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

Francesca Piras[1], Francesca Biagi[1], Anna Rita Taddei[2], Anna Maria Fausto[3], Vittorio Farina[3], Marco Zedda[4], Antonello Floris[3], Piero Franzoi[5], Marcella Carcupino[1]

1. Dipartimento di Scienze dalla natura e del Territorio, Università di Sassari.
2. Centro Interdipartimentale di Microscopia Elettronica (CIME). Università della Tuscia, Viterbo.
3. Dipartimento per l'Innovazione nei sistemi Biologici, Agro-alimentari e Forestali, Università della Tuscia, Viterbo
4. Dipartimento di Medicina Veterinaria, Università di Sassari.
5. Dipartimento di Scienze Ambientali, Informatica e Statistica, Università Ca' Foscari di Venezia.

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Corresponding Author

Marcella Carcupino, Dipartimento di Scienze della Natura e del Territorio,

Via Muroni 25, 07100 Sassari, Italy. Tel ++39 079 228664; Fax ++39 079 228665

E-mail: carcupin@uniss.it

Running head: Semicystic spermatogenesis in *H. guttulatus*

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.

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Introduction

Seahorses and their close relatives pipefishes and seadragons (family Syngnathidae) occupy a very interesting position in the field of reproductive biology of bony fishes. Several interesting features characterize syngnathids. They show:

ii) peculiar parental care, with male pregnancy (Breder and Rosen 1966);

ii) heterogeneous mating system, varying from monogamy to different types of polygamy associated with conventional or inverted sex roles) (c.f. Jones *et al.* 1999);

iii) an atypical organization of both female and male gonads (Begovac and Wallace 1987, 1988; Selman *et al.* 1991; Carcupino *et al.* 1999; Sogabe *et al.* 2008; Biagi *et al.* 2015).

The teleost testes is generally characterized by numerous seminiferous lobules or tubules, which are connected to the main sperm duct via an efferent duct system (Parenti and Greir 2004; Schulz *et al.* 2010). The efferent duct system collects and, sometimes stores, the spermatozoa. The syngnathids testes lack an of efferent duct system. Testes, at least in several species belonging to the *Syngnathus* genus, i.e. *Syngnathus abaster*, *S. typhle*, *S. tenuirostris*, *S. acus*, are constituted by a single seminiferous compartment of unrestricted lobular types, which continue into a sperm duct. The two sperm ducts converge posteriorly to form a single main duct, which runs parallel to the urethra and opens independently in the apex of a urogenital papilla. This latter is located caudal to the anus, hidden by numerous skin folds arranged radially to the anal opening (personal osbervation). Two sperm ducts, originating from the last portion of the testis and converging caudally to form a single main duct which opens in an urogenital papilla, also characterized the reproductive systems of other syngnathids species such as *Nerophis ophidion* and *Hippocampus guttuatus*, although the male gonad organization in these species has not been analysed in details.

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However, at least in *Hippocampus kuda*, testes have a morphology similar to that reported for the *Syngnathus* species (Laksanawimol 2004). In the testis of both non–brooding and brooding males of *H. kuda*, examined during the reproductive season, spermatogonia and primary spermatocytes were found along the entire length of the testis, whereas secondary spermatocytes and spermatids were only found inside the lumen. This seems to confirm that in syngnathids of *Hippocampus* genus, as well as those of *Syngnathus*, the testis organization is of unrestricted lobular type and the spermatogenetic process is of the semicystic type (Carcupino *et al*. 1999; Biagi *et al*. 2015).

This type of spermatogenesis is currently known in few species belonging to several teleost groups (Selman and Wallace 1986; Bazzoli and Godinho 1991; Mattei *et al.* 1993; Manni and Rasotto 1997; Yoneda *et al.* 1998a; Carcupino *et al.* 1999; Giacomello *et al.* 2008; Srivastava and Singh 1994; Andrade *et al*. 2001; Mazzoldi 2001; Muñoz *et al*. 2002; Sàbat 2002; Laksanawimol 2004; García-López *et al.* 2005; Hernández *et al*. 2005; Shahin 2006; Sàbat *et al*. 2009; Magalhaes *et al*. 2011). It consists of a precocious opening of the germinal cysts, causing an asynchronous maturation of spermatids and the simultaneous presence of germ cells at different developmental stages inside the testis lumen. Moreover, in *Syngnathus* species, developing germ cells inside the lumen are mono- and, more frequently, poly-nucleate and poly-flagellate cells. Individualization of mature sperm seems to occur at the end of spermiogenesis, so the cytokinesis seems to be abolished or at least delayed (Carcupino *et al.* 1999; Biagi *et al.* 2015). Poly-nucleate developing germ cells has not been reported in *H. kuda* (Laksanawimol 2004).

Syngnathids are also known to produce a very low number of sperm. The functional sperm : egg ratio was estimated to be about 191 : 1 in *S. abaster* (Dzyuba

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et al. 2008) and even much lower 5 : 1 in *Hippocampus kuda* (Van Look *et al*. 2007). These values are numerous orders of magnitude lower than estimated in the zebrafish (*Danio rerio*) (48000 : 1), which was considered to have one of the lower sperm concentration in fish (Stokley *et al.* 1996). Probably due to this low sperm concentration, no sperm were observed by Laksanawimol (2004) in the testis lumen of both non-brooding and brooding male of *H. kuda*, whereas dimorphic sperm were reported in the same species by Van Look *et al.* (2007). In this last study, however no data on sperm ultrastructure were shown. Sperm polymorphism was also reported in a freshwater population of *Syngnathus abaster* (Dzyuba *et al.* 2008), which belong to a monophyletic lineage within the urphorine subfamily including *Syngnathus* and to *Hippocampus* species (Wilson and Orr 2011). Recently we have demonstrated that in two population the brackish water form of the same species *S. abaster* mature sperm are great variable in their morphometric traits, but they can not be distinguished in different morphotypes (Piras *et al.* in press).

The aim of this paper was to analyse the male gonad morphology, the spermatogenetic process and both sperm traits and sperm ultrastructure in the seahorse *Hippocampus guttulatus*.

Materials and Methods

Sampling

Four adult male of *Hippocampus guttulatus* were sampled from Venice lagoon (Veneto), during the reproductive period (May-September 2013). Alive specimens, delivered to the laboratory within 3 h, were sacrificed by exposure to the anaesthetic 3-aminobenzoic acid ethyl ether (MS-222, Sigma-Aldrich) for 10 min, and then processed for microscopic analysis.

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Light microscopy:

Four testes, obtained by two specimens, were fixed in aqueous Bouin's fixative, dehydrated in a graded ethanol series, cleared in Bioclear and finally embedded in paraffin wax. Sections (5µm) were stained with Eosin and Mayer Emallume (Mazzi 1977) and processed for the morphological analysis using a Zeiss Axiophot light microscope (ZEISS, Oberkochen, Germany).

Gonads dissected by the fifth male were gently open in order to obtain aliquots of seminal fluid containing cells eventually free inside the testicular lumen. Aliquots of 20 µl each were seminal fluid fixed in 5% glutaraldehyde were placed on poly-lysine coated coverslips (1 mg mL-1; Sigma P1274) and air-dried. Then, samples were stained with 0.1% Toluidine Blue in aqueous solution and analysed with a Zeiss Axiophot light microscope.

Transmission electron microscopy (TEM)

Two additional male gonads were fixed for 2h in 4% paraformaldehyde-5% glutaraldehyde buffered with sodium cacodylate (0.1M and pH 7.2). Specimens were then rinsed overnight in the same buffer, post-fixed for 1h in 1% osmium tetroxide buffered with sodium cacodylate. After dehydrating in an ethanol series, samples were embedded in Epon 812 resin. Thin sections, of about 80 nm thick, were cut with a Reichert Ultracut ultramicrotome (Leica Microsystems, Wetzlar, Germany), and stained with uranyl acetate and lead citrate. Samples were then examined with a Jeol Tem 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan).

Morphometry

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Intact spermatozoa (N = 20), obtained from the gonads of one male stained with Toluidine blue (see above) were analysed in order to study spermatozoa morphometric, such as head length (including nucleus and midpiece) and the length of the flagellum. Abnormal, broken or difficult to measure spermatozoa were discarded.

Digital images of mature spermatozoa were acquired with a digital camera Nikon DS-FI1 connected with DS-L2 control unit and mounted on an optical microscope Nikon Eclipse 80i (Nikon, Shinjuku, Japan). The measurements were made using the program Tpsdig2.

Results

The paired testes were semi-translucent organs (Fig. 1A) adhering to the abdominal cavity by extensions of the mesentery. No accessory structures connected with the testes were recognizable analysing serial histological sections. However, when the two testes were dissected together with the anus and the connective tissue of the belonging urogenital region (as shown in Figure A), two large vesicles were evident. Each testis was characterized by a large central lumen and a thin wall (Fig.1B). The latter consisted of the germinal epithelium and a vascularized fibrous capsule, consisting of connective tissue rich in muscle fibres (Fig.1B-E). The tissue of the capsule is continuous and did not enter the organ where inter-germinal compartments were not observed (Fig. 1B).

The germinal epithelium showed the typical organization in spermatocysts resting on the basal membrane and formed by germ cells enveloped by Sertoli cells (Fig. 1 C-E). Along the entire length of the testis, the germinal epithelium contained spermatocysts inside which spermatogonia and primary spermatocytes were easily

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recognizable (Fig. 1 C-D). Developing spermatids and mature sperm were never observed inside the spermatocysts.

Two types of large mono-nucleate cells, aflagellate and flagellate cells, together with thin mature spermatozoa were observed inside the testis lumen (Figs 1F, 2-4). The aflagellate cells had irregular shape and were characterized by numerous cytoplasmic protrusions. Their nuclei were round in shape and showed a large eccentric spherical nucleolus (Figs 1F, 2A-B). Their cytoplasm was rich in rough endoplasmic reticulum, Golgi complexes and droplets of different size and density (Fig. 2B). Cells of similar appearances were visible emerging from the surface of the germinal epithelium facing the central lumen (Figs 1C, E, 2A). Some of these cells appeared unquestionably appear to be spermatids; in their cytoplasm a forming flagellum was recognizable (Fig. 2C-D)

In contrast, the flagellate cells were characterized by nuclei with nucleoli not more evident and cytoplasm containing a less amount of droplets and all components typical of the future sperm midpiece (Fig. 3). They were mitochondria, which surrounded a cytoplasmic anal, and an emerging flagellum, running inside the cytoplasmic canal. Patches of electron-dense material begin to accumulate in close association to the inner membrane of the cytoplasmic canal (Fig. 3B, insert, C).

Mature sperm of *Hippocampus guttulatu*s (Fig. 4) were anacrosomal and mono-flagellate cells of several tens of microns in length (51.35 ± 3.68 µm, n = 20). The spermatozoa consisted of three distinct portions: head, midpiece and flagellum. The head was cylindrical in shape and entirely occupied by the nucleus. (2.78 ± 0.19 µm, n = 20) (Fig. 4A). At the basal end of the nucleus, a deep nuclear fossa was present, and inside it, both the basal and the distal centrioles were localized. The midpiece was

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clearly marked under the nucleus by two mitochondrial rings. These were housed inside a cytoplasmic collar, which was separated from the first portion of the flagellum by a deep cytoplasmic canal (Fig- 4B-D). The plasma membrane of the collar lining the canal appeared closely associated to a sheath of electron-dense material arranged in ring-like structures regularly spaced. The flagellum had an internal "9+2" axoneme, originating from the distal centriole and surrounded by the plasma membrane which forms two lateral fins (Fig. 4E).

Discussion

Like other syngnathids (Carcupino *et al*. 1999; Biagi *et al*. 2015), the reproductive apparatus in the seahorses, *Hippocampus guttulatus*, is formed by the paired testes within which does not open any type of accessory organ. The testes are located inside the coelomic cavity between the intestines and the urinary system. In teleost, as well as in other Syngnathidae species previously analyzed (Biagi *et al*. 2015), the urinary system generally has a unique urinary bladder. The latter appears as a elongated vesicle, located in middle-dorsal position in respect to the testes (personal observations). In contrast, in *Hippocampus guttulatus*, or at least in the sample used to obtain the testes shown in Figure A, the typical urinary bladder appears to be absent. In its place, two large vesicles are present. Further studies are needed to determine whether these vesicles correspond to two bladders and if this unusual condition is typical of the species or it is an abnormal condition of an single individual.

Each testis is atypically constituted by a single and continuous germinal compartment, surrounded by a single and continuous somatic compartment. Each testis appears as a tubular organ characterized by a unique testicular lumen surrounded

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by two concentric layers, the tunica albuginea and the germinal epithelium, separated by the basement membrane.

The germinal compartment extends to the periphery of the testis and terminates blindly, and is formed by a germinal epithelium with the tripartite organization, which is typical of teleost testis; i.e. germ cells are surrounded by Sertoli cells forming spermatocysts, which rest on the basal lamina. Inside spermatocysts, spermatogonia are clearly distributed along the entire length of the testis. Based on these data, the testis organization in *H. guttulatus* may be attributed to the unrestricted lobular type, typically found throughout the Neoteleostei, including other syngnathids (Biagi *et al.* 2015; Laksanawimol 2004), except for the atherinomorphs (Parenti and Grier 2004).

In agreement with previous data on syngnathids testes (Carcupino *et al*. 1999; Biagi *et al.* 2015), the germinal spermatocysts of all reproductive males here analysed only contain spermatogonia and primary spermatocytes. Developing spermatids, which are always mono-nucleate cells, identifiable by both the presence of the emerging flagellum, and nuclei characterized by different degrees of chromatin condensation, are only visible inside the testis lumen together with mature sperm. The spermatogenetic process may therefore be attributed to the semicystic type. In the semicystic spermatogenesis, the cysts rupture at the spermatocyte or spermatid stage, so germ cells only partly develop inside them, producing an asynchronous maturation of spermatids and thereby reducing the number of simultaneously mature sperm (Mattei and Mattei 1978).

Because developing spermatids of *H. guttulatus* are always mono-nucleate cells, the semicystic spermatogenetic process seems to have the typical features, i.e. spermatocytes and/or spermatids are released after that cytokinesis is completed

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among isogenetic germ cells. Therefore inside the lumen, the germ cells advance individually through spermiogenesis. In contrast, in the *Syngnathus* species, the cytokinesis seems to be abolished or at least delayed. Indeed, as previously documented in *Syngnathus abaster* and *S. acus* (Carcupino *et al.* 1999), and recently confirmed in the same and in other species of the same genus (Biagi *et al.* 2015), developing germ cells inside the lumen of these species are mono- and, more frequently, polynucleate and polyflagellate cells. A possible functional explanation of the delayed cytokinesis in *Syngnathus* species will be discussed below.

The semicystic spermatogenesis was interpreted as a possible mechanism, evolved several times in different teleost taxa, able to reduce the cost of sperm production. Therefore, it seems to be crucial particularly in those species where a small ejaculate size is justified by their low fecundity, monogamous mating system, absence of sperm competition and presence of male parental care (Rasotto *et al*. 1992; Marconato and Rasotto 1993; Mazzoldi 2001). This is also the case of *H. guttulatus*, which has small ejaculate size, low fecundity, male parental care, absence of sperm competition, and strictly monogamous mating system (for references c.f. Sanna *et al.* 2008; Wilson *et al.* 2003).

Moreover, together with developing spermatids and mature spermatozoa, another type of mono-nucleate cells is present inside the testicular lumen of *H. guttulatus*. These cells are aflagellate and characterized by a large amount of cytoplasmic droplets. Large droplets-containing cells were also reported in several species of the *Syngnathus* genus, such as *S. schlegeli* (Watanabe *et al.* 2000), *S. abaster, S. acus, S. tenuirostris* and *S. typhle* (Carcupino *et al.* 1999; Biagi *et al.* 2015), although in these species these cells are polynucleate cells as well as developing spermatids. In both reproductive males of *H. guttulatus* and *Syngnathus*

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species however, the aflagellate cells are frequently observed both free into the testicular lumen and coming out from the epithelium. Moreover, like in the *Syngnathus* species, a smaller amount of droplets of different size and electron-density may be also recognizable in the cytoplasm of developing spermatids of *H. guttulatus*. All these data support the hypothesis first reported by Carcupino *et al.* (1999), and recently reformulated by Biagi *et al.* (2015), that these aflagellate cells represent the youngest germ cells released inside the lumen at the spermatocyte or a very early spermatid stage, after having accumulated a large amount of material in form of droplets. These droplets progressively reduce in size and number during the germ cell maturation.

It was speculated that the large amount of droplets could be involved in several function. (i) In the formation of an abundant and fibrous seminal fluid, having the function to trap the very low number of mature sperm produced by syngnathids, avoiding sperm loss during mating and (ii) in the metabolic supply to the developing germ cells, which are released in a very early spermatogenetic stage (Biagi *et al.* 2015).

As regards the first possible function, it must be said that in some teleost species (such as *Ophidion marginatum* and *Lophiomus setigerus*), which lay eggs in a gelatinous mass (Fahay 1992; Yoneda *et al.* 1998b) as syngnathids do, the semicystic spermatogenesis was thought to be somehow related to the secretion of abundant thick seminal fluid. The latter was reported to act in maintaining sperm together and facilitating fertilization of egg mass (Muñoz *et al.* 2002).

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About to the second function, it should not be forgotten that in the semicystic spermatogenetic process Sertoli cells cannot regulate and support the metabolites transfer towards the developing germ cells, when these are free inside the lumen.

If the functions of the large amount of droplets accumulated in the cytoplasm of syngnathids developing germ cells are the same, why are these cells in *Syngnathus* species polynucleate? The answer to this question is not so easy. The delayed cytokinesis observed in the semicystic spermatogenesis of *Syngnathus* species, was recently speculated to be correlated to the need in limiting the reduction of cytoplasm and organelles. An early cytoplasmic division among isogenetic cells could endanger the production and accumulation of a sufficient amount of material employed both in the energy requirements of each germ cell and the production of the seminal fluid (Biagi *et al.* 2015). This hypothesis seems not be supported by the absence of polynucleate cells in *H. guttulatus.* A possible explanation of these different data could be sought in a less need in *H. guttulatus* in producing a large amount of fibrous seminal fluid. Although *H. guttulatus* has lower concentration of sperm respect the *Syngnathus* species, it has a closed pouch and a monogamous mating system. Because of that *H. guttulatus* males mate much less frequently respect *Syngnathus* species, and not release sperm for a long period of time. These features could reduce the loss of sperm during fertilization. Indeed, the *Syngnathus* species apparently have a larger amount of sperm, but they have a semi-closed pouch and a polygamous mating system.

According to the ultrastructural analysis of all types of flagellated cells recognizable inside the testis lumen of *H. guttulatus*, we have identified only one type of mature sperm. They are characterized by an elongated head, completely occupied by a nucleus with condensed chromatin, a short midpiece characterized by two

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mitochondrial rings surrounding the first portion of the axoneme, and a long flagellum. This datum does not seem to support the presence of dimorphic sperm reported in *H. kuda* (Van Look *et al.* 2007*)*. In this latter species, type 1 spermatozoa, which were considered the only sperm type taking part in fertilization, seem to have similar morphology and morphometric traits of *H. guttulatus* sperm. The *total sperm* length is 51.35 ± 3.68 µm (mean ± standard deviation, N = 20) in *H. guttulatus* and 49.3 µm (median length of flaglellum N = 44) in *H. kuda*. The head length is 2.78 ± 0.19 µm (N = 20) in *H. guttulatus* and 3.7 µm (median length, N = 44) in *H. kuda.* In contrast, type 2 spermatozoa of *H. kuda,* that were interpreted a remnant population of the primitive externally fertilizing sperm type (aquasperm) no taking part in fertilization. were reported to have a very large spherical head.

However, the difference between our study and that of Van Look and co-authors (2007) could have been arisen by several reasons. First, the two studies differ in the methods performed to obtain measurable sperm. We used fixed spermatozoa, whereas Van Look *et al.* (2007) used living cells. Second, we measure only mature (apparently fully formed) and intact sperm (sperm with all their three portions clearly visible i.e. nucleus, midpiece and flagellum), whereas Van Look *et al.* (2007) measured all sperm, for which it was possible to obtain clear images. This could explain, for example, the high difference in the minimum and maximum values of flagellar length obtained in the two studies; 47.68 (minimum) and 61.49 (maximum) µm in our study for *H. guttulatus*, and 6.3 and 69.3 µm for *H. kuda*.

Last, but not least, the spermatogenetic process in *H. ku*da is not known. Indeed, if *H. kuda* has, like *H. guttulatus* and other syngnathids species (i.e. *Syngnathus abaster*, *S. typlhe*, *S. tenuirostris*, *S. acus* and *Phyllopteryx taeniolatus*) (Biagi *et al.*

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2015; Forsgren and Young 2008) a spermatogenetic process of semicystic type, it should be very likely that the type 2 sperm are developing spermatids.

Moreover, both the simultaneous presence of flagellate and aflagellate cells inside the testis lumen, determined by the semicystic spermatogenesis, and the difficulty to see the thin flagellum in the histological sections, may have induced other authors to interpret these cells as aflagellate spermatozoa. This could be the case of Miranda-Marure *et al.* (2004) for the aflagellate sperm reported in *Microphis brachyurus lineatus*.

Functional sperm with elongated head similar in morphology to those of *H. guttulatus* and *H. kuda* were also reported in other syngnathids species, such as *Syngnathus abaster*, *S. typhle*, *S. tenuirostris*, *S. acus* and *Nerophis ophidion* (Carcupino *et al.* 1999; Ah-king *et al.* 2006; Biagi *et al.* 2015 and Piras *et al*. in press). A similar type of sperm are also present in some Blenniidae (Lahnsteiner *et al*. 1990) in *Lepadogaster lepadogaster* (Mattei and Mattei 1978), and in *Ophidion barbatum* (Hernandez *et al*. 2005), all species with external fertilization and semicystic spermatogenesis. In general, spermatozoa with elongated heads are related to internal fertilization (Jamieson and Leung 1991), an explanation that does not match any of the all above mentioned species. According to Burns *et al*. (1995), the elongated nucleus may also facilitate the storage of the spermatozoa in the testicular ducts. Nevertheless, in the specific cases of *O. barbatum* (Hernandez *et al*. 2005), and syngnathids species (our personal observations) no packaging of spermatozoa were observed. On the base of these data, and according to the hypothesis first formulated by Biagi *et al.* (2015) a third possibility to explain the elongated head of syngnathids sperm could be related to their need to cross through the gelatinous mass of maternal origin to reach the eggs.

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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. Aflegellate 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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associated to the internal membrane of cytoplasmic canal (arrow); axoneme (Ax); spermatids cytoplasm (Cy); cytoplasmic canal (CC); droplets (D); mitochondria (M); nucleus (N). Scale bar: A= 1.5 µm; B = 1 µm; Insert = 625 nm; C = 200 nm.

Figure 4. **A**. Light microscopic image of *Hippocampus guttulatus* mature sperm. **B-E.** Transmission electron micrographs of mature spermatozoa showing (i) elongated anacrosomal sperm heads with completely condensed nuclei; (ii) deep nuclear fossa; (iii) distal and basal centrioles; (iv) "9+2" axoneme originating from distal centriole; (v) short midpiece characterized by the cytoplasmic collar occupied by two rings of mitochondria; (vi) cytoplasmic canal; (vii) numerous rings of electron-dense material between the mitochondria and the internal plasma membrane of the collar. Electron-dense material (arrow); axoneme (Ax); basal and distal centrioles inside the nuclear fossa (C); cytoplasmic canal (CC); cytoplasmic droplets (D); flagellum (F) sperm head (H) mitochondria (M); sperm midpiece (MP); nucleus (N).

Scale bar: A = 4.2 µm; B = 270 nm; C = 200 nm; D, E = 110 nm.

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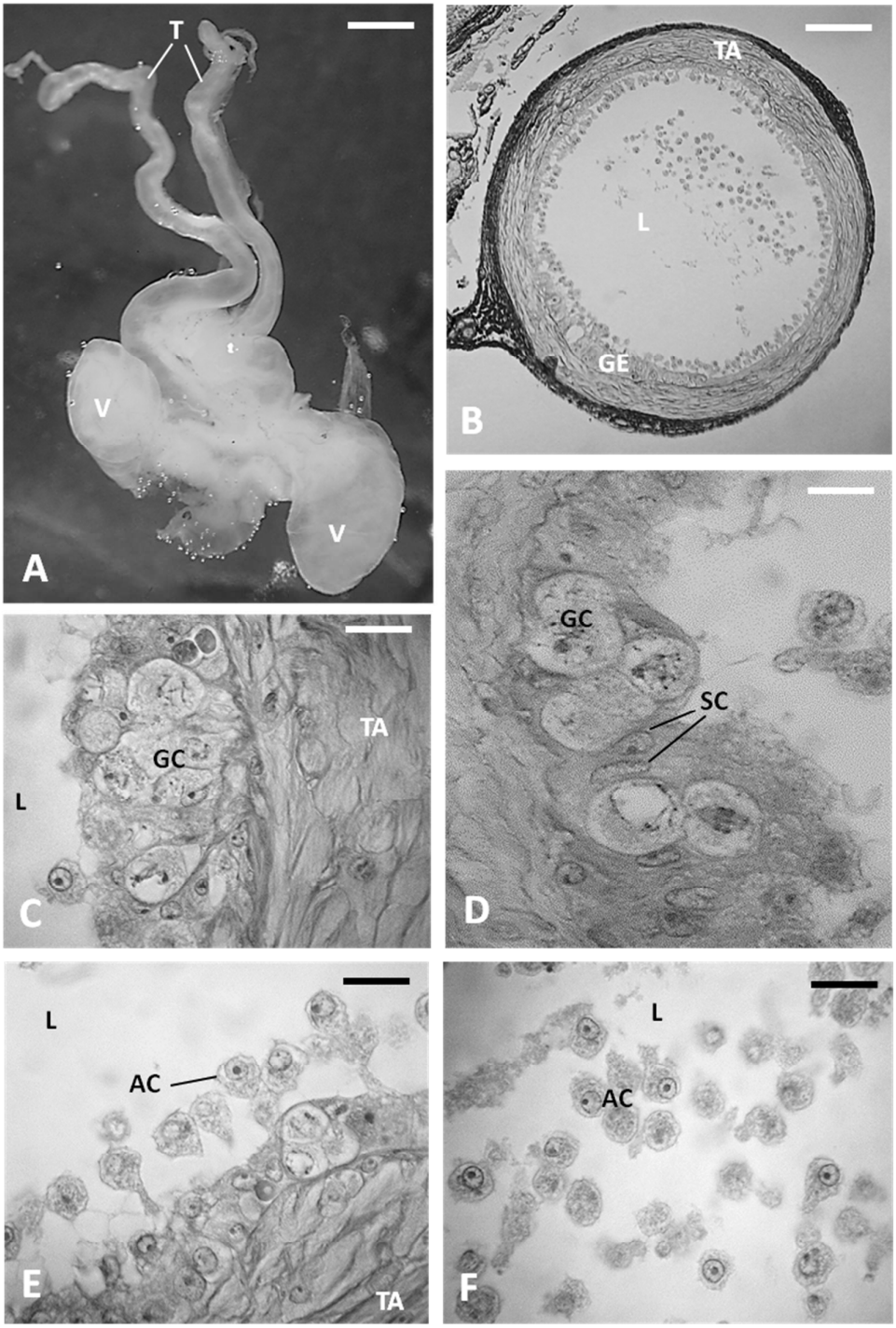

Fig. 1

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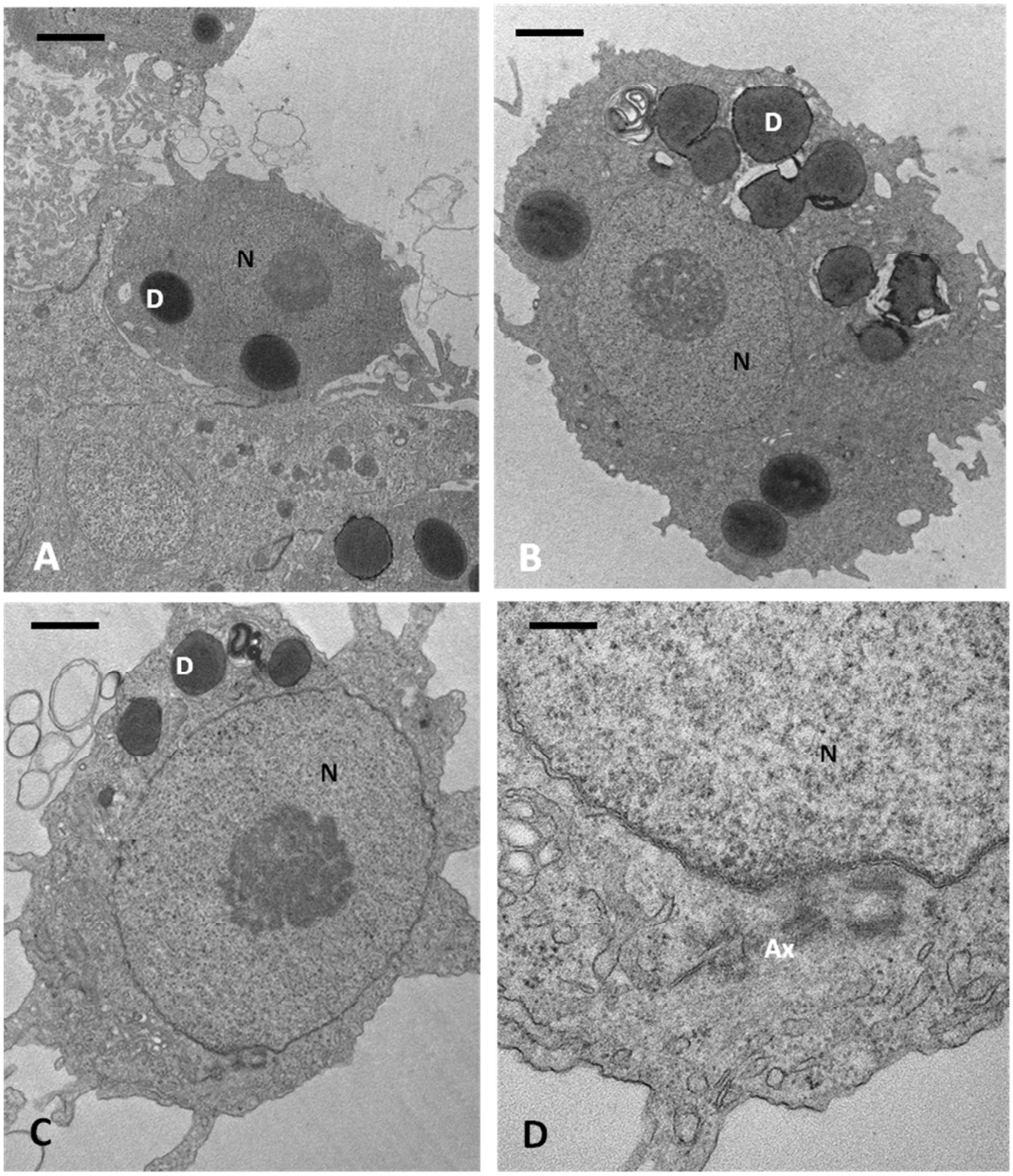

Fig. 2

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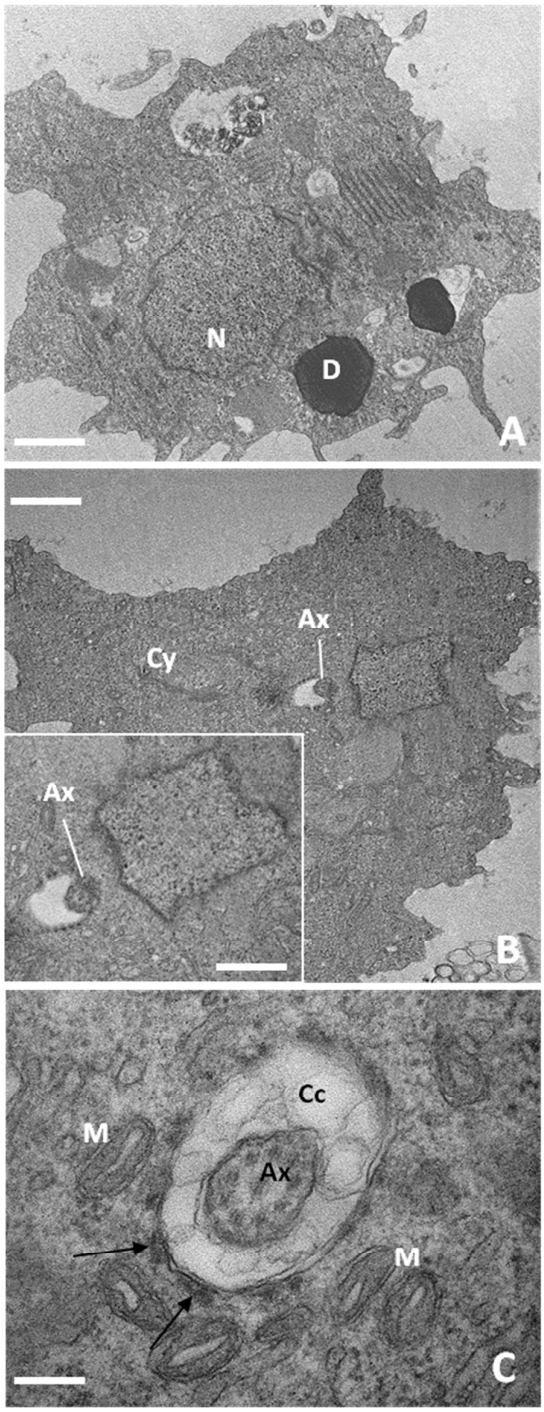

Fig. 3

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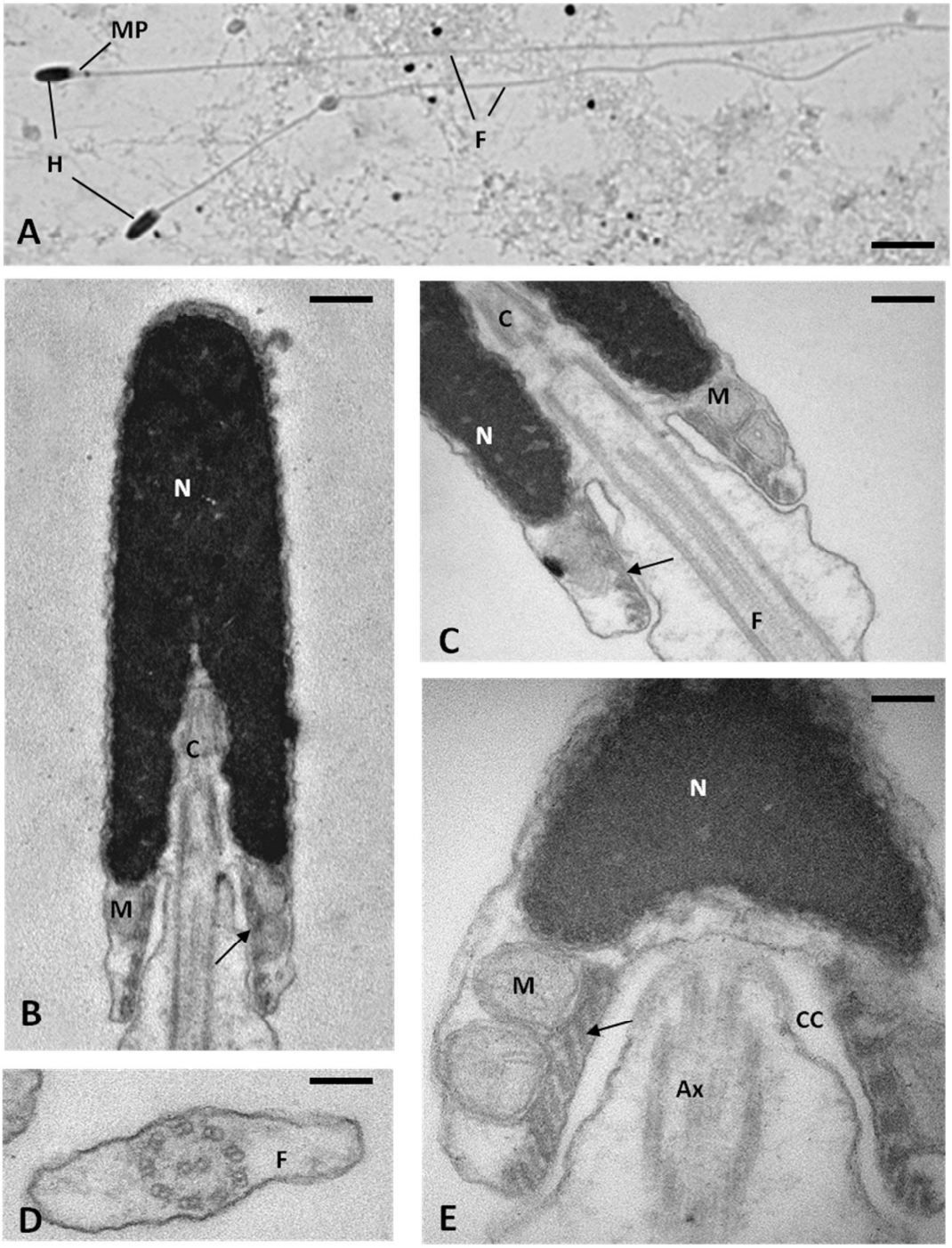

Fig. 4