

Sperm ultrastructure in three different families of weakly electric fishes (Teleostei: Gymnotiformes)

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This study presents details of sperm ultrastructure for *Gymnotus* aff. *carapo* (Gymnotidae), *Eigenmannia trilineata* (Sternopygidae), and three *Brachyhypopomus* species (*B. draco*, *B. bombilla*, and *B. gauderio* - Hypopomidae) from southern Brazil. Differences were found among the representatives of the different families. For example, nuclear rotation was present in *E. trilineata* and in the *Brachyhypopomus* species, but absent in *Gymnotus* aff. *carapo*, and the presence of flagellar fins was only observed in *E. trilineata*. Some intraspecific variations could also be noticed among the *Brachyhypopomus* species analyzed. Most of the characters found in the spermatozoa of the species studied herein are shared with species of Gymnotiformes previously analyzed.

Este estudo apresenta detalhes sobre a ultraestrutura do espermatozoide de *Gymnotus* aff. *carapo* (Gymnotidae), *Eigenmannia trilineata* (Sternopygidae), e três espécies de *Brachyhypopomus* (*B. draco*, *B. bombilla*, e *B. gauderio* - Hypopomidae) do sul do Brasil. Diferenças foram encontradas entre os representantes das diferentes famílias, como por exemplo a presença de rotação nuclear em *E. trilineata* e nas espécies de *Brachyhypopomus* e a ausência em *G. aff. carapo*, e a presença de aletas laterais no flagelo observada somente em *E. trilineata*. Algumas variações intraespecíficas também puderam ser registradas entre as espécies de *Brachyhypopomus* analisadas. A maioria das características encontradas nos espermatozoides das espécies aqui analisadas são compartilhadas com as demais espécies de Gymnotiformes previamente analisadas.

Key words: Gymnotidae, Hypopomidae, Spermatozoa, Sternopygidae.

Introduction

The order Gymnotiformes is restricted to Neotropical freshwaters, from Guatemala to Argentina, and also on the Caribbean island of Trinidad (Mago-Leccia, 1978). The order is comprised of five families and 185 species (Eschmeyer & Fong, 2011) and, although there are many hypotheses on the relationships among its families (Triques, 1993; Mago-Leccia, 1994; Alves-Gomes *et al.*, 1995; Albert & Crampton, 2005), they are still controversial.

According to Mattei (1991), since great structural diversity has been demonstrated for fish at all taxonomic levels, it has not been possible to construct spermiac models, even for monophyletic groups such as Actinopterygii or Teleostei. For this reason, results from sperm ultrastructure have provided valuable information on cellular modifications associated with reproductive habits and uncovered morphological characters useful in hypothesizing

phylogenetic relationships (Baccetti, 1987; Baicere-Silva *et al.*, 2011; Burns *et al.*, 1998; Burns *et al.*, 2002).

Ultrastructural studies on gymnotiform spermatozoa have been carried out on *Apteronotus albifrons* (Linnaeus, 1766) (cited as *Sternarchus albifrons*) (Jamieson, 1991), *Gymnotus* cf. *anguilaris* Hoedeman, 1962 and *Brachyhypopomus* cf. *pinnicaudatus* (Hopkins, 1991) (França *et al.*, 2007), and *Rhamphichthys* cf. *hahni* (Meinken, 1937), *Eigenmannia* cf. *virescens* (Valenciennes, 1842) and *Apteronotus* cf. *albifrons* (França *et al.*, 2009). However, many questions remain and more studies are needed to better understand sperm evolution in the order Gymnotiformes (França, 2006). The purpose of the present study is to provide details of sperm ultrastructure in *Gymnotus* aff. *carapo* Linnaeus, 1758, *Eigenmannia trilineata* López & Castello, 1966, and three *Brachyhypopomus* species (*B. draco* Giora, Malabarba & Crampton, 2008, *B. bombilla* Loureiro & Silva, 2006, and *B. gauderio* Giora & Malabarba, 2009) from southern Brazil.

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Material and Methods

The present study was conducted on adult males of *Brachyhypopomus bombilla*, *Brachyhypopomus draco*, *Brachyhypopomus gauderio*, *Eigenmannia trilineata*, and *Gymnotus* aff. *carapo* collected in Rio Grande do Sul State, southern Brazil. *Brachyhypopomus draco*, *B. gauderio*, and *G. aff. carapo* were collected at a flooded area near the arroio dos Ratos creek (29°57'31.9"S 51°33'10.1"W), Charqueadas Municipality, laguna dos Patos drainage. *Brachyhypopomus bombilla* and *E. trilineata* were collected at a creek that is part of the rio Uruguay drainage (30°12'42.8"S 55°03'17.5"W), near Rosário do Sul Municipality. The fishes were sacrificed by severing the spinal cord. Immediately afterwards, small pieces of testes were placed in modified Karnovsky's fixative (Ito & Karnovsky, 1968) and kept under refrigeration until the start of further processing. The fishes were deposited in the fish collection of the Universidade Federal do Rio Grande do Sul (*Brachyhypopomus gauderio* - UFRGS 9200; *Brachyhypopomus bombilla* - UFRGS 9284; *Brachyhypopomus draco* - UFRGS 6750, *Eigenmannia trilineata* - UFRGS 6635, *Gymnotus* aff. *carapo* - UFRGS 6859).

Testes were dehydrated in an ethanol series and critical-point dried for scanning electron microscopy (SEM). The dried tissue was then attached to stubs with carbon double-stick tape and teased apart with needles. The sample was sputter-coated with carbon and gold and viewed in a LEO 1430VP scanning electron microscope. For transmission electron microscopy (TEM), testes were cut into small pieces ($\pm 1 \text{ mm}^3$), rinsed in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer. Afterwards testes were rinsed in phosphate buffer, dehydrated in an ethanol series, infiltrated, and embedded in Araldite 502. Ultrathin sections were cut on a Sorvall MT5000 ultramicrotome, mounted on grids, and stained with aqueous uranyl acetate and lead citrate. Sections were examined with a JEOL JEM 1200 transmission electron microscope.

Results

Spermatozoa of *Brachyhypopomus draco*, *Brachyhypopomus bombilla* and *Brachyhypopomus gauderio* - Hypopomidae.

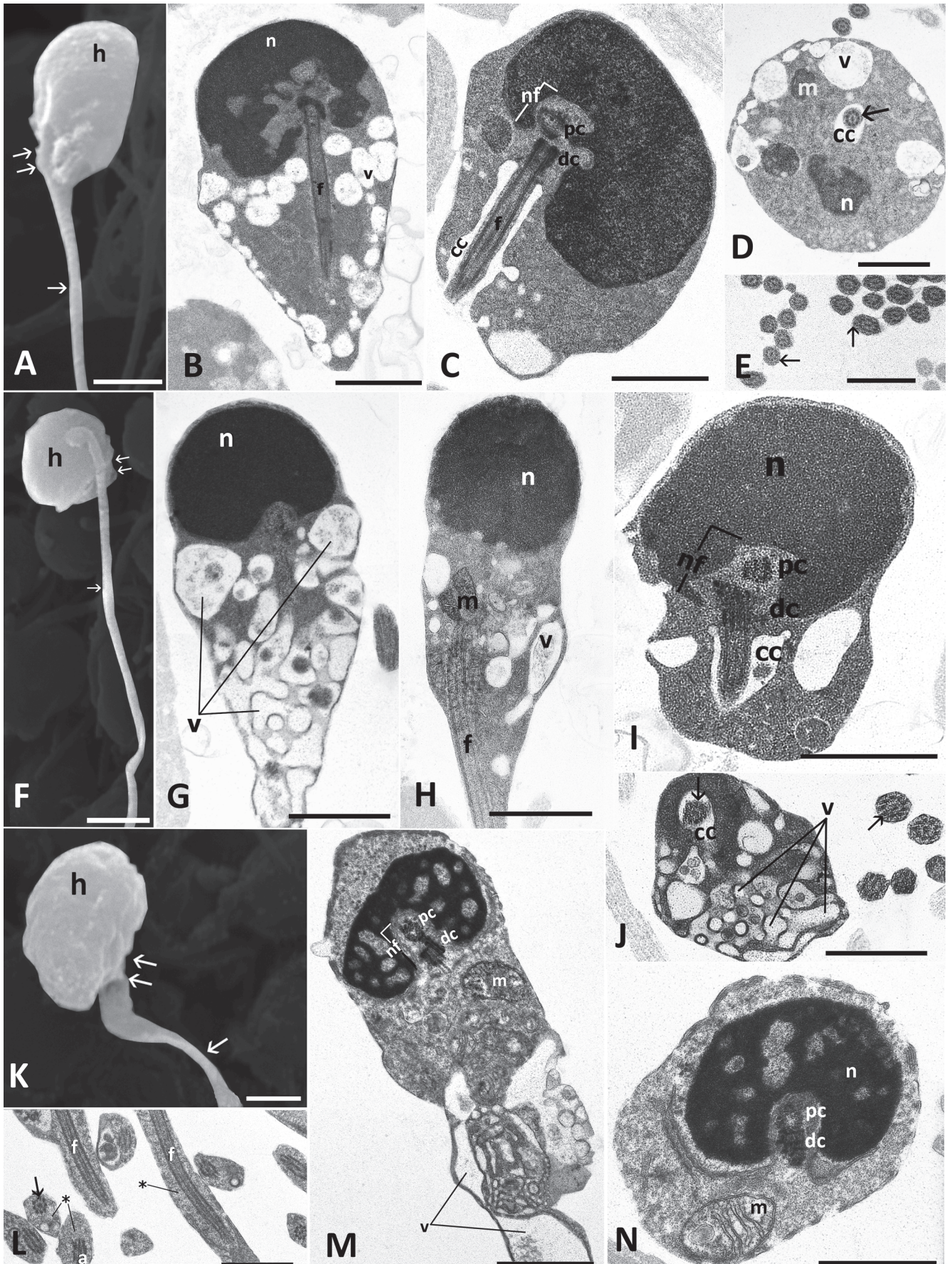
The spermatozoa of the three analyzed species are characterized by a well defined head, midpiece, and single flagellum. Both the head and nucleus tend to be spherical in *B. draco* (head, Fig. 1F; nucleus Fig. 1G-H-I), but ovoid in *B. gauderio* (head, Fig. 1A; nucleus, Fig. 1B-C) and *B. bombilla* (head, Fig. 1K; nucleus, Fig. 1M-N). Nuclear diameter is 1.0 μm for *B. draco*; length and width nuclear measurements are 1.7 x 2.8 μm for *B. gauderio* and 0.9 x 1.8 μm for *B. bombilla*. Nuclear chromatin is condensed and granular in all three species, with extensive areas of lighter chromatin seen only in *B. bombilla* (Fig. 1M-N). All species undergo nuclear rotation. The proximal centriole is anterior, slightly lateral and perpendicular to the distal. Electron-dense material is associated with both centrioles.

The proximal centriole and most of the distal are contained within a deep nuclear fossa (Fig. 1C-I-M). Only in *B. gauderio* does the fossa branch extensively throughout the nucleus (Fig. 1B). The midpiece contains several elongate mitochondria and a large number of vesicles, some of which contain stainable material (Fig. 1B-D-G-H-M). An open cytoplasmic canal is only observed immediately posterior to the centriolar complex in *B. gauderio* (Fig. 1C) and *B. draco* (Fig. 1I) but this may close more posteriorly. An open cytoplasmic canal is not apparent in *B. bombilla*. An area of more organized membranous structures is only observed in the midpiece of *B. draco* (Fig. 1G-J). The flagellum has the classic 9 + 2 microtubular pattern and does not show intratubular differentiation, both tubules of each peripheral doublet being electron-lucent (Fig. 1E-J-L). The flagellar membrane does not have lateral projections or fins in any of the species. However, there is substantially more cytoplasm between the axoneme and flagellar membrane in *B. bombilla* (Fig. 1L).

Spermatozoa of *Gymnotus* aff. *carapo* - Gymnotidae

The spermatozoa of *Gymnotus* aff. *carapo* are comprised of a head (Fig. 2A) containing a spherical nucleus (Fig. 2C), a

Fig. 1. (next page) **A-F-K:** Spermatozoa of *Brachyhypopomus gauderio*, *Brachyhypopomus draco*, and *Brachyhypopomus bombilla* respectively (SEM), h = sperm head, arrow = flagellum, double arrow = midpiece; **B-C:** Spermatozoa of *B. gauderio* in longitudinal section (TEM) showing ovoid nucleus (n), deep nuclear fossa (nf), centriolar arrangement (dc= distal centriole, pc= proximal centriole), open cytoplasmic canal (cc), and presence of vesicles (v) containing some electron-dense material, f = flagellum; **D:** Midpiece of spermatozoa of *B. gauderio* in cross section (TEM) showing cytoplasmic canal (cc) and elongate mitochondrion (m); **E:** Flagella of spermatozoa of *B. gauderio* in cross section (TEM) showing electron-lucent tubules of each peripheral doublet (arrow); **G-H-I:** Spermatozoa of *B. draco* in longitudinal section (TEM) showing spherical nucleus (n), centriolar arrangement (dc= distal centriole, pc= proximal centriole), open cytoplasmic canal (cc), elongate mitochondria (m), and presence of vesicles (v) containing some electron-dense material and forming an organized membranous structure; **J:** Midpiece and flagellum of spermatozoa of *B. draco* in cross section (TEM) showing vesicles (v) forming an organized membranous structure, and electron-lucent tubules of each peripheral doublet (arrow); **L:** Flagella of spermatozoa of *B. bombilla* in longitudinal and cross section (TEM) showing electron-lucent tubules of each peripheral doublet (arrow), and cytoplasm located between axoneme (a) and flagellar membrane (asterisk); **M-N:** Spermatozoa of *B. bombilla* in longitudinal section (TEM) showing ovoid nucleus with extensive areas of lighter chromatin (n), centriolar arrangement (dc= distal centriole, pc= proximal centriole), elongate mitochondria (m), and presence of vesicles (v) containing some electron-dense material. Scale bars = 1 μm .



well defined midpiece (Fig. 2C-E) and a single flagellum (Fig. 2A-B-C-E). Nuclear diameter is approximately 1.9 μm . Nuclear chromatin is condensed and granular (Fig. 2B-C). The nucleus does not appear to undergo rotation during spermiogenesis, thus resulting in a centriolar complex and flagellum that are lateral to the nucleus (Fig. 2B-C). The nucleus is indented by a double, shallow, and strongly eccentric nuclear fossa (Fig. 2B). The proximal centriole is anterior and perpendicular to the distal centriole, both are associated with electron-dense spurs and other material, and both are located to one side of the nucleus completely outside the nuclear fossa (Fig. 2B-C). The midpiece contains spherical to elongate mitochondria irregularly distributed, and abundant vesicles, most of which are concentrated in the posterior portion of midpiece (Fig. 2C-E). Many of these vesicles are elongate and often contain electron-dense material. The midpiece also contains a short cytoplasmic canal immediately posterior to the distal centriole (Fig. 2B-E), but this appears to close more posteriorly. The single flagellum has a 9 + 2 microtubular axoneme which does not show intratubular differentiation (Fig. 2D). The flagellum lacks lateral projections or fins (Fig. 2D). Some irregularly arranged accessory microtubules were present in the cytoplasm of the midpiece, but these were not evident on all micrographs.

Spermatozoa of *Eigenmannia trilineata* - Sternopygidae

The spermatozoa of *Eigenmannia trilineata* are comprised of a spherical head (Fig. 3A) containing an ovoid nucleus (Fig. 1B), a distinct midpiece (Fig. 3B-E), and single flagellum (Fig. 3A-B). Nuclear length measured approximately 1.3 μm and width 1.3 μm . The nucleus contains masses of electron-dense flocculent chromatin in a lighter matrix (Fig. 3B-E). Nuclear rotation takes place during spermiogenesis. The proximal centriole is anterior and perpendicular to the distal centriole and both centrioles are contained within a nuclear fossa (Fig. 3B). It was not possible to definitively identify a cytoplasmic collar in the region immediately posterior to the nucleus; however, more posteriorly a collar is lacking. The midpiece contains several elongate mitochondria (Fig. 3B-D-E) and relatively few vesicles (Fig. 3D-E). The flagellum has the usual 9 + 2 microtubular arrangement with no intratubular differentiation (Fig. 3C-D). Along most of its length the flagellum has a pair of lateral projections or fins (Fig. 3C).

Discussion

According to Mattei (1970) there are two basic types of teleost spermatozoa based on the process of nuclear rotation during spermiogenesis. In type I the nucleus rotates 90° in relation to the flagellar axis, resulting in the flagellum being located perpendicular to the nuclear axis and the centrioles contained within a nuclear fossa. In type II, nuclear rotation does not occur, resulting in a flagellum located lateral to the nucleus and centrioles positioned outside any nuclear fossa. Another type of spermiogenesis, type III, was described by Quagio-Grassiotto & Oliveira (2008), where the flagellum

develops at a central position in relation to the nucleus, the nucleus does not rotate during spermiogenesis, and formation of a nuclear fossa and cytoplasmic canal does not occur. Spermiogenesis type III is described for species of the family Pimelodidae (Quagio-Grassiotto & Oliveira, 2008; Quagio-Grassiotto & Carvalho, 2000), for some species of Callichthyidae e Loricariidae (Spadella, 2004), and for many species of the family Characidae (Baicere-Silva *et al.*, 2011). *Eigenmannia trilineata* and the three *Brachyhypopomus* species analyzed all resemble type I spermatozoa, the most common type among Teleostei (Mattei, 1970), which is also observed in most species of Characiformes (Quagio-Grassiotto *et al.*, 2003; Burns *et al.*, 2009), Cypriniformes (Bacceti *et al.*, 1984; Burns *et al.*, 2009) and Siluriformes (Poiret & Nicholson, 1982; Burns *et al.*, 2009) studied to date. The type I spermatozoon is presumed to be the primitive condition within Teleostei (Pecio, 2003). *Gymnotus aff. carapo*, on the other hand, produces spermatozoa more similar to Mattei's type II spermatozoon in that nuclear rotation does not occur during spermiogenesis resulting in a centriolar complex located outside any nuclear fossa. A similar spermatozoon has also been described in *Gymnotus cf. anguillaris* (França *et al.*, 2007). Other ostariophysan spermatozoa similar to Mattei's type II have been reported in the characiform families Acestrorhynchidae and Lebiasinidae, and the *incertae sedis* species *Bryconamericus stramineus* Eigenmann, 1908 and in the siluriform family Callichthyidae (Burns *et al.*, 2009). Given that all recent cladograms have ostariophysan species reporting "type II" spermatozoa nested within groups that produce type I spermatozoa, Burns *et al.* (2009) feel that ostariophysan "type II" spermatozoa may have arisen independently within this clade. Thus, the type II spermatozoa described in Perciformes by Mattei (1970) and the "type II" of ostariophysans appear to be examples of convergent evolution.

The shape, length, and width of the sperm cell nucleus may exhibit great variation among teleost species, with these characteristics frequently associated with the mode of fertilization. The basic structure of the spermatozoon of species that are externally fertilizing tends to be an anacrosomal aquasperm, defined as having a spherical to ovoid nucleus and a short midpiece (Jamieson, 1991). All species of gymnotiform fishes studied to date (Jamieson, 1991; França *et al.*, 2007; França *et al.*, 2009; Burns *et al.*, 2009), as well as those in the present study, produce a type of anacrosomal aquasperm suggesting that these fishes engage in external fertilization. Although the representatives of the three families in the current study all exhibit similar sperm ultrastructure, some differences are evident, particularly with regard to the position of the flagellum relative to the nucleus, position of the centriolar complex, condensation of nuclear chromatin, presence and characteristics of vesicles, and the presence of flagellar fins.

The sperm nucleus of *Eigenmannia trilineata* possesses dense flocculent chromatin in a lighter matrix, similar to that described for *Eigenmannia cf. virescens*, *Rhamphichthys cf.*

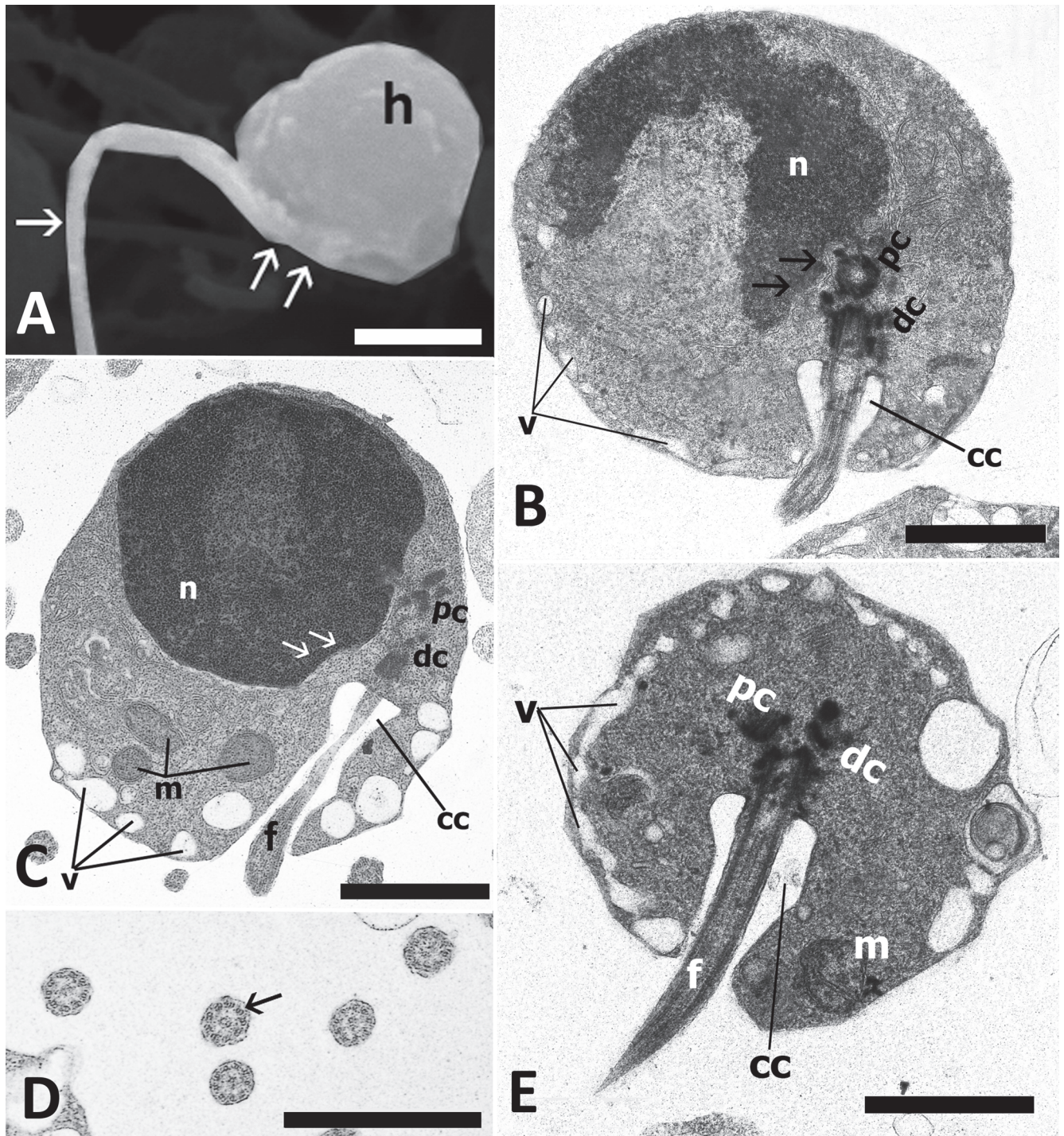


Fig. 2. **A:** Spermatozoon of *Gymnotus* aff. *carapo* (SEM), h = sperm head, arrow = flagellum, double arrow = midpiece; **B-C:** Spermatozoa of *G. aff. carapo* in longitudinal section (TEM) showing spherical nucleus (n), centriolar complex and flagellum (f) lateral to the nucleus (n), double nuclear fossa (double arrow), centriolar arrangement (dc= distal centriole, pc= proximal centriole), mitochondria (m), and presence of vesicles (v) in the posterior portion of midpiece, cc = cytoplasmic canal; **D:** Flagella of spermatozoa of *G. aff. carapo* in cross section (TEM) showing electron-lucent tubules of each peripheral doublet (arrow); **E:** Midpiece of spermatozoon of *G. aff. carapo* in longitudinal section (TEM) showing short cytoplasmic canal (cc) and vesicular arrangement (v). Scale bars = 1 μm.

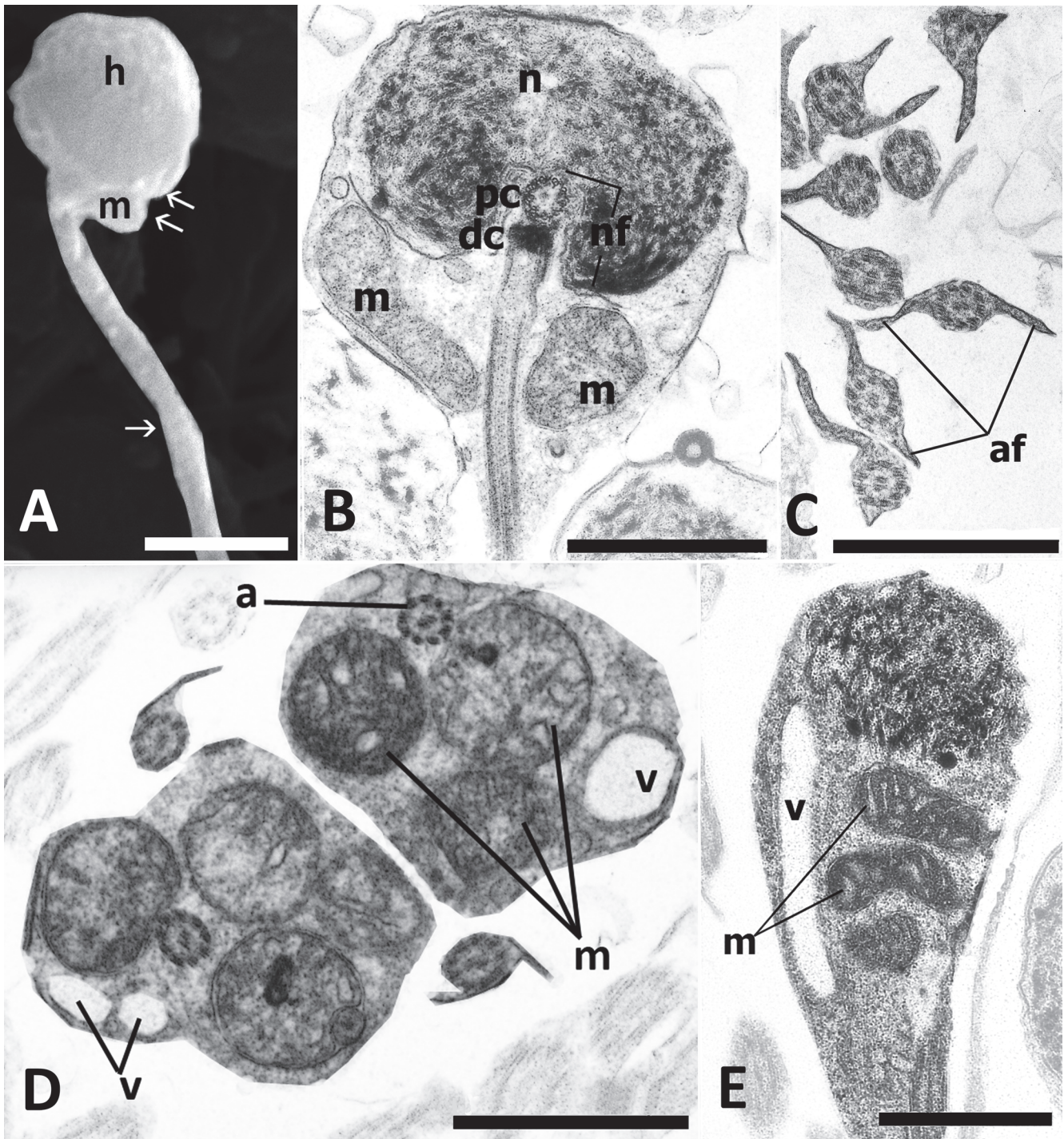


Fig. 3. **A:** Spermatozoon of *Eigenmannia trilineata* (SEM), h = sperm head, arrow = flagellum, double arrow = midpiece, m = mitochondrion; **B, E:** Spermatozoa of *E. trilineata* in longitudinal section (TEM) showing ovoid nucleus (n) with flocculent chromatin, centriolar arrangement (dc= distal centriole, pc= proximal centriole), and elongate mitochondria (m), v = vesicles; **C:** Flagella of spermatozoa of *E. trilineata* in cross sections (TEM) showing axonemal or flagellar fins (af); **D:** Midpiece of spermatozoa of *E. trilineata* in cross section (TEM) showing presence of vesicles (v) and mitochondria (m), and flagellar axoneme (a) with electron-lucent tubules of each peripheral doublet. Scale bars = 1 μ m.

hahni, and *Apteronotus cf. albifrons* (França *et al.*, 2009; Burns *et al.*, 2009). On the other hand, nuclear chromatin is condensed and granular in the three species of *Brachyhyopomus* and *Gymnotus aff. carapo*, as reported for *Brachyhyopomus cf. pinnicaudatus* and *Gymnotus cf. anguillaris* (França *et al.*, 2007; Burns *et al.*, 2009). Of the three species of *Brachyhyopomus* in the present study, only *B. gauderio* has a deep, branching nuclear fossa similar to that described in *Brachyhyopomus cf. pinnicaudatus* (França *et al.*, 2007; Burns *et al.*, 2009). The extensive areas of lighter chromatin within the nucleus of *B. bombilla* may be unique to this species.

With the exception of *Eigenmannia trilineata*, abundant vesicles were observed within the midpieces of the other species analyzed, similar to that previously reported for conspecifics (Burns *et al.*, 2009). Most of the vesicles of all the species herein analyzed contained some electron-dense material. It is possible that this material is the result of fixation artifact. However, the presence of such material in specimens fixed at different times and its consistent observation in the vesicles suggest that it may indeed be of cellular origin. Although numerous accessory microtubules have been reported in the spermatozoa of *Gymnotus cf. anguillaris* and *Brachyhyopomus cf. pinnicaudatus* (França *et al.*, 2007; Burns *et al.*, 2009), accessory microtubules were only observed in the midpiece of *Gymnotus aff. carapo*.

Prior to 2001, the presence of flagellar fins (axonemal fins) had not been reported for any taxon within Ostariophysi, and absence of such fins was interpreted as a secondary reduction and apomorphic character for this group of Teleostei (Jamieson, 1991). Since that time, Quagio-Grassiotto *et al.* (2001) described fins on the flagellar membrane of *Diplomystes mesembrinus* [= *Olivaichthys mesembrinus* (Ringuet, 1982)], considered to be a primitive representative of Siluriformes, and Pecio (2003) reported flagellar fins for the first time in a species of Characiformes. Within Gymnotiformes, flagellar fins have now been described in representatives of the families Rhamphichthyidae and Sternopygidae (França *et al.*, 2009), being absent in species of Apteronotidae (França *et al.*, 2009; Jamieson, 1991), Hypopomidae and Gymnotidae (França *et al.*, 2007). In the present study, flagellar fins were only observed in *E. trilineata*, similar to the finding of França *et al.* (2009) for *Eigenmannia cf. virescens*. The greater amount of cytoplasm between the axoneme and flagellar plasma membrane observed in *B. bombilla* was also reported for *Brachyhyopomus cf. pinnicaudatus* (França *et al.*, 2007; Burns *et al.*, 2009).

This study demonstrates the diversity of sperm ultrastructural characters not only among the families of Gymnotiformes, but also within its individual genera. Such information may be useful in hypothesizing phylogenetic relationships at numerous taxonomic levels.

Acknowledgements

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