ULTRASTRUCTURE OF SPERMATOCYTOGENESIS IN THE BIVALVE DONAX VITTATUS (MOLLUSCA, BIVALVIA)

ABSTRACT:
Ultrastructure of spermatogenesis in the bivalve Donax vittatus during the period from March 2010 to August 2010 was done. Investigated specimens were collected monthly from the northern Mediterranean Sea coast of Gamasa (31° 30' N and 31° 30' E), Egypt. The different stages of spermatogenesis were investigated. The results revealed that spermatogonia, primary and secondary spermatocytes and spermatids are similar to many other bivalves. Though, spermatozoa showed some deviation to the typical structure found in Herterodonta.

KEY WORDS:
Bivalvia, Donax, spermatogenesis, sperm, ultrastructure.

INTRODUCTION:
Germ cell differentiations during spermatogenesis and mature sperm ultrastructural features have been documented in many species of bivalve molluscs using both light and transmission electron microscopy (Franzén, 1970; Daniels et al., 1971; Franzén, 1983; Dorange and Le Pennec, 1989; Healy, 1989&1995a&b; Eckelbarger et al., 1990; Gaulejac et al., 1995; Eckelbarger and Davis, 1996; Chung and Ryou, 2000; Chung et al., 2007, Chung et al., 2010). It is well-known that spermatogenesis occurs through spermatogonic germ cell differentiations in acini of the testis. So, it is important to study the processes of germ cell differentiations by developmental stages throughout spermatogenesis to clarify the reproductive mechanism.

Several studies have been published on sperm ultrastructure within the Bivalves to verify the utility of sperm characters for taxonomic and phylogenetic analysis at and above the species level. Some authors have carefully analyzed bivalve sperm morphologies, covering undescribed families and providing important phylogenetic considerations (Bernard and Hodgson, 1985; Hodgson et al., 1990; Guerra et al., 1994; Sousa and Oliveira, 1994; Garrido and Gallardo, 1996; Healy, 1996; Komaru and Konishi, 1996; Kafanov and Drozdov, 1998; Healy and Keys, 1999; Healy et al., 2001; Erkan and Sousa, 2002; Gwo et al., 2002; Introini et al., 2004; Healy et al., 2008; Introini et al., 2009).

The specificity of sperm features in some families and sub-families of bivalves has been published in several literatures. Kafanov and Drozdov (1998) reported that the species of the recent Mytiloidea have been grouped into two subfamilies: Modiolinae and Mytilinae. Species lacking an acrosomal rod can be considered members of the subfamily Modiolinae, meanwhile the presence of this structure suggests that other species belong to the subfamily Mytilinae. Hence, electron microscopy of bivalve sperm cells has been widely employed as an important tool to solve taxonomic and phylogenetic questions, including those related to the family Mytilidae.

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Introini et al. (2004) and Introini et al. (2009) found significant differences between closely-related species using transmission and scanning electron microscopy. These differences recommended that characteristics of the acrosome could be a significant concern for taxonomic differentiation. Despite the fact that, spermatozoa of the same group usually share similar basic cytological features (Sousa et al., 1995), comparing cytological acrosomal staining of spermatozoa could distinguish between different sperm species.

The present study describes the ultrastructural features of the different stages of spermatogenesis with a special focus on the mature spermatozoon in *Donax vittatus* and compare them with those reported in previous studies of heterodont bivalves.

**MATERIAL AND METHODS:**

Sexually mature specimens of *Donax vittatus* from populations living in intertidal zones buried in sandy sediment were collected during the period from March to August 2010 from Gamasa beach, Egypt (31° 30’ N and 31° 30’ E). The sampled specimens were immediately immersed in seawater and were processed for analyses at a laboratory at the University of Mansoura, Egypt.

Small pieces of male gonads were prefixed with 2.5% glutaraldehyde buffered with 0.2M sodium cacodylate, containing 3% sucrose (pH 7.4) for 2 h. at 4°C. After fixation, they were washed with 0.2M sodium cacodylate buffer for 2 h at 4°C, and postfixed with 1% osmium tetroxide (OsO₄), in the same buffer, for 2 h at 4°C. Subsequently, samples were dehydrated in a graded acetone series and embedded in white London resin. Sections were stained with uranyl acetate and lead citrate and then examined with a JEOL 100 SX TEM at 60 kv.

**RESULTS:**

**Developmental stages of spermatogenesis:**

In general, spermatogenesis occurs in acini of testis and can be divided into four stages: (1) spermatogonia, (2) spermatocytes, (3) spermatids, and (4) spermatozoa. Generally, the process of spermatogenesis of *Donax vittatus* appears to be similar to those of other bivalve species.

**Spermatogonia:**

Spermatogonia of *Donax vittatus* are present along the acinus wall relatively large germ cells (approximately 8 to 11 μm in diameter), irregularly shaped cells having little amount of cytoplasm and a large spherical nucleus (about 6-9 μm in diameter) containing scattered chromatin materials. At this stage, a number of mitochondria and vacuoles appear in the cytoplasm, while the cytoplasm of this cell is largely devoid of other organelles (Figs 1-4).
Spermatocytes:

There are two stages of spermatocytes, presumed to be primary and secondary. The spermatogonia differentiate into primary spermatocytes which resemble spermatogonia except their larger diameter of nuclei. Primary spermatocytes (approximately 10-12 μm) are slightly bigger cells than that are distinguished by their nuclei (approximately 6.4-9.5 μm) which have abundant and darker staining of chromatins. At this stage, mitochondria appear in the cytoplasm. At this time, the nucleolus is no longer prominent (Figs 5-6).

Spermatids:

The secondary spermatocyte of *D. vittatus* (Fig. 7) transforms into spermatids as a result of the secondary meiotic division. For convenience, spermiogenesis has been classified into two stages: the early and late stages.

In the early stage of spermiogenesis, spermatids (approximately 4-4.4 μm in diameter) are oval in shape, and the nucleus is spherical and occupies the center of the cell. The nucleus (about 3 - 3.2 μm in diameter) contains scattered electron-dense granular heterochromatin (Fig. 7). The morphology of the spermatid nucleus changes gradually during the differentiation of the spermatid. At this time, small granules are
formed by the Golgi complex in the cytoplasm move to a position just in front of the nucleus, while mitochondria move to a position just behind the nucleus. After all, the morphologies of the spermatid nuclei were gradually elongated, and one or a few granules in the cytoplasm of the spermatid become a proacrosomal vesicle (Fig. 8).

The proacrosomal vesicle migrates to the presumptive anterior end of the nucleus of spermatid, where they unite to form a single electron-dense vesicle. The mitochondria become reduced in number but increase in size by mitochondrial fusion. However, the shape of the nucleus is modified and becomes significantly elongated. A proacrosomal vesicle becomes cone-like acrosomal vesicle on the nucleus. Thereafter, a cone-like acrosomal vesicle becomes an acrosome. The acrosome lying on the sperm nucleus becomes cone in shape (Fig. 9).

In the basal part of the nucleus, the mitochondria become reduced in number but increase in size by mitochondrial fusion. Posterior to the nucleus is the midpiece. This region consists of four spherical mitochondria surrounding a pair of centrioles. The cristae of each mitochondrion are randomly arranged (Figs 8&9). At this time, the two centrioles, at right angles, show the classic nine structure of microtubules. The proximal centriole lies at 90° relative to the distal centriole and sperm longitudinal axis. The distal centriole lies parallel to the sperm longitudinal axis and forms the point of origin for flagellar axoneme (Fig. 8).

In the late stage of spermiogenesis, the spermatid nucleus becomes obviously elongated, and the cytoplasm is greatly reduced. So, the ratio of nucleo-cytoplasm is high. An acrosomal vesicle is also gradually elongated (average 0.52 μm long). The acrosome is composed of electron-opaque part. The axial filaments are not found in the acrosomal lumen, while subacrosomal granular materials are present in the subacrosomal space between the anterior invaginated part of the nucleus and the acrosomal vesicle of the acrosome (Fig. 9).

The axial filament is not found in the subacrosomal materials in the acrosomal vesicle. The acrosomal vesicle is composed of well-developed basal rings, and the curved nucleus (angle of the nucleus: 45°) is adjacent to the plasma membrane. The anterior nuclear fossa is existing in front of an acrosomal vesicle and the posterior nuclear fossa is present near the proximal centriole and distal centriole with some spherical mitochondria in the sperm midpiece (Fig. 9).

**Spermatozoa:**

Sperm morphology is primitive and undergoes external fertilization most bivalve species as some characteristics of the acrosomal vesicle structures in an acrosome of this species, the basal and lateral parts of basal rings show electron opaque part (region), while the anterior apex part of the acrosomal vesicle shows electron lucent part (Fig. 9).

Mature spermatozoa of *D. vittatus* measure approximately 25-30 μm long, and consist of conical acrosome positioned at the
top of an elongated nucleus, a pair of centrioles surrounded by a ring of four spherical mitochondria, and a flagellum. The sperm head is about 5 μm long and comprises a long, electron-dense nucleus (about 4 μm long), with the anterior nuclear fossa, and an acrosome. The acrosomal vesicle is about 0.5 μm long, membrane-bound, and deeply invaginated. The acrosomal vesicle is a cone shape. The morphology of the sperm nucleus and the acrosomes of this species are of a curved cylindrical type (the angle of the nucleus: 45°) and a cone shape, respectively (Fig. 9).

Four spherical mitochondria with well-defined cristae occur near the posterior nuclear fossa of the nucleus (Figs. 8&9). A cross-sectioned tail flagellum shows that the axoneme is composed of a classic 9+2 microtubular substructure (nine peripheral doublets surrounding a central pair of singlet microtubules) enclosed by a plasma membrane (Fig. 10).

Fig. 10. Transmission electron micrographs of spermatogenesis in male Donax vittatus showing cross section through spermatozoon flagellum showing the 9+2 structure.

DISCUSSION:

The processes of germ cell differentiations and mature sperm ultrastucture of this species were similar to those in the species in bivalves (Chung et al., 2010). Therefore, the processes of spermatogenesis of the present species were similar to those in the species of bivalves (Sakker, 1984; Bernard and Hodgson, 1985; Chung, 2006; Chung et al., 2007; Kim et al., 2010a&b).

Spermatogonia of D. vittatus were multipliclated by the mitotic division and developed into the primary spermatocytes containing electron dense scattered chromatins in the nucleus. During spermiogenesis, according to concentration degree of chromatins in the spermatid nucleus, the morphology of the nucleus remarkably changed into the anterior and posterior elongations or right and left extentensions. Yasuzumi (1974) reported that the nuclei of spermatids can be classified into three morphological types according to the concentration morphology and degree of chromatins in the nuclei of spermatids as follows: (1) granular type, (2) fibrous type, and (3) layer type. In case of bivalves, it was a granular type in the early stage of chromatin concentrations; as the concentration of chromatins progresses, it was changed to be the fibrous type. Kim (2001) reported that the nuclear morphology of the species having wide nucleus from side to side (Ostreidae, Mytilidae) and that of the elongated species back and forth (Veneridae, Pectinidae, Corbiculidae, Mya arenaria, Panopea japonica) is elongated with the concentration of fibrous chromatins.

The acrosome is formed by various granular secretions secreted by the Golgi complex (Longo and Dornfeld, 1967; Sastry, 1979). In Mytilus coruscus, several small proacrosomal vesicles are formed by the Golgi complexes, and these vesicles were mixed with each other in the acrosomal vesicle during spermiogenesis (Kim et al., 2010a).

In the present study, the morphology of the spermatozoon of D. vittatus has a primitive type and is similar to those of other species in the subclass Heterodonta and most bivalves. The morphologies of the sperm nucleus and the acrosomal vesicle of this species are of cylindrical type and cap shape, respectively. Mature spermatozoa of this species contain subacrosomal material (embedded in a granular matrix), an elongated nucleus showing invaginated anteriorly and two centrioles surrounded by four spherical mitochondria.

Regarding the sizes of sperm nuclei (lengths) in the species of Veneridae in Subclass Heterodonta, Kim (2001) described that lengths of nuclei varied with the species of Veneridae: Meretrix lusoria is 1.49 μm, Cyclina sinensis 2.13 μm, and G. veneriformis 7.80 μm. Therefore, it is assumed that sperm nuclei could not be used for the taxonomical key because the sizes of sperm nuclei vary with the species (Healy, 1995 a&b).

Taking into account the published data on spermatozoa of the Heterodonta (Pochon-Masson and Gharagozou, 1970; Gharagozou et al., 1971; Popham, 1974&1979; Hylander and Summers, 1977; Franzén, 1983; O’ Foighil, 1985 a&b; van der Horst et al., 1986; Sousa and Azevedo, 1988; Sousa et al., 1989; Eckelbarger et al., 1990; Hodgson et al., 1990; Nicotra and Zappata, 1991; Sousa and Oliveira, 1994), it can be observed that there is spermatozoa with unusual features which slightly deviate from the classic primitive type (i.e. the Veneridae Ruditapes decussatus (long tapering nucleus, with the acrosomal vesicle shifted from the nuclear apex by a

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In the Lasaeidae spermatozoa of Lasaea subviridis have curved nucleus, laterally tilted acrosomal vesicle and those of MySELLa tumida the acrosome shifted from the nuclear apex (O’Foighil, 1985a).

In the Cardiinae spermatozoa have spiral pointed nucleus, with mitochondria slightly bulging laterally to the nucleus (Sousa and Azevedo, 1988).

In Scrobiculariidae sperm have very long nucleus, with mitochondria shifted laterally to the nucleus (Sousa et al., 1989), and these Callista chione have curved nucleus (Nicota and Zappata, 1991). Spermatozoa of Galeommatidae curved nucleus, laterally tilted acrosomal vesicle, and abundant free glycogen in midpiece (Eckelbarger et al., 1990). So, only primitive spermatozoa have been described in the Donaciidae, including Donax trunculus (van der Horst et al., 1986; Hodgson et al., 1990; Sousa and Oliveira, 1994).

Thus, ultrastructural characteristics of mature spermatozoa of D. vittatus showed somewhat different characteristics in many families in the subclass Heterodonta.

Sperm ultrastructure has long been viewed as a tool in assessing taxonomic problems and phylogenetic relationship in the Metazoa through the use of spermiocladistic analysis (Jamieson, 1991; Healy, 1989&1995 a&b). In this study, D. vittatus belongs to external fertilization species and the type of spermatozoa is the primitive type. Many species of Veneridae in the subclass Heterodonta have three sperm nuclear types (cylindrical type) according to the angle of curved sperm nucleus: (1) no curved nuclear type (angle of the nucleus: 0°), (2) slightly curved nuclear type (angle of the curved sperm nucleus: 5°-10°), and (3) largely curved sperm nuclear type (angle of the curved nucleus: 15°-80°). Of them, Protothaca jedoensis (0°) belongs to no curved nuclear type, and the three species; Gomphina veneriformis (5°), Meretrix lusoria (10°), and Cyclina sinensis (10°) belong to the slightly curved nuclear type, and the species Saxidomus purpuratus (15°), Ruditapes philippinarum (25°), Mercenaria stimpsoni (80°) belong to the largely curved nuclear type. In particular, the nuclear type of P. japonicus belongs to largely curved nuclear type because the angle of the curved sperm nucleus is 45°. Thus, the angle of the sperm nuclear types varies with the species in Veneridae of Subclass Heterodonta.

In the present work, the basal and lateral parts (regions) of the basal rings of the acrosomal vesicle of spermatozoa of D. vittatus, showed electron opaque part (region), while the anterior parts of the apex showed electron lucent part (region). Hodgson and Bernard (1986) reported that in case of the subclass Pteriomorphia, the morphology of sperm acrosomal vesicles shows the same cone shape, as in the subclass Heterodonta.

The sperm midpiece, satellite fibres and flagellum of D. vittatus present no unique features. The presence of four mitochondria surrounding a pair of orthogonally-arranged centrioles is very common, not only among bivalve spermatozoa but also in aquasperm of various "archaeogastropods" (Healy 1988&1996) and, more generally, other invertebrates (Baccetti and Afzelius 1976; Jamieson 1987; Rouse and Jamieson 1987). Only three mitochondria were observed in T. maxima (Healy and Keys, 1999). Hodgson and Bernard (1986) and Healy (1989) stated that the number of mitochondria in the sperm midpiece tends to be stable within any family or superfamilies varying from a maximum of 14 in the mytiloid Modiolus diffusus (Droz dov and Reunov, 1986) to a minimum of 4 (Healy, 1989, 1995a&b). In general, the number of sperm mitochondria in bivalves varies between 4 and 5 (often within a species, and usually with one number predominating), with some genera of Mytiloidea displaying as many as 14 mitochondria (Droz dov and Reunov, 1986), and 7 to 9 being characteristic of the Carditoidea (Healy 1995a&b &1996).

In this study, the number of mitochondria at the midpiece of the spermatozoa are four (common to many bivalve families. Regarding the results reported by some authors (Chung and Ryou, 2000; Kim, 2001; Chung et al., 2006), the number of mitochondria at the midpiece of sperm of bivalves were four in Veneridae, Solenidae, Corbiculidae in subclass Heterodonta and Ostreidae in subclass Pteriomorphia, while those of Arcidae were five mitochondria, Mytilidae in subclass Pteriomorphia and some species of Veneridae (Saxidomus purpuratus, Meretrix lusoria, Cyclina sinensis) and Atrina pinnata japonica. Healy (1989) reported that the number of mitochondria at the midpiece of sperm showed unconstant and irregular characteristics in the level of subclass however showed stable constant characteristics under the level of family or in superfamil. Accordingly, the results of the number of the mitochondria are coincided with that reported by Healy (1989).

Sperm ultrastructure characteristics have been used as tools in studies of the taxonomic and phylogenetic relationships of Bivalvea, including the Donaciidae and Mytilidae. So far, spermatozoa of Mytilidae have been described as a primitive type, which is characteristic of invertebrates that reproduce by external fertilization. In these species, the sperm acrosome is conical and can be either long- or short-shaped, the nucleus is quite small. The ultrastructural

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organization of the intermediate piece is similar to the one described here, in which spherical mitochondria are grouped as a ring encircling the distal and proximal centrioles. The sperm has a single flagellum in the posterior region (Kafanov and Drozdov, 1998; Reunov et al., 1999; Introni et al., 2004).

The spermatozoon morphology of the *D. vittatus* specimens studied in the present work is in agreement with the recorded characteristics of the family Mytilidae sperm. The presence of the long axial rod, extending from the sperm nucleus to the acrosomal vesicle, justifies inclusion of the genera *Perna* and *Mytilus* in the subfamily Mytilinae. However, various other genera, such as *Modiolus* and *Brachidontes*, do not have axial rods and are considered members of the subfamily Modiolinae (Introni et al., 2004).

Perhaps the most significant results concluded from the present study are the spermatozoal similarities between *Donax vittatus*, *Donax trunculus*, *Tridacna maxima*, the cardiid *Cerastoderma edule* and *Phacosoma japonica* (Sousa and Azévedo 1988; Sousa and Oliveira 1994, Healy and Keys, 1999), respectively. In these species, the nucleus is elongate (although anteriorly helical in *C. edule*; nucleus straight in *T. maxima*) and refined apically into a peg-shaped structure penetrating deep into the invagination of the acrosomal vesicle (Sousa and Azévedo 1988; Sousa et al. 1995). In addition, the acrosomal vesicle in both species has a blunt conical form with a prominent basal ring; admittedly, a common pattern among heterodont bivalves, but best developed in groups such as the Cardioidae and Veneroidae (Popham 1979; Healy 1995a &b & 1996). However, further data on other donacids, tridacnids and cardiids are necessary in order to adequately test their phylogenetic relationship.

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