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Spermathecae of the mangrove crab *Ucides cordatus*: a histological and histochemical view

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Ucides cordatus is the most commercially important mangrove crab in Brazil. In spite of its economic importance, there are few studies of its reproduction, in particular the female reproductive system. The present study describes the histology and histochemistry of the spermathecae of *U. cordatus*. Adult females were caught monthly from July 2004 through June 2005, at Iguape, State of São Paulo. The crabs were anaesthetized, and their spermathecae removed and fixed in Davidson's fluid, following the histological routine for paraffin. The slides were stained with HE, xylidine Ponceau, PAS, alcian blue (pH 1.0 and 2.5), Sudan black B and picrosirius-haematoxylin. Histologically, the spermathecae possesses a capsule of conjunctive tissue, rich in collagen fibres, which surrounds the secretory columnar epithelium. In the lumen, individual sperm packets are not observed; the spermatophores are intermixed with the seminal fluid and secretions of the spermathecae itself. A large proportion of the free spermatozoids and spermatophores are arranged in homogeneous masses in the proximal part of the spermathecae. The secretion produced by the columnar epithelium appears to promote the movement of the gametes to the fertilization chamber, in a ventral position, allowing fertilization of the oocytes. Histochemically, the secretion produced by the columnar epithelium was strongly positive for neutral polysaccharides, positive for acid polysaccharides, and weakly positive for proteins and lipids. This secretion forms a glycoprotein matrix which is associated with maintenance of the spermatophores, which can remain stored for long periods.

INTRODUCTION

Following copulation, females of brachiuran crabs store the spermatic secretion in paired, sac-shaped organs termed spermathecae, which are located just below the heart and hepatopancreas (Johnson, 1980). The spermatic secretion consists of a mixture of sperm and seminal fluid, which in females of the blue crab *Callinectes sapidus* Rathbun, 1896 can be stored up to two years and can fertilize multiple egg masses (Hines et al., 2003). Histologically, in the snow crab *Chionoecetes opilio* (Fabricius, 1788) the spermathecae are surrounded externally by conjunctive tissue, and internally by glandular epithelium which produces a secretion whose function is still uncertain (Beninger et al., 1993).

The role of this secretion is debatable because it is a mixture of secretions produced by the spermathecae, added to the secretions of the male introduced during insemination. Several functions are attributed to the spermathecae secretion, such as forming a gel with glycoproteins and neutral polysaccharides which may be important for storing and opening of the spermatophores in *C. opilio* (Beninger et al., 1993) and the field crab *Paratelphusa hydrodromus* (Herbst) (Anikumar & Adiyodi, 1977; Adiyodi, 1988). However, most of the functions attributed to the fluid present in the interior of the spermathecae are related to the seminal fluid of the male (Johnson, 1980; Beninger et al., 1988). In *Callinectes sapidus*, the hardening of the seminal fluid forms a sperm plug at the entrance of the female's vulva, which prevents future copulations with other males (Jivoff, 1997), and can prevent the loss of sperm from or the entry of harmful materials into the female reproductive tract (Johnson, 1980). Additionally, in the mud crab *Scylla serrata* (Forsskål) it can also act as an antimicrobial agent (Jayasankar & Subramonian, 1999), and in *Chionoecetes opilio* can promote sperm differentiation and maintenance during hibernation (Sainte-Marie & Sainte-Marie, 1999).

The basic function of the spermathecae is to receive the spermatophores and store them until the oocytes are fertilized (Lópes-Greco et al., 1999). Fertilization occurs in the fertilization chamber located in its lower part (Beninger et al., 1988). During egg laying, the spermatophores located in the proximal region of the spermathecae dissolve and become suspended in the fertilization chamber, where they encounter the oocytes and fertilize them (Diesel, 1989).

After fertilization, the spermatophores can be stored in two forms: (1) in individual sperm packets, which are defined as a mass composed of spermatophores, spermatozoids and seminal secretions, observed for the spider crab *Inachus*

phalangium (Fabricius 1775) by Diesel (1988), which, according to this author, can be quantified by histological analysis; or (2) after copulation, the male and female secretions are mixed, forming a mixed white secretion, like the pattern in the grapsid crab *Chasmagnathus granulata* Dana 1851 (López-Greco et al., 1997).

Ucides cordatus (L.) is the most commercially important species of crab in Brazil. In spite of this, knowledge of its reproductive apparatus is limited only to the analyses of the ovaries and testicles by Mota-Alves (1975) and Dalabona & Silva (2005), and of the ultrastructure of the spermatozoid, studied by Matos et al. (2000). In the present work, we characterize the morphology of the spermathecae of *U. cordatus*, and assess the pattern of sperm storage, and the histochemical composition of the tissues and the secretion that fills the spermathecae.

MATERIALS AND METHODS

Histology

Crabs were collected monthly, from July 2004 through June 2005, in the Iguape estuarine region (24°41'22.90"S 47°27'37.95"W), State of São Paulo, Brazil. In each collection, about 50 females with cephalothorax width over 43 mm were caught. In this region, females of this size were considered adults by Hattori (2002).

The crabs which had their spermathecae used for histological analysis were previously anaesthetized by thermal shock (0°C for 15 min), as suggested by López-Greco et al. (1999). The dissection was done by lifting the dorsal part of the cephalothoracic carapace, and then removing the heart, according to Johnson (1980).

After a rapid macroscopic inspection of spermathecae, where the distribution of the mass of spermatophores inside the spermathecae was observed, it was fixed in Davidson's solution (330 ml ethylic alcohol (98%)/300 ml of satured formol (40%)/115 ml of acetic acid and 335 ml of destilate water) for 24 h (Kiernan, 1999). After this period, the spermathecae were dehydrated in an ascending alcohol series (70 to 100%), diaphanized in an ascending series of xylol P.A., and then embedded in paraffin. Serial longitudinal sections of 8 μm thick were made at median part of spermathecae on a rotating microtome in the histology laboratories of the Department of Biology, IB, UNESP-Rio Claro, and the Department of Applied Biology, FCAV, UNESP-Jaboticabal.

The slides were deparaffinated, hydrated and stained with haematoxylin and eosin, according to Junqueira & Junqueira (1983). The images were captured with a Zeiss Axiolab® microscope and a Zeiss Stemi® SV-6 stereomicroscope, linked to the Computer Imaging Analysis System, using the Zeiss program KS-100® 3.0.

Histochemistry

One spermathecae from the same pair analysed histologically was submitted to histochemical techniques. All the slides were previously deparaffinated and hydrated, and the sections were submitted to the techniques of PAS for neutral polysaccharides with 1–2 glycol groups (McManus, 1946), alcian blue (pH 1.0) for acid polysaccharides (strongly sulphated) and simultaneous staining with alcian blue (pH 2.5)–PAS (Junqueira & Junqueira, 1983) for weakly sulphated polysaccharides, Sudan black B for total lipids (Junqueira & Junqueira, 1983), xylidine Ponceau, for protein cation radicals (Vidal, 1970), and picrosirius-haematoxylin trichrome, for proteins and collagen fibres (Junqueira & Junqueira, 1983). Following the histochemical procedures, the slides were mounted in Permount. The images were captured and digitized using a Leica DM2000 microscope, at the Department of Biology, UNESP – Rio Claro.

RESULTS

Histology of the spermathecae

The spermathecae of *Ucides cordatus* is a sac-shaped organ (Figure 1A,E–G), surrounded externally by a capsule of conjunctive tissue, which supports the inner columnar epithelium, responsible for the synthesis of large amounts of a secretion (Figure 1A–C). The spermatophores and spermatozoids are mixed in with this secretion; sperm packets are absent in this species (Figure 1A,D). Most of the spermathecae (62.66% of 557 spermathecae analysed) store the spermatozoids and spermatophores mixed with this secretion, which is completely translucent to the naked eye, and slightly eosinophilic (Figure 1A,B). The secretion appears to move the spermatophores and spermatozoids to the proximal region of the spermathecae, next to the fertilization chamber (Figure 1A,E). Other distribution patterns of the spermatophores and spermatozoids, (28.37% of 557 spermathecae analysed), were observed (Figure 1E–G). Spermathecae filled only with the translucent secretion, or completely filled with spermatophores, were seldom recorded: 3.05 and 4.49% respectively of the total analysed.

During the dissection, the presence of orange pigments in the distal (dorsal) part of the spermathecae could be observed in 190 individuals (Figure 2A, arrow). Histological analysis established that these orange pigments in the spermathecae (Figure 2B,C) are identical to the ovarian oocytes (Figure 2D, arrow). Spermathecae with oocytes present in their interior occurred in 34.11% of the 557 females analysed.

Histochemistry

The PAS histochemical technique revealed the presence of neutral polysaccharides (containing 1–2 glycol groups) in the conjunctive-tissue capsule surrounding the spermathecae, in the columnar epithelium, and in the secretion present in the lumen of the spermathecae of *U. cordatus*. The conjunctive-tissue capsule showed a strong positive reaction to PAS, principally in the basal membrane, that is, in the interface between the spermathecae and the haemolymph (Figure 3A). In the interior of the capsule were anastomosing lines that showed little affinity for PAS (Figure 3A, arrow). The inner columnar epithelium, as well as its basal lamina, were intensely stained by this technique (Figure 3A).

The luminal secretion of the spermathecae was positive to PAS (Figure 3B), more strongly in the regions near the spermatophores and free spermatozoids (Figure 4A,B). The secretion inside the spermatophores was strongly positive, but its surrounding capsule showed a comparatively less-intense reaction for carbohydrates containing 1–2 glycol groups (Figure 4A, arrow). The free spermatozoids, as well

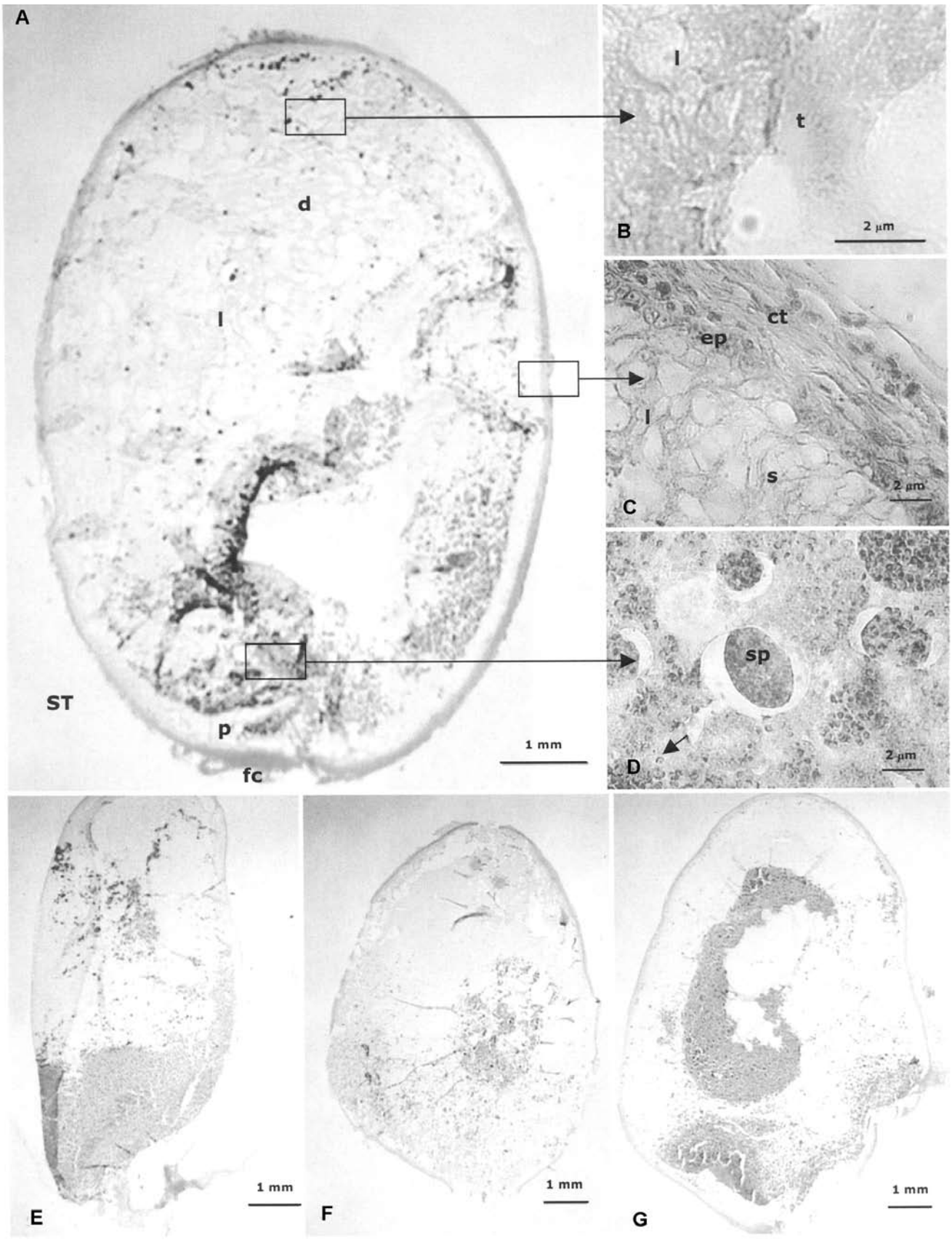


Figure 1. General aspect of the most common pattern of the spermathecae (ST) of *Ucides cordatus* (A) showing the higher concentration of spermatophores and spermatozoids in the proximal region (p) above the fertilization chamber (fc). In the distal portion (d) a large quantity of a translucent, slightly eosinophilic secretion can be seen; (B) detail of the translucent secretion (t) present in the lumen of the distal, slightly eosinophilic region (l); (C) detail of the spermathecae wall, with a capsule of outer conjunctive tissue (ct) surrounding the inner columnar epithelium (ep), which shows numerous secretion vesicles (s) which are released into the lumen (l); (D) spermatophores (sp) and free spermatozoids (arrow) in the proximal region of the spermathecae; (E, F ,G) less-frequent patterns of filling and arrangement of the spermatophores and spermatozoids in the spermathecae of *U. cordatus*.

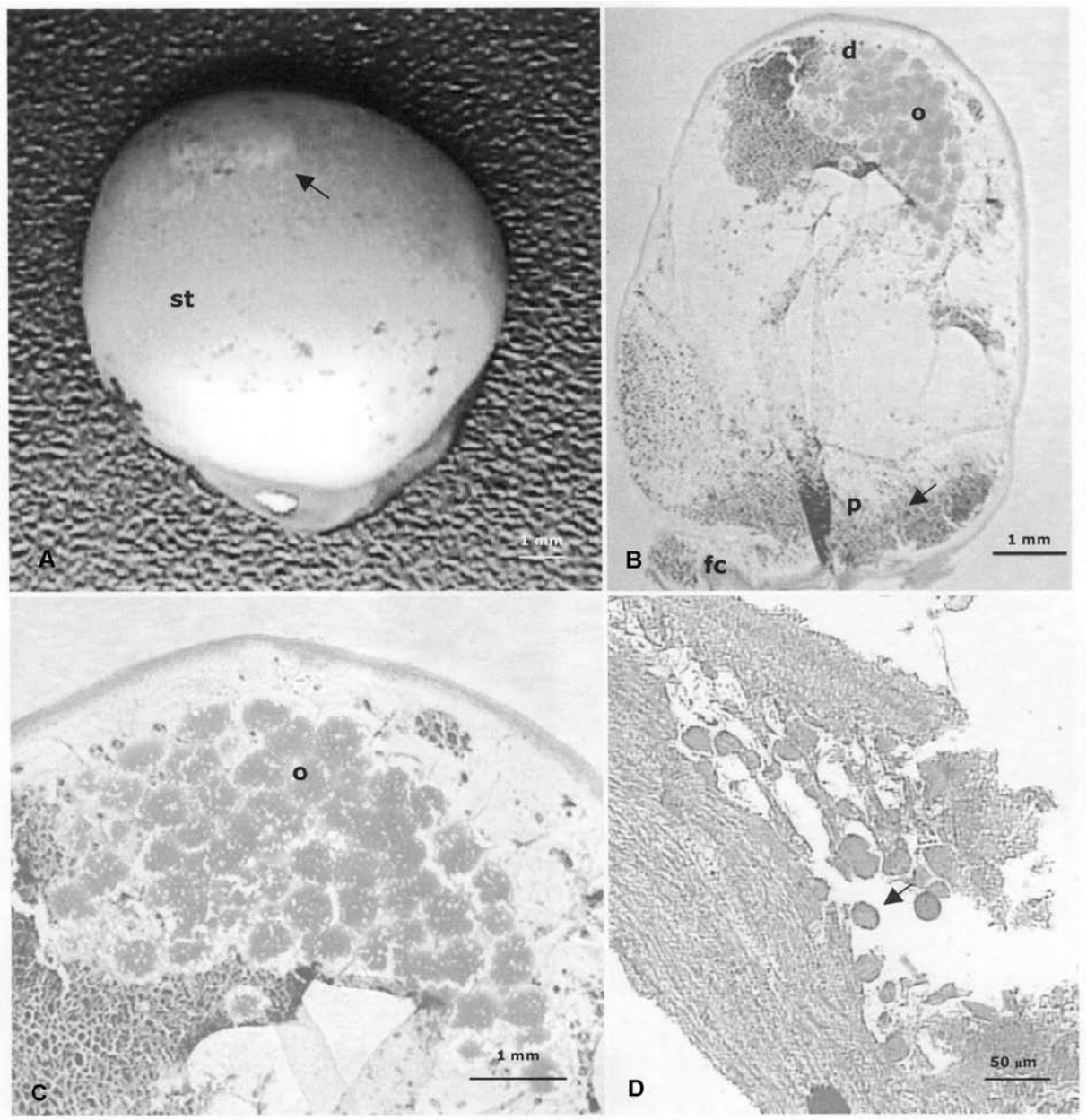
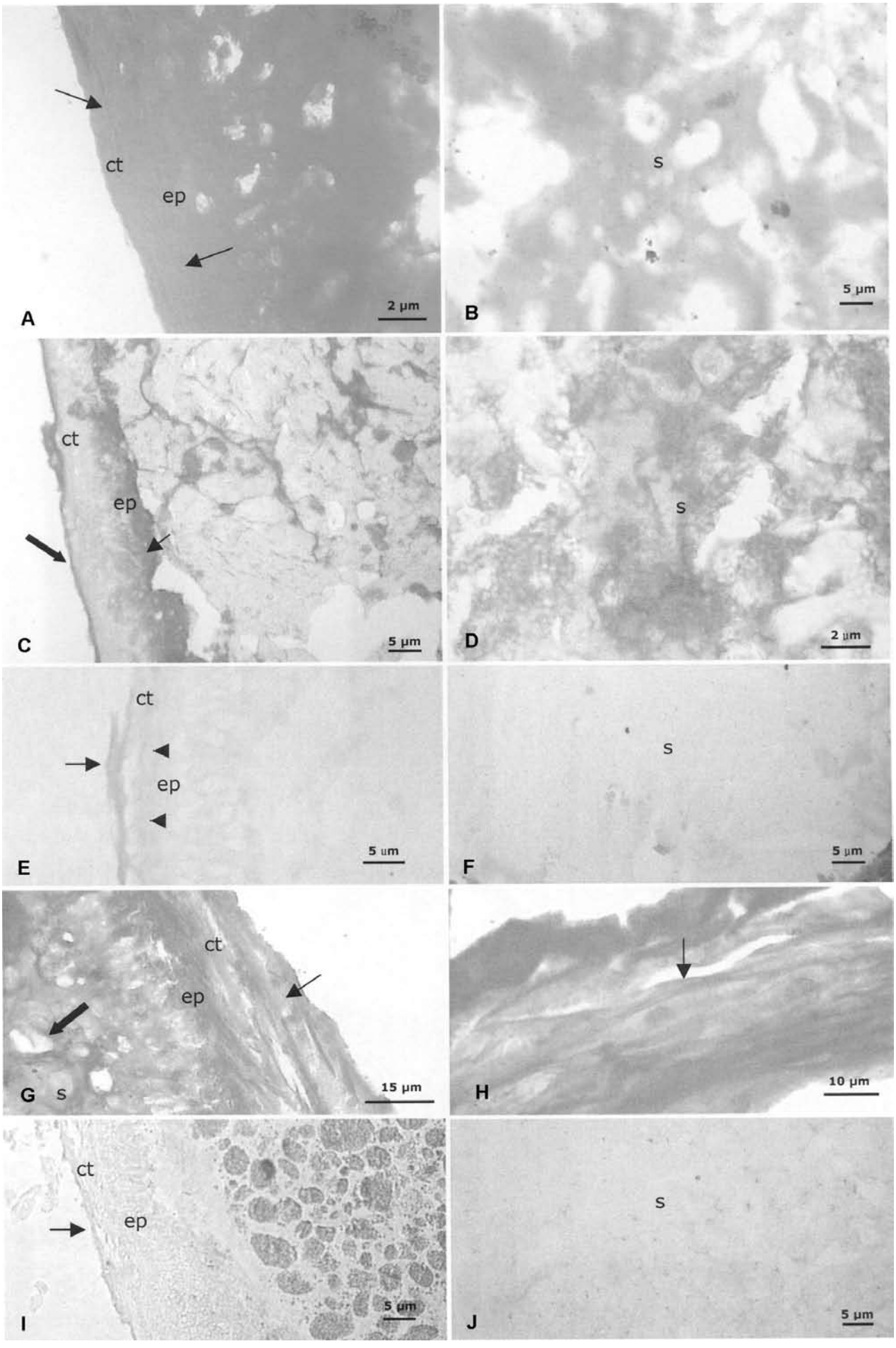


Figure 2. Spermathecae of *Ucides cordatus* with orange pigment present in their interior; (A) freshly dissected spermathecae (st) with orange pigment (arrow); (B) histological section of the spermathecae showing the orange pigment (o) in the distal region (d). Next to these, note the presence of spermatophores and spermatozoids (arrow), most of these found in the proximal region (p) next to the fertilization chamber (fc); (C) detail of the distal region, showing the orange pigment (o), very similar to the oocytes of *U. cordatus*; (D) general aspect of the oocytes (arrow) inside the fertilization chamber, which are identical to the orange pigment found inside the spermathecae.

Figure 3. Histochemical analyses of the tissues and the translucent secretion of the spermathecae of *Ucides cordatus*; (A) general aspect of the tissues of the spermathecal wall, showing strong marking for PAS. Observe the anastomosing lines (arrows) in the conjunctive tissue (ct), which are weakly positive; (B) general aspect of the translucent secretion (s), which is positive for carbohydrates containing 1–2 glycol groups; (C) spermathecal wall, treated with the alcian blue technique (pH 1.0 and 2.5), showing a positive reaction for the basal membrane (wide arrow); the rest of the tissue is negative. The columnar epithelium (ep) was also negative for this technique, except for the basal lamina and the apical region around the secretion vesicles (thin arrow); (D) luminal secretion of the spermathecae with positive reaction for acid polysaccharides; (E) spermathecal wall with strongly positive marking for xylidine Ponceau, next to the basal membrane and in the lines present in the conjunctive tissue (arrowhead); (F) luminal secretion weakly positive for total proteins; (G) spermathecal wall treated with the picrosirius-haematoxylin technique, showing strong marking next to the conjunctive-tissue capsule (ct), epithelium (ep) and luminal secretion. The secretion vesicles were negative for this technique (wide arrow); (H) conjunctive-tissue capsule, strongly positive for picrosirius-haematoxylin, with strong marking on the anastomosing fibres (arrow), indicating that these are collagen fibres; (I) spermathecae treated with Sudan black B, showing weakly positive marking in all parts. Note that the basal membrane (arrow) and the interior of the spermatophores show a positive reaction for total lipids; (J) secretion, weakly positive for total lipids.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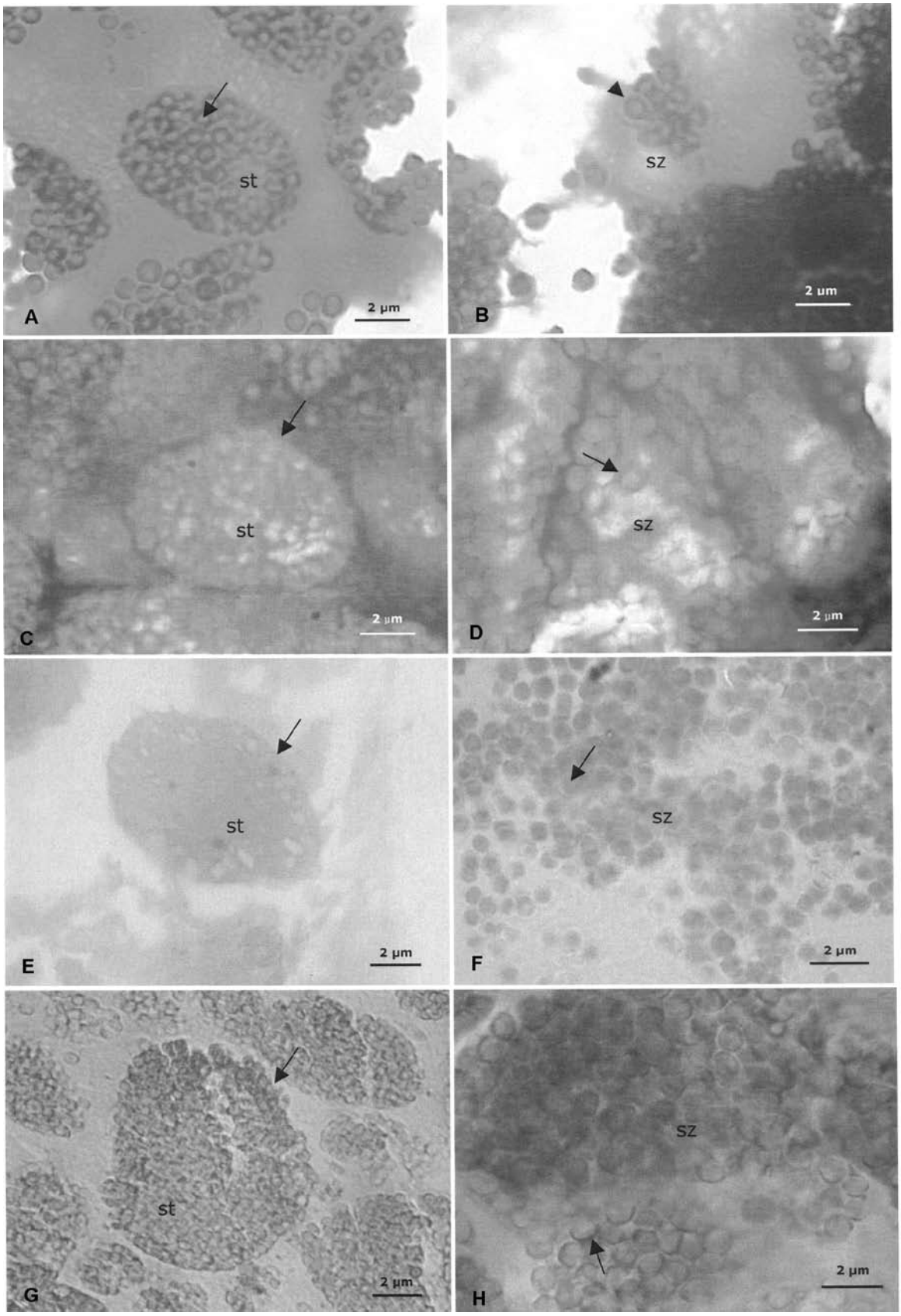

Figure 4. Histochemical analyses of the spermatophores and spermatozoids contained in the spermathecae of *Ucides cordatus*. (A, B) Spermatophores (st) and spermatozoids (sz) treated with PAS, showing the absence of markings for neutral polysaccharides in the acrosome (arrow), and the strongly positive reaction in the rest of the cytoplasm (arrowhead); (C) spermatophore (st) treated with alcian blue, with a positive reaction for its surroundings (arrow); (D) aspect of the spermatozoids (sz) with a positive reaction on the cell surface (arrow) and a negative reaction in other regions; (E) spermatophore (st) positive for xylidine Ponceau, with strongly positive markings in the surrounding capsule (arrow); (F) aspect of the spermatozoids (sz), with strong marking for proteins in the acrosome (arrow); (G) positive reaction for total lipids of the spermatophore (st), with its surrounding capsule negative for Sudan black B (arrow); (H) spermatozoids with positive marking for lipids, principally at the cell surface (arrow). The acrosome was negative for this technique.

Table 1. *Results of histochemical tests on the spermathecae of* Ucides cordatus.

Component	Epithelium	Connective tissue	Luminal secretion	Secretion close to Spermatophore
PAS	++	+++	++	+++
Alcian blue*	–	+++	++	++
Xylidine Ponceau	+	++	+	+
Sudan black	+	+	+	+
Picrosirius/Haematoxylin	+++	+++	+++	+++

+, weakly positive; ++, positive; +++, strongly positive; –, negative; *, the results of this technique refer to pH 1.0 and 2.5.

Table 2. *Results of histochemical tests on the spermatophores and free spermatozoids present in the spermathecae of* Ucides cordatus.

	Spermatophores		Spermatozoids	
Component	Spermatophore	Capsule	Acrosome	Cytoplasm
PAS	+++	++	–	+++
Alcian blue*	–	+++	–	–
Xylidine Ponceau	+++	+++	+++	+++
Sudan black	++	–	–	+
Picrosirius/Haematoxylin	+++	+++	–	++

+, weakly positive; ++, positive; +++, strongly positive; –, negative; *, the results of this technique refer to pH 1.0 and 2.5.

as those inside the spermatophore, were strongly positive to PAS, and the acrosomes were negative (Figure 4A,B, arrow).

With the alcian blue technique at pH 1.0 and 2.5 for acid polysaccharides, that is, for those compounds with repeated sulphate or carboxyl groups, there were no differences in coloration between the pH levels used. However, acid polysaccharides were distributed differently from neutral carbohydrates. The interface between the spermathecae and the haemolymph (basal membrane) showed a strongly positive reaction (Figure 3C, wide arrow). The basal lamina of the inner columnar epithelium was positive for alcian blue (Figure 3C, arrow), and the apex of this epithelium, principally between the secretion vesicles, was strongly positive for acid polysaccharides (Figure 3C, arrow). The luminal secretion was positive, including the parts nearest the spermatophores and free spermatozoids (Figure 3D). The interior of the spermatophores was negative for alcian blue; however, the capsule around the spermatophore was strongly positive for acid polysaccharides (Figure 4C, arrow). The acrosome and cytoplasm of the spermatozoids were negative for this technique (Figure 4C); however, the surface of the spermatozoids (basal lamina) was positive (Figure 4D, arrow).

With the xylidine Ponceau technique, positive markings in the entire capsule of conjunctive tissue could be seen (Figure 3E), and strongly positive markings were detected in the basal membrane (Figure 3E, arrow) and in the anastomosing lines (Figure 3E, arrowhead). The epithelium showed a less-intense reaction than the conjunctive tissue, i.e. weakly positive (Figure 3E). The secretion present in the lumen of the spermathecae was also weakly positive for total proteins (Figure 3F). The spermatophores (Figure 4E), as well as their surroundings, showed a strongly positive reaction for total proteins (Figure 4E, arrow). The cytoplasm and acrosome of the spermatozoids were intensely stained by xylidine Ponceau (Figure 4F). The strongly acidic stain of the picrosirius-haematoxylin technique revealed that the anastomosing lines present in the conjunctive-tissue capsule showed a strong affinity for the molecules of Direct Red (formerly Sirius Red F3B), indicating that these strongly positive lines are collagen molecules, oriented to form fibres (Figure 3 G,H, arrows).

For total lipids, the spermathecae of *U. cordatus* showed a weakly positive reaction in the conjunctive tissue, more precisely at the interface between the conjunctive tissue and the haemolymph (Figure 3I, arrow). In general, the rest of the conjunctive-tissue capsule, the epithelial tissue and the secretion present in the lumen stained weakly positive, showing a grey tone. The interior of the spermatophores was positive (Figure 4G). The cytoplasm of the spermatozoids showed a low-intensity reaction to this technique; the acrosome was completely negative (Figure 4H).

The results of the histochemical tests are summarized in Tables 1 and 2.

DISCUSSION

The spermathecae of *Ucides cordatus* are histologically similar to those of other brachiurans, such as *Callinectes sapidus* (see Johnson, 1980) and *Chionectes opilio* (see Beninger et al., 1988). However, Beninger et al. (1993) and Sainte-Marie & Sainte-Marie (1998), analysing the histology of the spermathecae of *C. opilio*, found a layer of chitin in their ventral part; such a layer does not occur in *U. cordatus*. Many studies have mentioned that the dorsal region of the

spermathecae is composed of glandular tissue (George, 1963; Ryan, 1967; Johnson, 1980; Diesel, 1989). However, in *U. cordatus* this specialized glandular tissue is absent; all of the inner columnar epithelium has secretory characteristics easily observed by numerous vesicles of secretion.

Spermatophores are sacs of spermatozoids; in decapod crustaceans, they are nonmotile and unflagellated (Brown et al., 1977). According to Matos et al. (2000), the spermatozoid of *U. cordatus* has three components: the prominent acrosome, the polymorphic nucleus and part of the cytoplasm containing cellular organelles, especially six short stalks, implanted at the base and consisting of a microtubular region. Few theories have attempted to explain the process of storage of spermatophores and spermatozoids in brachiurans. Diesel (1989) suggested that the enzymes responsible for opening the spermatophores are contained in the secretion produced by the epithelial tissue of the spermathecae.

Beninger et al. (1993), studying spermathecae of *C. opilio*, established that this substance is high in energy, and may be important in the storage and opening of the spermatophores. In the present study, the histochemical analyses revealed a pattern similar to that obtained by Beninger et al. (1993). Probably the energy characteristics of this secretion are associated with maintenance of the spermatophores which were stored for long periods in the spermathecae, similar to what occurs in *C. opilio* (Sainte-Marie & Sainte-Marie, 1999). Furthermore, it is supposed that the secretion aids in medium- and long-term hibernation of the spermatophores, and that changes in the chemical composition of this secretion induce the differentiation of immature spermatids and their transformation into spermatozoids within the spermathecae (Sainte-Marie & Sainte-Marie, 1999).

This event may be occurring in *U. cordatus*, although in this case related not to hibernation, but rather to maintenance of the spermatozoids during the year. This is supported by the seasonal reproduction of the species (Pinheiro & Sant'Anna, in preparation), which occurs only in the warmer months of the year, and by the records of spermatophores during the entire year in the spermathecae of *U. cordatus*, observed in the present study. Thus, females would carry spermatophores during the entire year, until the next reproductive period, and in case copulation did not occur, the female would have viable spermatozoids for the laying season.

The capsule of conjunctive tissue is composed of anastomosing lines, which are collagen fibres, as shown by the picrosirius-haematoxylin and xylidine Ponceau. The lines are composed mainly of type I collagen, which is weakly positive to PAS, as proposed by Junqueira & Carneiro (2004), because of the small quantity of polysaccharides in their composition compared to type III collagen. The presence of collagen fibres has been observed in *Callinectes sapidus* (Johnson, 1980), and the presence of elastic fibres appears to be more common in *Chionectes opilio* (Benninger, 1988). Thus, this element appears to be the principal compound which maintains the turgid shape of the spermathecae, and which at the same time allows it to expand during copulation.

Sainte-Marie & Sainte-Marie (1999) observed an orange pigment in the spermathecae of *C. opilio*. Analysing this pigment microscopically, they distinguished two subtypes, one which corresponds to corpuscles derived from the ovary, which the oviduct would have introduced into the spermathecae; and another substance, similar to the granules brought from cells surrounding the oviduct. Continuing their studies, Sainte-Marie et al. (2000) confirmed the presence of this orange substance, and suggested various origins for it: derived from the exterior of the ovary, from ovarian glands or from cells similar to leucocytes which are present around the oviduct. In *U. cordatus*, we also observed orange corpuscles (oocytes), derived from the ovary, inside the spermathecae. However, we suppose that these oocytes are introduced into the spermathecae in a different manner: at the moment of copulation, during the penetration of the pleopods, these would rupture the fertilization chamber and accidentally push the oocytes present in it, into the spermathecae.

In *Inachus phalangium*, Diesel (1988, 1990) recorded the presence of sperm packets, that is, packets of spermatophores derived from multiple copulations within the spermathecae, and forming several layers of sperm packets. He suggested that the last male to copulate would be the progenitor of the offspring. Urbani et al. (1998), in laboratory experiments with *C. opilio*, proved this theory through paternity tests. The theory is only valid for species which store sperm in the form of sperm packets.

The pattern of sperm storage in *U. cordatus* is similar to that of *Chasmagnathus granulata* (López-Greco et al., 1999), which does not form individual packets of spermatophores in the spermathecae, in the case to exist multiple copulations. For both of these crabs, sperm from different copulations are stored together. Thus in *U. cordatus* both the first or last male to copulate have, apparently, an equal chance to be the progenitor because the secretions are stored together, and there is no sperm competition.

In some portunids, such as *Callinectes sapidus* and the speckled swimming crab *Arenaeus cribrarius* (Lamarck, 1818) (Pinheiro & Fransozo, 2002; Wolcott et al., 2005), the spermathecae only remain turgid when they are full of male secretions (seminal fluid, spermatophores and water); they become smaller during the course of the reproductive period, and remain practically empty outside the reproductive period. In *U. cordatus*, all the fresh spermathecae analysed were turgid (full), even those which did not contain spermatophores. Thus, we can conclude that in adult individuals, the production of secretion from the inner columnar epithelium is continuous, independent of the reproductive season or the presence of spermatophores, and that this aids in directing the spermatophores toward the proximal region, juxtaposed to the fertilization chamber.

According to Hines et al. (2003), reproduction becomes sperm-limited when the number or quality of sperm received by females is insufficient to fertilize the total egg production. Hines et al. (2003) stated that the intense fishing pressure on the portunid *C. sapidus* affects its abundance, its size (males) and the sex ratio, which affects the quantity of sperm ejaculated. Similarly, the same phenomenon may be occurring with *U. cordatus*, which has been exploited for many years: mainly affecting the population of males, because of their larger size. However, the constancy of the record of spermatophores in the spermathecae throughout the

year associated with the possibility of multiple copulations without the formation of sperm packets may minimize the effects of such kind of human pressure in this species.

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