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SPERMATOGENESIS, SPERMATOPHORE, AND SEMINAL FLUID PRODUCTION IN THE ADULT BLUE CRAB *CALLINESTES DANAE* (PORTUNIDAE)

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ABSTRACT

Sperm and spermatophore production in *Callinectes danae* Smith, 1869 were examined by histochemistry and correlated with gonadosomatic (GSI) and hepatosomatic (HSI) indices. The GSI from developing (DE) and mature (MAT) males increased while the HSI decreased from DE to MAT, demonstrating that the maturation of the male reproductive system requires resources from the hepatopancreas. No histological or histochemical differences were observed between DE and MAT except for the larger amount of secretions produced in MAT. Spermatogenesis occurs in the seminiferous lobules surrounded by accessory cells. Each lobule is filled with cells at the same developmental stage. Spermatid maturation is characterized by an increase in the metachromatic acrosome. Sperm are released into seminiferous ducts, which moves them to the vas deferens divided into anterior (AVD), median (MVD), and posterior (PVD) regions. Spermatophore formation begins at the anterior part of AVD; sperm masses are separated and compacted in small packets by a basophilic and alcianophilic secretion. Small amounts of eosinophilic secretion, positive for proteins and neutral polysaccharides, are added around the sperm initiating the formation of the spermatophore wall. Mature round spermatophores are found in the posterior part of AVD and present a thick glycoproteinaceous wall, surrounded by acidic polysaccharides. The spermatophores are stored in MVD without size difference from DE to MAT. The MVD is filled with a granular secretion composed of glycoproteins. The secretion in PVD is fluid and homogeneous, facilitating the transference of the spermatophores. In conclusion, the hepatopancreas is related to the maturation of the male reproductive system in *C. danae*. DE males presented all histological conditions to fertilize females as MAT males, but the decrease in HSI and increase in GSI indices correlated with the vas deferens indicate that reserves are necessary to produce large amounts of seminal fluid in MAT males.

KEY WORDS: blue crab, gonadosomatic index, hepatosomatic index, histochemistry, male reproductive system

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INTRODUCTION

Reproductive effort is defined by the proportion of the energy an organism uses for reproduction and can be considered a key factor for the onset of the reproductive life span in animals (López-Greco and Rodríguez, 1999). The reproductive organs of decapod crustaceans apparently receive nutrients stored in the hepatopancreas; most studies suggest that in females they are used in the development of the ovaries (Chu, 1999; Castiglioni et al., 2006). However, in the male reproductive system, the energy requirements needed from the hepatopancreas apparently do not change during the development of testes when examined annually (Chu, 1999).

No information is available on the relationship between gonadosomatic (GSI) and hepatosomatic indices (HSI) during the adult development of the male reproductive system, and macroscopic patterns are poorly known. In Portunidae, the macroscopic development of the male reproductive system was examined in *Callinectes danae* Smith, 1869 (Costa and Negreiros-Fransozo, 1998), *Callinectes ornatus* Ordaway, 1863 (Mantelatto and Fransozo, 1999), *Arenaeus cribrarius* (Lamarck, 1818) (Pinheiro and Fransozo, 1998), and *Portunus spinimanus* Latreille, 1819 (Santos and Negreiros-Fransozo, 1999), but correlations with variations in GSI and HSI have not been examined to date. The correlation between macroscopic patterns and GSI/HSI can provide information on the physiology, development, and maturation of the male reproductive system. Macroscopically, the development of the male reproductive system is characterized by an increase in the volume of the vas deferens (Costa and Negreiros-Fransozo, 1998) and may be associated with the production of seminal fluid, which acts in the transfer of sperm (Hinsh, 1986, 1988a, b) and the formation of the sperm plug in the seminal receptacles of portunid females (Hartnoll, 1969; Johnson, 1980).

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The morphology of the reproductive system and spermatogenesis has been extensively studied in brachyurans (Binford, 1913; Fasten, 1918; Cronin, 1947; Ryan, 1967a; Johnson, 1980; Castilho et al., 2008; Erkan et al., 2009; Stewart et al., 2010). On the other hand, the many stages of spermiogenesis have been described mainly ultrastructurally (Langreth, 1969; Haley, 1986; Medina and Rodríguez, 1992; Medina, 1994; Simeó et al., 2010; Stewart et al., 2010), while few studies have used light microscopy for the identification of maturation stages. The first descriptions consisted of drawings representing spermiogenesis in *Menippe mercenaria* (Say, 1818) (Binford, 1913) and *Cancer magister* (Dana, 1852) (Fasten, 1918). Johnson (1980) and Stewart et al. (2010) reported preliminary descriptions of early spermatids and spermatids-spermatozoa in Portunidae. In *Chionoecetes opilio* (Fabricius, 1788), light microscopy spermatid maturation has been described in detail. However, unlike other brachyuran species, *C. opilio* spermatid maturation occurs outside the testicular lobules, inside spermatophores in the vas deferens (Sainte-Marie and Sainte-Marie, 1999).

The testis is continuous to the vas deferens, which can be divided into three portions: anterior or proximal (AVD), median or medial (MVD) and posterior or distal (PVD) (Johnson, 1980; Krol et al., 1992). Spermatophores are formed in the AVD; the MVD produces most of the granular seminal fluid and stores mature spermatophores, and the PVD exhibits lateral sacs and is involved in the production of seminal fluids (Johnson, 1980; Sainte-Marie and Sainte-Marie, 1999; Jivoff et al., 2007). In Portunidae, the formation of the spermatophore and the seminal fluid has been briefly described for *Callinectes sapidus* (Rathbun, 1896) by Johnson (1980).

The blue crab *C. danae* is of great economic importance in small-scale fisheries in Brazil (Severino-Rodrigues et al., 2001). This is the most abundant portunid in estuary-bay complexes with moderate to large influx of freshwater in Brazil (Pita et al., 1985; Costa and Negreiros-Fransozo, 1998; Branco and Masunari, 2000; Chacur et al., 2000). This species is also easy to keep under laboratory conditions and lends itself well to experimentation. Therefore, *C. danae* can be used as a model in Brazil just as *C. sapidus* is used in the USA. The aim of this study is to describe the relationship between the hepatosomatic and gonadosomatic indexes associated with the development of the reproductive system in adult males of *C. danae* and to characterize spermatogenesis, spermatophore production and seminal fluid with histochemical techniques.

Material and Methods

Male blue crabs were collected over the course of one year (March 2007 to March 2008) in the estuary-bay of São Vicente, southern Brazil by trawling during 20 minutes at eight sites (from 23°56′01.52″S/46°27′15.66″W to 23°58′52.19″S/46°21′23.51″W) using a semi-balloon net. Animals from each site were randomly chosen and transported alive in labeled plastic boxes to the laboratory for dissections. Others were maintained on ice and their reproductive maturation stage was determined macroscopically. The carapace width (CW) of all animals, including juveniles, was measured with a caliper (0.05 mm) and sex was determined based on abdominal characteristics common to the genus *Callinectes* (Pita et al., 1985; Van Engel, 1990). Animals missing or regenerating limbs were not included in analyses.

We used the Sturges formula: $k = 1 + \log 2\, n$ (K = number of width classes; n = number of individuals) (Sturges, 1926) to check the frequency distribution of sampled males at different stages of macroscopic development of their reproductive system.

We included only adult male blue crabs at hard-shelled intermoult condition C (Mantelatto and Fransozo, 1999). Animals were anesthetized by thermal shock (−20°C/15 minutes) (López-Greco et al., 1999), and the carapace was removed to access the reproductive system (testes and vasa deferentia) and hepatopancreas. The macroscopic development of the reproductive system was determined according to Costa and Negreiros-Fransozo (1998).

Each male crab and their reproductive system and hepatopancreas were weighed to determine the gonadosomatic index (GSI = weight of the reproductive system/animal weight) $*$ 100 and the hepatosomatic index (HIS = weight of the hepatopancreas/animal weight) $*$ 100 according to Kyomo (1988). Mean values and standard deviations for GSI and HSI were calculated for each developmental stage of the male reproductive system of *C. danae*. Averages were compared with the Student's T test ($\alpha = 0.05$) (Sokal and Rohlf, 1995).

For the histological and histochemical analysis testes and vasa deferentia of five developing (DE) and five mature (MAT), males of *C. danae* collected in different seasons were fixed in 4% paraformaldehyde prepared with water from the collection site and 0.2 M sodium phosphate buffer (pH 7.2), for 24 hours. After fixation, samples were dehydrated, embedded in methacrylate historesin Leica®, according to routine procedures. Serial sections of 5-8 μm in thickness were obtained with a rotary microtome. The material was stained with hematoxylin and eosin (H&E) according to Sant'Anna et al. (2010) for the general histological description, and toluidine blue pH 4.0 (Taboga and Dolder, 1991) for description of spermatogenesis.

The presence of proteins was demonstrated by mercuric-bromophenol blue staining (Pearse, 1960). Neutral polysaccharides with 1-2-glycol groups were detected by periodic acid Schiff (PAS) staining. The PAS was also conjugated to Alcian blue at pH 2.5 to acidic polysaccharides (Junqueira and Junqueira, 1983). The lipids were identified by Sudan black B (Junqueira and Junqueira, 1983). The samples fixed for Sudan black B staining were embedded directly in methacrylate avoiding dehydration steps and processed according to routine techniques proposed by Leica® protocol.

Three slides from different males were used to examine germ cells in different stages of spermatogenesis. In each slide, 30 nuclei were measured. Nuclear measurements were taken using the software Leica® IM50, with appropriate calibration for the objective lens used. We measured the longest nuclear diameter of germ cells stained with toluidine blue (pH 4.0). Averages as well as standard deviations were calculated using measurements from thirty cells per

stage. The results were compared with a Tukey's multiple comparison test ($\alpha = 0.05$) when a single-factor ANOVA indicated a significant difference among the size of nuclei being analyzed (Sokal and Rohlf, 1995). For spermatophore measurements, we performed MVD squashing and dilution in 5 ml of 4% paraformaldehyde taking 100 μl *per* slide from two DE and four MAT males. A total of 160 DE and MAT spermatophores were measured at the major axis using the same microscope and software as above. The Student's T test ($\alpha = 0.05$) was used to compare the average diameter between both groups (Sokal and Rohlf, 1995).

RESULTS

Relationship Between GSI and HIS

Of the 230 adult males of *C. danae* collected during 12 months, 32 were classified as DE and accounted for 14% of the individuals in the adult population. GSI indices varied significantly. The average GSI was 0.7 ± 0.2 for DE different from 3.5 ± 1 for MAT males ($t = -5.6938$, $P < 0.01$). HSI indices were significantly different ($t = -2.61428$, $P < 0.05$) between developmental stages. The average HSI indices were 4.5 ± 0.6 for DE and 3.5 ± 0.8 for MAT males (Fig. 1).

Gross Morphology

The male reproductive system of *C. danae* is a bilateral organ in the form of an "H" located in the cephalothoracic cavity. This organ is composed of paired testes and paired vasa deferentia (Fig. 2A-C). The gross morphology does not change during adult development (Fig. 2A-M), however, in the DE males the vasa deferentia are more slender (Fig. 2A and C-H) than in MAT males (Fig. 2B and I-M). The testis is a convoluted tube filled by seminiferous lobules (Fig. 2D and J). The testes are joined to each other by a transverse commissure near the vas deferens (Fig. 2A, B and I). As in Brachyura, the vas deferens is divided into AVD, MVD, and PVD sections (Fig. 2C and I). The AVD is tubular and convoluted filled by white secretion (Fig. 2E and K). In DE males the AVD, MVD and PVD are whitish (Fig. 2C-H) while in MAT the MVD is filled by pale pink secretion and the PVD is yellowish (Fig. 2B and I). Numerous lateral outpocketings (sensu Johnson, 1980) are visible in the MVD and PVD in both DE and MAT (Fig. 2G, H, L and M).

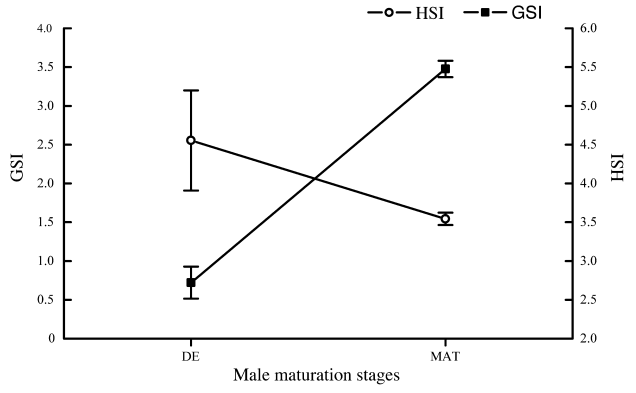

Fig. 1. Variations of gonadosomatic (GSI – line and circle) and hepatosomatic (HSI – line and square) indices in *C. danae* according to adult developmental stage of reproductive system, N = 109. Mean and standard error vertical bars depicted at each developmental stage. DE = developing male; MAT = mature male.

Histology and Histochemistry

Testis and Sperm – Spermatogenesis and Spermiogenesis.—Hard-shelled males displaying DE and MAT condition were found in almost all size classes ranging from 60 to 102 mm of CW (Fig. 3). No histological or histochemical differences were detected in the testes and the vasa deferentia of DE and MAT, including males analyzed during the same or different seasons. The only difference between DE and MAT adult males was the volume of the secretion produced by the vas deferens, which was qualitatively higher in MAT males (Fig. 2A-M).

The testes of DE (CW 60-100 mm) and MAT (CW 60-102 mm) (Fig. 2) adult males are lobular with many seminiferous or testicular lobules connected to a highly convoluted seminiferous duct. Seminiferous lobules are surrounded by a thin capsule of fibrous connective tissue and each lobule is delimited by accessory cells. Inside lobules, many germ cells at the same stage of meiosis are observed (Fig. 4A-I). The accessory cells show flat to ellipsoid nuclei and scarce cytoplasm (Fig. 4B and C). In lobules where spermatogenesis is more advanced (with developing spermatids) the accessory cells contain a large nucleus (Fig. 4D-F and H).

During spermatogenesis and spermiogenesis, the mean diameter of germ cell nuclei significantly decreased (ANOVA $F = 275.311$, $P < 0.001$). Spermatogonia are found in germinal centers, usually in the periphery of wide testicular lobules surrounded by flat accessory cells. The spermatogonia nuclei are large (7.5 ± 0.9 μm), with granules, and strongly stained with toluidine blue pH 4.0 (Fig. 4A). The spermatocytes and spermatids always occupy completely wide seminiferous lobules (Fig. 4A-F). The nuclei of primary spermatocytes remain large and in meiotic prophase, although significantly smaller (5 ± 0.4 μm) than those of spermatogonia (Fig. 4B) (Tukey $= 23.304$, $P < 0.01$). The nuclei of secondary spermatocytes are smaller (3.5 ± 0.3 μm), dense, and homogeneously stained with toluidine blue pH 4.0 (Fig. 4C). These nuclei are significantly different than those of the previous stage (Tukey $= 13.6823$, $P < 0.01$). Early spermatids are difficult to identify before the beginning of production of the acrosomal vesicle. This α-metachromatic vesicle arises in the cytoplasm and facilitates the identification of early spermatids, even when nuclei are still round (Fig. 4D). Nuclei are less dense than those of secondary spermatocytes. Developing or middle spermatids are characterized mainly by changes in the shape of the nucleus, from round to C-shaped over the acrosomal vesicle (Fig. 4E). In late spermatids, the nuclear cap (3.2 ± 0.2 μm) becomes more evident and surrounds most of the α-metachromatic vesicle. Nuclei are also narrower than those observed in previous stages (Fig. 4F). No differences were found between the diameter of these nuclei compared to those of secondary spermatocytes (Tukey $= 2.304$, $P > 0.05$). Sperm are characterized by nuclei surrounding almost completely the acrosome (Fig. 4G). However, despite morphological changes, the average diameter (3.0 ± 0.2 μm) was not significantly different than those of late spermatids (Tukey $= 1.8008$, $P > 0.05$). The average de-

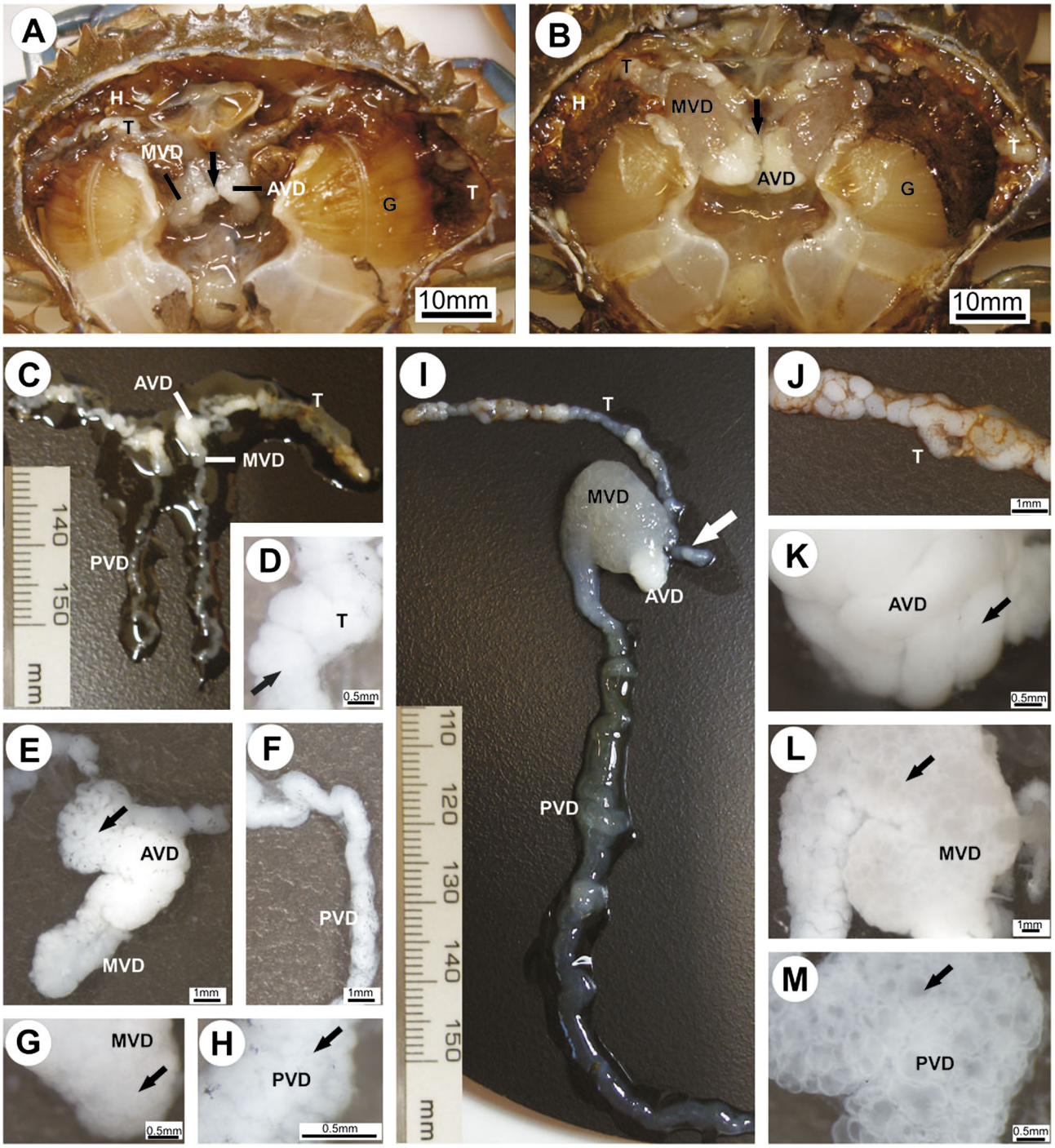

Fig. 2. *Callinectes danae* gross morphology and internal anatomy. A, Testes and vasa deferentia lie on hepatopancreas of DE male with CW = 70.4 mm; B, MAT male (CW = 74.6 mm) showing enlarged vas deferens [in both A and B, heart removed and only anterior and median region of vas deferens observed]; C, Complete reproductive system of DE male depicting pair of testis and vasa deferentia divided in anterior, median, and posterior regions; D, Detail of seminiferous loblules (arrow) in DE testis; E, Tubular anterior vas deferens (arrow) filled with sperm and white secretion and poorly developed median vas deferens; F, Convoluted posterior vas deferens from DE male; G and H, Detail of lateral out-pocketings (arrows) in both median (G) and posterior (H) vas deferens; I, General view of left reproductive system from MAT male [arrow depicts commissure joining both testis]; J, Detail of lobular testis in MAT individuals; K, Convoluted tubules (arrow) of anterior vas deferens; L and M, Enlarged median (L) and posterior (M) vas deferens from MAT males showing lateral out-pocketings (arrows). AVD = anterior vas deferens; H = hepatopancreas; G = gills; MVD = median vas deferens; PVD = posterior vas deferens; T = testis.

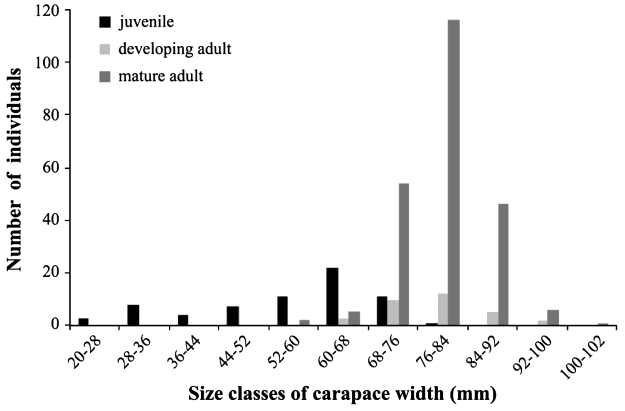

Fig. 3. Frequency distribution of males classified according to morphological and adult macroscopic development of reproductive system related to classes of carapace width using the Sturges formula (Sturges, 1926).

crease in nuclear diameter during spermatogenesis is illustrated in Fig. 5.

Although no significant differences were found regarding the diameter of germ cells of adult males (ANOVA $F = 1.7813$, $P = 0.1390$), the number of lobules with spermatozoa produced in MAT is apparently higher (Fig. 4H) than DE. The seminiferous duct is composed of a monostratified epithelium and the epithelial cells are columnar or cubic (Fig. 4G and I) and receives the spermatozoa from the testicular lobule (Fig. 4I).

Vas Deferens – Spermatophore Production and Seminal Fluid

Spermatozoa move within the seminiferous ducts and converge to a single collecting duct or vas efferens (Fig. 6A) which opens at the most anterior part of convoluted vas deferens.

In *C. danae*, the AVD was divided into two portions: proximal (AVDp), immediately after the vas efferens (Fig. 6A), and distal (AVDd), near MVD. The vas efferens receive the sperm from the seminiferous ducts and open into the AVD. Both vas efferens and AVDp show the same type of columnar epithelium (Fig. 6A-C). Associated with the inner epithelium is a single intermediary thin layer of musculature and an outer layer of connective tissue with fibroblast-like cells (Fig. 6C). In the AVDp lumen, many free spermatozoa are surrounded by a type I secretion which is highly basophilic (Fig. 6A and B). This secretion apparently aids the separation of spermatozoa into large distinct masses (Fig. 6B), by forming small ridges in between. A second secretion called type II is strongly acidophilic and penetrates into the ridges formed by the basophilic secretion (Fig. 6A-C), which will be part of the spermatophore wall. The wall of the AVDd is composed by the same architecture as the AVDp except the epithelial cells are cubic (Fig. 6D-I). The lumen of the AVDd exhibits the same two types of secretion, however the amount of secretion seems greater than in the AVDp. In the AVDd completely formed ovoid spermatophores are observed. The luminal secretion type I (basophilic) is strongly stained with Alcian blue (pH 2.5), while the acidophilic secretion (type II) is stained for neutral polysaccharides and seems to be gradually added to the spermatophores wall (Fig. 6D-F). The secretion type II is also positive for proteins (Fig. 6G) and form the glycoproteinaceous wall surrounding the sperm masses. The glycoproteinaceous spermatophore wall shows only one layer at light microscopy (Figs. 6C-I and 7A-D). Mercuric bromophenol blue staining demonstrates that the basophilic type I secretion is composed exclusively of acidic polysaccharides being negative to proteins (Fig. 7G). Both types I and II secretions present in the AVD do not contain lipids (Figs. 6H and 7C).

The secretion found in MVD is very different from that observed in AVD. This difference is clearly observed in the transition area between the two regions (Figs. 6I and 7A-C). The acidophilic secretion in MVD contains granules, while the secretion in AVD is more fluid and basophilic (Fig. 6I). In addition to the changes in staining patterns, polysaccharides and proteins present in the secretions are also different. The secretion found in MVD is strongly stained for neutral polysaccharides and proteins, while that of AVD is strongly positive for acidic polysaccharides and negative for proteins (Fig. 7A and B). The secretions found in MVD do not contain lipids (Fig. 7C).

MVD is characterized by several well-developed lateral out-pocketings filled with secretions that are discharged into a larger central duct (Fig. 7D). The columnar epithelium consists of cells with basal nuclei, which lie upon connective tissue. No histological differences were observed between the epithelium of the lateral out-pocketings and the central duct. The luminal secretion is composed mainly of acidophilic granules, although basophilic granules are also found (Fig. 7E). This difference was also observed with PAS and bromophenol blue staining, as luminal granules of MVD exhibit slightly different staining patterns for the two dyes (Fig. 7F and G). Both secretions were negative for acidic polysaccharides (Fig. 7A). Ovoid spermatophores are observed in the duct between the acidophilic and granular secretion. The average diameter of DE (270 ± 41 μm) and MAT (277 ± 32 μm) spermatophores was not different ($t = -1.6995$; $P > 0.05$). Moreover, both DE and MAT spermatophores depict great variation in diameter from 165 to 373 μm for DE while MAT ranging from 185 to 367 μm.

The posterior vas deferens also exhibits lateral out-pocketings as observed in MVD, associated with a central duct (Fig. 7H and I). However, the amount of lateral out-pocketings seems to decrease from closer to the MVD, which exhibit a mixture of granular and fluid secretion (Fig. 7I) to distal portions of the PVD, where the secretion becomes more fluid and lacks granules (Fig. 7H). In this region, the epithelium is flatter (Fig. 7H) and the secretion is less basophilic than that of MVD (Fig. 7D). Its compounds are negative for acidic polysaccharides, positive for neutral polysaccharides and proteins (Fig. 7J and K), and negative for lipids. The histochemical results for the vas deferens and spermatophore are summarized in Table 1.

DISCUSSION

During the maturation of the male reproductive system of *C. danae*, resources from the hepatopancreas are mobilized while GSI levels increase. This increase in the reproductive system weight is directly associated with the vas deferens, although DE and MAT adult males retain the same histological characteristics. The mobilization of resources from the

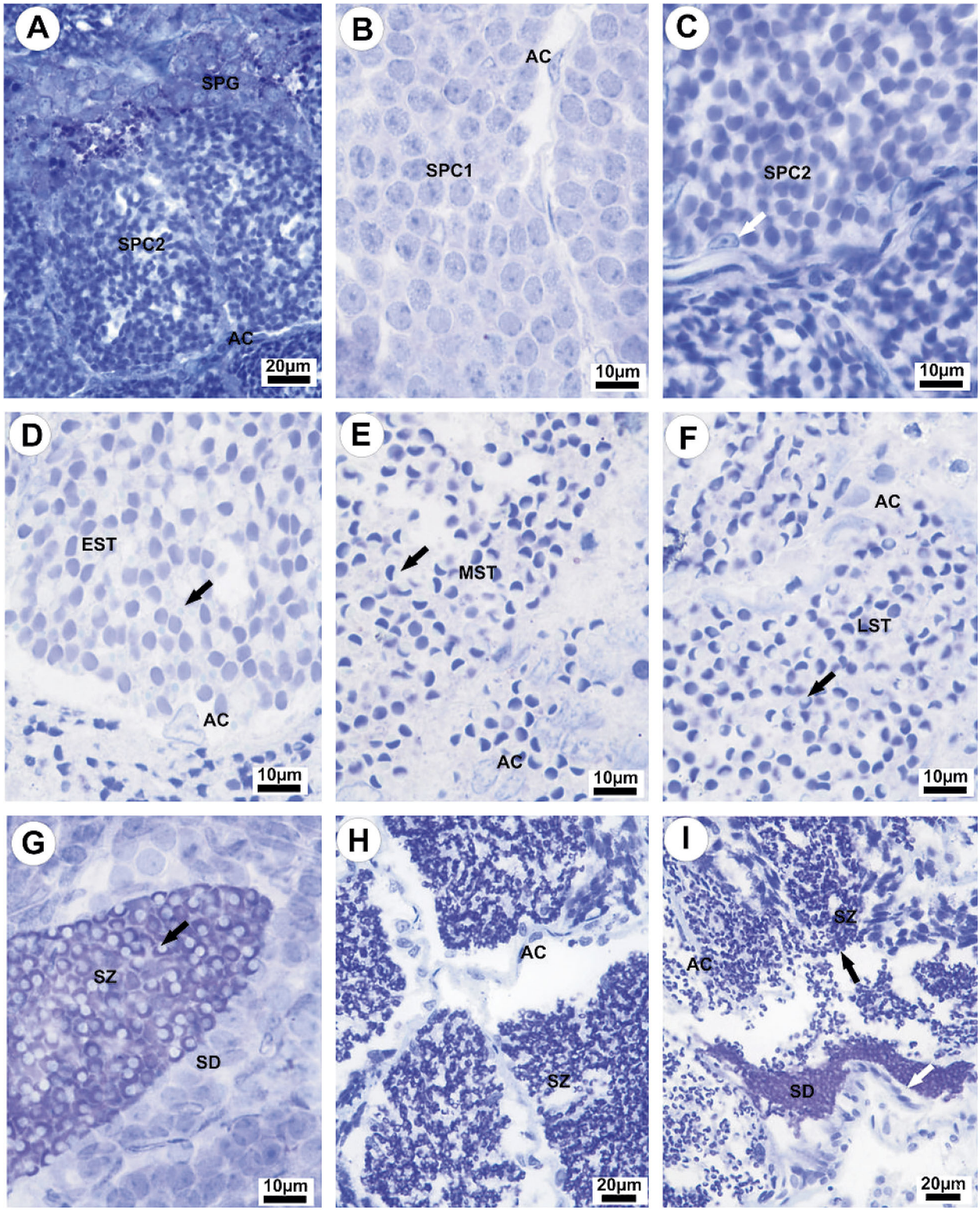

Fig. 4. Spermatogenesis in DE males of *C. danae*, Toluidine blue staining (pH 4.0). A, Germinal center of spermatogonia in periphery of two seminiferous lobules filled by spermatocytes II [both lobules delimited by accessory cells]; B, Seminiferous lobule of spermatocytes I with characteristics of meiotic prophase delimited by accessory cells with flat nuclei; C, Spermatocytes II with small and homogeneous nuclei and elliptical accessory cell (arrow); D, Early spermatids with round nuclei and proacrosomal vesicle with α metachromasia (arrow) [note increasing size of accessory cells during spermiogenesis]; E, Intermediary spermatids with nuclei forming cup on proacrosomal vesicle (arrow); F, Late spermatids with compact nuclear cup on acrosome (arrow); G, Seminiferous duct filled with spermatozoa with α-metachromatic acrosome (arrow); H, General view of several testicular lobules in MAT males filled with mature spermatozoa and accessory cells with condensed nuclei; I, Testicular lobule (arrow) with flat accessory cells releasing spermatozoa into seminiferous duct formed with cubical/columnar epithelium (white arrow). AC = accessory cell; EST = early spermatid; LST = late spermatid; MST = middle spermatid; SD = seminiferous duct; SPC1 = spermatocytes I; SPC2 = spermatocytes II; SZ = spermatozoa.

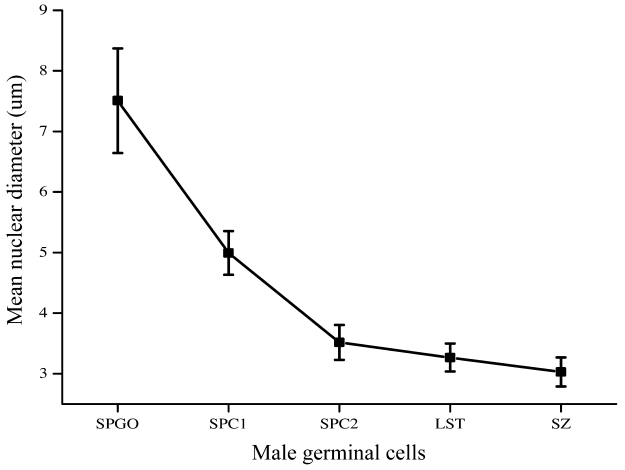

Fig. 5. Decrease in average nuclear diameter during spermatogenesis and spermiogenesis in adult males of *C. danae*. Mean and standard deviation bars depicted at each germinative cell type. LST = late spermatid; SPGO = spermatogonia; SPC1 = primary spermatocytes; SPC2 = secondary spermatocytes; SZ = spermatozoa.

hepatopancreas strongly suggests an investment in the reproduction of *C. danae*, also supported by the wide overlap of size classes of DE and MAT hard-shelled males. The development of the male reproductive system occurs rapidly, as the number of DE individuals found in the population was low (14%), with all of them displaying an HSI index significantly higher than MAT individuals. The mobilization of resources stored in the hepatopancreas for reproduction has been reported in many female crustaceans (Kyomo, 1988; Chu, 1999; López-Greco and Rodríguez, 1999; Castiglioni et al., 2006). However, most studies did not find a correlation between the hepatopancreas and the male reproductive system (Kyomo, 1988; Chu, 1999; Yamaguchi, 2001; Castiglioni et al., 2006; Sokolowicz et al., 2006). In general, studies on HSI and GSI have focused on the annual variation to characterize sexual maturity and reproductive periods (Kyomo, 1988; Chu, 1999; López-Greco and Rodríguez, 1999; Castiglioni et al., 2006; Sokolowics et al., 2006). The association between macroscopic developmental patterns of the male reproductive system similar to that described for several Portunidae (Costa and Negreiros-Fransozo, 1998; Pinheiro and Fransozo, 1998, 2002; Mantelatto and Fransozo, 1999; Santos and Negreiros-Fransozo, 1999) and GSI and HSI presented here, demonstrates for the first time the involvement of reserves from the hepatopancreas in the sexual maturation of adult males in *C. danae.*

The overlapping size classes between DE and MAT in *C. danae* support the different macroscopic conditions of the male reproductive system, specially the vas deferens and are in line with those found for other brachyuran sperm plug producers, such as Portunidae and Cancridae (Pinheiro and Fransozo, 1998; Mantelatto and Fransozo, 1999; Ungfors, 2007). A small percentage of intermoult DE males in the population of *C. danae* seems to be common in reports for other Portunidae (Mantelatto and Fransozo, 1999; Pinheiro and Fransozo, 2002; Ungfors, 2007). In *C. sapidus*, intermoult males showing extremely low vas deferens weight were considered sperm-depleted males (Kendall et al., 2001; Carver et al., 2005). Similarly, this hypothesis for DE intermolt animals of *C. danae* also being sperm-depleted or recovering males should not be dismissed. However, in *C. danae* the difference between DE and MAT individuals is in part due to their reproductive system development since the vas deferens from pre-copulatory and early post-copulatory males are very similar macroscopically (Zara, unpublished data).

The H-shaped and three-part reproductive system composed of testes and vas deferens of DE and MAT males of *C. danae* is similar to that observed for Podotremata (Minagawa et al., 1994) or other Eubrachyura (Fasten, 1918; Ryan, 1967a; Johnson, 1980; Moriyasu and Benhalima, 1998; Simeó et al., 2009; Stewart et al., 2010). The testes of *C. danae* can be classified as the lobular type (Nagao and Munehara, 2003; Simeó et al., 2009) and this is the most common term used for brachyuran crabs (Johnson, 1980; Batoy et al., 1989; Diesel, 1989; Moriyasu and Benhalima, 1998; Castilho et al., 2008; Erkan et al., 2009; Santos et al., 2009, Simeó et al., 2009). However, some authors refer to them as tubules (Fasten, 1918), testicular cysts with collecting ducts (Garcia and Silva, 2006), lobules and seminiferous tubules (Sal Moyano et al., 2009), lobules, cysts and seminiferous tubules for the same testicular structure (Santos et al., 2009). The use of these different terms could be misleading in the morphological analysis of testes. Simeó et al. (2009) provide the clearest explanation about the lobular and tubular types of testes found in brachyurans as proposed by Nagao and Munehara (2003). We suggest that the terms seminiferous lobules and seminiferous ducts be used for lobular testes, and consider lobules and cysts as synonyms.

In *C. danae*, spermatogonia are concentrated in the periphery of more advanced testicular lobules as observed in other lobular-type testis brachyuran (Johnson, 1980; Garcia and Silva, 2006; Santos et al., 2009; Stewart et al., 2010). The germ cell are not concentrated on one side of the periphery forming germinative zone, with sequential transition and evacuation zones, as proposed by Nagao and Munehara (2003) and observed in the tubular brachyuran testes species (Binford, 1913; Erkan et al., 2009; Simeó et al., 2009, 2010). Instead, after the spermatogonia in *C. danae*, the seminiferous lobule is filled by almost the same stage cells of spermatocytes or spermatids in both DE and MAT as widely described among Brachyura (Ryan, 1967a; Johnson, 1980; Hinsch, 1988b; Minagawa et al., 1994; Castilho et al., 2008) and non-brachyuran species (for review see Simeó et al., 2009). During spermatogenesis a significant reduction in the nucleus occurs, as has also been observed in Portunidae (Johnson, 1980; Stewart et al., 2010) and *Ucides cordatus* (Linnaeus, 1763) by Castilho et al. (2008). However no nuclear reduction was detected in *Maja brachydactyla* Balss 1922 (Simeó et al., 2010).

Inside the lobules of *C. danae* where the spermatogenesis is not yet advanced, accessory cells are similar to other *Callinectes* (Johnson, 1980) and *Portunus* (Ryan, 1967a; Batoy et al., 1989; Stewart et al., 2010) species with flat to round-shaped nuclei. In lobules filled by early and intermediary spermatids, these cells exhibit more cytoplasm and large round-shaped nuclei. According to Johnson (1980), the role of these cells is unknown in *C. sapidus*. In *C. danae*, they are apparently more active, indicating a participation

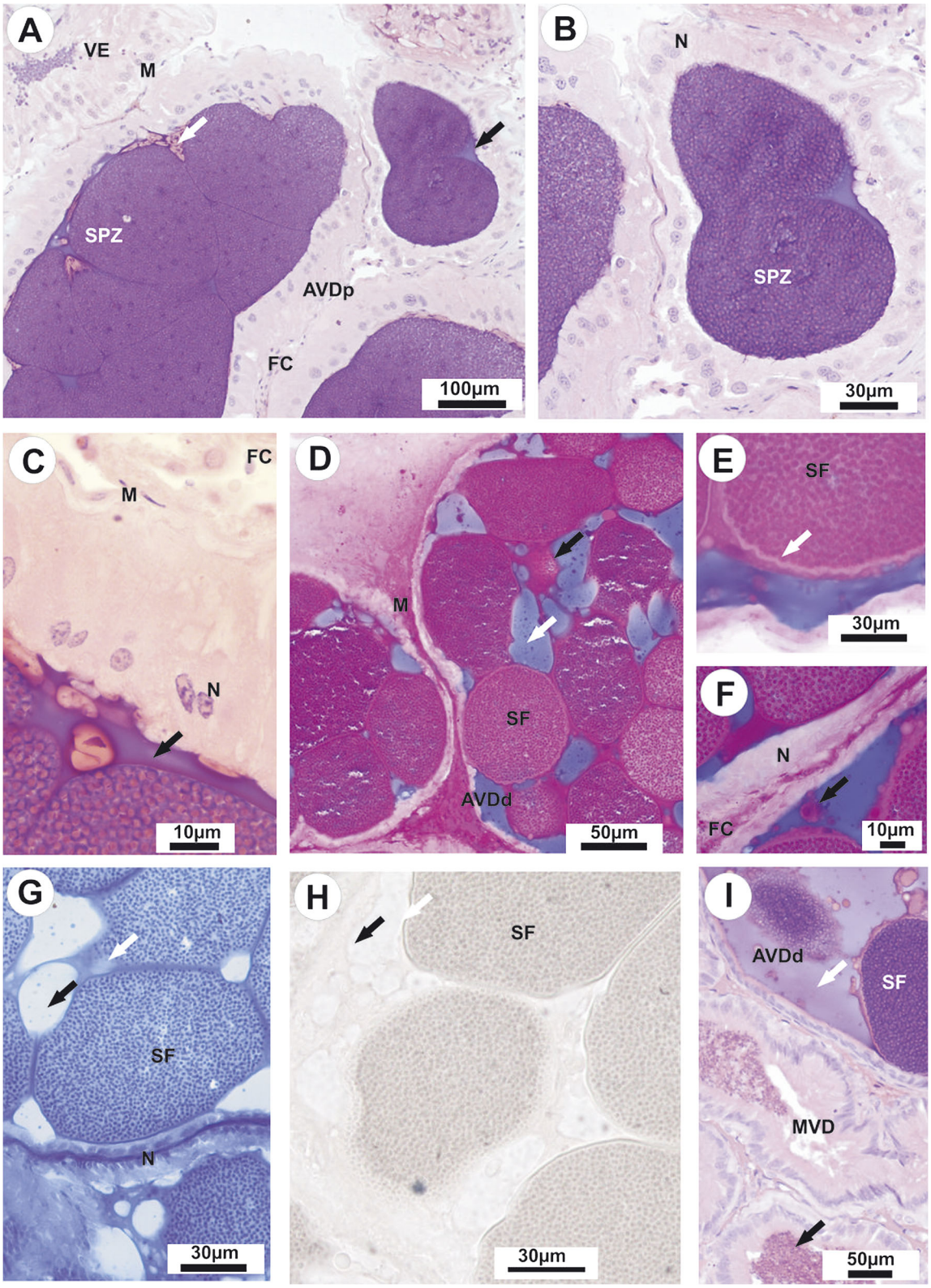

Fig. 6. AVD in DE males of *C. danae*. A, General view of vas efferens filled by sperm and proximal portion of AVD showing lumen filled with basophilic secretion (black arrow), which apparently compacts and separate the mass of free spermatozoa [in another portion of AVD, thick acidophilic secretion penetrates between masses of spermatozoa (white arrows)], H&E; B, Detail of lumen filled with spermatozoa compacted by basophilic secretion, H&E; C, Thick acidophilic secretion penetrating between spaces formed by basophilic secretion (arrow); D, Distal portion of AVD showing in lumen, acidophilic secretion strongly positive for acidic polysaccharides (white arrow), and basophilic secretion positive for neutral polysaccharides (black arrow) PAS/Alcian blue pH 2.5 staining; E, Detail of the PAS stained wall of the spermatophore (arrow) PAS/Alcian blue pH 2.5 staining; F, Detail of luminal secretion strongly stained for neutral polysaccharides (arrow), within in alcianophilic matrix, PAS/Alcian blue pH 2.5 staining; G, Lumen of distal region of AVD, basophilic

in spermiogenesis and release of cells into the seminiferous duct as also found in Majoidea (Simeó et al., 2010).

Spermiogenesis in *C. danae* is characterized by three cellular stages: 1) early spermatid with metachromatic proacrosomal vesicle and round-shaped nucleus, 2) middle or intermediary spermatids with large "C"-shaped nucleus around the proacrosomal vesicle, 3) and late spermatid with thin nucleus forming a nuclear cup around the acrosome. In *Portunus pelagicus* (Linnaeus, 1758), the three stages of spermatid maturation were characterized by light microscopy, revealing changes in nuclear blocks of chromatin; the proacrosomal vesicle or acrosomal vesicle was only observed by TEM (Stewart et al., 2010). Regardless of *C. danae* spermatids being smaller than *P. pelagicus*, their proacrosomal vesicle is always observed during the three maturation phases. These three phases, with many intermediate steps, are usually described in ultrastructural studies (Haley, 1986; Medina and Rodríguez, 1992; Medina, 1994; Simeó et al., 2010). However, most studies using light microscopy have not described spermiogenesis (Batoy et al., 1989; Diesel, 1989; Moriyasu and Benhalima, 1998; Garcia and Silva, 2006; Castilho et al., 2008; Erkan et al., 2009; Santos et al., 2009), making comparisons among groups difficult. At the end of spermiogenesis in *C. danae* only mature spermatozoa are released into seminiferous ducts, unlike that observed in the tubular testes of *Chionoecetes opilio* and *Eriphia verrucosa* (Forskål, 1775), in which spermatids are still observed in the lumen (Sainte-Marie and Sante-Marie, 1999; Erkan et al., 2009).

The anterior vas deferens in *C. danae* is different from that found in *C. opilio*, as it presents a monostratified columnar epithelium, instead of a simple cubic one (Moriyasu and Benhalima, 1998). Compared to the portunid crab *C. sapidus* (Johnson, 1980), the anterior region of these vasa deferentia are very similar, however, in *C. danae*, cells are not multinucleated. The AVD of *C. danae* can be divided into two portions, proximal (AVDp) and distal (AVDd), based on the type of luminal secretion as proposed for *Chionoecetes opilio* (Beninger et al., 1988; Sainte-Marie and Sainte-Marie, 1999). Johnson (1980) divided the AVD into three parts with the anteriormost region corresponding to the vas efferens, that receives sperm from the seminiferous ducts. The arrangement in *C. danae* is similar to that proposed for *C. sapidus* (Cronin, 1947). This same region was termed a typhlosole in *Portunus sanguinolentus* (Herbst, 1783) (Ryan, 1967a).

No histological or histochemical differences were observed between DE and MAT males of *C. danae* regarding the anterior (AVD), median (MVD), and posterior (PVD) regions of the vas deferens. However, qualitatively, the entire vas deferens (AVD, MVD and PVD) is much larger in MAT males. It is likely that the whole process of spermatophore formation promoted by the secretions types I and II are assisted by muscular contractions (Spalding, 1942; Cronin, 1947; Ryan, 1967a; Simeó et al., 2009). The basophilic and acidophilic substances in AVDp are a common characteristic reported for spermatophore production for both Portunidae (Spalding, 1942; Uma and Subramoniam, 1979; Johnson, 1980; Stewart et al., 2010) and Majoidea (Sainte-Marie and Sainte-Marie, 1999; Simeó et al., 2009). On the other hand, the presence of alcianophilic secretions (negative to PAS and bromophenol blue) in this region and the early formation of the spermatophore, has not been described for several brachyuran species (Johnson, 1980; Garcia and Silva, 2006; Castilho et al., 2008; Erkan et al., 2009; Santos et al., 2009). However, in *C. opilio* two types of secretion have been found. One type of secretion is PAS positive and aggregates masses of spermatids, while the other type, a PAS-negative secretion (Sainte-Marie and Sainte-Marie, 1999) may correspond to the alcianophilic secretion in *C. danae*. In the distal portion of AVD of *C. danae*, sperm masses already enclosed by the spermatophore wall continue to receive glycoproteins, always separated by acidic carbohydrates, as reported for other brachyuran species (Johnson, 1980; Garcia and Silva, 2006; Erkan et al., 2009). According to Garcia and Silva (2006) and Erkan et al. (2009), PAS-positive, alcianophilic compounds stained for proteins are produced continuously in other regions (MVD and PVD) of the vas deferens, unlike that observed in *C. danae*.

The spermatophores of *C. danae* showed a similar diameter in both DE and MAT males and are classified as type I, typical of crustaceans with internal fertilization with storage in seminal, round-shaped receptacles (Erkan et al., 2009). The PAS chemical composition is similar to those observed in other portunids (Uma and Subramoniam, 1979; Johnson, 1980), although the presence of proteins was found only in other non-portunids species (Garcia and Silva, 2006; Erkan et al., 2009). With this type of composition, the spermatophore of *C. danae* can be structurally resistant and at the same time permeable to low molecular weight molecules (Uma and Subramoniam, 1979). Thus, the spermatophore wall of *C. danae* is probably a more inert structure than that observed in oregoniid and eriphiid crabs where the maturation of spermatids in spermatozoa occurs inside the spermatophore and therefore is dependent on external compounds (Beninger et al., 1988; Sainte-Marie and Sainte-Marie, 1999; Erkan et al., 2009).

The MVD exhibits many lateral out-pocketings along the highly convoluted tubules, as also reported for other Portunidae (Cronin, 1947; Ryan, 1967a; Johnson, 1980). These lateral expansions of the vas deferens were also reported in Majoidea as cecae (Beninger et al., 1988; Diesel, 1989; Sainte-Marie and Sainte-Marie, 1999) or diverticula (Simeó et al., 2009). The secretion produced by lateral out-pocketings is added to the seminal fluid (Beninger et al., 1988; Diesel, 1989; Sainte-Marie and Sainte-Marie, 1999), and altered in the posterior vas deferens (Moriyasu et al., 2002). This also occurs in *C. danae* since the granular secretion changes in the PVD. The granular secretion of the MVD in *C. danae* is eosinophilic and displays the same

secretion negative for proteins (black arrow), while acidophilic secretion positive (white arrow) [note that spermatophore walls positive for proteins], mercuric bromophenol blue staining; H, AVD negative for lipids and spermatophores weakly positive, sudan black B staining; I, Transition between vas deferens with basophilic luminal matrix (white arrow) in AVD and acidophilic matrix (black arrow) in MVD, H&E. AVDd = distal portion of AVD; AVDp = proximal portion of AVD; M = muscles; N = nucleus; SF = spermatophores; SZ = spermatozoa; VE = vas efferens.

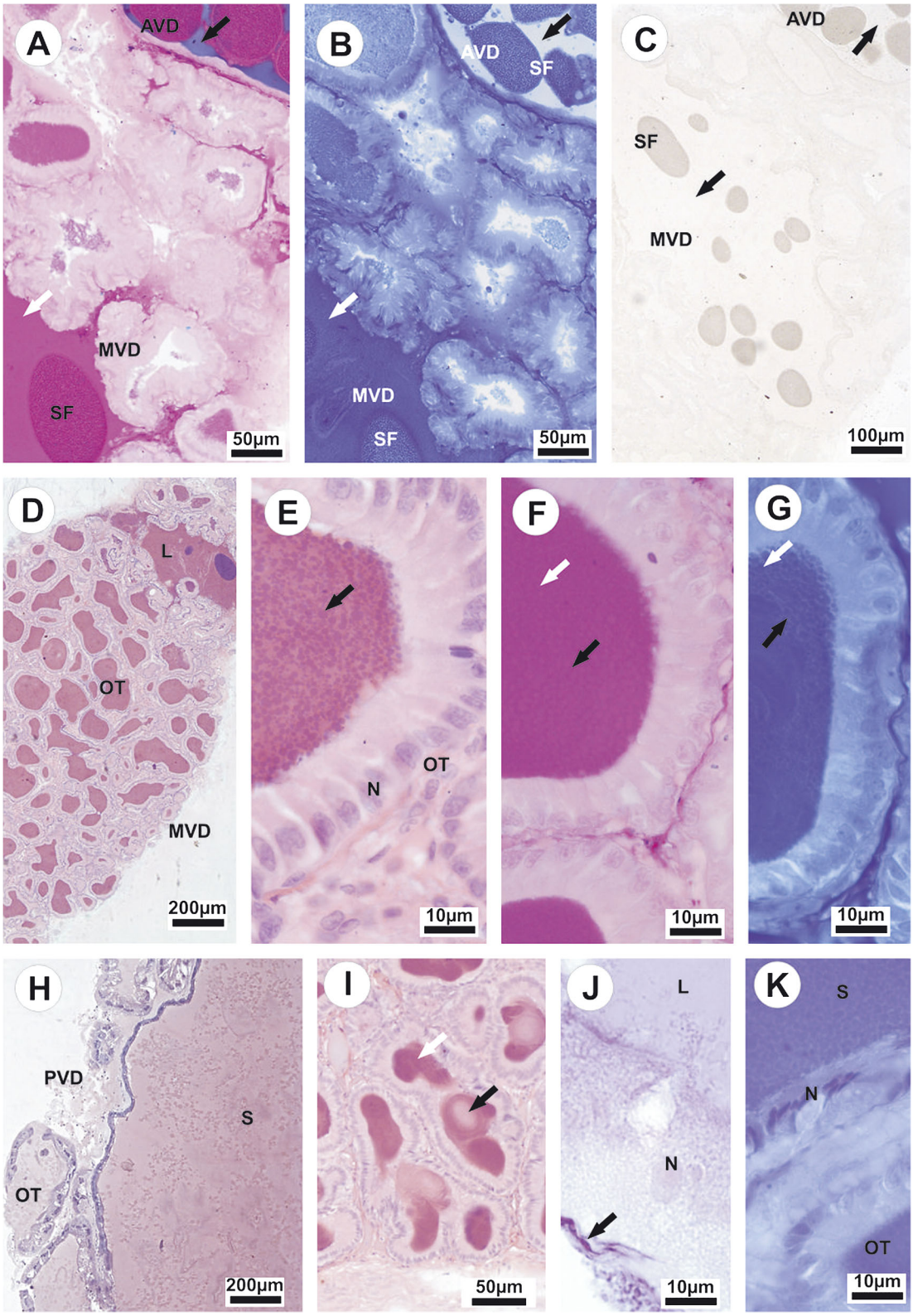

Fig. 7. Transition between AVD and MVD in MAT males (A-C). A, Differences in luminal secretion of distal portion of AVD rich in acidic polysaccharides (black arrow) while in MVD secretion positive for neutral polysaccharides (white arrow), PAS/Alcian blue pH 2.5 staining; B, Luminal secretion of distal portion of AVD negative for proteins (black arrow), except for that associated with spermatophores MVD with secretion strongly stained for proteins (white arrow), mercuric bromophenol blue staining; C, Both AVD and MVD negative for total lipids (arrows), sudan black B staining. MVD in MAT males (D-G); D, Portion of MVD with several lateral out-pocketings and central duct both showing same monostratified columnar epithelium; E, Detail of lateral out-pocketings columnar epithelium intermingled with connective tissue [luminal secretion composed of acidophilic granules exhibiting different staining patterns (arrow)], H&E; F and G, Differences in staining patterns of granules and matrix of secretion in MVD lumen for PAS/Alcian blue pH 2.5 and

Table 1. Histochemical data for secretion in anterior (AVD), medium (MVD) and posterior (PVD) regions of vas deferens and spermatophore wall in *Callinectes danae* males. +++ = strongly positive; ++ = positive, – = negative.

Secretion	Histochemical compound			
	Acid polysaccharides	Neutral polysaccharides	Proteins	Lipids
AVD type I	+++	–	–	–
AVD type II	–	+++	+++	–
MVD granules	–	+++	+++	–
MVD matrix	–	++	+++	–
PVD	–	++	+++	–
Spermatophore wall	–	+++	+++	–

characteristics as that in *C. sapidus* (Johnson, 1980). This is in contrast though to laminar secretions in *M. brachydactyla* (Simeó et al., 2009) or homogeneous seminal fluid in *Libinia spinosa* H. Milne Edwards, 1834 according to Sal Moyano et al. (2009). This secretion forms a matrix of glycoproteins, strongly positive for neutral polysaccharides and proteins, and negative for lipids. The presence of glycoproteins as observed in *C. danae* has only been previously reported in *C. opilio* (Benhalima and Moriyasu, 2000). Unlike the role of completing the formation of spermatophores (Beninger et al., 1988; Diesel, 1989; Sainte-Marie and Sainte-Marie, 1999) or inducing the differentiation of spermatids into spermatozoa in spermatophores (Sainte-Marie and Sainte-Marie, 1999) the granular secretion in *C. danae* apparently aids maintenance of spermatophores in MVD and is the main compound of the seminal fluid.

The PVD secretion in *C. danae* changes from granular and heterogeneous to smooth and homogeneous as also reported in *C. sapidus* (Johnson, 1980). The PVD has previously been described as a simple conduit for secretion and spermatophores (Johnson, 1980). However, this region seems to have an important role in making the seminal plasma more fluid, which might facilitate the transit of spermatophores into the female seminal receptacle during mating. This region shows less developed lateral out-pocketings than the MVD which make it different to Majoidea since PVD diverticula (named accessory glands) or caeca are well developed (Sainte-Marie and Sainte-Marie, 1999; Benhalima and Moriyasu, 2000; Simeó et al., 2009) and produce granular secretions (Diesel, 1989; Simeó et al., 2009). Thus, the PVD of Portunidae resembles the MVD of the species of Majoidea in light microscopy and the ion exchange function proposed for the MVD of *M. brachydactyla* (Simeó et al., 2009) could be speculated for *C. danae* PVD. The fluid matrix still contains neutral glycoproteins similar to the seminal plasma of *Scylla serrata* (Forskål, 1775) as found by Jeyalectumie and Subramoniam (1991) and Jayasankar and Subramoniam (1997). In grapsid and eriphiid crabs, this secretion contains neutral and acidic polysaccharides (Garcia and Silva, 2006; Erkan et al., 2009), but is PAS negative in oregoniid crabs (Sainte-Marie and Sainte-Marie, 1999). This histochemical variation noticed among brachyuran families may be associated with the different functions of seminal fluid during sperm transfer and/or sperm storage in the female seminal receptacle.

The sperm transfer in *C. danae* during mating occurs by muscular contraction as described for other Brachyura (Hartnoll, 1969). The thin muscles that surround the epithelium of the vas deferens probably play a role in this process. However, they seem to be more important in AVD, during the formation of spermatophores. The vas deferens seminal fluid is stored in the seminal receptacle of the portunid female (Ryan, 1967a, b; Johnson, 1980; Jivoff et al., 2007). These compounds, in addition to those of the receptacle, probably play important roles that have not yet been identified. These roles could include the mechanical conduction of spermatophore and sperm to the female (Adiyodi and Adiyodi, 1975; Johnson, 1980; Adiyodi, 1988; Sainte-Marie and Sainte-Marie, 1998; Benhalima and Moriyasu, 2000) avoiding loss of sperm (Spalding, 1942; Ryan, 1967b; Diesel, 1989); dehiscence of spermatophores by wall rupture (Ryan, 1967b; Diesel, 1989; Sainte-Marie and Sainte-Marie, 1998); formation of the sperm plug to prevent other males from transferring their spermatophores to the female (Hartnoll, 1969; Johnson, 1980; Diesel, 1989, 1991) preventing sperm competition (Diesel, 1990; Sal Moyano et al., 2009); dorsally displacing of old ejaculates (Diesel, 1989, 1990, 1991; Sal Moyano et al., 2009); proteolitic activity related to formation and or dissolution of sperm plug (Jayasankar and Subramoniam, 1997); acting as a "nuptial gift" of energetic value to the female (Hines et al., 2003); sperm nourishment in seminal receptacles (Spalding, 1942; Ryan, 1967b; Diesel, 1989; Jeyalectumie and Subramonian, 1991) especially during long-term storage (Sal Moyano et al., 2009); acting as bacteriostatic or antibacterial agent minimizing bacterial infection (Johnson, 1980; Beninger et al., 1993; Sainte-Marie and Sainte-Marie, 1998; Jayasankar and Subramoniam, 1999; Benhalima and Moriyasu, 2000) acting as

bromophenol blue (white and black arrows, respectively). PVD in MAT males of *C. danae* (H-K); H, General aspect of the fluid secretion without granules in the lumen stained in hematoxilin and eosin [PVD with lateral out-pocketings show same cubical epithelium of central duct due to large amount of secretion; I, Detail of lateral out-pocketings with different acidophilic secretion (arrows), H&E; J, Muscular layer (arrow) and luminal secretion positive for neutral polysaccharides, PAS/Alcian blue pH 2.5 staining; K, Luminal secretion reactive to mercuric bromophenol blue for proteins. L = lumen; M = muscular layer; N = nucleus; OT = lateral out-pocketings; S = secretion; SF = spermatophore.

an important factor in ovarian development (Wolcott et al., 2005) and, finally, acting as a mechanical agent by distending the seminal receptacle.

In conclusion, the hepatopancreas in *C. danae* is related to the maturation of the male reproductive system, and variation in HSI/GSI are indications of the degree reproductive effort. The reproductive system seems to develop quickly and both DE and MAT adult males displaying the same CW size are capable of reproducing, although the amount of seminal fluid is smaller in DE males. These results indicate that future studies on sperm plug formation and sperm competition might reveal functional aspects of reproductive success in DE and MAT males. The spermatophore production in *C. danae* is very similar to that observed for Portunidae and Majoidea. In contrast, the vas deferens shows differences in the MVD and PVD mainly in external morphology and lateral out-pocketings, caeca or diverticula, also found in found in Inachidae, Oregoniidae, and Majidae (Beninger et al., 1988; Diesel, 1989; Sainte-Marie and Sainte-Marie, 1999; Benhalima and Moriyasu, 2000; Simeó et al., 2009). In addition, the histochemical data reported here are different from that described in Grapsidae, Sesarmidae, and Eriphiidae, with the seminal secretion consisting of glycoproteins and being more fluid in the PVD.

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