Development of the male reproductive system in *Callinectes ornatus* Ordway, 1863 (Brachyura: Portunidae)

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Abstract - This study describes the histology and histochemistry of the male reproductive system in *Callinectes ornatus*, comparing juvenile and adult developmental stages. We also analyzed changes in the gonadosomatic (GSI) and hepatosomatic (HSI) indices, and the weights of the testis and vas deferens during the development. The results showed that all stages, beginning with the juvenile (JUV), through developing (DEV) and mature (MAT) adult males of *C. ornatus* produce sperm and spermatophores. During development, testicular lobes showed the same characteristics of production and release of sperm into the seminiferous duct. The vas deferens showed little histological and histochemical change in the epithelium in juvenile and adult males. The differences consisted of the larger amount of secretion in MAT males compared to JUV and DEV ones. The chemical composition of the seminal fluid was similar, but MAT males produced a more homogeneous secretion. Morphological and physiological maturation are not synchronized in *C. ornatus*, since JUV males produced spermatophores similar to those in DEV and MAT males. However, these JUV are not yet able to reproduce, since they still have the abdomen attached to the cephalothoracic sternum. The increase of the GSI during development was significant for MAT males, and is related to the production of sufficient volume of seminal fluid to form the sperm "plug" in the female seminal receptacle. The HSI decreased from DEV to MAT adult stages, indicating that reserves from the hepatopancreas are used to develop the reproductive system after the pubertal molt.

Key words: Gonadosomatic index, histochemistry, seminal fluid, spermatogenesis, spermatophore

Introduction

The swimming crab *Callinectes ornatus* Ordway, 1863 is one of the most abundant species on the coast of southeastern and southern Brazil (Branco and Lunardon-Branco, 1993a,b; Mantelatto and Fransozo, 1996, 1997, 1999; Negreiros-Fransozo *et al.*, 1999). This crab supports an important small-scale fishery, as a bycatch of shrimp fishing (Severino-Rodrigues *et al.*, 2001), and shows continuous reproductive activity with a peak during the summer and autumn (Branco and Lunardon-Branco, 1993a; Mantelatto and Fransozo, 1999).

The male reproductive system in crabs is bilateral, H-shaped, and consists of paired testes and vasa deferetia with different regions (Krol *et al.*, 1992). Despite the great diversity of brachyuran species (Ng *et al.*, 2008), there are relatively few studies on the male

reproductive system. The male reproductive system has been studied in some brachyurans, including Portunidae (Cronin, 1947; Johnson, 1980; Stewart *et al.*, 2010), Ucididae (Castilho *et al.*, 2008), Eriphiidae (Erkan *et al.*, 2009), Geryonidae (Hinsch and McKnight, 1988), Cancridae (Moriyasu *et al.*, 2002), Grapsidae (Garcia and Silva, 2006), Epialtidae (Hinsch and Walker, 1974; Sal Moyano *et al.*, 2009), Oregoneiidae (Beninger *et al.*, 1988; Moriyasu and Benhalima, 1998; Sainte-Marie and Sainte-Marie, 1999), Inachidae (Diesel, 1989) and Majidae (Simeó *et al.*, 2009, 2010).

The brachyuran testes can be classified as tubular, as observed in different species of Grapsoidea, Majoidea and Xanthoidea (Simeó *et al.*, 2009); or as lobular which is seen in other brachyuran superfamilies (Simeó *et al.*, 2009). In Portunidae the testes are characterized by multiple seminiferous lobules (Zara *et al.*, 2012) and each seminiferous lobule is enclosed by accessory cells and releases the spermatozoa into the seminiferous duct (Cronin, 1947; Jivoff *et al.*, 2007; Ryan, 1967; Johnson, 1980; Stewart *et al.*, 2010; Zara *et al.*, 2012). The portunid vas deferens is divided into three main regions: anterior (AVD), middle (MVD) and posterior (PVD) (Jivoff *et al.*, 2007). The main function of the AVD is packing the spermatozoa into spermatophores, while the MVD region produces a large part of the seminal fluid and stores the spermatophores from the AVD. In portunids as other brachyuran species, the PVD is generally bulky and also provides storage for the spermatophores; the seminal fluid becomes more liquid dissolving the dense and granular secretion from MVD to aid in transferring the spermatophores to the female seminal receptacle (Ryan, 1967; Johnson, 1980; Zara *et al.*, 2012).

In crustaceans, morphological and physiological maturity are not always synchronized (Sastry, 1983). The presence of spermatophores before the pubertal molt was described for the portunid *Arenaeus cribrarius* (Lamarck, 1818) (Pinheiro and Fransozo, 1998), which suggests that physiological maturity could be reached before morphological maturity in this family. The physiological maturation of the male reproductive system was studied macroscopically in some species of Portunidae, with respect to color and the volume occupied by the organ in the cephalothoracic cavity (Costa and Negreiros-Fransozo, 1998; Santos and Negreiros-Fransozo, 1999; Mantelatto and Fransozo, 1999). Other criteria also used to determine physiological maturity are the gonadosomatic (GSI) and hepatosomatic (HSI) indices (Mantelatto, 1995), in addition to histological techniques (Johnson, 1980). The study of the relationship between the reproductive system and the hepatopancreas in many crustacean species has shown that the hepatopancreas reserves are used to develop the female adult reproductive system (Adiyodi, 1969; Adiyodi and Adiyodi, 1972; Kyomo, 1988; Lawrence and Castille, 1989; López-Greco and Rodríguez, 1999, Zara *et al.*, 2013). On the other hand, Griffen *et al.* (2012) found a negative relationship between GSI and HIS to *Hemigrapsus sanguineus* (De Haan, 1835) suggesting that energy used to reproductive output is probably derived from sources besides the hepatopancreas conferring an advantage for invasive crabs. In males, the GSI/HSI relationship has been studied only in adults of *Callinectes danae* Smith 1869; in this species, the HIS decreases at the same time that the GSI increases after the pubertal molt (Zara *et al.*, 2012). In general, the GSI and HSI indices, rather than physiological maturity, are more commonly used to determine female seasonal reproductive cycle (Kyomo, 1988; Chu, 1999; López-Greco and Rodríguez, 1999; Castiglioni *et al.*, 2006; Sokolowics *et al.*, 2006).

Here, we describe in detail the histology and histochemistry of the male reproductive system in juvenile and adult males of *C. ornatus*. Additionally, we investigated whether sexual maturity is reached before or after the pubertal molt. We also followed the changes in the GSI and HSI indices and the weights of the testes and vas deferens in juveniles and adults during the development of the reproductive system.

Material and Methods

Specimens of *Callinectes ornatus* were collected monthly by trawling at four different sites in depths between 6 and 15 m, from January to December 2009 in the Estuary-Bay of São Vicente, São Paulo State, Brazil. The crabs were transported alive to the laboratory and the sex and morphological developmental stage (juvenile or adult) were determined following the criteria proposed by Van Engel (1990). According to this author, all males showing the T inverted morphology of abdomen attached to the thoracic sternites were classified as juveniles. The carapace width (CW) was measured to 0.05mm using a caliper. The crabs were anesthetized by thermal shock (-20ºC/15 min) (López-Greco *et al.*, 1999) followed by removal of the testis, vas deferens and hepatopancreas.

The male reproductive system was classified macroscopically by the color and the size relative to the hepatopancreas. The adults were further divided into developing (DEV) or mature (MAT) based on Costa and Negreiros-Fransozo (1998). However the stages rudimentary and developing from these authors were analyzed and classified together in DEV stage according Zara *et al.* (2012). The macroscopic criteria used was: testes visible only by magnifying or when visible occupies the anterolateral margin of cephalothorax; the vasa deferentia are very thin behind the stomach or thin with both MVD and PVD clearly less voluminous than observed in MAT ones; the reproductive system/hepatopancreas ratio is 1:4. The juveniles (JUV) were sorted by a morphological criterion (abdomen attached to cephalothorax) (Van Engel, 1990). Only males with CW around 50 mm ranging since 45 to 61 mm (the largest CW classes preceding the pubertal molt) and in hard-shelled intermolt condition C (Mantelatto and Fransozo, 1999) were examined.

The testes and vas deferens from crabs in each stage of maturation were weighed on an analytical balance (0.001 g). The GSI (testes plus vasa deferentia) and HSI indices were obtained for each crab dividing the mass of the hepatopancreas or the reproductive system by the body weigh the crab, respectively.

The tissues from at least five crabs for each developmental stage were fixed in 4% paraformaldehyde prepared with salt water. After fixation for 24 h, the samples were washed twice in 0.2 M sodium phosphate buffer (pH 7.2), dehydrated in an increasing ethanol series (70-95%), and embedded in Leica® methacrylate resin for histological examination. Serial sections of testes and vasa deferentia at 5 and 7 μm in thickness were obtained with a microtome and stained using hematoxylin and eosin (H&E) according to Junqueira and Junqueira (1983) and by avoiding ethanol and xylene baths (Sant'Anna *et al.*, 2010). Spermatogenesis was observed in sections stained with toluidine blue, pH 4.0 (Taboga and Dolder, 1991).

The following histochemical techniques were used: mercuric-bromophenol blue (Pearse, 1985) and Xylidine ponceau (Mello and Vidal, 1980) for proteins; toluidine blue (pH 2.5 and 4.0) acidic substrates (Pearse, 1985) and Alcian blue (pH 1.0 and 2.5) (Junqueira and Junqueira, 1983) for acid polysaccharides; periodic acid of Schiff (PAS) (Junqueira and Junqueira, 1983) for neutral polysaccharides; and the conjugated technique of PAS/ Alcian Blue (pH 2.5) (Junqueira and Junqueira, 1983) for acid and neutral polysaccharides. The samples stained with Sudan black B (Pearse,1985) for lipids were not dehydrated, and were embedded directly in methacrylate resin for 24 h (Zara *et al.*, 2012).

The diameter of 30 nuclei of germ cells per slide stained in toluidine blue according to Zara *et al.* (2012) were measured using Leica IM50 software in three crabs for each maturation stage.

Data were normalized by the Kolmogorov-Smirnov test. The Dunn comparison method ($P \leq 0.05$) was used when the Kruskal-Wallis test for non-parametric data indicated a significant difference between developmental stages, or the germ cell sizes (Sokal and Rohlf, 1995).

Results

Lobular behavior during sperm production

The testes of *Callinectes ornatus* contain numerous seminiferous lobules, where spermatogenesis and spermiogenesis occur (Fig. 1). Between the lobes is the highly convoluted seminiferous duct filled with mature sperm (Fig. 1). The lobules are surrounded by connective tissue and are separated from each other by accessory cells with a thin layer of connective tissue (Figs. 2, 3 and 4). Each lobe is filled with germ cells at the same stage of spermiogenesis, but the stages of maturation vary among the lobules (Fig. 2). The spermatogonia can fully occupy the lobule (Figs. 3 and 4), or in others that contain meiotic maturation cells, the spermatogonia form isolated germinal centers close to the seminiferous duct (Fig. 3) or at the periphery of the lobule (Figs. 5 and 9). In lobules filled with spermatogonia, the presence of mitotic figures is common (Fig. 6). Lobules in development are observed in JUV and in both DEV and MAT adult males (Figs. 3 and 4). Once filled with spermatogonia, the lobules begin spermatogenesis synchronously (Fig. 5). Primary spermatocytes contain chromosomes at different stages of prophase I (Fig. 7), and some lobules are filled synchronously by numerous metaphase plates (Fig. 8). The secondary spermatocytes are smaller than the primary spermatocytes, and some are in the second meiotic division (Fig. 8), originating the initial spermatids (Fig. 9). Lobules containing germ cells are surrounded by large accessory cells, with a nucleus that ranges from flat to round, and with little heterochromatin seen by toluidine blue stain (Fig. 8).

During spermiogenesis, the spermatids undergo cell differentiation, which can be divided into three distinct stages under light microscopy: early (EST), intermediate (IST) and late spermatid (LST) (Figs. 9 to 13). Mature sperm is released to the lumen of the seminiferous duct, which is formed by monostratified cubic epithelium (Figs. 14 and 15).

The testis and vas deferens showed almost no morphological, histological or histochemical variation at different stages of maturation. The only changes were the weights of the testis and vas deferens during the maturation process. Although testis weight increased in the different stages (Fig. 16), the differences were significant (KW: $H = 13.6774$) only between the DEV and MAT stages (Dunn: $z = 2.9287$; $P \leq 0.05$). The vas deferens increased in weight significantly (KW: $H = 22.6884$) between JUV and MAT (Dunn: $z = 3.0285$; $P \leq 0.05$) and also between DEV and MAT (Dunn: $z = 3.8126$; $P \leq 0.05$) (Fig. 16). Table 1 summarizes the average changes in carapace width (CW), total body weight, and testes and vas deferens weights during the developmental stages of the reproductive system.

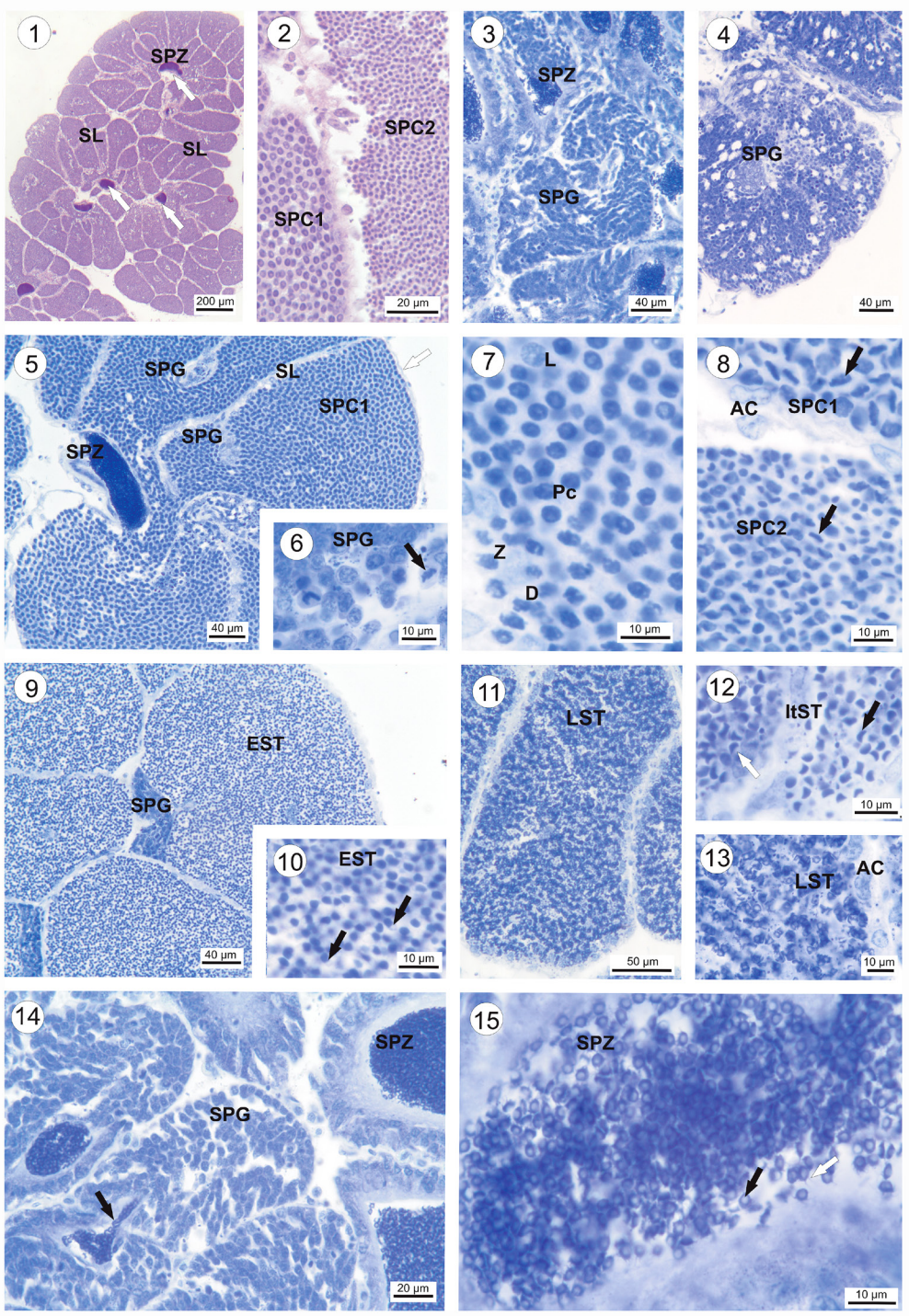
Spermatogenesis

The spermatogonia (Figs. 3, 5 and 9) have a large nucleus (4.9 ± 0.8 μm) filled with heterochromatin blocks and a clear nucleolus (Fig. 6). The cytoplasm is also reactive to toluidine blue pH 4.0, although less intensely than the nucleus (Figs. 3–6 and 14). The primary spermatocytes have a smaller nucleus compared to the spermatogonia (3.8 ± 0.2 μm) and are strongly basophilic (Fig. 2). In these cells, the leptotene, pachytene and diplotene phases of meiosis are very distinctive (Fig. 7). Metaphase I is marked by the appearance of the metaphase plate, which is intensely reactive to toluidine blue (Fig. 8). Secondary spermatocytes are characterized by the small-

Table 1. Mean ± standard deviation of cephalothoracic carapace width (CW) and body, testis and vas deferens weights (g) during development of the reproductive system in *Callinectes ornatus*.

	JUV*(n = 55)	DEV*(n = 19)	MAT*(n = 61)
CW	54.9 ± 4.3	58.8 ± 5.8	63.2 ± 5.8
Body weight	22.6 ± 5.5	28.9 ± 8.0	37.7 ± 10.1
Testis weight	0.08 ± 0.03	0.1 ± 0.08	0.19 ± 0.07
Vas deferens weight	0.04 ± 0.01	0.4 ± 0.5	1.3 ± 0.5

*Macroscopic classification of the male reproductive system: JUV=juvenile; DE= developing adult; MAT= mature adult



Figures 1 – 15. Spermatogenesis and spermiogenesis: (1) seminiferous lobules (SL), general aspect, with spermatozoa (SPZ) inside the seminiferous duct (arrows); (2) Detail of seminiferous lobules, showing the size difference between primary spermatocytes (SPC1) and secondary spermatocytes (SPC2); (3) testicular lobules filled with spermatogonia (SPG); (4) testicular lobules in juvenile male, similar to those observed in mature male; (5) general view of testes, with germinal center peripheral to seminiferous lobules and seminiferous duct filled with sperm. The arrow indicates the conjunctive tissue capsule; (6) spermatogonia in mitosis (arrow); (7) primary spermatocytes in prophase I showing leptotene (L), zygotene (Z), pachytene (Pc) and diplotene (D) phases; (8) testicular lobes in different meiosis stages. Note the difference in size between primary and secondary spermatocytes. The black arrows indicate metaphase I and II; (9) seminiferous lobule at the beginning of spermiogenesis, filled with early spermatids (EST); (10) Early spermatids marked by the appearance of pro-acrosomal vesicle (arrow), with round nucleus; (11) lobule with late spermatids, LST; (12) intermediate spermatids, ItST, marked by voluminous C-shaped nucleus (black arrow), and increase of acrosomal vesicle (white arrow); (13) Detail of LST in the lobe interiors; (14) spermatozoa being released to the seminiferous duct (arrow); (15) mature sperm in the lumen of the seminiferous duct, with large central acrosomal vesicle (black arrow) and nuclear arms (white arrow). AC, accessory cells. Figures 1 and 2, stained with hematoxylin and eosin; Figures 3 – 15, stained with toluidine blue pH 4.0.

sized nucleus (2.02 ± 0.27 μm), clearly smaller than in the previous stage, with strongly basophilic and homogeneous features (Fig. 2). During meiosis II, the processes of metaphase II can be observed, with a clearly smaller metaphase plate compared to metaphase I (Fig. 8).

Despite the reduction in the nucleus during spermatogenesis, there was no size difference between the spermatogonia and primary spermatocytes. Differences were observed in the spermatogenesis process (*KW*: $H = 162.5358$) between primary and secondary spermatocytes (Dunn: $z = 7.3913$; $P \leq 0.05$). During spermiogenesis, differences were observed between the initial and intermediate spermatid (Dunn: $z = 3.3621$; $P \leq 0.05$) and between the initial and late spermatid (Dunn: $z = 4.9958$; $P \leq 0.05$).

Spermiogenesis begins in the early spermatids (Fig. 9), which are characterized by the appearance of the acrosomal vesicle under light microscopy. The acrosomal vesicle shows weak α-metachromasia (green) for toluidine blue pH 4.0, a characteristic that persists until the sperm is formed. Early spermatids have a rounded and homogeneous nucleus (1.93 ± 0.23 μm) (Fig. 10). The nucleus of intermediate spermatids (2.37 ± 0.32 μm) changes from round to C-shaped. The acrosomal vesicle becomes larger compared to the previous stage (Fig. 12). Late spermatids show slender nuclei with β-metachromasia (blue - purple) (2.68 ± 0.41 μm) that almost completely surrounds the acrosomal α-metachromatic vesicle (Fig. 13). Similar to late spermatids, mature sperm is located within the seminiferous duct, showing slender β–metachromatic cup-shaped nuclei (2.21 ± 0.27 μm) and almost completely surrounding the acrosomal vesicle. At this stage, small radial arms formed by nuclear expansions strongly stained by toluidine blue can be observed (Fig. 15).

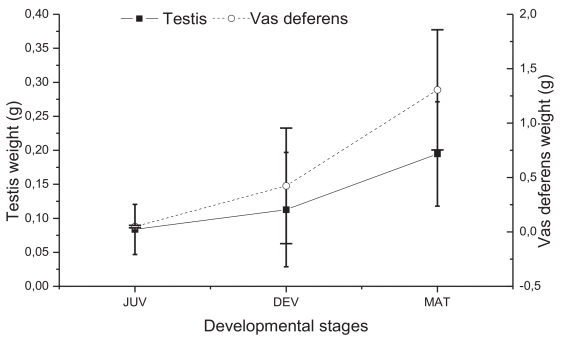
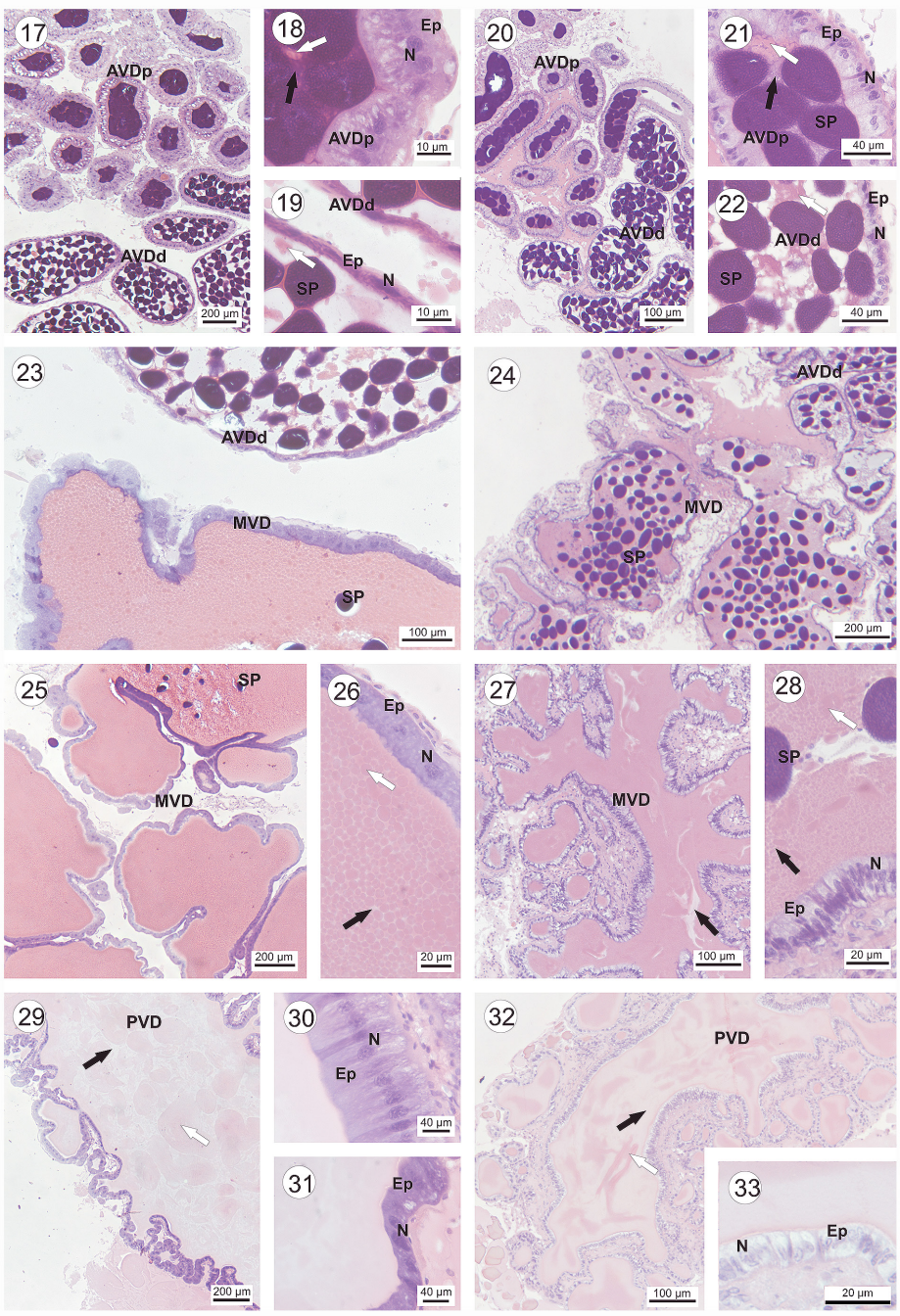


Figure 16. Weight change (g) of testis and vas deferens among different developmental stages of the male reproductive system, *N=123*. Mean and standard deviation are plotted for each developmental stage. JUV= juvenile; DEV= developing adult; MAT= mature adult.

Histology of spermatophores and seminal fluid formation

Mature spermatozoa in the lumen of the seminiferous duct are conducted to the AVD (Figs. 17 to 22). The vas deferens is organized similarly (Figs. 16 to 32) at all developmental stages, JUV (Figs. 17 to 19), DEV and MAT males (Figs. 20 to 22). AVD was divided into two distinct histological and histochemical regions. The proximal AVD (AVDp) receives the sperm from the seminiferous duct and stores it in the lumen, where sperm masses are compacted by basophilic secretion (Figs. 17 and 20). AVDp is formed by the columnar epithelium with irregular and basal nuclei (Figs. 18 and 21). Among the basophilic secretion is the acidophilic secretion, which is added to the periphery of the sperm mass, forming the spermatophores (Figs. 18 and 21). The distal portion of AVD (AVDd) is filled with spermatophores already formed in the large lumen (Figs. 17 and 20). In JUV and DEV males, the AVDd is formed by a monostratified columnar epithelium with cells having a basal nucleus and basophilic cytoplasm (Fig. 19). In MAT males, this region has a monostratified squamous epithelium with basophilic cytoplasm (Fig. 22).

The spermatophores produced in the AVD are sent to the MVD, where they are stored in a large amount of seminal fluid (Figs. 23 to 28). In JUV (Fig. 23) and MAT (Fig. 24) males the lumen contains large numbers of spermatophores. Isolated spermatophores are easily seen in JUV and DEV because of the smaller volume of secretion (Fig. 23). The MVD is characterized by the presence of outpocketings (*sensu* Johnson, 1980), which are qualitatively smaller in JUV and DEV



Figures 17 – 33. Vas deferens (Juvenile and Mature) – H&E staining: (17) Anterior vas deferens (AVD) in juvenile males (JUV) separated into proximal (AVDp) and distal (AVDd) regions; (18) JUV male showing AVDp with columnar epithelium and basophilic (black arrow) and acidophilic (white arrow) secretions; (19) AVDd of JUV male showing cubic epithelium (Ep) and acidophilic secretion (arrow) in the lumen, the same found in the spermatophore wall; (20) AVD in mature males (MAT), showing proximal and distal regions; (21) AVDp in MAT male with columnar epithelium (Ep) and basophilic (black arrow) and acidophilic (white arrow) secretions; (22) AVDd of MAT male showing squamous epithelium and acidophilic secretion (arrow), the same surrounding the spermatophores (SP); (23 and 24) Transition between AVDd and the middle region of the vas deferens (MVD) in JUV and MAT males, respectively; (25) MVD of JUV male, filled with acidophilic secretion (arrow); (26) Detail of the columnar epithelium of MVD of JUV male, and spermatophores immersed in the acidophilic secretion (arrows); (27) MVD of MAT male, characterized by large amount of acidophilic secretion and completely formed spermatophores; (28) Detail of the previous micrograph, showing the cubic epithelium and the acidophilic secretion formed by granules and immersed in matrix (arrows); (29 and 30) general view (29) and detail (30) of posterior vas deferens (PVD) in JUV male, very similar to what is observed in MAT male, although without morphological variation of the epithelium, which is always columnar due to the smaller secretion volume; (31) PVD in MAT male, showing less-acidophilic, more-homogeneous and fluid secretion, compared to MVD (arrows); (32 and 33) Squamous and columnar epithelium in the PVD of MAT male, where the change is due to the large volume of the secretion pressing certain regions of the epithelium. N, nucleus.

individuals (Fig. 25) compared to MAT (Fig. 27). The epithelium of these outpocketings in JUV and DEV males is columnar, with elongated nuclei and a weakly basophilic cytoplasm (Fig. 26); while in MAT males the epithelium varies from cubic to squamous, with round to irregular nuclei (Fig. 28). The luminal secretion of the MVD contains large quantities of homogeneous acidophilic granules, dispersed in a fine matrix, which is also homogeneous and less acidophilic than MAT males (Figs. 26 and 28).

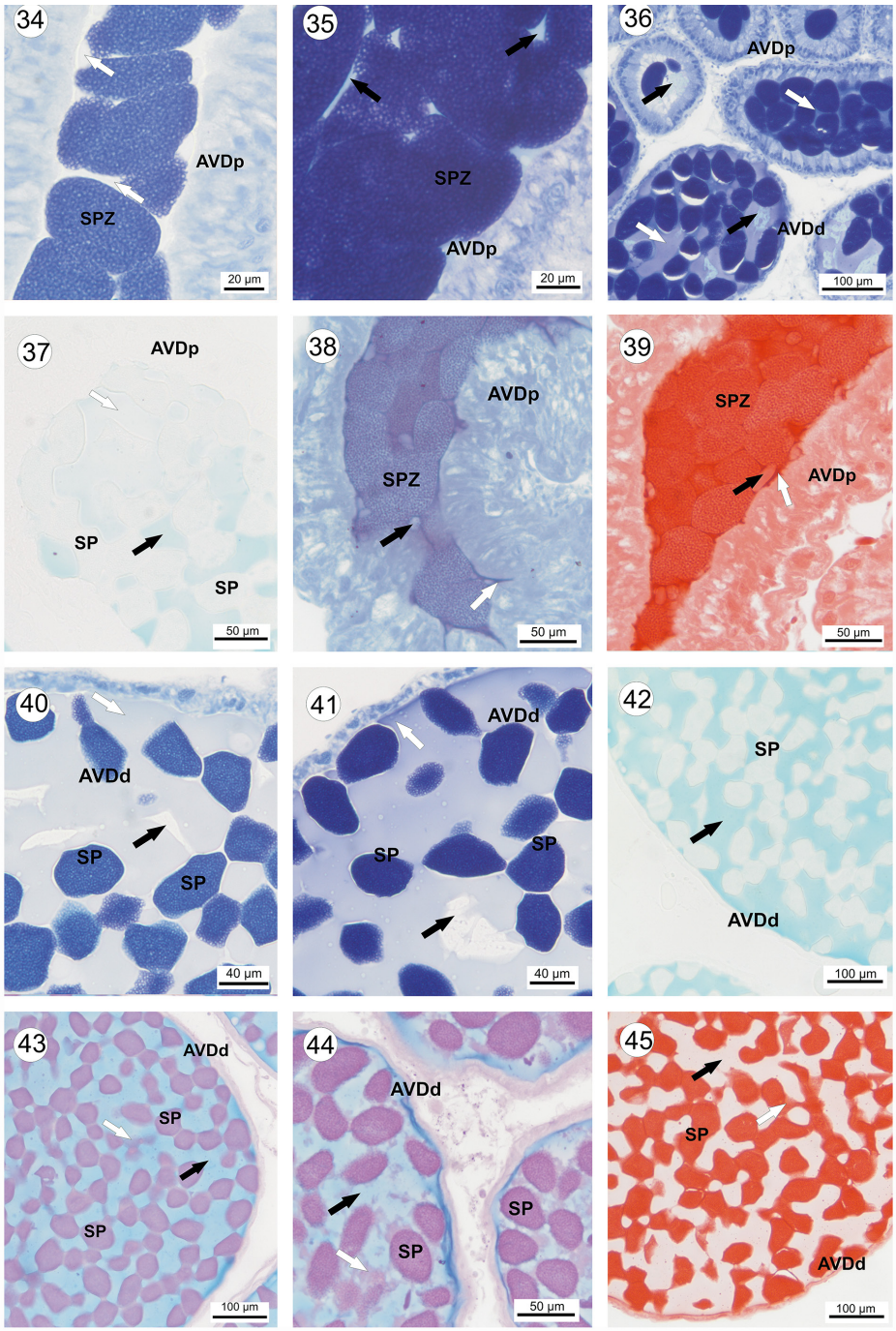
The PVD has side pockets and the lumen of these outpockets contains an acidophilic fluid secretion without the acidophilic granules, but consisting of two elements: one homogeneous and the other coagulated and slightly eosinophilic (Figs. 29 to 33). Throughout the PVD, JUV males have the columnar epithelium with cells having a basal nucleus and weakly basophilic cytoplasm (Fig. 30). MAT males display a sometimes columnar and sometimes cubic-squamous epithelium, due to the increasing volume of luminal secretion (Figs. 32 and 33).

Histochemistry of spermatophores and seminal fluid formation

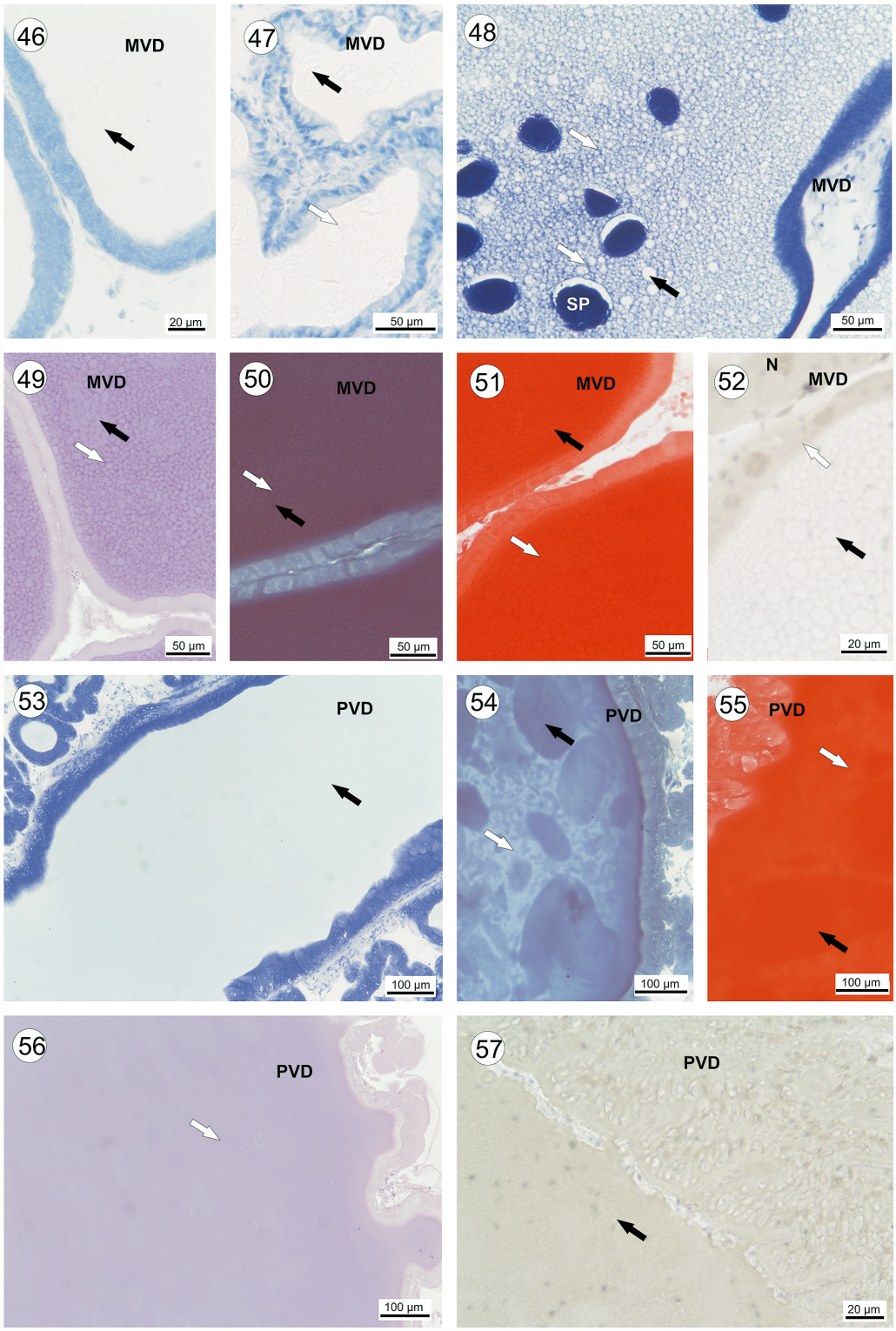
The secretion in AVDp MAT and JUV males shows weak α-metachromasia for toluidine blue pH 2.5 (Fig. 34). However, the luminal secretion of MAT males is clearly α-metachromasic (greenish blue) to toluidine blue pH 4 (Fig. 35), while JUV males display β- and α-metachromasia (Fig. 36). Metachromasia α and β occur in acidophilic and basophilic secretions stained with H&E, respectively (Fig. 18). These characteristics indicate the presence of an acid secretion consisting of acid polysaccharides. This result was supported by Alcian blue staining, where the secretions were negative in pH 1.0 and positive in pH 2.5 (Fig. 37). Secretions with α-metachromasia were negative for Alcian blue in both pHs (Fig. 37).

Still in this region, intensive reactivity to protein is noted in the secretion near the epithelium, which is basophilic in H&E (Fig. 21); while the masses of acidophilic secretions (Fig. 21) were positive for protein (Figs. 38 and 39). In JUV and MAT, the AVDd stained with toluidine blue at pH 2.5 exhibits two types of secretion, one homogeneous with weak β–metachromasia, and the other negative (Fig. 40). With the same stain at pH 4, the β-metachromasia (purplish blue) is more evident (Fig. 41), and the secretion is acidophilic in H&E (Figs. 19 and 22). The secretion with β-metachromasia in the AVDd showed a strong reaction for acid glycosaminoglycans in Alcian blue at pH 2.5 (Fig. 42), but was negative at pH 1.0. On the other hand, secretions with α–metachromasia that were acidophilic to H&E (Figs. 19 and 22) and reactive for proteins from the spermatophores, were negative to Alcian blue. This secretion forms the spermatophore wall and was strongly reactive for neutral polysaccharides using combined staining of PAS/Alcian blue at pH 2.5. This reaction was detected precisely in those regions that were negative to toluidine blue pH 4, always close to the spermatophores in MAT (Fig. 43) and more dispersed among the acid glycosaminoglycans in JUV males (Fig. 44). The secretions reactive to PAS were also positive for protein (Fig. 45), while the secretions that were alcianophilic at pH 2.5 (Figs. 43 and 44) were negative to both Xylidine ponceau (Fig. 45) and mercuric-bromophenol blue. Both portions of the AVD were negative for lipids as tested by Sudan black B.

Toluidine blue pH 2.5 did not cause a reaction in the granules and luminal matrix of the MVD for either adult or juvenile males (Figs. 46 and 47). Similarly to the result for toluidine blue pH 4.0, no reaction was detected in the granules for both maturation stages. However, the luminal matrix showed intense β-metachromasia in MAT males (Fig. 48). The granules and luminal matrix were reactive for neutral polysaccharides and negative for acid polysaccharides, using Alcian blue pH 1.0 and combined PAS/Alcian blue pH 2.5 (Fig. 49). On the other hand, the luminal matrix and granules reacted intensely to proteins in both Xylidine ponceau and mercuric-bromophenol blue stains (Figs. 50 and 51). The MVD secretion was negative for lipids, while the



Figures 34 – 45. Histochemistry of AVD secretion: (34) AVDp of MAT male with luminal secretion marked by weak metachromasia α (white arrow) stained with toluidine blue pH 2.5; (35) Strong metachromasia α (black arrow) in AVDp of MAT male stained with toluidine blue pH 4.0; (36) AVDp and AVDd of JUV male for toluidine blue pH 4.0. Both regions show secretion with metachromasia α (black arrow) and metachromasia β (white arrow); (37) Alcian blue pH 2.5 in AVDp of MAT male, with positive secretion (black arrow), and negative (white arrow) for acid polysaccharides, and spermatophores (SP) also negative for Alcian blue; (38 and 39) AVDp of MAT male showing strong reaction for protein inside the lumen (black arrow) and positive (white arrow) in the compact section close to the epithelium, as well as in the spermatophores; (40 and 41) AVDd of MAT male for toluidine blue pH 2.5 and 4.0, respectively, showing luminal secretion with homogeneous metachromasia β (white arrow), and more intense for pH 4.0. The more-compact secretion did not react for both pH's (black arrow); (42) Secretion strongly positive for Alcian blue pH 2.5 (black arrow), showing non-reactive spermatophores in AVDd of MAT male; (43) AVDd of MAT make showing secretion strongly positive for combined technique PAS/Alcian blue pH 2.5. The homogeneous matrix (black arrow) depicts the presence of acid polysaccharides, more intense close to the epithelium. The white arrow indicates the secretion reactive to neutral polysaccharides, close to spermatophores, which were also reactive for PAS; (44) JUV male for PAS/Alcian blue pH 2.5 in the AVDd with matrix positive for acid polysaccharides, more intense close to the epithelium (black arrow), and large amount of a diffuse secretion positive for neutral polysaccharides, associated with the spermatophores, which are also positive; (45) AVDd for Xylidine ponceau, showing lack of reaction in the matrix of the luminal secretion (black arrow), and positive reaction of the more-compact secretion close to the spermatophores (white arrow). SPZ, spermatozoa; Figure 38, mercuric-bromophenol blue; Figure 39, xylidine ponceau.



Figures 46 – 57. Histochemistry of MVD and PVD secretions: (46 and 47) MVD of MAT and JUV males, respectively, showing luminal secretion non-reactive for toluidine blue pH 2.5 (arrow); (48) MAT male, showing MVD filled with secretion consisting of granules negative to toluidine blue pH 4.0 (black arrow), surrounded by matrix with metachromasia β; (49) Matrix and granules of MVD, showing exclusive reaction to neutral polysaccharides, (arrows) by the conjugated technique PAS/Alcian blue pH 2.5; (50 and 51) Lumen of MVD showing strong reaction to proteins (arrows) by mercuric-bromophenol blue and xylidine ponceau, respectively; (52) MVD of MAT male, showing no reaction for lipids in the secretion (black arrow), and weak reaction in the cytoplasm of the epithelium (white arrow); (53) Toluidine blue pH 4.0 of MAT male, showing the homogeneous luminal secretion of PVD and metachromasia α (arrow); (54 and 55) PVD of MAT male stained with mercuric-bromophenol blue and Xylidine ponceau, respectively, showing matrix with positive secretion (white arrows) and more intensely stained clumps of secretion (black arrows); (56) Secretion of PVD of MAT male, homogeneous and positive for neutral polysaccharides (white arrow) for conjugated technique PAS/Alcian blue pH 2.5; (57) Absence of lipids in the PVD of MAT male (black arrow) with Sudan black B stain. N= nucleus.

epithelium was weakly stained by Sudan black B (Fig. 52).

PVD showed homogeneous α-metachromasia (Fig. 53) and was strongly positive for proteins, but some lumps in the seminal fluid were even more reactive (Figs. 54 and 55). This same secretion was not reactive to Alcian blue stain at both pHs, and was homogeneously reactive for neutral polysaccharides in PAS/Alcian blue pH 2.5 (Fig. 56). The luminal secretion of the PVD was negative for lipids (Fig. 57).

Gonadosomatic and hepatosomatic relationship during development

A total of 135 males were dissected and classified macroscopically as JUV (n = 55), DEV (n = 19) and MAT (n = 61). The mean weights of the body, hepatopancreas, reproductive system, GSI and HSI are listed in Table 2. The GSI increased throughout the developmental stages (Fig. 58). However, a significant difference (*KW:* H = 23.8519) was observed between JUV and MAT stages (Dunn: z = 2.9337; $P \leq 0.05$) and between DEV and MAT (Dunn: z = 4.0383; $P \leq 0.05$). On the other hand, HSI increased between the JUV and DEV stages despite the absence of statistic difference; and decreased significantly in MAT males (Dunn: z = 3.9905; $P \leq 0.05$).

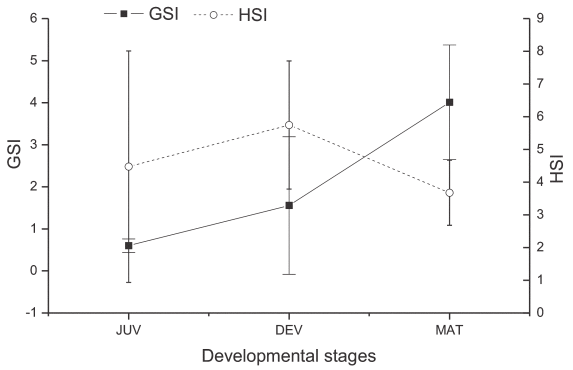


Figure 58. Mean variation of the gonadosomatic index (GSI) and hepatosomatic index (HSI) according to developmental stage of the reproductive system, N = 135. Bars = standard deviation. JUV= juvenile; DEV= developing adult; MAT= mature adult.

DISCUSSION

In *Callinectes ornatus,* the male reproductive system is divided into the testis and vas deferens, arranged in the "H" shape, as described for other portunids *Callinectes sapidus* (Rathbun, 1896) (Cronin, 1947; Johnson, 1980), *Portunus hawaiiensis* (Herbst, 1783) (Ryan 1967 as *Portunus sanguinolentus*), *Portunus pelagicus* (Linnaeus, 1758) (Batoy *et al.*, 1989; Stewart *et al.*, 2010) and *Callinectes danae* (Zara *et al.*, 2012). This seems to be the most commonly observed pattern in Podotremata and Eubrachyura (Zara *et al.*, 2012). The testis is lobular with a highly convoluted seminiferous duct, as in *C. danae* (Zara *et al.*, 2012) and observed in different brachyuran families (Simeó *et al.*, 2009).

In the genus *Callinectes*, in the seminiferous lobules containing developing spermatids, the cytoplasmic volume of accessory cells enlarges, indicating the role of these cells in sperm formation (Johnson, 1980; Zara *et al.*, 2012), as observed for *C. ornatus*. These accessory cells were not observed in the germinal centers in *C. sapidus* (Johnson, 1980), although in *C. ornatus* they are present in the germinal centers, and are more easily observed in lobules completely filled with spermatogonia, in both juvenile and adult males. Pochon-Masson (1983) compared these accessory cells to the Sertoli cells of vertebrates, and the present observations for *C. ornatus* are in agreement with this statement. The histological behavior of the

Table 2. Mean ± standard deviation of total body weight, male reproductive system and hepatopancreas weights, gonadosomatic index (GSI) and hepatosomatic index (HSI) during development of the reproductive system in *Callinectes ornatus*.

	JUV*(n = 55)	DEV*(n = 19)	MAT*(n = 61)
Body weight (g)	22.7 ± 5.5	28.9 ± 8	37.7 ± 10.9
Male reproductive system weight (g)	0.13 ± 0.04	0.6 ± 0.5	1.5 ± 0.6
Hepatopancreas weight (g)	0.8 ± 0.5	1.5 ± 0.5	1.4 ± 0.5
GSI	0.6 ± 0.2	1.5 ± 1.6	4.0 ± 1.4
HSI	4.5 ± 3.5	5.7 ± 1.9	3.7 ± 1.0

*Macroscopic classification of the male reproductive system: JUV=juvenile; DEV= developing adult; MAT= mature adult

seminiferous lobules and germ cells during spermatogenesis was similar in juvenile and adult males in *C. ornatus* and is similar to DEV and MAT adults in *C. danae* (Zara *et al.*, 2012). At the beginning of spermatogenesis, the proliferation of spermatogonia starts in the germinal centers, which are located close to the seminiferous ducts and on the periphery of the seminiferous lobules, suggesting a germinal-center pattern, as observed in other portunid crabs (Ryan, 1967; Johnson, 1980; Stewart *et al.*, 2010; Zara *et al.*, 2012). However, in *C. ornatus* the proliferation in the germinal centers occurs soon after the sperm are released into the lumen of the seminiferous ducts by spermatogonial mitosis, restarting the formation of a new seminiferous lobule. Once formed, the spermatogonia begin meiosis to become spermatocytes, and groups of spermatogonia remain peripheral to form germinal centers, with no sign of mitosis. The release of spermatozoa seems to be the trigger for the germinal center to proliferate into new spermatogonia, restarting the lobular cycle. This synchronous cell cycle found in juveniles and adults of *C. ornatus* was also described for adult males of other members of *Callinectes* (Johnson, 1980; Zara *et al.*, 2012). On the other hand, the seminiferous lobules of *P. pelagicus* are described as asynchronous, showing germinal, proliferative and evacuation zones (Stewart *et al.*, 2010); or as synchronous, filled with cells in the same meiotic stage (Ravi *et al.*, 2012). Other genera such as *Arenaeus*, *Achelous* (Portuninae) and *Charybdis* (Thalamitinae) should be studied in order to determine the usual behavior of the seminiferous lobules during spermatogenesis in Portunidae.

During spermatogenesis and spermiogenesis, there was a clear reduction of the cellular nucleus from the spermatogonia until the mature sperm. This reduction has also been observed in other crab species such as *Ucides cordatus* (Linnaeus, 1763) and in particular, in portunids such as *C. sapidus, P. pelagicus* and *C. danae* (Johnson, 1980; Castilho *et al.*, 2008; Stewart *et al.*, 2010; Ravi *et al.*, 2012; Zara *et al.*, 2012). However, in *Maja brachydactyla* Balss, 1922 no nuclear reduction was observed during spermiogenesis (Simeó *et al.*, 2010). *Callinectes ornatus* did not show a difference between spermatogonia and primary spermatocytes, although the cells were smaller, differing from *C. danae* (Zara *et al.*, 2012). In *U. cordatus* the nuclear volume is slightly reduced (Castilho *et al.*, 2008). In *C. ornatus* the nuclear volume did not change, indicating that in prophase I of meiosis, the genetic material remain dispersed as in the spermatogonia, despite the events of chromosome condensation. The nuclear morphology of the spermatogonia, primary and secondary spermatocytes have the same histological features described for other Brachyura (Ryan, 1967; Johnson, 1980; Garcia and Silva, 2006; Castilho *et al.*, 2008; Santos *et al.*, 2009; Stewart *et al.*, 2010; Zara *et al.*, 2012).

Spermiogenesis in Portunidae has been recently studied by means of light microscopy (Stewart *et al.*, 2010; Zara *et al.*, 2012), following the earlier study by Johnson (1980). In *C. ornatus* the sequence of spermatid maturation, including three developmental stages, is very similar to that described for *C. danae* by Zara *et al.* (2012), where in early spermatids the round nucleus progressively develops and the pro-acrosomal vesicle is noticed in the cytoplasm. The nucleus of intermediate spermatids changes into the C-shape below the acrosome. The nucleus of late spermatids is slender and forms a cup, surrounding the acrosome almost completely. Mature sperm were observed only in the lumen of the seminiferous duct, as reported for cancrids (Fasten, 1918) and portunids (Johnson, 1980; Stewart *et al.*, 2010; Ravi *et al.*, 2012; Zara *et al.*, 2012).

Juvenile males of *C. ornatus*, as well as adult males, produced sperm continuously. However, *C. ornatus* juvenile males cannot reproduce successfully, because the abdomen is still attached to the thoracic sternum and does not allow copulation and the insertion of copulatory pleopods into the female genital opening (Van Engel, 1990). On the other hand, *C. ornatus* DEV adult males may have a chance to transfer their genetic material in

spite of the smaller volume of seminal fluid compared to mature males. In *C. danae,* the DEV males had spermatophores with a similar diameter and identical in all histological characteristics to those of MAT males (Zara *et al.*, 2012). The DEV males of *C. ornatus* are able to copulate and have spermatophores and spermatozoa, but the volume of the seminal fluid is probably insufficient to form a complete sperm plug in the female receptacle. In Portunidae and Cancridae the female seminal receptacle is sealed by a sperm plug, which is formed by hardening of the male seminal fluid (Hartnoll, 1969). The sperm plug prevents sperm competition, by blocking the transfer of sperm from other males (Hartnoll, 1969; Diesel, 1990; Bauer and Min, 1993; Jivoff *et al.*, 2007, Zara *et al.*, 2012). It is assumed that in the genus *Callinectes* the females mate only once (Jivoff *et al.*, 2007; Van Engel, 1958). However, Jivoff *et al.* (1997, 2007) found that 12% of *C. sapidus* females had spermatophores from more than one male. If this also occurs in *C. ornatus*, part of the sperm may have originated from DEV male adults that failed to produce a complete sperm plug.

The vas deferens is divided into: anterior (AVD), middle (MVD) and posterior (PVD) as widely reported for Brachyura (Krol *et al.*, 1992). The AVD in *C. ornatus* was divided into two clearly differentiated regions with histological and histochemical differences, called the proximal (AVDp) and distal regions (AVDd) of the anterior vas deferens. In Brachyura, AVD divided into two regions was also reported for *Goniopsis cruentata* (Latreille, 1803) (Garcia and Silva, 2006) and *C. danae* (Zara *et al.*, 2012). However, in *C. sapidus* and *U. cordatus* this region was described as having three parts (Johnson 1980; Castilho *et al.*, 2008). The AVDp receives the sperm mass and packs them into spermatophores. The luminal secretion is heterogeneous, since two types of secretion can be seen: type I, basophilic, consisting of acid polysaccharides and type II, eosinophilic, which is glycoproteinaceous and contains neutral polysaccharides. The type I (basophilic) secretion forms a matrix that separates the sperm cells into sperm masses; while the masses of type II (eosinophilic) secretion are observed among the sperm groups. This secretion surrounds the sperm mass to form the spermatophore wall. The presence of eosinophilic secretions of AVDp and the spermatophore formation are very similar to the description for *C. danae* by Zara *et al.* (2012). The eosinophilic secretion has been reported only in the middle portion of the AVD in *C. sapidus* (Johnson, 1980) and in the AVDd of *U. cordatus* (Castilho *et al.*, 2008). The secretions in *C. ornatus* AVDd and AVDp have the same chemical characteristics. However, in the AVDd the spermatophores are already completely surrounded by the glycoproteinaceous wall, although this compound is still added to the spermatophores. A key aspect of this portion is the large quantity of polysaccharides separating the spermatophores. The presence of these two types of compounds in the AVDd was also detected in both closely and distantly related species (Johnson, 1980; Sainte-Marie and Sainte-Marie, 1999; Garcia and Silva, 2006; Castilho *et al.*, 2008; Erkan *et al.*, 2009; Stewart *et al.*, 2010; Zara *et al.*, 2012). Portions of the epithelium of the AVD undergo changes in MAT males of *C. ornatus,* from columnar in the AVDp to squamous in the AVDd. This change was also detected in the AVD of *C. sapidus* (Johnson, 1980) and *U. cordatus* (Castilho *et al.*, 2008), but in *C. sapidus* this change occurs between the distal and medial compartments of the AVD. However, this epithelial change was not observed in JUV and DEV adult males of *C. ornatus*, which have columnar epithelium along the AVD. Thus, in *C. ornatus* and probably other species, the epithelial change is associated only with the secretion volume, which is significantly larger in MAT. In JUV and DEV adult males the general behavior of the secretion in both regions of AVD was the same as in MATs, including the general histochemical aspect. The histochemistry of the AVD during these developmental stages has not been examined in other Brachyura, except for developing males of *C. danae* (Zara *et al.*, 2012). In this species,

the results were the same as for *C. ornatus,* and we suppose that this could be a general pattern, at least in members of the genus *Callinectes.*

The MVD is responsible for producing part of the seminal fluid, showing several lateral outpocketings (Ryan, 1967; Johnson, 1980; Zara *et al.*, 2012). The completely formed spermatophores are accumulated in the transition between the AVD and MVD of *C. ornatus,* and no new components are added. This region is characterized by the change from acid to neutral polysaccharides in the matrix and the occurrence of large eosinophilic granules in the seminal fluid. These granules and the matrix between them have intensely reactive proteins and no lipids, similarly to *C. danae* (Zara *et al.*, 2012). In *C. ornatus* the disappearance of acid polysaccharides coincides with the spermatophore maturation, which indicates that the spermatophore wall formation occurs only in the presence of acidic polysaccharides. Both the formation of spermatophores in the AVD and their accumulation in the MVD are widely reported for Brachyura (Johnson, 1980; Beninguer *et al.*, 1988; Sainte-Marie and Sainte-Marie, 1999; Moriyasu *et al.*, 2002; Castilho *et al.*, 2008; Zara *et al.*, 2012). The histochemical composition of the MVD lumen did not change between *C. ornatus* juvenile and adult males, but the volume was qualitatively larger in MAT males. Although the secretions have not been studied in juveniles of other species, the presence of eosinophilic granules, PAS (Johnson, 1980; Diesel, 1989; Benhalima and Moriyasu, 2000; Castilho *et al.*, 2008; Zara *et al.*, 2012) and protein positive (Benhalima and Moriyasu, 2000; Garcia and Silva, 2006; Zara *et al.*, 2012) was detected in MAT males of other species of Brachyura.

The PDV also has outpocketings, which are common in Portunidae (Ryan, 1967; Johnson, 1980; Zara *et al.*, 2012). As in *C. sapidus* (Johnson, 1980), *C. danae* (Zara *et al.*, 2012) and *Inachus phalangium* (Fabricius, 1775) (Diesel, 1989), the PVD in *C. ornatus* does not have large numbers of spermatophores and seems to have only a secretory function, and may produce most of the seminal fluid (Beninguer *et al.*, 1988). In *C. ornatus* this secretion is fairly homogeneous, giving a fluid appearance to the granular matrix found in the MVD, as also seen in other Brachyura (Johnson, 1980; Diesel, 1989; Zara *et al.*, 2012). Thus, the seminal liquid becomes fluid in the PVD, allowing the transfer of spermatophores to the female seminal receptacle. The liquid nature of the PVD secretion probably results from ion exchange in the epithelial cells, as speculated for *C. danae* (Zara *et al.*, 2012). This secretion is also a glycoprotein that reacts strongly to PAS, mercuric-bromophenol blue and Xylidine ponceau. Positive reactions in this region to PAS and proteins were also detected in Majoidea (Diesel, 1989; Beninguer *et al.*, 1988) and Portunidae (Johnson, 1980; Zara *et al.*, 2012). The histochemistry of PVD secretion in *C. ornatus* was similar in JUV, DEV and MAT despite of the amount of substance being bigger in MAT ones. This PVD secretion was different from the waxy, dense and composed by concentric layers, which is the main compound of the sperm plug in *Cancer borealis* (Moriyasu *et al.*, 2002). According to this authors this secretion were found only in mature males different to *C. ornatus*. Thus, in *C. ornatus*, the sperm plug is formed with a mixture of secretions produced along of the vas deferens as reported to other *Callinectes* (Zara *et al.*, 2012).

During the development of the male reproductive system, the GSI showed significant changes between the two earlier stages and MAT. These observations reinforce the hypothesis that the volume of seminal fluid increases during development, and is essential to ensure that only the genetic material from this particular male is inserted into the female seminal receptacle. Thus, the high GSI observed in these individuals seem to be related to both sperm production and seminal fluid, but the latter forms the sperm plug that prevents other males from depositing their sperm (Diesel, 1989, 1990; Jivoff *et al.*, 2007) and ensures the male's reproductive success. The development of the reproductive system, characterized by

increasing GSI, as well as testis and vas deferens weight, is accompanied by a reduction of the HSI throughout the JUV and developing adult stages, with a significant difference between DEV and MAT stages. This HSI reduction may be related to the use of part of the reserves accumulated in the hepatopancreas for the development of the reproductive system of *C. ornatus*. The use of the reserves from the hepatopancreas to develop the reproductive system in crustaceans has also been observed for the brachyurans *Sesarmops intermedius* (De Haan, 1835) (Kyomo 1988 as *Sesarma intermedia*) and *Spiralothelphusa hydrodroma* (Adiyodi, 1969 as *Paratelphusa hydrodromus*) females and for adult males of *C. danae* (Zara *et al.*, 2012); and for the shrimps *Farfantepenaeus aztecus* (Ives, 1891) and *Litopenaeus setiferus* (Linnaeus, 1767) (Lawrence and Castille, 1989 as *Penaeus aztecus* and *Penaeus setiferus*). On the other hand, Chu (1999) suggested that testis development in the Portunidae *Charybdis affinis* Dana, 1852 is not affected by HSI, because of the low production cost of male germ cells. However, this approach does not concord with the observations for *C. ornatus* and also for *C. danae* (Zara *et al.*, 2012). Further studies using other portunid genera and species are required to elucidate whether the reduction of HSI during the male reproductive system development is a general pattern for the family or is limited to the genus *Callinectes*.

In conclusion, males of *Callinectes ornatus* reach physiological maturity before the pubertal molt, since the sperm and spermatophore production did not differ during the developmental stages of JUV and DEV and MAT adults. The spermatophore formation, chemical composition of the seminal fluid, and GSI/HIS variation during the reproductive system development are very similar to *C. danae,* and these traits seem to form a pattern that could be expected at least in other species of *Callinectes*. The significant increase in the seminal volume of juvenile and developing adult males compared to mature males indicates that in the presence of an available female, a developing adult male may attain some degree of reproductive success. However, the efficiency of the sperm plug produced by a developing male should be tested in further studies.

Acknowledgements - FAN and FJZ are grateful to FAPESP (São Paulo Research Foundation; grants IC 2009/01348-5; JP 2005/04707-5; Temático BIOTA 2010/50188-8) for financial support, and to CNPq (*Conselho* Nacional de Desenvolvimento Científico e Tecnológico; FJZ grants PQ2 308215/2010-9; Universal 486337/2013-8). Thanks are also due to Drs. Bruno Sampaio Sant'Anna, Fernando L. Mantelatto and anonymous reviewers for their critical reading of early version of the manuscript and Janet Reid (JWR Associates) for editing the English text. This work was conducted according to applicable Brazilian regulations (FJZ MMA-ICMBio, license #34587-1).

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Submitted 26 September 2013
Accepted 06 December 2013