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Spermatogenesis and spermatozoal ultrastructure in *Metapenaeus monoceros* (Fabricius, 1798)

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ABSTRACT

Spermatogenesis in the 'speckled shrimp' *Metapenaeus monoceros* (Fabricius, 1798) was studied employing histological and transmission electron microscopic techniques. Male specimens of the species were classified into three maturity stages based on gross morphology. Spermatozoa of *M. monoceros* was observed to be unistellate with a spherical main body and a spike. The main body was made up of a central nuclear region, a peripheral cytoplasmic band and an acrosomal cap, which overlaid the nuclear region anteriorly. From the acrosomal cap projected an extended spike towards the anterior of the spermatozoa. The spike and the acrosomal cap together were called the acrosomal vesicle. The average length of the spermatozoa was 6µ m. The main body had a mean diameter of 3.4µ m with the spike measuring 2.6µ m in length.

Introduction

It is a known fact that the reproductive quality of male plays an important role in the productivity of broodstock of captive penaeids (Diaz *et al.*, 2002). Unlike females, the male shrimps provide no grossly visible clues as to the physiological state of the gonad. Hence very few workers have described well-defined maturity stages in males. Using the small variations in opacity and size of the testes in relation to size of the animal, Subrahmanyam (1965) and Joseph (1996) described five maturity stages in male *Penaeus indicus* and *Penaeus monodon* respectively. Parnes *et al.* (2004) classified *Penaeus vannamei* males into four categories based on external appearance of the spematophores as observed in the terminal ampoule.

Histological studies on spermatogenesis of penaeids have been attempted in *Penaeus setiferus* (King, 1948); *P. indicus* (Subrahmanyam, 1965; Mohamed and Diwan, 1993) and *Metapenaeus dobsoni* (Vasudevappa, 1992). Of late, spermatozoal ultrastructural studies of decapod crustaceans have been receiving a lot of attention due to their use in spermiotaxonomy - application of sperm ultrastructure to phylogeny and taxonomy (Sclezo and Medina, 2003). Such studies have been conducted in *Penaeus aztecus* and *Sicyonia ingentis* (Clark *et al.*, 1973); *P. indicus* (Mohamed and Diwan, 1993); *Parapenaeus longirostris* (Medina, 1994); *Penaeus japonicus* and *Penaeus kerathurus* (Medina *et al.*, 1994b). According to Scelzo and Medina (2003), an investigation of the sperm ultrastructure of Indian Ocean genus of

Metapenaeus could provide a deeper insight into the dendrobranchiate sperm ultrastructure and phylogeny.

Metapenaeus monoceros commonly called as 'speckled shrimp' is one of the commercially important penaeid species found along both the coasts of India. With an average annual landing of around 10,000 t, it accounts for 7-10 % of the total penaeid shrimp catch of the country (Sukumaran *et al.*, 1993). It also forms an important component of the shrimp catch from the Pokkali fields of Kerala in India and the rice-prawn filtration units in Bangladesh. *M. monoceros* attains a maximum length of about 190 mm and has high export potential. There is very good scope for this species to be taken up for semi-intensive culture practices in India due to their larger size among the *Metapenaeus* spp. The present study on spermatogenesis and spermatozoal ultrastructure of *M. monoceros* was carried out considering its importance as a potential candidate species for shrimp culture diversification.

Materials and methods

Specimens of *M. monoceros* were collected during November 2001- June 2003 from trawlers operating from Kalamukku and Murikkumpadam fish landing centers of Vypeen island (Latitude 10.08° N, Longitude 76.21° E) in Kerala. Live adult shrimps of size ranging from 90 mm to 160 mm were used for the study. Twenty five litre plastic bins and aerators were used for live transport of shrimp from the fishing ground to the landing centers. Shrimps were then transported live to CMFRI laboratory where they were segregated sexwise and kept in 1 t fibre glass tanks with aeration. Total length and carapace length of the shrimps were measured to the nearest mm and total weight to the nearest mg. The specimens were subjected to careful examination of the terminal ampoule, and the petasma and testes were carefully dissected out proceeding from the terminal ampoule. The various parts of the male reproductive system, viz. the testicular lobes, proximal vas deferens, median vas deferens and distal vas deferens were observed under light microscope. Based on external morphology, the male specimens were grouped into three maturity stages.

Spermatophores were extruded by subjecting mature male specimens to 12 V electric current at the base of the fifth walking leg, using the electrodes of an electrocautery. Spermatophores thus extracted and testicular lobes of shrimps belonging to the three maturity stages were fixed for both light microscopy and transmission electron microscopy. For light microscopy tissues were fixed in Bouin's fluid for 24 - 48 h. Tissue preparation for light microscopy was performed according to Bell and Lightner (1988). Briefly, the tissues were washed overnight to remove excess picric acid, dehydrated in propanol series (30-100%), cold impregnated in a mixture of wax and chloroform (1:1), tissue blocks were prepared, serial sections of 5-6µ m were affixed to glass slides and subjected to routine haematoxylin-eosin staining. Stained sections were repeatedly washed in an ascending series of propanol grades to remove excess eosin and cleared in xylene. Sections were then mounted with DPX and examined under a LEICA MPS 60 binocular microscope. Photomicrographs were taken using a LEICA camera attached to the microscope.

For electronmicroscopy, the testicular lobes and spermatophores from male specimens were fixed in 3% buffered glutaraldehyde solution for 2 h at 4°C, washed with buffer (0.1 M sodium cacodylate), post-fixed in 1% osmium

tetroxide, dehydrated in acetone series, infiltrated in Spurr's resin (Spurr, 1969) and blocks were prepared. From the polymerized blocks, ultra-thin sections (60-90 nm) were taken, double-stained with Uranyl actetate and Lead citrate, mounted on grids and the images were observed and photographed in a Hitachi H 600 Transmission Electron Microscope.

Results

Based on gross external morphology, male specimens of *M. monoceros* are classified into three stages of maturity, viz. immature, maturing and mature.

Immature stage: Testis is thin, translucent and extremely delicate organ in the cardiac region. Testicular lobes not differentiated. Vas deferens appears like a translucent thread like structure and the different components are not differentiated. The terminal ampoule appears like a delicate membranous bag. The secondary sexual characteristics (petasma and appendix masculina) are not developed.

Maturing stage: Testis is lobed and vas deferens thicker and tubular. The terminal ampoule increased in thickness. The entire vas deferens including the terminal ampoule is translucent without any spermatophore. The endopods of the first pair of pleopods are modified and partially united to form the petasma. Appendix masculina appeared as small bud at the base of the second pair of pleopods.

Mature stage: All internal and external reproductive organs are well developed; testes appeared milky white in colour; testicular lobes separated and distinguishable. Different components of vas deferens (proximal, median and distal vas deferens) could be well differentiated. Terminal ampoule is a thick, muscular, membranous bag and appears white in colour due to the presence of fully developed spermatophores inside. The endopods of the first pair of pleopods are linked by a series of minute hook-like structures, forming the petasma and with well developed appendix masculina.

The entire testis is covered by a thin wall of outer epithelium. Histological sections of testis at different maturity stages revealed that each testicular lobe is composed of innumerable testicular acini held together by connective tissue (Fig. 1). In immature animals the testicular acini are completely empty. The acinar

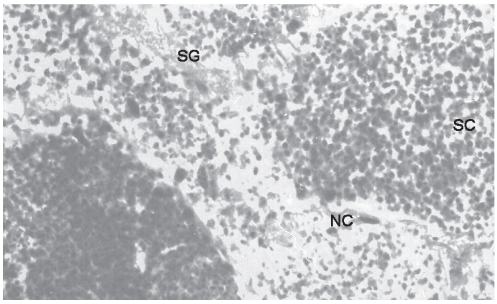

Fig. 1. Light micrograph of a maturing testis with spermatogonia (SG) in germinal zone, spermatocytes (SC) and nurse cells (NC) X 400

wall was found thicker in immature animals and contained only a small germinal zone with non-differentiated germ cells. In testicular lobes of maturing animals, germinal zone is shifted to the periphery and contained spermatogonial cells (Fig. 1 & 2). Diakinetic stages of spermatocytes are noticed in the centre of the acini. In fully mature males, the germinal zone is very much restricted and acini are fully occupied with cells of a particular type like spermatids and spermatozoa (Fig. 3 & 4).

Formation of mature spermatozoa from germinal cells in the lumen of testicular acini is called spermatogenesis. In histological sections of testicular lobes of *M. monoceros* a germinal zone contain-

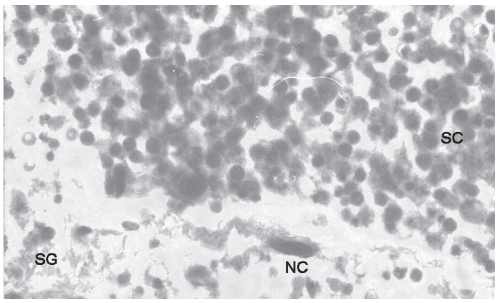

Fig. 2. Higher magnification of a maturing testis showing diakinetic stages of spermatocytes (SC) (SG – spermatogonia, NC – nurse cells) X 1000

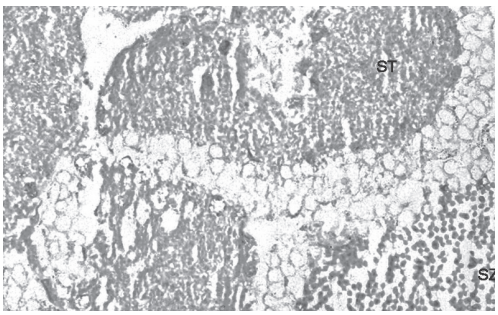

Fig. 3. Light micrograph of a mature testis showing spermatids (ST) and spermatozoa (SZ) X 400

ing spermatogonial cells and nurse cells is apparent (Fig. 1). Spermatogenesis always progressed from the periphery of acini to the centre and therefore subsequent developmental stages are found towards the centre in a graded manner. Spermatogonia pass through a period of quick growth to become primary spermatocytes, which undergo meiosis to form two secondary spermatocytes. The two spermatocytes divide mitotically to form four spermatids from each primary spermatocyte, and the spermatids modified into spermatozoa without further division (Fig. 3 & 4).

Spermatogonial cells had a round vesicular nucleus with diffused chromatin. Cell boundaries are not clearly demarcated. Nurse cells are found dispersed in between spermatogonia (Fig. 1 & 2). These are elongate cells measuring 9-10 ìm in length and 3-4 ìm in width. By virtue of their close association with gonial cells, they are assumed to have a nutritive and supportive role. The cell boundaries of spermatocytes, spermatids and spermatozoa are distinct. Spermatocytes have a prominent basophilic nucleus and a thin rim of eosinophilic cytoplasm around it. These spermatocytes are usually the dividing

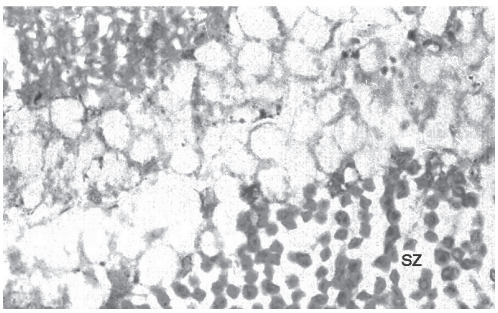

Fig. 4. Higher magnification of a mature testis showing spermatozoa with main body and spike X 1000.

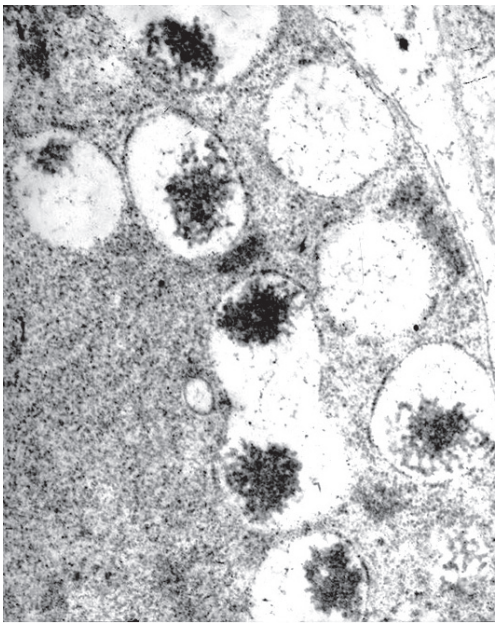

Fig. 5. Electron micrograph of testicular acinus showing cells in active division X 25000

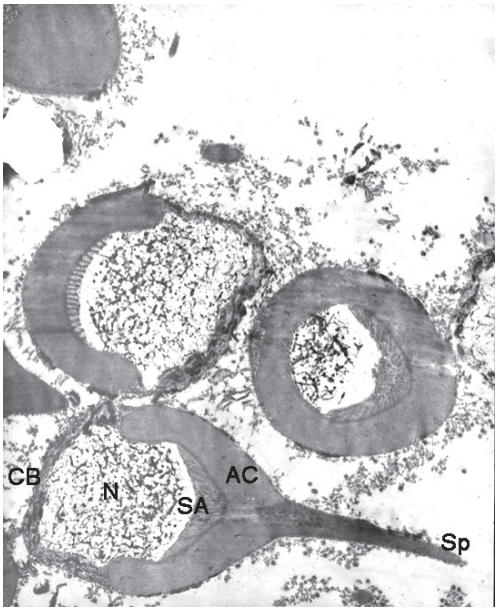

Fig. 6. Electron micrograph of spermatozoa from a fully formed spermatophore showing main body with nucleus (N) and cytoplasmic band (CB) and acrosomal complex with acrosomal cap (AC), sub-acrosomal material (SA) and spike (Sp) X 10000

cells in the testis and diakinetic stages characteristic of dividing cells were frequently observed among them. Electron micrographs of testicular acini showed actively dividing cells (Fig. 5 & 6). Spermatids were smaller than spermatocytes with condensation of chromatin matter. The spermatozoa developed from these cells through cellular differentiation (Fig. 3). In histological sections spermatozoa appeared almost circular in outline with basophilic condensed chromatin matter. A 'Y' shaped acrosome vesicle was apparent at the apical region with a less clear spike (Fig. 4).

Fully mature spermatozoan of *M. monoceros* consisted of a spherical main body and a spike (unistellate) (Fig. 6). The main body was made up of the central nuclear region, a peripheral cytoplasmic band and an acrosomal cap, which

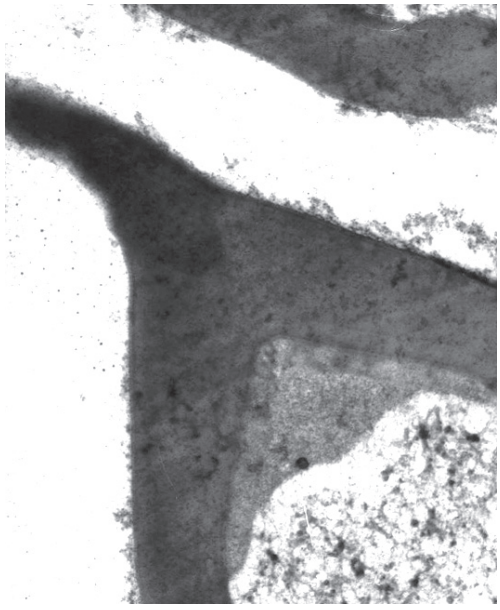

Fig. 7. Electron micrograph of a mature spermatozoan X 25000

overlaid the nuclear region anteriorly. From the acrosomal cap projected the extended spike. The spike and the

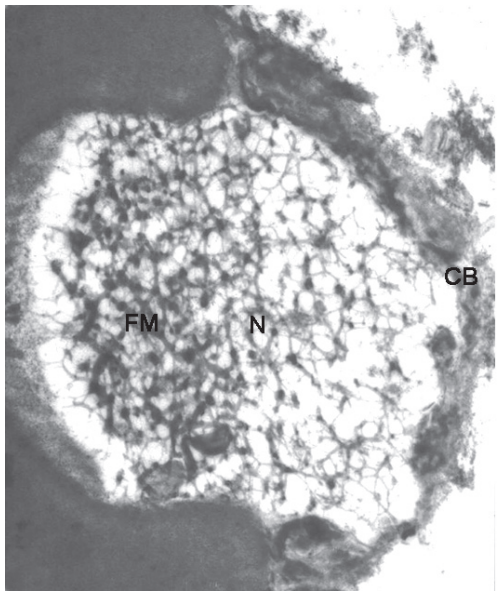

Fig. 8. Electron micrograph of main body of mature spermatozoan showing nucleus with fibrillar material (FM) and cytoplasmic band (CB) X 30000

acrosomal cap are together called the acrosomal vesicle. The average length of the spermatozoa was 6 ìm. The main body had a mean diameter of 3.4 ìm with the spike measuring 2.6 ìm in length.

The acrosomal structure was complex, consisting of the membrane bound acrosomal vesicle and the sub-acrosomal substance (Fig. 7 & 8). The acrosomal vesicle was made up of two distinct elements surrounded by a continuous membrane: the acrosomal cap, which overlaid the anterior surface of the sperm cell and the spike, which projected anteriorly. Over the outer surface of the acrosomal vesicle, the sperm plasma membrane and the acrosomal membrane were closely joined, so that both the spike and convex side of the acrosomal cap were surrounded by a double membrane. The spike was composed of a limiting membrane and the internal spike material. The spike material was more electron dense than the constituents of the acrosomal cap (Fig. 7 & 8). Beneath the inner surface of the acrosomal vesicle was a homogeneous, electron-lucent subacrosomal substance separating the acrosome from the nuclear region.

The central region of the sperm body occupied by the nucleoplasm, is not surrounded by a membrane (Fig. 8). The nuclear material is in direct contact with the cell membrane as a discrete nuclear membrane is not present. Inside the nucleus, chromatin formed a network of fibrillar material. The nucleus outlined postero-laerally by the cytoplasmic band, which extended up to the edge of the acrosomal cap.

Discussion

Unlike females, the male penaeids give few externally visible clues regarding the stages of development of the testis. Moreover, the size at first maturity in males is much smaller than that for females and information regarding the maturity stages in male shrimps is scanty. Therefore very few workers have described any well-defined maturity stages in males of penaeid shrimp. Subrahmanyam (1965) described five maturity stages in *P. indicus* by relating variations in the opacity and size of the testis to the animal size. Castille and Lawrence (1991) classified males of *P. aztecus* and *P. setiferus* into three maturity stages viz. immature, developing and mature, based on the size and appearance of the terminal ampoule. Parnes *et al*. (2004) used the appearance of spermatophores through terminal ampoule for classification of the males of *L. vannamei*. In the present study, male specimens of *M. monoceros* were classified into three stages viz. immature, maturing and mature based on the external morphology of testes, vas deferens, petasma and appendix masculina. Of the different criteria used, the size and colour of the terminal ampoule, appendix masculina and petasma have been found to be more reliable and useful as the same can be observed externally without sacrificing the shrimp. Joseph (1996) also classified *P. monodon* males into three stages.

The process of spermatogenesis in *M. monoceros* is similar to that reported in other decapod crustaceans (Pochon – Masson, 1983). Spermatogenesis begins in the peripheral germinative layer of the testicular acini when spermatogonia enter into the prophase of meiosis (King, 1948). This pattern of spermatogenesis wherein the spermatogonia become spermatozoa through stages like spermatocytes and spermatids is noticed in *P. setiferus* (King, 1948); *M. dobsoni* (Vasudevappa, 1992) and *P. indicus* (Mohamed and Diwan, 1993). As in the present study, there are many reports of

crustacean testis having nurse or nutritive cells among the spermatogonia, which perform important functions like providing nourishment, support and some hormones during spermiogenesis (Hinsch, 1969). In *M. monoceros* a single testicular acinus was found to contain sperm cells at various stages of development. This synchronous pattern of spermatozoan development has also been observed in other penaeids (Pochon–Masson, 1983).

The spermatozoal ultrastructure of *M. monoceros* conformed to the ground plan of unistellate sperm present in most dendrobranchiates and consisted of a spherical main body and a single spike as seen in *M. dobsoni* (Vasudevappa, 1992), *P. indicus* (Mohamed and Diwan, 1993), *P. monodon* (Joseph, 1996) and *Artimesia longinaris* (Sclezo and Medina, 2003). The main body of the spermatozoan consisted of the central nuclear region, a perinuclear cytoplasmic band and an anterior acrosomal cap. *M. monoceros* share with other panaeoids several presumed plesiomorphic features such as the non– membrane–bound filamentous chromatin, the perinuclear arrangement of the cytoplasmic mass and the absence of centrioles and radial arms (Medina, 1995).

Like any typical decapod sperm, the sperm of *M. monoceros* lacks a nuclear envelope. The nucleus of dendrobranchiate sperm is confined to the main body and is decondensed (Clark *et al.*, 1973). According to Beach and Talbot (1987) a decondensed nucleus may be necessary in decapods to accommodate the unusual acrosome reaction of this group, where the nucleus is rapidly thrust forward to the egg during the reaction. It is probable that the decondensed state of the decapod nucleus and its relatively fluid state facilitate movement of DNA toward the oocyte during the acrosome reaction. Bulk of the main body of the spermatozoa in *M. monoceros* is composed of loosely packed fibrils (non-membrane-bound nucleus) which is postero–laterally bordered by an amorphous cytoplasmic band as in other penaeids (Clark *et al.*, 1973; Medina, 1994).

Acrosomal complex of *M. monoceros* exhibited structures similar to those described in other penaeids like *P. aztecus* (Clark *et al.*, 1973); *P. indicus* (Mohamed and Diwan, 1993); *P. longirostris* (Medina, 1994); *P. monodon* (Joseph, 1996) and *A. longinaris* (Sclezo and Medina, 2003). Shigekawa and Clark (1986) used the term 'cap region' to denote the combination of acrosomal cap and sub–acrosomal substance. In *M. monoceros* the outermost surface of the acrosomal vesicle is bound by a double membrane consisting of the membrane of the acrosomal vesicle proper and the plasma membrane as seen in all the other penaeids. In *P. longirostris* (Medina, 1994) and *A. longinaris* (Sclezo and Medina, 2003), a central protruberance at the inner surface of the acrosomal cap, located immediately opposite the spike has been reported. The present study shows that such a central protuberance is absent in *M. monoceros*.

Like in other penaeids, the spike occurred as an anterior extension of the acrosomal complex as both were encompassed in a single membrane (Clark *et al.*, 1973). In *M. monoceros* the spike appeared straight but in *P. longirostris*, Medina (1994) reported bent spike which he attributed to the dense packing of sperm in the ampulla. In the penaeid shrimp *A. longinaris*, Sclezo and Medina (2003) observed that the spike showed a filamentous structure internally due to the organization of the spike materials

into a bundle of filaments that are coiled into a loose helix. No such organelles were observed in the fully mature spermatozoa of *M. monoceros*. The degeneration of organelles has already been reported in many decapod crustaceans (Clark *et al.*, 1973). It is not only in decapods, among Malacostraca itself the spermatozoa show considerable modification and loss of organelles (Adiyodi, 1985).

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