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Research article

Spermatozoon of the wild scalloped perchlet, *Ambassis nalua* (Hamilton, 1822): Ultrastructure and morphometric analysis

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Abstract

The description of sperm morphology is fundamental in the reproductive biology of fishes, but this information is limited in the family Ambassidae. Our report hence focused on the ultrastructure and morphometric analysis of spermatozoa in a pelagic fish *Ambassis nalua*. All fish (n = 75) were obtained during January and March 2017 from the Estuarine Pranburi River, Thailand. The standard length of fish used in this study was 3.4 ± 0.12 cm (mean ± standard deviation). All specimens were considered mature based on the abundance of spermatozoa in the testis. The testicular organs were collected and observed using standard histology and transmission electron microscopy (TEM). Ultrastructural observation associated with morphometric analysis showed that spermatozoa are structurally long cells of approximately 51.17 ± 4.54 µm total length, composed of a head, a midpiece and a tail. The head had no acrosome, and the granular structure of condensed chromatin was observed within the ovoid nucleus. The midpiece consisted of a short cylindrical region with the length of 1.29 ± 0.87 µm in diameter, having the centriolar complex organization and eight mitochondria (approx. 0.32 ± 0.02 µm each). The uniflagellar tail was clearly identified with a classical 9+2 arrangements of microtubules. Based on these characteristics, the spermatozoon of wild scalloped perchlet are considered as uniflagellate anacrosomal aquqsperm. The morphological features, including the number of mitochondria, may be used for further cryopreservation and in the evolutionary biology of this species.

Keywords: Unioflagellar sperm, Sperm morphology, Scalloped perchlet, Thailand

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INTRODUCTION

Pioneering observations on the morphology of fish spermatozoa morphology were documented by Geiger (1955) and Mattei (1970), in which varying ultrastructural features of fish spermatozoa were reported from a reproductive point of view. Later studies from an evolutionary perspective have shown that the morphology of fish spermatozoa is highly conserved, but they have been morphologically classified into aquasperm and introsperm (Baccetti et al., 1984; Rouse & Jamieson, 1987; Jamieson and Leung, 1991; Mattei, 1991; Cosson et al., 2008). The two types of spermatozoa are considered to reflect the different modes of fertilization/reproduction. The aquasperm spermatozoa have a round head, a short neck region (mid-piece), and a few mitochondria (Jamieson, 1991). This type of spermatozoa is associated with external fertilization (Jamieson, 1991), which has been found in 97% of all teleosts (Lahnsteiner et al., 1990; Jamieson, 1991; Quagio-Grassiotto et al., 2005; Maricchiolo et al., 2007), as reported in several species such as *Lates calcarifer* (Jamieson, 1991), *Pagrus major* (Hara and okiyama, 1998), *Pagellus bogaraveo* (Maricchiolo et al., 2010), *Rastrelliger brachysoma* (Senarat et al., 2018a) and *Allenbatrachus grunniens* (Sukkhee et al., 2021). The introsperm spermatozoa have an elongated head, but the mid-piece is known to be missing (Mattei, 1991; Jamieson and Grier, 1993; Jamieson, 1991). Nuclear rotation and fewer numbers of mitochondria have also been reported for this type of spermatozoa. The introsperm spermatozoa have been found in fishes of at least 24 teleostean families (Reznick et al., 2002; Burns & Weitzman, 2005) that undergo internal fertilization (Jamieson and Grier, 1993; Jamieson, 1991).

The wild scalloped perchlet, *Ambassis nalua* (Hamilton, 1822) is a small species widely distributed in estuarine waters including the Estuarine Pranburi River, Thailand. Arianti et al. (2017) reported that wild scalloped perchlet spawn throughout the year with a reproductive peak in September. The sex ratio of mature scalloped perchlet was 1:1.9 (male: female). Yet, little is still known on the structure and gametogenic differentiation of wild scalloped perchlet. Morphological characterization of the sperm is a starting point to understanding the male reproductive biology of this species. The goal of this study was to conduct ultrastructural observations and morphometric analysis of the spermatozoa of the wild scalloped perchlet using transmission electron microscopy (TEM). The results from this study will provide baseline information for future studies related to taxonomic classification and reproduction.

MATERIALS AND METHODS

Study area and fish collection

Live male specimens of adult *Ambassis nalua* (n = 75 individual fish) were randomly obtained during January to March 2017 by using beach seines from Estuarine Pranburi River (EPR), Thailand (N12º 24.314' E099º 58.597'). The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1723004).

Tissue preparation and light microscopic observation

Fish specimens were euthanatized by a rapid cooling method (Wilson et al., 2009) and then measured for standard length, shown as the mean ± standard deviation (SD). Testicular tissues (0.5 x 0.5 cm, n = 75 individual fish) were collected from the peritoneal cavity of all fish and fixed with a standard fixative (Davidson's fixative) for routine histological examinations. The testicular paraffin blocks were cut in transverse orientation into 5 µm thickness, stained with Harris's hematoxylin and eosin (H&E) and then mounted in DPX (Presnell and Schreibman, 1997; Suvarna et al., 2013; Senarat et al., 2018b; Senarat et al., 2019). The histological sections were observed for testicular development and spermatogenic differentiation and photomicrographed using a Leica DM750 light microscope (Leica, Germany).

Transmission electron microscopy

Fragmented testis from five specimens (n = 5) were prepared for the TEM using a two-step fixation protocol. The first prefixation was performed in 2.5% glutaraldehyde solution in phosphate buffer pH 7.4 for about 24 h, and the second fixation was performed in 1% osmium tetroxide. All fixed testicular tissues were processed using a standard ultrastructural method. The plastic blocks were cut with a diamond knife into ultrathin sections of 90 nm thickness and then stained with uranyl acetate and lead citrate. The stained ultrathin sections were observed in both sperm composition and its organelles under a TEM (Philips/TECNAI 20). The quantitative morphometric characterization of sperm ultrastructure including sperm length, head length, mid-piece length and flagellum length was carried out using an image analysis program [Image J (Version 4.0.1 for MS windows, 1998)] and reported as means ± SD. An illustration of sperm morphology of *A. nalua* was created using Adobe Illustrator CS5.

RESULTS

The standard length of wild male scalloped perchlet specimens were 2.3 to 5.9 cm with the average of 3.4 ± 0.1 cm. It was histologically confirmed that all fish samples had mature or maturating testis, which consisted of developing sperm cells in the germinal compartment (i.e., under "spermatogenesis"). The spermatogenic cells of the wild scalloped perchlet were classified into spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid and spermatozoa having a head and tail (Figure 1A). The spermatogonia were widely distributed along the seminiferous lobule, being designated as "unrestricted spermatogonial testis" (Figure 1B). Many spermatozoa were migrating toward the lumen of seminiferous tubules (Figures 1A-1B).

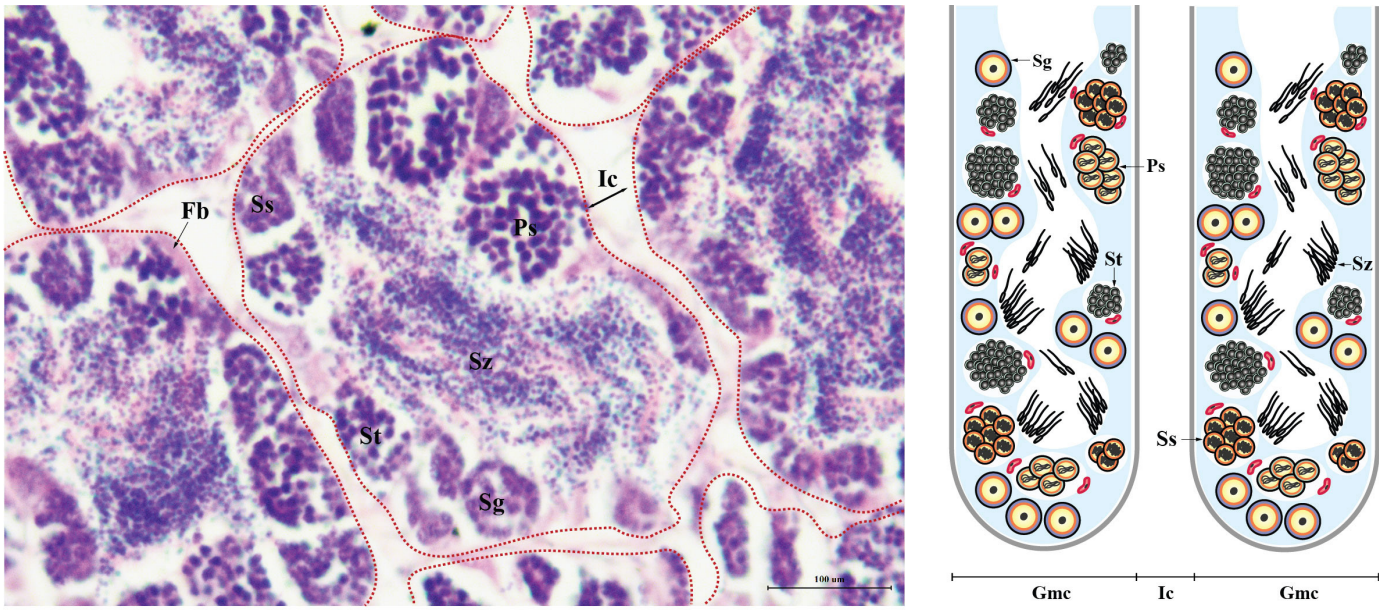

Figure 1 Light micrograph showing spermatogenesis in the testis of wild scalloped perchlet, *Ambassis nalua*. A: A Cross-sectional histological image of testis having a seminiferous lobule. The different stages of sperm could be classified into spermatogonia (Sg), primary spermatocyte (Ps), secondary spermatocyte (Ss), spermatid (St) and spermatozoa (Sz). Abundant Sz were clearly found in the lumen. B: Schematic illustration of the spermatogonial testis. Abbreviations: Ic=interstitial compartment, Gmc=germinal compartment, Fb=fibroblast.

Results of ultrastructural observations and the morphometric analysis of mature spermatozoa are shown in Figures 2-3 and Table 1, respectively. The total length of spermatozoa was 51.17 ± 4.54 µm, and a spermatozoon was determined to consist of three main regions: head, mid-piece, and tail (flagellum) (Figure 2, Table 1). The head region had a symmetrical ovoid shape with a length of 3.31 ± 0.05 µm. The nucleus rotation and granular structures of electron-dense chromatin elements were clearly observed in the nucleus in the head of almost all of the wild scalloped perchlet spermatozoa (for example Figures 2B-2D).

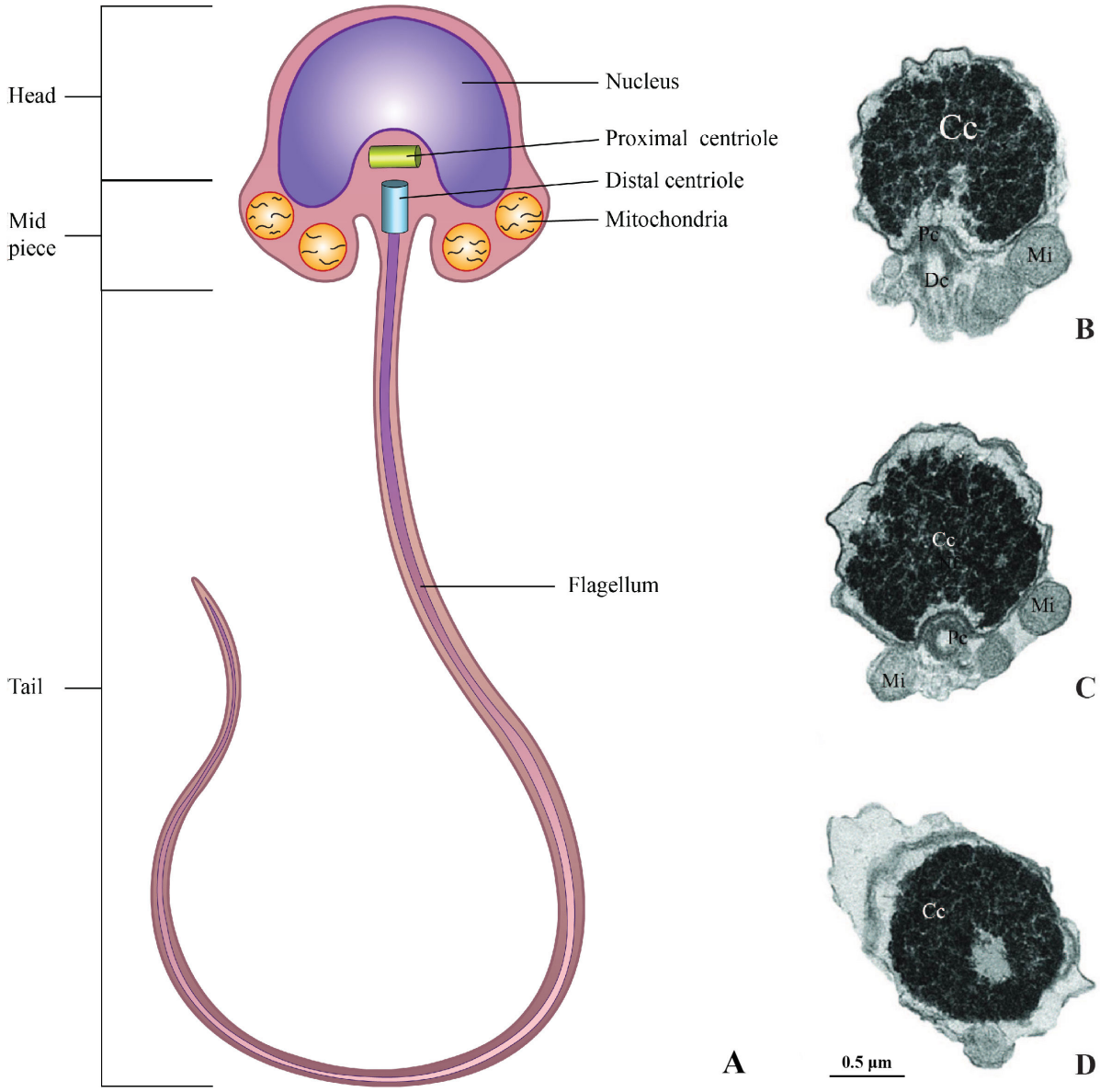

Figure 2 Schematic diagram (A) and transmission electron micrograph from several views (B-D) of the spermatozoa in wild scalloped perchlet, *Ambassis nalua*. The sperm had three regions including the head (He), mid-piece (Mp) and tail (Ta). Abbreviations: Ax = axoneme, Cc = chromatin condensation, Dc = distal centriole, Mi = mitochondria, Nf = nuclear fossa, Pc = proximal centriole.

Table 1 Morphometric analysis of spermatozoon in wild scalloped perchlet, *Ambassis nalua*.

Spermatozoa	Variables			
Sperm size (µm)	Total length; 51.17 ± 4.54			
Head (µm)	Length; 1.31 ± 0.04	Width; 1.44 ± 0.05	Nucleus length; 1.14 ± 0.05	Nucleus width; 1. .21 ± 0.09
Mid-piece (µm)	Length; 0.27 ± 0.08	Width; 0.57 ± 0.08	Mitochondria diameter; 0.32 ± 0.02	Mitochondria number; 8
Flagellum (µm)	Length; 49.86 ± 4.52	Width; 0.25 ± 0.03	Axoneme width; 0.27 ± 0.03	
Axonemal pattern	A classical 9+2 arrangements of microtubules			

Electron microscopy also showed a short cylindrical mid-piece of wild scalloped perchlet spermatozoa with the length of 0.27 ± 0.08 µm (Table 1). At the posterior end of the head, the centriolar complex was found inside the nuclear implantation fossa (Figure 3A). The mid-piece was composed of (i) the proximal centriole within the nuclear fossa and (ii) the distal centriole that was differentiated into the basal body and directly connected to an axoneme (Figures 3A-3B). Eight mitochondria (approx. 0.32 ± 0.02 µm in diameter each) (Table 1) were documented in the wild scalloped perchlet of our study. All mitochondria had irregular cristae and a moderately electron-dense surrounding matrix (Figure 3C).

The flagellar of wild scalloped perchlet spermatozoa consisted of a long cylindrical tail (or uniflagellar) within the cytoplasmic membrane (Figure 2A). Each flagellar axis shown in the cross-sectional views consisted of a circle of nine triplet's doubles of peripheral microtubules and axonemal doublet of the microtubules, which represented the classical 9+2 arrangements of microtubules. Each of the nine doublets was associated with a structural complex of radial spokes and dynein arms (Figure 3D and Table 1).

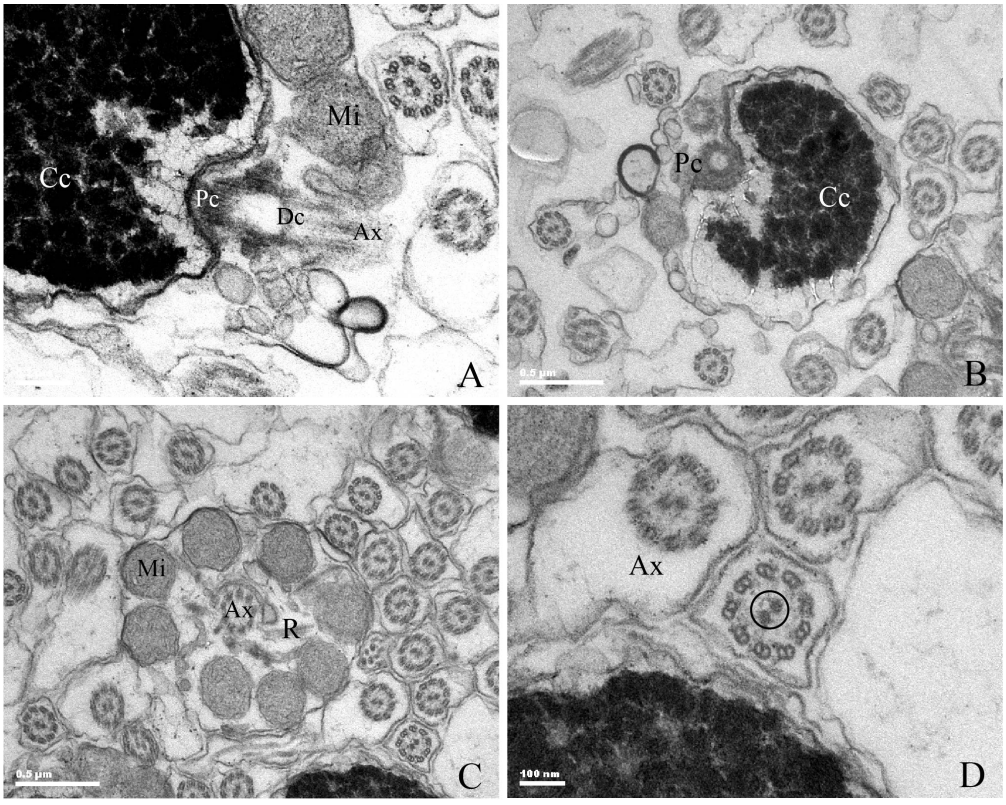

Figure 3 Transmission electron micrograph of different regions of spermatozoa from wild scalloped perchlet, *Ambassis nalua*. A-B: The midpiece showed the proximal (Pc) and distal (Dc) centriole. C: The eight mitochondria were identified in the midpiece. D: The doublet microtubules were structural components of the axoneme (Cycle). Abbreviations: Ax = axoneme, Cc = chromatin condensation, He = head, Mp = midpiece, Ta = tail, Mi = mitochondria, Nf = nuclear fossa, R = radial spokes.

DISCUSSION

Our observations showed the testicular morphology of wild scalloped perchlet captured during January to March 2017. Considering that all male specimens had mature testes, the maturation size of this fish species was first estimated to be above 2.3 cm. This finding also indicates that the breeding period of wild scalloped perchlet in this area covers at least January to March, although it is possible that the actual breeding period much more protracted. Further investigations of the reproductive cycle and spawning season of this fish will be done in the near future.

The morphology of teleost spermatozoa is related to the mode of fertilization, which has previously been classified into aquasperm and introsperm (Jamieson and Grier, 1993; Jamieson, 1991). Based on our results, the spermatozoa of wild scalloped perchlet are likely aquasperm, because they had an oval shaped head, nucleus rotation, and nucleus outsides the fossa, whereas the centrioles remained within the shallow nuclear fossa formation. This is similar to that observed in Perciformes (Mattei, 1991; Abascal et al., 2002) and Siluriformes (Poirer and Nicholson, 1982), the morphology of which has been associated with external fertilization (Mattei, 1991; Biagi et al., 2016).

The shape of the spermatozoon head is well known to be highly variable among teleosts (Jamieson, 1991). Our observations showed a constricted symmetrical morphology of an ovoid nucleus with the absence of acrosome in the anterior region, which is a characteristic of simple spermiogenesis (Grier, 1981; Jamieson, 1991; Maricchiolo et al., 2004). In contrast, an elongated head region is derived from a more complex differentiation process of spermiogenesis (Grier, 1981; Jamieson, 1991; Maricchiolo et al., 2004). It is reflected as an advanced morphological sperm feature, which is found many internally fertilized teleosts (Jamieson and Grier, 1993). On the other hand, the absence of acrosomes has been associated with the presence of egg micropyle, a protrusion of the chorion (Medina et al., 2000; Chung, 2008). The head of sperm is related to hydrodynamic abilities of swimming performance and velocity (Malo et al., 2006).

The short mid-piece of wild scalloped perchlet spermatozoa is likely reflective of external fertilization, which has been commonly reported in teleosts (Jamieson, 1991; Maricchiolo et al., 2004; Vergílio et al., 2013). Interspecific variation of the number of mitochondria in the mid-piece was noted (Baccetti et al., 1984; Mattei, 1991). Our study found eight mitochondria in A. naula spermatozoa. However, the number of mitochondria varies depending on the species; for example only a single mitochondrion is in *Perca fluviatilis* (Retzius, 1906), 2 in *M. cephalus* (Fahmy et al., 2007), 4 to 6 in *Barbus barbus* (Alavi et al., 2008), 6 in *Thynnus thynnus* (Abascal et al., 2002), 11 in *A. grunniens* (Sukkhee et al., 2021) and more than 20 in *Idus melanotus* (Ginsburg, 1968). Since the major role of mitochondria is to produce ATP for spermatozoa motility (Christen et al., 1987; Cosson et al., 1999), the presence of fewer mitochondria may be related to lower energy compared to sperm having more mitochondria (Lahnsteiner and Patzner, 1995; Billard et al., 1995; Billard et al., 2000). In this regard, the number of mitochondria observed in this study, eight, may indicate the high energy available that enables efficient movement and successful fertilization.

There are three types of spermatozoon flagella (aflagellate, uniflagellate and biflagellate species) in teleosts (Jamieson, 1991; Jamieson, 2009; Quagio-Grassiotto et al., 2011). The wild scalloped perchlet spermatozoa were uniflagellate sperm with a classical 9+2 structure of microtubules as similarly described in *S. australasicus* (Hara and Okiyama, 1998), *R. brachysoma* (Senarat et al., 2018a) and *A. grunniens* (Sukkhee et al., 2021). However, the functional significance of the number of sperm flagella is not fully resolved and warrants further study.

CONCLUSION

Our observation showed for the first time that wild scalloped perchlet spermatozoa had an oval head, eight mitochondria and the axoneme of the sperm tail (classical 9+2 arrangements of microtubules). The present finding suggests that sperm in wild scalloped perchlet are of a uniflagellate anacrosomal aquasperm. The morphometric data provides basic reproductive information of the species and helps to identify the taxonomy among the family. The physiological aspects, such as the activity and motility of sperm, can also be associated with sperm morphology to understand the reproductive biology of this species of fish.

AUTHOR CONTRIBUTIONS

Conceptualization, N.S. and J.K; Methodology, N.S., T.M., T.K. and S.S.; Formal Analysis, N.S. and S.S.; Investigation, N.S.; Resources, N.S., S.S. and J.K.P.; Writing – Original Draft Preparation, N.S.; Writing – Review & Editing, T.M., T.K., S.S., N.K., G.K. and J.K.; Supervision, J.K.; Project Administration, J.K.; Funding Acquisition, N.S. and J.K. All authors have read and approved of the final manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

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