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Research paper

Ultrastructure of spermatophores and spermatozoa of intertidal crabs *Pachygrapsus transversus*, *Pachygrapsus gracilis* and *Geograpsus lividus* (Decapoda: Grapsidae)

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ABSTRACT

Pachygrapsus transversus, *P. gracilis*, and *Geograpsus lividus* are intertidal crabs, whose ultrastructure of spermatophores and spermatozoa are unknown. In this work, we characterized the ultrastructure of the spermatophores and spermatozoa of these three species comparing them to the results of previous research on molecular phylogeny of Grapsidae. Fragments of the posterior vas deferens were processed for routine in transmission electron microscopy. *Pachygrapsus transversus* and *G. lividus* exhibit coenospermic spermatophores, while *Pachygrapsus gracilis* presents cleistospermic spermatophores. The spermatozoa of all three species present the typical pattern seen in Thoracotremata. The acrosome is wider than long, with an acrosome length to width ratio of 0.82 ± 0.022 in *P. transversus*, 0.75 ± 0.008 in *P. gracilis*, and 0.82 ± 0.024 in *G. lividus*. The main specific sperm differences were observed in the perforatorial chamber, the operculum and the concentric acrosomal layers. *Pachygrapsus transversus* has a flask-shaped perforatorial chamber with a prominent narrowing under the apex, while *Pachygrapsus gracilis* exhibits a flask-shaped perforatorial chamber with a slight narrowing under the apex, thus wider than that of *P. transversus*. *Geograpsus lividus* presents a club-shaped perforatorial chamber with a conical apex. The acrosome of *P. transversus* has a narrow inner layer while the outer layer presents peripheral concentric lamellae near the opercular pole. In *P. gracilis*, the inner acrosomal layer of the acrosome is shifted toward the apical portion of the spermatozoon and the outer layer exhibits peripheral concentric lamellae throughout its extension. *Geograpsus lividus* shows a narrow and electron-dense inner layer shifted toward the opercular pole and curved operculum, unlike the pattern found for the *Pachygrapsus* species. The differences in the morphology of spermatophores suggest different strategies of transfer and/or storage of spermatozoa between the studied species. The spermatozoal ultrastructure supports the phylogenetic proximity of *Pachygrapsus* species and also demonstrate that spermiotaxonomy is a useful tool to be associated to molecular data.

1. Introduction

The structure and morphology of the spermatophores and spermatozoa of the Decapoda have been frequently used as a complementary tool to elucidate phylogenetic relationships among species (Benetti et al., 2008; Jamieson, 1991, 1994; Jamieson and Tudge, 2000; Tudge, 1991, 2009; Tudge and Jamieson, 1991). In Brachyura the spermatophores are not pedunculate and are considered one of the simplest spermatophore morphologies among decapods (Uma and Subramonian, 1979). The brachyuran spermatophores are usually spherical or elliptical and consist of a mass of spermatozoa immersed in an extracellular matrix delimited by an acellular wall (Erkan et al., 2009; Klaus et al., 2009). According to the available literature, marine and estuarine crabs exhibit coenospermy – several spermatozoa delimited by a spermatophore wall (Anilkumar et al., 1999; Benetti et al., 2008; Cuartas and Sousa, 2007; El-Sherief, 1991; Guinot et al.,

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1997; Jamieson, 1994) while many freshwater crab species exhibit coenospermy, in which each spermatophore contains a single spermatozoon (Guinot et al., 1997; Klaus et al., 2009; Klaus and Brandis, 2011). Surprisingly, we have recently observed coenospermy under light microscopy in *Pachygrapsus gracilis* (Saussure, 1858), a marine/estuarine representative of Grapsidae (Tiseo et al., 2014), what motivated us in search of new answers on this topic.

The ultrastructure of spermatozoa in the Crustacea is very diverse (Jamieson, 1991). The presence or absence of ultrastructural characters has proven useful to determine phylogenetic relationships among different taxa (Tudge, 2009). All pleocyemate decapods share common morphological characters of the spermatozoon such as: immotile aflagellate cells, wide acrosomal vesicle with concentric layers, posterior nucleus with lateral arms and cytoplasm that may contain mitochondria, lamellar complex, centrioles, and microtubules (Jamieson and Tudge, 2000; Tudge, 2009). The brachyuran acrosome is spherical and consists of a central perforatorial chamber (a *perforatorium* equivalent) and adjacent concentric layers or zones. Above the acrosome is the operculum and underneath it is the subopercular zone (Jamieson, 1994; Tudge, 2009). The nucleus surrounds part of the posterior region of the acrosome and exhibits a variable number of lateral arms depending on the species (Benetti et al., 2008; Klaus et al., 2009; Medina, 1992; Medina and Rodriguez, 1992; Tudge, 2009). The composition of the nucleus may consist of only chromatin, as well as elements of the cytoskeleton, such as microtubules (Hinsch, 1991; Tudge, 2009).

1.1. Taxonomic status of the target species

The Grapsoidea includes a variety of members, occurring in marine, freshwater, and terrestrial habitats. This superfamily was considered, by morphological analysis, as a monophyletic group (Ng et al., 2008), but its status has been changed to a polyphyletic superfamily according to alternative molecular proposals (Schubart et al., 2005; Tsang et al., 2014). The family Grapsidae MacLeay, 1838 consists of 8 genera and 40 species (Davie et al., 2015; De Grave et al., 2009). Most of species are inhabitants of intertidal rocky shores and at least four genera are found on the Brazilian coast (Melo, 1996). According to Ng et al. (2008), both *Geograpsus* Stimpson, 1858 and *Pachygrapsus* Randall, 1840 belong to the subfamily Grapsinae MacLeay, 1838 with 5 and 14 species, respectively. Based on morphological and molecular analysis, *Pachygrapsus* is the genus with contrasting characters, and is classified as a polyphyletic group (Ip et al., 2015; Poupin et al., 2005; Schubart, 2011). Poupin et al. (2005) used 15 morphological characters to establish similarity relationships among 13 species of *Pachygrapsus*, with *Pachygrapsus transversus* (Gibbes, 1850) and *P. gracilis* positioned in distinct complexes of species. Schubart (2011) presented a molecular phylogenetic tree consisting of eight genera of Grapsidae in seven distinct lineages. In this work, based on a single mitochondrial gene, *Pachygrapsus* was the most problematic genus, with its members split into six of the seven phylogenetic lineages. Schubart (2011) supported the maintenance of *P. transversus* in the same genus with six more species of *Pachygrapsus*, and proposed the inclusion of *P. gracilis* in the genus *Geograpsus*. Recently, a more comprehensive analysis for Grapsidae phylogeny was carried out using seven genes including both mtDNA and nDNA. In this work, the monophyly of *Pachygrapsus* was rejected and *P. gracilis* is not closely related to the monophyletic *Geograpsus* clade (Ip et al., 2015).

Considering that the comparison of spermatophores and spermatozoa has been rarely undertaken in intertidal brachyuran crabs, and given the large diversity of species that occupy this transitional habitat, we describe the ultrastructure of spermatophores and spermatozoa of *Pachygrapsus transversus*, *P. gracilis* and *Geograpsus lividus* (Milne Edwards, 1837). We also compare them to identify similarities that support or reject the phylogenetic positioning of *P. gracilis* in the genus *Geograpsus* proposed by Schubart (2011) and Ip et al. (2015). Thus, we demonstrate that sperm ultrastructure can be used as an additional tool to elucidate phylogenetic problems arising from molecular studies. Additionally, we compared our findings with those reported in the sperm ultrastructure literature on the Thoracotremata to examine possible synapomorphies in the Grapsoidea.

2. Material and methods

Specimens of *P. transversus*, *P. gracilis*, and *G. lividus* were collected during the low tide in two seasons of the year: summer (December to March) and winter (June to September) at the research station of the Oceanographic Institute of the University of São Paulo (25° 1′13.14“S; 47°55′28.87“O) in the estuary of Cananéia, São Paulo, Brazil, from July 2012 to July 2013. The live animals were then transported to the Invertebrate Morphology Laboratory of the Department of Biology, FCAV, UNESP Jaboticabal.

For transmission electron microscopy (TEM), fragments of 1 mm^3 of the posterior vas deferens (PVD) of five mature males (Benetti et al., 2008) were fixed in a Karnovsky solution (modified Karnovsky, 1965) consisting of 2.5% glutaraldehyde with 0.08% paraformaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4). Samples were fixed for four hours at 4 °C. Post-fixation was carried out with 1% osmium tetroxide in the same buffer, and the samples were contrasted “en bloc” with 1% uranyl acetate (overnight at 4 °C), dehydrated in ethanol (70–100%), and embedded in Epon-Araldite resin. Semi-thin and ultrathin sections were obtained with a Leica® UC7 ultramicrotome. Copper grids with sections were contrasted with uranyl acetate and lead citrate, and later examined and photographed using a Jeol J1010 TEM at the Laboratory of Electron Microscopy of FCAV UNESP – Jaboticabal, operated at 80 kV. For the observation of lateral arms, fragments of PVD were macerated in 5 ml of buffered 2.5% glutaraldehyde or seawater from the collection site. Then 100 μl of this solution was placed on a slide and covered with a coverslip (Zara et al., 2012). Slides were later examined using a differential interference contrast (DIC) microscope Zeiss Axio Imager Z2. The description of spermatozoa and spermatophores was based on the nomenclature proposed by Benetti et al. (2008, 2013), Jamieson (1991, 1994), Jamieson and Tudge (2000), Klaus et al. (2009) and Klaus and Brandis (2011). For spermatophore measurements the diameters of at least 60 (*P. transversus*, n = 89; *P. gracilis*, n = 61; *G. lividus*, n = 84) spermatophores from three individuals per specie were measured on a Leica® stereomicroscope using the Leica® IM50 program, calibrated for the magnification used (Zara et al., 2012; Nascimento and Zara, 2013).

3. Results

3.1. Morphology of spermatophores

Pachygrapsus transversus exhibits coenospermic spermatophores (several spermatozoa per spermatophore) with spermatozoa immersed in a slightly electron-dense matrix that maintains these cells apart from each other. The general morphology of the spermatophore varied from elliptical to spherical (19.39 ± 6,51 μm) most commonly the latter (Fig. 1A). Spermatophores are immersed in two types of secretion contained in the seminal fluid: a homogeneous matrix and another one formed by granules of various sizes. These granules show

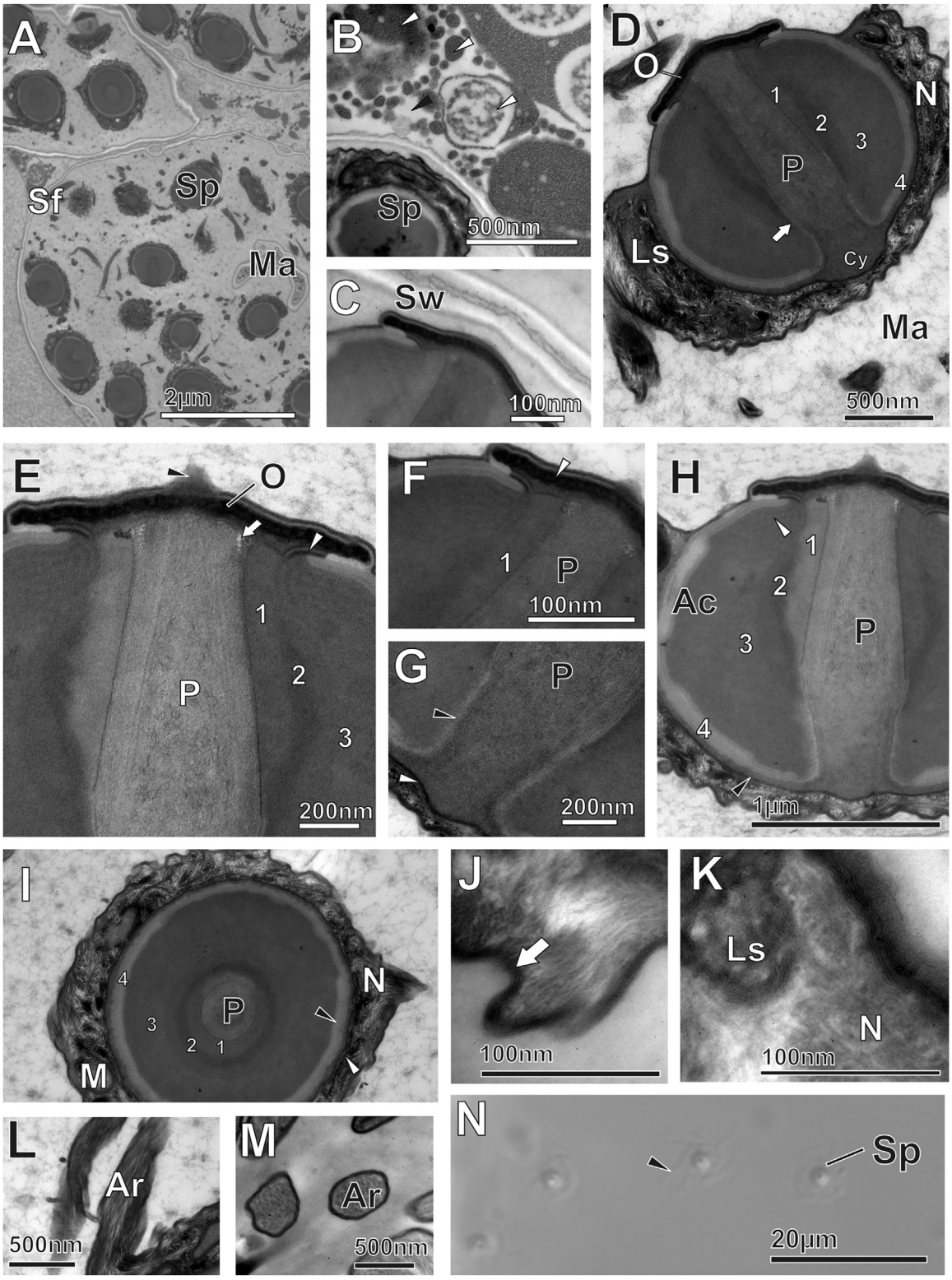

Fig. 1. Spermatophores and spermatozoa of *Pachygrapsus transversus*. A. General view of spermatophores of *Pachygrapsus transversus* in coenospermy. B. Spermatophores are surrounded by two types of secretion, a homogeneous matrix (black arrowhead) and granules of different sizes (white arrowhead). C. Detail of the wall of the electron-dense spermatophore. D. Longitudinal section of a spermatozoon immersed in the extracellular matrix. The white arrow indicates narrowing toward the base of the perforatorial chamber E. Perforated, flat, and slightly centrally round operculum with apical button (black arrowhead). Note the presence of a reduced accessory opercular ring (white arrowhead). The perforatorial chamber is flask-shaped and round, but slightly flat at the apical portion and exhibits small electron-lucent vesicles (white arrow). F. Detail of the operculum showing slight segmentation, with electron-dense regions alternated with electron-lucent regions (white arrowhead). G. Detail of the base of the perforatorial chamber, continuous with the capsule and modified and reduced thick ring (black arrowhead). The nucleus with fibrous chromatin is delimited by the nuclear envelope (white arrowhead). H. Detail of the acrosome with four concentric layers (1, 2, 3, and 4). The outer layer is characterized by peripheral concentric lamellae (white arrow). I. Cross-section of a spermatozoon with the perforatorial chamber in the center and acrosome with four concentric layers (1, 2, 3, and 4) of different electron-densities. Note the fourth electron-lucent layer (black arrowhead). The nuclear material is separated by two unit membranes (white arrowhead). J. Detail of the nuclear envelope (white arrow). K. Detail of the lamellar complex inside the vestigial cytoplasm. L. Longitudinal section of lateral arms filled with fibrous chromatin. M. Detail of a cross-section of lateral arms.

distinctive patterns of electron-density and ultrastructural composition (Fig. 1B). The wall of the spermatophore is thin, consisting of four adjacent layers: an outer thin electron-dense layer, followed by a layer of intermediary electrondensity, an electron-lucent layer, and an innermost electron-dense and thicker layer (Fig. 1C).

Pachygrapsus gracilis presents elliptical ($3.67 \pm 0.57\ \mu m$) cleistospermic spermatophores (one spermatozoon per spermatophore). Each spermatozoon is delimited by a moderately electrondense wall of various patterns, containing seven adjacent layers with the outermost one as the most electron-dense (Fig. 2A, B and C). Spermatophores are immersed in a granular electrondense secretion and are compressed by a second type of secretion forming large masses that are less electron-dense and thinly granular (Fig. 2B and D). When exposed to seawater, spermatophores dehisced, characterized by the disappearance of the wall and by the presence of conspicuous lateral arms on the spermatozoa, as clearly observed in the DIC (Fig. 2E).

In *Geograpsus lividus*, spermatophores are coenospermic, ranging from small to large ($30.2 \pm 11.1\ \mu m$), and exhibit a few spermatozoa near to one another, immersed in a moderately electron dense matrix (Fig. 3A). The secretion of the posterior vas deferens is composed of two types of granules, one large and moderately electron-dense, and another one small and electron dense, both immersed in a homogeneous matrix (Fig. 3A). The wall of the spermatophore is thin and electron dense with three adjacent layers of different electron densities (Fig. 3B).

3.2. General morphology of the spermatozoon

The spermatozoa of the three study species show the typical pattern observed in Thoracotremata. The acrosome presents four concentric layers, surrounded almost completely by a cup-like nucleus. The perforatorial chamber is filled with perforatorial tubules and there is an absence of the thickened ring from the basal portion. The electron-dense operculum is perforated at the central portion and exhibits an apical button. The nucleus is filled with chromatin and lateral arms are present (Figs. 1–3).

3.2.1. Pachygrapsus transversus

The acrosome of the spermatozoon of *P. transversus* exhibits four concentric layers (Fig. 1D), it is wider than long, with an acrosome length to width ratio of 0.82 ± 0.02. The internal layer of the acrosome is moderately electron-dense, shifted toward the opercular pole and inside is the perforatorial chamber. The perforatorial chamber is flask-shaped, narrow, filled with perforatorial tubules (Fig. 1D) and its apex is flat, slightly round, with prominent narrowing toward the base of the chamber. At the apex, small electron-lucent vesicles are present, which are not observed in sections more peripheral to the perforatorial chamber (Fig. 1F). The base of the perforatorial chamber is not surrounded by a thickened ring (Fig. 1G). The intermediate acrosomal zone is electron-dense and located in the equatorial region of the spermatozoon (Fig. 1D, E, and H) while the outer layer of the acrosome (Fig. 1D) exhibits peripheral concentric lamellae near the opercular pole (Fig. 1H). Adjacent to this structure, a fourth electron-lucent layer is present also with concentric lamellae (Fig. 1H). The electron dense striated, flat and centrally round operculum is perforated at its central portion and exhibits an apical button (Fig. 1D–F and H). The subopercular layer is electron-dense, with an accessory opercular ring which is reduced and bent (Fig. 1E). The acrosome is delimited by a single unit membrane (Fig. 1G and H), as well as a nuclear envelope (Fig. 1G and I). The nucleus surrounds the acrosome almost completely (Fig. 1H), but between the nuclear envelope and the acrosome, a thin strip of cytoplasm is observed with a lamellar structure and mitochondria devoid of cristae (Fig. 1I, J, and K). The nucleus contains fibrous chromatin (Fig. 1K and L) and the four to six lateral arms are observed to be filled with DNA (Fig. 1L, M, and N).

3.2.2. Pachygrapsus gracilis

The spermatozoon of *P. gracilis* has an acrosome length to width ratio of 0.84 ± 0.112. The inner acrosome layer is less electron-dense than the remaining ones and is displaced toward the anterior portion of the spermatozoon (Fig. 2F). The intermediate acrosome zone is electron-dense and is located in the equatorial portion of the acrosome, adjacent to the outer layer, which is filled by peripheral concentric lamellae. Next to this layer, a fourth electron-lucent layer is present, which narrows toward the equatorial region of the acrosome, near the capsule (Fig. 2F and I). The electron-dense operculum is striated, flat, centrally round and perforated at the central portion with the apical button continuous with the subopercular region (Fig. 2F and G). In the subopercular region, a reduced accessory opercular ring is present, continuous with the unit membrane that separates the subopercular region of the acrosome and the perforatorial chamber (Fig. 2H). The perforatorial chamber is flask-shaped, but wider along its entire extension. The apex is rounder and slightly tapered toward the base of the perforatorial chamber (Fig. 2F). This structure exhibits organized and dispersed perforatorial tubules (Fig. 2I). The perforatorial chamber is continuous with the thin cytoplasm and near it is a unit membrane of the acrosome (Fig. 2I and J). The nucleus contains a fibrous chromatin (Fig. 2K) and is delimited by a single unit membrane (Fig. 2J) and flanked by lateral arms (Fig. 2E, L, and M). The number of lateral arms varies from four to five as observed under DIC (Fig. 2E). In the vestigial cytoplasm, a reduced lamellar complex is observed with degenerate mitochondria (Fig. 2O).

3.2.3. Geograpsus lividus

The spermatozoon of *G. lividus* has an acrosome length to width ratio of 0.82 ± 0.024, thus slightly wider than long. The acrosome has four concentric layers. The innermost one is more electron-dense than the intermediate layer, and both are narrow and shifted toward the opercular pole (Fig. 3C and D). The outer layer is wide with prominent concentric lamellae (Fig. 3D and E). A fourth electron-lucent and outermost layer is also observed (Fig. 3C and E). The operculum resembles a "W" centrally protruding and round, also perforated at its central portion exhibiting an apical button (Fig. 3F). The subopercular layer is electron dense and sinuous on both acrosome and operculum sides (Fig. 3F). Around the margin of the operculum is the periopercular ring (Fig. 3D and F) and underneath it, is noticed the accessory opercular ring (Fig. 3F). Internal to the acrosome vesicle is the club-shaped perforatorial chamber, with a narrow conic apex and slightly pointed operculum (Fig. 3C to F). The base of the perforatorial chamber is marked by the absence of any thickened ring (Fig. 3G) and the periacrosomal cytoplasm is very thick (Fig. 3G and H), with mitochondria without cristae (Fig. 3I) and lamellar complexes (Fig. 3J). The nucleus is filled by fibrillar chromatin and exhibits lateral arms (Fig. 3K).

N. Spermatozoa with lateral arms (black arrowhead) under differential interference contrast (DIC) microscopy. Ac = acrosome, Ar = lateral arms; Cy = cytoplasm; Ls = lamellar complex; M = mitochondria; Ma = extracellular matrix; N = nucleus; O = operculum; P = perforatorial chamber; Sf = spermatophore; Sp = spermatozoon; Sw = spermatophore wall.

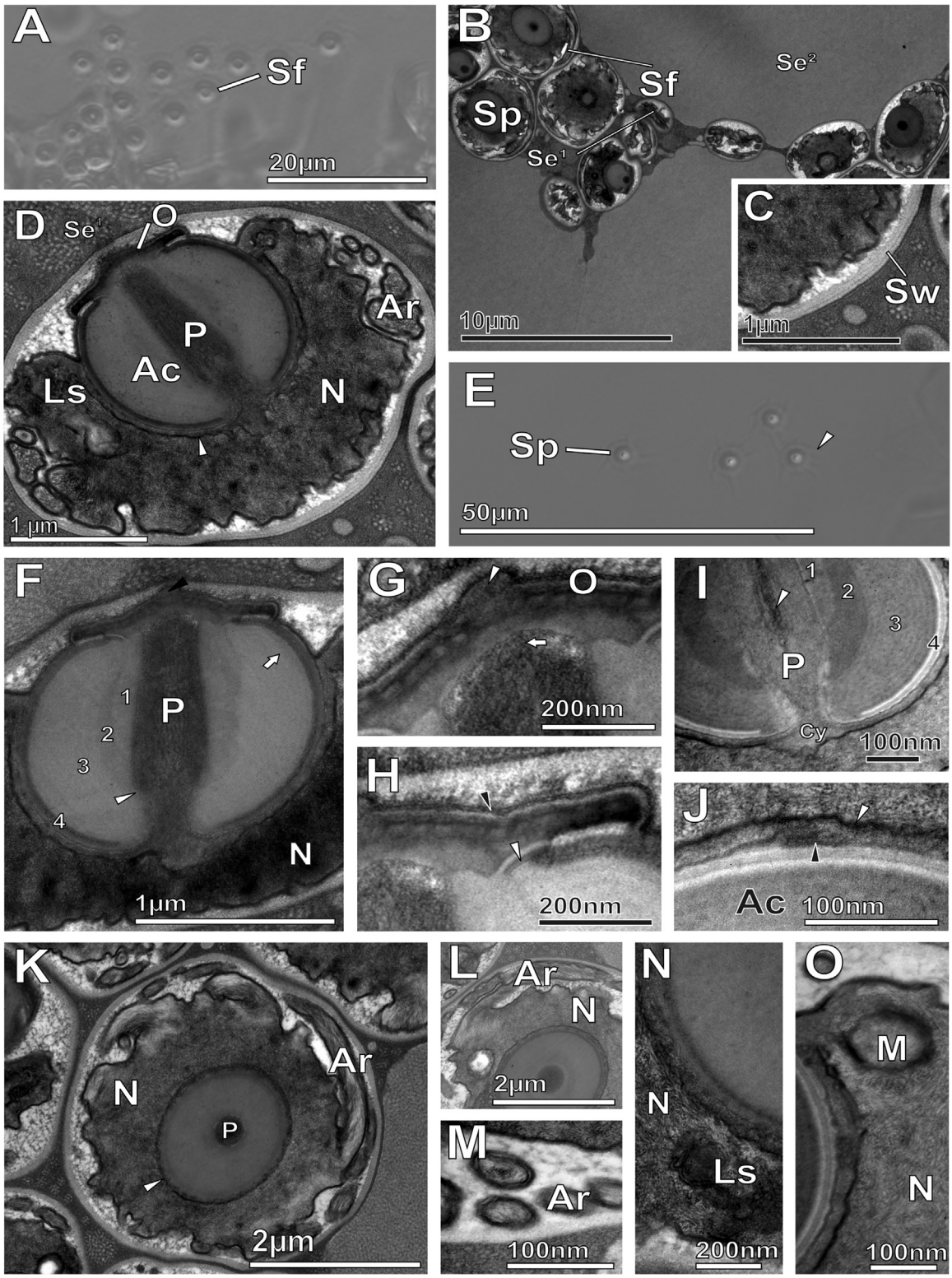

Fig. 2. Spermatophores and spermatozoa of *Pachygrapsus gracilis*. A. Cleistospermic spermatophores observed under DIC. B. Spermatophore are surrounded by two different types of secretion that form the seminal fluid (Se1 and Se2). C. Detail of the electron-dense spermatophore wall. D. Longitudinal section of a spermatozoon immersed in electron-lucent extracellular matrix. The acrosome is delimited by one-unit membrane (white arrowhead) that separates it from the nuclear material. E. Detail of spermatozoa under DIC showing radial arms (white arrowhead). F. Longitudinal section of the acrosome composed of four concentric layers (1, 2, 3, and 4). The outer layer exhibits peripheral concentric lamellae (white arrow). The flat and centrally round operculum is perforated by the apical button (black arrowhead) and segmented with electron-dense regions alternated with electron-lucent ones. Note the flask-shaped perforatorial chamber with slight narrowing toward the base of the perforatorial chamber (white arrowhead). G. Longitudinal section of a detail of the apical button (white arrowhead) and the apex of the round perforatorial chamber with small electron-lucent vesicles (white arrow). H. Detail of the accessory opercular ring (white arrowhead) and the capsule (black arrowhead). I. Longitudinal section of a detail of the base of the perforatorial chamber filled with dispersed and organized microtubules (white arrowhead). Adjacent to the perforatorial chamber is the cytoplasm. J. Detail of the nuclear envelope (white arrowhead) and the acrosome membrane (black arrowhead). K. The nucleus is irregular and separated from the acrosome by two unit membranes (white arrowhead). L. Longitudinal section of the lateral arm. M. Cross-section of a detail of the lateral arms. N. Detail of the lamellar complex. O. Detail of mitochondria devoidof cristae. Ac = acrosome, Ar = lateral arms; Cy = cytoplasm; Ls = lamellar complex; M = mitochondria; Ma = extracellular matrix; N = nucleus; O = operculum; P = perforatorial chamber; Se1 = secretion type I; Se2 = second type II; Sf = spermatophore; Sp = spermatozoon; Sw = spermatophore wall.

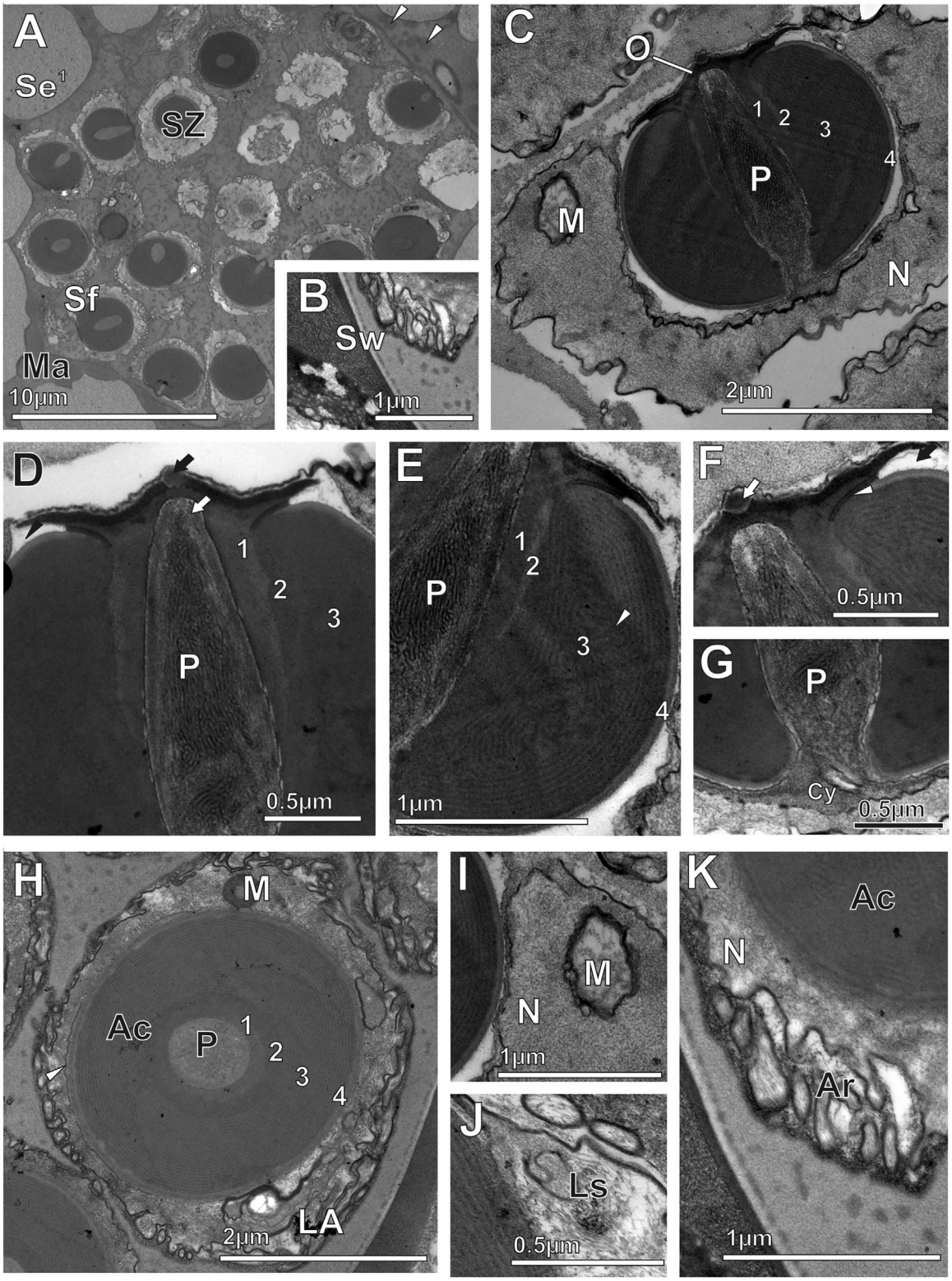

Fig. 3. Spermatophores and spermatozoa of *Geograpsus lividus* A. Coenospermic spermatophore of *G. lividus*. Spermatophores are surrounded by secretion containing two types of granules: one large and moderately electron-dense (Se1) and another small and electron-dense (white arrowhead), both immersed in homogeneous matrix. B. Detail of the spermatophore wall. C. Longitudinal section of a spermatozoon immersed in extracellular matrix. The acrosome exhibits four concentric layers (1, 2, 3, and 4) of different electron densities. Note in the center the club-shaped perforatorial chamber. D. The club-shaped perforatorial chamber is filled with perforatorial tubules and is characterized by a conical apex (white arrow). The centrally protruding and round operculum is perforated with an apical button (black arrow). Notice the periopercular ring (black arrowhead). E. Detail of acrosomal layers. In the external layers (3), peripheral concentric lamellae are present (white arrowhead). F. Detail of the perforated operculum with apical button (white arrow). Note the presence of a reduced accessory opercular ring (white arrowhead) and the presence of the periopercular ring (black arrow). G. Detail of the base of the perforatorial chamber continuous to the capsule and the thin layer of vestigial cytoplasm. H. Cross-section of the spermatozoon showing the acrosome with four concentric layers (1, 2, 3, and 4). The nucleus with fibrous chromatin is delimited by the nuclear envelope (white arrowhead) and exhibits lateral arms. I. Detail of mitochondria without cristae. J. Detail of the lamellar complex. K. Detail of a longitudinal section of the lateral arms. Ac = acrosome; Cy = cytoplasm; LA = lateral arms; Ls = lamellar complex; M = mitochondria; Ma = extracellular matrix; N = nucleus; O = operculum; P = perforatorial chamber; Se1 = Large moderately electron-dense granule; Sf = spermatophore; Sp = spermatozoon; Sw = spermatophore wall.

4. Discussion

The spermatophores of the three studied species exhibit different ultrastructural characteristics, with a strong indication that the reproductive structure fits with previous phylogenetic positioning proposal. In *Pachygrapsus transversus* and in *Geograpus lividus* spermatozoa are grouped in coenospermic spermatophores which is the typical arrangement found in many marine brachyurans (Benetti et al., 2008; Guinot et al., 1997; Jamieson, 1994; Zara et al., 2012), including other grapsoids (Anilkumar et al., 1999; Garcia and Silva, 2006; Nicolau et al., 2012; Santos et al., 2009). On the other hand, *P. gracilis* exhibits cleistospermic spermatophores (one spermatozoon per spermatophore), and this is the first ultrastructural report of this condition in the Thoracotremata, corroborating with our previous light microscope data (Tiseo et al., 2014).

Pachygrapsus transversus and *G. lividus* have ovoid or elliptical coenospermic spermatophores variable in size. The spermatophores of *Geograpsus lividus* are bigger than *P. transversus* and both presented high values of standard deviation as Benetti et al. (2013) also report for *Cardisoma ganhumi* (Latreille, 1828). Differently, in *P. gracilis* we observed only small elliptical cleistospermic spermatophores showing low standard deviations. The high deviation values in coenospermic spermatophores of *P. transversus* and *G. lividus* can be explained by a variation in the shape and number of spermatozoa inside the spermatophores.

Guinot et al. (1997) proposed that the occurrence of cleistopermia was restricted to the freshwater crab species of the family Potamidae Ortmann, 1896. However, this type of spermatophore has also been reported for other members of freshwater crabs of the family Gecarcinucidae Rathbun, 1904 (Klaus et al., 2009). Hartnoll (1964), Guinot et al. (1997) and Jamieson and Tudge (2000) proposed that the occurrence of cleistospermic spermatophores could be an adaptation to avoid polyspermy, reducing the rates of oocyte wastage, since freshwater crabs have bigger eggs. However, this is not applicable to *P. gracilis* as the characteristics of eggs, typically with around 0.5 mm diameter, from marine and estuarine species of Grapsidae are different to those of freshwater crabs, typically with around 1.5–2.0 mm diameter, (Hartnoll, 1964). Tiseo et al. (2014) reported that the diameter of eggs of *P. transversus*, which exhibits coenospermic spermatophores, is larger than those of *P. gracilis*. In the latter species, the presence of a more acidic seminal fluid and cleistospermic spermatophores seems to be associated with differences in spermatophore dehiscence (Tiseo et al., 2014), as also suggested by Beninger et al. (1993) for *Chionoecetes opilio* (Fabricius, 1788) and Klaus et al. (2009) for some species of Potamoidea.

Comparatively, spermatozoa of *P. transversus* and *G. lividus* are immersed in a slightly electron-dense matrix and apart from each other, while *P. gracilis* spermatozoa are very close to the spermatophore wall. According to Klaus et al. (2009), the small space between the spermatozoa and spermatophore wall might be seen as a packing device for sperm transfer in Potamidae and Gecarcinucidae, corroborating our findings. Tiseo et al. (2014) described the process of spermatophore formation in *P. transversus* and *P. gracilis* and suggested that the distinct structures of the spermatophore, and characteristics of seminal fluid found in both species are indications of physiological differences associated with sperm transference and storage in the seminal receptacle. Additionally, Klaus and Brandis (2011) demonstrated that the occurrence of cleistospermia is a variable trait found in phylogenetically unrelated species within the same family. Based on evidence and variabilities detected among these characters, we conclude that the differences between the types of spermatophores of the studied species (spermatophore with several spermatozoa and spermatophore with a single spermatozoon) are not robust enough to be used to analyse phylogenetic relationships. Thus, to understand the transition from the marine environment to freshwater, further studies relating to the reproductive system of various species (including spermatophores and spermatozoa) associated with the type of embryonic and post-embryonic development and molecular tools will be required to have a clear picture of the conquest of the freshwater environment.

The ultrastructural evidence of sperm cells from *P. transversus*, *P. gracilis* and *G. lividus* corroborate the utility of spermiotaxonomy as a systematic tool and can therefore, along with other morphological and molecular studies, help to solve existing taxonomic problems in *Pachygrapsus* genus. Both studied species of *Pachygrapsus* show very similar spermatozoa.

The main differences were small and found mainly in the morphology and thickness of the apex of the flask-shaped perforatorial chamber; thickness and shape of the inner and middle acrosome layers; and distribution and position of the concentric lamellae in the outer layer of the acrosome. When comparing spermatozoa of both species of *Pachygrapsus* with that of *G. lividus*, the main differences are also associated with the morphology of the perforatorial chamber and the operculum. In *G. lividus*, the perforatorial chamber is club-shaped with a conical and narrow apex; the thickened ring is absent in the basal portion; a more protruding and rounded operculum than those of both species of *Pachygrapsus*; and the presence of a peripercular rim. In addition to the characteristics described above, peripheral concentric lamellae are observed throughout the extension of the outer acrosome layer of *G. lividus*. The small morphological variation in the apex of the perforatorial chamber, which maintains the flask-shaped pattern for *Pachygrapsus* and club-shaped for *Geograpsus*, seems to be very significant, mainly at the specific level. This structure has the role to help penetrate the egg chorion during fertilization in the Brachyura (Brown, 1966; Medina, 1992; Medina and Rodriguez, 1992). Additionally, it has been shown that myosin and kinesins (signal-regulated kinases – ERKs k and KIFC1–a C terminal kinesin motor) are gradually accumulated in the perforatorial chamber apex and in the operculum during spermatogenesis in the Grapsoidea *Eriocheir sinensis* Milne Edwards, 1853. The former is responsible for forming the acrosome during spermatogenesis and the latter is responsible for the eversion of the perforating structure. Both motor proteins are involved in acrosome reaction of the Chinese mitten crab (Yu et al., 2009; Sun et al., 2010, 2015). Since this region of the perforatorial chamber is where differences are observed, we speculate that they might be associated with specific recognition of the egg chorion. If the species of *Pachygrapsus* from our study belonged to different genera, more prominent differences on the perforatorial chamber structure would be expected.

According to the ultrastructural characteristics of spermatozoa of the species examined in the present study, the morphology of the spermatozoa of *P. gracilis* is more similar to that of *P. transversus* than to that of *G. lividus*. Poupin et al. (2005), based on morphological characters, established phylogenetic relationships for 13 species of *Pachygrapsus*. These authors reported that *P. transversus* and *P. gracilis* are in different species complexes within the same genus. On the other hand, the molecular phylogeny of the Grapsidae proposed by Schubart (2011) has seven distinct lineages. Of the eight genera of grapsid crabs analyzed, *Pachygrapsus* was the most problematic and a taxonomic review of the genus seems necessary. In this work, the author proposed to maintain the species *P. transversus*, *Pachygrapsus crassipes* Randall, 1840, *Pachygrapsus minutus* Milne-Edwards, 1873, *Pachygrapsus socius* Stimpson, 1871, *Pachygrapsus loveridgei* Chace, 1966, and *Pachygrapsus propinquus* De Man, 1908 in the same genus, while the species *P. gracilis* should be included in the genus *Geograpsus*, as these species

Table 1
Comparison of spermatophores and spermatozoa of *Pachygrapsus transversus*, *Pachygrapsus gracilis*, and *Geograpsus lividus* with those of other Thoracotremata described in the literature.

	Thoracotremata											
	Grapsidae						Varunidae		Sesarmidae		Gecarcinidae	
Characteristic	*Pachygrapsus transversus*[1]	*Pachygrapsus gracilis*[1]	*Geograpsus lividus*[1]	*Grapsus albolineatus*[2]	*Metopograpsus messor*[3]	*Cyclograpsus punctatus*[4]	*Varuna literatta*[5]	*Eriocheir japonicus*[6]	*Parasesarma erythrodactyla*[2]	*Sesarma catenata*[4]	*Cardisoma carnifex*[3]	*Cardisoma guanhumi*[7]
1. Type of spermatophore	Coenospermy[a*]	Cleistospermy [b*]	Coenospermy[a*]	N	Coenospermy[a]	N	N	N	N	N	N	Coenospermy[a]
2. Spermatophore size (μm)	19.39 ± 6.51	3.67 ± 0.57	30.2 ± 11.1	N	N	N	N	N	N	N	N	179 ± 41.4
3. Acrosome ratio	0.82 ± 0.022	0.84 ± 0.112	0.82 ± 0.024	N	1.2	1.03	0.907	N	N	N	0.9	0.94
4. Acrosome zonation	Concentric [a]	Concentric [a]	Concentric [a]	Concentric [a?]	Concentric [a?]	Concentric [a?]	Concentric [a]	Concentric [a?]	Concentric [a?]	Concentric [a?]	Concentric [a]	Concentric [a]
5. Operculum	Perforated with apical button [a*]	Perforated with apical button [a*]	Perforated with apical button [a*]	Perforated with apical button [a]	Continuous [b]	Perforated [a]	Perforated [a]	Perforated [a?]	Perforated with apical button [a]	Perforated with apical button [a]	Perforated with apical button [a]	Perforated with apical button [a]
6. Opercular projections	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]
7. Continuity of the operculum with the capsule	Discontinuous [a]	Discontinuous [a]	Discontinuous [a]	Discontinuous[a?]	Discontinuous[a?]	Discontinuous[a?]	Discontinuous[a]	Discontinuous[a?]	Discontinuous[a?]	Discontinuous[a?]	Discontinuous[a]	Discontinuous[a?]
8. Operculum thickness	Moderate [a]	Moderate [a]	Moderate [a]	Moderate [a]	Thick [b]	Moderate [a?]	Moderate [a]	Moderate [a]	Moderate [a]	Moderate [a?]	Moderate [a]	Moderate [a?]
9. Operculum length	Not wide [a]	Not wide [a]	Not wide [a]	Not wide [a?]	Not wide [a?]	Not wide [a?]	Not wide [a?]	Not wide [a?]	Not wide [a?]	Not wide [a?]	Not wide [a?]	Not wide [a?]
10. Protuberance of the subopercular material in the operculum	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]
11.Periopercular rim	Absent [a*]	Absent [a*]	Present [b*]	Absent [a?]	Present [b?]	Absent [a?]	Present [b]	Present [b?]	Weakly developed [c?]	Present [b?]	Weakly developed [c]	Present [b]
12. Accessory opercular ring	Present [a]	Present [a]	Present [a]	N	Present [a]	N	Present [a]	Absent [b?]	Present [a?]	N	Present [a]	Present [a]
13. Acrosome ray zone	Absent [a]	Absent [a]	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]
14. Outer acrosome zone	Wide [a]	Wide [a]	Wide [a]	Wide [a?]	Wide [a?]	Wide [a?]	Wide [a]	Wide [a]	Wide [a?]	Wide [a?]	Wide [a?]	Wide [a]
15. Antero-lateral pale zone	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]
16. Flange-like lower acrosome zone	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]
17. Inner acrosome zone	Narrow/straight[a*]	Wide/straight [b*]	Narrow/curved[a*]	Narrow/straight[a?]	Narrow/straight[a?]	Wide/round [c?]	Narrow/straight[a?]	Wide/round [c?]	Narrow/straight[a?]	Narrow/straight[a?]	Wide/straight[b?]	Narrow/straight[a?]
18. Electron-dense subopercular horizontal layer	Present [a]	Present [a]	Present [a]	Present [a?]	Present [a]	Absent [b?]	Absent [b?]	Absent [b?]	Present [a?]	Absent [b?]	Present [a]	Present [a]
19. Xanthidae ring	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Present [b?]	Present [b?]
20. Morphology of the perforatorial chamber	Flask-shaped [a*]	Flask-shaped [a*]	Club-shaped [b*]	Club-shaped [b?]	Flask-shaped [a?]	Finger-shaped[c?]	Club-shaped [b?]	Flask-shaped [a?]	Flask-shaped [a?]	Club-shaped [b?]	N	Finger-shaped [c?]
21. Apex of the perforatorial chamber	Round/wide [a*]	Round/wide [a*]	Conical [b*]	Round [a?]	Conical [b?]	Round [a?]	Round [a?]	Round [a?]	Round [a?]	Round [a?]	Conical [b?]	Round [a?]
22. Corrugations of the perforatorial chamber wall	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent a[?]	Absent [a?]	Absent [a?]	Absent [a?]
23. Lateral arms	Present [a]	Present [a]	Present [a]	Present [a]	Present [a]	Present [a]	Present [a?]	Present [a]	N	Present [a]	Present [a]	Present [a]
24. Composition of lateral arms	DNA [a]	DNA [a]	DNA [a]	DNA [a?]	DNA [a]	DNA [a?]	DNA [a]	DNA [a]	DNA [a?]	DNA [a?]	DNA [a]	DNA [a]
25. Centrioles	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a]	Present [b?]	Present [b]	N	Absent [a?]	N	Present [b]	Present [a]
26. Chromatin	Granular [a*]	Granular [a*]	Fibrillar [b*]	Granular [a?]	Reticulated [c]	Granular [a?]	Granular [a?]	Granular [a?]	Granular [a?]	Granular [a?]	Fibers [b]	Fibers [b]
27. Median process of the nucleus	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]
28. Thick ring	Present (modified and reduced) [a*]	Present (modified and reduced) [a*]	Absent [b*]	Absent [b]	Absent [b]	Absent [b?]	Present [c]	N	Absent [b]	N	Absent [b?]	Absent [b?]
29. Concentric lamellae in acrosome	Little evident [a*]	Evident [b*]	Evident [b*]	N	Evident [b]	Evident [b]	Little evident [a]	Evident [b]	Evident [b]	N	Evident [b]	Evident [b]
30 Capsule chamber	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a?]
31. Capsule projections	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent a[?]
32. Flange-like capsule	Absent [a]	Absent [a]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]	Absent [a]	Absent [a?]	Absent [a?]
33. Mitochondria	Present [a]	Present [a]	Present [a]	N	N	Present [a]	Present [a?]	Present [a]	N	N	Present [a]	Present [a]
34. Lamellar structure	Present [a*]	Present [a*]	Present [a*]	N	N	Present [a]	Present [a?]	Present [a]	N	Present [a]	N	Present [a]

[1] Present study; [2] Jamieson (1991); [3] Anilkumar et al., (1999); [4] Jamieson and Tudge (2000); [5] Jamieson et al. (1996); [6] Yasuzumi (1960); [7] Benetti et al. (2013).
Different letters indicate variation among species; * = difference among species of the present study; N = undescribed; ? = undescribed and observed in the published figure.

were grouped in the same phylogenetic lineage. Based on a new Grapsidae phylogeny, the monophyly of *Pachygrapsus* was also rejected. Out of the four species of the *Pachygrapsus* studied, *P. gracilis* is the most basal species of the clade and presents no affinity to the monophyletic clade of the genus *Geograpsus* (Ip et al., 2015). Although our study did not present a phylogenetic analysis, the great similarity found between the spermatozoa of *Pachygrapsus* species conflicts with the proposition of Schubart (2011), but agrees with the molecular results found by Ip et al. (2015).

In general, the spermatozoa of the three studied species follow the pattern observed for Thoracotremata with three main synapomorphies: loss of the acrosome ray zone, development of the apical button, and concentric lamellae in the acrosome (Jamieson and Tudge, 2000). When compared to spermatozoa of eleven species from four families of Grapsoidea described in the literature [Grapsidae *P. transversus*, *P. gracilis*, *Grapsus albolineatus* Lamarck, 1818 (Jamieson, 1991) and *Metopograpsus messor* (Forskål, 1775) (Anilkumar et al., 1999); the Varunidae *Cyclograpsus punctatus* Milne Edwards, 1837 (Jamieson and Tudge, 2000), *Varuna litterata* (Fabricius, 1798) (Jamieson et al., 1996) and *Eriocheir japonicus* De Haan, 1835 (Yasuzumi 1960); the Sesarmidae *Parasesarma erythodactyla* (Hess, 1865) (Jamieson, 1991) and *Sesarma catenata* (Ortmann, 1897) (Jamieson and Tudge, 2000) and the Gecarcinidae *Cardisoma carnifex* (Herbst, 1796) (Jamieson et al., 1996) and *Cardisoma guanhumi* (Benetti et al., 2013)], we noted that all grapsoids exhibit concentric acrosomes with peripheral concentric lamellae in the outer acrosome zone and lateral arms as extensions of the nucleus all filled with nuclear material. Except for *M. messor*, all have perforated operculum and in *P. transversus*, *P. gracilis*, *G. albolineatus*, *P. erythrodactyla*, *S. catenata*, *C. carnifex* and *C. guanhumi*, an apical button is present (Table 1). The main variable characteristics in the Grapsoidea were associated with the structure of the perforatorial chamber, which varied from flask-shaped, and finger-shaped, to club-shaped; the continuity of the operculum, which ranged from perforated to continuous; and presence or absence of the apical button; indicating again the importance of these structures for the differentiation of spermatozoa among species (Table 1). Comparing the morphology of the spermatozoon of *P. gracilis* with those of other freshwater crabs of the Heterotremata assemblage that exhibit cleistospermia [i.e., Potamidae *Potamon fluviatile* (Herbst, 1785), *Potamon ibericum* (Bieberstein, 1808) (Guinot et al., 1997), *Johora singaporensis* (Ng, 1986), and *Thaiphusa sirikit* (Naiyanetr, 1992) (Klaus et al., 2009) and the Gecarcinucidae *Sartoriana spinigera* (Wood-Mason, 1871), *Heterothelphusa fatum* Ng, 1997 and *Sayamia bangkokensis* (Naiyanetr, 1982)], there is no single shared structure, but rather exclusive traits of *P. gracilis* not shared with Potamidae and Gecarcinucidae such as the existence of peripheral concentric lamellae in the outer acrosomal zone. Likewise, there are traits common to the Heterotremata, but not present in the spermatozoon of *P. gracilis*, such as the acrosomal ray zone and the periopercular rim. Therefore, based on the characteristics of the spermatozoon, the proximity of *Pachygrapsus gracilis* to Potamidae and Gecarcinucidae cannot be confirmed. However, the existence of typical structures of Thoracotremata clearly separates them.

4.1. Conclusions

In conclusion, the distinct morphology of spermatophores among the three species is associated to different mechanisms of transference and/or spermatic storage found in the same genus, as proposed for *Pachygrapsus* (Tiseo et al., 2014). Thus, using the morphology of spermatophores in taxonomic studies on the Grapsidae, and perhaps the Brachyura, should be considered with caution, as demonstrated for families of freshwater crabs, since cleistospermia, as well as coenospermia may occur in phylogenetically unrelated species (Klaus et al., 2009; Klaus and Brandis, 2011, present study). Therefore, a broad analysis of spermatophores and spermatozoa for the families of Thoracotremata, especially Grapsidae, Sesarmidae and Varunidae is needed to confirm this hypothesis. The spermatozoa of *P. transversus* and *P. gracilis* follow the pattern observed in Thoracotremata and have very similar structures with some minor differences. On the other hand, significant differences in the contact regions of the spermatozoon and oocyte, mainly the operculum, apex, and general morphology of the perforatorial chamber differentiate the spermatozoon of *Pachygrapsus* from that of *Geograpsus* and these structures should be highlighted in further studies. Thus, this research confirms that the spermatozoal ultrastructure is an important additional tool to clarify taxonomic problems within the Grapsidae and their relationships to other crabs.

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