

Male gonads morphology, spermatogenesis and sperm ultrastructure of the seahorse *Hippocampus guttulatus* (Syngnathidae)

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4 **Male gonads morphology, spermatogenesis and sperm ultrastructure of the**
5 **seahorse *Hippocampus guttulatus* (Syngnathidae).**
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56 Running head: Semicystic spermatogenesis in *H. guttulatus*
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Abstract

Testes morphology, spermatogenetic process and mature sperm ultrastructure were analysed in *Hippocampus guttulatus*, using both light and transmission electron microscopy. Both testis were organized in a single large germinal compartment, with a central lumen. Spermatocysts only contained spermatogonia and primary spermatocytes. Inside the testis lumen, together with mature sperm, two types of large mono-nucleate cells, flagellate and aflagellate, were present. Both types of cells were interpreted as developing germ cells precociously released inside the testis lumen, where their maturation was completed. According to the different morphological features of the nuclei, such as chromatin condensation degree, aspect of the nuclear fossa and others, the flagellate cells were unquestionably developing spermatids. On the contrary, the developmental stage of the aflagellate were more difficult to interpreted. They could be secondary spermatocytes of young spermatids. No dimorphic sperm were recognizable, the only sperm type observed have features typical of the intro-sperm reports in other syngnathids species. They had a cylindrical head, a short midpiece, characterized by two mitochondrial rings housed inside a cytoplasmic collar, and a long flagellum. These and previous data about the same topic reported on other syngnathids species were compared and discussed.

Introduction

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3 Seahorses and their close relatives pipefishes and seadragons (family
4 Syngnathidae) occupy a very interesting position in the field of reproductive biology
5 of bony fishes. Several interesting features characterize syngnathids. They show:
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10 ii) peculiar parental care, with male pregnancy (Breder and Rosen 1966);
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13 ii) heterogeneous mating system, varying from monogamy to different types of
14 polygamy associated with conventional or inverted sex roles) (c.f. Jones *et al.* 1999);
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18 iii) an atypical organization of both female and male gonads (Begovac and
19 Wallace 1987, 1988; Selman *et al.* 1991; Carcupino *et al.* 1999; Sogabe *et al.* 2008;
20 Biagi *et al.* 2015).
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26 The teleost testes is generally characterized by numerous seminiferous lobules or
27 tubules, which are connected to the main sperm duct via an efferent duct system
28 (Parenti and Greir 2004; Schulz *et al.* 2010). The efferent duct system collects and,
29 sometimes stores, the spermatozoa. The syngnathids testes lack an of efferent duct
30 system. Testes, at least in several species belonging to the *Syngnathus* genus, i.e.
31 *Syngnathus abaster*, *S. typhle*, *S. tenuirostris*, *S. acus*, are constituted by a single
32 seminiferous compartment of unrestricted lobular types, which continue into a sperm
33 duct. The two sperm ducts converge posteriorly to form a single main duct, which runs
34 parallel to the urethra and opens independently in the apex of a urogenital papilla. This
35 latter is located caudal to the anus, hidden by numerous skin folds arranged radially to
36 the anal opening (personal osbervation). Two sperm ducts, originating from the last
37 portion of the testis and converging caudally to form a single main duct which opens
38 in an urogenital papilla, also characterized the reproductive systems of other
39 syngnathids species such as *Nerophis ophidion* and *Hippocampus guttuatus*, although
40 the male gonad organization in these species has not been analysed in details.
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3 However, at least in *Hippocampus kuda*, testes have a morphology similar to that
4 reported for the *Syngnathus* species (Laksanawimol 2004). In the testis of both non-
5 brooding and brooding males of *H. kuda*, examined during the reproductive season,
6 spermatogonia and primary spermatocytes were found along the entire length of the
7 testis, whereas secondary spermatocytes and spermatids were only found inside the
8 lumen. This seems to confirm that in syngnathids of *Hippocampus* genus, as well as
9 those of *Syngnathus*, the testis organization is of unrestricted lobular type and the
10 spermatogenetic process is of the semicystic type (Carcupino *et al.* 1999; Biagi *et al.*
11 2015).
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24 This type of spermatogenesis is currently known in few species belonging to
25 several teleost groups (Selman and Wallace 1986; Bazzoli and Godinho 1991; Mattei
26 *et al.* 1993; Manni and Rasotto 1997; Yoneda *et al.* 1998a; Carcupino *et al.* 1999;
27 Giacomello *et al.* 2008; Srivastava and Singh 1994; Andrade *et al.* 2001; Mazzoldi
28 2001; Muñoz *et al.* 2002; Sábát 2002; Laksanawimol 2004; García-López *et al.* 2005;
29 Hernández *et al.* 2005; Shahin 2006; Sábát *et al.* 2009; Magalhaes *et al.* 2011). It
30 consists of a precocious opening of the germinal cysts, causing an asynchronous
31 maturation of spermatids and the simultaneous presence of germ cells at different
32 developmental stages inside the testis lumen. Moreover, in *Syngnathus* species,
33 developing germ cells inside the lumen are mono- and, more frequently, poly-nucleate
34 and poly-flagellate cells. Individualization of mature sperm seems to occur at the end
35 of spermiogenesis, so the cytokinesis seems to be abolished or at least delayed
36 (Carcupino *et al.* 1999; Biagi *et al.* 2015). Poly-nucleate developing germ cells has not
37 been reported in *H. kuda* (Laksanawimol 2004).
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55 Syngnathids are also known to produce a very low number of sperm. The
56 functional sperm : egg ratio was estimated to be about 191 : 1 in *S. abaster* (Dzyuba
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3 *et al.* 2008) and even much lower 5 : 1 in *Hippocampus kuda* (Van Look *et al.* 2007).
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5 These values are numerous orders of magnitude lower than estimated in the zebrafish
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7 (*Danio rerio*) (48000 : 1), which was considered to have one of the lower sperm
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9 concentration in fish (Stokley *et al.* 1996). Probably due to this low sperm
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11 concentration, no sperm were observed by Laksanawimol (2004) in the testis lumen
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13 of both non-brooding and brooding male of *H. kuda*, whereas dimorphic sperm were
14
15 reported in the same species by Van Look *et al.* (2007). In this last study, however no
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17 data on sperm ultrastructure were shown. Sperm polymorphism was also reported in a
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19 freshwater population of *Syngnathus abaster* (Dzyuba *et al.* 2008), which belong to a
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21 monophyletic lineage within the urphorine subfamily including *Syngnathus* and to
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23 *Hippocampus* species (Wilson and Orr 2011). Recently we have demonstrated that in
24
25 two population the brackish water form of the same species *S. abaster* mature sperm
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27 are great variable in their morphometric traits, but they can not be distinguished in
28
29 different morphotypes (Piras *et al.* in press).
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35 The aim of this paper was to analyse the male gonad morphology, the
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37 spermatogenic process and both sperm traits and sperm ultrastructure in the seahorse
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39 *Hippocampus guttulatus*.
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42 **Materials and Methods**

43 *Sampling*

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48 Four adult male of *Hippocampus guttulatus* were sampled from Venice
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50 lagoon (Veneto), during the reproductive period (May-September 2013). Alive
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52 specimens, delivered to the laboratory within 3 h, were sacrificed by exposure
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54 to the anaesthetic 3-aminobenzoic acid ethyl ether (MS-222, Sigma-Aldrich) for
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56 10 min, and then processed for microscopic analysis.
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3 *Light microscopy:*
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6 Four testes, obtained by two specimens, were fixed in aqueous Bouin's fixative,
7
8 dehydrated in a graded ethanol series, cleared in Bioclear and finally embedded in
9
10 paraffin wax. Sections (5µm) were stained with Eosin and Mayer Emallume (Mazzi
11
12 1977) and processed for the morphological analysis using a Zeiss Axiophot light
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14 microscope (ZEISS, Oberkochen, Germany).
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18 Gonads dissected by the fifth male were gently open in order to obtain aliquots
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20 of seminal fluid containing cells eventually free inside the testicular lumen. Aliquots
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22 of 20 µl each were seminal fluid fixed in 5% glutaraldehyde were placed on poly-
23
24 lysine coated coverslips (1 mg mL⁻¹; Sigma P1274) and air-dried. Then, samples were
25
26 stained with 0.1% Toluidine Blue in aqueous solution and analysed with a Zeiss
27
28 Axiophot light microscope.
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32 *Transmission electron microscopy (TEM)*
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35 Two additional male gonads were fixed for 2h in 4% paraformaldehyde-5%
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37 glutaraldehyde buffered with sodium cacodylate (0.1M and pH 7.2). Specimens were
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39 then rinsed overnight in the same buffer, post-fixed for 1h in 1% osmium tetroxide
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41 buffered with sodium cacodylate. After dehydrating in an ethanol series, samples
42
43 were embedded in Epon 812 resin. Thin sections, of about 80 nm thick, were cut with
44
45 a Reichert Ultracut ultramicrotome (Leica Microsystems, Wetzlar, Germany), and
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47 stained with uranyl acetate and lead citrate. Samples were then examined with a Jeol
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49 Tem 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan).
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53 *Morphometry*
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3 Intact spermatozoa (N = 20), obtained from the gonads of one male stained with
4 Toluidine blue (see above) were analysed in order to study spermatozoa
5 morphometric, such as head length (including nucleus and midpiece) and the length
6 of the flagellum. Abnormal, broken or difficult to measure spermatozoa were
7 discarded.
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14 Digital images of mature spermatozoa were acquired with a digital camera
15 Nikon DS-F11 connected with DS-L2 control unit and mounted on an optical
16 microscope Nikon Eclipse 80i (Nikon, Shinjuku, Japan). The measurements were
17 made using the program Tpsdig2.
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23 24 25 **Results**

26
27 The paired testes were semi-translucent organs (Fig. 1A) adhering to the
28 abdominal cavity by extensions of the mesentery. No accessory structures connected
29 with the testes were recognizable analysing serial histological sections. However,
30 when the two testes were dissected together with the anus and the connective tissue of
31 the belonging urogenital region (as shown in Figure A), two large vesicles were
32 evident. Each testis was characterized by a large central lumen and a thin wall
33 (Fig.1B). The latter consisted of the germinal epithelium and a vascularized fibrous
34 capsule, consisting of connective tissue rich in muscle fibres (Fig.1B-E). The tissue of
35 the capsule is continuous and did not enter the organ where inter-germinal
36 compartments were not observed (Fig. 1B).
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50 The germinal epithelium showed the typical organization in spermatocysts
51 resting on the basal membrane and formed by germ cells enveloped by Sertoli cells
52 (Fig. 1 C-E). Along the entire length of the testis, the germinal epithelium contained
53 spermatocysts inside which spermatogonia and primary spermatocytes were easily
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3 recognizable (Fig. 1 C-D). Developing spermatids and mature sperm were never
4
5 observed inside the spermatocysts.
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9 Two types of large mono-nucleate cells, aflagellate and flagellate cells, together
10 with thin mature spermatozoa were observed inside the testis lumen (Figs 1F, 2-4).
11 The aflagellate cells had irregular shape and were characterized by numerous
12 cytoplasmic protrusions. Their nuclei were round in shape and showed a large
13 eccentric spherical nucleolus (Figs 1F, 2A-B). Their cytoplasm was rich in rough
14 endoplasmic reticulum, Golgi complexes and droplets of different size and density
15 (Fig. 2B). Cells of similar appearances were visible emerging from the surface of the
16 germinal epithelium facing the central lumen (Figs 1C, E, 2A). Some of these cells
17 appeared unquestionably appear to be spermatids; in their cytoplasm a forming
18 flagellum was recognizable (Fig. 2C-D)
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31 In contrast, the flagellate cells were characterized by nuclei with nucleoli not
32 more evident and cytoplasm containing a less amount of droplets and all components
33 typical of the future sperm midpiece (Fig. 3). They were mitochondria, which
34 surrounded a cytoplasmic canal, and an emerging flagellum, running inside the
35 cytoplasmic canal. Patches of electron-dense material begin to accumulate in close
36 association to the inner membrane of the cytoplasmic canal (Fig. 3B, insert, C).
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45 Mature sperm of *Hippocampus guttulatus* (Fig. 4) were anacrosomal and mono-
46 flagellate cells of several tens of microns in length ($51.35 \pm 3.68 \mu\text{m}$, $n = 20$). The
47 spermatozoa consisted of three distinct portions: head, midpiece and flagellum. The
48 head was cylindrical in shape and entirely occupied by the nucleus. ($2.78 \pm 0.19 \mu\text{m}$, n
49 $= 20$) (Fig. 4A). At the basal end of the nucleus, a deep nuclear fossa was present, and
50 inside it, both the basal and the distal centrioles were localized. The midpiece was
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3 clearly marked under the nucleus by two mitochondrial rings. These were housed
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5 inside a cytoplasmic collar, which was separated from the first portion of the flagellum
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7 by a deep cytoplasmic canal (Fig- 4B-D). The plasma membrane of the collar lining
8
9 the canal appeared closely associated to a sheath of electron-dense material arranged
10
11 in ring-like structures regularly spaced. The flagellum had an internal “9+2” axoneme,
12
13 originating from the distal centriole and surrounded by the plasma membrane which
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15 forms two lateral fins (Fig. 4E).
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19 Discussion

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21 Like other syngnathids (Carcupino *et al.* 1999; Biagi *et al.* 2015), the
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23 reproductive apparatus in the seahorses, *Hippocampus guttulatus*, is formed by
24
25 the paired testes within which does not open any type of accessory organ. The
26
27 testes are located inside the coelomic cavity between the intestines and the
28
29 urinary system. In teleost, as well as in other Syngnathidae species previously
30
31 analyzed (Biagi *et al.* 2015), the urinary system generally has a unique urinary
32
33 bladder. The latter appears as a elongated vesicle, located in middle-dorsal
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35 position in respect to the testes (personal observations). In contrast, in
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37 *Hippocampus guttulatus*, or at least in the sample used to obtain the testes shown
38
39 in Figure A, the typical urinary bladder appears to be absent. In its place, two
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41 large vesicles are present. Further studies are needed to determine whether
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43 these vesicles correspond to two bladders and if this unusual condition is typical
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45 of the species or it is an abnormal condition of an single individual.
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52 Each testis is atypically constituted by a single and continuous germinal
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54 compartment, surrounded by a single and continuous somatic compartment. Each
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56 testis appears as a tubular organ characterized by a unique testicular lumen surrounded
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3 by two concentric layers, the tunica albuginea and the germinal epithelium, separated
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5 by the basement membrane.
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8 The germinal compartment extends to the periphery of the testis and terminates
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10 blindly, and is formed by a germinal epithelium with the tripartite organization, which
11
12 is typical of teleost testis; i.e. germ cells are surrounded by Sertoli cells forming
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14 spermatocysts, which rest on the basal lamina. Inside spermatocysts, spermatogonia
15
16 are clearly distributed along the entire length of the testis. Based on these data, the
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18 testis organization in *H. guttulatus* may be attributed to the unrestricted lobular type,
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20 typically found throughout the Neoteleostei, including other syngnathids (Biagi *et al.*
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22 2015; Laksanawimol 2004), except for the atherinomorphs (Parenti and Grier 2004).
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27 In agreement with previous data on syngnathids testes (Carcupino *et al.* 1999;
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29 Biagi *et al.* 2015), the germinal spermatocysts of all reproductive males here analysed
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31 only contain spermatogonia and primary spermatocytes. Developing spermatids,
32
33 which are always mono-nucleate cells, identifiable by both the presence of the
34
35 emerging flagellum, and nuclei characterized by different degrees of chromatin
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37 condensation, are only visible inside the testis lumen together with mature sperm. The
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39 spermatogenetic process may therefore be attributed to the semicystic type. In the
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41 semicystic spermatogenesis, the cysts rupture at the spermatocyte or spermatid stage,
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43 so germ cells only partly develop inside them, producing an asynchronous maturation
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45 of spermatids and thereby reducing the number of simultaneously mature sperm
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47 (Mattei and Mattei 1978).
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51
52 Because developing spermatids of *H. guttulatus* are always mono-nucleate cells,
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54 the semicystic spermatogenetic process seems to have the typical features, i.e.
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56 spermatocytes and/or spermatids are released after that cytokinesis is completed
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3 among isogenetic germ cells. Therefore inside the lumen, the germ cells advance
4 individually through spermiogenesis. In contrast, in the *Syngnathus* species, the
5 cytokinesis seems to be abolished or at least delayed. Indeed, as previously
6 documented in *Syngnathus abaster* and *S. acus* (Carcupino *et al.* 1999), and recently
7 confirmed in the same and in other species of the same genus (Biagi *et al.* 2015),
8 developing germ cells inside the lumen of these species are mono- and, more
9 frequently, polynucleate and polyflagellate cells. A possible functional explanation of
10 the delayed cytokinesis in *Syngnathus* species will be discussed below.
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21 The semicyclic spermatogenesis was interpreted as a possible mechanism,
22 evolved several times in different teleost taxa, able to reduce the cost of sperm
23 production. Therefore, it seems to be crucial particularly in those species where a
24 small ejaculate size is justified by their low fecundity, monogamous mating system,
25 absence of sperm competition and presence of male parental care (Rasotto *et al.* 1992;
26 Marconato and Rasotto 1993; Mazzoldi 2001). This is also the case of *H. guttulatus*,
27 which has small ejaculate size, low fecundity, male parental care, absence of sperm
28 competition, and strictly monogamous mating system (for references c.f. Sanna *et al.*
29 2008; Wilson *et al.* 2003).
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42 Moreover, together with developing spermatids and mature spermatozoa,
43 another type of mono-nucleate cells is present inside the testicular lumen of *H.*
44 *guttulatus*. These cells are aflagellate and characterized by a large amount of
45 cytoplasmic droplets. Large droplets-containing cells were also reported in several
46 species of the *Syngnathus* genus, such as *S. schlegeli* (Watanabe *et al.* 2000), *S.*
47 *abaster*, *S. acus*, *S. tenuirostris* and *S. typhle* (Carcupino *et al.* 1999; Biagi *et al.*
48 2015), although in these species these cells are polynucleate cells as well as
49 developing spermatids. In both reproductive males of *H. guttulatus* and *Syngnathus*
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3 species however, the aflagellate cells are frequently observed both free into the
4 testicular lumen and coming out from the epithelium. Moreover, like in the
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7 *Syngnathus* species, a smaller amount of droplets of different size and electron-
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10 density may be also recognizable in the cytoplasm of developing spermatids of *H.*
11
12 *guttulatus*. All these data support the hypothesis first reported by Carcupino *et al.*
13
14 (1999), and recently reformulated by Biagi *et al.* (2015), that these aflagellate cells
15
16 represent the youngest germ cells released inside the lumen at the spermatocyte or a
17
18 very early spermatid stage, after having accumulated a large amount of material in
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20 form of droplets. These droplets progressively reduce in size and number during the
21
22 germ cell maturation.
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26 It was speculated that the large amount of droplets could be involved in several
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28 function. (i) In the formation of an abundant and fibrous seminal fluid, having the
29
30 function to trap the very low number of mature sperm produced by syngnathids,
31
32 avoiding sperm loss during mating and (ii) in the metabolic supply to the developing
33
34 germ cells, which are released in a very early spermatogenetic stage (Biagi *et al.*
35
36 2015).
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40 As regards the first possible function, it must be said that in some teleost species
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42 (such as *Ophidion marginatum* and *Lophiomus setigerus*), which lay eggs in a
43
44 gelatinous mass (Fahay 1992; Yoneda *et al.* 1998b) as syngnathids do, the semicyclic
45
46 spermatogenesis was thought to be somehow related to the secretion of abundant
47
48 thick seminal fluid. The latter was reported to act in maintaining sperm together and
49
50 facilitating fertilization of egg mass (Muñoz *et al.* 2002).
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3 About to the second function, it should not be forgotten that in the semicyclic
4 spermatogenic process Sertoli cells cannot regulate and support the metabolites
5 transfer towards the developing germ cells, when these are free inside the lumen.
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10 If the functions of the large amount of droplets accumulated in the cytoplasm of
11 syngnathids developing germ cells are the same, why are these cells in *Syngnathus*
12 species polynucleate? The answer to this question is not so easy. The delayed
13 cytokinesis observed in the semicyclic spermatogenesis of *Syngnathus* species, was
14 recently speculated to be correlated to the need in limiting the reduction of cytoplasm
15 and organelles. An early cytoplasmic division among isogenetic cells could endanger
16 the production and accumulation of a sufficient amount of material employed both in
17 the energy requirements of each germ cell and the production of the seminal fluid
18 (Biagi *et al.* 2015). This hypothesis seems not be supported by the absence of
19 polynucleate cells in *H. guttulatus*. A possible explanation of these different data
20 could be sought in a less need in *H. guttulatus* in producing a large amount of fibrous
21 seminal fluid. Although *H. guttulatus* has lower concentration of sperm respect the
22 *Syngnathus* species, it has a closed pouch and a monogamous mating system. Because
23 of that *H. guttulatus* males mate much less frequently respect *Syngnathus* species, and
24 not release sperm for a long period of time. These features could reduce the loss of
25 sperm during fertilization. Indeed, the *Syngnathus* species apparently have a larger
26 amount of sperm, but they have a semi-closed pouch and a polygamous mating
27 system.
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51 According to the ultrastructural analysis of all types of flagellated cells
52 recognizable inside the testis lumen of *H. guttulatus*, we have identified only one type
53 of mature sperm. They are characterized by an elongated head, completely occupied
54 by a nucleus with condensed chromatin, a short midpiece characterized by two
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3 mitochondrial rings surrounding the first portion of the axoneme, and a long flagellum.
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5 This datum does not seem to support the presence of dimorphic sperm reported in *H.*
6
7 *kuda* (Van Look *et al.* 2007). In this latter species, type 1 spermatozoa, which were
8
9 considered the only sperm type taking part in fertilization, seem to have similar
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11 morphology and morphometric traits of *H. guttulatus* sperm. The *total sperm* length
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13 is $51.35 \pm 3.68 \mu\text{m}$ (mean \pm standard deviation, $N = 20$) in *H. guttulatus* and $49.3 \mu\text{m}$
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15 (median length of flagellum $N = 44$) in *H. kuda*. The head length is $2.78 \pm 0.19 \mu\text{m}$
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17 ($N = 20$) in *H. guttulatus* and $3.7 \mu\text{m}$ (median length, $N = 44$) in *H. kuda*. In contrast,
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19 type 2 spermatozoa of *H. kuda*, that were interpreted a remnant population of the
20
21 primitive externally fertilizing sperm type (aquasperm) no taking part in fertilization.
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23 were reported to have a very large spherical head.
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29 However, the difference between our study and that of Van Look and co-
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31 authors (2007) could have been arisen by several reasons. First, the two studies differ
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33 in the methods performed to obtain measurable sperm. We used fixed spermatozoa,
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35 whereas Van Look *et al.* (2007) used living cells. Second, we measure only mature
36
37 (apparently fully formed) and intact sperm (sperm with all their three portions clearly
38
39 visible i.e. nucleus, midpiece and flagellum), whereas Van Look *et al.* (2007)
40
41 measured all sperm, for which it was possible to obtain clear images. This could
42
43 explain, for example, the high difference in the minimum and maximum values of
44
45 flagellar length obtained in the two studies; 47.68 (minimum) and 61.49 (maximum)
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47 μm in our study for *H. guttulatus*, and 6.3 and $69.3 \mu\text{m}$ for *H. kuda*.
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52 Last, but not least, the spermatogenetic process in *H. kuda* is not known. Indeed,
53
54 if *H. kuda* has, like *H. guttulatus* and other syngnathids species (i.e. *Syngnathus*
55
56 *abaster*, *S. typlhe*, *S. tenuirostris*, *S. acus* and *Phyllopteryx taeniolatus*) (Biagi *et al.*
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3 2015; Forsgren and Young 2008) a spermatogenetic process of semicystic type, it
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5 should be very likely that the type 2 sperm are developing spermatids.
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10 Moreover, both the simultaneous presence of flagellate and aflagellate cells
11 inside the testis lumen, determined by the semicystic spermatogenesis, and the
12 difficulty to see the thin flagellum in the histological sections, may have induced
13 other authors to interpret these cells as aflagellate spermatozoa. This could be the
14 case of Miranda-Marure *et al.* (2004) for the aflagellate sperm reported in *Microphis*
15 *brachyurus lineatus*.
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23 Functional sperm with elongated head similar in morphology to those of *H.*
24 *guttulatus* and *H. kuda* were also reported in other syngnathids species, such as
25 *Syngnathus abaster*, *S. typhle*, *S. tenuirostris*, *S. acus* and *Nerophis ophidion*
26 (Carcupino *et al.* 1999; Ah-king *et al.* 2006; Biagi *et al.* 2015 and Piras *et al.* in press).
27 A similar type of sperm are also present in some Blenniidae (Lahnsteiner *et al.* 1990)
28 in *Lepadogaster lepadogaster* (Mattei and Mattei 1978), and in *Ophidion barbatum*
29 (Hernandez *et al.* 2005), all species with external fertilization and semicystic
30 spermatogenesis. In general, spermatozoa with elongated heads are related to internal
31 fertilization (Jamieson and Leung 1991), an explanation that does not match any of
32 the all above mentioned species. According to Burns *et al.* (1995), the elongated
33 nucleus may also facilitate the storage of the spermatozoa in the testicular ducts.
34 Nevertheless, in the specific cases of *O. barbatum* (Hernandez *et al.* 2005), and
35 syngnathids species (our personal observations) no packaging of spermatozoa were
36 observed. On the base of these data, and according to the hypothesis first formulated
37 by Biagi *et al.* (2015) a third possibility to explain the elongated head of syngnathids
38 sperm could be related to their need to cross through the gelatinous mass of maternal
39 origin to reach the eggs.
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Review Copy

Figures legends

Figure 1. **A.** Entire reproductive apparatus of *Hippocampus guttulatus* mature male showing paired testes of uniform external morphology. **B.** Transverse paraffin section of testis appearing as a hollow tube. **C-E.** High magnification of sections obtained by the same testis reported in B, showing: (i) spermatocysts formed by germ cells (spermatogonia and spermatocytes) enveloped by Sertoli cells; (ii) aflagellate mono-nucleate cells protruding from the germinal epithelium. **F.** Aflagellate mono-nucleate cells free inside the lumen. Aflagellate cells (AC); germ cells (GC); germinal epithelium (GE); Lumen (L) Sertoli cells (SC); tunica albuginea (TA); testis (T); vesicles (V). Scale bar: A= 1 mm; B = 62 μm ; C = 17 μm ; D = 9 μm ; E = 11 μm ; F = 15 μm .

Figure 2. Transmission electron micrographs of both aflagellate (**A-B**) and flagellate cells of *Hippocampus guttulatus* testis (**C-D**). **A.** Aflagellate cells protruding from the germinal epithelium. **B.** Aflagellate cells free inside the testicular lumen. **C.** High magnification of the centrioles region of the same cells reported in figure C. Flagellate cells free inside the testicular lumen showing both distal and proximal centriole and the forming axoneme. **D.** High magnification the same cells reported in C. Axoneme (Ax); cytoplasmic droplets (D); nucleus (N). Scale bar: A= 2.5 μm ; B = 133 nm; C = 117 nm; D = 310 nm.

Figure 3. Transmission electron micrographs of developing flagellate cells (spermatids) of *Hippocampus guttulatus* testis. **A.** Young Spermatids. **B.** more advanced spermatids. Insert. High magnification of spermatid cytoplasm showing the midpiece formation region. **C.** Midpiece region. Electron-dense material closely

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3 associated to the internal membrane of cytoplasmic canal (arrow); axoneme (Ax);
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5 spermatids cytoplasm (Cy); cytoplasmic canal (CC); droplets (D); mitochondria (M);
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7 nucleus (N). Scale bar: A= 1.5 μm ; B = 1 μm ; Insert = 625 nm; C = 200 nm.
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13 Figure 4. **A.** Light microscopic image of *Hippocampus guttulatus* mature sperm. **B-E.**

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15 Transmission electron micrographs of mature spermatozoa showing (i) elongated
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17 anacrosomal sperm heads with completely condensed nuclei; (ii) deep nuclear fossa;
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19 (iii) distal and basal centrioles; (iv) “9+2” axoneme originating from distal
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21 centriole; (v) short midpiece characterized by the cytoplasmic collar occupied by
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23 two rings of mitochondria; (vi) cytoplasmic canal; (vii) numerous rings of electron-
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25 dense material between the mitochondria and the internal plasma membrane of the
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27 collar. Electron-dense material (arrow); axoneme (Ax); basal and distal centrioles
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29 inside the nuclear fossa (C); cytoplasmic canal (CC); cytoplasmic droplets (D);
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31 flagellum (F) sperm head (H) mitochondria (M); sperm midpiece (MP); nucleus (N).
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36 Scale bar: A = 4.2 μm ; B = 270 nm; C = 200 nm; D, E = 110 nm.
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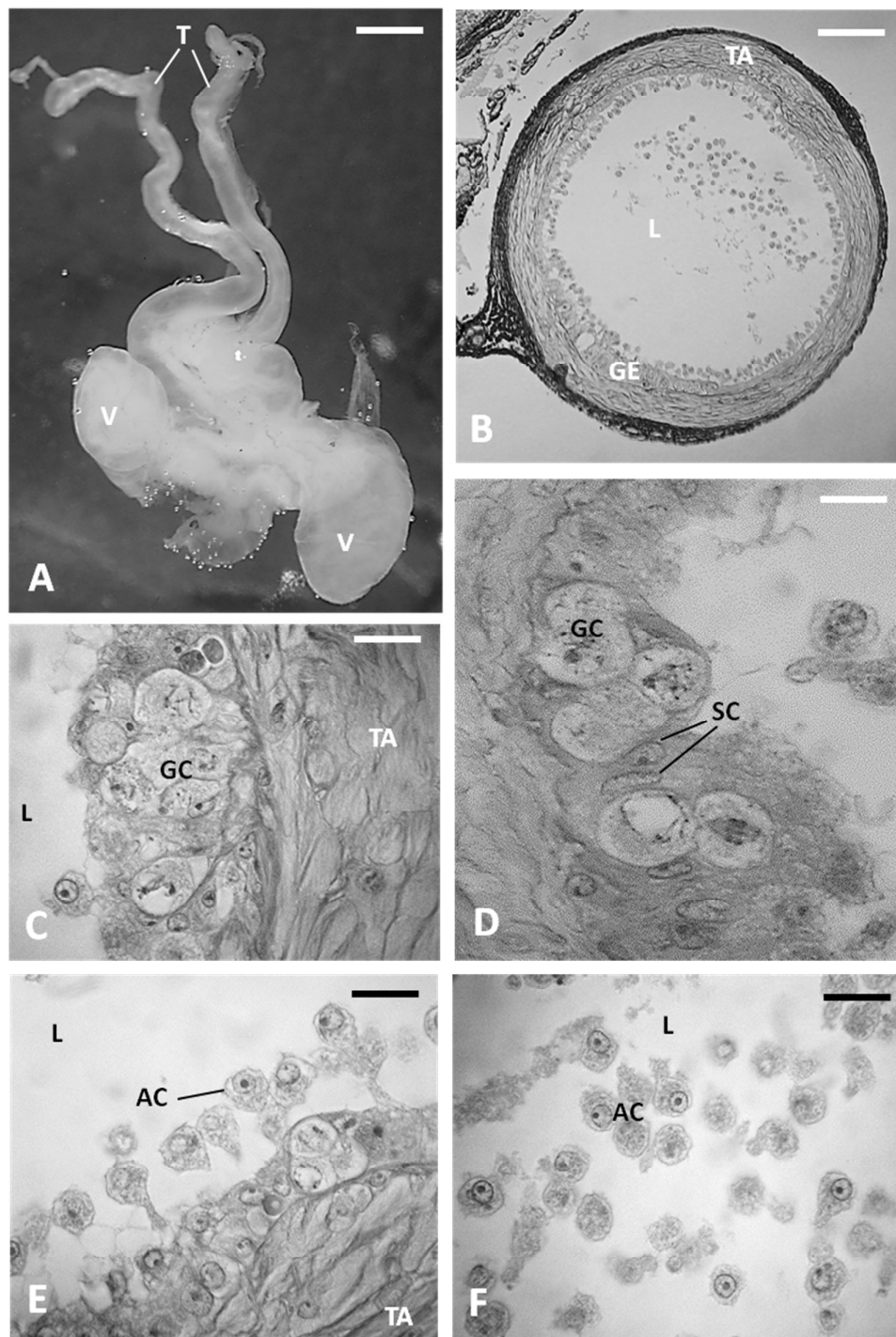


Fig. 1

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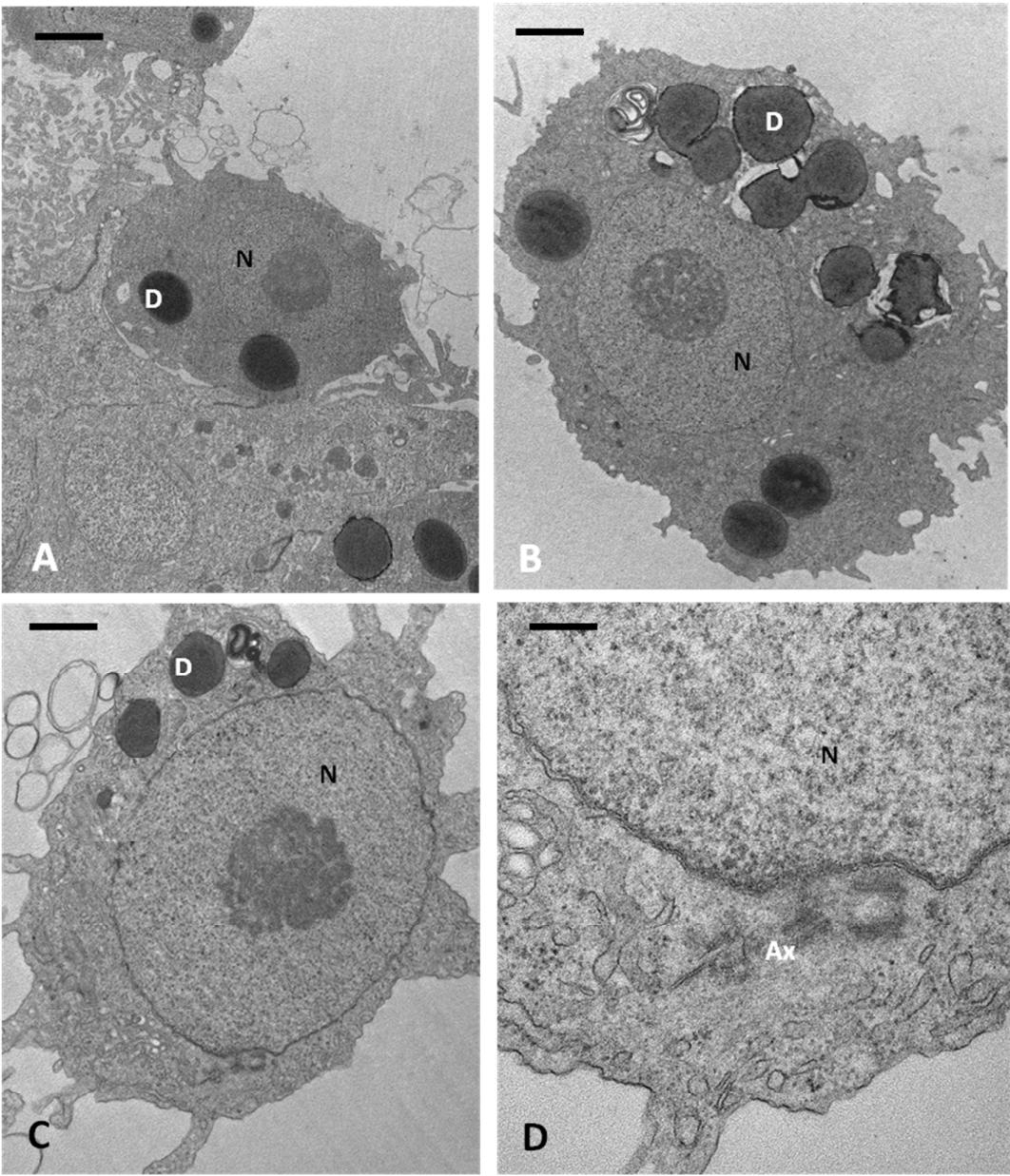


Fig. 2

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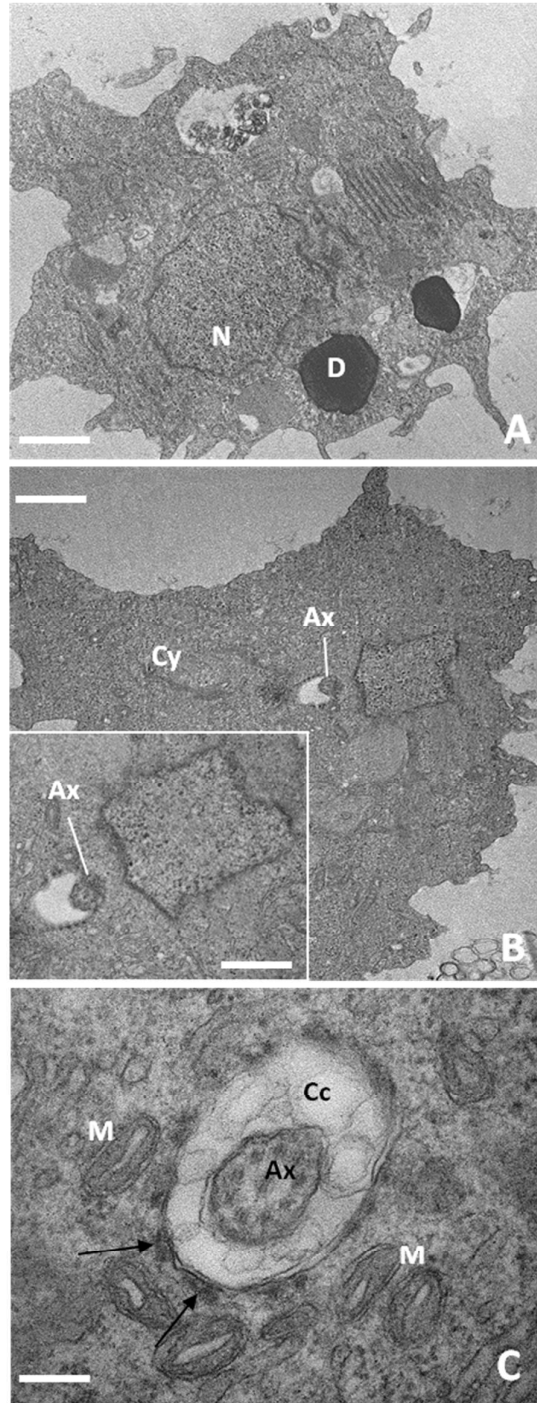


Fig. 3

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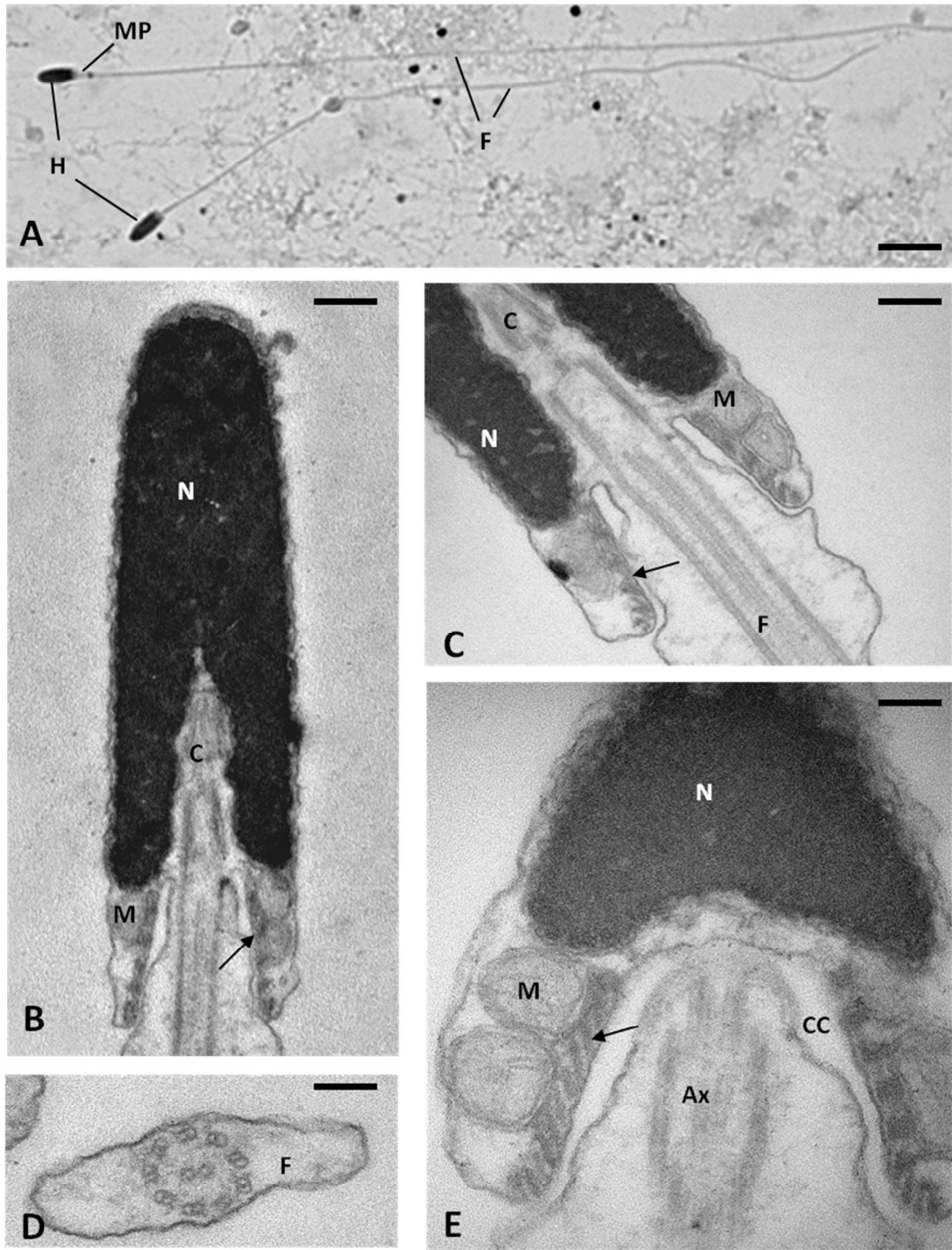


Fig. 4