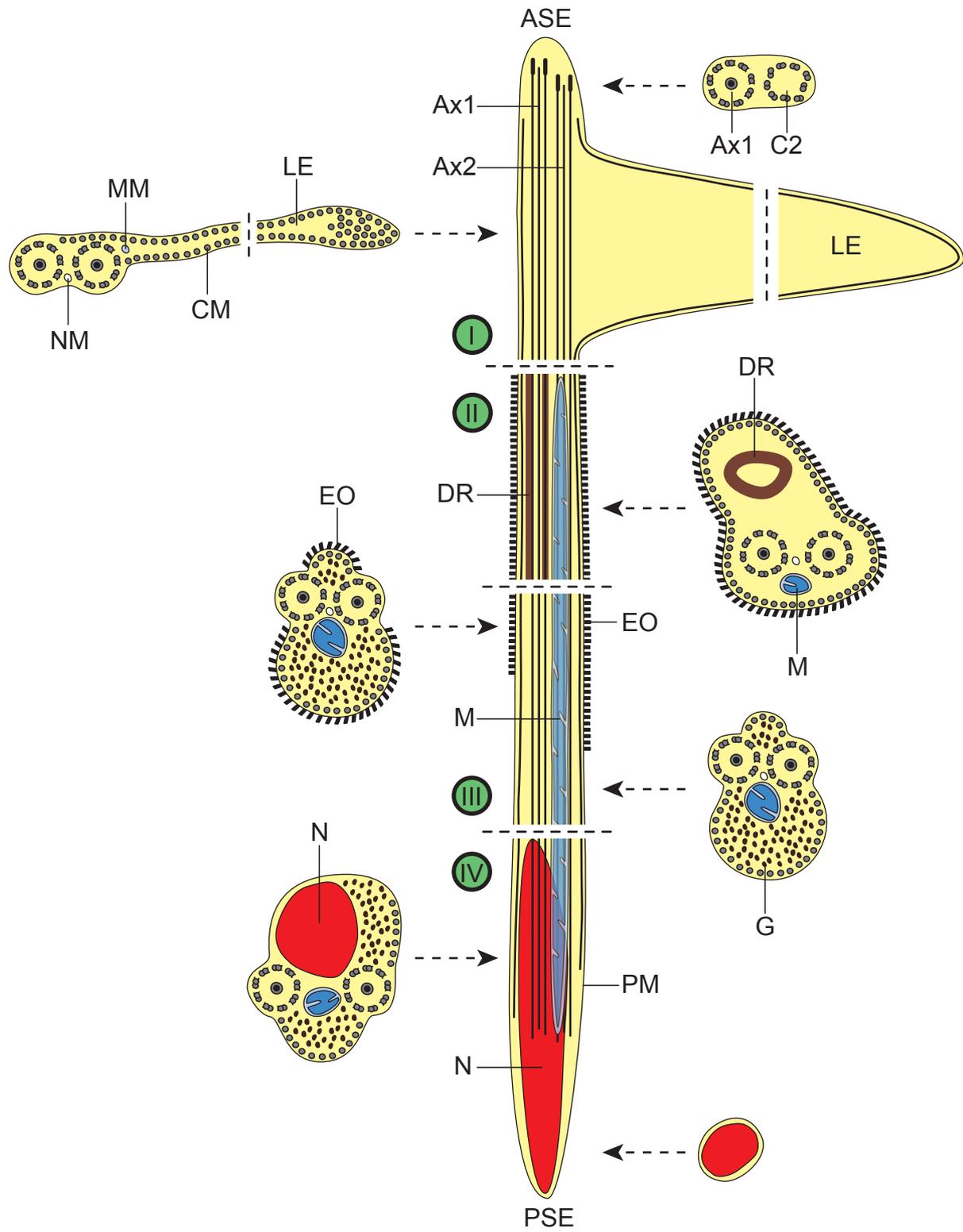
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Figure 1

Figure 3

Figure 4

1   **Sperm characters of the aspidogastrean *Rohdella amazonica* (Aspidogastridae, Rohdellinae),**

2   **a parasite of the banded puffer fish *Colomesus psittacus***

3

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Abstract

24

25   The ultrastructural characteristics of the mature spermatozoon of the aspidogastrean *Rohdella*

26   *amazonica* (Aspidogastridae, Rohdellinae) were studied by means of transmission electron

27   microscopy. The sperm cell shows two axonemes of the 9+'1' trepaxonematan pattern of

28   Platyhelminthes, parallel cortical microtubules, a well-developed lateral expansion, external

29   ornamentation of the plasma membrane, one mitochondrion, an electron-dense ring, a nucleus and

30   granules of glycogen. The present results were compared with those observed in the

31   aspidogastreans studied to date and in other Platyhelminthes. The lateral expansion and the

32   electron-dense ring are typical characters for aspidogastreans. Although a lateral expansion has

33   been described in other Platyhelminthes such as monogeneans and digeneans, the Aspidogastrea

34   show a much larger lateral expansion with both peripheral and internal microtubules. The dense

35   ring is observed as a cylinder in a longitudinal view and shows a more granular appearance in

36   sperm cells from the seminal vesicle in comparison to a more electron-dense appearance in sperm

37   cells from the seminal uterine receptacle.

38

39   **Keywords:** *Rohdella amazonica*; Aspidogastrea; Aspidogastridae; Rohdellinae; sperm characters;

40   ultrastructure

41

42   **Introduction**

43   The Aspidogastrea is a small group of Neodermata that occupies a basal position within the class

44   Trematoda. It comprises species that infect freshwater and marine molluscs, chondrichthyans,

45   teleosts and freshwater turtles as final hosts. Their known life-cycles include some molluscs as

46   intermediate hosts. Aspidogastreans are composed of four families, the Aspidogastridae,

47   Multicalycidae, Rugogastridae and Stichocotylidae. With respect to the Aspidogastridae, this

48 family includes three subfamilies: Aspidogastrinae, Cotylaspidinae and Rohdellinae (Rohde 1994,

49 2002).

50   Morphological and molecular studies have shown that the Aspidogastrea are the sister group

51 of the Digenea (Littlewood et al. 1999; Litvaitis and Rohde 1999; Rohde 2001). Moreover,

52 phylogenetic studies do not support the currently accepted classification of the family

53 Aspidogastridae, revealing that the subfamilies Aspidogastrinae and Cotylaspinae are

54 monophyletic groups whereas the subfamily Rohdellinae is a paraphyletic group (Zamparo and

55 Brooks 2003). *Rohdella amazonica* Giese et al., 2014 belongs to the subfamily Rohdellinae and is

56 the only aspidogastrean described to date in fish from the Amazon biome (Alves et al. 2015).

57   Currently, the usefulness of the ultrastructural analysis of the spermatozoon as a source of

58 characters and as an important tool to elucidate relationships between the Platyhelminthes is well

59 known and accepted (Justine 1991a, b, 1998, 2001; Levron et al. 2010; Quilichini et al. 2010, 2011;

60 Bakhoum et al. 2017; Justine and Poddubnaya 2018). In this sense, the ultrastructural study of the

61 spermatozoon in aspidogastreans is of great interest because of their phylogenetic position as a key

62 taxon linking parasitic and free-living Platyhelminthes (Schludermann et al. 2005). To date, there

63 are only a few studies on spermatogenesis and the sperm ultrastructure in the Aspidogastrea. Thus,

64 some data is available on the sperm cell ultrastructure of the Aspidogastridae *Aspidogaster*

65 *conchicola*, *A. limacoides*, *Lobatostoma manteri* and *Multicotyle purvisi* (Bakker and Diegenbach

66 1973; Rohde et al. 1991; Watson and Rohde 1991; Levron et al. 2009), all of them included in the

67 subfamily Aspidogastrinae. Data is also available on the sperm cell ultrastructure of the

68 Rugogastridae *Rugogaster hydrolagi* (Watson and Rohde 1992).

69   In the present work, we described for the first time the ultrastructural sperm characteristics

70 and their organisation in the mature spermatozoon in an aspidogastrid of the subfamily

71 Rohdellinae, *R. amazonica*, thus contributing to expanding the current knowledge of sperm

72 ultrastructure in the Aspidogastrea.

73

74 **Materials and methods**

75 *Specimens*

76 Live adult specimens of *Rohdella amazonica* Giese et al., 2014 were collected from the intestinal

77 tract of a naturally infected banded puffer fish *Colomesus psittacus* (Bloch & Schneider, 1801)

78 captured in Bay of Marajó, at the mouth of the Paracauari river, Marajó (Pará, Brazil) in February

79 2019.

80

81 *Transmission electron microscopy (TEM)*

82 For TEM, several live adult worms were immediately rinsed with a 0.9% NaCl solution and fixed

83 in cold (4 °C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum

84 of 2 h. Worms were then rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4

85 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in

86 Milli-Q water (Millipore Gradient A10) and dehydrated in an ethanol series and propylene oxide.

87 After dehydration, worms were embedded in Spurr's resin and polymerised at 60 °C for 72 h.

88 Ultrathin sections (60–90 nm thick) at the level of the seminal vesicle and the uterine seminal

89 receptacle were obtained using a Reichert-Jung Ultracut E ultramicrotome. Sections were placed

90 on 200 μm mesh copper and gold grids. Sections placed on copper grids were double-stained with

91 uranyl acetate and lead citrate according to the Reynolds (1963) procedure. Copper grids were

92 examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of

93 80 kV, in the 'Centres Científics i Tecnològics' of the University of Barcelona (CCiTUB).

94

Cytochemistry

96   Sections placed on gold grids were treated according to the Thiéry (1967) test to reveal the presence

97   of glycogen at an ultrastructural level. Thus, they were treated in periodic acid (PA),

98   thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-

99   Q water, 24 h in TCH, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark

100   and rinsed in Milli-Q water. Sections were examined in a JEOL 1010 transmission electron

101   microscope in the CCiTUB.

102

103   **Results**

104   The observation of mature spermatozoa of *Rohdella amazonica* from the seminal vesicle and the

105   uterine seminal receptacle by means of TEM allowed establishing four well-defined regions with

106   characteristic features (I to IV). The detailed description of the ultrastructural arrangement of the

107   spermatic cell components is illustrated in Figures 1 to 4. The male gamete has two trepaxonematan

108   axonemes, parallel cortical microtubules, a well-developed lateral expansion, external

109   ornamentation of the plasma membrane, one mitochondrion, an electron-dense ring, a nucleus and

110   granules of glycogen.

111   Region I (Figs. 1a-g and 4I) is the anterior extremity of the spermatozoon. It is characterised

112   by the appearance of both axonemes, which are slightly longitudinally displaced (Fig. 1a, b).

113   Submembraneous parallel cortical microtubules are observed when both axonemes are fully

114   formed (Fig. 1c, d). The posterior part of region I exhibits a well-developed lateral expansion (Fig.

115   1e-g). In areas at maximum development, the lateral expansion measures more than 2.5 µm (Fig.

116   1g). In this expansion, cortical microtubules are arranged as a submembraneous layer and there are

117   also some cortical microtubules randomly distributed in the cytoplasm (Fig. 1e-g). It is interesting

118   to note the presence of nuclear membrane as remnants of the nuclear migration during

119   spermiogenesis (Fig. 1e, g) and also the external membrane of the mitochondrion (Fig. 1e).

120   Region II (Figs. 1h-l and 4II) is mainly characterised by the presence of the external

121   ornamentation of the plasma membrane (Fig. 1h-j, l), the well-developed mitochondrion (Fig 1h,

122   j, l) and an electron-dense material (Fig. 1h, j-l). This dense material shows the shape of a ring in

123   cross-sections (Fig. 1h, j, k) and describes a cylinder in a longitudinal view (Fig 1l). This character

124   presents a granular appearance in spermatozoa from the seminal vesicle and a more electron-dense

125   appearance and more well-defined margins in spermatozoa from the uterine seminal receptacle

126   (compare Fig. 1h and Fig. 1j, k). In this region, the external ornamentation surrounds all the gamete

127   and it is associated with cortical microtubules, which describe a continuous layer under the plasma

128   membrane (Fig. 1h, j). Tangential sections at the level of the external ornamentation show its

129   particular aspect, with alternating electron-dense and electron lucent transversal rows. There are

130   two types of electron-dense rows, a thin band and a thicker one. The latter is formed by regularly

131   arranged electron-dense patches (Fig. 1i).

132   Region III (Figs. 2a-d, 3 and 4III) is characterised by the absence of the electron-dense ring

133   and by the discontinuous arrangement of the external ornamentation and cortical microtubules in

134   ventral and dorsal sides of the spermatozoon (Fig. 2a-d). The external ornamentation disappears

135   progressively, first in the ventral side and later in the dorsal side of the sperm cell (Fig. 2a-c). A

136   large amount of granular material is also observed throughout this region, which is also observed

137   in-between cortical microtubules (Fig. 2a,b-d). This material was identified as glycogen by means

138   of the cytochemical test of Thiéry (Fig. 3).

139   Region IV (Figs. 2e-j, 3 and 4IV) is the posterior and nuclear region of the spermatozoon. In

140   the anterior part of this region, the sperm cell presents nucleus, mitochondrion and granules of

141   glycogen (Fig. 2e-h). The nucleus is the only character present in the posterior spermatozoon

142   extremity (Fig. 2i-j). Additionally, the number of cortical microtubules decreases progressively

143   throughout this region. It is interesting to remark that the disorganisation of the axonemes and the

144   posterior extremity of the mitochondrion were not observed. Considering the high number of

145   observed specimens and sections, we assume that the area where they occur is extremely reduced.

146

147   **Discussion**

148   *Anterior and posterior spermatozoon extremities*

149   The anterior extremity of the mature spermatozoon of *Rohdella amazonica* is characterised by the

150   appearance of two axonemes that are slightly longitudinally displaced from one another. These

151   axonemes present the 9+'1' trepaxonematan pattern established by Ehlers (1984). Thus, they have

152   the typical nine peripheral doublets arranged around a central element or central core that is formed

153   by twisted fibres clearly visible in a longitudinal view. This particular central core is the most

154   characteristic feature in both axonemes and flagella in the sperm cells of trepaxonematan

155   Platyhelminthes. The sole exceptions are schistosomes (with a 9+'1' special pattern) and some

156   didymozoids (with 9+0 axonemes) (see Justine and Mattei 1982; Jamieson and Justine 2017).

157   In the male gamete of *R. amazonica* the posterior axonemal extremities have not been

158   observed. However, they occur before the posterior nuclear extremity. The area presenting the

159   disorganisation of both axonemes appears to be very reduced in relation to the total length of the

160   mature spermatozoon, as explained in the results section. Several descriptions were provided in the

161   remaining studied aspidogastreans, although details of axonemal disorganisation were never

162   clearly shown in any species. In *Aspidogaster limacoides* (Levron et al. 2009) the ending of

163   axonemes also occurs before the disappearance of the nucleus. However, similarly to the present

164   study on *R. amazonica*, the axonemes disorganization itself was never observed in *Aspidogaster*

165   *limacoides* (Levron et al. 2009). In *Multicotyle purvisi* the disruption of axonemes seems to occur

166 simultaneously and posteriorly to the disappearance of both nucleus and mitochondrion (Watson

167 and Rohde 1991). A year later, the same authors showed a great variability in the posterior

168 axonemal extremities in the case of *Rugogaster hydrolagi* spermatozoa: (i) simultaneous disruption

169 of both axonemes before and (ii) after the disappearance of the nucleus, (iii)

170 asynchronous/unmatched disruption of axonemes or (iv) axonemal disorganisation in the

171 mitochondrial region (see Watson and Rohde 1992). Finally, the axonemes' posterior extremity

172 was not described neither in *Aspidogaster conchicola* nor in *Lobatostoma manteri* (see Bakker and

173 Diegenbach 1973 and Rohde et al. 1991, respectively).

174   Considering all these studies, the posterior spermatozoon extremity is only well illustrated in

175 *A. limacoides* and *R. amazonica* (Levron et al. 2009; present study). In both aspidogastreans the

176 nucleus is the only character present in the posterior tip of the sperm cell.

177

178 *Lateral expansion*

179 The presence of a well-developed lateral expansion is a common characteristic of the mature sperm

180 of most aspidogastreans (Rohde et al. 1991; Watson and Rohde 1992, 1995; Levron et al. 2009;

181 present study). Only *A. conchicola* apparently lacks this structure (see Bakker and Diegenbach

182 1973). A lateral expansion is clearly visible in *R. hydrolagi* illustrations although there is no

183 mention of the structure in the publication (Watson and Rohde 1992). A lateral flange formation is

184 described during spermiogenesis in *M. purvisi* (Watson and Rohde 1995). However, such structure

185 was not mentioned in the previous work on *M. purvisi* spermatozoa (Watson and Rohde 1991).

186   A lateral expansion presenting peripheral microtubules under the plasma membrane has also

187 been described in other Platyhelminthes such as monogeneans and digeneans. Nevertheless,

188 monogeneans or digeneans do not present internal microtubules in this ultrastructural character.

189 Concerning the Monogenea, several authors reported this character in some species of the

190   Polyopisthocotylea, e.g. *Concinnocotyla australensis*, *Discocotyle sagittata*, *Gonoplasius* sp. and

191   *Gotocotyla acanthura* (Justine and Mattei 1985; Rohde and Watson 1994; Watson et al. 1995;

192   Cable and Tinsley 2001). The lateral expansion was named "undulating membrane" by some

193   authors although only Justine and Mattei (1985) described "in vivo" the undulating high-frequency

194   movement of the lateral expansion in the spermatozoon of *G. acanthura*. So, if there is no clear

195   evidence of the functional role of these structures, it is preferable to name them lateral expansions

196   rather than "undulating membranes". Within the Digenea, some species possess a lateral expansion

197   in their spermatozoa particularly those belonging to the families Bucephalidae, Diplodiscidae,

198   Echinostomatidae, Fasciolidae and Paramphistomidae (Ndiaye et al. 2003, 2018; Seck et al. 2008;

199   Bakhoum et al. 2011; Miquel et al. 2018). However, in the majority of these species the lateral

200   expansions are smaller than in aspidogastreans. Only spermatozoa of *Diplodiscus subclavatus*

201   (Bakhoum et al. 2011) exhibit a well-developed lateral expansion, which is morphologically

202   different from that of aspidogastreans. In fact, a lateral expansion with cortical microtubules

203   associated with an external ornamentation of the plasma membrane is present in the *D. subclavatus*

204   sperm cell, while the lateral expansion in aspidogastreans presents no ornamentation. Moreover,

205   the lateral expansion of aspidogastreans has internal microtubules and also a higher number of

206   peripheral microtubules. In digenean sperm cells with lateral expansion, as well as in

207   aspidogastreans, the maximum number of cortical microtubules is observed in the lateral expansion

208   region. Around 75 cortical microtubules were present in this region in *D. subclavatus* (Bakhoum

209   et al. 2011)*,* which represents the highest number of cortical microtubules in digenean sperm cells.

210   In the present study, the lateral expansion region of *R. amazonica* presented over 150 peripheral

211   microtubules, a much higher number than that present in digenean spermatozoa.

212

213   *External ornamentation*

214   The external ornamentation of the plasma membrane has been observed in the sperm cell of several

215   groups of Platyhelminthes (see Bakhoum et al. 2017; Justine and Poddubnaya 2018). This

216   ornamentation is usually associated with cortical microtubules, but in some digeneans another type

217   of external ornamentation not associated with peripheral microtubules has been described (see

218   Bakhoum et al. 2017). In the aspidogastreans, an external ornamentation associated with cortical

219   microtubules occurs in *A. conchicola*, *A. limacoides*, *L. manteri*, *R. amazonica* and *R. hydrolagi*

220   (Bakker and Diegenbach 1973; Rohde et al. 1991; Watson and Rohde 1992; Levron et al. 2009;

221   present study). Rohde et al. (1991) describe external rod-like structures and an internal granular

222   rod (analogue to the dense ring, see below) in a particular region of the *L. manteri* spermatozoon.

223   The respective TEM micrographs (see Rohde et al. 1991, Figs. 5-7 and 9) show similarities to the

224   structures observed in the present study, indicating that they probably are tangential sections of the

225   ornamented area of the male gamete.

226

227   *Electron-dense ring*

228   An electron-dense ring is present in the mature spermatozoon of *R. amazonica* as in most of the

229   studied aspidogastreans (Bakker and Diegenbach 1973; Rohde et al. 1991; Watson and Rohde

230   1991; Levron et al. 2009). Justine (1995) emphasizes the presence of electron-dense rings as a

231   possible synapomorphy for the Aspidogastrea. Among aspidogastreans, only the sperm cell of *R.*

232   *hydrolagi* lacks this structure (Watson and Rohde, 1992). The dense ring is present in the

233   spermatozoon's mitochondrial region, which also shows an external ornamentation. The dense ring

234   has been described under different names such as dark rods (Watson and Rohde 1991), granular

235   rods (Rohde et al. 1991) or electron-dense zone (Levron et al. 2009). In *A. conchicola,* Bakker and

236   Diegenbach (1973) misinterpreted the electron-dense ring as a cylindrical part of the nucleus.

237   However, it is clear that this dense ring lacks any membrane. Levron et al. (2009) described a dense

238 ring with three layers of different electron density in *A. limacoides*, although this was not observed

239 in the remaining species.

240   It is noteworthy that electron-dense rings in *R. amazonica* show different morphological

241 appearances depending on the observation area. Spermatozoa in the seminal vesicle have electron-

242 dense rings with a granular appearance, while those in the uterine seminal receptacle show this

243 structure to be more compact, more electron-dense and with more well-defined margins. These

244 differences could be related to the capacitation process of mature sperm cells. In this respect, Ishida

245 et al. (1991) described diverse ultrastructural changes in spermatozoa of several species of tricladid

246 turbellarians. These authors observed ultrastructural differences in spermatozoa from testis,

247 ovovitelline duct and seminiferous tubule associated to spermatozoa movement pattern changes

248 observed "in vivo" in each area, which were attributed to the capacitation of sperm cells (see Ishida

249 et al. 1991).

250

261

262 **Compliance with ethical standards**

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

264

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378

379   **Figure captions**

380 **Fig. 1** Mature spermatozoon of *Rohdella amazonica*: regions I (**a-g**) and II (**h-l**). **a-c** Progressive

381 appearance of the two axonemes. **d** Cross-section showing a complete row of submembraneous

382 cortical microtubules. **e-g** Progressive development of the lateral expansion. **h-l** Sections of region

383 II. Note the morphology of the external ornamentation in a tangential section (i) and the electron-

384 dense ring both in cross- and longitudinal sections (j-l). Ax axoneme, Ax1 and Ax2 first and second

385 axoneme, C1 and C2 centrioles of the first and second axoneme, CM cortical microtubules, DR

386 electron-dense ring, EO external ornamentation of the plasma membrane, LE lateral expansion, M

387 mitochondrion, MM mitochondrial membrane, NM nuclear membrane, tEO tangential section of

388 the external ornamentation, Scale bars (a-h, j, l) 300 nm; (i) 200 nm; (k) 100 nm.

389 **Fig. 2** Mature spermatozoon of *Rohdella amazonica*: regions III (**a-d**) and IV (**e-j**). **a-d** Cross-

390 sections showing the arrangement of the external ornamentation in two bundles and its progressive

391 disappearance. Note the presence of a large amount of granules of glycogen. **e-i** Sections showing

392 the transition of characters along the nuclear region (region IV) towards the posterior spermatozoon

393 extremity. **j** Longitudinal section of the posterior spermatozoon tip. CM cortical microtubules, EO

394 external ornamentation of the plasma membrane, G granules of glycogen, M mitochondrion, N

395 nucleus, NM nuclear membrane, PSE posterior spermatozoon extremity, Scale bars 300 nm.

396 **Fig. 3** Mature spermatozoon of *Rohdella amazonica*: cytochemical test of Thiéry evidencing

397 glycogen. G granules of glycogen, M mitochondrion, N nucleus, Scale bar 300 nm.

398 **Fig. 4** Schematic diagram illustrating the ultrastructural organisation of the mature spermatozoon

399 of *Rohdella amazonica*. In order to make the diagram clearer, granules of glycogen and remnants

400 of nuclear and mitochondrial migration are not shown in longitudinal sections. ASE anterior

401 spermatozoon extremity, Ax1 and Ax2 first and second axoneme, C2 centriole of the second

402 axoneme, CM cortical microtubules, DR electron-dense ring, EO external ornamentation of the

403 plasma membrane, G granules of glycogen, LE lateral expansion, M mitochondrion, MM

17

404   mitochondrial membrane, N nucleus, NM nuclear membrane, PM plasma membrane, PSE

405   posterior spermatozoon extremity.