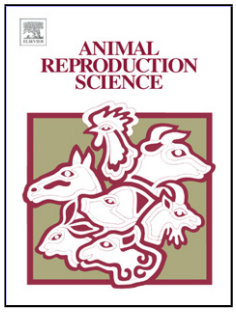


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# Testicular structure and development of germ cells of *Hypophthalmus marginatus* Valenciennes 1840 (Siluriformes: Pimelodidae)

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**ABSTRACT**

The present study was conducted to assess the testicular structure and germ cell ultrastructure of *Hypophthalmus marginatus* during spermatogenesis. Semen and sections of the mid-region of the testis were collected, processed, and analyzed using optical and electron microscopy. Macroscopically, the testes of *H. marginatus* were filiform, and the testicular parenchyma was composed of spermatogenic cells that proliferated, organized within spermatic cysts. During spermiogenesis, spermatids had no nuclear rotation. The proximal centriole was perpendicular to the distal centriole, characteristic of type III spermiogenesis. Spermatozoa were released into the lumen of the seminiferous tubules and had an ovoid head without an acrosome, condensed nucleus, and shallow nuclear fossa. The midpiece was short, with a single long flagellum. The flagellum had the typical axoneme structure, with nine pairs of peripheral and a central pair of microtubules. The thin end piece comprised only peripheral microtubules. Spermatogenesis in *H. marginatus* features filiform testes, cystic spermatogenesis, and type III spermiogenesis.

**Keywords**: Histology; Pimelodidae; Spermatozoa; Teleost; Testis

## 1. Introduction

Knowledge of the testis anatomy and spermatogenesis aids in understanding important aspects of the reproductive physiology of fish (Santos et al., 2001; Nóbrega et al., 2009). Based on the organization of the germinal compartment and distribution of spermatogonia within the epithelium, the testes of teleost fishes are classified as having three types: anastomosed tubular, unrestricted spermatogonial lobular, and restricted spermatogonial lobular. In anastomosed tubular testes, the seminiferous tubules connect to form a continuous tubular system, characteristic of primitive fish, including Siluriformes (Grier, 1993). In unrestricted spermatogonial lobular testes, the spermatogonia are located along the entire length of the lobules (Grier et al., 1980; Grier, 1993); and in restricted spermatogonial lobular

testes, spermatogonia are restricted to the terminal end of the lobules (Grier and Uribe-Aranzábal, 2009; Mazzoni et al., 2014). Lobular testes are characteristic of derived Teleostei (Parenti and Grier, 2004; Grier and Uribe-Aranzábal, 2009).

In fish, spermatogenesis can be cystic or semicystic. Cystic spermatogenesis is completed within cysts, where germ cells are attached to cytoplasmic residues; these cysts are surrounded by cytoplasmic processes of Sertoli cells (Mattei et al., 1993; Matta et al., 2002; Schulz et al., 2010). At the end of spermiogenesis, the cysts rupture and the spermatozoa are released into the lumen of the seminiferous tubules. These types of fish have synchronous development of germ cells derived from a single spermatogonium. In contrast, in semicystic spermatogenesis, the spermatids are released into the lumen of the seminiferous tubules, and these cells undergo asynchronous development (Mattei et al., 1993; Schulz et al., 2010).

Teleost fish have three types of spermiogenesis, based on morphological characteristics of the spermatozoa, specifically in the presence or absence of nuclear rotation and the location of the flagellar axis (Mattei, 1970; Quagio-Grassiotto and Oliveira, 2008). With type I spermiogenesis, the flagellar axis is perpendicular to the nucleus, and the centriole complex penetrates into the nuclear fossa, as observed in *Diplomystes mesembrinus* (Quagio-Grassiotto et al., 2001), *Microglanis aff. parahybae* (Quagio-Grassiotto et al., 2005), *Callichthys callichthys* (Spadella et al., 2007), and *Astyanax bimaculatus* (Melo et al., 2017). With species that have type II spermiogenesis, the nucleus does not rotate and the centriole complex remains parallel to the nucleus, with a smaller nuclear fossa, as described for members of the genera *Lebiasina* and *Piabucina* (Santana et al., 2013). With those species that have type III spermiogenesis, the nucleus does not rotate, the flagellum develops centrally and perpendicular to the nucleus, and no nuclear fossa is formed (Quagio-Grassiotto and Oliveira, 2008; Melo et al., 2017).

The freshwater mapará catfish *Hypophthalmus marginatus* (Valenciennes, 1840), a member of the family Pimelodidae, order Siluriformes (Teugels, 1996), is a valuable source of income and nutrition for communities in the Amazon region (Juras et al., 2004). Knowledge of spermatogenesis and spermatid differentiation in fish species is important for phylogenetic studies, as well as providing morphological data on spermatozoa for studies of semen cryopreservation (Nóbrega et al., 2009; Schulz et al., 2010). Because little is known regarding the development of male gametes in *H. marginatus*, the present study was conducted to characterize the testicular structure and describe spermatogenesis in this species, based on histological and ultrastructural analyses of spermatogenic cells during sexual development.

## 2. Materials and methods

### *2.1. Sample collection*

Adult males (n = 39) of *H. marginatus* were captured from the Tocantins River downstream from the Tucuruí Hydroelectric Dam (3°49'48.9"S, 49°39'07.0"W), in the eastern Amazon (Brazil). The fish were caught with gillnets, 100 m long and 3.0 m high, with an 8-9 cm mesh size. Captured fish were anesthetized with benzocaine (0.1 g/L) and euthanized by intraperitoneal administration of sodium pentobarbital (60 – 100 mg/kg), following the Guidelines of the National Council of Animal Experimentation Control (process 27884-2), and was approved by the Ethics Committee on Animal Use of the Federal University of Pará (protocol CEUA 6045280219). A ventral incision was subsequently performed to remove the testes, which were analyzed macroscopically to describe the shape and location within the coelomic cavity. Each testis was divided into three sections; the middle region of each testis was cut into small fragments and immediately fixed either in Bouin’s or in Karnovsky's solution (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3).

Three males were selected and anesthetized for semen collection, which was performed by applying mild abdominal pressure. Semen was collected in 1-ml syringes and fixed in Karnovsky's solution. After fixation, the biological material was packed in an isothermal box with ice, transported to the laboratory of Cell Ultrastructure of the Federal University of Pará (UFPA), and subjected to histological procedures.

*2.2. Light microscopy*

Fragments of testis were fixed in Bouin's solution for 24 h and subjected to routine histological processing for paraffin embedding (Prophet et al., 1995). Samples were subsequently sectioned into 5-µm-thick sections, using a RM2245 microtome (Leica Microsystems, Germany), and stained with hematoxylin-eosin. Stained sections were analyzed, and photomicrographs were obtained using an Eclipse Ci-S light microscope (Nikon, Japan) connected to a Nikon DS-Ri1 digital camera.

*2.3. Transmission electron microscopy (TEM)*

Fragments of the testis were fixed in Karnovsky's solution for 24 h and then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h, and contrasted in block in 1% uranyl acetate. The tissues were then dehydrated in acetone, and infiltrated and embedded in Epon 812. To define the study area, 0.5 µm semithin sections were prepared on a UC6 ultramicrotome (Leica Microsystems, Mannheim, Germany). The sections were subsequently stained with toluidine blue and analyzed and photo-micrographed using an Eclipse Ci-S light microscope connected to a DS-Ri1 digital camera). The 70-nm ultrathin sections were subsequently analyzed and photographed using a TEM LEO 906E electron microscope (Carl Zeiss, Oberkoshen, Germany).

*2.4. Scanning electron microscopy (SEM)*

Some of the testicular sections as well as 500-µl aliquots of raw semen were fixed in Karnovsky´s solution for 24 h at 4 °C. Then, a drop of the fixed semen sample was placed on a poly-L-lysine-coated coverslip. The samples were subsequently post-fixed in a 1% osmium tetroxide solution buffered with sodium cacodylate (0.1 M, pH 7.3) for 2 h at room temperature and dehydrated in a graded ethanol series. The slides were subsequently mounted on conductive tabs and vacuum-dried in a CPD 030 BAL TEC Critical Point Dryer, followed by sputter-coating with gold for 2 min. Images were obtained using a LEO 1430 scanning electron microscope (LEO-ZEISS, Cambridge, England).

*2.5. Morphometry and statistical analysis*

Nuclei ($n$ = 100) were measured for each type of germ cell (spermatogonia, spermatocyte, spermatid and spermatozoa). To assure that each cell was counted only once, the nuclei were measured only when the central region of the nucleus was visible in the image. Samples were cut in approximately 24 serial sections of 0.5 µm thickness. This method for quantifying the cell diameter was adapted from Rodrigues et al. (2017). The slides were evaluated under a photomicroscope with the software NIS-elements BR (4.00.07-bit), and measurements were made at 40X magnification. Means were assessed for normality using the Shapiro-Wilk test and analyzed using the Kruskal-Wallis test ($P < 0.05$) (Zar, 1999). All analyses were performed using the R Development Core Team Program (2016).

## 3. Results

*3.1. Testis morphology*

Macroscopically, the testes of *H. marginatus* are paired, filiform organs of equal size. The testes are fused caudally, forming a single sperm duct leading to the urogenital papilla

(Fig. 1A). Results from histological analyses indicated the tunica albuginea invaginated within the testis, resulting in anastomosed seminiferous tubules (Fig. 1B). When assessed using TEM, the seminiferous tubules were found to consist of a germinal epithelium. This epithelium has Sertoli cells, and spermatogenic cells in different stages including spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. The spermatogenic cells were organized in cysts (Fig. 1C and 1E). Blood vessels and Leydig cells were observed among the seminiferous tubules, in the interstitial space (Fig. 1D).

*3.2. Proliferative phase*

Spermatogonial germ cells in the testis of *H. marginatus* could be characterized as being of two types, primary (type SgA) and secondary (type SgB) spermatogonia, distributed randomly along the tubular wall. These spermatogonial types differed in chromatin condensation, number of nucleoli, and cell size.

Type SgA spermatogonia were the largest germ cells had a mean diameter of 7.79 ± 0.44 μm. These cells were located in the basal portion of the germinal epithelium, surrounded by cytoplasmic extensions of Sertoli cells. These spermatogonia were ovoid and contained a nucleus with evenly distributed euchromatin and a well-defined nucleolus. The clear and abundant cytoplasm contained ribosomes; mitochondria of various shapes; and nuages, electron-dense structures associated with the nuclear membrane and/or with mitochondria (Fig. 2A and 2B).

Type SgB spermatogonia were grouped in cysts. The nucleus was round, with heterochromatin, and contained one to three nucleoli. The mean diameter of these nuclei was 5.17 ± 0.21 μm. In the cytoplasm, mitochondria were segregated to one of the cell poles (Figs. 2C-E).

The Sertoli cells surrounding the cysts contained an elongated nucleus with euchromatin and a prominent nucleolus (Fig. 2A and 2B). A zonula adherens was visible between the Sertoli cells and spermatogonia (Fig. 2F and 2G).

*3.3. Meiotic phase*

Primary spermatocytes in prophase I were identifiable by the chromatin undergoing condensation and the cell size. Primary spermatocytes in the leptotene/zygotene stage had a mean diameter of 4.22 ± 0.32 μm. In the leptotene stage, the nucleus was ovoid, with fine granular chromatin beginning to condense (Fig. 3A). In the zygotene stage, nucleoli were absent and the synaptic complex began to form near the nuclear membrane (Fig. 3B). In the pachytene stage, the primary spermatocytes showed many synaptonemal complexes (Fig. 3C). The mean nuclear diameter was 4.14 ± 0.40 μm. In the diplotene stage, the nuclear chomatin reached its maximum degree of condensation; the mean nuclear diameter was 3.76 ± 0.41 μm. At diakinesis, the chromatin became dense and irregularly shaped; the mean nuclear diameter was 3.56 ± 0.36 μm. At metaphase I, primary spermatocytes were centrally located, had condensed chromatin (Fig. 3D, inset) with a mean nuclear diameter of 3.08 ± 0.42 μm. Anaphase and telophase were not observed.

Secondary spermatocytes (SII) were identified by the sparse cytoplasm, which contained vesicles, centrioles, and a few mitochondria. The nucleus contained condensed chromatin (Fig. 3E, inset).

*3.4. Spermiogenesis phase*

Three phases of spermatids were identified in *H. marginatus*: initial (E1), intermediate (E2), and final (E3). These phases were classified based on the extent of chromatin compaction, elimination of cytoplasm, and flagellum formation.

Phase E1 spermatids were smaller than the spermatocyte (Fig. 6). The nucleus was ovoid, with condensed chromatin, and measured 2.55 ± 0.20 μm. The cells were interconnected by cytoplasmic bridges; these cells contained vesicles and mitochondria, and had a few flagella in the initial stage of development (Fig. 4A). In phase E2 spermatids, the nucleus had a dimeter of 1.81 ± 0.14 μm and the chromatin was more condensed and the nucleus did not rotate. The proximal centriole was located near the nucleus and perpendicular to the distal centriole. Some spermatids were still interconnected by cytoplasmic bridges; mitochondria and vesicles could be observed in the remaining cytoplasm (Fig. 4B, inset-E). Phase E3 spermatids had a thin layer of cytoplasm surrounding the nucleus, a centriolar complex surrounded by mitochondria, and vesicles. The largest elimination of the residual cytoplasm inside the cysts occurred at this stage. The developing flagellum was perpendicular to the nucleus (Fig. 4F and 4G). The nucleus diameter was 1.52 ± 0.15 μm. From the morphometric data for each germ cell ($n$ = 100), there appeared to marked differences in reduction in nuclear diameter during spermatogenesis ($H_{(10,\ n\ =\ 1,100)}$ = 1039, 642; $P$ = 0.001; Fig. 6).

Assessments of scanning electron micrographs indicated that during the final stage of spermiogenesis, cysts containing E3 spermatids had an irregular surface, where these differentiated into spermatozoa. When the cytoplasmic processes of the Sertoli cells ruptured, there was release of numerous spermatozoa into the lumen of the seminiferous tubules (Fig. 5A-G).

A combination of histology, TEM and SEM assessments was used to detail the structure of mature spermatozoa (Figs. 1C, inset; 4H-L, and 5E-G). These cells were the smallest germ cells, being 1.30 ± 0.12 μm in diameter. Spermatozoa had an ovoid head with an irregular surface membrane and lacked an acrosome. The nucleus was condensed, and a shallow nuclear fossa partially contained the proximal centriole. The flagellum was formed of

three sections: a short midpiece containing mitochondria and vesicles of various shapes; a long principal piece consisting of an axoneme formed by nine peripheral and one central pair of microtubules; and a thin end piece with nine pairs of peripheral microtubules.

## 4. Discussion

In the present study, there was analysis of the anatomy and process of spermatogenesis in *H. marginatus* with detailed microscopy studies. Macroscopically, the testes were filiform and slightly sinuous, most likely a result of germ-cyst enlargement. The testis morphology differed from that in other members of Pimelodidae, which in general possess a fringed testis, as observed in *Iheringichthys labrosus* (Santos et al., 2001), *Conorhynchus conirostris* (Lopes et al., 2004), and *Pseudoplatystoma fasciatum* (Batlouni et al., 2006). It is suspected that this feature may have contributed to the evolutionary differentiation of *H. marginatus* from other Pimelodidae. Structurally, the testes of *H. marginatus* had anastomosed tubules that are present in other teleost fish, including Characiformes, Siluriformes and Cypriniformes. This characteristic differs from neoteleost fish, which possess only restricted or unrestricted lobular testes, as observed in Cyprinodontiformes and Perciformes (Parenti and Grier, 2004).

In *H. marginatus*, spermatogonia were observed along the entire length of the seminiferous tubules (i.e., were unrestricted), as previously described by Grier et al. (1980). This is also a characteristic in other members of Siluriformes, such as *P. fasciatum* (Batlouni et al., 2006) and *R. quelen* (Melo et al., 2017), as well as in other teleost groups, including Characiformes such as *Leporinus macrocephalus* (Muñoz et al., 2011) and Cypriniformes such as *Danio rerio* (Rupik et al., 2011). These observations indicate that *H. marginatus*, while a Neotropical species, retains morphological features of the testes that are present in primitive fish. These observations are considered to infer that this structural organization of the testicular parenchyma promotes spermatogenic efficiency, considering the presence of

scattered spermatogonia throughout the seminiferous tubule may result in larger numbers of germinal cysts and consequently in greater production of sperm.

Spermatogenesis in *H. marginatus* was cystic, with synchronous release of spermatozoa into the lumen of the seminiferous tubules, as described for other species such as *Liposarcus anisitsi* (Cruz et al., 2005), *C. callichthys* (Spadella et al., 2007), and *Hypoptopoma guentheri* (Spadella et al., 2012). Spermatogonia were the largest cells in the seminiferous tubules of *H. marginatus*, and this was also observed in other teleosts (Leal et al., 2009; Rupik et al., 2011; De Melo Dias et al., 2017). Primary spermatogonia have been classified into several types, based on cell size, nuclear morphology, and the presence of nucleoli (Leal et al., 2009; Schulz et al., 2010). In the present study, there were primary spermatogonia with different mitotic stages and secondary spermatogonia in *H. marginatus*. The morphology of these cells is consistent with descriptions for other members of Pimelodidae, including *P. fasciatum* (Batlouni et al., 2006). During the transition from primary to secondary spermatocytes, there were morphological changes in the nucleus, such as the presence of synaptonemal complexes and the absence of a nucleolus. The morphological features were similar to those observed in other teleost groups (Billard, 1984; Grier, 2002; Batlouni et al., 2006; De Melo Dias et al., 2017). Unfortunately, there were no spermatocytes detected in anaphase or telophase, probably because these stages progress very rapidly.

Based on the extent of chromatin condensation, the formation of the midpiece and flagellum, and the elimination of the cytoplasm, spermatids from *H. marginatus* were classified into three phases, E1, E2 and E3, similarly to the descriptions of this process in *Alcolapia grahami* (Papah et al., 2013), *D. rerio* (Schulz et al., 2010), and *Prochilodus lineatus* (De Melo Dias et al., 2017). The process of spermiogenesis in fish is classified into three types (I, II, and III) based on the presence or absence of nuclear rotation and the position

of the flagellar axis in relation to the nucleus (Mattei, 1970; Quagio-Grassiotto and Oliveira, 2008). Results from present study indicate the flagellum initially developed centrally, the nucleus did not rotate, and the centriolar complex did not migrate, consistent with *H. marginatus* having spermiogenesis type III. Quagio-Grassiotto and Oliveira (2008) reported that this type of spermiogenesis is characteristic of Pimelodidae. In general, the process of spermatogenesis and spermiogenesis of *H. marginatus* is similar to that of *P. fasciatum* and *Pimelodus maculatus* (Batlouni et al., 2006; Quagio-Grassiotto and Oliveira, 2008).

The sperm head of *H. marginatus* lacked an acrosome, which is common in spermatozoa where there is external fertilization (Mattei, 1991). These spermatozoa consisted of a short midpiece, a principal piece with the classic 9+2 axonemal pattern, and a thin end piece consisting of 9+0 pairs of microtubules. The midpiece possessed vesicles, as described for other Pimelodidae such as *I. labrosus* (Santos et al., 2001), *P. fasciatum*, and *P. maculatus* (Quagio-Grassiotto and Oliveira, 2008). In addition, in *H. marginatus* the spermatozoa had an elongated flagellum perpendicular to the nucleus, similarly to the description of *Pimelodella gracilis* (Quagio-Grassiotto et al., 2005). The presence of a shallow nuclear fossa in this species contrasts with other members of Pimelodidae such as *P. fasciatum* and *P. maculatus* (Quagio-Grassiotto and Oliveira, 2008), but is similar to the structure in *Surubim lima* (Quagio-Grassiotto and Carvalho, 2000).

Sertoli cells function mainly to provide protection, nourishment, and support to developing germ cells (Grier, 1993; Schulz et al., 2010; Hai et al., 2014). In the cystic spermatogonial model, Sertoli cells provide the physical structure within which germ cells develop in synchrony, with the number of supporting cells increasing in the initial stages of spermatogenesis and in a species-specific manner (Matta et al., 2002). Because spermatogonia are the largest cells and proliferate rapidly, the largest increase in cyst size and Sertoli cell proliferation usually occurs during the mitotic stage (Matta et al., 2002; Schulz et al., 2005).

Notably, zonulae adherens were observed between Sertoli cells and between Sertoli cells and spermatogonia. This membrane specialization is also observed in other members of the Pimelodidae, such as in *P. fasciatum* (Batlouni et al., 2005). This finding indicates that the Sertoli cell provides a cystic microenvironment for the development and maturation of germ cells to mature sperm cells (Schulz et al., 2005; Rodrigues et al., 2017).

## 5. Conclusion

Regarding the spermatogenesis, *H. marginatus* conserves some of the characteristics of the Pimelodidae, such as the structure of the testicular parenchyma (anastomosing tubular type), unrestricted spermatogonial development, cystic spermatogenesis, uniflagellar sperm, and spermiogenesis type III. This information aids in establishing precise phylogenetic relationships among species of Pimelodidae. Furthermore, these findings may lead to an enhanced interest in the development of biotechnological techniques for aquaculture production as well as the sustainable management of this species.

## Authorship

All authors contributed to the drafting of this review and approved it before submission.

## Conflict of interest

None of the authors have any conflict of interest to declare.

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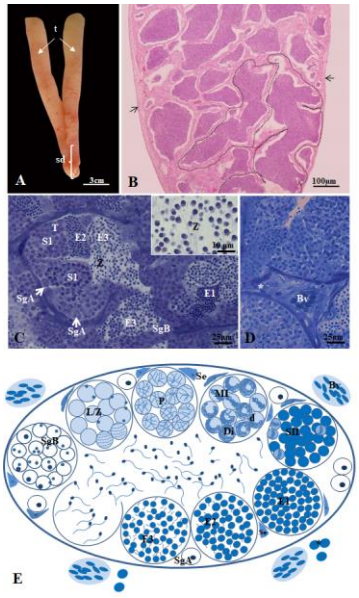


**Fig. 1.** Morphology of the testis of *H. marginatus*. (A) macroscopically, the testes (t) are filiform and are fused caudally, forming a single spermatic duct (sd); (B) transverse section with tubular anastomosis (black dotted line) and tunica albuginea (arrow); (C) testicular parenchyma displaying the seminiferous tubules (T) containing primary spermatogonia (arrowhead), secondary spermatogonia (SgB), primary spermatocytes (SI), spermatids (E1, E2, E3) and spermatozoa (Z, inset); (D) interstitial space with Leydig cells (asterisk) and blood vessels (Bv) (white dotted line); (E) Schematic representation of the seminiferous tubule and interstitial compartment of *H. marginatus*; Note the seminiferous tubule with primary spermatogonia, secondary spermatogonia, primary spermatocytes in leptotene/zygotene (L/Z), pachytene (P), diplotene (d), diakinesis (Di), Metaphase I (MI), secondary spermatocytes (SII), spermatids (E1, E2, E3) and spermatozoa (Z). (B) Hematoxylin+eosin; (C, inset, D) Toluidine blue-stained TEM semithin sections

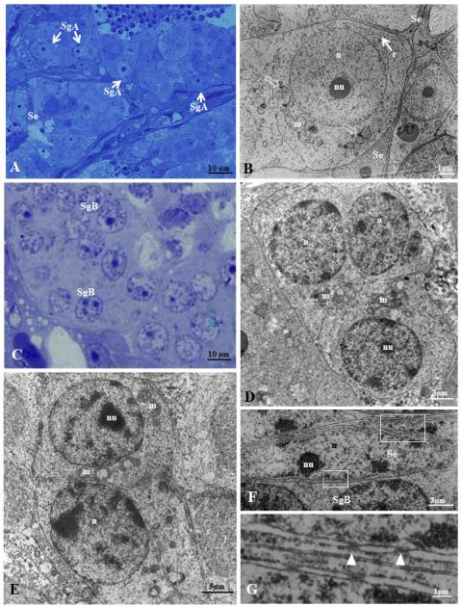


**Fig. 2.** Proliferative phase of spermatogenesis in *H. marginatus*. (A-B) primary spermatogonia (SgA), with ovoid shape, nucleus with evenly distributed euchromatin, and nucleolus; the cytoplasm contained ribosomes and mitochondria of variable shape and nuage appearance (empty arrow); SgA were surrounded by cytoplasmic extensions of Sertoli cells (Se). (C-E) secondary spermatogonia (SgB), the nucleus acquires a rounded shape with heterochromatin, and contained one to three nucleoli; (F) Sertoli cell, elongated nucleus with euchromatin and prominent nucleolus, in addition to cell junction with zonula adherens (white detail); (G) cell junction with zonula adherens (white arrowhead) between spermatogonia and Sertoli cells; (m) mitochondria; (n) nucleus; (nu) nucleoli; (r) ribosomes; (A,C) Toluidine blue-stained TEM semithin sections; (B,D,E,F,G) TEM images

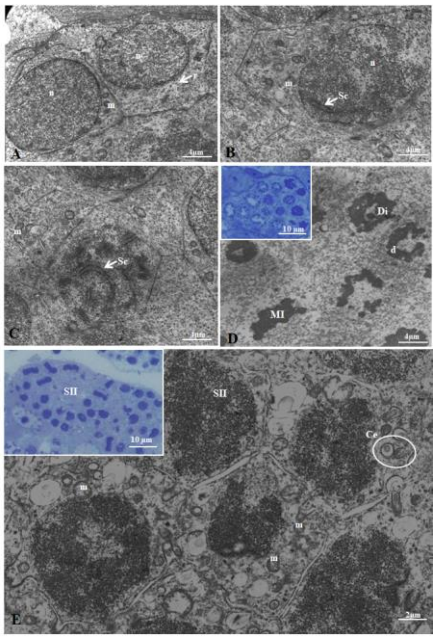


**Fig. 3.** Meiotic phase of spermatogenesis in *H. marginatus*; (A-D) Primary spermatocytes in prophase I; (A) leptotene, beginning process of chromatin condensation; (B) Zygotene, synaptonemal complex (Sc) formation begins close to the nuclear membrane; (C) Pachytene, several well-developed synaptonemal complexes are present; (D-inset) Diplotene (d), chromatin agglomerates organize adjacent to the nuclear membrane; Diakinesis (Di), nuclear membrane disappears and chromatin thickens; and Metaphase I (MI), chromatin locates in the middle portion of the cell; (E-inset) Secondary spermatocytes (SII), with sparse cytoplasm containing vesicles, centrioles and few mitochondria (Ce) centrioles; (m) mitochondria; (r) ribosomes; (v) vesicle; Insets: Toluidine blue-stained TEM semithin sections

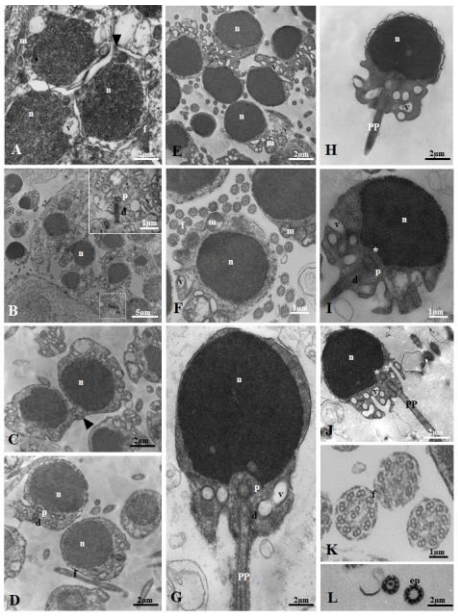

**Fig. 4.** Spermiogenesis in *H. marginatus*. (TEM); (A) initial spermatids (E1), interconnected by cytoplasmic bridges (arrowhead), with incipient flagella (f), mitochondria (m) and vesicles (v). (B,C) intermediate spermatids (E2), with proximal (p) and distal (d) centrioles (inset); (D-G) final stage spermatid (E3), have a round and/or ovoid and voluminous nucleus, surrounded by a thin layer of cytoplasm, midpiece with proximal (p) and distal (d) centrioles, and the principal piece (pp); (H-J) sperm with an ovoid head (h) and membrane with an irregular surface, along with the midpiece (mp) and principal piece (pp); (K) transverse section of the principal piece of a flagellum, with nine pairs of peripheral and a central pair of microtubules (f); (L) Transverse section of the end piece (ep) with only the peripheral microtubules; (n) nucleus; (asterisk) nuclear fossa

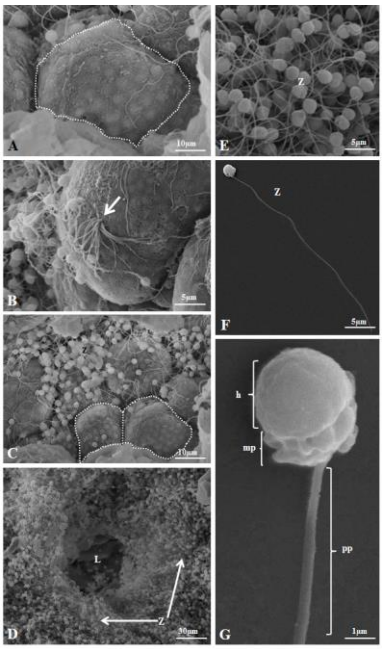


**Fig. 5.** Detail of the seminiferous tubules in *H. marginatus* (SEM); (A) Intact germinal cyst (white dotted line) with irregular surface; (B) Ruptured cyst releasing sperm; (C) Intact germinal cysts (white dotted lines) and cysts during rupture, releasing sperm (Z); (D) Seminiferous tubule lumen (L) with large numbers of free sperm; (E-G) Detail of spermatozoa extracted from semen, with an ovoid head with an irregularly surfaced membrane (H), and the midpiece (mp) and principal piece (pp) of the flagellum

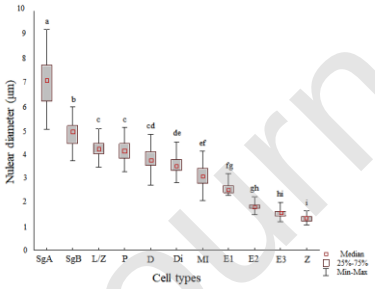


**Fig. 6.** Nuclear diameters (µm) of the germ cell in *H. marginatus*. SgA - primary spermatogonia; SgB - secondary spermatogonia; L/Z - spermatocytes in leptotene/zygotene; P - spermatocytes in pachytene; D - spermatocytes in diplotene; Di - spermatocytes in diakinesis; MI - spermatocytes in metaphase I; E1 - initial spermatid; E2 - intermediate

spermatid; E3 - final spermatid and Z – spermatozoa; Different lower-case letters indicate that the mean values differ ($P = 0.001$)