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Ultrastructural demonstration of the model of *Litopenaeus vannamei* (Crustacea, Penaeidae) male sexual maturation and spermatozoal capacitation

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ABSTRACT

The present study demonstrates ultrastructurally the model of *Litopenaeus vannamei* male sexual maturation and spermatozoal capacitation. The results show that phase 1 of the model occurred in the seminiferous tubules and includes spermatogenesis. In this phase, throughout differentiation of spermatogonia into late spermatids the following processes were observed: (1) decondensation of chromatin; (2) rupture of the nuclear envelope; (3) reduction of the cytoplasm and degeneration of organelles; (4) formation of the acrosome via fusion of cytoplasmic vesicles. Phase 2 comprised of spermatozoal maturation, a process that started with the transfer of late spermatids into the seminiferous ducts and ended with the formation of the acrosomal spike in the terminal ampoules. During this phase, development of the subacrosomal region and lateral electron-dense particles occurred in the seminiferous ducts, which is a novel finding of this species. Phase 3 was observed after spermatophore placement on the female thelycum and was mainly characterized by ultrastructural changes in the nucleus and the subacrosomal region. These results are in agreement with the model of male sexual maturation and spermatozoal capacitation proposed for *L. vannamei*.

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Introduction

Morphological studies on *Litopenaeus vannamei* (Boone, 1931), the most important aquaculture shrimp species worldwide, have been reported frequently to improve knowledge of its biology and develop techniques that could enhance its performance in captivity (Oliveira Cesar et al. 2006; Suita et al. 2015). Studies focused on morphology of reproductive structures and processes have been also published (Diamond et al. 2008; Ceballos-Vázquez et al. 2010; Alfaro-Montoya & Hernández 2012). From these studies *L. vannamei* was shown to have a unistellate spermatozoon, which has a main body and a spike extending from the acrosome (Alfaro et al. 2007; Braga et al. 2013).

Alfaro-Montoya (2010) proposed a model to explain male sexual maturation and spermatozoal capacitation in *Litopenaeus*, in which ionic, biochemical and morphological changes undergone by spermatozoa after a period of spermatophore placement on the female thelycum, equip the cells with fertilization capacity (Braga et al. 2013). The model includes three phases: (1) spermatogenesis in the testes, where spermatogonia are differentiated into spermatids via meiotic divisions; (2) spermatozoal maturation,

which starts when the late spermatids are transferred to the vas deferens and ends with formation of the acrosomal spike; (3) spermatozoal capacitation after spermatophore placement (Alfaro-Montoya 2010).

Some of the reproductive processes of the model have been used to develop techniques in aquaculture. For example, there are efforts to improve economic gains in shrimp farming via controlling sexual differentiation owing to higher growth rates in female penaeids (Pérez-Rostro & Ibarra 2003; Moss & Moss 2006). As part of this effort, Alfaro-Montoya et al. (2016) evaluated androgenic gland ablation as an alternative technology for monosex female culture. No sex reversal was observed, but it was concluded that spermatozoal maturation was stopped based on the phases of the model because only early spermatids were observed in seminiferous tubules and ducts of andrectomized males.

The mechanisms of spermatozoal capacitation are poorly understood, preventing *in vitro* reproduction of shrimp in laboratories. Some authors have demonstrated low *in vitro* fertilization rates using spermatophores collected from the terminal ampoules (Bray & Lawrence 1992; Alfaro et al. 1993). The main reason for

low *in vitro* fertilization rates is that mature spermatozoa are not equipped with fertilization capacity if the spermatophore was not previously attached to the thelycum (Wikramanayake et al. 1992; Alfaro et al. 2003, 2007). Therefore, understanding the mechanisms of capacitation could be useful to the development of biotechnologies that would allow spermatozoal induction without spermatophore placement on the thelycum (Braga et al. 2014).

Despite the relevance of the reproductive processes involved in the model for aquaculture research, the ultrastructure of the model for *L. vannamei* has not been demonstrated. These processes have been only reported fragmentarily. Xu et al. (2010) described the ultrastructure of spermatogenesis and histological observations were briefly reported by Muhammad and Shafi (2013) and Alfaro-Montoya (2013). The ultrastructure of spermatozoal capacitation has been reported separately (Alfaro et al. 2007; Aungsuchawan et al. 2011). Therefore, this study aimed to demonstrate ultrastructurally the model of *L. vannamei* male sexual maturation and spermatozoal capacitation.

Material and methods

Animals

L. vannamei juveniles were haphazardly selected from commercial semi-intensive culture in earthen ponds from Golfo de Nicoya, Costa Rica, and transferred to Estación de Biología Marina (EBM), Universidad Nacional, for further growth. Adult males and females were then transferred to the maturation laboratory of EBM under conventional controlled conditions for reproduction as described previously (Alfaro-Montoya 2013).

Tissue sampling

For transmission electron microscopy, male sexual organs were dissected under cold anesthetization from six cultured young specimens of *L. vannamei* (body weight (b.w.) = 9–12 g). Additionally, spermatophores from two adult males (b.w. = 30 g) were collected by manual ejaculation and two inseminated *L. vannamei* females (b.w. = 37 g) from maturation tanks were isolated in spawning tanks for 4–5 h before removing the attached sperm mass.

Regions of sexual organs and compact spermatophores were fixed whole. Sperm masses were expelled from spermatophores collected from female thelyca before fixation. Tissues were fixed with a solution of paraformaldehyde (2.0%), glutaraldehyde (2.5%), and sucrose (5.0%) in 0.1 M sodium cacodylate buffer at pH 7.4, according to Ro et al. (1990). The samples were transferred to the Centro de Investigación en Estructuras Microscópicas (CIEMIC), University of Costa Rica, where microscopy was conducted

separately for each collected region of the male reproductive system and sperm mass.

Tissue ultrastructure

While in fixative, samples were observed under light microscopy to identify sperm masses and eliminate unnecessary material. After three washes in fresh 0.1 M cacodylate buffer, samples were postfixed in 1% OsO₄ in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 2 h. Samples were then rinsed with 0.1 M cacodylate buffer and distilled water several times, dehydrated in an ascending series of ethanol, then embedded, via propylene oxide, in Spurr's resin. Ultrathin sections (40–60 nm thickness) were cut with an ultramicrotome using a diamond knife, picked up on copper grids, stained sequentially with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Hitachi H-7700) at 100 kV. As a reference, histological sections of testes stained with hematoxylin and eosin were also processed as described previously (Alfaro-Montoya et al. 2016).

Results

Phase 1: spermatogenesis in testes

Testicular lobules are composed of convoluted seminiferous tubules where different stages of spermatogenesis are present at the same time (Figure 1). When seen in cross-section, each seminiferous tubule has three regions: a germinal region with spermatogonia located at the periphery of the tubules, a large and central spermatogenic center with spermatocytes and spermatids, and a layer of elongate nurse cells, surrounding the spermatogenic center (Figure 1(A)). The phases of spermatogenesis are mostly synchronized in the spermatogenic center within seminiferous tubules, but different phases are developing in adjacent tubules (Figure 1(B)). The external layer of seminiferous tubules is formed by connective tissue (Figure 1(C)). Seminiferous ducts for collecting immature spermatozoa can be seen within seminiferous tubules.

The spermatogonium is a large oval-shaped cell with a large nucleus (mean $\pm$ S.D. nuclear diameter, n.d., = $7.4 \pm 0.3 \mu\text{m}$), containing a nucleolus. The cytoplasm has mitochondria and small electron-dense bodies at the periphery of the nuclear surface (Figure 1(C)).

Primary spermatocytes are spherical cells with a nucleus mostly composed of uncondensed chromatin (Figure 1(D)–(F)). The nucleus is similar in size (n.d. = $7.9 \pm 1.3 \mu\text{m}$) to those of spermatogonia. Mitochondria and ribosomes are abundant in the cytoplasm, which has its volume mainly occupied by rough endoplasmic reticulum, proliferating parallel to the nuclear envelope (Figure 1(D) and (F)). A

Figure 1. Light microscopy of *Litopenaeus vannamei* seminiferous tubules (A, B) and ultrastructure of spermatogonia (C), primary (D–E) and secondary spermatocytes (F) contained in testes.

Notes: ct: connective tissue; cy: cytoplasm; cv: cytoplasmic vesicles; esd: early spermatid; eb: electron-dense bodies; pg: electron-dense vesicle; gc: germinal region; ln: layer of nurse cells; m: mitochondria; nu: nucleoli; n: nucleus; nc: nurse cell; sy1: primary spermatocyte; rb: ribosomes; rr: rough endoplasmic reticulum; sy2: secondary spermatocyte; sc: spermatogenic region; sg: spermatogonia. Scale bars = A, B: 20 μ m; C, D, F: 2 μ m; E: 800 nm.

membrane-bound electron-dense vesicle of eosinophilic nature under light microscopy is embedded in the endoplasmic reticulum vesicles and located near to the nuclear region (Figure 1(B), (D)–(F)). Secondary spermatocytes develop after the first meiotic division, generating a smaller nucleus composed of highly condensed

chromatin and limited by the envelope (Figure 1(F); n.d. = $3.4 \pm 0.2 \mu$ m). The volume of the cytoplasmic region, the amount of rough endoplasmic reticulum, and number of mitochondria are reduced, resulting in the presence of vesicles, and the electron-dense vesicle is absent in secondary spermatocytes (Figure 1(F)).

Figure 2. Fine structure of early (A, B), mid- (C, D) and late spermatids (E, F) in *L. vannamei* testes.

Notes: av: acrosomal vesicle; ag: anterior granule; ap: apical protuberance; cac: central acrosomal cap; cb: cytoplasmic band; esd: early spermatid; el: electron-dense inner layer; ga: Golgi-like body; lsd: late spermatid; lac: lateral acrosomal cap; msd: mid-spermatid; m: mitochondria; ne: nuclear envelope; n: nucleus; nc: nurse cell; pv: proacrosomal vesicle; rb: ribosomes; rr: rough endoplasmic reticulum; black arrowhead: electron-denser nuclear region; unfilled arrowhead: electronically less-dense nuclear region; white arrowhead: rupture of the nuclear envelope. Scale bars = A–C, F: 1 μ m; D, E: 2 μ m.

Early spermatids have a spherical nucleus with condensed chromatin and some heterochromatin, producing a granular pattern (Figure 2(A), (B); $n. d. = 4.3 \pm 0.0 \mu$ m). In the cytoplasmic region, strands of rough endoplasmic reticulum with abundant associated ribosomes parallel to the nuclear envelope are present and Golgi-like bodies and mitochondria are occasionally observed. Abundant

electron-lucent proacrosomal vesicles are characteristic of early spermatids (Figure 2(A) and (B)).

In mid-spermatids, the nucleus is composed of condensed chromatin, although it is possible to observe that decondensation has begun, which results in two lateral regions distinguished by differences in electron density (Figure 2(C)). At this stage, the breakdown of the nuclear

envelope occurs and the nuclear content is mixed with the cytoplasm in some areas (Figure 2(C) and (D)). The cytoplasm is considerably reduced and Golgi-like bodies, rough endoplasmic reticulum, and mitochondria are present, although numerically reduced. The cytoplasmic region surrounds the posterior region of the nucleus and is anteriorly limited by an acrosomal vesicle, which was formed via fusion of the proacrosomal vesicles. An anterior granule is observed within the acrosomal vesicle, which is ultrastructurally a spherical and more electron-dense region (Figure 2(C) and (D)).

The most advanced stage of spermiogenesis in testes was observed in late spermatids, which have a nucleus composed of decondensed chromatin with a flocculent appearance and surrounded posterolaterally by a thin cytoplasmic band. The perinuclear cytoplasm has a granular pattern and is composed of some mitochondria and abundant vesicles. The acrosomal vesicle has undergone significant changes, assuming a slight internal concave shape with an apical protuberance. Also, the acrosomal vesicle includes the acrosomal cap and the anterior granule at this stage. The acrosomal cap is composed of two regions: two lateral acrosomal caps and a central cap. The lateral caps have heterogeneous material with electron-dense granules and have a clear electron-dense inner layer at the border with the nucleus. The central cap is composed of more homogeneous material. The anterior granule is more electron-dense in comparison with mid-spermatids and has migrated to the apical region of late spermatids (Figure 2(E) and (F)).

Phase 2: spermatozoal maturation in the vas deferens

Late spermatids are transferred to seminiferous ducts, where spermatozoal maturation begins. Figure 3 shows transitional stages between late spermatids and early immature spermatozoa in the seminiferous ducts. These cells have a similar ultrastructure to the late spermatids within testicular lobules, but some new structures have begun to develop. These include a subacrosomal region and lateral electron-dense particles of the anterior granule, which is accompanied by anterior granule expansion into the central acrosomal cap (Figure 3).

The maturation of spermatozoa continues in the vas deferens and ends only at the level of terminal ampoules, where spermatophores contain mature spermatozoa. The cells observed in the spermatophore are composed of a spherical main body and a single appendage extending from the acrosome, the spike (Figure 4(A)). Spermatozoa have chromatin in a decondensed state, but with a fibrous pattern made up of a granular network and filaments (Figure 4(B)). The perinuclear cytoplasmic band has not

undergone significant changes. The internal concavity of the acrosomal vesicle is more evident and the vesicle is composed of a subacrosomal region, acrosomal cap, and spike (Figure 4(A)). The subacrosomal region has extended and includes two regions: a filamentous meshwork and a clear zone (Figure 4(B)). The lateral acrosomal caps keep the inner electron-dense layer, whereas the central cap is more electron-dense (Figure 4(B)). The anterior granule is no longer visible and has turned into the spike, keeping the lateral electron-dense particles at the base of the spike (Figure 4(A) and (B)).

Phase 3: spermatozoal capacitation after spermatophore attachment to the thelycum

Spermatozoa removed from female thelyca after attachment have the same ultrastructure as spermatozoa removed from the spermatophore, including a main body composed of a nucleus posterolaterally surrounded by the cytoplasmic band and an acrosomal vesicle expanded anteriorly into a spike (Figure 4(C)). However, some ultrastructural developments were observed 3–4 h after spermatophore attachment, especially in the nucleus and the acrosomal vesicle. In the nucleus, the chromatin is decondensed, but the granular network and filaments are less abundant, resulting in a more electron-translucent pattern (Figure 4(D)). In the acrosomal vesicle, the internal concavity is decreased. The degree of electron density is increased in the acrosomal cap and spike. The filamentous meshwork has proliferated to cover most of the subacrosomal region (Figure 4(C) and (D)).

Discussion

The seminiferous tubules of *L. vannamei* contained only spermatogonia, spermatocytes and spermatids. Spermatozoal maturation began in the seminiferous ducts and ended in the terminal ampoules with the formation of the acrosomal spike. After spermatophore attachment to the thelycum, mature spermatozoa had undergone ultrastructural changes. Therefore, the results reported in this study are in agreement with the model proposed by Alfaro-Montoya (2010).

The model of male sexual maturation and spermatozoal capacitation in *L. vannamei* is in disagreement with previous observations for *Fenneropenaeus chinensis*, *Metapenaeus monoceros*, and *Parapenaeus longirostris* (Medina 1994; Abraham et al. 2007; Kang et al. 2008). Spermatozoa with an acrosomal spike were observed in the seminiferous tubules of these species. By contrast, the most advanced stage of germ cells in the testes of *Litopenaeus setiferus*, *Litopenaeus stylirostris*, *Litopenaeus schmitti*, and *Sicyonia ingentis* was the late spermatid,

Figure 3. Ultrastructure of spermatozoal maturation in *Litopenaeus vannamei* seminiferous ducts.

Notes: av: acrosomal vesicle; ag: anterior granule; cac: central acrosomal cap; cb: cytoplasmic band; ed: electron-dense particles of the anterior granule; el: electron-dense inner layer; gm: germ cells at transitional stages between late spermatids and early immature spermatozoa; lac: lateral acrosomal cap; m: mitochondria; n: nucleus; sr: subacrosomal region. Scale bars = A: 2 μ m; B: 1 μ m; C, D: 400 nm.

which continue their maturation in the vas deferens (King 1948; Shigekawa & Clark Jr. 1986; Alfaro 1994; Fransozo et al. 2016). Thus, the model that consists of spermatogenesis in the testes, spermatozoal maturation in the vas deferens, and spermatozoal capacitation after spermophore attachment is most likely an apomorphic feature for the genus *Litopenaeus*, shared homoplasically with sicyoniids. However, further descriptions for species of Dendrobranchiata are required to test this hypothesis.

Alfaro-Montoya (2013) and Muhammad and Shafi (2013) reported histological descriptions of the morphological organization of the germ cells within the seminiferous tubules of *Litopenaeus*, where phase 1 occurs. Our study has revealed that the cells are organized into three different regions: a germinal region located at the periphery of the tubules and composed of spermatogonia, a central spermatogenic center composed of spermatocytes

and spermatids, and a layer of elongate nurse cells. This zonation of the germ cells within *L. vannamei* seminiferous tubules was not previously reported.

The zonation of germ cells within seminiferous tubules of shrimp-like decapods has been described in the literature. The presence of spermatogonia within a peripheral germinal region and more advanced cellular phases within a spermatogenic region has been observed for other penaeids (Chow et al. 1991; Medina 1994). Likewise, nurse cells have been observed in crustacean seminiferous tubules, providing nourishment and some hormones during spermatogenesis (Hinsch 1969). However, the positioning of the nurse cells in the seminiferous tubules is not related to phylogeny of shrimp-like decapods. The presence of nurse cells in a layer at the tubule edge, as observed in the present study, is congruent with the pattern described by Arsenault et al. (1979) for *Crangon*

Figure 4. *Litopenaeus vannamei* mature (A, B) and capacitated spermatozoa (C, D).

Notes: ac: acrosomal cap; av: acrosomal vesicle; cac: central acrosomal cap; cz: clear zone; cb: cytoplasmic band; ed: electron-dense particles; fm: filamentous meshwork; lac: lateral acrosomal cap; mb: main body; n: nucleus; s: spike; sr: subacrosomal region. Scale bars = A: 2 μ m; B, C: 1 μ m; D: 500 nm.

septemspinosa. However, nurse cells were observed associated with spermatogonia in the germinal region of the caridean *Macrobrachium rosenbergii*, and the penaeids *Penaeus indicus* and *M. monoceros* (Mohamed & Diwan 1993; Abraham et al. 2007; Poljaroen et al. 2010). Thus, further comparative studies are required to improve knowledge of the role of the positioning of nurse cells within seminiferous tubules.

Differentiation of spermatogonia into late spermatids via meiotic divisions was observed inside seminiferous tubules. This can be summarized into the following processes: (1) decondensation of chromatin, which was preceded by condensation from spermatogonia to secondary spermatocytes and, then, gradual decondensation in the more advanced stages of germ cells; (2) rupture of the nuclear envelope in mid-spermatids, which resulted in mixing of nucleoplasm and cytoplasmic contents; (3) reduction of the cytoplasm and degeneration of organelles, which results in an abundant population of vesicles in early spermatids; (4) formation of the acrosome, beginning with fusion of the cytoplasmic vesicles. The proacrosomal vesicle is observed in mid-spermatids and undergoes significant changes in late spermatids. The ultrastructure of phase 1 of the model observed in this study is congruent with the previous report on *L. vannamei* spermatogenesis (Xu et al. 2010).

The ultrastructure of the phase 2 was characterized by development of new structures, such as the subacrosomal region and lateral electron-dense particles of the anterior granule, after transfer of the late spermatids to the seminiferous ducts. The presence of these structures has been reported only in *L. vannamei* mature spermatozoa present in spermatophores collected from the terminal ampoule (Xu et al. 2010; Alfaro-Montoya 2013). Thus, the conclusion that the development of the subacrosomal region and lateral electron-dense particles occurs in the seminiferous ducts is a novel finding reported for this species. These electron-dense particles have been also observed in *L. schmitti* spermatozoa from the median vas deferens (Fransozo et al. 2016). Most of the studies on the ultrastructure of shrimp spermatogenesis available in the literature are restricted to observations from seminiferous tubules and spermatophores and, therefore, more attention to the full male reproductive system is required to determine the ultrastructural changes from seminiferous ducts to terminal ampoule.

At the end of phase 2, spermatozoal maturation is completed and the cells consist of the following sections: a spherical main body composed of a nucleus with a fibrous pattern made up of a granular network and filaments posterolaterally surrounded by a cytoplasmic band; and the spike extending from the acrosome, which also includes the acrosomal cap and the subacrosomal region divided into a filamentous meshwork and a clear zone. In phase 3, the mature spermatozoa undergo changes mainly related to the nucleus (granular network and filaments are less abundant) and subacrosomal region (enlargement of the filamentous meshwork). The ultrastructure of both mature and capacitated spermatozoa is in agreement with previous reports for *L. vannamei* (Alfaro et al. 2007; Aungsuchawan et al. 2011). However, it has also been indicated that spike elongation occurs after transfer to female thelyca (Xu et al. 2010).

The ultrastructural changes undergone by distinct germ cells throughout the phases of *L. vannamei* male sexual maturation and spermatozoal capacitation are most likely related to fertilization. Although the ultrastructure of *L. vannamei* fertilization is unknown, it is widely accepted that this process in dendrobranchiates begins with a visible acrosomal reaction, consisting of biochemical, ionic and morphological changes in the acrosomal complex necessary for spermatozoal incorporation into the egg (Kruevaisayawan et al. 2008; Braga et al. 2013). The penaeid acrosomal reaction is initiated with spike depolymerization via acrosomal vesicle rupture and ends with exocytosis of the cell contents (Braga et al. 2013). The reduction of the cytoplasm and the degeneration of the organelles suggest decreasing metabolism throughout spermatogenesis (Kang et al. 2008). However, as demonstrated in our study,

changes undergone by the cytoplasm are also involved in differentiation of the acrosomal vesicle, which plays a role in egg recognition and attachment via the spike tip (Braga et al. 2013). The development of the subacrosomal region (phase 2) and the subsequent enlargement of the filamentous meshwork (phase 3) are most likely related to exocytosis because it becomes progressively denser and larger throughout the acrosomal reaction, forcing the rupture of the acrosomal vesicle (Alfaro et al. 2003, 2007). Finally, the pattern of chromatin decondensation and absence of the nuclear envelope have been suggested as factors that facilitate exocytosis at the end of the acrosomal reaction (Shigekawa & Clark 1986).

Complementary biochemical evidence (phosphorylation activity) to further support the ultrastructural changes associated with the capacitation of *L. vannamei* spermatozoa were given by Aungsuchawan et al. (2011). Also in *L. occidentalis*, supporting evidence by inducing the acrosome reaction *in vitro* was provided by Alfaro et al. (2007). Additional evidence of capacitation from other *Litopenaeus* species (Franzoso et al. 2016) will contribute to an understanding and control of this limiting *in vitro* fertilization factor.

The present study demonstrated ultrastructurally the model of *L. vannamei* male sexual maturation and spermatozoal capacitation. The model is composed of three phases: (1) spermatogenesis in testes, a process that comprises differentiation of spermatogonia to late spermatids in seminiferous tubules and is characterized by decondensation of the chromatin, rupture of the nuclear envelope, reduction of cytoplasm via degeneration of organelles, and development of the acrosomal vesicle; (2) spermatozoal maturation, which begins when late spermatids are transferred to seminiferous ducts, where the subacrosomal region is developed, and ends only with the formation of the acrosomal spike in the terminal ampoules; (3) spermatozoal capacitation, in which mature spermatozoa undergo significant ultrastructural change mainly related to the nucleus and subacrosomal region after spermatophore attachment to the female thelycum.

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Disclosure statement

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