Animal Reproduction Science 149 (2014) 325–334

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Post-mating morphological changes in the spermatozoon and spermatophore wall of the crayfish *Astacus leptodactylus*: Insight into a non-motile spermatozoon

Hamid Niksirat*, Antonín Kouba, Pavel Kozák

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic

ARTICLE INFO

Article history:
Received 29 November 2013
Received in revised form 5 July 2014
Accepted 11 July 2014
Available online 27 July 2014

Keywords:
Crayfish
Filament/droplet structure
Morphology
Spermatozoon capacitation
Subacrosome zone
Ultrastructure

ABSTRACT

Morphology of the crayfish spermatozoon and of the spermatophore wall during three stages of final maturation including freshly ejaculated, post-mating, and after spermatozoa release was studied and compared. The crayfish spermatophore consists of a sperm mass enveloped by a three layered spermatophore wall. After mating, the thickness of the outer layer of the spermatophore is increased. The matrix in the middle layer of the spermatophore becomes reticulated, and granules inside this layer release their contents. Fibers in the inner layer degrade to small particles. The spermatozoon capsule swells and the space between the capsule and the spermatozoon appears. The area of the plasma membrane is increased by wrinkling of the surface and alteration from a single to a multilayered structure at the anterior part of the acrosome. The density of the subacrosome zone increases in the vicinity of the main body of the acrosome. With the onset of fertilization, the layers of the spermatophore are dissolved by female glair gland secretions. The spermatozoon extracellular capsule, plasma membrane, and membranous lamellae are eliminated, and bundles of filaments are released from anterior part of the acrosome. The subacrosome zone loses electron density and retracts. The electron-dense material of the innermost layer of the acrosome is discharged and, together with acrosome filaments, forms a filament/droplet structure at the anterior part of the spermatozoon. The most important change is observed in the subacrosome zone, which may play a key role in the fertilization. Also, morphological changes of the spermatozoon that occur after release from the capsule, especially formation of the filament/droplet structure, may contribute to the mechanism of egg-spermatozoon binding in the crayfish, representative of animals with non-motile spermatozoa.

1. Introduction

Freshwater crayfish comprise a relatively diverse and large group of both ecologically and commercially important animals currently comprising 3 families, 33 genera, and 640 known species (Crandall and Buhay, 2008). Due to the high market price for crayfish and the high demand there is a considerable interest in crayfish aquaculture (Skurdal and Taugbøl, 2002).

In the crayfish, spermatozoa are packaged into spermatophores (Galeotti et al., 2012) that are transferred from the male to female during mating. Crayfish spermatophores are deposited either on the ventral surface (in Astacidae and Parastacidae) of the female or into the *Annulus*

* Corresponding author. Tel.: +420 720 527 501.
E-mail address: niksirat@frov.jcu.cz (H. Niksirat).

http://dx.doi.org/10.1016/j.anireprosci.2014.07.017

ventralis (in Cambaridae). The spermatophores remain for several days with the female before initiation of ovulation and subsequent fertilization (Vogt, 2002). This is accompanied by morphological changes (Dudenhausen and Talbot, 1983; López-Greco and Lo Nostro, 2008), suggesting that final maturation and capacitation of a decapod spermatozoon takes place in the seminal receptacle of the female (Alfaro et al., 2003, 2007; Aungsuchawan et al., 2011; Vanichviriyakit et al., 2004).

Study of the reproductive biology in the crayfish can help development of new techniques for artificial reproduction in aquaculture. Additionally, the decapod spermatozoon lacks a true flagellum and is non-motile (Jamieson and Tudge, 2000; Tudge, 2009), providing opportunities for comparison with motile spermatozoa.

The present study was designed to investigate the morphological changes of the freshly ejaculated spermatozoon and spermatophore wall of narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) during post-mating stages including storage on the body of the female, and after release of spermatozoa from the spermatophore at the beginning of the fertilization.

2. Materials and methods

Narrow-clawed crayfish (*A. leptodactylus*) were obtained from a flooded gravel pit in South Bohemia, Czech Republic in autumn, transferred to the research facilities and kept in an outdoor pond under natural temperature and photoperiod conditions. Freshly ejaculated spermatophore samples were obtained from 3 males by electrical stimulation (AC250K2D, Diametral, Czech Republic; Jerry, 2001), and fixed immediately after ejaculation. Post-mating samples were obtained during two different stages. In the first post-mating stage, samples were collected manually from the ventral surface of three naturally mated females after one week of mating. Samples of the second post-mating stage were obtained from 3 females after their natural glair secretions and release of spermatozoa from the spermatophore. This stage happened ten days after mating.

Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48 h at 4 °C, washed in buffer, and post-fixed in 4% osmium tetroxide for 2 h, washed in buffer, dehydrated through an acetone series (30, 50, 70, 90, 95, and 100% for 15 min each), and embedded in resin (EPON). A series of ultra-thin sections were cut using an UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on the copper grids, double-stained with uranyl acetate and lead citrate, and examined with a 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV.

Samples for scanning electron microscopy (SEM) were processed in the same manner as for TEM procedure from beginning to the dehydration step. Then, samples were dried with a Pelco CPD 2 critical point dryer (Ted Pella, Inc., Redding, CA, USA), coated with gold under vacuum with a SEM coating unit E5100 (Polaron Equipment Ltd., England) and examined using a JSM 7401 F scanning electron microscope (JEOL Ltd., Tokyo, Japan).

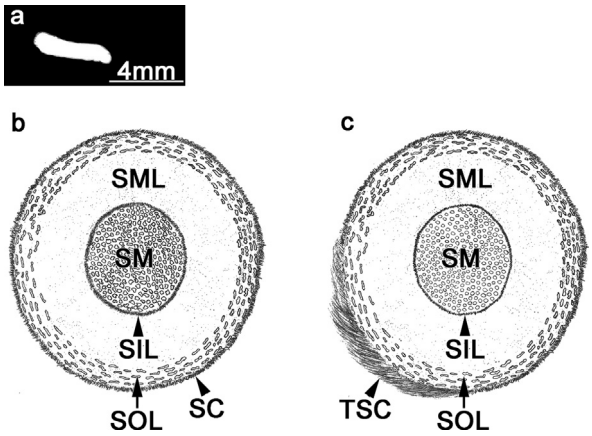

Fig. 1. (a) A general view of *A. leptodactylus* spermatophore; schematic drawings of the cross section of the spermatophore, (b) before, and (c) after mating. The sticky coat of the spermatophore is thickened at the site of attachment. SC: sticky coat; SM: sperm mass; SIL: spermatophore inner layer; SML: spermatophore middle layer; SOL: spermatophore outer layer; TSC: thickened sticky coat.

3. Results

3.1. General morphology of the spermatophore

The spermatophore is cylindrical, 2–10 mm long and approximately 1 mm in diameter (Fig. 1a). The crayfish spermatophore consists of a central sperm mass, enclosed by a three layered spermatophore wall (Fig. 1b).

3.2. Ultrastructure of the wall of the freshly ejaculated spermatophore

The surface of the spermatophore outer layer is covered by a sticky coat originating from elongated electron-lucent bodies (Fig. 2a). Beneath the sticky coat, the spermatophore outer layer is smooth and porous. At high magnification, small particles of the spermatophore outer layer matrix are visible (Fig. 2b). The outer layer of the spermatophore wall contains elongated electron-lucent bodies (Fig. 2c) and granules (Fig. 2d).

Spherical granules containing electron-dense material with small electron-lucent spots at the periphery are scattered within the grainy matrix of the middle layer of the spermatophore wall (Fig. 2e). The inner layer of the spermatophore wall consists of dense parallel fibers which separate the sperm mass from the middle layer of the spermatophore wall (Fig. 2f).

3.3. Ultrastructure of the freshly ejaculated spermatozoon

The sperm mass consists of spermatozoa which are closely packed together (Fig. 3a and b). In each of the spermatozoon, the acrosome and nucleus are located at the anterior and posterior parts of the cell, respectively. The nucleus contains fibers, dense granules and extensions of radial arms. Arms originating from the nucleus encircle the cell (Fig. 3c and d). The acrosome complex is divided into

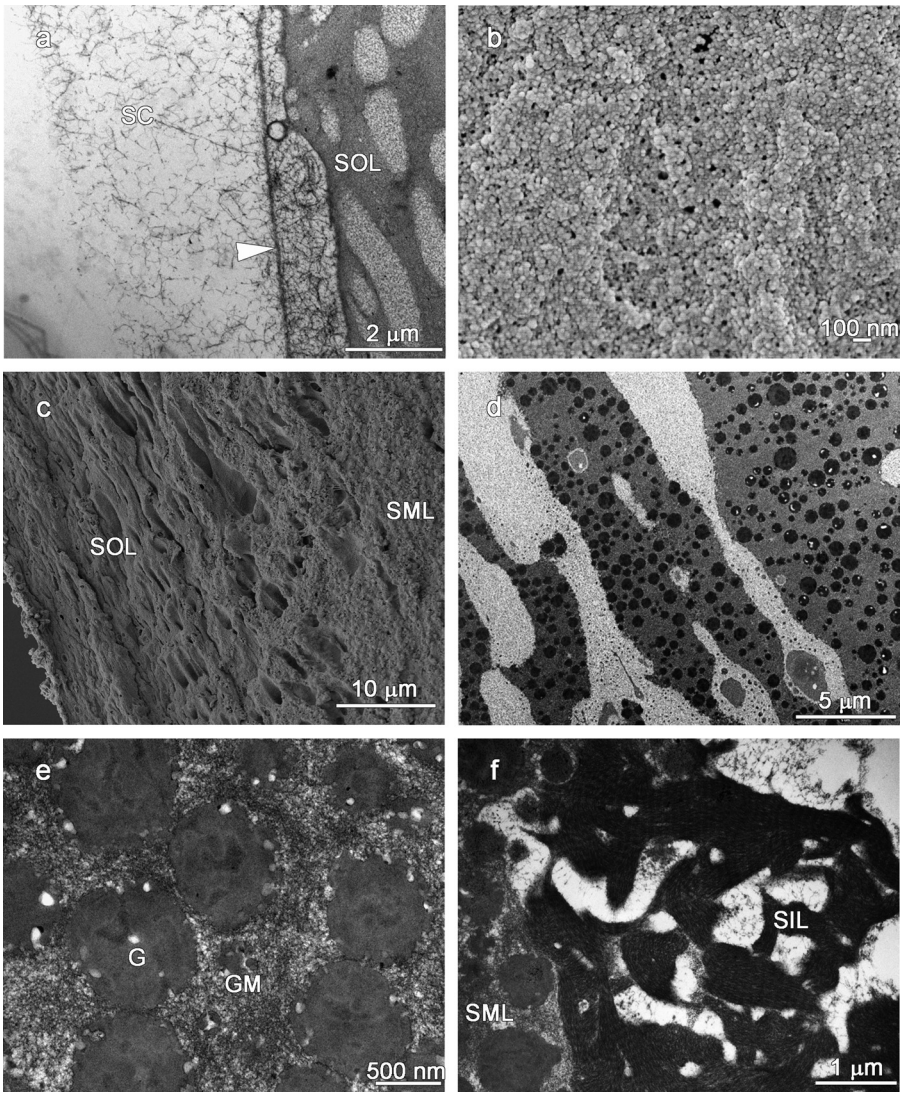

Fig. 2. Transmission and scanning electron micrographs of the wall layers of the freshly ejaculated spermatophore of *A. leptodactylus*. (a) The sticky coat of the spermatophore outer layer originating from elongated electron-lucent bodies. Arrow head shows the part which is described in (b); (b) small particles and pores beneath the sticky coat of the spermatophore outer layer; (c) the elongated bodies in the spermatophore outer layer; (d) the granules and elongated electron-lucent bodies in the spermatophore outer layer; (e) the granules in the spermatophore middle layer; (f) the fibers of the spermatophore inner layer. G: granule; GM: grainy matrix; SC: sticky coat; SIL: spermatophore inner layer; SML: spermatophore middle layer; SOL: spermatophore outer layer.

the main body of the acrosome and the subacrosome zone (Fig. 3d). The main body of the acrosome consists of three layers with different electron densities (Fig. 3e).

The middle layer of the acrosome, with lower electron density, contains scattered sections of hollow small diameter tubes coated by material with an electron density higher than the middle layer (Fig. 3e). The outermost acrosome layer with density higher than the middle layer occupies mainly the anterior periphery of the acrosome (Fig. 3e). An opening in the anterior part of the main body of the acrosome contains bundles of curved or straight filaments within a moderately electron-dense background. This region is called the apical zone (Fig. 3f). The filaments extend into the electron-dense matrix of the innermost layer of the acrosome (Fig. 3e). The subacrosomal zone comprises two distinct regions. The largest area is occupied by flocculent and electron-lucent material, but the density of mass is reduced more in the vicinity of the inner part of the main body of the acrosome (Fig. 3g).

A conspicuous membranous lamellae consisting of both electron-lucent and electron-dense concentric layers is located lateral or posterior to the acrosome and is connected to the plasma membrane (Fig. 3h).

Three distinguishable sub-domains form the plasma membrane. The first sub-domain, which forms most of the plasma membrane surrounding the acrosome, consists of a multi-layers part extending from the membranous lamellae. In the margin of the anterior part of the acrosome, it is separated from the second sub-domain of the plasma membrane by a narrow connection between the plasma

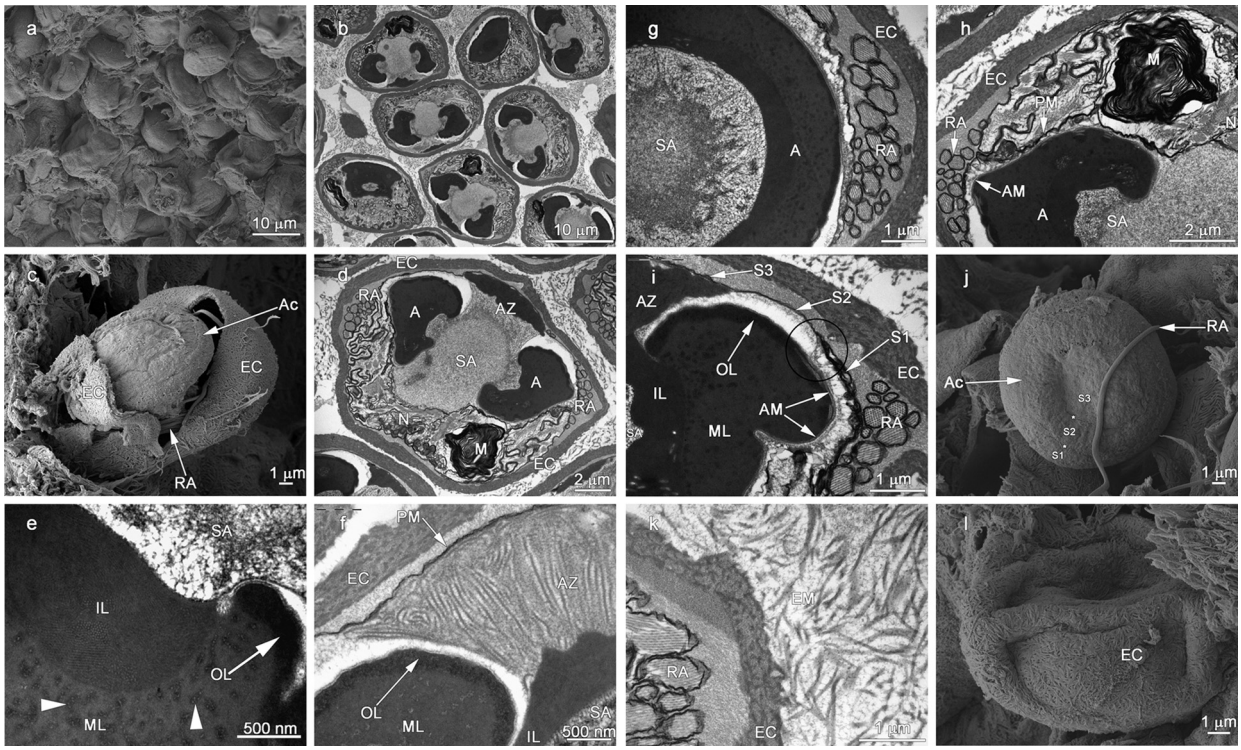

Fig. 3. Transmission and scanning electron micrographs of the freshly ejaculated spermatozoa of *A. leptodactylus*. (a and b) Spermatozoa within the spermatophore; (c) anterior lateral view of a single spermatozoon with artificially broken extracellular capsule. Note the exposed acrosome and radial arms; (d) the spermatozoon sagittal section showing contents of the spermatozoon; (e) layers of the acrosome main body and filaments inside the acrosome innermost layer. Arrowheads show sections of hollow small diameter tubes coated by electron dense material within the acrosome middle layer; (f) the apical zone in the anterior part of the spermatozoon; (g) the spermatozoon cross section showing the acrosome main body and subacrosome zone; (h) the membranous lamellae and their connection to the plasma membrane; (i) the sagittal section of different sub-domains of plasma membrane and its connection with the acrosome membrane (circle); (j) the anterior part of the plasma membrane of the spermatozoon with artificially broken extracellular capsule revealing its acrosome. Stars show the borders of the three membrane sub-domains; (k) the extracellular capsule and fibrous components originating from spermatozoal capsules and form the extracellular matrix; (l) the spermatozoon with an intact extracellular capsule. A: acrosome main body; Ac: acrosome complex; AM: acrosome membrane; IL: acrosome innermost layer; ML: acrosome middle layer; OL: acrosome outermost layer; AZ: apical zone; EC: extracellular capsule; EM: extracellular matrix; M: membranous lamellae; N: nucleus; PM: plasma membrane; RA: radial arms; SA: subacrosome zone; S1, S2, S3: sub-domains of plasma membrane.

membrane and the acrosome membrane which is visible in sagittal section (Fig. 3i). Two further sub-domains of the plasma membrane are located at the acrosome anterior part, appearing as concentric circles (Fig. 3j). One starts from the place of plasma membrane and acrosome membrane connection and extends to the beginning of apical zone. The third sub-domain covers the apical zone and filament bundles (Fig. 3i). Although obvious borders between the sub-domains are visible, the plasma membrane is continuous (Fig. 3i and j). The spermatozoon is tightly enclosed by an extracellular capsule. The space surrounding spermatozoa is occupied by fibrous component originating from spermatozoal extracellular capsules. Those fibers form the extracellular matrix (Fig. 3k and l).

3.4. Ultrastructural changes of the spermatophore wall after mating

After mating the spermatophore changes from a soft and sticky to a hardened structure attached to the ventral surface of the female and to other spermatophores using sticky coat of the spermatophore which is thickened at the site of attachment (Figs. 1c, 4a and b). The matrix of the outer spermatophore layer is reticulated and the granules release their content (Fig. 4c), The grainy matrix surrounding the granules of the spermatophore middle layer changes from a dense to a reticulated electron-lucent texture and a substantial change takes place in the granules. The contents of the granules are released and large electron-lucent spaces appear inside the granules (Fig. 4d).

The fibers of the inner layer of the spermatophore wall now appear to transform to small particles of reduced electron density (Fig. 4e).

3.5. Ultrastructural changes of the spermatozoon after mating

The main changes happen in the subacrosome zone, plasma membrane and extracellular capsule (Fig. 5a). The most anterior parts of the plasma membrane wrinkle and become multilayered, thus increasing the membrane surface covering the apical zone of the acrosome (Fig. 5b and c). The density of subacrosome is increased in the vicinity of the main body of the acrosome (Fig. 5d and e). The

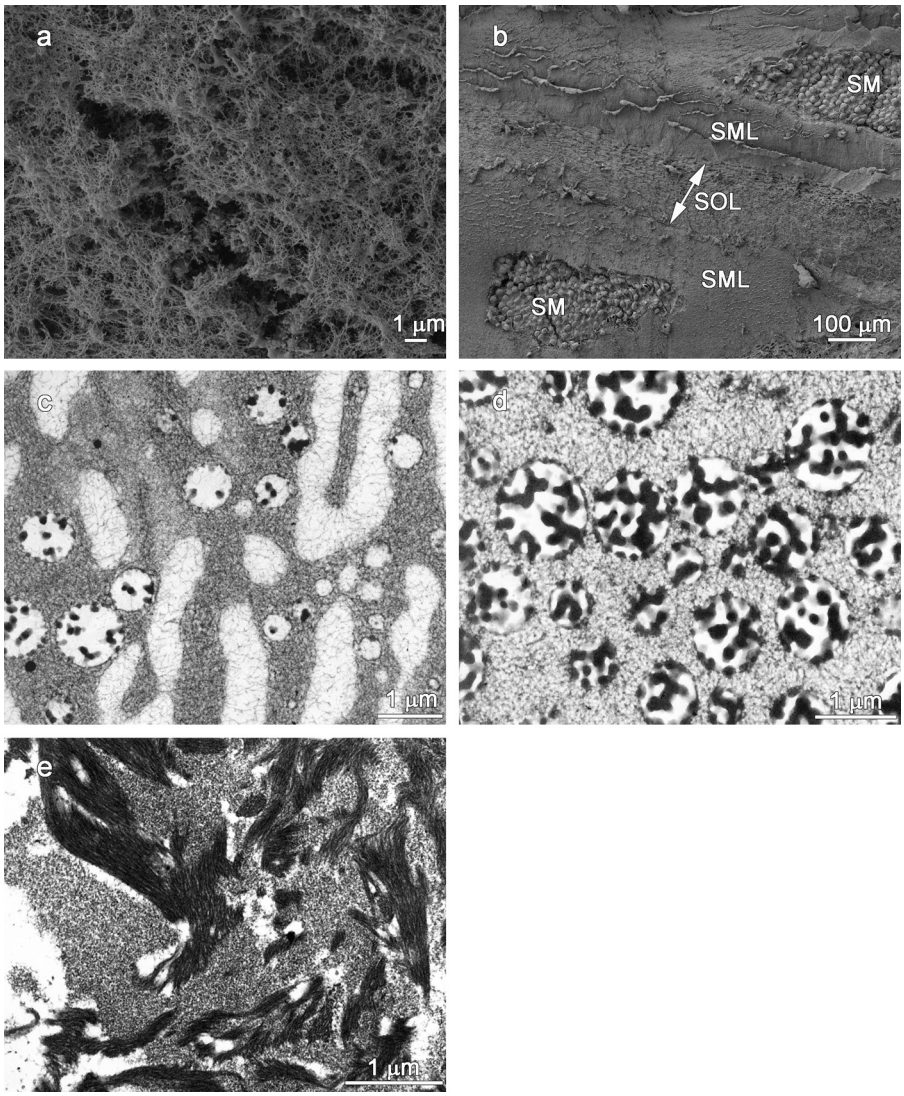

Fig. 4. Transmission and scanning electron micrographs of the spermatophore wall layers of *A. leptodactylus* after mating. (a) The thickened sticky coat of the spermatophore outer layer; (b) the section of the conjoined outer layers of two adjacent spermatophores; (c) granules and elongated bodies within the outer layer of the spermatophore; (d) the spermatophore middle layer granules which released their contents; (e) the spermatophore inner layer fibers transforming to small particles of reduced electron density. SM: sperm mass; SML: spermatophore middle layer; SOL: spermatophore outer layer.

capsules of spermatozoa swell and become nearly spherical. In sections empty spaces between the capsule and the spermatozoon are visible. The volume of the components of the spermatozoal capsule of the extracellular matrix is increased and they are joined together (Fig. 5a and f).

3.6. *Ultrastructural changes of the spermatozoon and spermatophore wall after release of the spermatozoon*

The layers of the spermatophore wall are dissolved by the female glair glands secretions (Fig. 6a). The extracellular capsule and plasma membrane in the anterior part of the acrosome are eliminated, bundles of filaments are released, and a cavity appears at the anterior part of the acrosome (Fig. 6b and c). The subacrosome zone separates from the main body of the acrosome, loses electron density and retracts. The membranous lamellae detach from the free spermatozoon (Fig. 6d).

The electron-dense matrix that covers the filaments in the innermost layer of the acrosome before and after mating is discharged from the spermatozoon and, together with the acrosome filaments, forms a droplet/filament structure in the anterior part of the spermatozoon (Fig. 6d and e). The filaments of the acrosome innermost layer are better visible after discharge of electron-dense matrix (Fig. 6f). The main body of the acrosome shows secretion activity. Some small pores and droplets are observed on the surface of the acrosome (Fig. 6g and h). The nuclear material of the spermatozoon at this stage is less condensed than in spermatozoa at earlier stages (Fig. 6i).

Morphological changes occurring during three stages of reproduction in the spermatophore wall and the

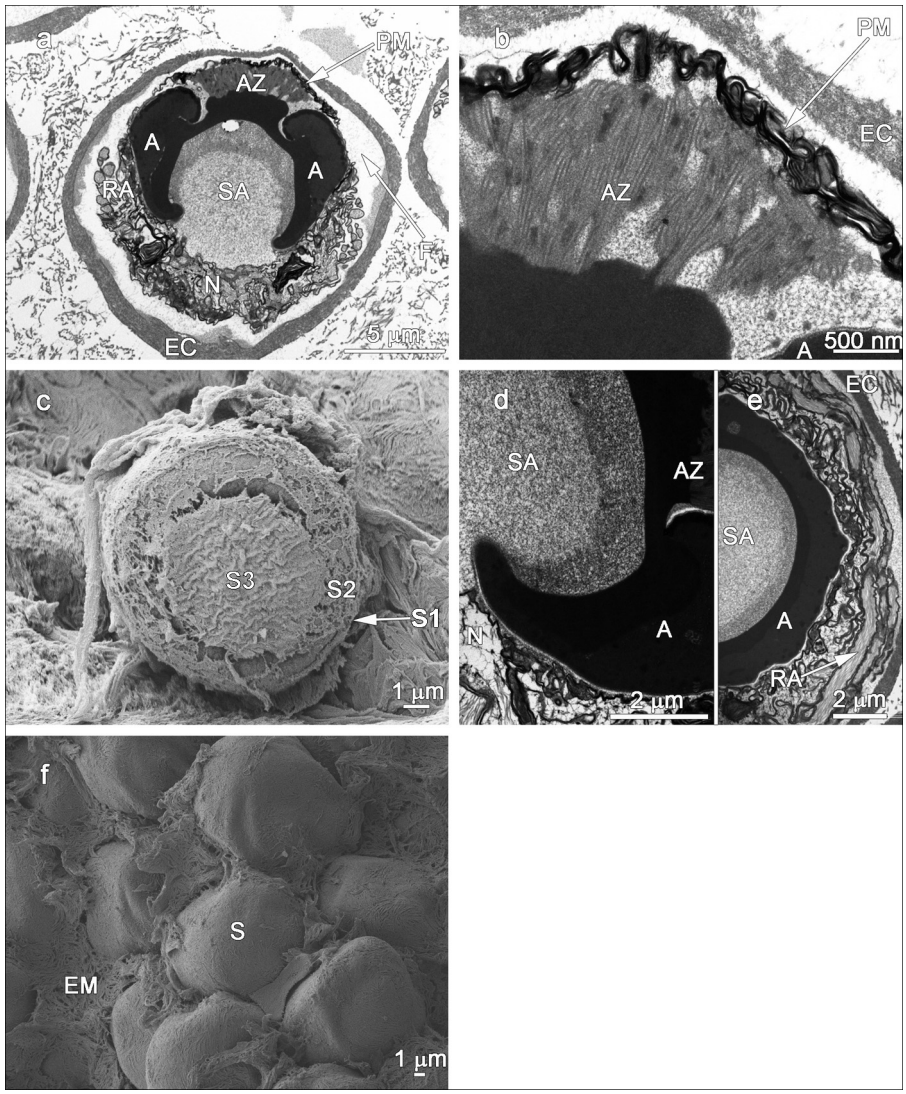

Fig. 5. Transmission and scanning electron micrographs of the spermatozoon of *A. leptodactylus* after mating. (a) The general view of the spermatozoon after mating; (b) the wrinkled plasma membrane in the anterior part of the spermatozoon; (c) the wrinkled plasma membrane in the anterior part of the spermatozoon with artificially broken extracellular capsule; (d and e) the sagittal and cross sections of subacrosome zone showing increased subacrosome density in the vicinity of the acrosome main body; (f) the sperm mass showing spermatozoa with swollen extracellular capsules and matrix. A: acrosome main body; AZ: apical zone; EC: extracellular capsule; EM: extracellular matrix; F: free space; N: nucleus; PM: plasma membrane; RA: radial arms; S: spermatozoon; S1, S2, S3: sub-domains of plasma membrane.

spermatozoon of narrow-clawed crayfish *A. leptodactylus* are summarized in Table 1.

4. Discussion

4.1. The morphological changes of the spermatophore wall

Substantial changes have been observed in the morphology of the narrow-clawed crayfish spermatophore layers after mating. Dudenhausen and Talbot (1983) reported that after mating in *Pacifastacus leniusculus*, the granules in the middle layer of the spermatophore wall appeared to have diffused a large portion of their contents. A similar situation was observed in the present study for *A. leptodactylus*.

Upon transfer from the testis into the *vas deferens*, spermatozoa become first surrounded by secretions that consolidate spermatozoa into a compact mass. Thereafter, during transit of the sperm mass to the distal portion of the *vas deferens*, a spermatophore wall is added by secretions of the *vas deferens* epithelium (Vogt, 2002). A three layered spermatophore wall was reported for crayfish (Dudenhausen and Talbot, 1983; Galeotti et al., 2012). Although the basic function of the spermatophores is the transfer of spermatozoa from male to female (Dudenhausen and Talbot, 1983), the complex ultrastructure of the spermatophore layers and their post-mating morphological changes may indicate

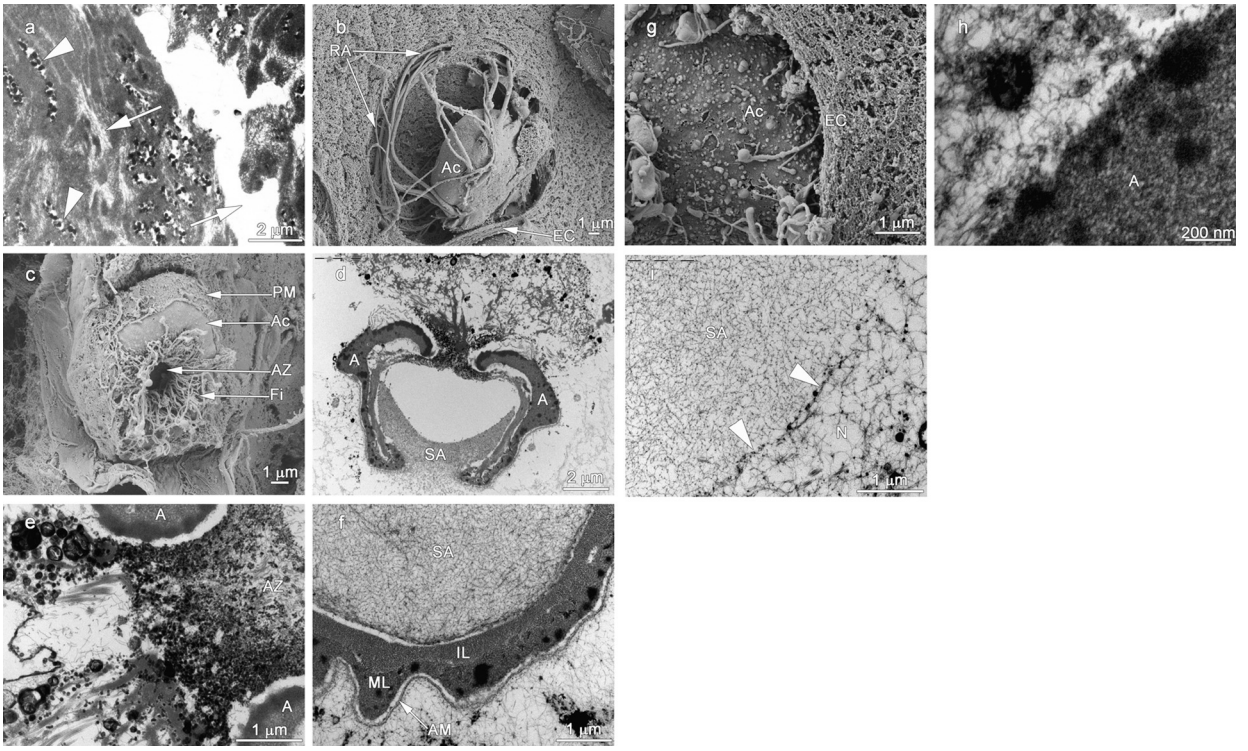

Fig. 6. Transmission and scanning electron micrographs of the spermatophore wall and the spermatozoon of *A. leptodactylus* after release of spermatozoa. (a) Distorted granules (arrowheads) within the dissolving wall of the spermatophore. Arrows show examples of two places which were penetrated and dissolved by glair secretions; (b) the spermatozoon released from extracellular capsule. Radial arms are visible; (c) the acrosome anterior part showing cavity appeared after release of spermatozoon from its extracellular capsule; (d) the sagittal section of a free spermatozoon showing the filament/droplet structure of the acrosome and retracted subacrosome zone; (e) the electron-dense material from innermost layer of the acrosome discharging toward outside via apical zone; (f) the cross section of acrosome layers and subacrosome zone after discharge of electron-dense material; (g) the anterior view of the acrosome surface. Numerous droplets showing its secretion; (h) the acrosome sagittal section revealing the acrosomal secretion activity. Note the electron-dense vesicles close to the inner acrosome surface (right part of the micrograph) and their extruded contents outside the acrosome (left upper part of the micrograph); (i) the subacrosome zone and nucleus. Arrowheads show the border of subacrosome zone and nucleus. A: acrosome main body; Ac: acrosome complex; AM: acrosome membrane; AZ: apical zone; EC: extracellular capsule; IL: acrosome innermost layer; ML: acrosome middle layer; Fi: filaments; N: nucleus; PM: remnant of plasma membrane; RA: radial arms; SA: subacrosome zone.

additional functional significance in crayfish. The final maturation of spermatozoa of ticks (*Ornithodoros moubata* and *Dermacentor variabilis*, Arthropoda: Ixodida), after transfer to the female, is induced by secretions of the male accessory gland which are added to the spermatophore during ejaculation (Shepherd et al., 1982). In the nematode *Ascaris suum* (Nematoda: Ascaridida), post-mating maturational changes can be induced by secretions of the glandular *vas deferens* (Foor and McMahon, 1973).

We observed that the middle layer of the spermatophore wall becomes hardened and reticulated after mating, when it comes in contact with water. The significance of this is unclear. Dudenhausen and Talbot (1983) suggested that it may be related to permeability properties of the hardened wall, which could facilitate gas exchange or transport of low molecular weight components across the wall during storage. Selective permeability was observed in the spermatophore wall of the giant mud crab *Scylla serrate* (Crustacea: Decapoda) (Uma and Subramoniam, 1979). It can be hypothesized that the post-mating contact of water with the crayfish spermatophore wall induces reticulation of the grainy matrix. Therefore, contents of the granules can be released and reach the sperm mass via porous texture of the reticulated grainy matrix, and the substances released may be acting synergistically with factors from the female reproductive tracts (Vanichviriyakit et al., 2004) or independently for final maturation and capacitation of spermatozoa.

The hardening of the spermatophore wall after mating probably promotes spermatozoon survival in the presence of environmental stressors and mechanical damages. Uma and Subramoniam (1979) demonstrated that the outer layer of the spermatophore of *Scylla serrata* is resistant to acidic and basic stress but the inner layer easily shrinks or disrupts under such treatment. Observations by Dudenhausen and Talbot (1983) demonstrated that the middle layer of the spermatophore is the site of hardening, since this layer, which forms the bulk of the spermatophore wall, becomes reticulated after mating and retains this shape during storage on the body surface of the female crayfish. While the chemical basis of post-mating hardening has not been characterized in crayfish, it was shown in the spermatophore of shrimp *Penaeus trisulcatus* (Crustacea: Decapoda), that hardening results from phenolic tanning, an enzymatic cross-linking reaction associated

Table 1
Summarized morphological changes during three stages of reproduction in the spermatophore of narrow-clawed crayfish *A. leptodactylus*.

Spermatophore layers or spermatozoon organelles	Freshly ejaculated spermatophore	Post-mating stages	
		After mating	After release of spermatozoa from the spermatophore
Spermatophore outer layer	– Sticky coat, elongated bodies, and granules within grainy matrix	– Sticky coat is thickened, matrix is reticulated, granules release their contents	– Dissolved
Spermatophore middle layer	– Granules within grainy matrix	– Matrix is reticulated, granules release their contents	– Dissolved
Spermatophore inner layer	– Dense parallel fibers	– Transform to small particles of reduced electron density	– Dissolved
Extracellular matrix	– Fibrous components	– Volume increases and attaches to the female and other spermatophores	– Dissolved
Extracellular capsule	– Tightly encloses spermatozoon	– Swells and becomes spherical	– Dissolved
Membranous lamella	– Electron-lucent and electron-dense layers connected to the plasma membrane	– No change	– Detach from cell
Plasma membrane	– Three sub-domains that connected together to form a continuous plasma membrane	– Anterior part wrinkles and becomes multilayered	– Eliminated from cell
Apical zone	– Contains bundles of curved or straight filaments	– No change	– Bundles of filaments are released, and a cavity appears at the anterior part of the acrosome
Innermost layer of the acrosome	– Filaments within electron-dense matrix	– No change	– Electron-dense matrix is discharged and, together with the filaments, forms a droplet/filament structure in the anterior part of the spermatozoon
Subacrosome zone	– Flocculent and electron-lucent material, with less density in the vicinity of the main body of the acrosome	– Density is increased in the vicinity of the main body of the acrosome	– Separates from the main body of the acrosome, loses electron density and retracts
Nucleus	– Nucleus materials and extensions of radial arms	– No change	– Loses electron density

with hardening of the chitin complex (Malek and Bawab, 1971).

4.2. The morphological changes of the spermatozoon

The present study revealed that the narrow-clawed crayfish spermatozoa undergo morphological changes during storage on the spermatophoric plate of the female, and after release of spermatozoa.

In the post-mating spermatozoon of the shrimp *Sicyonia ingentis* (Crustacea: Decapoda) the membrane pouches appear swollen and more electron-lucent. The crenulated nature of the cap region is greatly reduced and the spiral configuration of appendages lost. A new structure, the extended saucer, develops in the granular region and bridges the saucer plate and the crystalline lattice (Wikramanayake et al., 1992).

In the crayfish, spermatozoa possess a subacrosome zone (Niksirat et al., 2013a,b). Morphological changes in the subacrosome zone were observed in the crayfish spermatozoon in the present study. Alfaro et al. (2007) reported that in the whiteleg shrimp *Litopenaeus vannamei* (Crustacea: Decapoda), a region called the filamentous meshwork, analogous to the subacrosome zone in the crayfish, is synthesized in the male reproductive system, but seems to complete development after mating. A decondensed nucleus, larger and denser filamentous meshwork, and cytoplasmic particles in post-mating spermatozoa of *L. vannamei* were reported by Aungsuchawan et al. (2011). The subacrosomal zone is adjacent to the nuclear material. The perforatorial chamber in other decapod spermatozoa is analogous to this subacrosomal zone. It projects forward and becomes the leading part of the reacted spermatozoon during acrosome reaction in the fiddler crab, *Uca tangeri* (Crustacea: Decapoda) (Medina and Rodriguez, 1992), apparently drawing nuclear material toward the egg. This suggests that morphological changes in the subacrosome zone during post-mating and after release of spermatozoa from the extracellular capsule are required for crayfish spermatozoa to become functional.

The membranous lamella is formed by alteration and transformation of several mitochondria during spermatogenesis, which still capable to supply energy to the crayfish spermatozoon (Anderson and Ellis, 1967). An earlier research showed the positive staining of Janus green B, indicator of mitochondria, in the same area of the crayfish spermatozoon (André, 1962). The elimination of the membranous lamellae as a source of energy after release of the crayfish spermatozoon reinforces earlier findings of immotility of crayfish and other decapods spermatozoa by several researchers (Jamieson and Tudge, 2000; Moses, 1961a,b; Tudge, 2009; Tudge et al., 2001; Reynolds, 2002).

Since there is an interval of several hours between secretions from glair glands and release of eggs (Andrews,

1906), it seems that spermatozoa remain as free cells within glair secretions and the acrosome reaction is not triggered prior to the contact of the spermatozoon with the egg.

To compensate for the lack of motility in spermatozoa, crayfish have developed mechanisms to facilitate egg-spermatozoon binding. The female lies with its ventral surface upward and starts secreting from its glair glands. Secretions from the female glair glands dissolve the spermatophore wall and distribute spermatozoa across the ventral abdomen a few hours before egg releasing. This secretion is of sufficient density to prevent spermatozoa from being washed away until completion of fertilization. The female forms a brood chamber by curling the abdomen to protect the spermatozoon and egg floating into glair secretions (Andrews, 1906). The spermatozoon possesses a filament/droplet complex at its anterior part as a passive mechanism to increase the chance of egg-spermatozoon binding. The electron-dense droplets, originating from the innermost layer of the main body of the acrosome, combined with the extruded filaments may provide a sticky structure that enables the spermatozoal body to adhere to the egg surface for fertilization. In addition, the process of fertilization is assisted by the continuous mixing of eggs and spermatozoa inside the glair secretions by the female's pleopods, which increases the chance of egg-spermatozoon binding.

We observed that after mating, especially during release of spermatozoa from their capsules, the nucleus loses its electron-density. Poljaroen et al. (2010) reported that nuclei of spermatozoa of the giant freshwater prawn, *Macrobrachium rosenbergii* (Crustacea: Decapoda), continuously lose histone proteins during spermatogenesis. These proteins aid the DNA compaction in the nucleus. Therefore, it is possible that loss of these basic nuclear proteins may result in decondensation and flexibility of chromatin that facilitates its passage through the narrow perforatorial chamber of the acrosome during fertilization.

5. Conclusion

In conclusion, obvious changes were observed in the morphology of spermatozoa and spermatophore layers during storage on the body of the female crayfish. The most important change is observable in the subacrosome zone which is assumed to play the key role in the fertilization process in decapods. Morphological changes of the spermatozoon after release from its capsule, especially formation of the filament/droplet structure, can contribute to the mechanism of egg-spermatozoon binding in the crayfish as representative of animals with non-motile spermatozoa.

Conflict of interest

None declared.

Acknowledgments

The authors express their sincere appreciation to the staff of the Laboratory of Electron Microscopy, Institute of Parasitology, Biology Center of ASCR for their valuable contribution. Our appreciation is extended to the Lucidus Consultancy for English improvement. The Czech Science Foundation supported this work through project P502/12/P177. Partial funding was also provided by projects CENAKVA (CENAKVA CZ.1.05/2.1.00/01.0024), CENAKVA II (the results of the project LO1205 were obtained with a financial support from the MEYS of the CR under the NPU I program), and 087/2013/Z of the Grant Agency of the University of South Bohemia. Open access was covered by the project CZ.1.07/2.3.00/20.0024 Strengthening of excellence scientific teams in USB FFPW.

References

Alfaro, J., Muñoz, N., Vargas, M., Komen, J., 2003. Induction of sperm activation in open and closed thelycum shrimps. Aquaculture 216, 371–381.
Alfaro, J., Ulate, K., Vargas, M., 2007. Sperm maturation and capacitation in the open thelycum shrimp *Litopenaeus* (Crustacea: Decapoda: Penaeoidea). Aquaculture 270, 436–442.
Anderson, W.A., Ellis, R.A., 1967. Cytodifferentiation of the crayfish spermatozoon: acrosome formation, transformation of mitochondria and development of microtubules. Z. Zellforsch. Mikrosk. Anat. 77, 80–94.
André, J., 1962. Contribution á la connaissance du chondriome. J. Ultrastruct. Res. 6 (Suppl. 1), 1–185.
Andrews, E.A., 1906. Egg-laying of crayfish. Am. Nat. 40, 343–356.
Aungsuchawan, S., Browdy, C.L., Withyachumnarnkul, B., 2011. Sperm capacitation of the shrimp *Litopenaeus vannamei*. Aquacult. Res. 42, 188–195.
Crandall, K.A., Buhay, J.E., 2008. Global diversity of crayfish (Astacidae, Cambaridae, and Parastacidae-Decapoda) in freshwater. Hydrobiologia 595, 295–301.
Dudenhausen, E.E., Talbot, P., 1983. An ultrastructural comparison of soft and hardened spermatophores from the crayfish *Pacifastacus leniusculus* Dana. Can. J. Zool. 61, 182–194.
Foor, W.E., McMahon, J.T., 1973. Role of the glandular vas deferens in the development of *Ascaris* spermatozoa. J. Parasitol. 59, 753–758.
Galeotti, P., Bernini, G., Locatello, L., Sacchi, R., Fasola, M., Rubolini, D., 2012. Sperm traits negatively covary with size and asymmetry of a secondary sexual trait in a freshwater crayfish. PLoS ONE 7 (8), e43771.
Jamieson, B.G.M., Tudge, C.C., 2000. Crustacea-Decapoda. In: Jamieson, B.G.M. (Ed.), Progress in Male Gamete Ultrastructure and Phylogeny, vol. 9. Part c of Adiyodi, K.G., Adiyodi, R.G. (Eds.), Reproductive Biology of the Invertebrates. John Wiley & Sons, Chichester, pp. 1–95.
Jerry, D.R., 2001. Electrical stimulation of spermatophore extrusion in the freshwater yabby (*Cherax destrctor*). Aquaculture 200, 317–322.
López-Greco, L.S., Lo Nostro, F.L., 2008. Structural changes in the spermatophore of the freshwater red claw crayfish *Cherax quadricarinatus* (Von Martens, 1898) (Decapoda, Parastacidae). Acta Zool. 89, 149–155.
Malek, S.R.A., Bawab, F.M., 1971. Tanning in the spermatophore of a crustacean (*Penaeus trisulcatus*). Experentia 27, 1098.
Medina, A., Rodriguez, A., 1992. Spermiogenesis and sperm structure in the crab *Uca tangeri* (Crustacea, Brachyura), with special reference to the acrosome differentiation. Zoomorphology 111, 161–165.
Moses, M.J., 1961a. Spermiogenesis in crayfish (*Procambarus clarkii*). I. Structural characterization of the mature sperm. J. Biophys. Biochem. Cytol. 9, 222–228.
Moses, M.J., 1961b. Spermiogenesis in crayfish (*Procambarus clarkii*). II. Description of the stages. J. Biophys. Biochem. Cytol. 10, 301–333.
Niksirat, H., Kouba, A., Pšenicka, M., Kuklina, I., Kozák, P., 2013a. Ultrastructure of spermatozoa from three genera of crayfish *Orconectes*, *Procambarus* and *Astacus* (Decapoda: Astacoidea): new findings and comparisons. Zool. Anz. 252, 226–233.
Niksirat, H., Kouba, A., Rodina, M., Kozák, P., 2013b. Comparative ultrastructure of the spermatozoa of three crayfish species: *Austropotamobius torrentium*, *Pacifastacus leniusculus*, and *Astacus astacus* (Decapoda: Astacidae). J. Morphol. 274, 750–758.
Poljaroen, J., Vanichviriyakit, R., Tinikul, Y., Phoungpetchara, I., Linthong, V., Weerachatyanukul, W., Sobhon, P., 2010. Spermatogenesis and distinctive mature sperm in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879). Zool. Anz. 249, 81–94.
Reynolds, J.D., 2002. Growth and reproduction. In: Holdich, D.M. (Ed.), Biology of Freshwater Crayfish. Blackwell Science, UK, pp. 152–191.

Shepherd, J., Oliver Jr., J.H., Hall, J.D., 1982. A polypeptide from male accessory glands which triggers maturation of tick spermatozoa. Int. J. Invertebr. Reprod. Dev. 5, 129–137.

Skurdal, J., Taugbøl, T., 2002. Crayfish of commercial importance. In: Holdich, D.M. (Ed.), Biology of Freshwater Crayfish. Blackwell Science, UK, pp. 465–670.

Tudge, C.C., Scheltinga, D.M., Jamieson, B.G.M., 2001. Spermatozoal morphology in the symmetrical hermit crab, *Pylocheles* (*Bathycheles*) sp. (Crustacea, Decapoda, Anomura, Paguroidea, Pylochelidae). Zoosystema 23, 117–130.

Tudge, C.C., 2009. Spermatozoal morphology and its bearing on decapod phylogeny. In: Martin, J.W., Crandall, A., Felder, D.L. (Eds.), Crustacean issues: decapod crustacean phylogenetics. Francis & Taylor/CRC Press, Boca Raton, pp. 101–119.

Uma, K., Subramoniam, T., 1979. Histochemical characteristics of spermatophore layers of *Scylla serrata* (Forskal) (Decapoda: Portunidae). Int. J. Invertebr. Reprod. Dev. 1, 31–40.

Vanichviriyakit, R., Kruevaisayawan, H., Weerachatyanukul, W., Tawipreeda, P., Wthyachumnarnkul, B., Pratoomchat, B., Chavade, J.J., Sobhon, P., 2004. Molecular modification of *Penaeus monodon* sperm in female thelycum and its consequent responses. Mol. Reprod. Dev. 69, 356–363.

Vogt, G., 2002. Functional anatomy. In: Holdich, D.M. (Ed.), Biology of Freshwater Crayfish. Blackwell Science, UK, pp. 53–151.

Wikramanayake, A.H., Uhlinger, K.R., Griffin, F.J., Clark W.H.JJr., 1992. Sperm of the shrimp *Sicyonia ingentis* undergo a bi-phasic capacitation accompanied by morphological changes. Dev. Growth Differ. 34, 347–355.