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a ochrany vod
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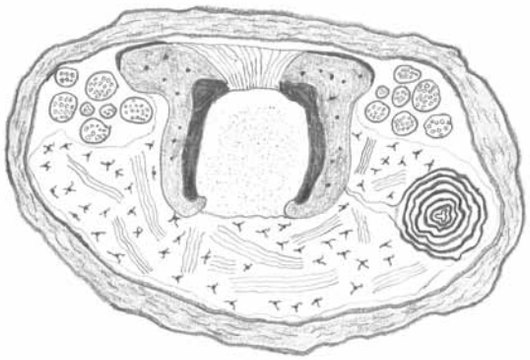
Biology of reproduction in the crayfish

Biologie rozmnožování u raků



Hamid Niksirat

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Supervisor
Assoc. Prof. Dipl.-Ing. Kozák Pavel, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Consultant
Dipl.-Ing. Kouba Antonín, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Head of Laboratory of Ethology of Fish and Crayfish
Assoc. Prof. Dipl.-Ing. Pavel Kozák, Ph.D.

Dean of Faculty of Fisheries and Protection of Waters
Prof. Dipl.-Ing. Otomar Linhart, D.Sc.

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Prof. Dr. Laura Susana López Greco – international referee

Date, hour and place of Ph.D. defense:
18th September 2014 at 12:30 in USB, FFPW, RIFCH, Vodňany

Name: Hamid Niksirat

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. IMPORTANCE OF BASIC BIOLOGY OF REPRODUCTION

Freshwater crayfish belong to the largest crustacean taxon, the Decapoda, comprising 3 families, 33 genera, and over 640 known species (Crandall and Buhay, 2008). Crayfish are the largest, mobile freshwater invertebrates, some such as *Astacopsis gouldi* reaching over 4.5 kg in weight; however, others are relatively minute, e.g. *Gramastacus* (Holdich, 2002). Their distribution is worldwide with the exception of continental Africa and Antarctica (Hobbs, 1988).

Crayfish represent unquestionable indirect worth to human beings, including recreational, cultural, ethical, aesthetic, scientific, technological, and educational values (Gherardi, 2011). The reproductive biology of crustaceans is crucial for crustacean industry. The decline in commercial crustacean fisheries around the world is widely known. Major factors contributing to the steady decline in wild crustacean population number include inadequate legislation providing protection for these species, increases in the harvest rate, and decrease in the size of the crustaceans and increases in worldwide consumption. One way to maintain suitable crustacean populations in the wild is by reproducing of crustacean in the hatcheries and farms for further cultivation and restocking of wild populations (Nagaraju, 2011). Understanding of basic structures and functions of reproductive system help better management of them in the wild or aquaculture facilities. Therefore, there has been increasing need for detailed study in case of different aspects of crustacean's reproductive biology.

1.2. GENERAL ANATOMY OF REPRODUCTIVE ORGANS

Most crustaceans have separate sexes, and these can be distinguished by appendages on the abdomen called pleopods. The first (sometimes the second) pair of pleopods are longer on the male than on the female. These abdominal pleopods have become modified into copulatory organs that transfer the spermatophores from the male penises to the female sexual openings. Female decapod crustaceans store the spermatophores for different periods in their thelycum before laying of eggs (Nagaraju, 2011).

The testes lie dorsally in the thorax between the floor of the pericardial sinus and hindgut. Their size and appearance depend on the age and reproductive condition of the individuals. In the breeding season the internal reproductive organs enlarge significantly. The testes take a milky-white color due to production of sperm. The testis is the site of spermatogenesis and spermiogenesis. In astacids, it consists of a paired anterior lobe and elongated, unpaired posterior lobe, whilst in parastacids there are two parallel testicular lobes that are grown together in the middle (Rudolph, 1995 a, b). A highly convulated *vas deferens* arises from each side of the testis and opens at the gonopore on the basis of the fifth preopod. The *vas deferens* is responsible for packaging the spermatozoa into spermatophores and for conducting the spermatophores to the gonopods. Crayfish have an external fertilization. During the mating period the spermatophores are deposited either on the ventral surface of the female or, in cambarids, into the *annulus ventralis*. When the mature eggs are released through the gonopores the wall of spermatophores dissolve and the spermatozoa become free to fertilize eggs (Vogt, 2002).

The testis of crayfish is covered by a cortex of connective tissue and is composed of numerous seminiferous tubules that include sertoli cells and various developmental stages of germ cells. Also, the testis of red swamp crayfish *Procambarus clarkii* is composed of numerous tubules containing sertoli cells and various developmental stages of germ cells. The seminefrous tubules fuse to form collecting ducts which terminate in the *vas deferens*

(Poljaroen et al., 2010). Spermatogenic cells and nurse cells constitute two major types of cells in the epithelium of the seminefrous tubules, whereas mature spermatozoa are found in the lumen (Vogt, 2002).

The ovary of *Procambarus clarkii* has a maximum length of 4 cm and a width of 5 mm and is composed of two anterior and one posterior sacs. The mature oocytes leave the ovary through the two ovarian ducts that originate from the bases of the paired anterior ovarian sacs. The oviducts are composed of a single-layered epithelium surrounded by connective tissue with interspersed muscle cells. They are first wound up in the ovary, but run then straight down to the gonopores that are located on the bases of third walking legs (Ando and Makioka, 1998; Vogt, 2002).

1.3. THE SPERMATOZOON ULTRASTRUCTURE

The spermatozoon ultrastructure has been successfully employed in studies on taxonomy and phylogeny in different animal taxa, including the Cructacean (Felgenhauer and Abele, 1991; Jamieson, 1991; Jamieson et al., 1995a, b; Medina, 1994; Medina et al., 1994a, b; Tudge, 1995; Jamieson and Tudge, 2000; Martin and Davis, 2001).

The spermatozoon of crustacean do not need to travel by swimming a long distance in the female tract, such as in case of other animals with internal fertilization, or in an aqueous medium as in case of animal with external fertilization. As a result, the spermatozoon lack tails and are non-motile (Tudge, 2009; Poljaroen et al., 2010).

During spermiogenesis in *P. clarkii*, the mitochondria become aggregated on one side of the spermatid cytoplasm, and then appear to degenerate when spermatids differentiate into mature spermatozoon (Moses, 1961a, b). Eventually, the mitochondria are totally absent in the mature sperm, and this may be due to the fact that, as spermiogenesis progresses, spermatids have a decreased demand for ATP from mitochondria (Moses, 1961b). In this crayfish, the size of residual spermatid mitochondria and the number of cristae are very small, corresponding to a low metabolic need of the late stage of spermatids, and finally the spermatozoon. It has been reported that there is a large reduction in the number, and even total loss, of mitochondria in other palaemonids (Koehler, 1979; Butcher and Felder, 1994; Kim et al., 2003) and dendrobranchiates (Shigekawa and Clark, 1986; Medina, 1994; Medina et al., 1994a, 1994b; Pongtippatee et al., 2007). As well, the *Macrobrachium rosenbergii* spermatozoa are non-motile and appear to need little energy from mitochondria. Thus, in a fully mature spermatozoon of this species there are very few mitochondria remaining in comparison with those present in the spermatid precursors, and most mitochondria degenerate into large vacuoles or whorls of membranes in the posterior cytoplasm.

In *Procambarus* species the spermatids develop into mature spermatozoa by formation of the acrosome and radial arms, alternation of the nucleus and elimination of considerable part of the cytoplasm inclusive of cell organelles (Moses, 1961a, b).

The spermatozoa of crayfish are aflagellate and follow in principle the general scheme published for the spermatozoon of reptantian decapods (Krol et al., 1992). In *Astacus astacus*, the most striking feature of the spermatozoon is the complicated acrosome, the decondensed nucleus and radial arms that include extensions of the nucleus and bundles of microtubules. Such arms are present in the Astacidae and Cambaridae but are absent in *Cherax tenuimanus* and *Cherax albidus* (Beach and Talbot, 1987). Their numbers varies greatly among species: there are four arms in *P. clarkii* (Moses, 1961a, b) but more than 20 in *A. astacus*. In the testis and *vas deferens* the radial arms are tightly wrapped around the spermatozoon, and the spermatozoon in inclusive of the arms is enveloped by a capsule. These capsules are thought to confine the radial arms and permit tighter packaging of the sperm in the spermatophore

(Dudenhausen and Talbot, 1983). This interpretation is supported by the absence of such capsule in *Cherax* species where radial arms are lacking (Beach and Talbot, 1987).

Traditionally, decapod spermatozoa are classified into two categories based on the presence of spikes or side appendages (Talbot and Summers, 1978; Lynn and Clark, 1983b; Kim et al., 2003). The unistellate spermatozoon exhibits a single spike which is extended from the dense plate that covers the anterior end of the nucleus. The spermatozoon in this group belong to natantian decapods, which comprise members of the suborder Pleocyemata, infraorder Caridea, families Palaemonidae and Pandalidae, which are represented by *M. rosenbergii* and *Palaemonetes paludosus*, and suborder Dendrobranchiata includes the families Sicyoniidae (represented by *Sicyonia ingentis*) and Penaeidae (represented by *Penaeus monodon*). In addition to a single spike, spermatozoa of the caridean decapods have a cup shape (Koehler, 1979; Butcher and Felder, 1994; Kim et al., 2003), while spermatozoa of dendrobranchiates possess a spherical shape (Shigekawa and Clark, 1986; Medina, 1994; Medina et al., 1994a, b; Pongtippatee et al., 2007). Furthermore, spermatozoa of all these species exhibit decondensed chromatin, with the caridean spermatozoon showing total decondensation while dendrobranchiate spermatozoon still possess some thicker chromatin fibers or granules. However, spermatozoa of certain dendrobranchiates, including those in families Sergestidae and Aristeidae, do not have spike or any extensions, even though they have fully formed acrosomes. These include spermatozoa of *Aristeus antennatus* (Demestre et al., 1997), *Peisos petrunkevitchi* (Scelzo and Medina, 2004), *Aristeus varidens* and *Aristaeopsis edwardsiana* (Medina et al., 2006). In contrast, multistellate spermatozoa are the characteristics of the remaining members of Pleocyemata, traditionally classified as reptantian decapods, and comprising members of the infraorders Astacidea (lobsters, crayfishes) and Brachyura (crabs). These multistellate spermatozoa show several appendages or arms of nuclear or cytoplasmic origin, and the acrosome which is enwrapped by the nucleus whose chromatin is completely decondensed (Krol et al., 1992; Vogt, 2002). In *P. paludosus,* the spike originates from an electron-dense structure in the cytoplasm of spermatids, called the lamellar body (Koehler, 1979). Only few studies investigated the components of the spikes in palaemonid spermatozoa. Microfilaments and tubulin-like protein have been detected in rays and spines of the *Rhynchocinetes typus* spermatozoon, but not in the spike and its base plate (Pérez et al., 1991). In *M. rosenbergii*, the origin, chemical composition, and precise role of the spikes are still obscure and need further studies. In contrast, the spike of a dendrobrachiate spermatozoon is a well-defined structure which arises from the condensed material in the anterior part of the proacrosomal vesicle, while the posterior part forms the acrosomal sacs. Hence the spike is an integral part of the acrosome complex (Shigekawa and Clark, 1986; Medina, 1994; Medina et al., 1994a, 1994b). In multistellate spermatozoa of reptantian decapods spikes are actually extensions of the nucleus or cytoplasm, and contain microtubules (Talbot and Summers, 1978; Talbot and Chanmanon, 1980). However, the microtubules are not arranged in the 9 + 2 axonemal pattern, which is typical in flagellated tails of other animal spermatozoa; thus they are probably not involved in the spermatozoon movement. In contrast to dendrobranchiate and other reptantian spermatozoa, the formation and boundary of the acrosome in caridean spermatozoa, including giant freshwater prawn, *M. rosenbergii* (Poljaroen et al., 2010), is still unclear. In the spermatozoon of *P. paludosus*, the acrosome complexes comprise multiple layers of perinuclear vesicles which are formed by the evagination of the outer layer of the nuclear membrane (Koehler, 1979). It is assumed that these vesicles or sacs may contain acrosomal enzymes. In *M. rosenbergii*, similar acrosomal sacs at the edge of the spike base plate of mature sperm taken from the ejaculatory bulb were observed (Poljaroen et al., 2010). In contrast, Lynn and Clark (1983a, b) did not observe these perinuclear sacs in *M. rosenbergii* spermatozoon, so they suggested that the spike may

function as the acrosome and is possibly covered with acrosomal enzymes which can help the spermatozoon to penetrate the egg investment. Instead, Poljaroen et al. (2010) proposed that the acrosomal sacs could be the major part of the acrosome that is closely associated with the base of the spike. However, immunocytochemical evidence for the presence of the acrosome enzymes in these sacs is needed before it could be claimed that these sacs are really components of the acrosome (Poljaroen et al., 2010).

The radial arms of spermatozoon in *A. astacus* are formed from the nucleus itself at the cell's equator. At the base of these structures numerous microtubules and fibers can be observed, but in their interior, where they separated from the body of the gamete, only microtubules and the nuclear and plasmatic membranes can be observed; although the later almost unites with the external membrane of nucleus. Within each radial arm about 30 to 40 microtubules can be found. The size of each microtubule varies between 180 and 200 Å. The thickness of radial arms varies between 0.3 and 0.4 μm in the most distal extreme (Lopez-Camps et al., 1981).

The significant function of these nuclear extended spikes (radial arms) has not been explained (Yasuzumi, 1960; Yasuzumi et al., 1961; Talbot and Summers, 1978). The electron-dense content of the spike may be a kind of contractile protein such as actin or other cytoskeleton proteins having depolymerization or repolimerization ability. The mechanism behind the curling and shortening of the spike is probably by depolymerization of the cytoskeletal protein (Pongtppatee et al., 2007).

Lopez-Camps et al. (1981) described the acrosome region of *A. astacus*. The region consists of a complex invaginated vesicle whose opening is oriented toward the nuclear region. The acrosome can be divided into two well-differentiated parts: 1. The vesicle, which is a thick, helmet-shaped body, consists of three sections (ring like formation resembling a thick doughnut, apical formation and amorphous matrix), 2. the central canal which is delimited by the invagination of the vesicle. Altogether the acrosome is a spherical organelle, whose greatest diameter varies from 11 to μm and whose smallest diameter varies from 5 to 8 μm.

Klaus et al. (2009) investigated the ultrastructure of spermatozoa of 19 palaeotropical freshwater crab species [12 species of the Gecarcinucidae, 6 of the Potamidae (Potamiscinae), and 1 species of the Potamonautidae (Deckeniinae: Hydrothelphusini)]. The acrosome in the Gecarcinucidae is much smaller and spherical, while the larger acrosome in the Potamiscinae has the tendency to be depressed. In the Potamiscinae, an additional middle acrosomal zone evolved between the acrosome ray zone and the outer acrosomal zone. Within the Gecarcinucidae, a differentiation into two groups (Gecarcinucinae and Parathelphusinae) is not supported by the present spermatological data. The spermatozoal morphology of *Hydrothelphusa* aff. *madagascariensis* (Potamonautidae: Deckeniinae) differs from *Potamonautes sidneyi* (Potamonautidae: Potamonautinae) in acrosomal size and shape, and in the absence of a periopercular rim. A closer relationship of Deckeniinae and Gecarcinucidae cannot be confirmed by spermatology.

1.4. MORPHOLOGY OF THE SPERMATOPHORE

Klaus and Brandis (2011) divided spermatophores into two groups: coenospermia spermatophores with several spermatozoa in one spermatophore and cleistospermia with only a single sperm cell per spermatophore. In *vas deferens* the sperm masses are packaged into spermatophores that function in the transfer of sperm from male to female during mating. Upon enterance from the testis into *vas deferencs*, the spermatozoa become first surrounded by epithelial 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 *vas deferens* epithelium. This secretory epithelium is single-layered and composed of cylindrical cells and is surrounded by a sheath of connective tissue. Formation of the spermatophore has been described in detail for *Pacifastacus leniusculus* on the basis of electron microspical investigations (Dudenhausen and Talbot, 1983).

The mature, unextruded spermatophores of *P. leniusculus* consist of two main parts, the central sperm mass and a three-layered spermatophore wall. In this form, the pliable and sticky spermatophores are stored until mating in the distal part of *vas deferens*. Following extrusion from the male and attachment to the female the spermatophore wall becomes gradually hardened in the water. Extruded and attached spermatophores are simple cylindrical structures measuring 4-9 mm in length and less than 1mm in diameter. The wall of such attached spermatophore is resistant to environmental stress that is necessary because of the temporal delay between mating and egg lying in crayfish (Dudenhausen and Talbot, 1983; Vogt, 2002).

In *Somanniathelphusa* sp. no evidence of spermatophore could be identified, with the spermatozoa floating uncoated in the *vas deferens* (Klaus et al., 2009).

The mocus type of spermatophore was biochemically analysed by Jeyalectumie and Subramoniam (1987) for *Spiralothelphusa hydrodroma* and showed to contain protein, free carbohydrates, and lipids.

The morphological differentiation in more and less electron-dense areas within mocous spermatophores points to a biochemically different composition of these areas. This could be due to different primary functions of the matrix types, for example, nutrition or protection. Such a morphological differentiation of the spermatophore matrix was already described in marine brachyrans like *Scylla serata* (Uma and Subramoniam, 1979), *Chacean fenneri* (Hinsch, 1991). The densely packed spermatophores of the potamiscinae, leaving very little space not only between the individual spermatozoa but also between spermatozoa and spermatophore wall, argue for a main function as a transfer device. At least a nutritional function can most probably be excluded (Klaus et al., 2009).

1.5. THE SPERMATOZOON CAPACITATION IN CLOSED AND OPEN THELYCA CRUSTACEAN

Spermatozoa of the closed thelyca shrimps *Farfantepenaeus aztecus* and *S. ingentis* undergo a capacitation process, as in mammalians, where matured spermatozoa experience physiological changes to facilitate their reactivity. The spermatozoon must be transferred and stored within the seminal receptacle of a female for a period of time before they achieve the ability to fertilize (Clark et al., 1984; Clark and Griffin, 1988; Griffin and Clark, 1990). Uncapacitated spermatozoa of *S. ingentis* have extremely low Ca^{2+} levels, which increase during capacitation (Lindsay and Clark, 1992).

In *S. ingentis*, spermatozoa mature in the female thelylicum, where further ultrastructural development within the cell takes place (Clark et al., 1984; Shigekawa and Clark, 1986). It has also been demonstrated for *Trachypenaeus byrdi* and *Xiphopenaeus riveti* (closed thelyca shrimps) that only spermatozoa from females, but not from males, react against conspecific egg water, indicating that further maturation or capacitation is required in seminal receptacle (Alfaro et al., 2003).

In open thelycum shrimps, it has been assumed that spermatophores within terminal ampules contain fully matured and capacitated spermatozoa, but no scientific observations have been published to improve knowledge on this crucial topic. It has been stated that spermatozoa of open thelyca penaeoideans do not appear to undergo capacitation after transfer to the female (Clark and Griffin, 1993), but recently, it has been proposed that

final spermatozoal maturation on the external surface of the thelycum may be required for fertilization in open thelyca shrimp (Alfaro et al., 2003).

1.6. MORPHOLOGICAL EVIDENCES FOR SPERMATOZOON CAPACITATION

It was discovered that a region named filamentous meshwork (FM), located between the nucleus and hemispherical cap of the spermatozoon, develops differently in three closely related species. In *Litopenaeus vannamei*, the FM is synthesized in the male reproductive system, but seems to complete its formation after mating in thelycum. In *L. stylirostris*, the FM region was not present in spermatophores collected from males or in the spermatozoon from thelycum. In *L. occidentalis*, the FM region is fully developed in male spermatozoon (Alfaro et al, 2007).

In *Penaeus monodon*, it was found that the nuclear material of the spermatozoon taken from the female thelycum (T-sperm) is less condensed than that of the spermatozoon taken from the male terminal ampoule of the *vas deferens* (S-sperm) (Aungsuchawan et al., 2011).

Also, for *Litopenaeus vannamei*, the most distinctive difference between the ultrastructure of the S- and T-sperm was in the nuclear material. Chromatin fibers in the nucleus of the T-sperm were more de-condensed than that of the S-sperm. This made the nucleus of T-sperm more vesicular than that of the S-sperm. In most cases, the filamentous meshwork of the T-sperm was larger and denser than those of S-sperm, and a few cases, it was difficult to distinguish the difference; however, it has never been observed that the filamentous meshwork of the S-sperm is larger than that of T-sperm. In the hemispherical rim of cytoplasmic particles containing several small and large electron-dense granules, the T-sperm occasionally contained large vesicles, which were not found in S-sperm. Also, a spiral line on the external surface of the spike and a basal circular line on the external surface of the cap region of S-sperm, especially those that were embedded in the spermatophoric matrix, were observed. The two lines were less prominent in T-sperm, compared with those of S-sperm. The immunofluorescent localization of tyrosine phosphorylation proteins on intact free S- and T-sperm revealed a difference in the area staining positive. S-sperm showed positive fluorescent signals on their plasma membrane and spike, but T-sperm was positive on the plasma membrane and filamentous meshwork, and on the base of spikes of very few spermatozoa. In addition, a bright spot of red fluorescence was present at a central part of the FM of many T-sperm, but not in the S-sperm. The positive signal of tyrosine phosphorylated proteins at the spike was decreased from S- to T-sperm and it disappeared completely when the bright red spot appeared on the FM (Aungsuchawn et al., 2011).

1.7. EGG ULTRASTRUCTURE AND ACTIVATION

The oocyte of lobster is covered by envelope 1. The cortex of oocyte contains four types of vesicles. Following *in vitro* fertilization, contents of granules are released sequentially, and at least two of them coalesced in the perivitelline space to form a new coat called envelope 2 (Talbot and Goudeau, 1988). In crab, *Carcinus maenas*, two populations of cortical vesicles were observed that release their contents during the reaction, successively (Goudeau and Jacqueline, 1982). During egg activation in penaeid shrimp, the cortical rods begin to emerge from the crypts on the periphery of the egg, and elevate the thin investment coat that covers the surface of the egg. Then, the cortical rods begin to break up and form the jelly layer around the egg. Finally, a new coat that is called hatching envelope is form around penaeid eggs (Pongtippatee-Taweepreda et al., 2004).

AIMS

1. To study and compare ultrastructure of spermatozoa in the six species of crayfish including: *Astacus astacus, Astacus leptodactylus, Pacifastacus leniusculus, Austropotamobius torrentium, Orconectes limosus,* and *Procambarus clarkii*.
2. To study the morphological changes of the spermatophore wall and spermatozoon during post-mating storage on the body surface of female of narrow-clawed crayfish *Astacus leptodactylus.*
3. To study egg ultrastructure and its morphological changes during egg activation in noble crayfish *Astacus astacus.*
4. Those species were selected based on their availability and importance for aquaculture industry.

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CHAPTER 2

ULTRASTRUCTURE OF CRAYFISH SPERMATOZOA

2.1. Niksirat, H., Kouba, A., Pšenička, M., Kuklina, Y., Kozák, P., 2013. Ultrastructure of spermatozoa from three genera of crayfish *Orconectes, Procambarus* and *Astacus* (Decapoda: Astacoidea): New findings and comparisons. Zoologischer Anzeiger 252, 226–233.

2.2. Niksirat, H., Kouba, A., Rodina, M., Kozák, P., 2013. Comparative Ultrastructure of the spermatozoa of three crayfish species: *Austropotamobius torrentium*, *Pacifastacus leniusculus*, and *Astacus astacus* (Decapoda: Astacidae). Journal of Morphology 274, 750–758.

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Ultrastructure of spermatozoa from three genera of crayfish *Orconectes*, *Procambarus* and *Astacus* (Decapoda: Astacoidea): New findings and comparisons

Hamid Niksirat*, Antonín Kouba, Martin Pšenička, Iryna Kuklina, 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

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ABSTRACT

Ultrastructure of spermatozoa of three crayfish genera including *Orconectes limosus* (Rafinesque, 1817), *Procambarus clarkii* (Girard, 1852), and *Astacus leptodactylus* (Eschscholtz, 1823) was studied using transmission electron microscopy. The length of the acrosome was significantly greater in *A. leptodactylus* (4.5 ± 0.65 μm) than *O. limosus* (1.7 ± 0.2 μm) and in *O. limosus* compared to *P. clarkii* (1.46 ± 0.16 μm). The width of the acrosome was significantly narrower in *P. clarkii* (2.45 ± 0.2 μm) than *O. limosus* (4.77 ± 0.51 μm) and in *O. limosus* compared to *A. leptodactylus* (8.23 ± 0.9 μm). Also, the length:width ratio was significantly greater in *P. clarkii* (0.6 ± 0.07) than *A. leptodactylus* (0.55 ± 0.08) and in *A. leptodactylus* compared to *O. limosus* (0.36 ± 0.05). The acrosome complex and nucleus are located at the anterior and posterior of the spermatozoon, respectively. The acrosome complex organelle is divided into two main parts: the main body of the acrosome that is a dense inverted cup-shaped structure and organized into three layers of differing electron densities and extended parallel filaments, and the sub-acrosome zone occupying the central part of the acrosome complex, which is divided into two electron dense areas. The spermatozoon of *Orconectes limosus* is described for the first time. In addition, an acrosome spike in the spermatozoon of *Procambarus clarkii* is described. Morphological and biometrical traits of spermatozoa may be used as tools for systematic studies in crayfish, as already reported for many other crustacean taxa.

1. Introduction

Spermatozoal cell ultrastructure has been successfully used in taxonomic and phylogenetic studies across animal taxa, including crustaceans (Felgenhauer and Abele, 1991; Jamieson, 1991,1994; Jamieson et al., 1995a,b; Medina, 1994; Tudge, 1995; Jamieson and Tudge, 2000; Martin and Davis, 2001; Tirelli et al., 2008; Tudge, 2009).

Most of the studies investigating decapods crustacean sperm morphology have been published in the last two decades and cover 100% of the decapod infraorders, 50% of the families, and approximately 10% of the extant genera, but only 2% of the described, extant species (Tudge, 2009).

Apart from its taxonomic and phylogenetic importance, knowledge of spermatozoal morphology can contribute to understanding the complex mechanisms of acrosome reaction and gamete fertilization (Simeó et al., 2010).

The spermatozoa of crustaceans do not swim long distances in the female tract, as is the case with other animals having internal fertilization, or in an aqueous medium as in animals with external fertilization. As a result, decapods crustacean sperm cells lack a true flagellum and are non-motile (Tudge, 2009; Poljaroen et al., 2010).

The acrosome is obviously the most complex organelle in the non-flagellated spermatozoa of decapods and provides the movement necessary for the sperm nucleus to penetrate the oocyte cytoplasm (Brown, 1966). The acrosome has been a focus of attention by researchers, and most of the published research is concentrated on this organelle.

Freshwater crayfish are highly diverse and commercially important animals currently comprising 3 families, 33 genera, and 640 known species (Crandall and Buhay, 2008). Limited research has been carried out on the ultrastructure of crayfish spermatozoa compared to other decapods such as crabs. Different aspects of spermatogenesis studied in *Cambaroides japonicus* (De Haan, 1841) (Kaye et al., 1961; Yasuzumi et al., 1961; Yasuzumi and Lee, 1966). Moses (1961a,b) described spermatogenesis and spermatozoa ultrastructure in *Procambarus clarkii*. Anderson and Ellis (1967) studied acrosome formation, transformation of mitochondria, and development of microtubules in *Cambarus* sp., and López-Camps et al. (1981) examined the spermatozoon ultrastructure of *Astacus astacus*. Beach and Talbot (1987) compared morphology of sperm from two species of *Cherax*. Dudenhausen and Talbot (1979, 1982)

* Corresponding author. Tel.: +420 387 774 603.
E-mail address: niksirat@frov.jcu.cz (H. Niksirat).

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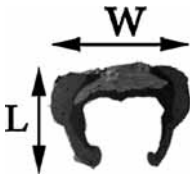

Fig. 1. The dimensions used for measurement of acrosome. *L*: length, *W*: width.

studied spermiogenesis and ultrastructure of mature sperm in *Pacifastacus leniusculus* (Dana, 1852). However, no comparative study of the ultrastructure of crayfish spermatozoa has been conducted among different genera of crayfish.

The aim of the present study was to describe and compare spermatozoal ultrastructure in three crayfish genera including *Orconectes*, *Procambarus* and *Astacus* through biometric and morphological analysis of transmission electron micrographs.

2. Material and methods

Narrow-clawed crayfish (*Astacus leptodactylus*) (Family Astacidae) males were obtained from a pond in South Bohemia (Czech Republic) in autumn 2010. The location is confidential due to conservation reasons. Specimens of spiny-cheek crayfish (*Orconectes limosus*) (Family Cambaridae) were captured in the channel Zlatá Stoka (Třeboň, Czech Republic; 49°0′N, 14°5′E) in early spring 2011. Red swamp crayfish (*Procambarus clarkii*) (Family Cambaridae) were purchased from Singapore in spring 2011. All crayfish appeared healthy and possessed a fully developed reproductive system.

Sperm samples were obtained from 3 males of each genus by electrical stimulation (AC250K2D, Diametral, Czech Republic; Jerry, 2001) or dissection. For dissection, each specimen was anesthetized on ice for at least 10 min until they did not respond to external stimuli. Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 days 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), and embedded in resin. A series of ultra-thin sections were cut using an UCT ultramicrotome (Leica, Microsystem, Japan, Tokyo), double-stained with uranyl acetate and lead citrate, and observed with a 1010 transmission electron microscope (JEOL, Japan, Tokyo) operating at 80 kV. The length (*L*) and width (*W*) of the acrosome and their ratio (*L*:*W*) (Fig. 1, Klaus et al., 2009) were determined from the TEM micrographs using Olympus Micro Image software (version 4.0.1 for Windows). For *P. clarkii*, the length of the acrosome spike was measured separately. Differences were assessed by the Kruskal–Wallis test, with a significance set at $P < 0.05$. The data are presented as means ± s.d.

3. Results

3.1. Comparison of measurements

The length of the acrosome was significantly ($P < 0.05$) greater in *A. leptodactylus* (4.5 ± 0.65 μm, range 3.12–6.50, $n = 98$) than *O. limosus* (1.7 ± 0.2 μm, range 1.34–2.70, $n = 139$) and in *O. limosus* compared to *P. clarkii* (1.46 ± 0.16 μm, range 1.11–1.90, $n = 115$). The width of the acrosome was significantly ($P < 0.05$) narrower in *P. clarkii* (2.45 ± 0.2 μm, range 2.00–2.98) than *O. limosus* (4.77 ± 0.51 μm, range 3.58–5.95) and in *O. limosus* compared to *A. leptodactylus* (8.23 ± 0.9 μm, range 5.52–11.04). Also, the L:W ratio was significantly ($P < 0.05$) higher in *P. clarkii* (0.6 ± 0.07, range 0.47–0.80) than *A. leptodactylus* (0.55 ± 0.08, range 0.41–0.85) and in *A. leptodactylus* compared to *O. limosus* (0.36 ± 0.05, range 0.25–0.56).

3.2. Morphological features

3.2.1. General ultrastructure of the sperm in crayfish

The acrosome complex is located at the anterior of the sperm cell and is divided into two main parts: (1) the main body of the acrosome is a dense inverted cup-shaped structure organized into three layers of differing electron densities and is composed of extended parallel filaments and (2) the subacrosome zone occupying the central area of the acrosome complex, with two separate electron-dense parts. Nucleus is located in the posterior part of sperm cell. Arms are originated from nucleus and wrapped around sperm cell. Whole sperm cell is covered by an extracellular capsule.

3.2.2. Orconectes limosus

The acrosome is visible as a depressed structure at the anterior of the sperm cell (Fig. 2A). Three to four short protrusions are present at the anterior of the acrosome body. Viewed in sagittal section, the two sides of the acrosome extend symmetrically, laterally and posteriorly, and then curve inward as two short arms forming a space within the body of the acrosome called the sub-acrosome zone. Two separate areas in the subacrosome zone are distinguished: an inner electron-dense portion and a thin outer layer of lower electron density. The main body of the acrosome comprises three layers of differing electron density. The middle layer with lower density extends from the base of the acrosome to the anterior protrusions (Fig. 2B). Filaments with a parallel arrangement are detected in this layer (Fig. 2C). This middle layer is enveloped by layers of higher density consisting of two parts and originating at the base of the acrosome. The first part forms the innermost layer and extends toward the anterior of the acrosome filling inside the protrusions of the acrosome crest. The second part of this layer is observed between the middle and outermost layer (Fig. 2B). The outermost layer is more visible in the lower part than in the upper part of the main body of acrosome. The acrosome structure is covered by an electron-dense plasma membrane with the acrosome membrane of lower density beneath it (PM&AM, Fig. 2B). The electron-dense membrane sometimes connects to a mass of dense material consisting of membranous lamella, underneath or lateral sides of the acrosome (Fig. 2D).

In sagittal view, radial arms of differing diameters are located on each side of the acrosome and nucleus. Each arm consists of a sheath randomly filled with numerous microtubules. Signs of penetration of nuclear material into the radial arms are visible at their base (Fig. 2E).

The nucleus is located posterior to the acrosome and bordered by the nuclear envelope and plasma membrane. Dense osmiophilic granules are observed in the nucleoplasm at the junction of chromatin filaments. The entire sperm cell structure was enveloped by a stratified fuzzy extracellular capsule (Fig. 2A and B).

3.2.3. Procambarus clarkii

As in other species, the entire sperm cell is covered by an extracellular capsule. A single projection named spike is present at the anterior of the acrosome and connects to it via an electron lucent platform appearing as a thin white area at its base. The diameter of the spike is greater at its base. An electron lucent area is also observed at the most distal point of the spike. The mean length of the spike found in *P. clarkii* is 0.48 ± 0.2 μm (range 0.23–0.91) (Fig. 3A).

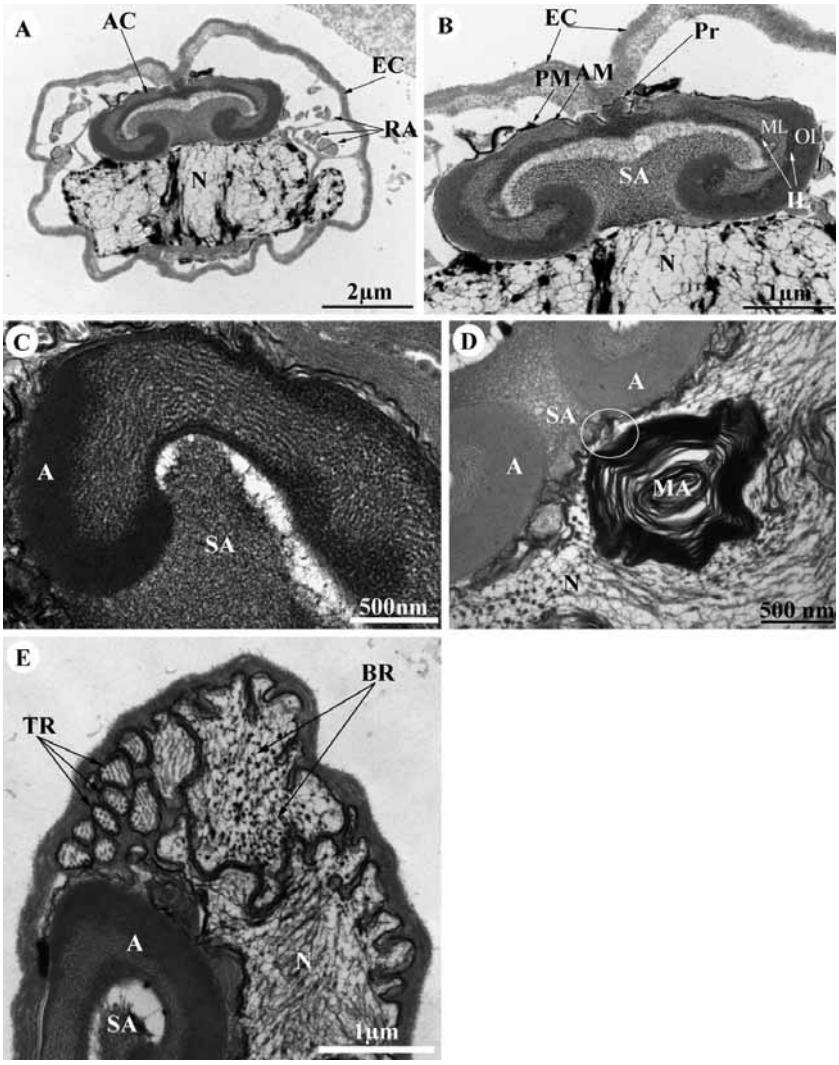

Fig. 2. Transmission electron micrographs of *O. limosus* spermatozoa. (A) Longitudinal sagittal view, (B) acrosome complex including acrosome main body and subacrosome zone, (C) filaments inside main body of acrosome, (D) circle shows connection between membranous lamella and the membrane in the sperm cell, and (E) penetration of chromatin from nucleus inside base of radial arms. A: acrosome main body, AC: acrosome complex, AM: acrosome membrane, BR: base of radial arm, EC: extracellular capsule, IL: innermost layer of acrosome, MA: membranous lamella, ML: middle layer of acrosome, N: nucleus, OL: outermost layer of acrosome, PM: plasma membrane, Pr: protrusion, RA: radial arms, SA: subacrosome zone, TR: tip of radial arms.

The acrosome consists of three layers with the middle layer having lower electron density compared to the other layers. The outermost layer originats at the base of the acrosome and ends at the beginning of the upper part (Fig. 3A and B). Parallel filaments extend from the base of the main body of acrosome toward the base of the spike within the innermost layer of the acrosome (Fig. 3C). The posterior part of subacrosome zone is filled with electron dense material, and in the more anterior region a larger area of lower density is filled with granules and filaments. A dense structure is observed in the anterior-most portion of the sub-acrosome zone (Fig. 3A).

An electron dense membranous lamella (MA) is connected with the dense plasma membrane and is also observed in varying thicknesses around the acrosome (Fig. 3A and D).

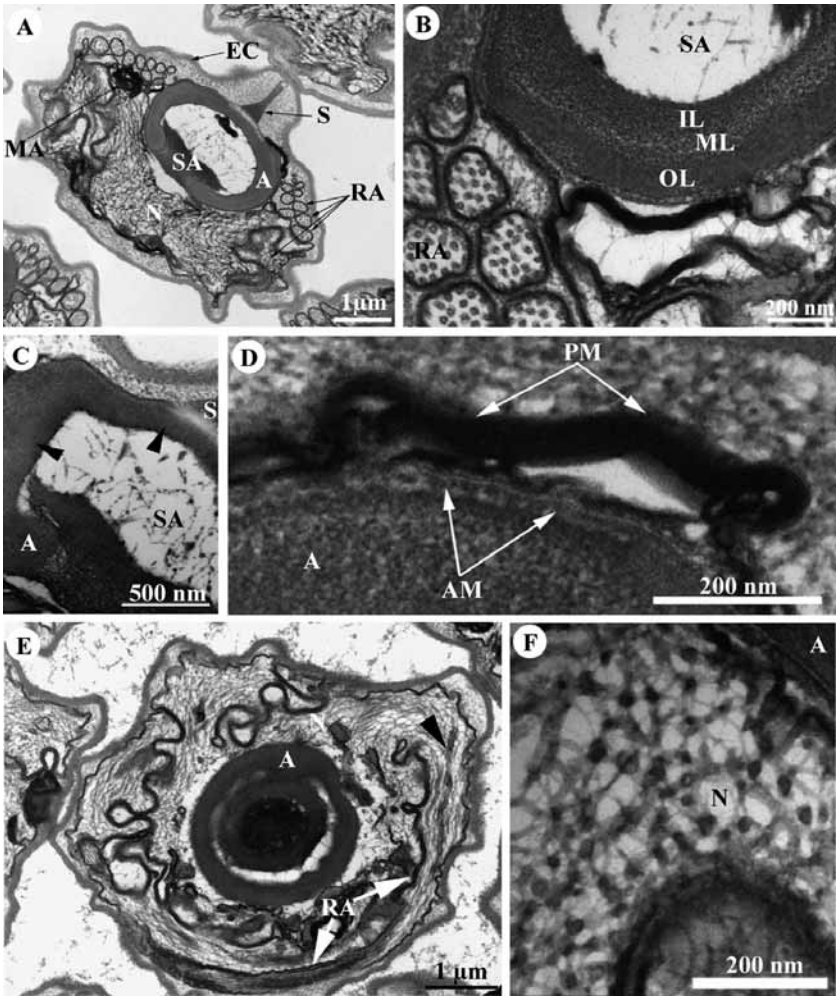

Fig. 3. Transmission electron micrographs of *P. clarkii* spermatozoa. (A) Longitudinal sagittal view, (B) different layers of acrosome, (C) filaments inside the main body of acrosome, (D) membranes of acrosome, (E) sperm cell in cross section, arrowhead showing thin chromatin filaments extending into the base of the radial arms wrapped clockwise around the acrosome (arrowheads), and (F) nucleus. A: acrosome main body, AM: acrosome membrane, EC: extracellular capsule, IL: innermost layer of acrosome, MA: membranous lamella, ML: middle layer of acrosome, N: nucleus, OL: outermost layer of acrosome, PM: plasma membrane, RA: radial arms, S: Spike, SA: subacrosome zone.

The nucleus is located posterior to the acrosome and contains fibers and dense granules (Fig. 3F) and is separated from other parts of the cell by a membrane (Fig. 3A and E).

Radial arms are visible on each side of the acrosome or nucleus in sagittal view (Fig. 3A) and wrap around the spermatozoon (Fig. 3E). Each radial arm consists of microtubules extending along the axes of the radial arms in parallel bundles within a sheath (Fig. 3B). The radial arms are observed to have different diameters in TEM micrographs of sagittal sections (Fig. 3A and B). At the base of each radial arm originating from the nucleus, nuclear materials are seen, but towards the tip microtubules are observed (Fig. 3A and B).

3.2.4. Astacus leptodactylus

The mature spermatozoon of *A. leptodactylus* possesses a large acrosome in the anterior part of cell similar to an inverted cup (Fig. 4A) and exhibiting several zones of varying electron density(Fig. 4B). The innermost layer of the acrosome is electron-dense and extends from the base to an electron-lucent region at the anterior of the acrosome. In this region, filaments are observed

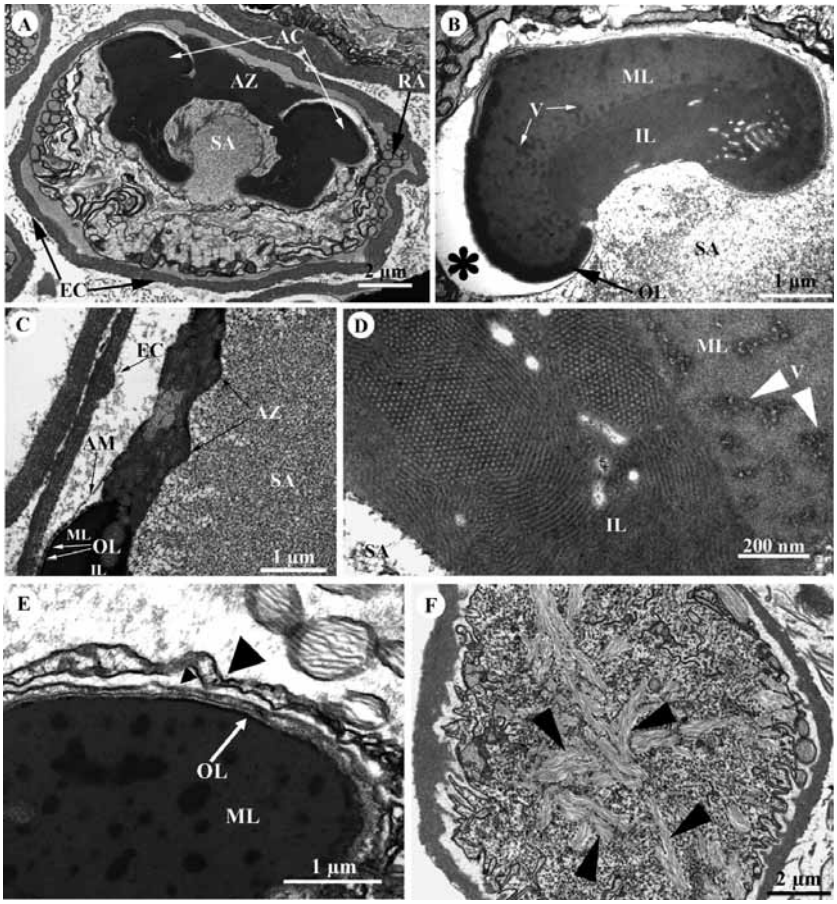

Fig. 4. Transmission electron micrographs of *A. leptodactylus* spermatozoa. (A) Longitudinal sagittal view of sperm cell, (B) different layers of acrosome (star shows the anterior part of acrosome), (C) apical zone located at the anterior part of acrosome, (D) extension of filaments from apical zone into the innermost layer of acrosome, (E) membranes of acrosome (arrowheads), and (F) nuclear components including chromatin and parallel filaments (microtubules) of arms (arrowheads). AC: acrosome, AM: acrosome membrane, AZ: apical zone, IL: innermost layer of acrosome, ML: middle layer of acrosome, N: nucleus, OL: outermost layer of acrosome, EC: extracellular capsule, RA: radial arms, SA: subacrosome zone, V: vesicle.

in bundles arranged in straight or curved patterns similar to a fingerprint within a moderately electron-dense background. The area is covered at the anterior part by a thin membrane (AZ, Fig. 4C). Extensions of these parallel filaments are visible through whole part of the innermost layer (Fig. 4D). In contrast with the other two species described here, the filamentous zone is located in a layer of higher electron density. The middle acrosome layer with lower electron density contains several vesicles (Fig. 4B and D). The outermost layer appears to be formed by the accumulation of these vesicles and is seen as a narrow band beneath the acrosome membrane (Fig. 4B). The subacrosomal zone possesses two distinct regions. Most of the subacrosomal zone is occupied by flocculent and electron-lucent material, but the mass is reduced more in the vicinity of the inner part of the main body of the acrosome (Fig. 4A–C). Parallel layers of osmophilic material membranes cover the main body of the acrosome (Fig. 4E), probably representing at least the acrosome membrane and plasma membrane.

The nucleus contains both fibers and dense granules. Extensions of radial arms penetrate into and pass through the nucleus and are observed as parallel networks of microtubules within granular material of the nucleus (Fig. 4F). An obvious membrane separates the nucleus from the acrosome and subacrosome zone (Fig. 4A).

The spermatozoal cell parts including nucleus, acrosome, and radial arms are covered by a capsule which tightly envelopes cell (Fig. 4A).

4. Discussion

The results of the present study reveal both a conserved general pattern of sperm morphology within the studied group and variability among them at least at the genus level.

Moses (1961b) considered the acrosome of crayfish to be far more complex than the acrosome of species with flagellate sperm. The acrosome is present in all decapods studied to date, except for some of dendrobranchiate shrimp (Aristeidae and Sergestidae) and the basal pleocyemate shrimp *Stenopus*, in the family Stenopodidae. Also, several investigated genera in the order Euphausiacea have acrosome-less spermatozoa (Scelzo and Medina, 2004; Medina et al., 2006; Tudge, 2009).

Jamieson (1991) and Tudge et al. (2001) used length:width ratio of acrosome to divide crustaceans into three different categories including depressed (<1), spherical (1) and elongated (>1). The three genera of crayfish at the present study fall into the depressed acrosome category and shared this depressed shape with a few thoracotreme and heterotreme brachyurans, all investigated podotreme brachyurans, and some astacid, palinurid and enoplometopid lobsters (Jamieson, 1991), and *Pylocheles* (*Bathycheles*) from Anomura (Tudge et al., 2001).

The dimensions of the acrosomes measured here show significant differences within the genera and provide a reliable tool to separate the three crayfish genera. The acrosome in *Procambarus* is smaller and less depressed, while the acrosome of *Astacus* is larger and more depressed than previous one. The acrosome in *Orconectes* is the most depressed with a smaller subacrosomal zone and appears kidney-shaped in 2D micrographs. Acrosomal dimensions and shapes are distinctly different between *Orconectes* and *Procambarus*, although both genera belong to the family Cambaridae (Crandall and Buhay, 2008).

No mitochondria were observed in mature spermatozoa of the crayfish examined. In general, decapod sperm have only small amounts of cytoplasm and, therefore, often low numbers of recognizable organelles. Mitochondria can even appear to be totally absent in mature sperm (Brachyura) or having few recognizable cristate, non-cristate or non-functunal (Tudge, 2009). Since mitochondria in flagellate spermatozoa presumably serve as an energy source for motility (Christen et al., 1987), it is not surprising that they are absent in aflagellate, non-motile, forms (Moses, 1961a), and are used to power the dynamic process of spermiogenesis only, rather than cell motility (Tudge, 2009). Pearson and Walker (1975) showed that activity of cytochrome C oxidase activity as an indicator of mitochondrial activity diminished as mitochondrial morphology changed over spermiogenesis in the crab *Carcinus maenas* (Linnaeus, 1758). Anderson and Ellis (1967) reported that in *Cambarus* sp. during the early spermatid stage some mitochondria lose their matrix and transform into membranous lamella. They proposed that these drastically altered mitochondria are still capable of supplying energy to the spermatozoon. Simeó et al. (2010) reported a similar region in the spermatozoa of *Maja brachydactyla* (Decapoda: Brachyura), called the SO-complex, consisting of membranes, microtubules, and mitochondria and proposed it as an anchor point for chromatin during the acrosomal reaction.

No centrioles are observed in mature spermatozoa of any of the species in this study. In the decapoda with true microtubular arms in sperm cell, microtubuls are grown from centrioles in the cytoplasm of the developing sperm cell, but once they become externalized they appear as either originating from the cytoplasm (e.g., all anomurans studied to date) or from the nucleus (e.g., Thalassinidae, Astacidae, and Palinura). This differing origin may have some phylogenetic significance (Tudge, 1997). On the other hand, many brachyuran crab spermatozoa which mostly do not retain microtubules in the mature sperm cells show a pair of orthogonally arranged centrioles beneath the acrosome vesicle (Tudge, 2009). Anderson and Ellis (1967) found centrioles in the mature spermatozoa of *Cambarus* sp. However, Moses (1961a, b) and Yasuzumi et al. (1961) reported disintegrated centriols by maturity in *P. clarkii* and *Cambaroides*, respectively. Pochon-Masson (1968) did not observe centriols in the mature sperm cell of *Astacus astacus* (Linnaeus, 1758). It seems that these organelles are not functional after crayfish spermatozoa reach maturity and are eliminated. On the other hand, since animals with aflagellate spermatozoa evolved from animals with flagellate spermatozoa (Morrow, 2004), the centrioles in crayfish spermatozoa, may be vestigial structures that have seemingly lost all or most of their original function through evolution.

Several studies carried out on spermatozoal ultrastructure and spermatogenesis in *Procambarus* (Moses, 1961a,b; Hinsch, 1992, 1993a,b) do not report an acrosomal spike. According to our observations, the main part of spike growth takes place in the *vas deferens* and it is absent, or small, in sperm cells inside the testis. This may be the reason why previous studies, that focused on the testis, did not report an acrosomal spike.

A similar acrosomal spike structure has also been reported in *Cambarus* sp. (Anderson and Ellis, 1967) and in *Cambaroides japonicus* (Yasuzumi and Lee, 1966). Also, members of the suborder Pleocyemata, infraorder Caridea, families Palaemonidae (Koehler, 1979), and Pandalidae (Kim et al., 2003) and suborder Dendrobranchiata includes the families Sicyonidae (Shigekawa and Clark, 1986) and Penaeidae (Pongtippatee et al., 2007) and Solenocridae (Medina et al., 1998) exhibit an acrosome spike in the anterior part of sperm cell.

The function of the acrosomal spike in the fertilization process in crayfish is unknown. The acrosomal spike presumably makes initial contact with the egg plasma membrane and substances inside it probably play a key role in egg–sperm interaction during fertilization (Brown, 1966; Anderson and Ellis, 1967). Studies in other crustaceans confirm a critical role during acrosome reaction and fertilization. The acrosome reaction in *Penaeus monodon* (Decapoda, Penaeidae) begins with curling, shortening, and disappearance of the spike (Pongtippatee et al., 2007; Kruevaisayawan et al., 2008).

The present study shows the penetration of nuclear material into the base of the radial arms in *P. clarkii* and *O. limosus*. The appearance of the material they contain suggests their continuity with the body of the nucleus. Simeó et al. (2010) observed that the chromatin fibers of the nucleus extend along the radial arms in spermatozoa of the crab *M. brachydactyla*. This was also previously reported for crayfish (Moses, 1961a,b; Anderson and Ellis, 1967). However, Talbot and Summers (1987) and López-Camps et al. (1981) did not report the extension of nuclear materials into the radial arms in *Panulirus argus* and *Astacus astacus*, respectively. The arms may be composed of nuclear material, or microtubules, or both. In the Anomura, the arms always contain microtubules, while in Brachyura they are composed of nuclear material, except for some members of the Majidae that are reported (Hinsch, 1969, 1973) to have microtubular elements in the nuclear arms. The function of microtubules in crayfish has yet to be determined as the arms appear to be immotile (Jamieson and Tudge, 2000).

The radial arms are present in the Astacidae and Cambaridae but are absent in *Cherax tenuimanus* (Smith, 1912) and *C. albidus* (Beach and Talbot, 1987). These number four in *Cambaroides* and *Procambarus clarkii* but exceed 20 in *P. leonensis* and, five, six, or seven in *Cambarus viridis* (Moses, 1961a,b; Felgenhauer and Abele, 1991; Jamieson and Tudge, 2000). In the testis and *vas deferens* the radial arms are tightly wrapped around the spermatozoon, and the entire spermatozoon inclusive of the arms is enveloped by a capsule. There is evidence that Sertoli cells involved in producing and secreting the mucopolysaccharide capsules (Hinsch, 1993b).

These capsules are thought to confine the radial arms and permit tighter packaging of the sperm in spermatophores (Dudenhausen and Talbot, 1983). This interpretation is supported by absence of such capsules in *Cherax* species where radial arms are lacking (Beach and Talbot, 1987; Vogt, 2002; Hobbs et al., 2007). The radial arms may be involved in the acrosomal reaction (Brown, 1966; Hinsch, 1971; Medina and Rodríguez, 1992) via increasing the contact surface between gametes, which may be necessary to stimulate ion transport that triggers the acrosomal reaction (Nanshan and Luzheng, 1987; Medina and Rodríguez, 1992).

Except for the Remipedia (Yager, 1989), and maxillopodans including Cirripedia, Branchiura, Pentastomida, Mystacocarida and Ascothoracica (Grygier, 1982; Healy & Anderson, 1990; Tudge, 2009), most crustaceans have aflagellate, immotile sperm. Some apparently flagellate crustacean spermatozoa, such as the long and filamentous ostracod, amphipod, mysid, cumacean, and isopod sperm cells, are considered pseudoflagellate, and their tail is most often a long straight extension of the acrosome (Tudge, 2009). Jamieson (1991) referred to this as a pseudoflagellum or striated tail-like appendage and regarded it as a synapomorphy for these peracarids.

Moses (1961a,b) considered mature spermatozoa of crayfish as immotile cells. The microtubules within each arm are not arranged in the 9+2 axonemal pattern typical of the flagellated tails of spermatozoa of other animals. Therefore, they are not responsible for sperm movement (Tudge, 2009; Poljaroen et al., 2010). On the other hand, movement of the processes arms of decapod crustacean spermatozoa was reported by an early researcher (Wilson, 1928). The fact that some motile cilia and flagella do not have the 9+2 arrangement of microtubules indicates that a precise pattern of tubules is not essential for movement (Shapiro et al., 1961). Moreover, nematodes produce aflagellate but motile spermatozoa, and larger spermatozoa of nematodes move more quickly (LaMunyon and Ward, 1998, 1999). To investigate possible movement of aflagellate spermatozoa of crayfish more researches are needed (e.g., an experiment designed using presumed natural inducers such as the secretions produced by female glair glands during oviposition and fertilization).

Since crayfish evolved from older Metazoa possessing motile gametes (Moses, 1961b; Morrow, 2004), the question arises as to why their aflagellate spermatozoa evolved. Morrow (2004) hypothesized that the evolution of aflagellity is associated with a monandrous mating system where competition is absent. In species in which spermatozoa do not compete for fertilization there is no selection pressure on males to produce motile gametes. Thus, sperm cells simply lose their flagella. However, it seems that this does not apply to crayfish, since inter-male competition for a female has been observed in both Cambaridae (Berrill and Arsenault, 1982; Snedden, 1990) and Astacidae (Mason, 1970). More comparative studies of flagellate and aflagellate spermatozoa types are needed to reveal reasons for the difference.

Spermatozoa with fully decondensed chromatin appear to be characteristic of decapod crustaceans. A nucleus with decondensed chromatin could be advantageous, since its nucleus could fuse immediately with the egg's nucleus when it enters the egg (Poljaroen et al., 2010). It makes the nucleus more mobile and flexible for the dynamic and explosive acrosome reaction, where the nucleus passes through the narrow perforatorial chamber inside the acrosome vesicle.

Parallel filaments are observed within the main body of the acrosome of the studied species. This structure might be related to the acrosome reaction. Filaments connected to the base of the acrosome may be responsible for eversion of the acrosome during sperm–egg contact.

The function(s) of many organelles in the sperm cell of crayfish during fertilization are unknown. Therefore, sets of experiments are being planned to explore and clarify the role of each organelle of the sperm cell during the acrosome reaction and fertilization.

5. Conclusion

Despite the overall uniformity in astacoid crayfish spermatozoal structure, remarkable disparities were observed, especially in the morphology and dimensions of the acrosome, that allowed separation of distinct sperm cell patterns in the genera examined. Therefore, measurement of dimensions of the acrosome may be used as methods for systematic studies in crayfish.

The observation of an acrosome spike in spermatozoa of *P. clarkii* raises questions about the process of forming and subsequent function of this structure especially during fertilization process.

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Comparative Ultrastructure of the Spermatozoa of Three Crayfish Species: *Austropotamobius torrentium*, *Pacifastacus leniusculus*, and *Astacus astacus* (Decapoda: Astacidae)

Hamid Niksirat,* Antonín Kouba, Marek Rodina and Pavel Kozák

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

ABSTRACT This study reports about the spermatozoal ultrastructure of three species of astacid crayfish, i.e., the stone crayfish *Austropotamobius torrentium*, signal crayfish *Pacifastacus leniusculus*, and noble crayfish *Astacus astacus*. The acrosome is a cup shaped and electron-dense structure at the anterior of the spermatozoon and comprises three layers of differing electron densities filled with parallel filaments that extend from the base to the apical zone. The acrosome was significantly longer in *A. astacus* than in *P. leniusculus* and the shortest acrosome belongs to *A. torrentium*. The width of the acrosome was significantly narrower in *A. torrentium* than in *P. leniusculus* and the widest acrosome belongs to *A. astacus*. The L:W ratio was significantly greater in *A. torrentium* than in *P. leniusculus* and the lowest ratio belongs to *A. astacus*. Radial arms are visible on each side of the acrosome or nucleus in sagittal view and wrap around the spermatozoon. Each radial arm comprises a parallel bundle of microtubules arranged along the long axis within a sheath. The nucleus, with decondensed material, is located in the posterior of the cell. All parts of the spermatozoon are tightly enclosed within an extracellular capsule. Despite a well-conserved general structure and similarity of pattern among these spermatozoa, differences in the dimensions of the acrosome within the studied species may be useful to help distinguish the different crayfish species. J. Morphol. 274:750–758, 2013.

KEY WORDS: acrosome; decapods; spermatozoon; stone crayfish; transmission electron microscopy

INTRODUCTION

The diversity in spermatozoal morphology within crustaceans, and especially decapods, has long been used as a tool for phylogenetic studies (Felgenhauer and Abele, 1991; Jamieson, 1991, 1994; Medina, 1994; Jamieson et al., 1995a,b; Tudge, 1995; Jamieson and Tudge, 2000; Martin and Davis, 2001; Medina et al., 2006a,b; Tirelli et al., 2008; Klaus et al., 2009; Tudge, 2009; Klaus and Brandis, 2011). Studies during the past two decades include all major clades of the decapodes, and approximately 10% of the extant genera, but only 2% of the described, extant species (Tudge, 2009).

Except for the Remipedia, and Maxillopoda including Cirripedia, Branchiura, Pentastomida, Mystacocarida, and Ascothoracica, most crustaceans have aflagellate, immotile sperm (Grygier, 1982; Yager, 1989; Healy and Anderson, 1990; Tudge, 2009). Some apparently flagellate crustacean sperm cells (Ostracoda, Amphipoda, Mysidacea, Cumacea, and Isopoda) are considered to have a pseudoflagellum (Jamieson 1991; Tudge, 2009).

The most prominent organelle in the spermatozoon of decapods is the acrosome, which is located in the anterior of the cell and has been extensively studied in many species (Jamieson, 1991; Jamieson and Tudge, 2000; Tudge, 2009). The acrosome is present in all decapods with the exception of some of the dendrobranchiate shrimp (Aristeidae and Sergestidae) and the basal pleocyemate shrimp *Stenopus*, in the family Stenopodidae. Also, several genera of the Euphausiacea have spermatozoa lacking an acrosome (Scelzo and Medina,

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*Correspondence to: Hamid Niksirat, Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice Zátiší 728/II, 389 25 Vodňany, Czech Republic.
E-mail: niksirat@frov.jcu.cz

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2004; Medina et al., 2006b; Tudge, 2009; Simeó et al., 2010). The acrosome plays an important role during fertilization. Knowledge of acrosome structure will help us to understand the process of acrosome reaction when the nucleus of the spermatozoon passes through the narrow chamber inside the acrosome vesicle and enters the egg cell (Jamieson and Tudge, 2000).

Freshwater crayfishes comprise a relatively large group of highly diverse and commercially important animals currently comprising 3 families, 33 genera, and 640 known species (Crandall and Buhay, 2008). Several aspects of crayfish spermatogenesis and spermatozoal ultrastructure have been studied. The ultrastructure of spermatogenesis of *Cambaroides japonicus* was studied (Kaye et al., 1961; Yasuzumi et al., 1961; Yasuzumi and Lee, 1966). Moses (1961 a,b) and Anderson and Ellis (1967) illustrated the process of spermatogenesis and spermatozoon ultrastructure in *Procambarus clarkii* and *Cambarus* sp., respectively. The ultrastructure of the spermatozoon of the noble crayfish was described by López-Camps et al. (1981). A comparative study was carried out on the spermatozoa of two species of *Cherax* (Beach and Talbot, 1987). Dudenhausen and Talbot (1979, 1982) studied spermiogenesis and ultrastructure of mature spermatozoon in the signal crayfish. Niksirat et al. (in press) carried out a comparative study on the spermatozoal ultrastructure of the spiny cheek, narrow clawed, and red swamp crayfish. However, the ultrastructure of spermatozoa in many crayfish remains unknown, and more studies are needed to improve our knowledge of crayfish phylogenetic and reproductive biology.

Austropotamobius torrentium and *Astacus astacus* are critically endangered species in Europe (Kozák et al., 2002, 2011), and basic reproductive information is important for future conservation plans. *Pacifastacus leniusculus* is a North American species that was introduced to Europe (Perdikaris et al., 2012). Therefore, the aim of the present study was to provide morphological comparison of the spermatozoal ultrastructure in the three astacid crayfish species: *Austropotamobius torrentium* (Schrank, 1803), *Pacifastacus leniusculus* (Dana, 1852) and *Astacus astacus* (Linnaeus, 1758) through biometric and morphological analysis using transmission electron microscopy.

MATERIALS AND METHODS

Male signal crayfish (n = 3; *Pacifastacus leniusculus* Dana, 1852) were obtained from the Babačka Brook (Sklené nad Oslavou, Czech Republic; 49° 26' N, 16° 2' E). Male noble crayfish (n = 3; *Astacus astacus* Linnaeus, 1758) were collected from the Kramata Reservoir (Hrabice, Czech Republic, 49° 4' N, 13° 43' E) and male stone crayfish (n = 3; *Austropotamobius torrentium* Schrank, 1803) came from the Zubřina Brook (Havlovice, Czech Republic; 49° 25' N, 12° 54' E). Because of the conservation status of noble and stone crayfish, special permissions were required before obtaining sperm samples in autumn (Permission no. NPS 03232/2011 provided by the Administration of the Protective Landscape Area of Šumava for the noble crayfish, and Permission no. ŽP/2450/2011 provided by the Regional office of the Plzeň Region for the stone crayfish). All crayfish appeared healthy and possessed fully developed spermatophores. The native species were returned unharmed to the environment after collection.

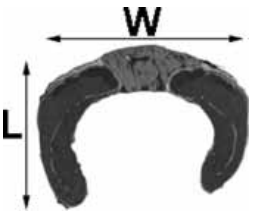

Fig. 1. The dimensions used for measurement of the acrosome. L: Length, W: Width.

We used electrical stimulation (Diametral, Czech Republic; Jerry, 2001) for extrusion of spermatophore. Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer for two days at 4°C, washed in buffer and postfixed 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), and embedded in resin. A series of ultra-thin sections were cut using an UCT ultramicrotome (Leica Microsystem, Wetzlar, Germany), double-stained with uranyl acetate and lead citrate, and observed with a 1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. The length (L) and width (W) of the acrosome and their L:W ratio (Fig. 1, Jamieson, 1991; Klaus et al., 2009) were determined from the TEM micrographs using Olympus Micro Image software (version 4.0.1 for Windows). Differences were assessed by the Kruskal–Wallis test, with significance set at $P<0.05$. The data are presented as means ± s.d.

RESULTS

General Structure of the Spermatozoon in the Crayfish

The acrosome is an electron-dense helmet shaped structure and is located in the anterior part of the spermatozoon. The nucleus with decondensed material is located posterior to the acrosome. The radial arms can be observed at both sides of the cell in sagittal section. An obvious membranous lamellae is observed in the spermatozoa of all studied species. All parts of the sperm cell are covered by an extracellular capsule (Figs. 2A, 3A, and 4A).

Acrosome Dimensions

The acrosome shape is subtly different in each species (Figs. 2A, 3A, and 4A). The length of the acrosome was significantly ($P < 0.01$) longer in *A. astacus* (5.13 ± 0.71 μm, range 3.68–7.26, n = 128 spermatozoa from three males) than in *P. leniusculus* (4.48 ± 0.70 μm, range 3.42–6.74, n = 54 spermatozoa from three males) and in *P. leniusculus*

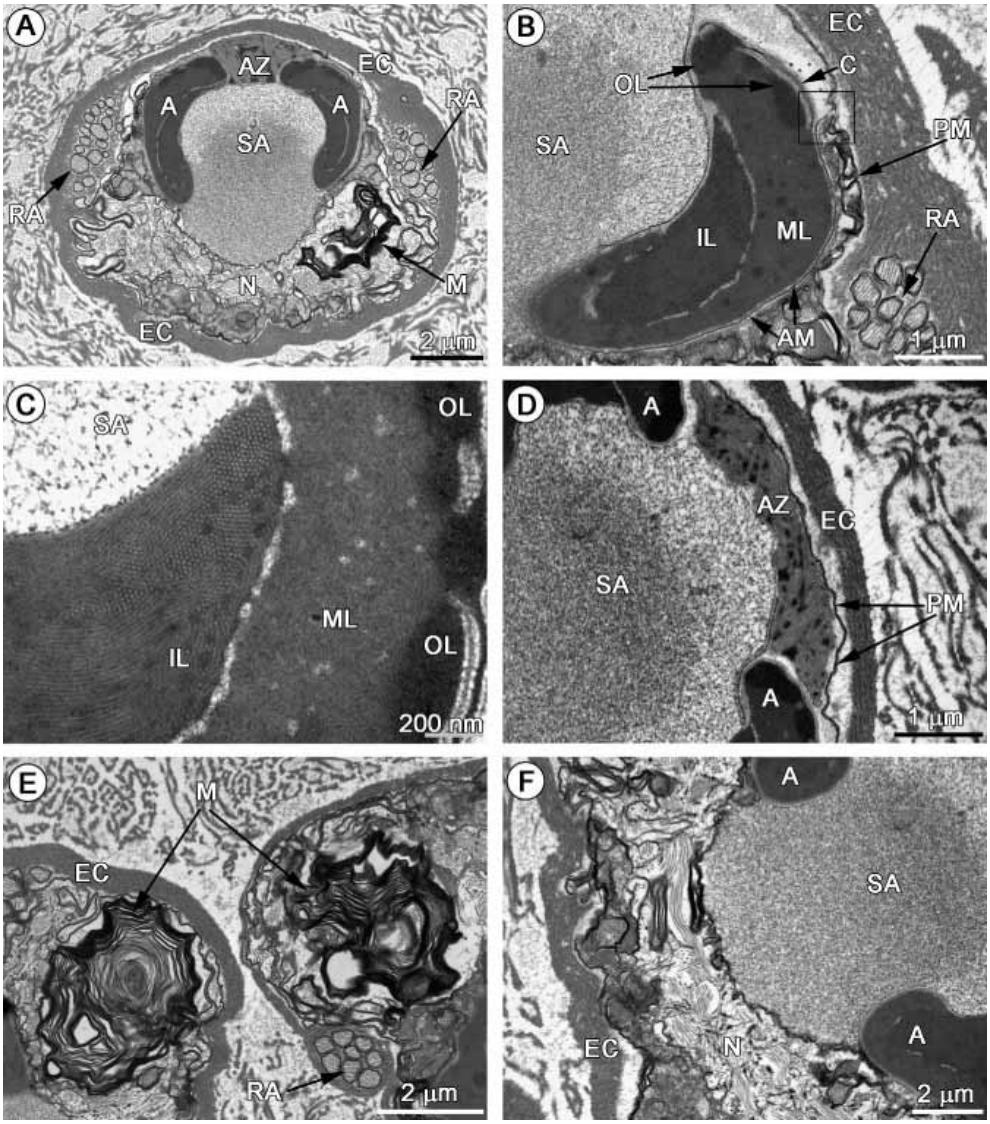

Fig. 2. Transmission electron micrographs of *Austropotamobius torrentium* spermatozoon. **A**: longitudinal sagittal view of the entire spermatozoon, **B**: layers of the main body of the acrosome, square shows the connection between plasma and acrosome membranes. **C**: filaments within the innermost layer of the main body of acrosome, **D**: apical zone, **E**: membranous lamellae of two spermatozoa, **F**: nucleus. A: acrosome main body, AM: acrosome membrane, AZ: apical zone, C: curved structure, EC: extracellular capsule, IL: innermost layer of acrosome, M: membranous lamella, ML: middle layer of acrosome, N: nucleus, OL: outermost layer of acrosome, PM: plasma membrane, RA: radial arms, SA: subacrosome zone.

compared to *A. torrentium* (4.13 ± 0.43 µm, range 3.08–5.53, n = 86 spermatozoa from three males). The width of the acrosome was significantly ($P <$ 0.01) narrower in *A. torrentium* (8.01 ± 0.92 µm, range 5.95–9.95) than in *P. leniusculus* (9.30 ± 1.23 µm, range 7.19–12.10) and in *P. leniusculus* com-

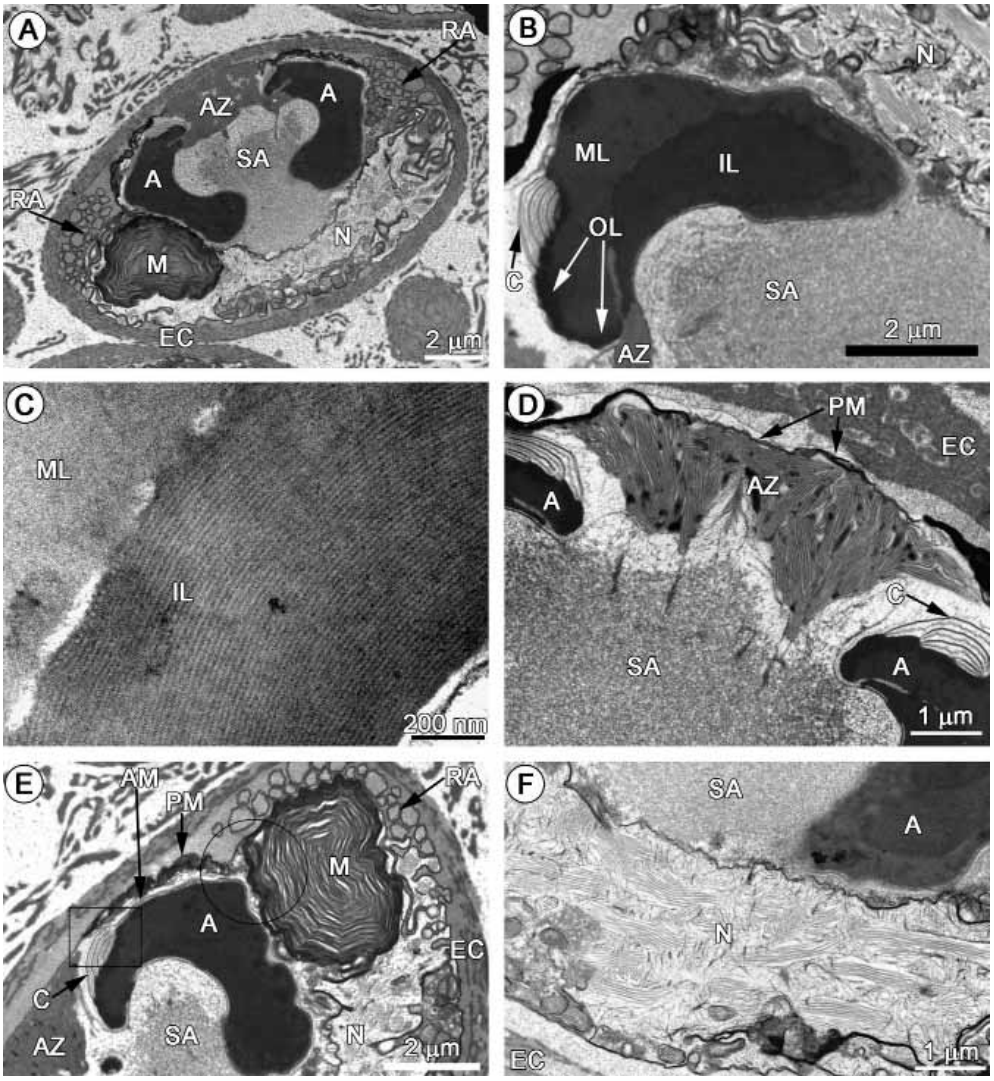

Fig. 3. Transmission electron micrographs of *Astacus astacus* spermatozoon. **A**: longitudinal sagittal view of the entire spermatozoon, **B**: layers of main body of acrosome, **C**: filaments within the innermost layer of main body of acrosome, **D**: apical zone, **E**: membranous lamella, rectangular shows the connection between plasma and acrosome membranes, circle shows the connection between the plasma membrane and membranous lamellae, **F**: nucleus. A: acrosome main body, AM: acrosome membrane, AZ: apical zone, C: curved structures, EC: extracellular capsule, IL: innermost layer of acrosome, M: membranous lamella, ML: middle layer of acrosome, N: nucleus, OL: outermost layer of acrosome, PM: plasma membrane, RA: radial arms, SA: subacrosome zone.

pared to *A. astacus* (11.66 ± 1.05 μm, range 8.48–14.62). The L:W ratio was significantly ($P < 0.01$) higher in *A. torrentium* (0.52 ± 0.06, range 0.37–0.67) than in *P. leniusculus* (0.49 ± 0.08, range 0.32–0.72) and in *P. leniusculus* compared to *A. astacus* (0.44 ± 0.06, range 0.32–0.58).

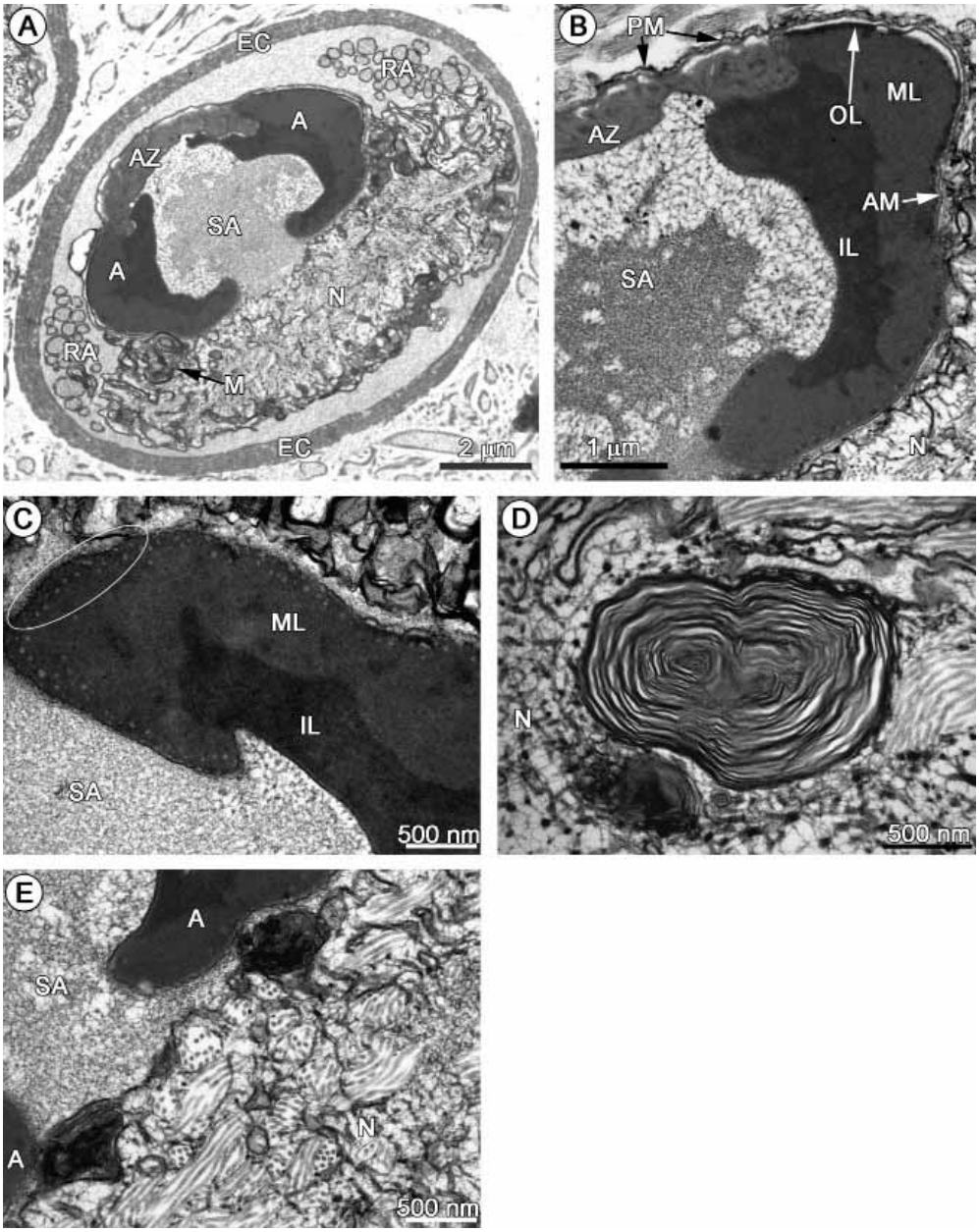

Fig. 4. Transmission electron micrographs of *Pacifastacus leniusculus* spermatozoon. **A**: longitudinal sagittal view of the entire spermatozoon, **B**: layers of main body of the acrosome, **C**: a set of electron lucent pores in the base of main body of the acrosome, **D**: membranous lamella, **E**: nucleus. A: acrosome main body, AM: acrosome membrane, AZ: apical zone, EC: extracellular capsule, IL: innermost layer of acrosome, M: membranous lamella, ML: middle layer of acrosome, N: nucleus, OL: outermost layer of acrosome, PM: plasma membrane, RA: radial arms, SA: subacrosome zone.

Morphological Features

The main body of the acrosome and the subacrosome zone are two distinct parts of the acrosome complex (Figs. 2A, 3A, and 4A). The main body of the acrosome is a dense inverted cup shaped structure located in the anterior of the spermatozoon and consists of three layers of differing electron densities (Figs. 2B, 3B, and 4B). The innermost acrosome layer with high electron density contains parallel filaments (Figs. 2C and 3C) that extend from the base to the anterior of the acrosome where they can be observed as straight or curled lines within a moderately electron dense background referred to as the apical zone (Figs. 2D, 3D, and 4B). The middle acrosome layer with lower electron density contains dense vesicles (Fig. 2B). The outermost acrosome layer with density higher than the middle layer occupies mainly the anterior of the acrosome. In the spermatozoon of *P. leniusculus* a set of electron lucent pores are observed mainly at the margins of the main body of the acrosome base (Fig. 4C).

The subacrosome zone is a chamber filled with flocculent and electron-lucent material with less electron density in the vicinity of main body of acrosome. The subacrosome zone abuts the nucleus (Figs. 2A, 3A, and 4A), but is clearly separated by a double membrane (Figs. 2F, 3F, and 4E).

A conspicuous membranous lamella is an asymmetric structure consisting of both electron lucent and dense concentric lines and is located lateral to or posterior of the acrosome (Figs. 2A,E, 3A,E, and 4A,D) in most spermatozoa. The spermatozoal plasma membrane is continuous with the membranous lamella (Fig. 3E). Another membrane envelops the acrosome which is thinner than the plasma membrane (Figs. 2B, 3E, and 4B). The acrosome membrane is connected to the plasma membrane in the anterior of the acrosome. The connection can be observed in longitudinal section (Figs. 2B and 3E). The transmission electron micrographs show a set of curved structures in the anterior of the acrosome near the apical zone which is more prominent in *A. astacus* than in *A. torrentium* (Figs. 2B and 3B,D,E).

Radial arms are visible on each side of the acrosome or nucleus in sagittal view and wrap around the spermatozoon. Each radial arm comprises parallel bundles of microtubules extending along the axis of the radial arm within a sheath. The radial arms appear to differ in diameter when viewed in sagittal sections (Figs. 2A, 3A, and 4A).

The nucleus with decondensed material is located in the posterior of each spermatozoon. Extensions of the radial arms penetrate into and pass through the nucleus and are observed as parallel networks of microtubules within the granular material of the nucleus (Figs. 2F, 3F, and 4E). An obvious membrane separates the nucleus from other parts of the cell (Figs. 2F, 3F, and 4E). The whole spermatozoon is tightly enclosed within an extracellular capsule (Figs. 2A, 3A, and 4A). The capsular width are 0.4 $\pm$ 0.1 μm, 0.5 $\pm$ 0.2 μm, 0.7 $\pm$ 0.3 μm for *P. lenisculus*, *A. torrentium*, and *A. astacus*, respectively. The cytoplasmic organelles such as centrioles and mitochondria are absent in all studied spermatozoa.

DISCUSSION

The results of the present study reveal similarity in the general spermatozoal ultrastructure, but differences in the dimensions of the acrosome within the studied species that may be useful to help distinguish the different crayfish species. The measurements reveal that while the acrosome in *A. astacus* is the largest, it has the most depressed shape compared to *P. leniusculus* and *A. torrentium*. On the other hand, the acrosome of *A. torrentium* with the smallest size showed a less depressed shape compared to other studied species.

According to the classification suggested by Jamieson (1991) and Tudge et al. (2001), the crayfish in the present study fall into the depressed acrosome category and share this shape with a few thoracotreme and heterotreme brachyurans, all investigated podotreme brachyurans, and some astacid, palinurid, and enoplometopid lobsters (Jamieson, 1991), anomuran *Pylocheles* (*Bathycheles*) (Tudge et al., 2001), and spiny cheek crayfish *Orconectes limosus* (Niksirat et al., in press).

Moreover, the size of acrosome in *Cherax* as a representative of Parastacidae is smaller than the representatives of Astacidae including *Astacus*, *Pacifastacus,* and *Austropotamobius*. The representatives of Cambaridae (*Orconectes* and *Procambarus*) are intermediate between the previously mentioned families (Jamieson, 1991; Niksirat et al., in press).

Also, Klaus and Brandis (2011) reported that the acrosome size is useful to show evolutionary trend in Potamiscinae. They hypothesized that increase in the acrosome size during evolution might be the result of sperm competition. Thus, if larger acrosome would give an advantage in the fertilization of oocytes, selection could drive such an increase in size.

The main body of the acrosome in astacid crayfish consists of a dense inverted cup-shaped structure with a heterogeneous and often striated apical zone in *Astacus, Pacifastacus* (Jamieson and Tudge, 2000) and *Austropotamobius* (present study). This apical differentiation is not observed in the all crayfish spermatozoa. A spike has been reported at the anterior of the acrosome in *Cambaroides japonicus* (Yasuzumi and Lee, 1966), *Cambarus* sp. (Anderson and Ellis, 1967), and *Procambarus clarkii* (Niksirat et al., in press). In *Cherax albidus*, some apical whorled material is present within the vesicle but is absent from

C. tenuimanus (Beach and Talbot, 1987; Jamieson, 1991). Also, some protrusions have been observed in the anterior of the acrosome of *Orconectes limosus* (Niksirat et al., in press).

It seems that the apical zone in these above mentioned crayfish spermatozoa is a clearly differentiated zone that acts as a passage way and allows evacuation and penetration of the subacrosomal, and subsequent nuclear, materials into the egg cell during acrosome reaction and fertilization as observed in other decapods such as the spider crab *Libinia emarginata* (Hinsch, 1971).

The filaments inside the innermost layer of the main body of the acrosome may be important during acrosome reaction. They are probably the source of enzymes that would be in direct contact with the chorion of the egg after reversion of the acrosome (Talbot and Chanmanon, 1980).

The subacrosomal zone is adjacent to the nuclear material. The perforatorial chamber in other decapod spermatozoa is equivalent to this subacrosomal zone. It projects forward and becomes the leading end of the reacted spermatozoon during acrosome reaction in the fiddler crab *Uca tangeri* (Medina and Rodriguez, 1992). Therefore, it seems to draw nuclear material toward the egg. Similar reaction/role is yet to be observed in astacid crayfish.

Although membranous lamellae were observed in the cytoplasm of the spermatozoa in all three species of this study, López-Camps et al. (1981) did not report a similar structure in the crayfish, *Astacus astacus*. Anderson and Ellis (1967) reported that, in *Cambarus* sp. during the early spermatid stage, some mitochondria lose their matrix and transform into membranous lamellae. They proposed that these highly altered mitochondria are still capable of supplying energy to the spermatozoon. André (1962) noted that staining with Janus green B, as an indicator of mitochondria, is strongly positive in this area of the spermatozoon of *Astacus* sp., but the same test was negative in *P. clarkii* (Moses, 1961a,b), *Cancer borealis* (Langreth, 1969), and *Palaemonetes paludoses* (Koehler, 1979). Also, Pearson and Walker (1975) reported that activity of cytochrome C oxidase as an indicator of mitochondrial activity in the spermatozoon of *Carcinus maenas* is absent in the mature spermatozoa. Hinsch (1971) proposed that association between the microtubules and the mitochondria in this region could be an indication of a possible locomotory apparatus of spermatozoa in the superfamily *Oxyrhyncha*. However, no decapod spermatozoon has ever been shown to have any locomotory capabilities (Tudge, 2009).

As in other decapods, the chromatin forms a fine, weakly osmiophilic network of fibrils varying from 20 to 200 Å in crayfish (Moses, 1961a; Yasuzumi and Lee, 1966; Pochon-Masson, 1968). Decondensation of chromatin could be useful during fertilization because it could easily pass through the narrow subacrosomal chamber and fuse immediately with the egg nucleus (Jamieson and Tudge, 2000; Poljaroen et al., 2010).

In the equatorial plane, the nucleus is extended to form radial arms or rays. These number four to six in *Cambaroides* and four to five in *Procambarus clarkii* but exceed 20 in *P. leonensis* and five to seven in *Cambarus viridis* (Moses, 1961a,b; Felgenhauer and Abele, 1991; Jamieson and Tudge, 2000). In *Cherax* species, radial arms are lacking (Beach and Talbot, 1987; Vogt, 2002; Hobbs et al., 2007).

The spermatozoon of crayfish is considered immotile in spite of the presence of radial arms (Moses, 1961a,b). The microtubules within each radial arm of crayfish scatter asymmetrically and do not have the 9 + 2 axonemal pattern typical of the flagellated tails of spermatozoa of other animals, so this may be responsible for the immobility of spermatozoa in crayfish; and in fact, all decapods (Tudge, 2009). Some authors have claimed that the extensive and explosive acrosome reaction seen in decapod sperm cells (Brown 1966; Talbot and Chanmanon, 1980) constitutes a form of cell motility, but even though it appears to annex new ground for the expanding cell, it does not qualify as independent swimming motion typically associated with sperm cell motility (Tudge, 2009).

In conclusion, the present study provides basic information about the spermatozoon ultrastructure of the two critically endangered species. Despite a well-conserved general structure and similarity in the pattern of spermatozoa of different crayfish species, the acrosome dimensions may be useful for separating the studied species. A comparison among the acrosome dimensions of studied crayfish species show that representative of Parastacidae has a smaller acrosome compared to Cambaridae. The representatives of Astacidae show the largest acrosome within three families of the crayfish. In addition, morphological features such as the presence of apical zone in spermatozoon can help for separating of different species.

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CHAPTER 3

POST-MATING MORPHOLOGICAL CHANGES

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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

Corresponding author. Tel.: +420 389 034 745
E-mail address: niksirat@frov.jcu.cz (H. Niksirat)

ABSTRACT

Morphology of the crayfish spermatozoon and spermatophore wall during three stages of final maturation including freshly ejaculated, post-mating, and after spermatozoa release are 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 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 glands secretions. The capsule, plasma membrane, and membranous lamellae are eliminated, and bundles of filaments are released. The subacrosome zone loses electron density and retracts. The electron-dense material of the innermost layer of the acrosome is discharged and, together with 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 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.

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

1. INTRODUCTION

Freshwater crayfishes 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 crayfish, spermatozoa are packaged into spermatophores that function in the transfer of spermatozoa from male to the female during mating. Crayfish spermatophores are deposited either on the ventral surface (in Astacidae and Parastacidae) of the female or into the *annulus 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 La Nostro, 2008), suggesting that final maturation of a decapod spermatozoon takes place in the seminal receptacle of the female (Alfaro et al., 2003; Alfaro et al., 2007; Aungsuchawan et al., 2011; Vanichviriyakit et al., 2004).

Study of the biology of reproduction in the crayfish can help development of new techniques for artificial reproduction in aquaculture. Additionally, studies of the spermatozoon biology in animals such as decapods will expand our understanding of evolutionary conservation and alteration of pathways of reproduction process. Furthermore, 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 (*Astacus leptodactylus*) were obtained from a flooded gravel pit in South Bohemia, Czech Republic in autumn and transferred to the research facilities and kept in 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. Samples of the second post-mating stage were obtained from 3 females after their natural glair secretions and release of spermatozoa from the spermatophore.

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, Microsystem, Gmbh, Austria), 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).

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 the 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. 2b, c) and granules (Figs. 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 separates 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 (Figs. 3a, 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 (Figs. 3c, d). The acrosome complex is divided into 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 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 and acrosome membranes 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 (Figs. 3i, j). The spermatozoon is tightly enclosed by an extracellular capsule. The space surrounding spermatozoa is occupied by fibrous component originating from spermatozoal capsules. Those fibers form the extracellular matrix (Fig. 3k, l).

3.4. Ultrastructural changes of the spermatophore wall after mating

At this time 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, b). The matrix of the outer spermatophore layer is reticulated and the granules release their content (Figs. 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 happened 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 (Figs.5b, c). The density of subacrosome is increased in the vicinity of the main body of the acrosome (Figs. 5d, e). The 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, 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 (Figs. 6b, 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 filaments, forms a droplet/filament structure in the anterior part of the spermatozoon (Figs. 6d, 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 (Figs. 6g, h). The nuclear material of the spermatozoon at this stage is less condensed than those of spermatozoa of earlier stages (Fig. 6i).

Morphological changes during three stages of reproduction in the spermatophore wall and the spermatozoon of narrow-clawed crayfish *A. leptodactylus* are summarized in Tab. 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). 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 additional functional significance in crayfish. The final maturation of spermatozoa of ticks (*Ornithodoros moubata* and *Dermacentore variabilis*), 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). The factor responsible for maturation in spermatozoa of those species is a polypeptide from the male accessory gland with molecular weight of about 12.5 kDa. The mode of action of this factor is hormone-like, and a few minutes exposure of spermatozoa to the factor is sufficient to trigger the entire maturation sequence, which takes 12–24 h *in vitro* (Shepherd et al., 1982). In the nematode *Ascaris suum*, 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 serrata* (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 to 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 of spermatozoa.

The hardening of the spermatophore wall after mating probably promotes spermatozoon survival in the presence of environmental stressors. 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*, that hardening results from phenolic tanning, an enzymatic cross-linking reaction associated 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 body surface of the female, and after release of spermatozoa.

In the post-mating spermatozoon of the shrimp *Sicyonia ingentis* 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., 2013 a, 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*, 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 *Litopenaeus 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 end of the reacted spermatozoon during acrosome reaction in the fiddler crab, *Uca tangeri* (Medina and Rodriguez, 1992), apparently drawing nuclear material towards 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, 1961 a, 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 back and starts secreting from her 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 rich 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 and, 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*, 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.

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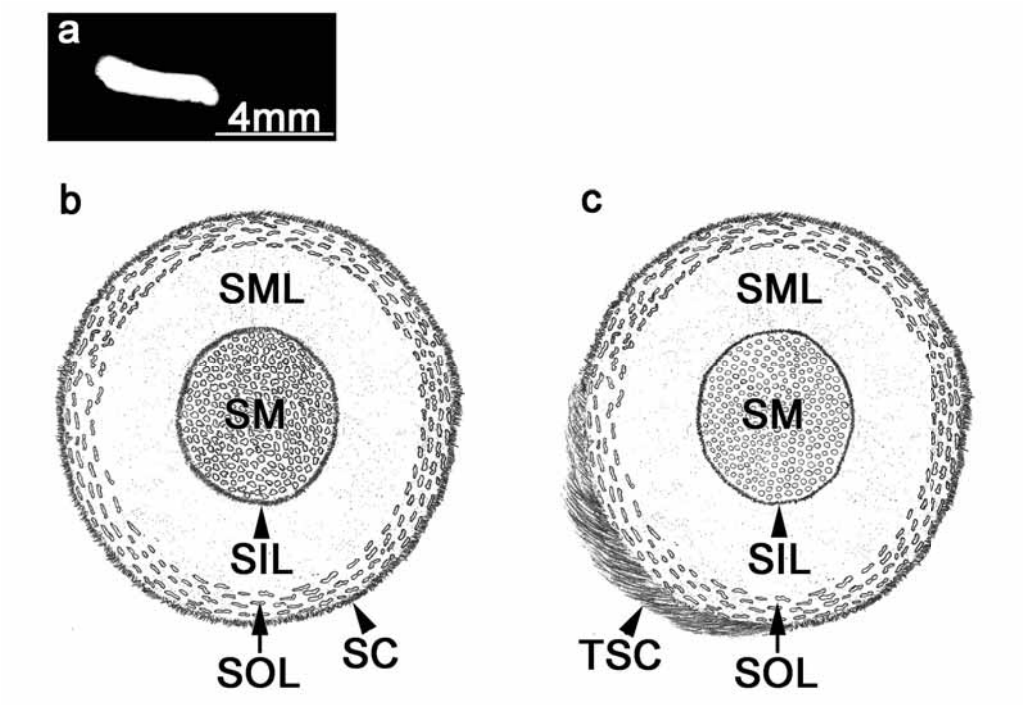

Fig. 1. *a – A general view of the narrow-clawed crayfish spermatophore; schematic drawings of the cross section of the spermatophore, b – before, and c – after mating. The sticky coat of the spermatophore is thickened at the site of attachment. SC: sticky coat; SM: sperm mass; SIL: spermatophore inner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

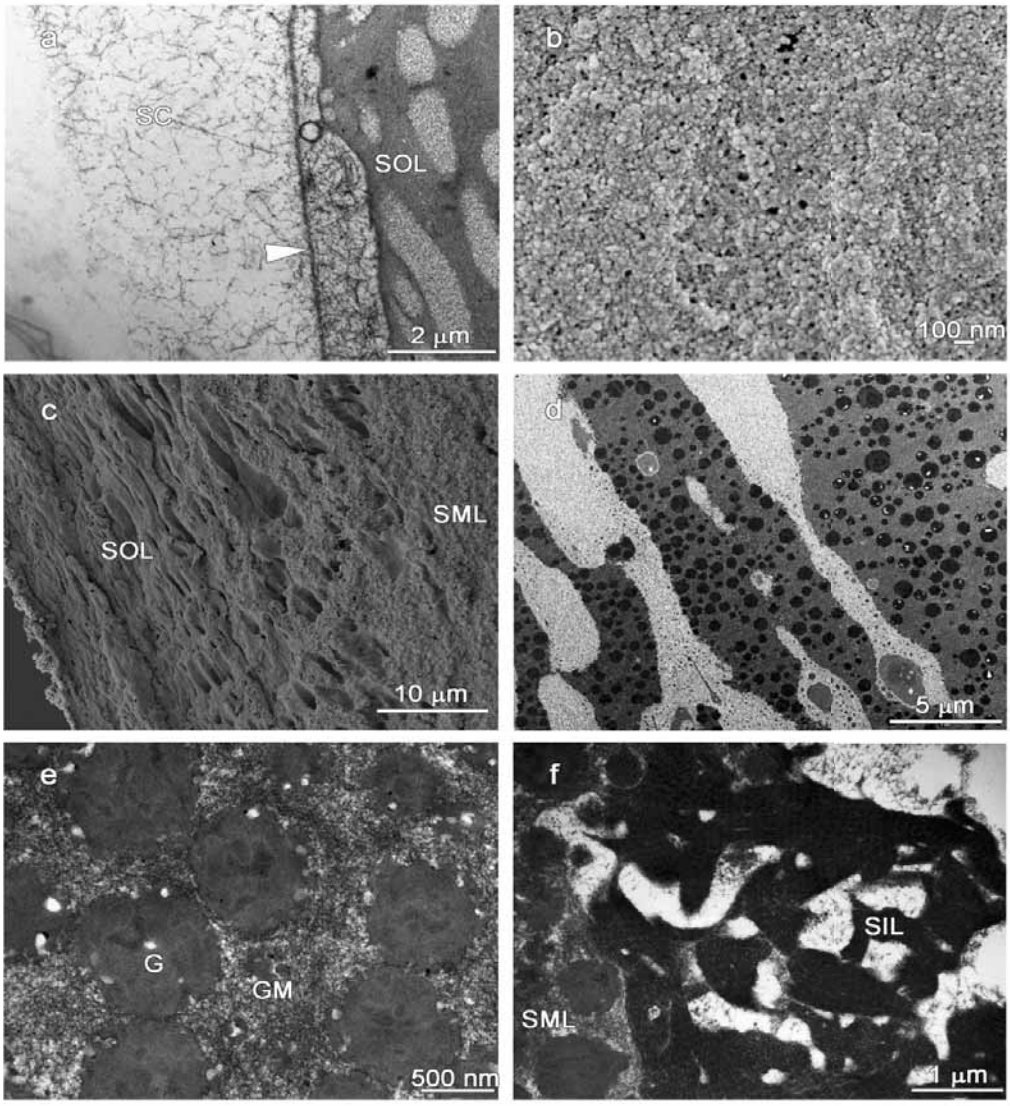

Fig. 2. *Micrographs of the wall layers of freshly ejaculated spermatophore of A. leptodactylus. a – transmission electron micrograph of the sticky coat of the spermatophore outer layer originating from elongated electron-lucent bodies. Arrow head shows the part which is described in Fig. 2b; b – scanning electron micrograph of small particles and pores beneath the sticky coat of the spermatophore outer layer; c – scanning electron micrograph of the elongated bodies in the spermatophore outer layer; d – transmission electron micrograph of granules and elongated electron-lucent bodies in the spermatophore outer layer; e – transmission electron micrograph of the granules in the spermatophore middle layer; f – transmission electron micrograph of 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.*

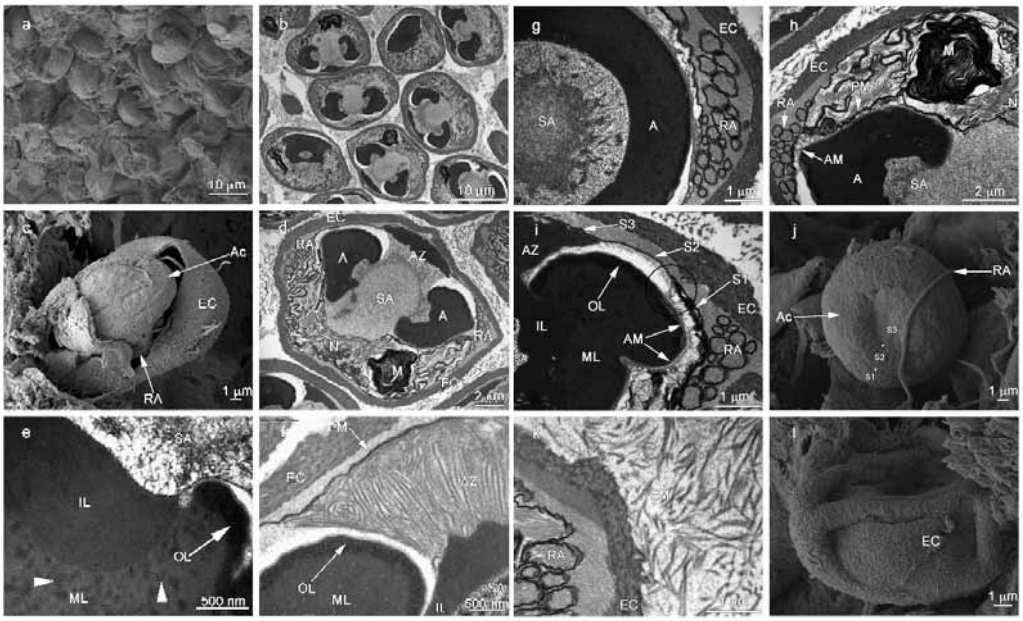

Fig. 3. *Micrographs of the freshly ejaculated spermatozoa of A. leptodactylus. a – scanning electron micrograph of spermatozoa within the spermatophore; b – transmission electron micrograph of spermatozoa within the spermatophore; c – scanning electron micrograph of anterior lateral view of a single spermatozoon with artificially broken extracellular capsule. Note the exposed acrosome and radial arms; d – transmission electron micrograph of the spermatozoon sagittal section showing contents of the spermatozoon; e – transmission electron micrograph of the 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 – transmission electron micrograph of the apical zone in the anterior part of the spermatozoon; g – transmission electron micrograph of the spermatozoon cross section showing the acrosome main body and subacrosome zone; h – transmission electron micrograph of the membranous lamellae and its connection to the plasma membrane; i – transmission electron micrograph of the sagittal section of different sub-domains of plasma membrane and its connection with the acrosome membrane (circle); j – scanning electron micrograph of 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 – transmission electron micrograph of the extracellular capsule and fibrous components originating from spermatozoal capsules and form the extracellular matrix; l – scanning electron micrograph of 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.*

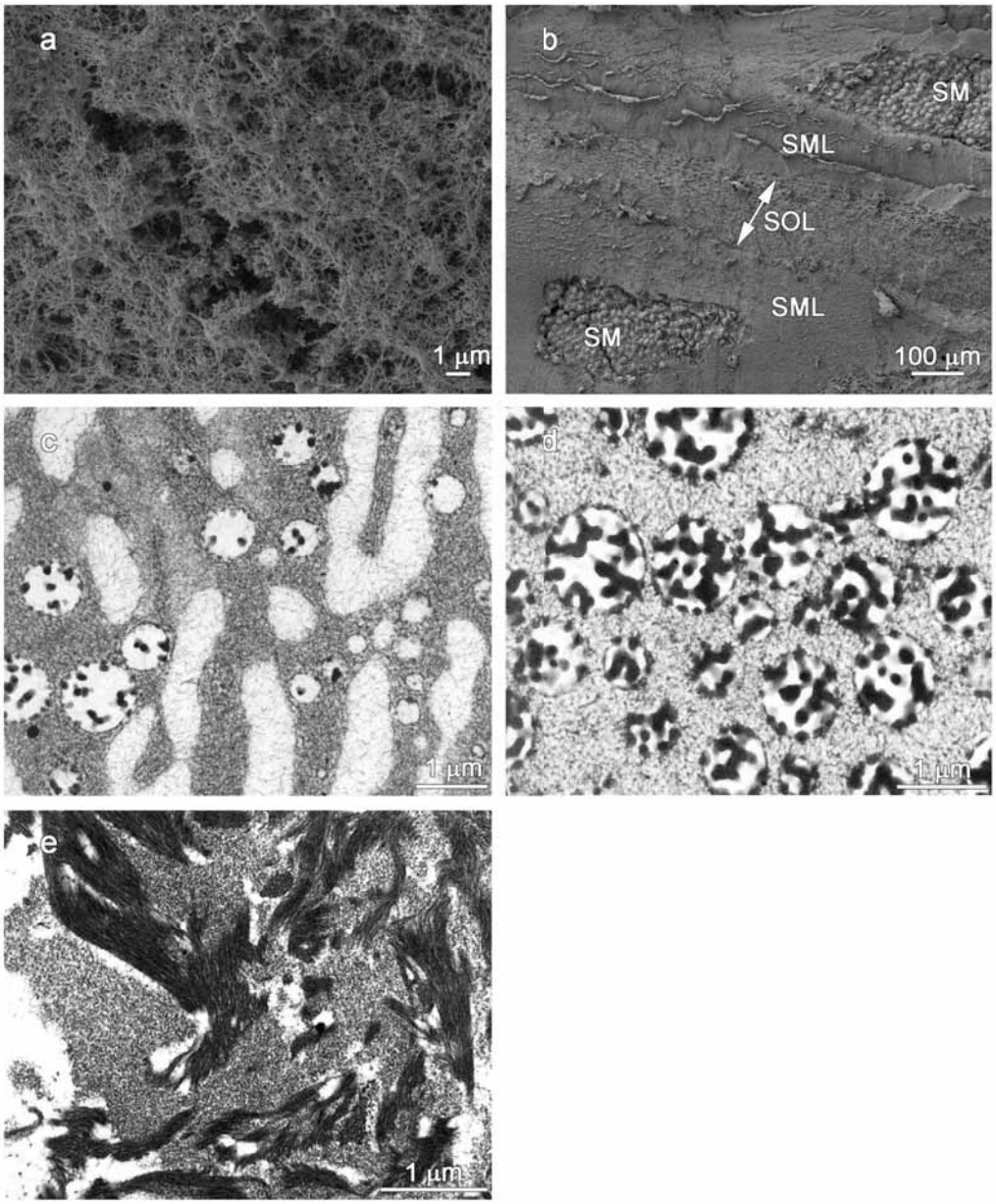

Fig. 4. *Micrographs of the spermatophore wall layers of A. leptodactylus after mating. a – scanning electron micrograph of the thickened sticky coat of the spermatophore outer layer; b – scanning electron micrograph of the section of the jointed outer layers of two adjacent spermatophores; c – transmission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

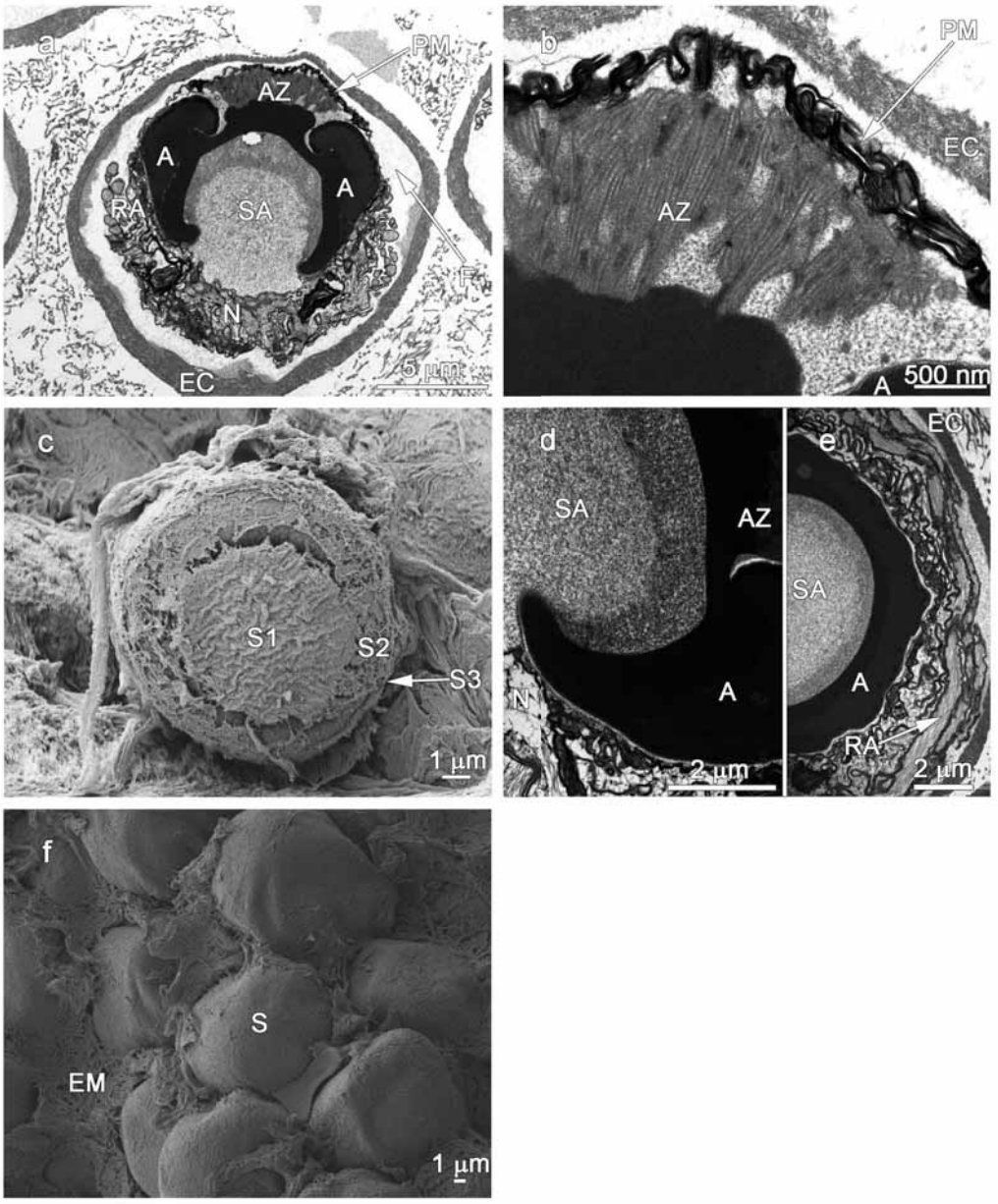

Fig. 5. *Micrographs of the spermatozoon of A. leptodactylus after mating. a – transmission electron micrograph of the general view of the spermatozoon after mating; b – transmission electron micrograph of the wrinkled plasma membrane in the anterior part of the spermatozoon; c – scanning electron micrograph of the wrinkled plasma membrane in the anterior part of the spermatozoon with artificially broken extracellular capsule; d, e – transmission electron micrographs of the sagittal and cross sections of subacrosome zone showing increased subacrosome density in the vicinity of the acrosome main body; f – scanning electron micrograph of 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.*

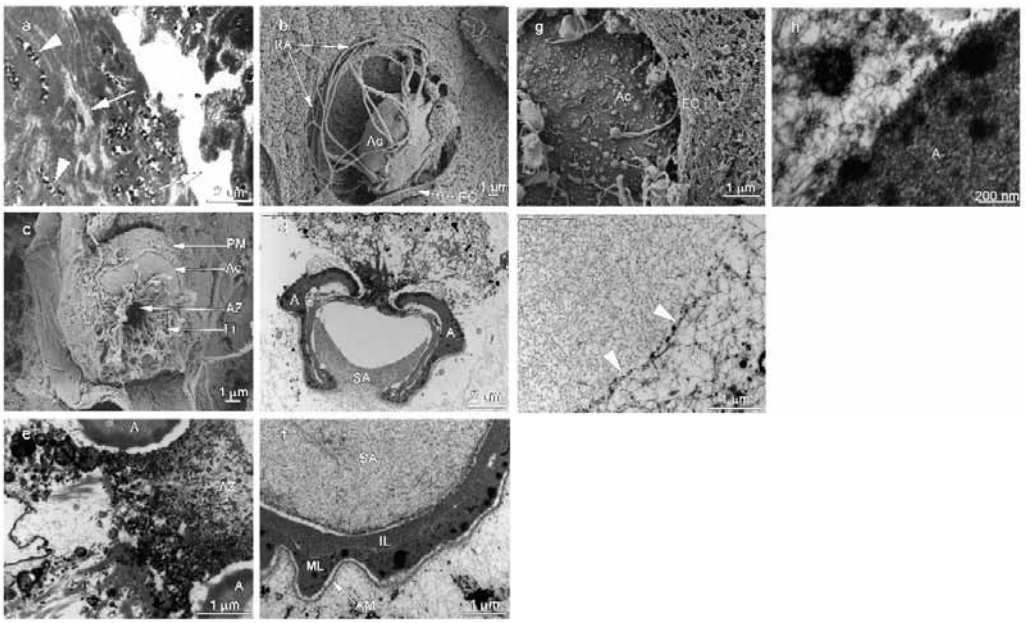

Fig. 6. *Micrographs of the spermatophore wall and the spermatozoon of A. leptodactylus after release of spermatozoa. a – transmission electron micrograph of distorted granules (arrowheads) within the dissolving wall of the spermatophore. Arrows show examples of two places which were penetrated and dissolved by glair secretions; b – scanning electron micrograph of the spermatozoon released from extracellular capsule. Radial arms are visible; c – scanning electron micrograph of the acrosome anterior part showing cavity appeared after release of spermatozoon from its extracellular capsule; d – transmission electron micrograph of the sagittal section of a free spermatozoon showing the filament/droplet structure of the acrosome and retracted subacrosome zone; e – transmission electron micrograph of the electron-dense material from innermost layer of the acrosome discharging towards outside via apical zone; f – transmission electron micrograph of the cross section of acrosome layers and subacrosome zone after discharge of electron-dense material; g – scanning electron micrograph of the anterior view of the acrosome surface. Numerous droplets showing its secretion; h – transmission electron micrograph of the acrosome sagittal section revealing the acrosomal secretion activity. Note the electron-dense vesicles close to the inner acrosome surface (right lower part of the micrograph) and their extruded contents outside the acrosome (left upper part of the micrograph); i – transmission electron micrograph of 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.*

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 increased and joined together	- 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	- consistent and consists of three sub-domains	- 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

CHAPTER 1

EGG ACTIVATION

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EGG ACTIVATION AND CORTICAL REACTION IN THE NOBLE CRAYFISH *ASTACUS ASTACUS*

Hamid Niksirat*, Antonín Kouba, Pavel Kozák

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

**Corresponding author's email address: niksirat@frov.jcu.cz*

ABSTRACT

Morphological changes in the oocyte of noble crayfish during four stages were studied. The ovarian mature oocytes are surrounded by first envelope that is formed by two different layers. Numerous pores are visible on the surface of outer layer of first envelope. The inner layer of the first envelope contains bottlebrush-shaped objects. Three types of cortical vesicles including high and moderate electron dense vesicle and multi-layered vesicle were observed in the cortex of the oocytes. The pores were gradually disappeared from outer layer of first envelope in freshly ovulated oocytes. The bottlebrush-shaped objected in the inner layer of first envelope were replaced by a spongy shaped electron lucent texture containing scattered electron dense vesicles. After one hour of spawning, the first envelope especially its inner layer started to be condensed. The high and moderate dense vesicles discharge their contents into perivitelline space forming second envelope around the oocyte. After 24 hours of ovulation, second envelope can be observed in previtelline space and a part of oocyte cortex. The high and moderate electron dense vesicles still can be observed to mix their contents to form second envelope. Also, results of present study showed that attachment stalk is derived from outer layer of first envelope of crayfish oocyte. In conclusion, fertilization coat in noble crayfish comprises from first and second envelopes that are constructed by follicular cells of ovary and cortical reaction of vesicles in the oocyte cortex, respectively.

Keyword: *attachment stalk, egg envelope, egg cortex, fertilization coat, perivitelline space*

1. INTRODUCTION

Freshwater crayfishes 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 astaciculture, use of several methods such as artificial incubation may lead to better production rates, provided costs are economically justifiable (Reynolds, 2002). During spawning, female crayfish lies back and positions her ventral surface up. Eggs are directed into brood chamber formed by curling of the abdomen toward openings of oviduct in the base of third walking legs. The brood chamber already is filled by secretion from glair glands. Eggs are attached to the body of female till hatching (Andrews, 1906). Study of the biology of reproduction in the crayfish spermatozoa (Tudge, 2009; Niksirat et al., 2013a, b) and eggs (Pérez et al., 1999; Nakata and Goshima, 2004; Nakata et al., 2004; Sáez-Royuela et al., 2009;

Kouba et al., 2012) can provide new knowledge for development of new techniques and approaches to solve the problems of artificial reproduction in aquaculture. Egg activation is believed to be responsible for the prevention of polyspermy, and may also establish a microenvironment to support embryo development (Clark et al., 1980; Wong and Wessel, 2006). Morphological changes during egg activation have been reported in several decapods such as *Penaeus aztecus* (Clark et al., 1980), *Sicyonia ingentis* (Pillai and Clark, 1990), *Homarus americanus* and *H. gammarus* (Talbot and Goudeau, 1988), *Trachypenaeus similis* and *Sicyonia ingentis* (Lynn et al., 1992), and *Penaeus monodon* (Pongtippatee-Taweepreda et al., 2004). An ultrastructural study of egg activation and water hardening can provide detailed information that might be beneficial for the improvement of methods of the crayfish artificial reproduction.

Therefore, the aim of the present study was to investigate the morphological changes during egg activation in noble crayfish *Astacus astacus* (Linnaeus, 1758), using transmission and scanning electron microscopy.

2. MATERIALS AND METHODS

Noble crayfish (*Astacus astacus* Linnaeus, 1758) were collected from the Kramata Reservoir (Hrabice, Czech Republic) shortly before beginning of reproduction season, and transferred to research facilities that consist of outdoor unites supplied with natural light and temprature conditions.

Mature ovarian oocytes samples were obtained from females after ten days of their natural mating. For dissection, three females were anesthetized on ice for at least 10 min until they did not respond to external stimuli. Freshly ovulated oocytes were collected from the base of third walking legs of three females during their natural spawning. Additional samples of oocytes were obtained after approximately 1 and 24 hours of spawning from abdominal part of female crayfish body.

Samples for transmission electron microscopy 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 cryo-scanning electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48 h at 4 °C. Samples were dried carefully by filter paper and glued-on an aluminum target which was mounted on a holder for 10 mm stubs. The sample was extremely fast (< 10^{-3} K/s) frozen in slushy nitrogen. After freezing the samples were transferred to a high vacuum preparation chamber (ALTO 2500, Gatan). The surfaces of the samples were then sublimated by -95 °C for 2 minutes. After sublimation the samples were coated with 3 nm layer of platina at a temperature of -135˚C. Coated samples were inserted into the chamber of the Field Emission Scanning Electron Microscope (JSM-7401F, JEOL). Images were obtained by the secondary electron signal at an accelerating voltage of 2 kV using GB low mode.

3. RESULTS

Ultrastructure of cortical vesicles:

The cortex of ovarian oocyte contains three types of vesicles. The high and moderate electron dense vesicles were observed in different sizes (Figs. 1a, b). Small high electron dense particles are visible within the texture of moderate electron dense vesicles (Fig. 1b). Also, multi-layered vesicles containing moderate electron dense materials are scattered in this part of the oocyte (Fig. 1c). The space between different vesicles is filled with a grainy matrix when oocytes are in the ovary (Fig. 1d).

Ultrastructure of first envelope in mature ovarian oocyte

Follicle layer is visible surrounding oocyte. Mature ovarian oocytes are covered by first envelop with different diameters (Fig. 2a). First envelope can be divided into two layers according to their electron densities. Outer layer of the first envelope is formed by a high density material and contains pores on the surface (Fig. 2b). Inner layer of first envelope contains elements resemble to bottle brushes that are located within a grainy matrix (Fig. 2c). The perivitelline space is located between first envelope and cortex of oocyte and contains several protrusions extended from the cortex (Fig. 2d).

Ultrastructure of the first envelope in freshly ovulated crayfish oocyte

Freshly ovulated oocyte of noble crayfish shows approximately an identical diameter in its first envelope. The pores of outer layer of first envelope gradually are disappeared. The bottle brushes objects are replaced by a spongy electron lucent texture with scattered electron dense granules that altogether form inner layer of the first envelope of freshly ovulated oocyte (Figs. 3a, b). The grainy matrix that fills empty spaces between cortical vesicles is gradually disappeared in the freshly ovulated oocyte (Fig. 3c).

Ultrastructure of the first envelope in crayfish oocyte after one hour of spawning, and formation of second envelope by cortical reaction

The first envelope especially its inner layer started to be condensed. The protrusions extended from cortex into the perivitelline space are no longer visible (Fig. 4a). The high electron dense vesicles started to be de-condensed and release their contents that consist of small dense granules (Fig. 4b). Also, moderate electron dense vesicles are divided into smaller vesicles (Fig. 4c). Those electron dense and lucent particles cross plasma membrane into perivitelline space and seem to mix each other to construct second envelope beneath the first one (Fig. 4d).

Ultrastructure of fertilization envelope and attachment stalk in crayfish oocyte after 24 hours of spawning

The thickness of layers of first envelope decreased compared to freshly ovulated oocyte as a result of condensation that caused by formation of second envelope. Second envelope as a thick layer appears heterogeneous initially but gradually condenses to form a compact homogeneous layer. Second envelope was developed in the perivitelline space and in the

outer part of cortex (Figs. 5a, b). The plasma membrane is visible within this newly formed layer (Fig. 5c). The granules released from electron dense vesicle mix with smaller particles of electron lucent vesicles to form second envelope (Figs. 5d–f). The attachment stalk is derived from first envelope (Figs. 6a–c).

4. DISCUSSION

The results of present study showed the morphological modification of first envelope of crayfish egg and formation of a second envelope in perivitelline space and a part of egg cortex after egg activation.

Egg activation was described in some members of decapods. During egg activation in penaeid shrimp, the cortical rods begin to emerge from the crypts on the periphery of the egg, and elevate the thin investment coat that covers the surface of the egg. Then, the cortical rods begin to break up and form the jelly layer around the egg. Finally, a new coat that is called hatching envelope is form around penaeid eggs (Pongtippatee-Taweepreda et al., 2004). In contrast, in lobster a new layer that is called envelope 2 is formed beneath envelope 1 by cortical reaction of cortical vesicles located in the egg cortex (Talbot and Goudeau, 1988). Therefore, egg activation in crayfish shows more similarities to lobsters rather than other decapods such as shrimps.

All metazoans that reproduce through the fusion of two haploid gametes have adopted a means to establish a post-fertilization barrier between the zygote and the environment, because successful embryonic development is enhanced when the zygote is protected from lethal agents such as additional spermatozoa (polyspermy) and microorganisms including parasites etc. (Wong and Wessel, 2006). In some of decapods such as lobsters fertilization is very fast (Tsai and Talbot, 1993), implying the presence of a rapid mechanism for blocking of polyspermy. There are evidences for an electrical polyspermy block in eggs of decapods (Gould and Stephano, 2003). This barrier is established by a shift in the egg membrane potential following spaermatozoon entry in lobster *Homarus gammarus* and crab *Maia squinado* (Goudeau and Goudeau, 1986; Goudeau and Goudeau, 1989a). In the crab, the egg membrane potential shift is very rapid and occurs in less than 400 msec that can stop entering additional spermatozoa to fertilized egg. The egg membrane can keep the new potential at least for 5 hours (Goudeau and Goudeau, 1989b), the time that seems to be enough for construction of a permanent barrier by means of subsequent cortical vesicle reaction. Since crabs and lobsters have a close phylogenetic relationship with crayfish, electrical barrier could also be possible for crayfish because no immediate morphological changes detected in our micrographs for polyspermy prevention.

Also, our results showed that the cortex of noble crayfish egg consists of three types of vesicles that at least two of them actively participate in the formation of second envelope after fertilization. Cortical vesicles are secretory organelles synthesized during oogenesis, concentrated in the outermost region of the egg's cortex, subjacent to the plasma membrane and released following egg-spermatozoon fusion (Wong and Wessel, 2006). Two and four types of cortical vesicles were reported from hourseshoe crab *Limulus polyphemus* (Bannon and Brown, 1980), and lobster (Talbot and Goudeau, 1988), respectively. Second envelope in the egg of crab *Carcinus maenas* is formed from one cortical granule product (Goudeau and LaChaise, 1980a). Also, it has been shown that in lobster cortical granules are released sequentially after fertilization and the contents of two of these granules are shown to coalesce in the perivitelline space to form a new coat that fuses with the original ovarian coat and forms the fertilization envelope (Talbot and Goudeau, 1988). However, our observations on the egg

of noble crayfish show that cortical vesicles release their contents simultaneously throughout formation of second envelope that can be considered as a species-specific difference with lobster.

Our observation showed that cortical vesicle reaction and releasing of their content can last for at least 24 hours in the noble crayfish, suggesting that the contribution of these vesicles, to an immediate physical block to polyspermy is negligible, and more likely it mainly protects the prolonged development of embryo while attached to the pleopods as suggested by Talbot and Goudeau (1988) in case of lobster. However, removal of spermatozoon receptors from egg surface by enzymes released from cortical granules following fertilization was reported in several animals (reviewed in Wong and Wessel, 2006), that indicates the possible involvement of early cortical vesicle secretion in establishing a protective barrier against polyspermy.

In crayfish fertilized eggs are attached to the pleopods of female until hatching (Andrews, 1906). Our results showed that attachment stalk is derived from first envelope of egg in noble crayfish. Some researchers speculated that fertilized eggs of decapods are attached to the body of female by cement secreted from female's secretory glands at the time of egg-laying (Andrews, 1906; Yonge, 1937; Stephens, 1952; Aiken and Waddy, 1982). For example, Fisher and Clark (1983) reported that the outermost coat around pleopodal eggs of the shrimp, *Paleamon macrodactylus* is originated from a secretion of the pleopods. In contrast, other studies indicated that attachment stalk in decapods is originated from mature eggs after fertilization (Cheung, 1966, Goudeau and LaChaise, 1980b, 1983; Saigusa et al., 2002). During egg attachment in the lobster, the pleopods beat vigorously and cause envelope 1 to stretch and form attachment stalks. Beating probably also causes the attachment stalks to twist and wrap around the ovigerous setae (Goudeau et al., 1987).

The first envelope of crayfish ovarian oocyte contains bottlebrush-like objects that are disappeared after spawning. Similar objects with same pattern of disappearance were observed in the envelope of lobster oocyte. These objects are synthesized in follicular cells and transferred to oocyte envelope during spermatogenesis (Talbot and Goudeau, 1988).

Numerous pores were observed on the surface of noble crayfish oocyte that gradually disappeared after spawning. Same structures were reported from the lobster oocyte surface, processes from the oocyte and follicle cells extend into these pores prior to ovulation (Talbot and Goudeau, 1988). This observation implies the involvement of these pores in the oogenesis.

We observed that freshly ovulated crayfish eggs are very sensitive and can be easily broken during handling for artificial reproduction purposes, and eggs should be retained on the body surface of female till completion of water hardening before transferring to artificial incubators.

In conclusion, our results showed that oocyte of crayfish is surrounded by first envelope, and its cortex contains three types of vesicles. Extensive morphological changes in the crayfish oocyte were observed during activation. Second envelope is formed by cortical reaction in the pervitelline space and a part of oocyte cortex. Cortical reaction last at least for 24 hours. Fertilization coat in noble crayfish comprises from first and second envelopes that are constructed by follicular cells of ovary and cortical reaction of vesicles in the oocyte cortex, respectively. In addition, results of present study showed that the attachment stalk is derived from first envelope of crayfish oocyte. The finding of this research offers basic knowledge about egg activation in crayfish that can be helpful for development and improvement of artificial reproduction of crayfish in aquaculture industry.

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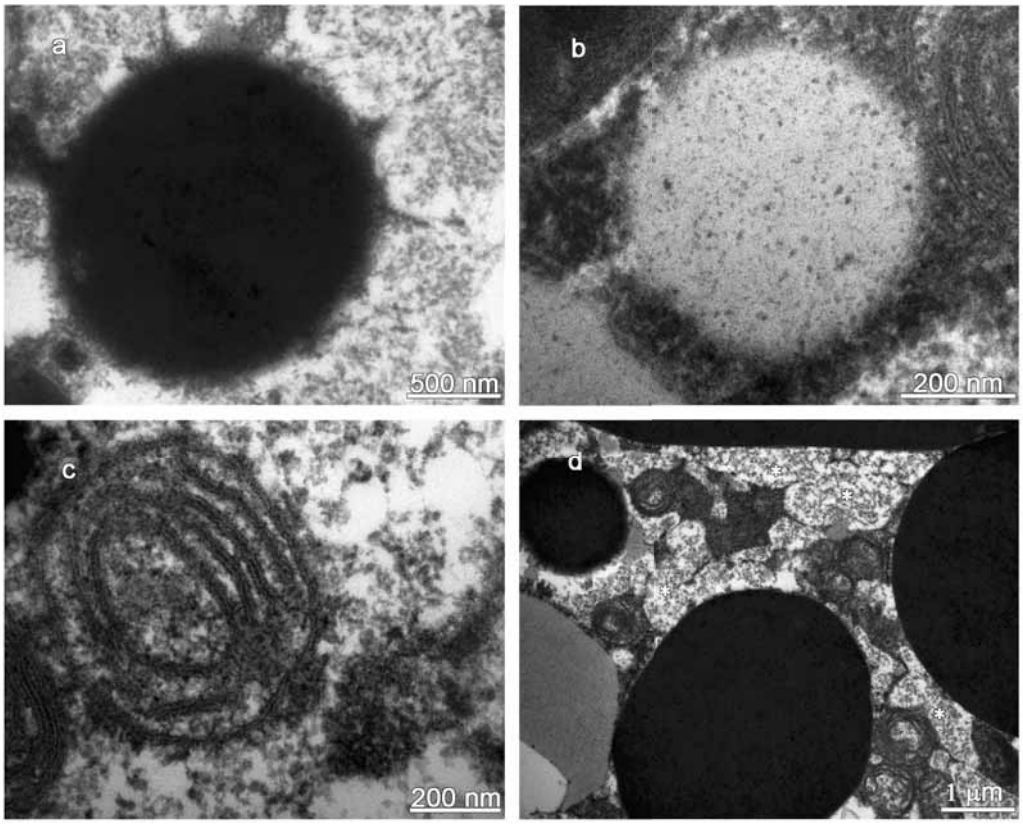

Fig. 1. *Transmission electron micrographs of different vesicles in the cortex of noble crayfish oocyte, a – high electron dense vesicle, b – moderate electron dense vesicle, c – multi-layered vesicle, d – different types of vesicles in the cortex of an ovarian oocyte, stars show the grainy matrix that fills space between vesicles.*

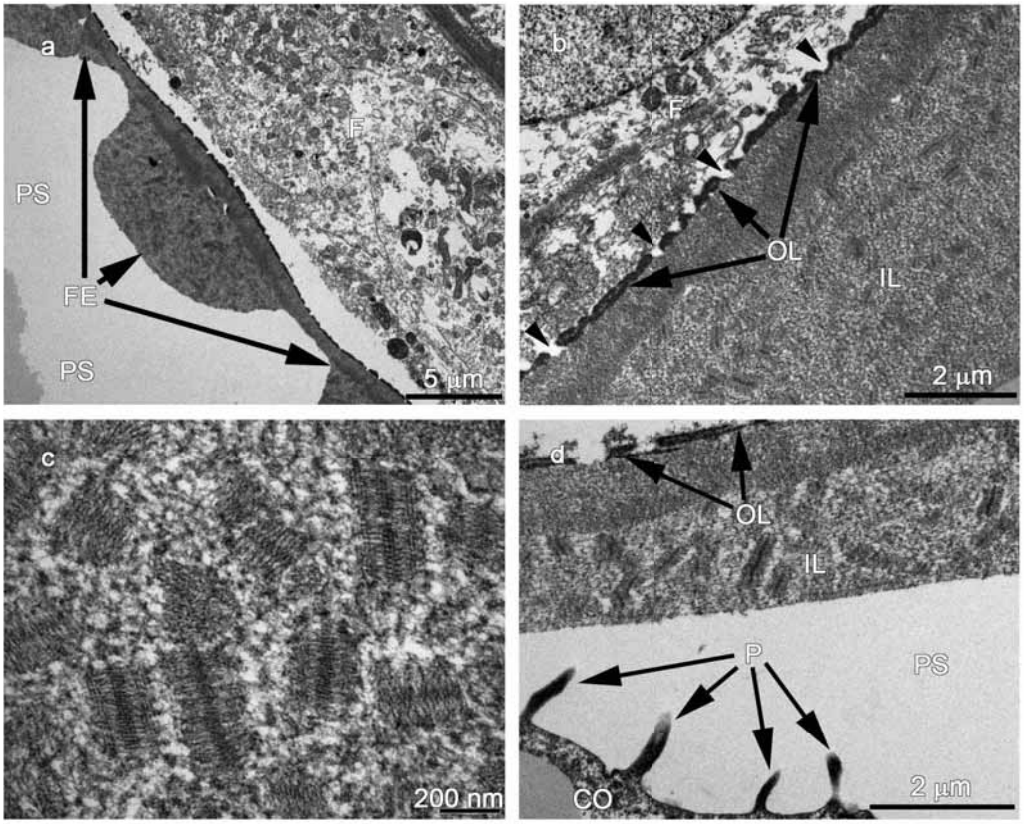

Fig. 2. *Transmission electron micrographs of the ovarian oocyte of noble crayfish, a – first envelope with different diameters with follicular layer cover oocyte, b – a micrograph with higher magnification from first envelope, arrowheads show pores in the outer layer of first envelope, c – bottlebrush objects in the inner layer of the first envelope of oocyte, d – protrusions in the oocyte cortex are extended into the perivitelline space. CO: cortex of oocyte, F: follicular layer, FE: first envelope, IL: inner layer of the first envelope, OL: outer layer of the first envelope, P: protrusion, PS: perivitelline space.*

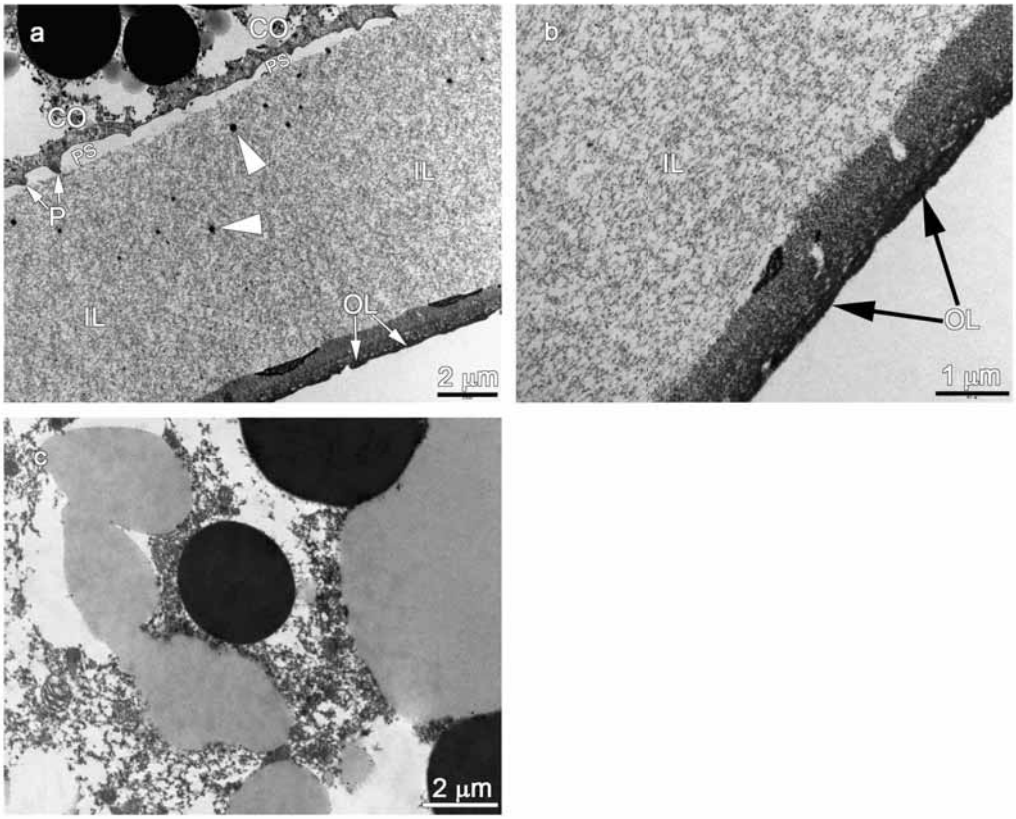

Fig. 3. *Transmission electron micrographs of the noble crayfish freshly ovulated oocyte, a – first envelope and cortex of the freshly ovulated oocyte of crayfish, arrowheads show electron dense granules in the inner layer of first envelope, b – higher magnification micrograph of the spongy electron lucent of inner layer and electron dense outer layer of first envelope, c – grainy matrix that fills space between cortical vesicles gradually is disappeared in the freshly ovulated oocyte. CO: cortex of oocyte, IL: inner layer of the first envelope, OL: outer layer of the first envelope, P: protrusion, PS: perivitelline space.*

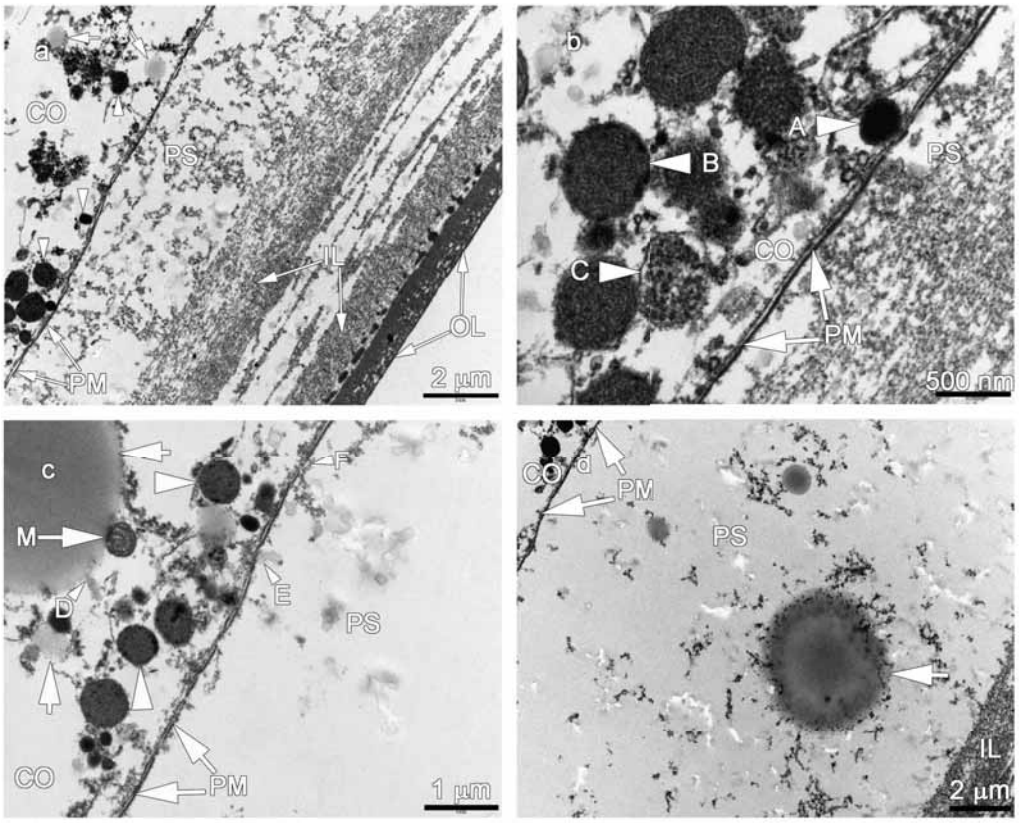

Fig. 4. *Transmission electron micrographs of the noble crayfish oocyte after one hour of spawning, a – condensation of first envelope, elimination of protrusions of the cortex, and cortical reaction, arrows and arrowheads show moderate and high electron dense vesicles in the cortex of egg, respectively, b – electron dense vesicles in different states of condensations, A, B and C show gradual decondansation of electron dense vesicle, c – arrows and arrowheads show moderate and high electron dense vesicles in the cortex of egg, respectively. Arrowhead D shows the place that a smaller size moderate electron dense vesicle is separated from a bigger moderate electron dense vesicle. Arrowheads F and E show places that contents of the high and moderate electron dense vesicles cross plasma membrane into the perivitelline space, respectively, d – arrow shows the aggregation of high and moderate electron dense vesicles in the perivitelline space. CO: cortex of oocyte, IL: inner layer of the first envelope, M: multi-layered vesicle, OL: outer layer of the first envelope, PM: plasma membrane, PS: perivitelline space.*

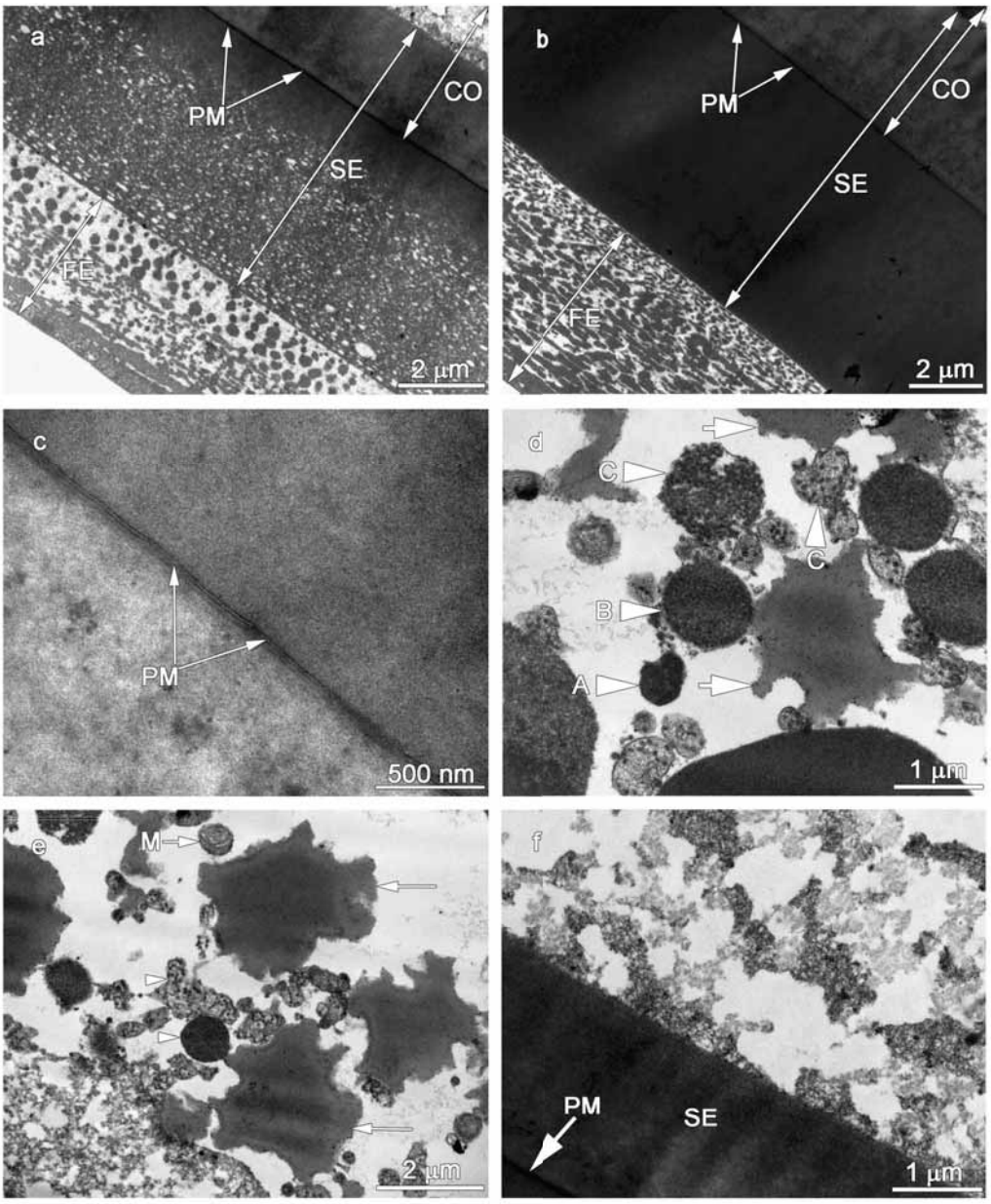

Fig. 5. *Transmission electron micrographs of the noble crayfish oocyte after 24 hours of spawning, a – development of second envelope in the perivitelline space and a part of cortex, b – a fully developed second envelope with a compact homogeneous layer, c – arrows show plasma membrane inside the second envelope, d – arrows and arrowheads show moderate and high electron dense vesicles in the cortex of egg, respectively. A, B and C show gradual de-condensation of electron dense vesicle, e – contents released by high and moderate electron dense vesicles are visible in lower-left side of the micrograph. Arrows and arrowheads show moderate and high electron dense vesicles in the cortex of egg, respectively, f – contents of high and moderate electron dense vesicles are forming the second envelope. CO: cortex of oocyte, FE: first envelope, PM: plasma membrane, SE: second envelope.*

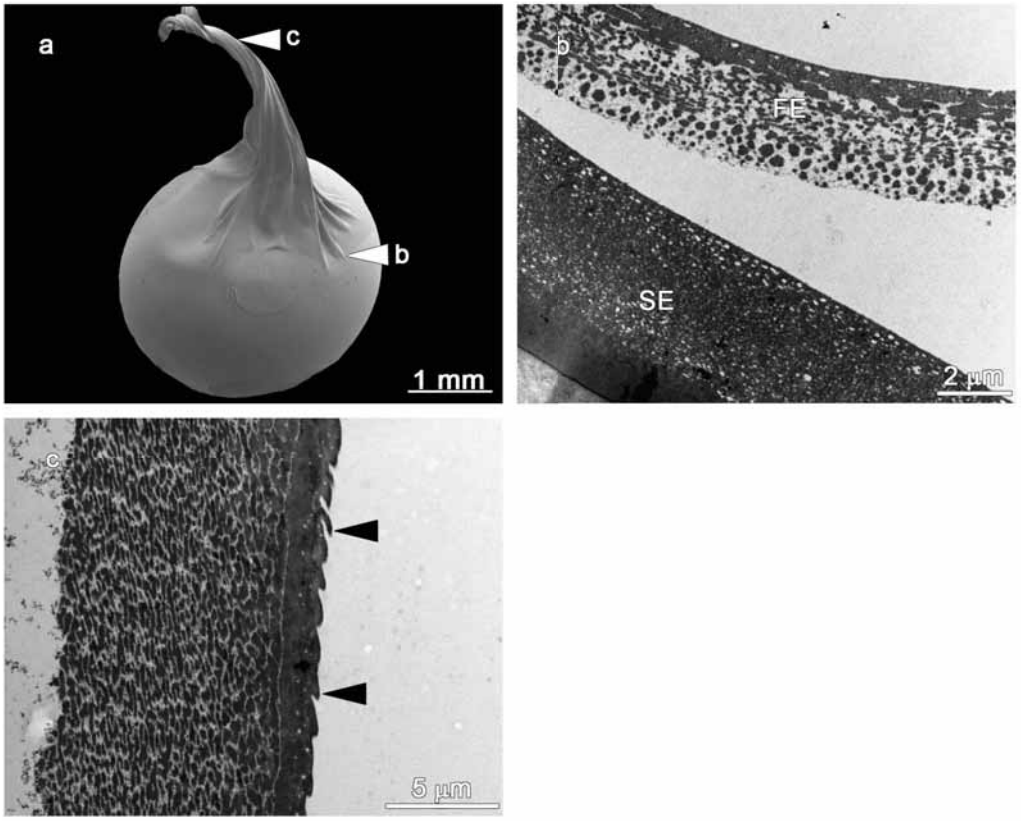

Fig. 6. *Scanning and transmission electron micrographs of attachment stalk in the oocyte of the noble crayfish. a – a fully developed attachment stalk, places showed by arrowheads a and b are described with more details in the next micrographs of this figure, b – the place that the attachment stalk is derived from first envelope, c – ultrastructure of upper part of attachment stalk, arrowheads show serrated margin on the surface of attachment stalk. FE: first envelope, SE: second envelope.*

CHAPTER 5

GENERAL DISCUSSION

Spermatozoal cell ultrastructure has been successfully used in taxonomic and phylogenetic studies across animal taxa, including crustaceans (Felgenhauer and Abele, 1991; Jamieson, 1991, 1994; Jamieson et al., 1995a, b; Medina, 1994; Tudge, 1995; Jamieson and Tudge, 2000; Martin and Davis, 2001; Tirelli et al., 2008; Tudge, 2009). Most of the studies investigating decapods crustacean spermatozoal morphology have been published in the last two decades and cover 100% of the decapod infraorders, 50% of the families, and approximately 10% of the extant genera, but only 2% of the described, extant species (Tudge, 2009). We reported dimensions and morphological features of spermatozoa from six species of freshwater crayfish including *Astacus astacus, Astacus leptodactylus, Pacifastacus leniusculus, Austropotamobius torrentium, Orconectes limosus, Procambarus clarkii*. Jamieson (1991) and Tudge et al. (2001) used length:width ratio of acrosome to divide crustaceans into three different categories including depressed (< 1), spherical (1) and elongated (> 1). The crayfish in the present study fall into the depressed acrosome category and share this shape with a few thoracotreme and heterotreme brachyurans, all investigated podotreme brachyurans, and some astacid, palinurid, and enoplometopid lobsters (Jamieson, 1991), anomuran Pylocheles (Bathycheles) (Tudge et al., 2001). Despite a well-conserved general structure and similarity in the pattern of spermatozoa of different crayfish species, the acrosome dimensions may be useful for separating the studied species. A comparison among the acrosome dimensions of studied crayfish species show that representative of Parastacidae has a smaller acrosome compared to Cambaridae. The representatives of Astacidae show the largest acrosome within three families of the crayfish. In addition, morphological features such as the presence of apical zone in the spermatozoon can help for separating of different species. Several studies carried out on spermatozoal ultrastructure and spermatogenesis in *P. clarkii* (Moses, 1961a, b; Hinsch, 1992, 1993a, b) do not report an acrosomal spike. According to our observations, the main part of spike growth takes place in the *vas deferens* and it is absent, or small, in spermatozoa inside the testis. This may be the reason why previous studies, that focused on the testis, did not report the acrosomal spike.

Also, we studied the spermatophore formation in narrow-clawed crayfish *A. leptodactylus* and its morphological changes during storage on the body surface of female. In crayfish, spermatozoa are packaged into spermatophores that function in the transfer of spermatozoa from male to the female during mating. Crayfish spermatophores are deposited either on the ventral surface (in Astacidae and Parastacidae) of the female or into the *annulus ventralis* (in Cambaridae). The spermatophore remains 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 La Nostro, 2008), suggesting that final maturation of a decapod spermatozoon takes place in the seminal receptacle of the female (Alfaro et al., 2003; Alfaro et al., 2007; Aungsuchawan et al., 2011; Vanichviriyakit et al., 2004). 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 *P. 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*. The hardening of the spermatophore wall after mating probably promotes spermatozoon survival in the presence of environmental stressors. 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*, that hardening results from phenolic tanning, an enzymatic cross-linking reaction associated with hardening of the chitin complex (Malek and Bawab, 1971). The most important change is observable in the subacrosome zone of the spermatozoon 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.

Egg ultrastructure of noble crayfish *A. astacus* in four different stages of activation was described. Egg activation was described in some members of decapods. During egg activation in penaeid shrimp, the cortical rods begin to emerge from the crypts on the periphery of the egg, and elevate the thin investment coat that covers the surface of the egg. Then, the cortical rods begin to break up and form the jelly layer around the egg. Finally, a new coat that is called hatching envelope is form around penaeid eggs (Pongtippatee-Taweepreda et al., 2004). In contrast, in lobster a new layer that is called envelope 2 is formed beneath envelope 1 by cortical reaction of cortical vesicles located in the egg cortex (Talbot and Goudeau, 1988). Therefore, egg activation in crayfish shows more similarities to lobsters rather than other decapods such as shrimps. In crayfish fertilized eggs are attached to the pleopods of female until hatching (Andrews, 1906). Our results showed that attachment stalk is derived from first envelope of egg in noble crayfish. Some researchers speculated that fertilized eggs of decapods are attached to the body of female by cement secreted from female's secretory glands at the time of egg-laying (Andrews, 1906; Yonge, 1937; Stephens, 1952; Aiken and Waddy, 1982). For example, Fisher and Clark (1983) reported that the outermost coat around pleopodal eggs of the shrimp, *Paleamon macrodactylus* is originated from a secretion of the pleopods. In contrast, other studies indicated that attachment stalk in decapods is originated from mature eggs after fertilization (Cheung, 1966, Goudeau and LaChaise, 1980, 1983; Saigusa et al., 2002). During egg attachment in the lobster, the pleopods beat vigorously and cause envelope 1 to stretch and form attachment stalks. Beating probably also causes the attachment stalks to twist and wrap around the ovigerous setae (Goudeau et al., 1987).

In conclusion, this thesis provided some basic knowledge about biology of the crayfish egg and spermatophore. That knowledge can be used for development of new techniques and approaches to solve the problems of artificial reproduction of crayfish in aquaculture industry.

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ENGLISH SUMMARY

Biology of reproduction in crayfish

Hamid Niksirat

The ultrastructure of spermatozoa from six crayfish species were described. The acrosome complex and nucleus are located at the anterior and posterior of the spermatozoon, respectively. The acrosome complex organelle is divided into two main parts: the main body of the acrosome that is a dense inverted cup-shaped structure and organized into three layers of differing electron densities and extended parallel filaments, and the sub-acrosome zone occupying the central part of the acrosome complex, which is divided into two electron dense areas. The spermatozoon of *Orconectes limosus* is described for the first time. In addition, an acrosome spike in the spermatozoon of *Procambarus clarkii* is described. Despite a well conserved general structure and similarity of pattern among these spermatozoa, differences in the dimensions of the acrosome within the studied species may be useful to help distinguish the different crayfish species.

The ultrastructure of the spermatozoon and spermatophore wall of the narrow clawed crayfish *Astacus leptodactylus* during three stages including freshly ejaculated, post-mating, and after release of the spermatozoon were described 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 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 glands secretions. The capsule, plasma membrane, and membranous lamellae are eliminated, and bundles of filaments are released. The subacrosome zone loses electron density and retracts. The electron-dense material of the innermost layer of the acrosome is discharged and, together with 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 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.

The ultrastructure of egg in noble crayfish *Astacus astacus*, during different stages of activation were described. The ovarian mature oocytes are surrounded by first envelope that is formed by two different layers. Numerous pores are visible on the surface of outer layer of first envelope. The inner layer of the first envelope contains bottlebrush-shaped objects. Three types of cortical vesicles including high and moderate electron dense vesicle and multi-layered vesicle were observed in the cortex of the oocytes. The pores were gradually disappeared from outer layer of first envelope in freshly ovulated oocytes. The bottlebrush-shaped objected in the inner layer of first envelope were replaced by a spongy shaped electron lucent texture containing scattered electron dense vesicles. After one hour of spawning, the first envelope especially its inner layer started to be condensed. The high and moderate dense vesicles discharge their contents into perivitelline space forming second envelope around

the oocyte. After 24 hours of ovulation, second envelope can be observed in previtelline space and a part of oocyte cortex. The high and moderate electron dense vesicles still can be observed to mix their contents to form second envelope. Also, results of present study showed that attachment stalk is derived from outer layer of first envelope of crayfish oocyte. In conclusion, fertilization coat in noble crayfish comprises from first and second envelopes that are constructed by follicular cells of ovary and cortical reaction of vesicles in the oocyte cortex, respectively.

CZECH SUMMARY

Biologie rozmnožování u raků

Hamid Niksirat

Byla popsána ultrastruktura spermií u šesti druhů raků. Akrozomální komplex je lokalizován v přední, kdežto jádro se nachází v zadní části buňky. Akrozomální komplex je tvořen dvěma hlavními částmi, kterými jsou vlastní tělo akrozomu a subakrozomální zóna. Tělo akrozomu má tvar obrácené misky a je tvořen třemi vrstvami lišícími se svou hustotou a obsahem paralelních vláken. Subakrozomální zóna zaujímá centrální část komplexu a je rozdělena do dvou oblastí s rozdílnou elektrodenzitou. Spermie raka pruhovaného *Orconectes limosus* byla popsána vůbec poprvé a u spermií raka červeného *Procambarus clarkii* byl nově nalezen i hrot na vrcholu vlastního akrozomu. I přes dobře zachovanou obecnou strukturu a podobnost spermií studovaných raků byly nalezeny rozdíly v rozměrech akrozomu, které tak mohou být využité pro odlišení jednotlivých druhů.

Dále byla popsána a porovnána ultrastruktura spermií a stěny spermatoforů raka bahenního, *Astacus leptodactylus*, a to ve třech stádiích: ihned po ejakulaci, po páření a po uvolnění spermií ze spermatoforů. Račí spermatofory jsou tvořeny masou spermií obalených třívrstevnou stěnou. Po páření se zvětšuje šířka vnější stěny spermatoforu. Matrix střední vrstvy začíná být retikulovaný a granule uvnitř této vrstvy uvolňují svůj obsah. Vlákna ve vnitřní vrstvě se rozkládají na malé částice. Zvětšuje se extracelulární kapsule a objevuje se volný prostor mezi spermií a touto kapsulí. Plocha plazmatické membrány se zvyšuje zvrásnění svého povrchu a změnami, které vedou k tvorbě vícevrstvé struktury na přední části akrozomu. Hustota subakrozomální zóny se zvyšuje v blízkosti těla akrozomu. S nástupem ovulace jsou stěny spermatoforu rozpuštěny působením sekretu ze žláz nacházejících se na spodní straně zadečku samice samice. Poté dochází k eliminaci extracelulární kapsuly, plazmatické membrány a membránové lamely. Subakrozomální zóna ztrácí svou elektrodenzitu a zatahuje se. Z nejvnitřnější vrstvy akrozomu je uvolněn materiál s vysokou elektrodenzitou a vlákny, které spolu na vrcholu akrozomu vytvářejí rozložitou strukturu. Nejdůležitější změny jsou pozorovány v subakrozomální zóně. Tyto mohou hrát klíčovou roli při oplodnění vajíčka. Rovněž morfologické změny spermie po propuštění extracelulární kapsuly, zejména tvorba rozložité struktury z materiálu s vysokou elektrodenzitou a vláken mohou hrát roli v navázání spermie s vajíčkem raků, jejichž spermie jsou nepohyblivé.

Popsána byla taktéž ultrastruktura vajíček raka říčního *Astacus astacus* během několika stádií. Zralé ovariální oocyty jsou obklopeny primárním obalem, který je tvořen dvěma vrstvami. Na povrchu vnější vrstvy jsou viditelné četné póry. Vnitřní vrstva primárního obalu obsahuje objekty ve tvaru kartáče na lahve. Tři typy kortikálních vezikul (se střední a vysokou elektrodenzitou a mnohovrstevné vezikuly) se nacházení v kortexu vajíčka. U čerstvě ovulovaných vajíček postupně mizí póry na vnější vrstvě primárního obalu. Objekty ve tvaru kartáče na lahve jsou ve vnitřní vrstvě primárního obalu nahrazeny houbovitou texturou s nízkou elektrodenzitou, která obsahuje rozptýlené vezikuly s vysokou elektrodenzitou. Primární obal (především jeho vnitřní vrstva) začíná do hodiny po oplození kondenzovat. Vezikuly se střední a vysokou elektrodenzitou uvolňují svůj obsah do perivitelinního prostoru, čímž dávají vzniknout sekundárnímu obalu vajíčka. Jeden den po ovulaci je možné pozorovat prostup sekundárního obalu vajíčka do perivitelinního prostoru a části kortexu vajíčka. Výsledky této studie také potvrdily, že stopka vajíčka umožňující jeho připevnění na samičí pleopody je odvozena od primárního obalu vajíčka. Závěrem lze tedy říci, že obal vajíček raka říčního se skládá z primární a sekundární vrstvy, První je odvozena od vlastního folikulu, kdežto druhá vzniká kortikální reakcí zapojující vezikuly v kortexu vajíčka.

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Hamid Niksirat
Research department	2010–2014 Laboratory of Ethology of Fish and Crayfish FFPW
Daily supervisor	Assoc. Prof. Dipl.-Ing Kozák Pavel, Ph.D.
Supervisor	Assoc. Prof. Dipl.-Ing Kozák Pavel, Ph.D.
Period	2010–2014

Ph.D. courses	**Year**
Basic of Scientific Communication	2011
Pond Aquaculture	2012
Applied Hydrobiology	2012
Ichthyology and Systematics of Fish	2012
English Language	2012

Scientific seminars	**Year**
Seminar days of RIFCH	2011 2012 2013 2014

International conferences	**Year**
conference DIFA II, September 24–26, 2013, Vodňany, Czech Republic	2013
Regional European Crayfish Meeting, Rovinj, Croatia	2013
19th Symposium of International Association of Astacology (IAA), Innsbruck, Austria (Oral presentation)	2012
AQUA 2012, Global Aquqculture, Prague, Czech Republic, September 1–5 (Poster presentation)	2012

Foreign stays during Ph.D.	**Year**
Prof. Peter James, Department of Immunotechnology, Lund University, Sweden (4 months)	2012-13

CURRICULUM VITAE

Surname: **Niksirat Hashjin**
First name: **Hamid**
Title: Master of Science (M.Sc.)
Born: 3. 5. 1979 Nowshahr, Iran
Nationality: Iranian
Contact: niksirat@frov.jcu.cz

PRESENT POSITION

Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW, www.frov.jcu.cz), Research Institute of Fish Culture and Hydrobiology (RIFCH), South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA), Laboratory of Ethology of Fish and Crayfish, Vodňany, Czech Republic

EDUCATION

1998–2002	B.Sc., Gorgan university of agricultural sciences and natural resources, Iran
2003–2006	M.Sc., University of Tehran, Iran
2010–2014	Ph.D. student, USB, FFPW, RIFCH, České Budějovice, Czech Republic

Ph.D. COURSES

Pond Aquaculture, Applied Hydrobiology, Ichthyology and Systematics of Fish, English Language, and Basic of Scientific Communication

KNOWLEDGE OF LANGUAGES

Persian, English

FOREIGN STAY DURING Ph.D. STUDY AT RIFCH AND FFPW

December 2012, March 2013, Prof. Peter James, Department of Immunotechnology, Lund University, Sweden