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Ultrastructure of grey mullet (*Mugil cephalus*, Linnaeus, 1758) spermatozoa as revealed from light, scanning and transmission electron microscopy

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Summary

Spermatozoa morphology and fine structure were studied in the grey mullet, *Mugil cephalus* using light, scanning and transmission electron microscopy. Light microscopy observations indicate a semi-cystic type of spermatogenesis in the testis. The electron microscopy micrograph showed that the spermatozoon of *M. cephalus* is uniflagellated (total length 5.78 ± 1.26 μm), differentiated into an ovoid-shaped head without acrosome (1.80 ± 0.35 μm in length and 1.91 ± 0.30 μm in diameter), with a short midpiece and a long cylindrical flagellar tail (length 3.60 ± 0.50 μm). The midpiece is characterized by the presence of four to five vacuoles, a cytoplasmic canal, two centriole and two spherical mitochondria having a flat type of cristae. Chromatin granules of the nucleus form an electron-dense homogeneous mass. The flagellum consists of nine peripheral microtubules and a central pair (9 + 2) surrounded by the plasma membrane with side fins. The results confirm that spermatozoa of *M. cephalus* are perciform or teleostean type II. Information generated from the present study will be useful in taxonomic classification, cryopreservation and breeding work.

Introduction

The patterns of spermatozoa structure are highly conserved within taxonomic groups, thus being a powerful tool for phylogenetic analysis in fish (Jamieson, 1991). Jamieson (1991) classified fish spermatozoa into aquasperm and introsperm. Aquasperm is divided into ect-aquasperm and ent-aquasperm. Ect-aquasperm can be divided into type I, type II, and type III sperm. This classification is based on whether or not nuclear rotation occurs during spermiogenesis (Mattei, 1970). In type I spermatozoa, nuclear rotation occurs, the flagellum becomes perpendicular and medial to the nucleus, and enters the nuclear fossa. In type II, there is no nuclear rotation; the flagellum remains parallel to the nucleus, and outside of the fossa. Type II spermatozoon is also referred to as the perciform type because of its widespread occurrence among the Perciformes (Abascal et al., 2002). Quagio-Grassiotto et al. (2005) and Shahin (2006) discovered a unique type of spermiogenesis called type III, which is intermediate between types I and II and characterized by an incomplete nuclear rotation, where the flagellum is eccentric to the nucleus. Within the family mugilidae, the spermatozoon ultrastructure has been examined in only a few species: *Liza dumerili* (Van der Horst and Cross, 1978); *Liza aurata,* (Brusle, 1981); *Mugil liza* and *Mugil platanus* (Eiras-Stofella and Gremski, 1991); and *Mugil curema* (Eiras-Stofella et al., 1993). There is no current information on the ultrastructure of *M. cephalus* sperm. However, El-Gharabawy et al. (2007) described the ultrastructure of steroidogenic secreting tissue. Therefore in the present experiment, the morphological and ultrastructure features of *M. cephalus* examine. The results will be useful in taxonomic classification, cryopreservation and as standards to troubleshoot problems during breeding.

Materials and methods

Sample collection

During the peak spawning season (November-December), oozing *M. cephalus* males (body length = 36 ± 8.5 cm and total weight = 380 ± 10 g; n = 6) were collected from the east coast of India (Kovalam, Chennai, lat. 12°49′ N; long. 80°15′ E). Immediately after collection, fish were anesthetized with 2-phenoxyehanol (150 ppm), urine was extruded by gently squeezing the fish near the genital pore, feces were carefully discarded, and the genital area was wiped with cotton. Semen was stripped by gentle pressure on the abdomen and collected in glass tubes. Another oozing male was dissected to cut open the testis, and a tissue sample was collected from the mid-portion of the testis and fixed in 10% neutral buffer formalin (NBF).

Light microscopy

A NBF-fixed testis was dehydrated by means of increasing ethanol concentrations (70–100%), followed by dipping in acetone and cleansing in benzene. The tissues were embedded in paraffin and serial sections were cut to 5 μm thickness using microtome. The sections of the testis were stained with haematoxylin and eosin, as described by Roberts (1989). The tissue slides were cleaned in xylene, mounted in DPX, and then observed under light microscope.

Scanning and transmission electron microscopy

Collected semen samples were immediately fixed in 2.5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer pH 7.4 (sodium cacodylate 2.14 g, calcium chloride 12.5 g, 0.2 N hydrochloric acid 1.25 ml and distilled water 100 ml) (primary fixative) for 4–8 h at 8°C. The primary fixative was decanted and then fixed in 0.1% osmium tetroxide prepared in the same buffer (secondary fixation). This post-fixation was carried out for 2 h at 8°C and centrifuged at 900 g for 10 min. Excess fixative was washed with the same buffer three times. The sperm samples were then processed for transmission (TEM) or scanning (SEM) electron microscopy. For TEM, tissues were dehydrated in graded series of acetone 30, 50, 70, 80, 90% for 10 min at each concentration, and with 100% acetone twice for 10 min, followed by propylene oxide treatment twice for 10 min each. The sections were then infiltrated with the resin mixture and acetone. The mixture consisted of epoxy 812 resin, DDSA (DoDecenyl Succinic Anhydride) and NMA (Nadiac Methyl Anhydride). The sections were then embedded using the same resin mixture with an added catalyst (DMP 30) in easy moulds at 60°C for 48 h. The resin blocks were removed from the moulds, trimmed, and sectioned using a Leica Ultracut R ultra-microtome fitted with glass knives. Initially, semi-thin sections were cut and stained with toluidine blue and screened using the light microscope to check for areas of interest in those sections from that particular block. Then ultrathin sections were cut, collected on copper grids and stained with saturated solutions of urinayl acetate followed by lead citrate for 10 min. After air-drying, the sections were screened in JEOL JEM 1400 transmission electron microscope at an accelerating voltage of 80 KV. Micrographs were taken with an Olympus Keen view CCD camera attached to the microscope. For SEM, fixed samples were glued on poly-l-lysine-coated coverslips. After dehydration through an ascending ethanol series, samples were air dried and coated with gold target by a coating unit and examined under a Cambridge Stereoscan 240 SEM operating at 10 KV. All measurements were carried out using Olympics Image software (Version 4.0.1 for MS windows, 1998) and expressed as mean ± standard error (SE).

Results

Macroscopic and histological observation

The testis of *M. cephalus* is bi-lobed and located in the posterior portion of the body cavity beneath the digestive tract, continues into a genital duct and ends in a genital pore immediately behind the anus. The Gonadosomatic Index (GSI %), total length and total weight of mature fish (n = 6) were 5.26 ± 1.6, 36 ± 8.5 cm and 380 ± 10 g, respectively. Bi-lobed testes are composed of many seminiferous lobules separated from others by a thin layer of interstitial tissue. Germ cells are arranged in clusters within the seminiferous lobules. Histological observation of mature testis shows the predominant presence of spermatozoa in the lumen of lobules, and a few spermatocytes and spermatids. Presence of spermatids in the lumen of the testicular tubules together with spermatozoa suggests that spermatogenesis is of the semi-cystic type (Fig. 1).

Scanning and transmission microscopy

The electron microscopy micrograph shows that the spermatozoon of *M. cephalus* is a uniflagellated cell, differentiated into a head without acrosome, a short midpiece, and a long cylindrical tail or flagellum (Figs 2 and 3). Total length of *M. cephalus* sperm (N = 30) was 5.78 ± 1.26 μm. The ovoid-shaped head measured 1.80 ± 0.35 μm in length and 1.91 ± 0.30 μm in width. The head was occupied almost totally by nucleus. The plasma membrane of the head tightly enclosed the nucleus (diameter 1.67 ± 0.25 μm) with a thin cytoplasmic layer between them (Fig. 3). The nucleus contains an electron-dense chromatin, which is

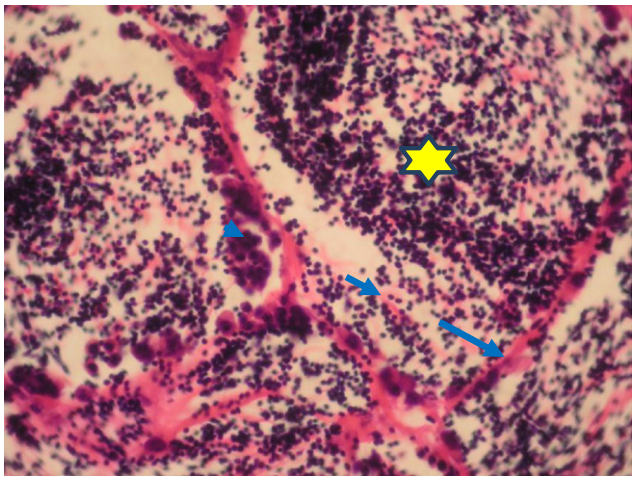

Fig 1. Light microscope micrographs of the testis of ripe male of *M. cephalus* showing: seminiferous lobules separated by interstitial tissue (large arrow), few spermatocytes (arrow head) and spermatids (small arrow) and predominantly spermatozoa (star) (100 X)

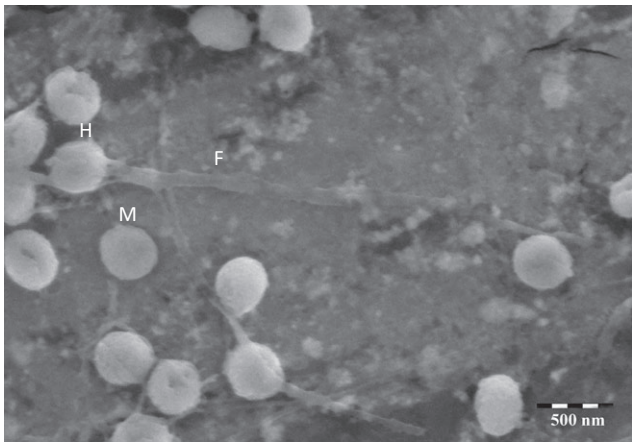

Fig 2. Scanning electron microscopy (SEM) micrograph of *M. cephalus* sperm showing head (H), middle piece (M), and flagellum (F)

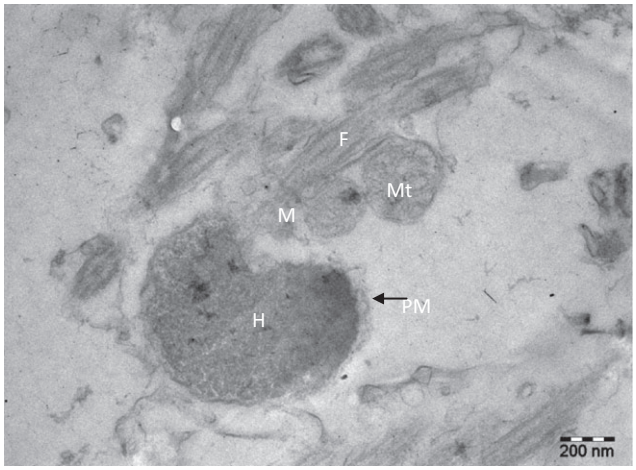

Fig 3. Transmission electron microscopy (TEM) micrograph of *M. cephalus* sperm longitudinal section showing distinct head (H), middle piece (M), mitochondria (Mt), plasma membrane (PM), and flagellum (F)

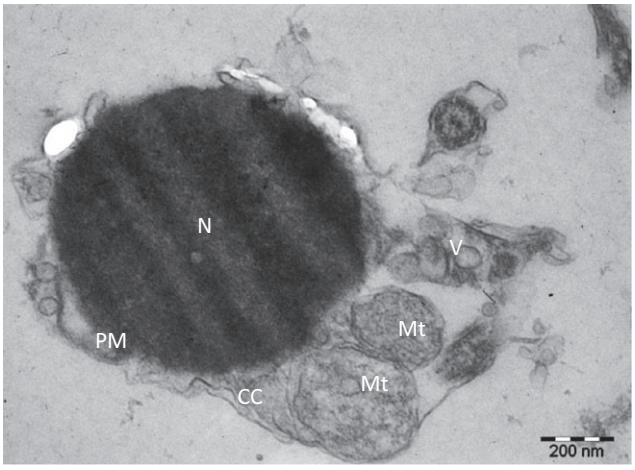

Fig 5. Transmission electron microscopy (TEM) micrograph of *M. cephalus* sperm, Cross section of nucleus (N), plasma membrane (PM), vacuoles (V) and mitochondria (Mt), and cytoplasmic canal (CC)

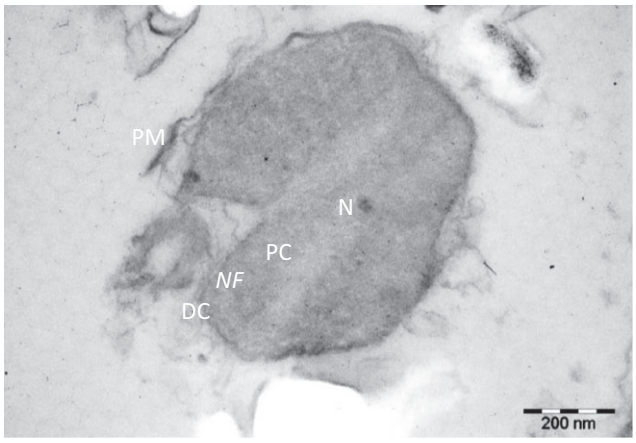

Fig 4. Transmission electron microscopy (TEM) micrograph of *M. cephalus* longitudinal section of sperm showing nucleus (N), plasma membrane (PM), proximal centriole (PC), distal centriole (DC), and nuclear fossa (NF)

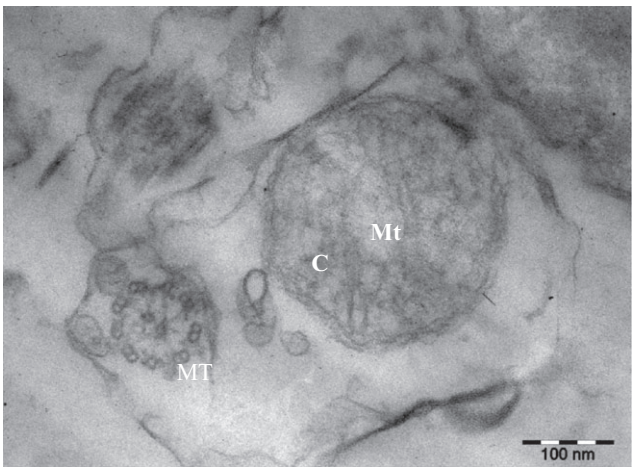

Fig 6. Transmission electron microscopy (TEM) micrograph of *M. cephalus* sperm mitochondria (Mt), irregular cristae (C) and axoneme with 9 + 2 microtubular arrangement (MT)

finely granular and homogeneously dispersed. At the posterior end of the nucleus, the nucleus invaginates and forms a depression known as nuclear fossa, to which axoneme is attached (Fig. 4). The mid-piece is characterized by the presence of two centrioles, four to five vacuoles and two mitochondria (Fig. 5). The anterior region of the nuclear fossa is occupied by the proximal centriole, whereas the distal centriole is located at the posterior (Fig. 4). The distal centriole becomes a basal body from which the axoneme emerges to form the sperm flagellum. The proximal and the distal centrioles are connected by a dense material, while the axoneme originates directly from the distal centriole (Fig. 4). Two mitochondria 0.34 ± 0.16 μm in diameter are found in the mid-piece region. The mitochondria have irregular cristae and a moderately electron-dense, granular matrix; they are separated from the axoneme by the cytoplasmic canal, which is formed by a deep invagination of the plasma membrane (Figs 5 and 6). In the distal centriole, a single axoneme with the conventional 9 + 2 arrangement, with nine peripheral microtubular doublets and a single central pair of microtubules was observed (Fig. 7). Each of the nine doublets consists of sub-fibers A and B. Two dynein arms arise from the sub-fiber (Fig. 7). The doublets are connected to each other by microfilaments and radial spokes. The flagellum, inserted perpendicular to the base of the nucleus, measured 3.60 ± 0.50 and 0.13 ± 0.01 μm in length and width, respectively. The flagellum has a cylindrical shape throughout its length and contains an unpaired side fin, which represents an evagination of the plasma lemma (Figs 8 and 9).

Discussion

Testis organization has two compartments, interstitial and tubular, as are present in all other vertebrates, from fish to

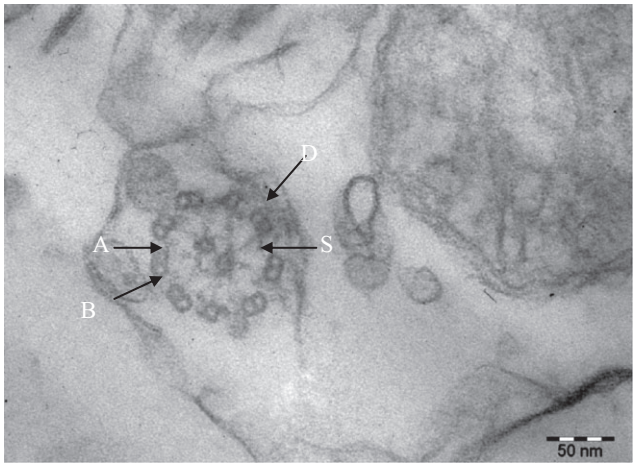

Fig 7. Transmission electron microscopy (TEM) micrograph of *M. cephalus* sperm axoneme with 9 + 2 microtubular arrangement. Microtubules A and B, radial spokes (S) and dynein arms (D) are visible

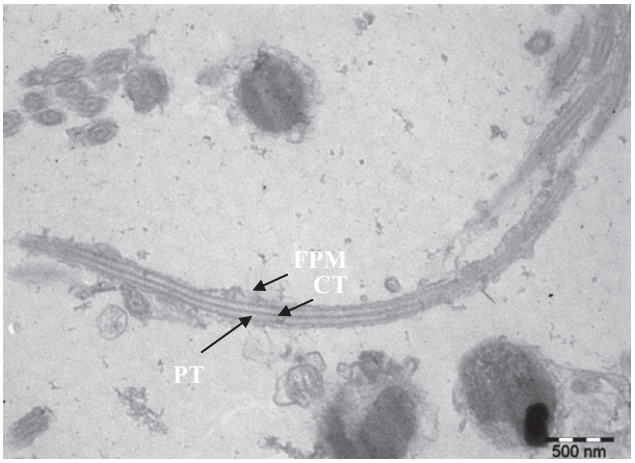

Fig 9. Transmission electron microscopy (TEM) micrograph of *M. cephalus* sperm longitudinal section of flagellum showing peripheral microtubules (PT), central microtubules (CT), and flagellar plasma membrane (FPM)

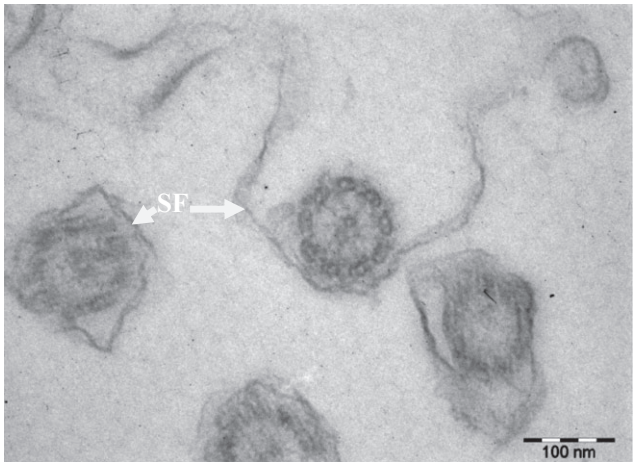

Fig 8. Transmission electron microscopy (TEM) micrograph of *M. cephalus* sperm cross-section of flagellum showing side-fins (SF)

mammals (Schulz et al., 2010). The interstitial compartment is composed of Leydig cells, blood/lymphatic vessels, macrophages, mast cells, neural and connective tissue cells (Koulish et al., 2002) and is continuous with the tunica albuginea, composed of fibrous tissue that surrounds the organ (Rupik et al., 2011). Seminiferous tubules contain spermatogenic cysts formed by Sertoli cells that sustain synchronously developing germ cells. In *M. cephalus*, the presence of spermatozoa in lumen of the testicular lobules suggests that spermatogenesis is of the semi-cystic type, as is reported in other mugilidae such as *Mugil liza* (Grier, 1981; Albieri and Araújo, 2010) and is common in fish and amphibians (Santos and Oliveira, 2008).

The spermatozoa of *M. cephalus* exhibit the configuration of uniflagellated anacrosomal aquasperm typically found in externally fertilizing fishes (Jamieson, 1991). Specifically, the sperm ultrastructure of *M. cephalus* conforms to the perciform sperm type or teleostean type II spermatozoon (Mattei, 1970), which occurs in as many as 29 out of 41 families of studied Perciformes (Mattei, 1991; Abascal et al., 2002), and is the result of a simplified (apomorphic) spermiogenic process in which rotation of the spermatid nucleus is lost (Mattei, 1970). The absence of nuclear rotation has also been described in an another mugilidae, *Liza aurata* (Brusle, 1981). Like other teleosts, *M. cephalus* spermatozoa lack acrosome. The loss of acrosome in most of the teleosts is a result of an evolutionary process compensated by the development of an opening in the zona pellucida of egg called the micropyle, which facilitates penetration of spermatozoa to access the oolemma (Medina et al., 2000). Different shapes of sperm heads occur in chondrostean and teleostean fishes with external fertilization. A spherical-shape spermatozoon head was found in mugilidae such as *Mugil curema* (Eiras-Stofella et al., 1993), *M. liza* and *M. planatus* (Eiras-Stofella and Gremski, 1991), *M. cephalus* (El-Gharabawy et al., 2007), *Liza aurata* (Brusle, 1981) and *L. dumerili* (Van der Horst and Cross, 1978). An ovoid or spherical-shaped head is a result of a simple spermiogenesis, whereas a more-or-less elongated head region is derived from a more complex spermiogenic process and is considered as an advanced morphological feature (Grier, 1981; Jamieson, 1991; Maricchiolo et al., 2004). The present finding suggests that the spermiogenesis and sperm in *M. cephalus* are of a simple and primitive type.

The length and width of a *M. cephalus* spermatozoa head are 1.80 ± 0.35 and 0.91 ± 0.30 μm, respectively, which are close to that of other mugilidae species viz. *M. liza* and *M. planatus,* measuring 1.50 μm (Eiras-Stofella and Gremski, 1991), but smaller than *Liza dumerii*, which has a spermatozoa head diameter of 2.4 μm (Van der Horst and Cross, 1978). Chromatin granules of the nucleus form an electron-dense homogeneous compact mass, probably attributed to the nuclear material that was not perfectly dehydrated during spermatogenesis (Guan et al., 1990). The kidney-shaped nucleus in *M. cephalus* is similar to *M. curema* and other mugilidae (Eiras-Stofella et al., 1993). The diameter of *M. cephalus* spermatozoa nucleus (1.67 ± 0.25 μm) is close

to that of *L. aurata* (1.5 μm) and *M. curema* (1.0 μm) (Eiras-Stofella et al., 1993). In *M. cephalus, the* proximal centriole is in an oblique position to the distal centriole, which is a common feature in the mugilidae family (Eiras-Stofella et al., 1993).

Sperm motility depends on the length of flagellum and number of mitochondria in spermatozoa (Lahnsteiner and Patzner, 2007). The flagellar movement of fish spermatozoa can be classified into two types according to sperm structure (Ishijima et al., 1998): (i) spermatozoa having an elongated mitochondria, and (ii) spermatozoa having a simple structure with rudimentary mitochondria. These two types appear in fish showing internal and external fertilization strategies, respectively. In general, longer spermatozoa of teleosts swim faster but with higher energy costs (Alavi et al., 2008). In Atlantic salmon, Vladic et al. (2002) demonstrated that the longer spermatozoa had more ATP available for swimming and achieved a higher fertilization success. Gage et al. (2004) also noted that sperm with higher velocities of spermatozoa were able to fertilize a greater number of eggs. Sperm size could be important in sperm competition because longer sperm may generate greater flagellar forces (Katz and Drobnis, 1990) and swim faster (Gomendio and Roldan, 1991). Smaller sperm may allow males to produce more gametes, which may be adaptive if sperm compete numerically (Parker, 1982).

In *M. cephalus* spermatozoa, a short midpiece is observed similar to many teleosts with external fertilization (Jamieson, 1991; Romagosa et al., 1999; Maricchiolo et al., 2004). A common source of interspecific variation in the sperm structure of fishes is the number of mitochondria contained in a midpiece (Baccetti et al., 1984; Mattei, 1991). In perch *Perca fluviatilis*, only a single mitochondrion was found (Retzius, 1906), while there are more than 20 mitochondria in the *Idus melanotus* (Ginsburg, 1968); in cyprinids, this varies from 2 to 10 (Baccetti et al., 1984); in mugilidae, four mitochondria were reported (Eiras-Stofella and Gremski, 1991; Albieri and Araújo, 2010). In another mugilidae species, *M. curema,* Eiras-Stofella et al. (1993) reported 9 to 10 mitochondria with an average diameter of 0.59 μm. However, in the present study, two mitochondria were observed in *M. cephalus* with an average diameter of 0.34 ± 0.16 μm, which is similar to that of *M. platanus* and *M. liza* (Eiras-Stofella and Gremski, 1991) but differs from that of *L. aurata* with 0.55–0.66 μm (Brusle, 1981) and *M. curema,* with 0.59 μm (Eiras-Stofella et al., 1993). Two mitochondria in *M. cephalus* were also reported by El-Gharabawy et al. (2007). There are three types of mitochondrial cristae: lamellar (flat), vesicular, and tubular. The majority of cells of higher plants and animals have lamellar mitochondrial cristae. In the present study, *M. cephalus* has a flat type of cristae, as reported in other mugilidae species such as *M. liza*, *M. platanus, L. aurata*, *L. dumerili,* and *M. curema* (Eiras-Stofella et al., 1993).

ATP produced by mitochondrial respiration is the main energy source for sperm motility. ATP is used by the dynein limbs, which give origin to a self-oscillatory bending behavior of the flagellar axoneme. The presence of a few mitochondria might indicate a lower energy-delivering capacity compared to those species possessing more mitochondria (Lahnsteiner and Patzner, 1995). Thus, the number of mitochondria might be a factor contributing to a more or less successful gamete preservation.

We observed vacuolated cytoplasm (vacuoles) around the nucleus and in the midpiece. A similar structure is observed in other mugilidae species viz. *M. liza* and *M. platanus* (Eiras-Stofella and Gremski, 1991) but the structure is rare in other mugilidae, *M. curema* (Eiras-Stofella et al., 1993). The function of this structure is to eliminate an excess of cytoplasm during spermiogenesis, as reported in *Brycon cephalus* and *B. microlepis* (Romagosa et al., 1999; Verissimo-Silveira et al., 2006). As reported in other mugilidae species, *M. cephalus* spermatozoa also possess a cytoplasmic canal, except that in *Liza dumerili* (Eiras-Stofella et al., 1993) and this is for the anchoring of flagella to the sperm head (Maricchiolo et al., 2007). Most teleost sperms, as in other vertebrates, possess single flagella, while a few teleosts, e.g. batrachids, bagrids, myctophids, gobiesocids and cichlids have been found with biflagellar sperms (Mattei, 1988; Matos et al., 2002). We observed the uniflagellate sperm in *M. cephalus*. The axoneme forms a cytoskeletal structure within the flagellum, providing support and structure to the flagellum primarily during movement. The axoneme of *M. cephalus* shows a typical eukaryotic organization, consisting of nine pairs of 'doublet' microtubules, forming a ring around a single central pair of microtubules, to create a '9 + 2' arrangement. Transverse sections at different levels of the flagellum show a radial spoke, tubules A and B of outer doublets of microtubules and dynein arms. This pattern is common in teleosts (Mattei, 1988), however, eel *Anguilla anguilla* has a '9 + 0' axoneme configuration (Shahin, 2006). The plasma membrane is folded into lateral fins or ridges along the flagellum length in *M. cephalus,* as found in other teleosts such as rainbow trout *Oncorhynchus mykiss*, eciliidae, Janysiidae, Patolontidae and Embiotocidae (Stanley, 1969; Van Deurs, 1975; Billard, 1983; Lahnsteiner et al., 1997). Lateral fins, which originate from the plasma membrane could accelerate velocity of the flagella to increase the efficiency of fertilization (Stoss, 1983; Zhang et al., 1993; Cosson et al., 2000). Flagellum with a 9 + 2 pattern and one or two lateral fins is common in primitive spermatozoon (Mattei, 1988).

Conclusion

The present study concludes that the spermatozoa of *M. cephalus* exhibit the configuration of the uniflagellated, anacrosomal aquasperm of type II. Presence of spermatozoa in the lumen of the testicular lobules suggests that the spermatogenesis is of the cystic type. The flagellar tail has 9 + 2 microtubular arrangement and the mid-piece is characterized by the presence of two mitochondria with a flat type of cristae.

Acknowledgements

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