Invertebrate Reproduction & Development

ISSN: 0792-4259 (Print) 2157-0272 (Online) Journal homepage: https://www.tandfonline.com/loi/tinv20

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To cite this article: V. Fransozo, A.B. Fernandes, L.S. López-Greco, F.J. Zara & D.C. Santos (2016) Functional morphology of the male reproductive system of the white shrimp *Litopenaeus schmitti* (Burkenroad, 1936) (Crustacea, Penaeidea) compared to other *Litopenaeus*, Invertebrate Reproduction & Development, 60:3, 161-174, DOI: 10.1080/07924259.2016.1174158

To link to this article: https://doi.org/10.1080/07924259.2016.1174158

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INVERTEBRATE REPRODUCTION & DEVELOPMENT, 2016
VOL. 60, NO. 3, 161–174
http://dx.doi.org/10.1080/07924259.2016.1174158

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Functional morphology of the male reproductive system of the white shrimp *Litopenaeus schmitti* (Burkenroad, 1936) (Crustacea, Penaeidea) compared to other *Litopenaeus*

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ABSTRACT

The male reproductive system of *L. schmitti* is described, including spermatozoon ultrastructure. The testis is composed of 8 to 14 lobules arranged as seminiferous tubules. The proximal vas deferens is short and filled with spermatozoa without spikes. The median vas deferens is larger than the other parts of the vas deferens and it shows two ducts separated by typhlosoles, the sperm and accessory ducts. The former is filled with sperm masses, while the accessory duct shows only secretions that are part of the spermatophore material. In this region, the spermatozoa have a spike, indicating the end of the spermatogenesis. The distal vas deferens is a short tube ending in the ampulla, composed of three parts. The spermatozoa have a round main body and an anterior spike associated with the acrosomal cap. The subacrosomal space has a centrosome-like structure above the nucleus. The features of the male reproductive system of *L. schmitti* share the pattern of penaeoidean shrimps with closed thelyca related to the median vas deferens and the ampulla. All the differences in spermatozoon morphology and vasa deferentia are discussed, and compared to other *Litopenaeus* species.

ARTICLE HISTORY
Received 15 December 2015
Accepted 24 March 2016

KEYWORDS
Histology; Penaeini; Penaeoidea; reproductive trait; seminal fluid; spermatozoon ultrastructure

Introduction

The reproductive morphology of penaeoidean shrimps has been studied in a number of species, particularly those in the genus *Litopenaeus* as follows: Pérez-Farfante (1975) species of the genus *Litopenaeus*; Ro et al. (1990) *Litopenaeus setiferus*; Bauer and Cash (1991) *L. setiferus*, *Farfantepenaeus duorarum* and *F. aztecus*; Díaz et al. (2002) *Pleoticus muelleri*; Fransozo (2008) *Xiphopenaeus kroyeri*; and Ceballos-Vázquez et al. (2010) *Litopenaeus vannamei*. Worldwide, the two best-studied penaeid shrimp species with reference to reproductive system morphology are *L. vanammei* and *L. setiferus*, owing to their economic importance (Young 1959; Muncy 1984; Browdy 1992; Alfaro 1994; Campos-Ramos et al. 2006; Rendón et al. 2007; Rodriguez et al. 2007; Garza-Torres et al. 2009; Alfaro-Montoya 2010; Ceballos-Vázquez et al. 2010). The reproductive morphology of a few penaeidean species that occur along the Brazilian coast has also been studied. Some studies were on the female reproductive system, e.g. Quintero and Garcia (1998) on *F. brasiliensis*; Peixoto et al. (2003) and Dumont et al. (2007) *F. paulensis* ; Dumont and D'Incao (2004) *Artemesia longinaris* Bate, 1888; de Campos et al. (2009) *X. kroyeri* and Machado et al. (2009) *L. schmitti* (Burkenroad 1936). Braga et al. (2013a) and Castelo-Branco et al. (2014) also carried out studies on the male reproductive system of *F. paulensis* and *F. subtilis*, respectively, which occur along the Brazilian coast. In both these studies, the aim was the analysis of spermatozoon ultrastructure by transmission (TEM) and scanning electron microscopy (SEM).

Detailed knowledge of the seminal fluid nature, formation of the spermatophores and its localization in the females' body are essential for understanding the mechanisms of sperm transfer, spermatozoa storage and their liberation during fertilization (Bauer & Min 1993). Penaeoideans produce spermatophores of varied complexities, which are transferred to the female thelycum

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(Bauer & Cash 1991; Bauer & Min 1993; Alfaro et al. 2003; Alfaro-Montoya 2010). Species with open thelyca usually receive spermatophores with complex accessory structures, in a winged shape, while in species with closed thelyca, the spermatophores are relatively simple masses of sperm, sometimes accompanied by relatively simple accessory substances (Shigekawa & Clark 1986; Bauer 1991; Bauer & Min 1993; Subramoniam 1995; Alfaro et al. 2003; Fransozo 2008; Alfaro-Montoya 2010).

In decapod crustaceans, the spermatozoa are immobile (Bauer 1991). Their morphological features are important in understanding insemination and mating system of penaeoidean shrimps (Bauer 1991; Medina 1995). Currently, at least, ten species of penaeoidean shrimps have had the ultrastructure of their spermatozoon described [(Medina (1995), Medina et al. (2005, 2006), who analyzed the sperm ultrastructure of Penaeidae, Scyoniidae, and Solenoceridae; and Braga et al. (2013b) provided a review on the evolution and fertilization of the unistellate sperm found in Penaeoidea and Caridea shrimps]. Although knowledge of shrimp spermatozoon ultrastructure is good, descriptions of the morphology and histology of reproductive systems are still necessary for aquaculture studies, mainly for sperm cryopreservation.

Very little data are available on the functional morphology of the male reproductive system of representatives of the genus *Litopenaeus* (Alfaro-Montoya 2010; Alfaro-Montoya & Hernández 2012). To better comprehend reproduction of *L. schmitti*, this study provides the morphological characterization of the male reproductive system by macroscopy, light microscopy, and ultrastructure in a comparative approach within Penaeoidea. Additionally, spermatozoon ultrastructure is described using TEM and SEM.

Material and methods

Male *L. schmitti* were collected in three different locations in Brazil: Sepetiba Bay, Rio de Janeiro state (43º 54'W; 23º 03'S) and two in São Paulo state near Ubatuba (44º 52'W; 23º 22'S) and Cananéia (47º 51'W; 25º 04'S) municipalities. The trawls were carried out using a small-scale shrimp fishery boat. Shrimp were caught and kept in thermic boxes with seawater from the sampling sites, under continuous aeration, until laboratory procedures were performed. Mature males were sampled during the reproductive period that coincides with the austral spring and summer seasons (Fransozo 2011).

At the laboratory, 10 adult male specimens (cephalothorax length larger than 31 mm and with large and whitish terminal ampullae [also known as the ejaculatory ducts (Bauer 1991); visible through the relatively transparent exoskeleton]) were anesthetized with cold seawater to remove the dorsal region of the cephalothorax, allowing dissection of the reproductive system. Some of the dissected specimens were used to macroscopically analyze the reproductive system, for which pictures were taken using a digital camera. The other specimens were used to prepare tissues for light, SEM, and TEM.

The samples from different regions of the male reproductive system were fixed with Karnovsky solution (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6) from 24 to 48 h at 4 °C. The fixed samples were dehydrated in an ascending ethanol series (70–95%) and embedded in metacrylate (historesin) Leica®, according to routine procedures. The sections (5 µm) were stained with toluidine blue (pH 4.0) and hematoxylin and eosin, avoiding xylene and the ethanol bath (Sant'Anna et al. 2010; Zara et al. 2012). The images were obtained and digitized with a Leica DM 500 light microscopy.

For electron microscopy, the fixed samples were post-fixed in 1% osmium tetroxide solution for 2 h, and dehydrated through a graded acetone series. For SEM, the dehydrated samples were critical-point-dried in a Balzers CPD 030 using CO_2. The dried samples were mounted on stubs and sputter-coated with gold in a SD Balzers SD50. The observations and digital images were obtained in a QUANTA-200 (FEI company) SEM at 10–20 kV. For TEM, after dehydration, the samples were embedded in Araldite® resin. The copper grids with ultra-thin sections were stained using uranyl acetate and lead citrate. The electron micrographs were obtained in a Philips CM100 and Jeol J1010 TEM at 80 Kv. The general spermatozoon description followed the terminology used by Medina (1994, 1995) and Medina et al. (2005, 2006).

Results

Gross morphology

Both testes are fused on the midline of the shrimp's cephalothorax between the heart and the stomach and appear as a multi-lobed white structure, with 8–14 lobules, although most typically with 8–10 lobules (Figure 1). Each lobule is approximately 10 mm long and 3 mm wide. The vasa deferentia are tubular structures approximately 35 mm long, which go from the testis to the terminal ampulla (at the coxa of the 5th pereiopods). The proximal vas deferens (PVD) is the smallest part of the vas deferens as a whole, and connects to the median vas deferens (MVD). The MVD is wider and longer than the PVD and the distal vas deferens (DVD). The MVD has, externally, a v-shaped morphology showing an ascending portion just after the PVD and a descending part that opens into the DVD. The DVD is connected to the most complex structure of the male reproductive system, the terminal ampulla. Each ampulla

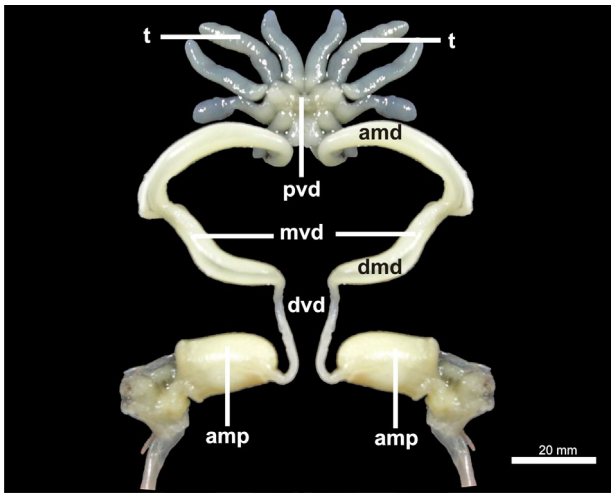

Figure 1. *Litopenaeus schmitti* gross morphology.
Notes: Complete reproductive system of male depicting testis and vas deferens divided into three proximal, medium, and distal regions and terminal ampoule. amd = anterior median deferens; amp = terminal ampoule; dmd = distal median deferens; dvd = distal vas deferens; mvd = median vas deferens; pvd = proximal vas deferens; t = testis.

is a bell-shaped structure measuring approximately 12 mm long and from 7 to 8 mm in width.

Testis and spermatogenesis

Each testis lobule has a digitiform structure surrounded by a layer of connective tissue (Figure 2 (A)). Inside the lobule, there is a convoluted net of seminiferous tubules (Figure 2 (B)). The seminiferous tubules show germinal cells and a somatic tube filled with spermatozoa (Figure 2 (C)). This tube is surrounded by a simple columnar epithelium that forms the seminiferous duct, filled with spikeless free spermatozoa (Figure 2 (D)). The spermatogonia form a germinal center at the periphery of the seminiferous tubule, usually at the opposite pole of the seminiferous duct (Figure 2 (E), (F)). The germinal center of the spermatogonia is normally found at the same time as spermatid maturation occurs (Figures 2 (E), (F), and 3 (A)). The seminiferous tubules are surrounded by accessory or Sertoli cells that increase their scarce cytoplasm during spermiogenesis, becoming eosinophilic and clearly visible (Figures 2F and 3 (A)–(C)). During spermatogenesis, the Sertoli cells have several mitochondria and smooth endoplasmic reticulum (Figure 3 (B) and (C)). Under TEM, the spermatogonia show a large nucleus depicting one or more nucleoli (Figure 3 (A)). The cytoplasm has a few organelles and is filled with some mitochondria, rough endoplasmic reticulum (RER) and in the perinuclear cytoplasm there are some small electron-dense bodies (Figure 3 (C)). The spermatogonia lie on thin connective tissue separating the germinal center from the area of developing spermatocytes (Figure 3 (A), (B), and (D)). The primary spermatocyte has large a nucleus and slightly larger cytoplasm than spermatogonia. Different stages of meiotic prophase are observed and evidenced through synaptonemal complexes. The cytoplasm has RER, and in some cells a large membrane-bound electron-dense vesicle can be observed (Figure 3 (E), (F)). This granule is eosinophilic and helpful for the identification of primary spermatocytes under light microscopy (Figure 2 (D)).

Spermiogenesis begins in the early spermatid that has large heterochromatin blocks that progressively become less compact. Several proacrosomal vesicles scattered in the cytoplasm merge with each other, increasing in size and migrating to the cell periphery. The cytoplasm contains several organelles such as the RER, Golgi bodies and mitochondria (Figure 4 (A), (B)). The early stages of the mid-spermatid show large proacrosomal vesicles at the opposite pole to the nucleus. The proacrosomal vesicle is considered as the acrosomal vesicle, although the cytoplasm is still large and with RER and heterochromatin blocks in the nucleus (Figure 4 (C)). In the mid-spermatid, the cytoplasm becomes filled with small vesicles; mitochondria that have few cristae and only thin filaments of chromatin are observed in the nucleus (Figure 4 (D)). The late spermatid is spikeless and is released into the seminiferous duct. In longitudinal sections, the acrosomal cap in the round main body has two conspicuous electron-dense areas that form the prospective spike base. The acrosomal cap spreads backwards, forming curved lateral expansions on the main body. The less electron-dense lateral expansion of the acrosomal cap and the spike base form the acrosomal vesicle in the late spermatid (Figure 4 (E)). Beneath the prospective spike base is granular material that is different from the filamentous chromatin of the central and large nucleus. The cytoplasm is restricted to a thin area in contrast to the acrosomal (Figure 4 (E)).

Vas deferens and terminal ampulla

With respect its gross morphology (Figure 1), each vas deferens is divided in three regions, showing histological traits very different to one another in appearance. Anteriorly, the PVD is very short and forms a simple duct (Figure 5 (A)–(C)). This region receives the sperm cells from each testis lobule (Figure 5 (B)). The PVD has a simple cubic-columnar epithelium lying on a thin muscle layer (Figure 5(A), (C)). The lumen shows little secretion and is filled with spikeless spermatozoa (Figure 5 (A)–(C)). The spermatozoa show an acidophilic area in the prospective region where the spike will appear at the acrosomal vesicle (Figure 5 (C)).

Both ascending and descending MVDs are similar in histology. In both portions, the MVD is composed by two ducts running side by side: the accessory duct and the sperm duct (Figure 6 (A)). The sperm duct is filled with

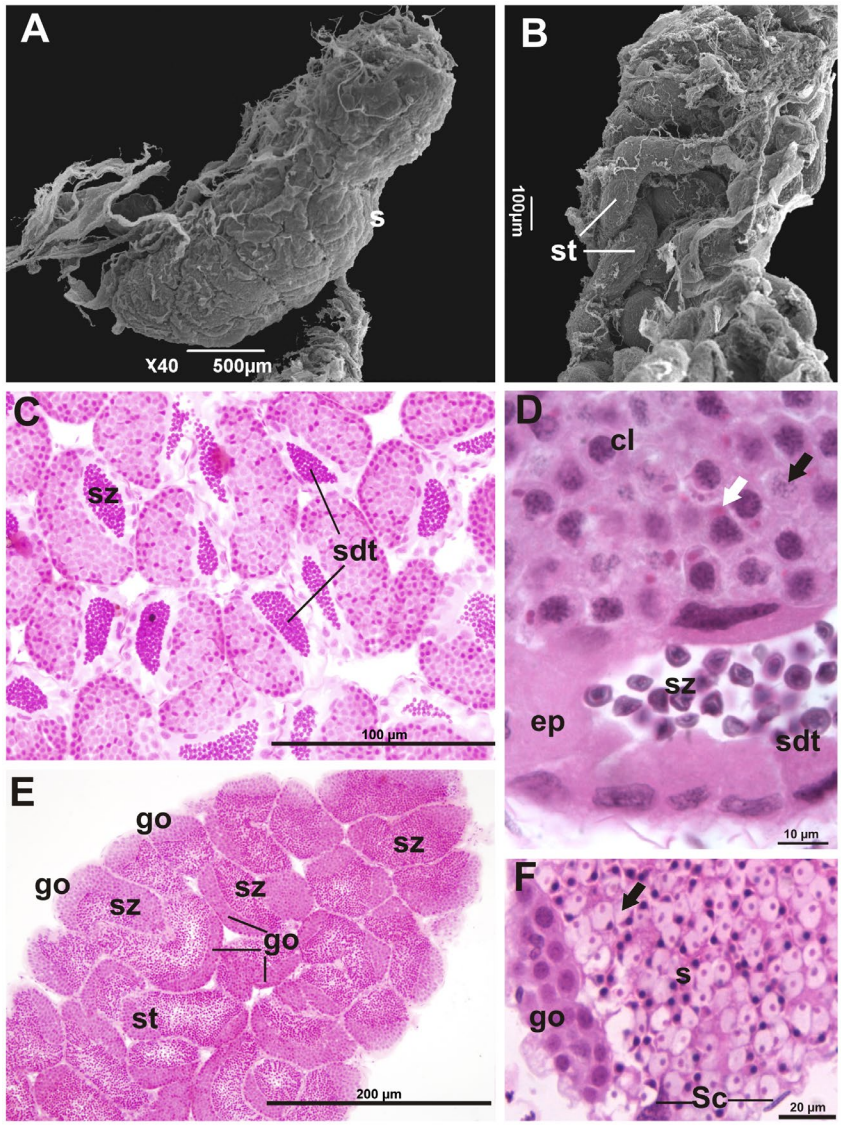

Figure 2. *Litopenaeus schmitti* scanning electron microscopy (SEM) and light microscopy of testis. The light microscopy sections were stained with Hematoxylin-Eosin.

Notes: A, General view of the testes surrounded by connective tissue. B, Detail of the testis without the connective tissue showing the seminiferous tubules. C, Seminiferous tubules showing the germinal cells and the seminiferous ducts filled with spermatozoa. D, Primary spermatocytes with large eosinophilic vesicle next to the nuclei (white arrow) and chromosomes in meiosis (black arrow). E, Germinal centre of spermatogonia in periphery of seminiferous tubules and spermatozoa released to the lumen of the seminiferous ducts. F, Germinal centre of spermatogonia and spermatid showing the acrosomal vesicle at the opposite pole to the nucleus (arrow). cl = primary spermatocyte; ep = epithelium; go = spermatogonia; s = spermatid; sc = Sertoli cells; sdt = seminiferous duct; sz = spermatozoa; st = seminiferous tubules; t = testis.

mature spiked sperm (Figure 6 (B), (C)). The sperm duct is isolated from the accessory or spermatophore duct by twofold or projections of the vas deferens epithelium lying on connective tissue to form typhlosoles I and II (Figure 6 (A), (D), (E)). The sperm duct contains a sperm mass surrounded by a secretion type 1 that is an acid substrate composed externally of an acid compound showing methacromasia γ (pink), while the internal matrix shows variable metachromasia β (blue) to orthochromasia (absence of color) with the toluidine blue stain (Figure 6 (D), (F)). The external compound was basophilic stained by hematoxylin, while the internal compound of type I secretion was acidophilic homogeneously stained by eosin (Figure 6 (G)). The accessory duct is filled with the secretion type 2 that shows homogenous metachromasia (pink), indicating an acid substrate (Figure 6 (D)). The MVD is surrounded by two muscular layers, which are continuous to the next region of the vas deferens (Figure 6 (G)).

The DVD is a narrow tube compared to the MVD, and typhlosoles I and II are found in this region still forming the sperm and accessory ducts (Figures 6 (D) and 7 (A), (B)). The epithelial cells from both typhlosoles are similar to those found in the MVD (Figure 7B). However, both ducts have a small amount of luminal secretion and seem to receive

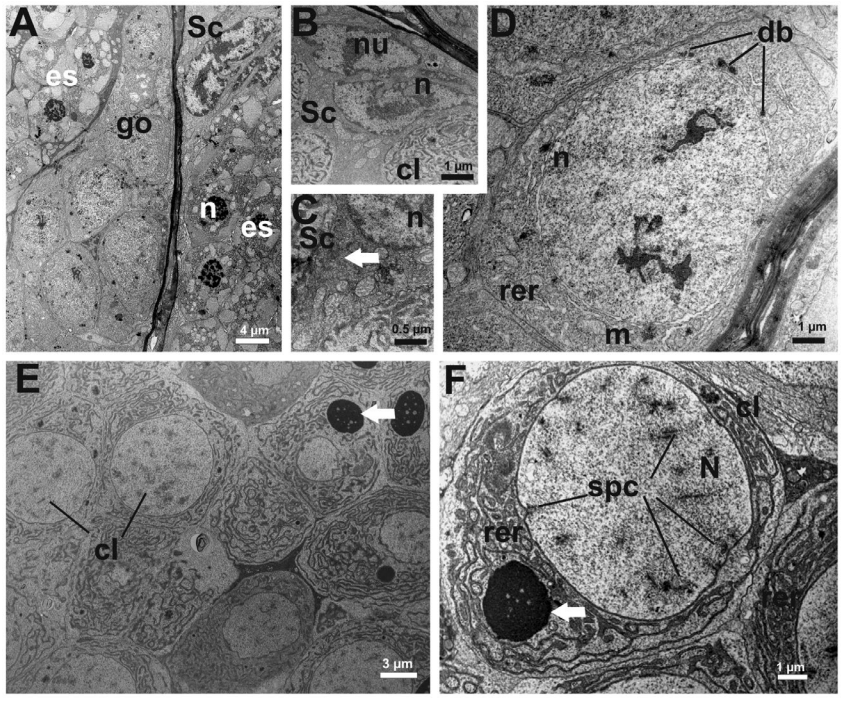

Figure 3. *Litopenaeus schmitti* TEM of testis.

Notes: A, The germinal centre of spermatogonia near the developing spermatids. B, Detail of Sertoli cell showing the flat nucleus depicting a clear nucleolus. C, Perinuclear cytoplasm of the Sertoli cell with many mitochondria and smooth endoplasmic reticulum (arrow). D, Spermatogonium with large nucleus and nucleolus. In the perinuclear cytoplasm some small electro-dense bodies and rough endoplasmic reticulum occupy large part of the cytoplasm. E, General view primary spermatocytes showing a large vesicle filled with electron-dense material (arrow). F, Detail of primary spermatocyte in zygotene showing the synaptonemal complex. The cytoplasm is filled with rough endoplasmic reticulum and a large membrane-bound electron-dense vesicle characterizes this cell (arrow). Cl = spermatocyte; db = electron-dense bodies; es = early spermatid; go = spermatogonium; m = mitochondria; n = nucleus; nu = nucleolus; rer = rough endoplasmic reticulum; sc = Sertoli cells; spc = synaptonemal complex.

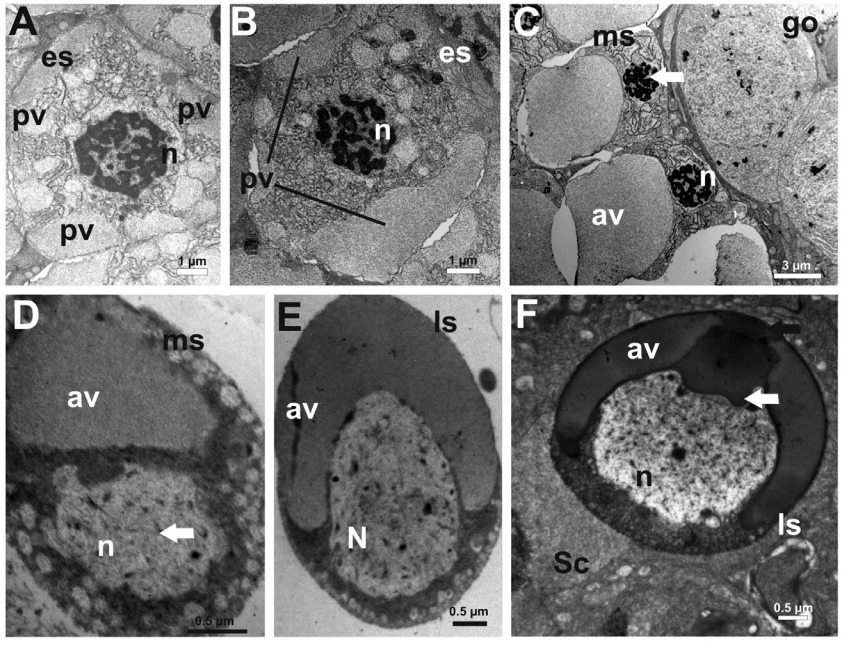

Figure 4. *Litopenaeus Schmitt*. TEM micrographs of spermiogenesis.

Notes: A, early spermatid showing many small proacrosomal vesicles scattered in the cytoplasm. B, early spermatid depicting enlarged proacrosomal vesicles at the cell periphery. C, Mid-spermatid showing only one large acrosomal vesicle at the opposite pole to the nucleus. The nucleus shows many heterochromatin blocks (arrow). D, Detail of mid-spermatid showing the loose chromatin in the nucleus (arrow) and many small vesicles in the cytoplasm. E, Late spermatid with the acrosomal vesicle occupying one pole and the cytoplasm concentrated in the opposite pole. F, Detail of late spermatid showing the acrosomal vesicle assuming the acrosomal cap and presumptive electron-dense spike base (black arrow). Notice the subacrosomal region is not formed (white arrow). av = acrosomal vesicle; es = early spermatid; go = spermatogonia; ls = late spermatid; ms = mid-spermatid; n = nucleus; pv = proacrosomal vesicles; sc = Sertoli cells.

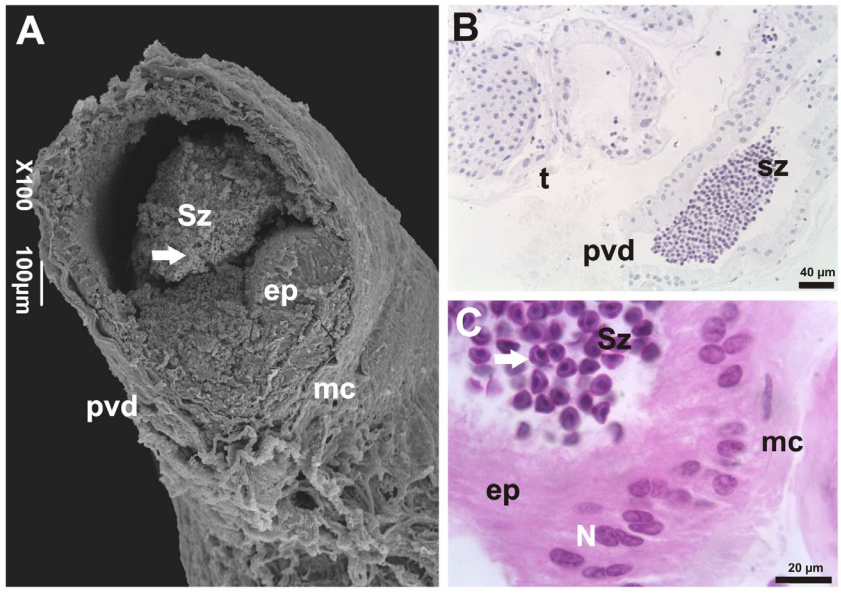

Figure 5. *Litopenaeus schmitti* proximal vas deferens under SEM and light microscopy, stained with Hematoxylin-Eosin.
Notes: A, Sperm mass within the proximal vas deferens (arrow). B, Proximal vas deferens with a sperm mass in the lumen. C, The spike-less spermatozoa (arrow). ep = epithelium; mc = muscle; n = nucleus; pvd = proximal vas deferens; sz = spermatozoa; t = testis.

secretion discontinuously (Figure 7 (B)). The DVD runs laterally and enters into an enlarged portion of the ejaculatory duct, the bell-shaped terminal ampulla (also known as the ejaculatory duct; Bauer 1991) (Figure 7 (C), (D)).

The ampulla is a highly modified region of the vas deferens, externally lined by a wide layer of muscular tissue (Mc) (Figure 7 (C)–(E)). In longitudinal and transverse sections, it is possible to recognize three chambers. Two are continuations from the sperm and accessory ducts of the vas deferens isolated by the two typhlosoles that are similar as already described histologically (Figure 7 (C)–(E)). The sperm duct has a stratified high epithelium and the spermatozoa are surrounded by acidophilic type I secretion, which is looser and homogeneous. Another acidophilic secretion is found externally to the previous and to consist of a more compact secretion forming a rigid structure (Figure 7 (E)). The glandular chamber (Gl) (Figure 7 (C)–(F)) shows the outer portion of the ampulla at the opposite side from the sperm duct, with the accessory duct in between (Figure 7 C, D). The Gl is a simple columnar epithelium producing acidophilic secretion, confirmed by a negative reaction to toluidine blue (Figure 7 (D)–(G)). In longitudinal sections, the Gl seems to be releasing secretion into the accessory duct that merges in the sperm ducts just before the ampulla terminates in the male gonopore, located on the coxa of the fifth pereiopod (Figure 7 (C)).

Spermatozoon ultrastructure

The morphologically mature spermatozoa of *L. schmitti* are found from the MVD, dispersed among seminal secretions of the sperm duct, similar to other species of Peneoidea (Figure 8 (A)). In longitudinal section, the length of the spermatozoon is 7.3 ± 1 µm, and the width of the main body measures 4.1 ± 0.5 µm giving a length: width ratio of 1.8 ($N = 6$). The main body is rounded, containing the nucleus and a thin cytoplasmic layer, whereas the acrosomal cap projects forward, forming the spike. Both acrosomal cap and spike form the acrosomal vesicle (Figure 8 (B)). The spike structure is rather smooth and internally dense, composed by a bundle of parallel microtubules clear to those observed in transverse sections (Figure 8 (C), (D)). In longitudinal sections, the spike base, just before the acrosomal cap in the main body, has a conspicuous swelling with two electron-dense spots on both sides (Figure 8 (B)–(F)). From the swelling base of the spike, the acrosomal cap spreads backward, forming curved lateral expansions covering a large area on both sides of the main body (Figure 8 (B)–(F)). The lateral expansions of the acrosomal cap are less electron-dense than the swelling base of the spike and show a striated arrangement (Figure 8 (E), (F)). The lateral expansions are a thin electron-dense inner layer that runs from the external side of the swelling base of the spike to the posterior end of the acrosomal vesicle (Figure 8 (E)–(I)). Beneath the swelling base of the spike is a concave subacrosomal space with a centrosome-like (filamentous meshwork) round structure with filaments attached to the acrosomal cap (Figure 8 (E)–(G)). In transverse sections, the acrosomal cap is a circular band around the nucleus, which occupies most of the volume of the main body (Figure 8 (B), (H)). The nucleus is centrally located and is filled with filamentous chromatin and several dense granules or clumps are observed in the nucleoplasm (Figure 8 (B), (E), (H)–(J)). An electron-lucent area appears to separate the

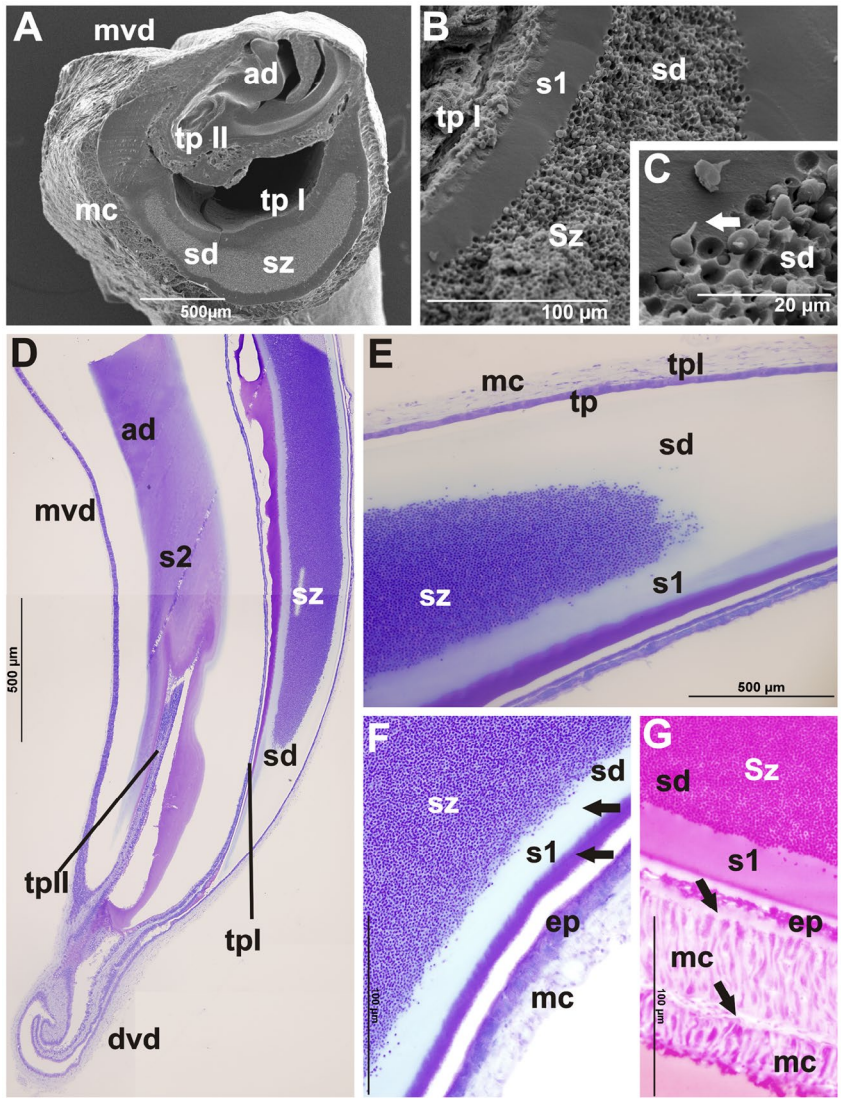

Figure 6. *Litopenaeus schmitti*. SEM (A–B) and light microscopy of median vas deferens stained with toluidine blue (pH 4.0) (D–E) and Hematoxylin-Eosin (F).

Notes: A, Cross section of the median vas deferens. B, Sperm duct filled with spermatozoa. C, Detail of the mature and spiked sperm surrounded by abundant secretion. D, Longitudinal section of the median vas deferens. The sperm and accessory ducts separated by typhlosoles I and II. E, The sperm duct is filled with secretion surrounding sperm cells. F, Detail of sperm duct exhibiting secretion type 1 metachromasia γ (arrow) and the internal matrix show variable metachromatic β at the toluidine blue stain (arrow). G, Detail of the muscular layer of the mvd and connective tissue (arrows). ad = accessory duct; dvd = distal vas deferens; ep = epithelium; mc = muscle; mvd = median vas deferens; s1 = secretion type 1; s2 = secretion type 2; sd = sperm duct; sz = spermatozoa; tp I = typhlosole I; tp II = typhlosole II.

subacrosomal space and the chromatin (Figure 8 (E), (F)). The nuclear envelope is disrupted, and composed of only one membrane unit (Figure 8 (I)). The cytoplasm is much reduced, located at the posterior part of the spermatozoon and its surface appears slightly rough (Figure 8 (B), (I)). The cytoplasm shows many membranous lamellae and mitochondria-like organelles with variable volumes between spermatozoa (Figure 8 (I), (J)).

Discussion

The white shrimp *L. schmitti* has a complex male reproductive system, as do other open thelyca penaeoidean shrimps. The male system has received less attention than the female system from morphological, endocrinological and functional points of view (Browdy 1992; Alfaro-Montoya 2010). The macroscopical features of the male reproductive system of *L. schmitti* follow the general pattern described for penaeoidean shrimps without variation among the tribes (Tuma 1967; Chow et al. 1991; Bauer & Min 1993; Alfaro-Montoya 2010).

The external morphology of the testis varies among the different groups of decapods (Díaz et al. 2002). In penaeoideans, including *L. schmitti*, the testes are composed of several separated lobules, which are connected to the vas deferens by means of a short and narrow

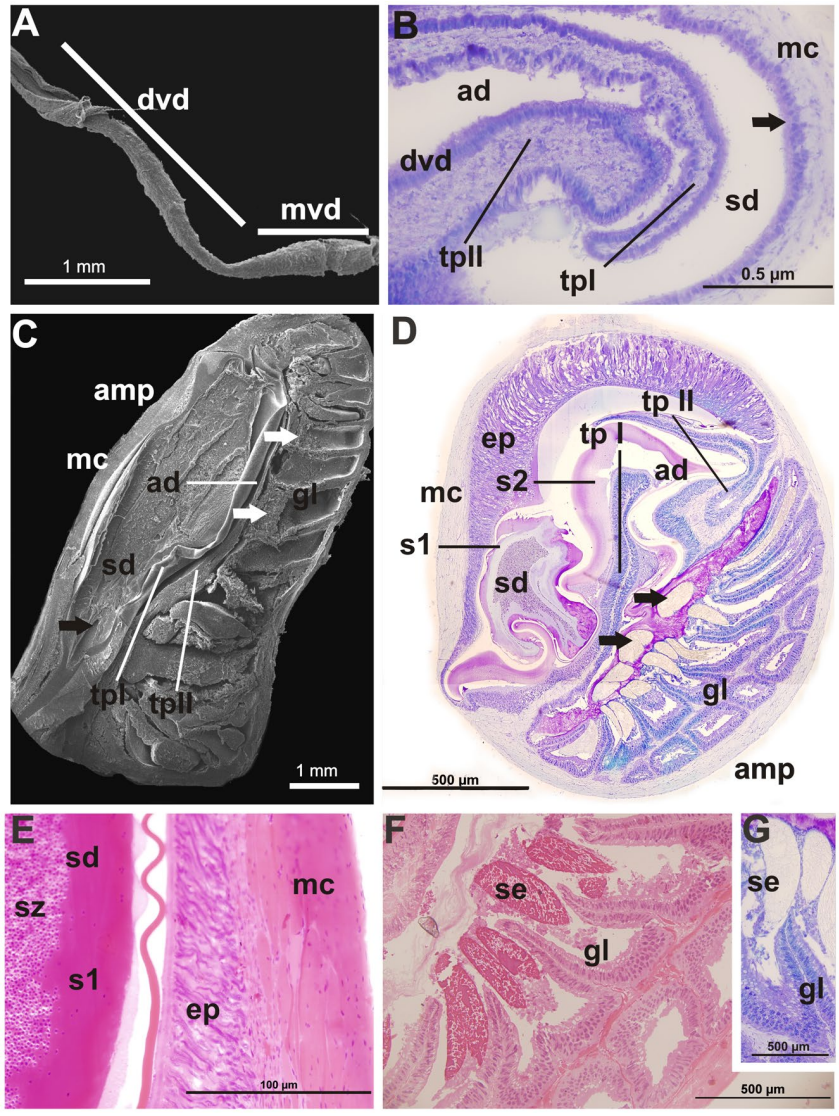

Figure 7. *Litopenaeus schmitti*. SEM of median and distal vas deferens (A–C) and light microscopy of distal vas deferens and terminal ampoule. B, D, G, toluidine blue (pH 4.0) stain; E, F, Hematoxylin-Eosin stain.

Notes: A, General view of the median and distal vas deferens. B, The distal vas deferens, luminal secretion (arrow). C, Longitudinal section of the terminal ampoule exhibiting the gland secretion (arrows). D, Longitudinal section of the terminal ampoule and binder secretion (arrows). E, Detail of the wall of the spermatophore and epithelium. F, The adhesive secretion being expelled from the glandular chamber. G, Detail of secretion and glandular chamber. ad = accessory duct; amp = terminal ampoule; dvd = distal vas deferens; ep = epithelium; gl = glandular chamber; mc = muscle; mvd = median vas deferens; s1 = secretion type 1; s2 = secretion type 2; sd = spermatophore duct; se = seminal fluid; sz = spermatozoa; tp I = typhlosole I; tp II = typhlosole II.

tubule (Ro et al. 1990). Histologically, each testis lobe is constituted by seminiferous tubules, which contain one spermatogenesis stage at the most proximal region and another distally. The adjacent lobules also show cells at the same stage of cellular development. This description characterizes the restricted type of testis (Grier 1993). This pattern was observed for *L. stylirostris* by Bell and Lightner (1988), and also in the caridean shrimp *Macrobrachium rosenbergii* by Okumura and Hara (2004), and the lobster *Nephrops norvergicus* by Rotllant et al. (2012). In Sicyonidae shrimps, each lobule has cells in different stages of spermatogenesis (Shigekawa & Clark 1986), similar to that which also occurs in the Parastacidae *Cherax quadricarinatus* (López-Greco et al. 2007).

There is a range of complexity in Dendrobranchiata reproductive traits that allows some trends to be delineated, as reviewed and explained by Bauer (1986). According to this author, in Aristeidae, Solenoceridae, and species of *Litopenaeus*, the male produces a complex spermatophore with a sperm core surrounded by various sperm-free substances produced into processes and appendages (Pérez-Farfante 1969, 1975, 1976). Each half of the final compound spermatophore is produced within the male vas deferens, and joined to the other half essentially unchanged when attached to the female. Males of these

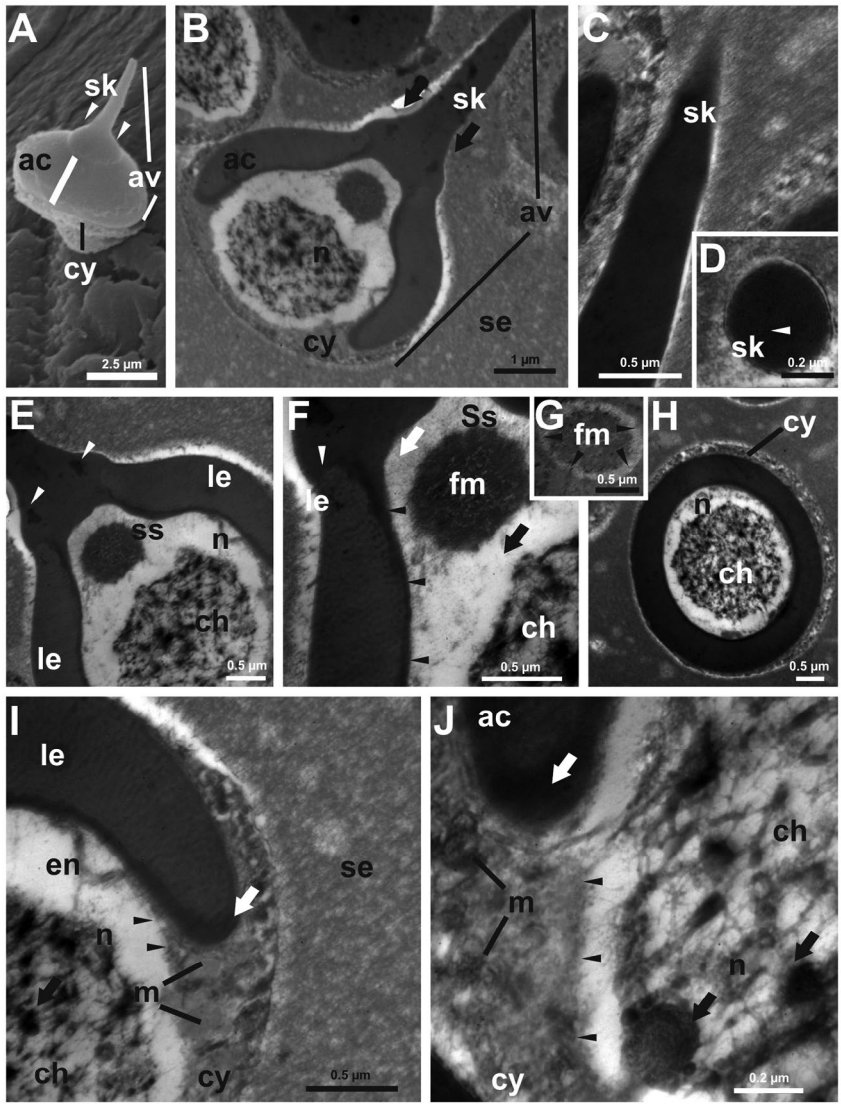

Figure 8. *Litopenaeus schmitti* electron microscopy of spermatozoa.

Notes: A, SEM of a sperm cell in the median vas deferens. Notice the developed spike and the acrosomal cap forming the acrosomal vesicle, covering the cytoplasm. The spike base shows a swelling in its base (arrows heads). B, Longitudinal section of the spermatozoon under TEM showing the acrosomal vesicle formed by the spike and acrosomal cap on the round main body. The spike shows a swelling at it base (arrows). C and D, Spike in longitudinal (C) and transverse (D) sections, showing the bundle of filaments with parallel arrangement (arrow head). E, Swelling at base of the spike showing two electron-dense dots (arrowheads) and less electron-dense lateral element symmetrically arranged on both sides of the sperm. F, Transition between swelling base of the spike and lateral element of the acrosomal cap. Notice the thin electron-dense inner layer that runs posteriorly (arrowheads) beneath the striated lateral element. The subacrosomal concave space shows a centrosome-like structure with filaments attached to the acrosomal cap (white arrow). An electron-lucent area separates the subacrosomal space and the nuclear chromatin (black arrow). G, Transverse section of the filamentous meshwork and its filaments (arrows head). H, Lateral element of the acrosome cap in transverse section forming a circular band around the nucleus with centrally positioned chromatin. I and J, Inner electron-dense layer reaching the apex posterior of the acrosomal vesicle (White arrow). The nuclear envelope is visible although it is disrupted (arrowheads) and there are several dense granules among the filamentous chromatin. The cytoplasm is filled with mitochondria-like organelles and derivatives membranes and its surface is slightly rough (black arrow). ac = acrosomal cap; av = acrosomal vesicle; fm = filamentous meshwork; ch = chromatin; cy = cytoplasm; en= nuclear envelope; le = lateral element of acrosomal cap; m = mitochondria; n = nucleus; sk = spike; se = seminal fluid; ss = subacrosomal space.

species have an open petasma (Burkenroad 1934, 1936) into which the compound spermatophore could conceivably fit during passage to the female body. Females have an open thelycum with coxal and sternal protuberances and depressions on the posterior thoracic segments. The complex spermatophore attaches to and fits within the open thelycum.

There are remarkable variations in the external and internal morphology of penaeoidean shrimps associated with insemination, i.e. sperm transference to females and its temporary or long-term storage until fertilization (Bauer & Cash 1991; Braga et al. 2013a). The open thelycum shrimp species, particularly in the genus *Litopenaeus*, have a male reproductive system that is much more complex than those of closed thelycum species. In *L. schmitti*, both the vas deferens and the ejaculatory duct have incomplete tissue folds, which keep their contents isolated in ducts. Such folds are named typhlosoles which allow the sperm

conduction and the non-cellular material to remain isolated throughout of the vas deferens until the ampulla at the ejaculatory duct. Thus, the existence of typhlosoles allows for the formation of a more complex spermatophore than in other shrimp species and seems to be a pattern to other *Litopenaeus* species (Bauer & Cash 1991; Chow et al. 1991; Ceballos-Vázquez et al. 2010).

The vas deferens of *L. schmitti* has a curve duct shape, very dilated in the median region that conducts the sperm from testis lobules to the ejaculatory duct and the gonopore. The white shrimp spermatozoa move through the vas deferens and a homogeneous non-cellular matrix different from that found in the accessory duct always surrounds these cells. According to Alfaro (1994), the maturation of the spermatozoa begins in the vas deferens, finishing only in the ampulla or even in the female thelycum after insemination (Díaz et al. 2002; Alfaro et al. 2007; Alfaro-Montoya 2010). In *L. schmitti*, the spermatozoa in the testes and PVD are not structurally mature, becoming spiked only in the MVD. This maturation event is distinct from that reported for *Parapenaeus longitostris* spermiogenesis, whose spike development is noticed in late spermatids inside the testis (Medina 1994). Similar morphological changes found in *L. schmitti* were reported for the spermatozoa of the congener *L. stylirostris* (described as *Penaeus stylirotris* by Alfaro 1994). However, this author uses the term spermatids to define these cells. In this paper, we used the term immature sperm cell since the accessory or Sertoli celsl have already been used to complete spermiogenesis and the secretions found in the MVD seems to promote spike formation. Therefore, both TEM and histochemical characterization of the secretions needs further investigation to clarify how the centrosome-like structure (filamentous meshwork) participates in these morphological changes in the spermatozoa of *Litopenaeus*.

At the terminal ampulla of *L. schmitti* a complex of ducts that show several adhesive glands open into the sperm duct. The adhesive glands are the most distinctive characteristic of the ampulla, and follow the pattern observed in other open thelycum species as *L. setiferus* and *L. vannamei* (Ro et al. 1990; Bauer & Cash 1991; Chow et al. 1991). Thus, as already postulated for other *Litopenaeus*, the ampulla in *L. schmitii* produce the main compound of the spermatophore surrounding the mass of spermatozoa from the vas deferens, and this adhesive substance plays the role of attaching the spermatophore at the female open thelycum. Concerning to the chemical nature of the substances found in the male reproductive system of decapods, Subramoniam (1991) provided a review on the chemical composition of their spermatophores, trying to correlate the functional significance with sperm storage, the adhesion of the spermatophore and its dehiscence. The histochemical properties of the spermatophores of decapods indicate the preponderance of glycosaminoglycans (GAGs), which are rich in acid and neutral polysaccharides. For some penaeoideans, already studied by the same author [*Fenneropenaeus indicus* and *Metapenaeus monodon*], the isolation of the GAGs by gel electrophoresis revealed two corresponding fractions, i.e. chondroitin sulfate and hyaluronic acid, both showing antimicrobial activities. The occurrence of a great quantity of GAGs (as mucopolysaccharides) in the spermatophores can preserve viable spermatozoa in a fluid medium until fertilization (Subramoniam 1991). Owing to the great structural complexity and the distinct types of matrix involving the spermatozoa of *L. schmitti* throughout the male reproductive system, a detailed study at the histochemical level is needed to clarify the nature and the function of the main components of each kind of matrix in the male reproductive system.

Spermatophores have evolved independently in several aquatic groups, both invertebrate taxa and some fishes (Proctor 1998). Many authors have considered fertilization via spermatophores a pre requisite to terrestrial invasion by the arthropods (Schaller 1979; Clark 1981; Proctor 1998). Penaeoidean shrimps show variable levels of complexity in the spermatophores which are deposited in the female's thelycum (Bauer 1991; Subramoniam 1995). Penaeoidean females with open thelyca differ from those with closed thelyca in relation to the number and shape of spermatophores. Some shrimps with closed thelyca show a great number of simple spermatophores, without complex addition structures (Shigekawa & Clark 1986; Bauer & Min 1993) while males of other closed thelyca species deposit two large but relatively simple spermatophores (e.g. *Farfantepenaeus* spp., Bauer & Cash 1991). Males of penaeoidean species with open thelyca females, such as *L. vannamei*, *L. setiferus*, and *P. muelleri*, produce two complex spermatophores, which can bear 'wings', as well as other accessories structures to fix them into the thelycum until fertilization (Pérez-Farfante 1975; Ro et al. 1990; Bauer 1991; Bauer & Cash 1991; Chow et al. 1991; Díaz et al. 2002; Alfaro-Montoya 2010).

According to Pérez-Farfante (1975), the genus *Litopenaeus* is worldwide represented by five species: *Litopenaeus occidentalis*, *L. schmitti*, *L. setiferus*, *L. stylirostris*, and *L. vannamei*, all of which have open thelyca. Those females bearing open thelyca (Pérez-Farfante 1969) have no seminal receptacles; instead, they show protuberances, projections, concavities or grooves and sometimes lamellae in the posterior cephalothoracic sternites (from XII to XIV), in which the spermatophores remain attached. The spermatophore is, in this way, exposed to the surrounding water and can be dislodged during fishery activities, as suggested by Burkenroad (1939). Nevertheless, the penaeoidean spermatophores usually show more complex

formation and morphology than carideans (Bauer 1991, 2004; Bauer & Min 1993), as the former pass through a capacitation process before fecundation (Alfaro et al. 2007; Aungsuchawan et al. 2011).

Considering the information above, immediately after copulation, the shrimp females of open thelyca species present a spermatic mass within a spermatophore, adhered to the ventral region of the thoracic sternite. However, it was not observed in the material studied here. Pérez-Farfante (1975) reported on the spermatophore of some species of the genus *Litopenaeus* and asserted that the lack of information on such structures is due, at least in part, to the fact that impregnated females are not readily found in nature (Weymouth et al. 1933; Burkenroad 1939; Heegaard 1953). Bueno (1990) verified that spermatophores remain adhered to the females' thelycum for a short time after mating, and are probably eaten by females after fecundation, as he did not find any spermatophores in the rearing tanks after mating and removal of the males. Nevertheless, such behavior may not occur in the natural environment and further studies should be undertaken to confirm this.

The Dendrobranchiata shows a very diverse spermatozoon ultrastructure. In general, the spermatozoon is a highly polarized cell with three distinct anatomic portions: the main body, the acrosomal cap, and acrosomal spike, the latter two found in a membrane as the acrosomal vesicle (Medina 1995; Medina et al., 2005). The SEM analysis showed that *L. schmitti* has a spermatozoon similar to other shrimp species of the families Penaeidae, Sicyoniidae, and Solenoceridae, which are characterized by a unistellate spermatozoon showing a conspicuous spike projected forward the acrosomal cap (Medina et al. 2005, 2006; Braga et al. 2013b). The *L. schmitti* spermatozoa were different from the Penaeidae *Rimapenaeus* (as *Trachypenaeus*) *similis* by the absence of a filament on the opposite side of the spike (Bauer & Min 1993) and posterior projections found in the SEM images of *Xiphopenaeus kroyeri*, *X. riveti*, and *Rimapenaeus* (as *Trachypenaeus*) *byrdi* (Alfaro et al. 2003; Alfaro-Montoya & Hernández 2012). However, a specific SEM feature found in *L. schmitti* spermatozoa is a conspicuous swelling at the spike base, which is absent in *Solenocera* (Medina et al., 2005) and in the Penaeidae *Farfantepenaeus paulensis* (Braga et al. 2013a). *Sicyonia ingentis* showed a slightly swelling at the spike base forming a rough surface, which is associated to the very complex acrosomal region (Kleve et al. 1980). In addition, the Solenoceridae *P. mulleri* also showed this swelling at the spike base, forming a smoother surface, although this morphological character is less conspicuous than in *L. schmitti*. Despite the presence in other families, the conspicuous enlargement of the spike base seems to be a character shared with some other species of *Litopenaeus* such as *L. occidentalis* (Alfaro et al. 2003) and *L. vannamei* (Aungsuchawan et al. 2011). In contrast, this region was not observed in TEM images of the sperm obtained from the spermatophore of *L. vannamei* and *L. stylirostris* (Alfaro et al. 2007). In the present study, the spermatozoa of *L. schmitti* obtained from MVD had swelling in both TEM and SEM images, indicating that this structure is not an artifact and occurs in different families of Dendrobranchiata.

Despite the SEM morphology, sperm ultrastructure of *L. schmitti* showed significant features compared to other Solenoceridae and Penaeidae sperm. The acrosomal vesicle is symmetrical and without separation of the plasma and acrosomal membranes at the spike base as already postulated for Solenoceridae (Medina et al., 2005). The acrosomal cap covering a large area above the main body differed from other Penaeidae such as *Marsupenaeus* (as *Penaeus*) *japonicus* and *F. paulensis* (Medina 1995; Braga et al. 2013a) and seems to be character of *Litopenaeus* (see Alfaro et al. 2007; Aungsuchawan et al. 2011). Two significant characters of *L. schmitti* are also found in the congeneric species: (1) the two electron-dense spots at the spike base; and (2) the concave-large subacrosomal space flied with a conspicuous filamentous meshwork or centrosome-like structure (Alfaro et al. 2003, 2007; Aungsuchawan et al. 2011). Both structures seem to be important during sperm capacitation and the acrosome reaction (Alfaro et al. 2007; Aungsuchawan et al. 2011). However, the significant features that distinguish the spermatozoa among the studied species of *Litopenaeus* is the filamentous meshwork in the subacrosomal space, which is, for instance, round and centrally concentrated in *L. schmitti* (see Alfaro et al. 2007; Aungsuchawan et al. 2011 for comparison) and could play a species-specific role during the acrosome reaction.

Research using TEM should identify possible differences at the cellular level among the spermatozoa of *L. schmitti* and other penaeoidean species. The existing literature on sperm ultrastructure shows that this kind of study is very useful in the elucidation of functional, taxonomic, and/or phylogenetic problems. Nevertheless, the great variety of shapes found among Dendrobranchiata spermatozoa (Medina et al. 2006) may indicate that several modifications originated throughout the evolution of this group that made comparisons much more complex. An example is the case of Solenoceridae (*Solenocera membranosa*, *S. africana*, and *P. muelleri)*, which shows considerable similarity (Medina et al. 2006) and supports the affinity between Penaeidae and Solenoceridae. Nevertheless, some ultrastructural sperm characters are variable and others are dubious in the penaeid group; the sperm ultrastructure (spermiotaxonomy) of *Farfantepenaeus*, *Rimapenaeus*, *Litopenaeus*, and *Xiphopenaeus* genera, using TEM analyses, is required to elucidate the function and evolution of these sperm characters in this family.

There is a consensus of the male reproductive quality on the productivity of the stock of penaoeideans from captivity (Díaz et al. 2002). Reproductive males of *L. schmitti* from nature are capable of fertilizing females and producing a higher quantity of nauplii than captive males (Ramos et al. 1995). Bueno (1990), studying maturation and spawning of *L. schmitti* under captive conditions, found a high rate of mating and fertilization from shrimps sampled in nature, without the necessity for artificial insemination. In this sense, this paper provides basic knowledge on the male reproductive traits of *L. schmitti* at an anatomical level by light microscopy and ultrastructural morphology (TEM and SEM) using shrimps directly obtained from the field.

Acknowledgments

Authors are also grateful to Dr Rafael A. Gregati for his help during the shrimp dissections and TEM routines. This work was carried out with support of CME (Electronic Microscopy Center, Biosciences Institute, UNESP, Botucatu, São Paulo) and IML (Invertebrate Morphology Laboratory, FCAV, UNESP, Jaboticabal, São Paulo). All shrimps were sampled following the Brazilian laws (FJZ permanent license #34587-1 from MMA/ICMBio/SISBIO).

Funding

This work was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) under doctoral fellowship to VF and grant CIMAR II [grant number #2014/1989] to FJZ; by São Paulo Research Foundation grants FAPESP [grant number #94/4878-8; #95/8520-3; #98/3134-6 and Biota#2010/50188-8].

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