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1 Comparative ultrastructure of the spermatogenesis of three species of Poecilosclerida (Porifera,
2 Demospongiae)

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24 Abstract.

25 The spermatogenesis of Porifera is still relatively poorly understood. In the past, it was accepted
26 that all species presented a primitive-type spermatozoon, lacking special structures and
27 acrosome. Nonetheless, a very peculiar spermatogenesis resulting in V-shaped
28 spermatozoon with acrosome was found in Poecilosclerida, calling into question the
29 reproductive and evolutionary aspects of Porifera. We investigated the ultrastructure of the
30 spermatogenesis of three different species of Poecilosclerida (Porifera, Demospongiae): *Iophon*
31 *proximum*, *I. piceus* and *Tedania ignis*. In all three species, spermatogenesis
32 was synchronized within the spermatogenic cysts. *Iophon proximum* and *T. ignis* presented elongated
33 sperm cells, but in *I. piceus* their shape was globular, suggesting a primitive spermatozoon. A
34 structure resembling an acrosome was only found in *T. ignis*. Apparently, the type of
35 spermatozoid in poecilosclerids is not dictated by the phylogeny nor the environment.
36 Nonetheless, we are proposing that the modified type of spermatozoon is the ancestral state in
37 Poecilosclerida.

38 Key-words: Reproduction, Sponges, Gametogenesis, Modified Spermatozoa; Acrosome

39 Introduction

40 Spermatogenesis is the process that gives rise to the male (usually motile) gamete of
41 animals, i.e. the spermatozoon. In general, this process is characterized by the establishment of
42 the germ-line, successively followed by a proliferation of germ cells, production of spermatids
43 by meiosis and finally the differentiation of the mature spermatozoa (L'Hernault 2006; White-
44 Cooper et al. 2009). Spermatozoa are among the most specialized of all metazoan cells. Their
45 diverse and sometimes intricate morphologies are adapted to a primary function – the
46 transmission of their own genetic contents to an egg of the same species (White-Cooper et al.
47 2009).

48 Sponges (Porifera) lack special organs or systems (Bergquist 1978; Simpson 1984) and
49 their spermatogenesis occurs through the transdifferentiation of somatic cells into germ cells
50 usually in non specific regions of the animal body (Boury-Esnault and Jamieson 1999;
51 Ereskovsky 2010). In Porifera, spermatocytes can either derive from archaeocytes or from
52 choanocytes, but in most Demospongiae, spermatocytes are usually derived from choanocytes
53 (Maldonado and Riesgo 2008; Ereskovsky 2010). In this class, choanocytes will form
54 aggregates progressively bordered by a follicular layer of pinacocyte-like cells, forming the so-
55 called spermatogenic cysts (Reiswig 1983; Boury-Esnault and Jamieson 1999). Spermatogenesis in
56 sponges may either occur synchronously in all spermatogenic cysts of a given specimen, or in a
57 given population, or only within individual spermatogenic cysts, independently from each

58 other(Reiswig 1983). In general, as in other animals, sponges also present four
59 spermatogenic stages: the first stage begins with spermatogonia, which suffer two consecutive
60 divisions resulting in primary spermatocytes and secondary spermatocytes, when the cells
61 remain interconnected by cytoplasmic bridges. During the third stage, these cells differentiate
62 into haploid spermatids. At the fourth stage, spermatozoa become mature (Riesgo and
63 Maldonado 2009; Ereskovsky 2010; Lanna and Klautau 2010).

64 Invertebrate spermatozoa can be divided into three types: (i) primitive, (ii) modified,
65 and (iii) aberrant, the first two types being the most common ones (Hodgson 1986; Reunov
66 2005). The main difference among these types are the position/presence of different
67 compartments of the cell, including acrosome, and the shape of the sperm. Although it has long
68 been assumed that Porifera presented a primitive type of spermatozoa, today a great diversity in
69 their morphology and size is indubitable. Reiswig (1983) described at least eleven different
70 shapes of sperm cells in Porifera. In most species of Poecilosclerida (Demospongiae)
71 investigated so far, mature spermatozoa are elongated cells (Ereskovsky 2010). However,
72 a different form of spermatozoa was recently described for this lineage: a sophisticated V-shaped
73 form in *Crambe crambe* (Schmidt, 1862) (Riesgo and Maldonado 2009).

74 The order Poecilosclerida is one of the most speciose in the phylum Porifera, comprising
75 four suborders, 25 families, 142 genera and more than 2500 species (van Soest et al. 2018).
76 However, neither the order, nor many of the inner clades are monophyletic (Redmond et al.
77 2013). As occurs in many other lineages of sponges, phylogenies based on molecular markers
78 provides groups that have no morphological characteristics to support them, probably related to
79 the homoplasy of the main characters used in the taxonomy of Porifera (Redmond et al. 2013).
80 This lack of congruence between molecules and morphology are not an exclusivity of the
81 sponges, but in other groups of metazoans, the use of the ultrastructure of the sperm cells in the
82 phylogenies has helped to better understand the relationship among the clades (reviewed in
83 Tudge 2009).

84 Apart from showing different types of spermatozoa, there is no pattern for the origin of
85 germ line in Poecilosclerida. In *Mycale fistulifera* (Row, 1911) and in the carnivorous sponge
86 *Lycopodina occidentalis* (Lambe, 1893) spermatocytes were suggested to originate from
87 archaeocytes (Meroz and Ilan 1995; Riesgo et al. 2007). On the other hand, in
88 *Hemimycale columella* (Bowerbank, 1874) and *Crelleaelegans* (Schmidt, 1862) spermatocytes
89 seem to originate from choanocytes (Pérez-Porro et al. 2012). In addition, it has been postulated
90 that the shape of the spermatozoon is influenced not only by the phylogenetic position of the
91 species, but also by the physiological and functional demands during dispersal of the gamete
92 and its subsequent fertilization process (Franzén 1956; Pitnick et al. 2009). In sponges, both the

93 functional and phylogenetic significance of the diverse morphologies remain poorly understood.
94 Expanding the number of species from different lineages of Poecilosclerida will help to
95 understand the evolution of the group and also the usefulness of the ultrastructure of sperm cells
96 to comprehend the phylogenetic relationship within this lineage of sponges. Therefore, in the
97 present study, we compared the ultrastructure of the spermatogenesis of three species distantly
98 related within the Poecilosclerida phylogeny (Vargas et al. 2015): *Tedania ignis* (Duchassaing &
99 Michelotti, 1864) (Fig. 1a), *Iophon proximum* (Ridley, 1881) (Fig. 1b), and *Iophon*
100 *piceus* (Vosmaer, 1881) (Fig. 1c), found in the tropical, temperate and polar zones of the world,
101 respectively.

102

103 Material and Methods

104 Tissue samples of *Tedania ignis* (with ca. 5 cm³) were collected at Porto da Barra,
105 Salvador, Bahia, Brazil (13°00'15.2"S 38°32'00.2" W) in October 2014, January and
106 November 2015, at 1-2 m depth. Specimens of *Iophon proximum* were collected in Comau Fjord,
107 Southern Chilean Patagonia (42°24'9.66"S – 72°25'14.01"W) in April 2004, at 20-25 m depth.
108 Five specimens of *Iophon piceus* were collected near the White Sea Marine Biological Station
109 «Belomorskaia» of St. Petersburg State University (Kandalaksha Bay, White Sea) (66°17'N,
110 33°39'E), at 16 m depth in July 2002. While *T. ignis* was collected by snorkeling, both species of
111 *Iophon* were sampled by SCUBA diving.

112 Specimens of *T. ignis* were processed for both light and electron microscopy. For light
113 microscopy (LM), specimens were fixed in a solution of saline formaline (4%) for 24 h and
114 processed for standard histology, as described in Lanna et al. (2018). For transmission electron
115 microscopy (TEM), fragments were fixed (for at least 24 h at 4 °C) in a solution of 25%
116 glutaraldehyde, 0.2 M of sodium cacodylate (pH 7.0) and filtered seawater (1:4:5) or fixed in a
117 solution of 25% glutaraldehyde, 0.4M PBS and 0.34M NaCl (1:4:5). For scanning electron
118 microscopy (SEM), fixed fragments were further rinsed with filtered seawater, dehydrated
119 through a graded ethanol series, fractured in liquid nitrogen, thawed in 100% ethanol at ambient
120 temperature and dried by the critical point method from carbon dioxide before being mounted
121 on aluminum stubs, coated with gold using a sputter coater and observed with a JEOL (JSM-
122 6390LV) scanning electron microscope at 12 kV. For transmission electron microscopy (TEM),
123 fixed fragments were rinsed three times in 0.2M sodium cacodylate buffer and post-fixed in a
124 solution of 1% osmium tetroxide for 30 minutes at room temperature. Later, specimens were
125 dehydrated in a graded acetone series and embedded in Epon. Semi-thin sections (1 µm thick)
126 were stained with toluidine blue. Ultrathin sections (60-90 nm thick) double-stained with uranyl

127 acetate and lead citrate (Reynolds 1963) were observed with a JEOL (JEM-1230) transmission
128 electron microscope.

129 Specimens of both species of *Iophon* were also fixed for light and electron microscopy,
130 but using different protocols. *Iophonpiceus* specimens prepared for light microscopy were
131 immersed in a Bouin fixative. Vouchers were then dehydrated through an ethanol series,
132 immersed in a celloidin-castor oil mixture and then in chloroform before embedding in paraffin.
133 Thick sections (6 µm thick) were mounted on glass slides and stained with Mayer's
134 hematoxylin, and eosin. For electron microscopy, fragments of about 1 mm³ were fixed in 2.5%
135 glutaraldehyde in phosphate buffer (pH 7.4) at room temperature for 1h. After fixation,
136 fragments were washed in phosphate buffer and postfixed in 1% osmium tetroxide in phosphate
137 buffer for 1 h. Samples were dehydrated through a graded ethanol series and embedded in Epon-
138 Araldite.

139 Vouchers of *Iophon proximum* (specimen RBINS-IG 32231-POR 8209 = MNRJ 8209)
140 were fixed when arriving at the surface in 4% glutaraldehyde in 0.2 M sodium cacodylate
141 buffer (pH 7.4) supplemented with 0.35 M sucrose and 0.1 M NaCl to obtain a final osmotic
142 pressure of 1105 mOsM for 24 h at 4°C. Vouchers were then washed six times for 10 min in 0.2
143 M sodium cacodylate buffer (pH 7.4) and post fixed for 1 h in 1% osmium tetroxide in 0.2 M
144 sodium cacodylate and 0.3 M NaCl, dehydrated through a graded ethanol series and embedded
145 in ERL 4206 according to Spurr (1969).

146 For both *Iophon* species, sections were obtained with a diamond knife on a Leica
147 Ultracut UCT ultramicrotome. Semi-thin sections (1 µm thick) were dried onto slides and
148 stained with methylene blue-borax. For TEM, thin sections double-stained with uranyl acetate
149 and lead citrate (Reynolds 1963) and later observed with a Tecnai 10 transmission electron
150 microscope. For SEM, specimens were fractured in liquid nitrogen, critical-point-dried, sputter
151 coated with gold-palladium, and observed with a Philips (XL30 ESEM) scanning electron
152 microscope.

153

154 **Institutional abbreviations and acronyms**

155 MNRJ = Museu Nacional da Universidade Federal do Rio de Janeiro, Brazil; RBINS = Royal
156 Belgian Institute of Natural Sciences

157

158 **Results**

159 *Tedania ignis*

160 *Tedania ignis* is a simultaneous hermaphrodite and its spermatogenesis occurs all year
161 round (Lanna et al. 2018). Spermatocysts were enveloped by a single layer of follicle cells

162 (Fig.2a-b), were rounded, and measured ca. 45µm in diameter(Fig.2b). They were spread in the
163 choanosome without any clear pattern of distribution. Different stages of maturation occurred in
164 the same individual, without synchronism between cysts, but the spermatic cells matured at the
165 same time within each cyst. Somatic cells were seldom observedinside the spermatic cysts
166 (Fig.2c).

167 The first stage of spermatogenesis was characterized by the presence of large cells
168 (3.6µm in diameter) with spherical shape, hyaline cytoplasm and a large nucleus (2.1 µm in
169 diameter). In this stage, the nucleus was rounded and its ratio to the cytoplasm was low. The
170 chromatin was partially condensed and spread evenly in the nucleus (Fig. 2d). The cytoplasm
171 presented a relatively well-developed Golgi apparatus and some spherical mitochondria (Fig.
172 2e).

173 The second stage of the spermatogenesiscomprised cells showinga volume reduction
174 (2.2µm in diameter) due to the division of the spermatogonia (Fig. 3a),which
175 apparentlyunderwent successive divisions toward primary and latersecondary spermatocytes.
176 Primary spermatocytes were rounded, with a hyaline cytoplasm filled with small clear vesicles,
177 a prominent Golgi apparatus and a rounded nucleus.The most important characteristic inthis
178 stage was the presence of synaptonemal complexes next to the nuclear envelope (Fig. 3b). In the
179 next stage (secondary spermatocyte), another roundof cell divisions occurred, but cytoplasmic
180 bridges kept the daughter cells connected. These cells were smaller (< 2.0 µm) than in the
181 previous stages, the nucleus ratio to the cytoplasm was ca. 1:1, and the cytoplasm presented
182 several small mitochondria. In this stage, the rounded nucleus started to condense, as some
183 heterochormatin spots were observed in different areas (Fig. 3d). Following the second division,
184 the secondary spermatocytes started to differentiateinto spermatids, still connected through
185 cytoplasmic bridges (Fig. 3c, f).However,dramatic changes occurred during this stage. The
186 volume of the cytoplasm started to decrease, probably by shedding clear vesicles into the lumen
187 of the spermatic cyst. Meanwhile, a flagellum started to emerge close to the nucleus, which was
188 still rounded (Fig. 3e). Later, while the condensation of chromatin progressed, the nucleus
189 became elongatedandan axoneme as well as some mitochondria were observednext to its basal
190 region (Fig. 3f). In this early stage, the spermatid could present a curved shape (Fig. 3c, f, h),
191 but when itfurther differentiated to become a sperm cell, the cytoplasm shedding induced the
192 elongation of the cell, withmitochondria still rounded and gathered in the posterior part of the
193 cell body, next to the flagellum insertion (Fig. 3g). In the late spermatid stage, the nucleus was
194 elongated, occupyingmost of the cytosol and an electron dense material,likely pre-acrosomal
195 vesicles, was present at the tip of the cell (between the plasma membrane and the apical portion
196 of the nucleus), resembling a pre-acrosomal complex (Fig. 3i). No fully mature spermatozoon
197 could be observed in electron microscopy despite the timing of the samples collections.

199 *Iophon proximum*

200 In April 2004, the investigated specimens of *Iophon proximum* were actively carrying
 201 out their spermatogenesis. As in *T. ignis* (and most Demospongiae), spermatogenesis in *I.*
 202 *proximum* took place in spermatogenic cysts. The shape of spermatogenic cysts were rounded, they
 203 measured from 6 to 11 μm and it was covered by follicle cells. Spermatogenic cysts were located in
 204 the choanosome of the sponges without any special localization (Fig. 4a). The follicle cells were
 205 flattened (Fig. 4b), with a lentil-like shape next to the nuclear region. The outward and inward
 206 surfaces of the follicle cells had numerous membrane protuberances (Fig. 4c). Maturation of the
 207 spermatogenic cells was not synchronous at the individual level but was evident within each
 208 spermatogenic cyst, encountered at different stages of spermatogenesis, from primary spermatocyte to
 209 mature spermatozoa. The early spermatogenic cysts development had already occurred when the
 210 sponges were collected and the spermatogonia origin could not be observed.

211 Primary spermatocytes presented numerous cytoplasmic inclusions and a flagellum. The
 212 cytoplasm presented several mitochondria (0.25 μm in diameter), which were round shaped with
 213 well-defined lamellar cristae. The Golgi apparatus was large (1.7 μm long) with an irregular shape
 214 and consisted of 3 to 4 cisternae. In addition, several small electron-clear vesicles were visible
 215 in this stage (Fig. 4d). The flagella derived from large cytoplasmic outgrowth on one side of the
 216 cell. The flagellar basal apparatus was composed of a long basal body (1.05 μm long, 0.2 μm in
 217 diameter) to which an associated accessory centriole (0.71 μm long, 0.2 μm in diameter) and alar
 218 sheets were found. A rootlet was absent. The basal body was situated near the Golgi apparatus
 219 (Fig. 4e-f). The cell shape of the primary spermatocyte was not regular (3.8 μm in diameter) and
 220 a rounded nucleus (2.5 μm in diameter) was observed during this stage. During the prophase I,
 221 the nucleus of the primary spermatocyte presented typical synaptonemal complexes (Fig. 4g).

222 The first meiotic division was not synchronous, primary spermatocytes in prophase I
 223 and secondary spermatocytes occurred at the same time within each cyst (Fig. 4g-h). Bi-
 224 nucleated secondary spermatocytes, deriving from incomplete cytoplasm division at the end of
 225 the first meiotic division were recognizable (Fig. 5a). The cytoplasm of the secondary
 226 spermatocytes was similar to the previous stage, but the cells were slight smaller. The chromatin
 227 formed electron-dense areas in the nucleus (1.4 μm in diameter). Bi-nucleated cells could be
 228 observed after the second meiotic division, indicating the transition from secondary
 229 spermatocyte to spermatid stages (Fig. 5a), along with the formation of lipid droplets (Fig. 5b).
 230 The Golgi complex was not as long as in the previous stage, but consisted also of 3 to 4 cisternae

231 (1.2 μm long). In this stage only, a cytoplasmic invagination, forming a pit in the plasma
232 membranearound the basal part of the flagellum was observed. Cytoplasmic bridges were still
233 observed connecting the spermatids (Fig. 5c).

234 Later, secondary spermatocytes evolved into spermatids, acquiring an elongated shape
235 (3.4 μm long, 1.3 μm in diameter) and being characterized by chromatin at different stages of
236 condensation as well as a stretched nucleus and the elongation and in eccentric position (2.4 μm
237 long, 0.9 μm in diameter). During this stage, pocket-like cytoplasmic invaginations arose around
238 the basal portion of the flagella (Fig. 5d-e). The spermiogenesis was accompanied by the full
239 compaction of the nuclear content altogether with the elongation of the nucleus. In
240 addition, residual cytoplasm was shed at the basal side of the cell or alongside the flagellum
241 reducing the overall volume of the cell (Fig. 5f-6a). Spermatozoa were elongated (up to 4.2 μm
242 long, with a diameter of 1.25 μm in basal and 0.83 μm in apical portion of the cell), with a long
243 flagellum arising from a cytoplasmic invagination of variable depth (Fig. 6a-b). The anterior part
244 of the nucleus presented a trapezoid shape in section. Mitochondria with constant spherical
245 shape and diameter (about 0.3 μm) occurred at the basal part of spermatozoa (Fig. 6b). No
246 particular orientation of spermatozoa within spermatocysts was noticeable.

247 *Iophon piceus*

248 *Iophon piceus* is a simultaneous hermaphrodite reproducing throughout the hydrological
249 summer, i.e. late April to early October (Ereskovsky 2000). The spermatogenesis of this species
250 was previously investigated in light and electron microscopy (Efremova et al. 1987) but the
251 samples collected in 2002 are providing more information about the ultrastructure of its
252 spermiogenesis, although only late stage of spermatid cysts were present (Fig. 7a). Spermatids
253 and spermatozoa were enveloped by flattened cells of the spermatid cyst (Fig. 7a). Spermatids
254 were rounded, measuring ca. 1.4 μm in diameter. The residual cytoplasm presented many large
255 electron clear vesicles and some tubules (likely resulting from the degenerating endoplasmic
256 reticulum) specially found in the residual cytoplasmic bridges uniting two or more spermatids
257 (Fig. 7b-c). At this stage, the nucleus was already fully condensed and started to stretch out, as
258 well as the cell, which also gained an elongated shape. The volume of the cell decreased and
259 large electron clear vesicles started to appear in the cytoplasm. (Fig. 7d). A single amorphous
260 crystalline inclusion was then observed in different regions of the cell, but usually close to the
261 nucleus (Fig. 7e-h). The flagellum of the spermatid/spermatozoon was long and free of
262 ornamentation (Fig. 7a-b, e). Even though the insertion of the flagellum was not observed in an
263 invagination of the cell membrane, small microvilli expansions were found at the basis of the
264 flagellum in several sections (Fig. 7e-f, h-i). The accessory centriole was located close to the

265 nucleus (Fig. 7d, f) and the basal body was positioned at the posterior end of the cell. Coarse
266 microtubule fibers, supposedly related to the rootlet of the flagellum, spread from the basal
267 body toward the nucleus. The nucleus reached ca. 3 μm in length and presented sharp endings in
268 some sections, indicating a truncated tip of the cell (Fig. 7h).

269 Discussion

270 We are here describing the morphology of several steps of the spermatogenesis of three
271 species belonging to two different genera of Poecilosclerida. These species inhabit different
272 regions of the globe (from tropics to polar regions), but still present sperm morphology with
273 stark resemblance to each other and to other previous studied poecilosclerids. Sampling was not
274 spread over time for the threepoecilosclerids described here and spermatogenesis was already in
275 progress in all samples examined. Therefore, the cell lineage of the spermatic cells could not be
276 determined. Nonetheless, based on the position, shape, and sizes of the spermatic cysts of the
277 three investigated species, we hypothesize that they also derive from choanocyte chambers, as
278 showed previously for other poecilosclerids (Pérez-Porro et al. 2012). The spermatogonia of
279 *Tedania ignis* (and also in *Iophon piceus* Efremova et al. 1987) seemed to lose their flagella
280 during the first division and to produce new ones at the later spermatid stage. However, in
281 *Iophon proximum*, as also occurs in the Homoscleromorpha investigated so far (Gaino et al.
282 1986; Riesgo et al. 2007b; Ereskovsky 2010) and some freshwater sponges (Paulus 1989), the
283 division of spermatocytes occurred without the loss of the flagellum. This situation is in
284 contradiction with most Metazoa that have the flagellum appearing only at the late stages of
285 gametogenesis (Reunov and Hodgson 1994). Besides sponges, such situation was only observed
286 in bivalves (Reunov and Hodgson 1994).

287 In the present study, the spermatozoa of all three species showed an elongate shape
288 (about 5.8x0.7 μm in *T. ignis*; 4.0 x0.9 μm in *I. piceus*; and 4.5x0.8 μm in *I. proximum*). The
289 morphology of their nucleus presented a striking similarity: in *T. ignis* as in the two *Iophon*
290 species it was cylindrical with a wavy surface and a trapezium-like anterior end.
291 Both *Iophon* species shared the presence of a shallow cytoplasmic invagination around the basal
292 part of their sperm flagellum and an unusually elongated basal body (in *I. piceus* it was about 1.7
293 μm and in *I. proximum* 1.1 μm). Within the order Poecilosclerida, former investigations in
294 electron microscopy were carried out on *Myxilla incrustans* (Johnston, 1842), *Iophon piceus*
295 (Efremova et al. 1987), *Lycopodina occidentalis* (Lambe, 1893) (Riesgo et al. 2007), *Crambe*
296 *crambe* (Maldonado and Riesgo 2008), *Hemimycale columella* (Pérez-Porro et al. 2012),
297 *Crellomima imparidens* and *Hymedesmia irregularis* (Ereskovsky 2010). The ultrastructure of the
298 spermatozoa of all three species investigated here was similar to that formerly observed for the

299 sperm of *I. piceus* (Efremova et al. 1987) and of *L. occidentalis* (Riesgo et al. 2007). In addition,
300 the presence of a shallow invagination in the flagellum insertion of *I. proximum* and *I. piceus* is
301 similar to that observed in the sperm cells of *H. columella* (Pérez-Porroet al. 2012).
302 Nevertheless, the very sophisticated spermatozoon observed in *C. crambe* (V-shaped) (Riesgo
303 and Maldonado 2009) was not observed in any of the three investigated species and is still a
304 peculiarity of this Mediterranean species. *Crambecrambeis*, apparently, in a sister-group of all
305 other poecilosclerids investigated so far (Hestetun et al. 2016). It could be, therefore, that this
306 sophisticated characteristic of the sperm of *C. crambe* is restricted to the lineage of the
307 Crambeidae, rather than widely spread in Poecilosclerida as a whole. We expect that new
308 investigations in other species of *Crambe* and of *Monanchora*, for example, are likely to answer
309 this question.

310 Elongated and V-shaped spermatozoa with elongated nucleus are considered as
311 ‘modified’ and are widely regarded as a derived character in Metazoa (Reunov 2005). The co-
312 occurrence of ‘primitive’ and ‘modified’ spermatozoa is a situation established in Porifera prior
313 to the emergence of higher metazoans. The occurrence of ‘modified’ spermatozoa among
314 Porifera sharing many ultrastructural traits with those of higher metazoans could be explained
315 by multiple cases of convergent evolution (Riesgo and Maldonado 2009). The same explanation
316 might also be useful for the acrosome. Most spermatozoa of Porifera described until the end of
317 the 1990’s lacked an acrosome (Boury-Esnault and Jamieson 1999) and it was commonly
318 admitted that they all were of the ‘primitive’ type and were more basic than the primitive sperm
319 of the majority of the other groups of metazoans. However, acrosomes were revealed in
320 spermatozoa of Homoscleromorpha (Ereskovsky 2010), of two *Calcarea* species (Nakamura et
321 al. 1997; Lanna and Klautau 2010) and also in several Demospongiae, especially
322 Poecilosclerida (Riesgo et al. 2007; Riesgo and Maldonado 2009; Ereskovsky 2010). The
323 spermatozoa of *T. ignis* presented small vesicles on top of the nucleus, that are likely pre-
324 acrosomal vesicles, as in other Poecilosclerida (*L. occidentalis*, *C. crambe*, and
325 *Crellomimaimparidens* (Rezvoi, 1925)) (Riesgo et al. 2007; Riesgo and Maldonado 2009;
326 Ereskovsky 2010). In contrast, in the spermatozoa of *I. piceus* and *I. proximum* these electron
327 dense granules were absent, as in *M. incrustans* (Efremova et al. 1987). Historically, the
328 presence of acrosome in Porifera brought the following question: is the acrosome a primitive
329 organelle that disappeared during evolution in most of the orders, or a new organelle
330 secondarily evolved in a few species? (Baccetti 1986). In the past, the sperm ultrastructure in
331 sponges supported the hypothesis presented by Afzelius (1972) that primitive spermatozoa may
332 have arisen twice, once in Porifera and another in all other metazoans. The sperm in Porifera
333 seems to have arisen primarily from choanocytes and has probably evolved independently of
334 sperm in Cnidaria and other groups (Franzén 1996). However, it is now being suggested that the

335 spermatozoon is homologous in all Metazoa and that the absence of acrosome in most sponges
336 could be a derived condition (Boury-Esnault and Jamieson 1999; Riesgo and Maldonado 2009).
337 Apparently, the general shape of the sperm might be related more to the fecundation physiology
338 of the animal, than being constrained by the phylogeny of the group. Although some
339 similarities could be observed amongst the three poecilosclerids investigated here, especially in
340 the elongated shape of the sperm cell, the morphology of the spermatozoa of these species still
341 present distinctive characteristics from each other. Expanding the investigations to other
342 poecilosclerids is mandatory if we are to understand whether the diversity of sperm cell
343 morphology is related to the phylogeny of the group or an adaptation to the fertilization process.
344 Nonetheless, till these new studies investigating a large variety of species in different lineages
345 of the group is carried out, we suggest that the “modified” sperm (elongated with a basal
346 flagellum) is the ancestral state in the lineage. Further modifications in the morphology of the
347 sperm, as the V-shaped ones of *C. crambe* (Riesgo and Maldonado 2009), are novelties within
348 Poecilosclerida. We conclude that the current data about spermatozoa ultrastructure in Porifera
349 as a whole (and in Poecilosclerida, particularly) is not sufficient to be used for taxonomy or
350 phylogeny as already proposed in other groups of metazoans, e.g. annelids, insects, birds (Tudge
351 2009). However, it indicates that the fertilization (which is largely unknown in Demospongiae,
352 Ereskovsky 2010) of these different species could be carried out in an apparently similar
353 pattern.

354

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- 469
- 470 Figure captions
- 471
- 472 Figure 1. In situ photographs of the investigated poecilosclerids: (a) *Tedania ignis* from Salvador
473 de Bahia, Brazil. (b) *Iophon proximum* from Comau Fjord, Chile. (c) *Iophon piceus* from White
474 Sea, Russia.
- 475
- 476 Figure 2. *Tedania ignis*: Spermatic cysts and spermatogonia. (a) Choanosome (ch) with different
477 spermatic cysts (sc) enveloped by a thin layer of follicle cells (cryofracture seen in SEM). (b)
478 Spermatic cyst (sc) with spermatic cells developing in synchrony (LM). (c) Spermatids (spd),
479 flagella (fl) and a large somatic cell (so) within a late stage spermatic cyst (TEM). (d)
480 Spermatogonium and its large nucleus (n) with compacting chromatin and clear cytoplasm (cy)

481 (TEM). (e) Mitochondria (mi) and a Golgi apparatus (ga) in the cytoplasm of a spermatogonium.
482 Arrows indicate follicle cells.

483

484 Figure 3. *Tedania ignis*: Spermatocytes and spermatids. (a) Spermatogonia (sp) and primary
485 spermatocytes (sc1) in a spermatid cyst (mes – mesohyl). (b) Primary spermatocyte with a large
486 nucleus (n) in prophase I (arrows indicate synaptonemal complex) clear cytoplasm (cy) and
487 Golgi apparatus (ga). (c) Spermatid cyst with secondary spermatocytes (sp2), spermatids (spd)
488 and spermatozoa (spz). (d) Secondary spermatocytes connected through cytoplasmic bridges
489 (cb) (mi – mitochondria; n – nucleus). (e) Secondary spermatocyte in a late stage of transition
490 towards spermatid, with compacting heterochromatin (hc) in the nucleus (n) (fl – flagellum; mi
491 – mitochondria). (f) Mid stage spermatid with cytoplasm containing many clear vesicles (cv).
492 An elongated nucleus (n) is above the axoneme (ax). (g) Spermatid in a late stage, with
493 stretched out nucleus (n) in the apical portion of the elongated cell. The base of the flagellum (fl)
494 appears in the cytoplasm. (h) Basal portion of the spermatid (neck) showing the flagellum (fl)
495 emerging from the cytoplasm, several mitochondria (mi) and clear vesicles (cv). (i) Tip of a late
496 stage spermatid showing the pre-acrosomal vesicles (arrow) (n – nucleus).

497

498 Figure 4. *Iophon proximum*: Spermatid cysts, spermatogonia, and primary spermatocytes. (a)
499 Spermatid cysts (sc) at different maturation stages in the choanosome. (b) Detail of a spermatid
500 cyst (sc), (fc - follicle cells; mes - mesohyl). (c) Detail of a follicle cell with membranous
501 protuberances (mb). (d) Spermatogonia with a large nucleus (n) and Golgi apparatus (ga). (e)
502 Primary spermatocyte showing basal body and accessory centriole (ac) of the flagellum (fl). (f)
503 Detail of the long basal body (bb) close to the Golgi apparatus (ga) and the accessory centriole
504 (ac). (g) Primary spermatocyte with nucleus (n) in prophase I (arrows indicate synaptonemal
505 complexes). (h) Secondary spermatocyte with a large nucleus (n) and compacting chromatin.
506 Mitochondria (mi) are abundant in all stages.

507

508 Figure 5. *Iophon proximum*: Secondary spermatocytes and spermatids of *I. proximum*. (a)
509 Secondary spermatocyte (spc2) soon after nuclear division. (b) Detail of a secondary
510 spermatocyte with a large nucleus. (c) Two secondary spermatocytes united by cytoplasmic
511 bridges (cb). (d) Spermatids with compacted and elongated nucleus and cytoplasm being shed.
512 Several mitochondria (mi) are located in the putative neck of the sperm cell and the flagellum
513 (fl) is emerging from a deep pit (arrows) in the cytoplasm. (e) Detail of the neck of the
514 spermatids with the flagellum insertion in the invagination of the cell (arrows). (f) Late
515 spermatid with stretched nucleus and residual cytoplasm (rc) being shed alongside the cell. (cy –
516 cytoplasm; mi – mitochondria; n – nucleus; spc1 – primary spermatocyte).

517

518 Figure 6. *Iophon proximum*: Spermatozoon. **(a)** Spermatozoon (spz) with long flagellum seen in
519 SEM. **(b)** TEM view of the spermatozoon showing compacted nucleus (n) with a trapezoid
520 apical shape (fl – flagellum; mi – mitochondria).

521

522 Figure 7. *Iophon piceus*: Spermatids. **(a)** Overview of a spermatid cyst (sc) with spermatids
523 (spd)(mes– mesohyl; fc – follicle cells). **(b)** Spermatids still connected by cytoplasmic bridges
524 (cb). **(c)** detail of the cytoplasmic bridge seen in b, with the presence of several microtubules.
525 **(d)** Spermatids containing important clear vesicles (cv). The centriole (ce) is inserted alongside
526 the compacted nucleus. **(e)** Late spermatid with globular shape. A crystalline amorphous
527 inclusion (ai) is located next to the compacted nucleus. The flagellum is inserted in the neck of
528 the cell attached to the basal body (bb). A fibrous rootlet (r) is connected to the nucleus. **(f)**
529 Detail of the flagellum (fl) insertion showing the basal body (bb) and the accessory centriole
530 (ac). **(g)** Amorphous inclusions are located in different regions of the cytoplasm. The tip of the
531 nucleus bears a trapezoid shape (arrow). **(h)** Elongated spermatid with long nucleus and
532 trapezoid apical shape (arrow). **(i)** Detail of the basal body (bb) and a microvilosity at the neck
533 of the spermatid. (ai – amorphous inclusion; fl – flagellum; mv – microvilosity; n – nucleus).













