



## Characterization of Testis and Sperm Morphology of *Sahyadria denisonii* (Day 1865) an Endemic and Threatened Ornamental Fish of the Western Ghats of India

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### Abstract

*Sahyadria denisonii* is an endemic Cyprinid of the Western Ghats of India that has great demand in the international ornamental fish market. The present study examined the morphology, histology and ultra structural biology of testis of the fish. Testicular structure and sperm morphology were studied using light and scanning electron microscopy techniques. Length and weight of testis varied from 16 to 41 mm and from 0.026 to 0.834 gm respectively. Germ cells are seen in different stages as primary and secondary spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa. A spermatozoon is characterized by ovoid head, a short mid piece, and a single flagellum, but the acrosome was absent. The present study described that *S. denisonii* has an anastomosing tubular type of testis with an unrestricted distribution of the spermatogonial cells. These descriptions are noteworthy for a better understanding of reproductive biology, for which information remain scarce in the literature.

**Keywords:** Electron microscopy, reproductive biology, spermatogenesis, western ghats, spermatozoa.

### Introduction

Reproductive strategies of fishes are extremely diverse (West, 1990) and gonad development differs intra-specifically or inter-specifically depending on morphology, or anatomy of gonad as well as environmental conditions (Rahemo and Al-Shatter, 2012). According to Magalhaes *et al.*, (2011), data on spermatogenesis and spermatogenic ultrastructure in teleosts are scarce and restricted to a few species. Therefore, a detailed study on the biological features of threatened species will be very important for implementing any fish conservation programme (Virjenhock *et al.*, 1998). The Redline Torpedo fish, *Sahyadria denisonii* (Day 1865) is distributed in twelve rivers of Western Ghats hotspot region of India (Mercy *et al.*, 2013). Recent IUCN Red List for freshwater fishes in the Western Ghats of India assessed the status of *S. denisonii* as Endangered (Molur *et al.*, 2011). Till to date, testicular maturation in *S. denisonii* has not been studied at the morphological or histological level, but very little information about its reproductive characteristics has been examined (Solomon *et al.*, 2011; Mercy *et al.*, 2013). In the present study to understand the testicular biology of *Sahyadria denisonii*, macroscopic as well as microscopic investigations were carried out.

### Materials and Methods

The Western Ghats, extending along the west coast of India, is one of 34 global biodiversity hotspots (Raghavan *et al.*, 2011). Monthly samples of the fish were collected from the catch for aquarium trade at collection sites in River Valapattanam, Kerala (Figure 1) during September 2012-August 2013. River Valapattanam lies in the Western Ghats region of South India (Latitude 11°93'N; Longitude 73°73'E) and has an overall passage length of 110 km. Total length and weight of fish as well as testis were recorded to nearest to mm and mg. After assessing the stage of maturation, the testes were preserved in 5% formalin for further studies. Quantification of maturity stages was done by following Mc Bride *et al.*, (2002) and scheme proposed by EL-Boray (2001). For the histological examination, small pieces of fresh tissues from the testis at different stages of maturity were fixed in Bouin's fluid. The bouin's fixed tissues were later processed by following standard procedures (Euphrasia, 2004; Sunesh Thamby, 2009) for histological studies and stained with haematoxylin-eosin. Testicular development were examined and photographed with a stereo microscope (LAS EZ, Leica Application Suite). For the scanning electron microscopic examination, milt was stripped off from

anaesthetized *S. denisonii* by manual pressure over the abdominal region and fixed in 1% glutaraldehyde and 3% paraformaldehyde in a buffer of 0.1M phosphate buffer (pH 7.3) for 24 hours and then were post-fixed with 1% osmium tetroxide in same buffer for 2 hours. After dehydrating in an ethanol series, critical point drying, and coating with gold, samples were examined and electro-micro graphed with Scanning Electron Microscope (JEOL Model JSM-6390LV).

## Results

The male reproductive system in *S. denisonii* which is characterized by a pair of testis lie in the body cavity ventral to the swim bladder (Figure 2), and is attached to the dorsal body by a mesorchium. The testes had two equal lobes which are broader at anterior region and tapered towards posterior. The two lobes were folded, but not branched. The testes ranged in length and weight from 1.6 to 4.1cm and

0.026 to 0.834gm respectively. Sexual maturation was characterized by its size and colour turning into milky white. Based on the shape, size, colour, texture and histological differentiations, eight maturity stages were recognized in *S. denisonii* as Immature virgin (Stage-I); Early developing (Stage-II); Late developing (Stage-III); Mature (Stage-IV); Ripe (Stage-V); Spawning (Stage-VI); spent (Stage-VII) and Developing recovery (Stage-VIII).

I. Immature virgin (Figure 3a): This is the first stage of testis development recorded in young individual, which has not yet spawned. It is thin, pale in colour, occupied a very small proportion of the body cavity and the left lobe was slightly longer than the right one. In this stage, all lobules of the testis were formed of primary spermatogonia ( $SG_1$ ) of different sizes and were distributed as single as well as groups.

II. Early developing (Figure 3b): Testis became firm, started developing its creamy white colour, increased in size, occupying about  $\frac{1}{4}$  of body cavity.

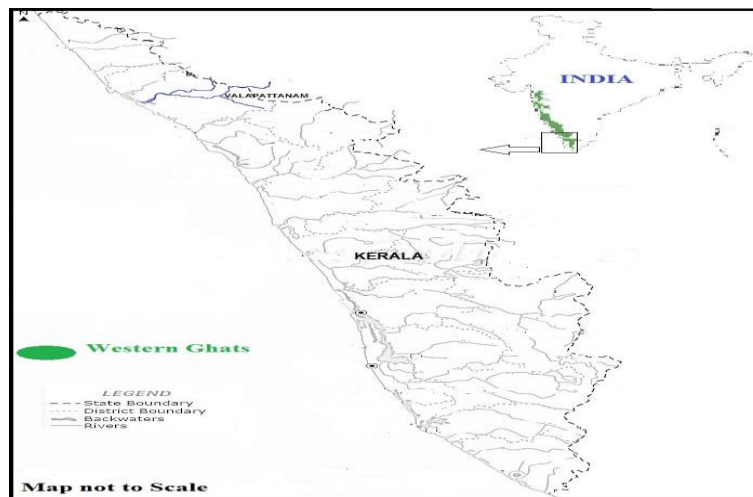


Figure 1. Location map of River Valapattanam.

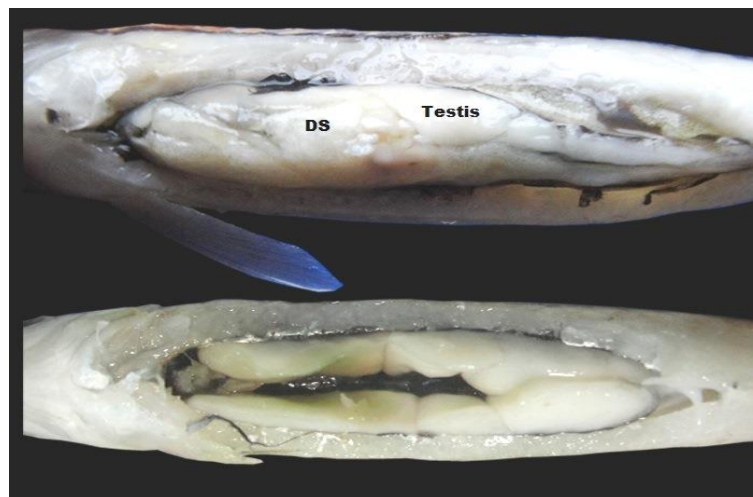


Figure 2. Gonadal system in male Sahyadria denisonii; DS-digestive system.

It was broader at its anterior and tapered towards the posterior end. This stage exhibited active spermatogenesis, where spermatogenic cells, including spermatogoniums (SGs) and primary spermatocytes (SC<sub>1</sub>) were found.

III. Late developing (Figure 3c): Testis became white, firm, occupied about ½ of the body cavity. This stage of maturation was characterized by the formation of lumen in the lobule. The secondary spermatocytes were predominant in this stage along with primary spermatocysts, spermatogoniums and a few spermatids.

IV. Mature (Figure 3d): Testis became creamy white, firm with irregular outer margin and occupied ¾ of the body cavity. In this stage, spermatids were predominant than other spermatogenic cells.

V. Ripe (Figure 3e): The testis became creamy white, soft lobed and reached their maximum size. Milt could be easily extruded upon exerting slight pressure on the belly. Testis contained abundant spermatozoa and spermatid in the outer portion of gonad and lobules were packed with spermatozoa. The spermatozoa were found nearer to the wall of the lobules and parachute shaped clumps were also noticed, where as those found in the lumen of the lobules became free. The spermatogonium, primary spermatocysts cells were less in number.

VI. Spawning (Figure 3f): The testis became very extensive, milky white in colour and milt could be easily extruded upon exerting slight pressure on the belly. Anterior portion of the testis lobe started to shrink due to discharge of milt.

VII. Spent (Figure 3g): Testis seemed to be dorso-ventrally flattened, thin and dull white in colour. The wall of the lobules became very thick, blood cells were found scattered and some lobules contained residual sperms or not.

VIII. Developing recovery (Figure 3h): In this stage, the spent testis entered into the regeneration phase with well-defined seminiferous tubules with spermatogonia and spermatocytes. Anterior and

posterior region of testis were broadened, than the early developing stage (Stage-I).

The internal testicular structure of *S. denisonii* was tubular type and intertubular stroma region contained loose connective tissue, blood capillaries and interstitial cells (Leyding cells). Active spermatogenesis was observed in *S. denisonii* and spermatogenesis occurred progressively during the annual reproductive cycle. Large amounts of spermatozoa were accumulated in the central system of ducts and after completion of spermatogenic process spermatozoa were released into the lumen.

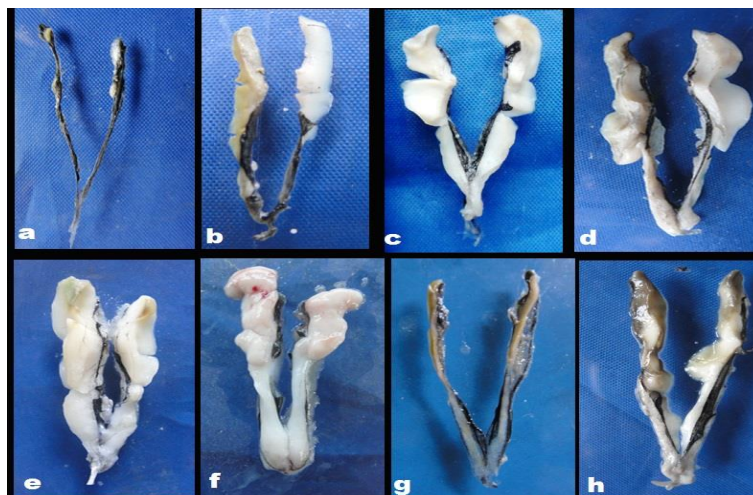
Each spermatogonium in the testis passed through different stages of maturation to form spermatozoa (Figure 4). The lobules were filled with discrete nests of spermatogenic cells in various stages of maturation and cell size became decreased gradually by the development from spermatogonia to spermatozoa (Figure 4). Six stages of maturation was recorded during spermatogenesis in *S. denisonii*,

1. Primary spermatogonia (SG<sub>1</sub>) (Figure 5.A,B): Spermatogonia were more or less spherical in shape and were distributed along the germinal epithelium. They could be found either as individual or as groups of cells.

2. Secondary spermatogonia (SG<sub>2</sub>) (Figure 5.A,B): Secondary spermatogoniums were formed by the mitotic division of primary spermatogonia (SG<sub>1</sub>) and were found as clusters of cells with intensely stained nuclei and clear cell membrane. They were similar to primary spermatogonia, except in size and darker colour.

3. Primary spermatocytes (SC<sub>1</sub>) (Figure 5.C): Secondary spermatogoniums were mitotically divided into primary spermatocytes and were smaller than secondary spermatogonia (SG<sub>2</sub>) with reduced cytoplasm. Cytoplasm was stained faintly, while nucleus was purple with haematoxylin-eosin stains.

4. Secondary spermatocytes (SC<sub>2</sub>) (Figure 5.C): Secondary spermatocytes were formed by the meiotic division of primary spermatocytes (SC<sub>1</sub>). They were

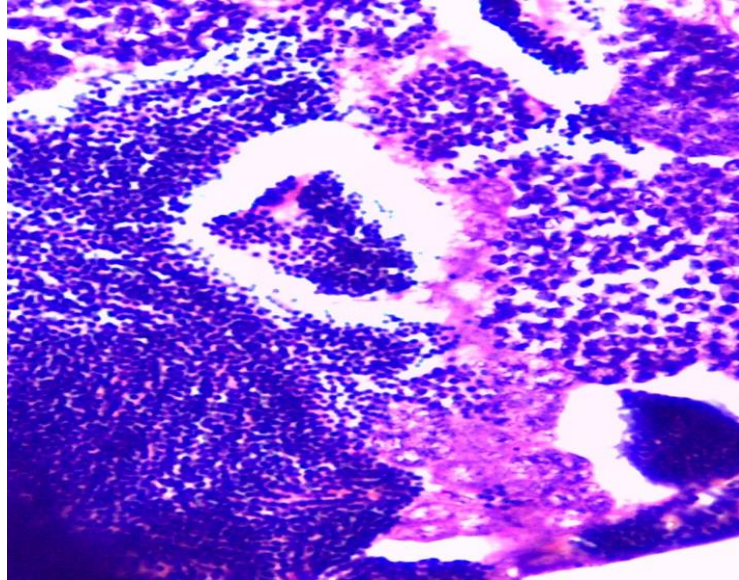


**Figure 3.** Testis maturity stages recognized in *Sahyadria denisonii*.

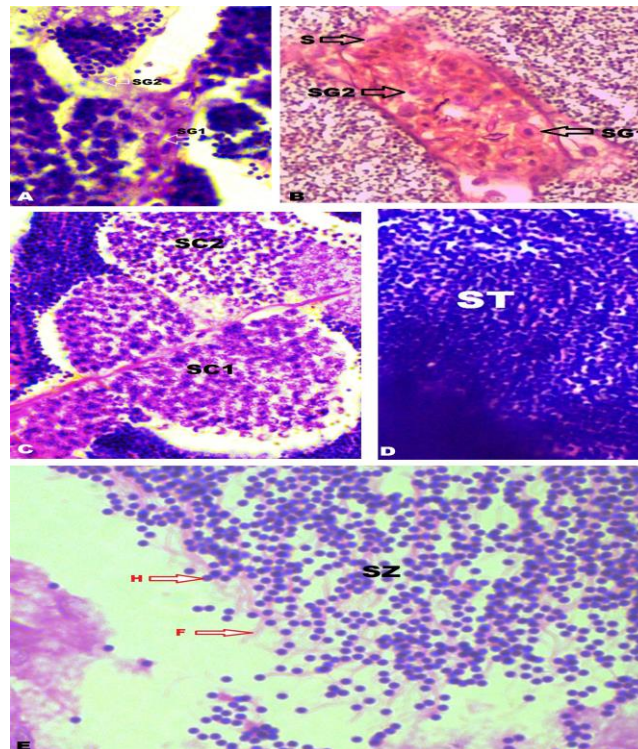
smaller and darker than primary spermatocytes. Cytoplasm was less and nucleolus was no longer visible.

5. Spermatids (STs) (Figure 5.D): The spermatids (STs) were produced by the second

meiotic division of the secondary spermatocytes (SC<sub>2</sub>). They were much smaller, compact, dark, dot-like structures and appeared as deeply stained with Haematoxylin-eosin. Spermatids were undergoing spermiogenesis to produce the spermatozoa.



**Figure 4.** Transverse section through testis showing, Spermatogonia (SG), Primary spermatocytes (SC1), Secondary spermatocytes (SC2), Spermatids (STs), Spermatozoa (SZ). H&E-40x.



**Figure 5.** Spermatogenesis in *Sahyadria denisonii*, Primary spermatogonia (SG1); secondary spermatogonia (SG2); Sertoli cells (S); Primary spermatocyst (SC1); Secondary spermatocyst (SC2); Spermatids (ST); Spermatozoa (SZ); Head (H); Flagellum (F). Figure. A-D (H&E, 40X) and Figure. E (H&E, 100X).

6. Spermatozoa (SZ) (Figure 5.E): Spermatozoa were the smallest spermatogenic cells with distinct tail and darkly stained nucleus. A mature spermatozoon consisted of two regions, ovoid head with elongated flagellum. After completing spermiogenesis, spermatozoa were released into the lumen and in ripe males lumen was richly packed with mature spermatozoa.

Scanning electron microscopy was used to investigate the fine structure of the spermatozoa of *S. denisonii*. Spermatozoa of *S. denisonii* were tightly packed in the lumen of the lobules (Figure 6). Spermatozoa consisted of an ovoid head, short cone shaped mid-piece and a long cylindrical flagellum (Figure 7). Spermatozoa had no acrosome and are aqua-sperm type.

## Discussion

In general fish reproductive physiologists have placed more importance on the study of histological changes in female than male (Schulz *et al.*, 2010).

Usually, gonad development was monitored on the basis of their macroscopic and microscopic appearance (Wang *et al.*, 2003; Martins *et al.*, 2012). Testis of numerous teleosts showed general structures with a paired testis is either fused along the entire length or completely separated or fused posteriorly (Selman and Wallace, 2005). The testis of *S. denisonii* had two equal lobes with folded structure but not branched, similar to other cyprinid fishes (Al-Daham and Bahatti, 1979; Bardakci *et al.*, 2000). The testes of *S. denisonii* were united at the posterior region to become the spermatoduct as similar to freshwater fishes like as *Barbus tor* (Rai, 1965); *Labeo fimbriatus* (Bhatnagar, 1972); *Schizothorax richardsonii* (Bisht, 1974); *Horaglanis krishnani* (Mercy *et al.*, 1982); *Brachydanio rerio* (Selman *et al.*, 1993); *Barbus longiceps* (Stoumboudi *et al.*, 1993); *Labeo dussumieri* (Kurup, 1994); *Barbus scateri* (Encina and Granado-Lorencio, 1997); *Pethia conchoniui* (Cek *et al.*, 2003); *Osteobrama bakerii* (Euphrasia, 2004); *Amblypharyngodon melettinus muriyadensis* (Teji, 2010); *Pethia pookodensis* (Augustine *et al.*, 2013) and

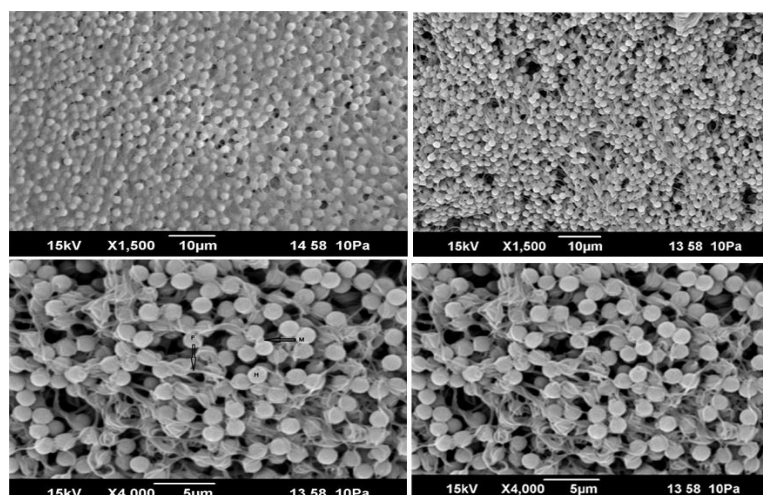


Figure 6. SEM photograph of ripe testis showing spermatozoa.

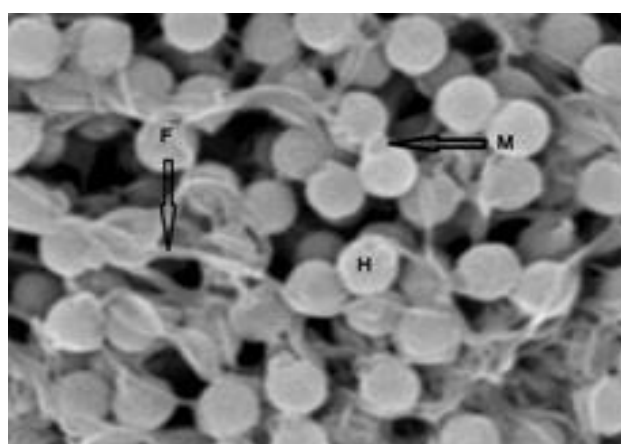


Figure 7. SEM image of spermatozoon showing Head (H), Middle piece (M) and Flagellum (F).

*Chela fasciata* (Indira *et al.*, 2014).

The testis of *S. denisonii* was attached to the dorsal body wall and was covered with tunica albuginea. The protrusion of tunica albuginea into the testicular parenchyma completely divided it into numerous seminiferous lobules. This is a common pattern found in the family Cyprinidae (Koulish *et al.*, 2002; Schulz *et al.*, 2010; Teji 2010; Montchowui *et al.*, 2012). Anatomical structure of testis in *S. denisonii* was found to be similar to other cyprinids that have external fertilization (Grier, 1993; Parenti and Grier, 2004). Present study showed that *S. denisonii* had lobular unrestricted type of testis, in which seminiferous tubules were grouped into many cysts.

Generally, spermatogenesis process in freshwater fishes was classified into four to six stages of development, depending on the species and the choice of criteria used (Nagahama, 1983; Joshi and Joshi, 1989; West, 1990; Rutaisire *et al.*, 2003; Unver and Unver Saraydin, 2004; Montchowui *et al.*, 2012). Six stages of spermatogenic cells were identified in *S. denisonii* as primary spermatogonia (SG<sub>1</sub>), secondary spermatogonia (SG<sub>2</sub>), primary spermatocyte (SC<sub>1</sub>), secondary spermatocyte (SC<sub>2</sub>), spermatid (ST) and spermatozoa (SZ). Spermatogenesis in *S. denisonii* appeared to be similar to (Joshi and Joshi, 1989); *Osteobrama bakerii* (Euphrasia 2004); *Garra surendranadini* (Sunesh Thamby, 2009); *Amplypharyngodon melettinus muriyadensis* (Teji, 2010). In the present study it was observed that all stages of spermatogenic cells were distributed asynchronously and this type of cell maturity resulted in the discharge of sperms intermittently during spawning period. It may be concluded that *S. denisonii* had a prolonged spawning season. However, the duration of spermatogenesis and level of testicular enlargement may vary with species as well as geographic location (West, 1990; Fraile *et al.*, 1992; Rutaisire *et al.*, 2003; Schulz *et al.*, 2010).

Spermatogenesis is a highly organized process, in which diploid spermatogonia got proliferated and differentiated in to mature spermatozoa (Schulz *et al.*, 2010). In many tropical species, reproduction is a seasonal or cyclic event related to environmental signals (Billard and Breton, 1978; Nash, 1998). Sperm motility is initiated only when the milt is diluted with an activating medium such as freshwater, saline media, pH, or ovarian fluid (Scott and Baynes, 1980; Billard, 1986; Sajan *et al.*, 2013), while Morisava and Morisava (1986) stated that spermatozoa acquire their motility when passing through the sperm duct. Spermatozoa within the seminiferous tubules are immotile and may lack fertilization capacity (Nagahama, 1983; Billard, 1986; Sajan *et al.*, 2013).

In the present study, scanning electron microscopy (SEM) was used to examine the fine structure of spermatozoa of *S. denisonii*. Spermatozoa of *S. denisonii* were characterized by a head, a short

mid piece, flagellum similar to other cyprinids (Baccetti *et al.*, 1984). Generally, shape of the spermatozoan head, mid-piece and flagellum varied between teleosts (Mattei, 1991; Maricchiolo *et al.*, 2004; Leal *et al.*, 2009). Spermatozoa of *Sahyadria denisonii* showed the typical organization of externally fertilizing fishes, as they have an ovoid nucleus, a small mid piece, and had no acrosome (Baccetti *et al.*, 1984; Jamieson, 1991; Vergilio *et al.*, 2012). The head of spermatozoa in fishes to family cyprinidae is usually spherical to ovoid in shape (Baccetti *et al.*, 1984), spherical heads were recorded in *Esox lucius* (Rotheli *et al.*, 1950) and *Carassius auratus* (Furbock *et al.*, 2009); ovoid in *Apogon imberbis* (Lahnsteiner and Patzner, 2008) and *Cyprinus carpio* and *Barbus barbus* (Furbock *et al.*, 2009), and banana-shaped in *Anguilla anguilla* (Todd, 1976). A short type mid piece was found in *S. denisonii* as common in teleosts with external fertilization (Nicander, 1970), rather than spermatozoa has a longer mid piece for internal fertilization (Jamieson, 1991).

Based on the presence or absence of acrosome, the spermatozoa of teleosts was categorized into acrosomal and anacrosomal type (Jamieson, 1991), *S. denisonii* had anacrosomal type of spermatozoa. This type of spermatozoa was also established by the presence of micropyle in the eggs of *S. denisonii* (Sajan, 2015). Micropyle is a channel structure through which an anacrosomal sperm can enter into the egg without proteolytic decomposition of the *zona pellucida* of the egg (Amanze and Iyvengar, 1990). The present study indicated the micropyle-dependent fertilization by which *S. denisonii* prevented the egg from polyspermy. Moreover, spermatozoa in fishes were classified into two forms as aqua-sperm and intro-sperm, according to external or internal mode of fertilization respectively (Jamieson, 1991). *S. denisonii* had anacrosomal and aqua-sperm type spermatozoa.

According to morphological and histological studies, it may be concluded that, *S. denisonii* had an extended spawning season. These descriptions are very important for a better understanding of reproductive biology, particularly of tropical fishes for which data remain scarce in the literature. This will also help in the development of captive breeding technology of this fish.

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*Sahyadria denisonii*, an endemic indigenous ornamental fish of Western Ghats of India.

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