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Comparison of spermiogenesis in the externally fertilizing *Hemigrammus erythrozonus* and the inseminating *Corynopoma riisei* (Teleostei: Characiformes: Characidae)

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Spermiogenesis and sperm ultrastructure were analyzed in two species of characids with different modes of fertilization: externally fertilizing *Hemigrammus erythrozonus* and inseminating *Corynopoma riisei*. Spermiogenesis in *H. erythrozonus* is characterized by lateral development of the flagellum, nuclear rotation, formation of a shallow nuclear fossa, condensation of the chromatin by elimination of the electron-lucent area from the peripheral region of the nucleus, and renewal of the nuclear membrane. Multilammelated membrane and multivesicular bodies were also observed during elimination of the excess cytoplasm. The spermatozoon exhibits characters typical of "aquasperm," *i.e.* a spherical head containing a spherical nucleus with highly condensed chromatin, several small mitochondria located at the base of the nucleus within a cytoplasmic collar that extends into a long cytoplasmic sleeve surrounding the anterior part of the single flagellum, which is contained within a cytoplasmic canal. The flagellum lacks fins. The proximal and distal centrioles are nearly parallel to one another, with the anterior tips of both located within shallow nuclear fossae. Spermiogenesis in *C. riisei* is characterized by nuclear elongation alongside the forming flagellum, formation of an elongate cytoplasmic canal, displacement and elongation of the mitochondria, and uniform condensation of chromatin throughout the nucleus through enlargement of the diameter of the chromatin granules. The spermatozoon has an elongate nucleus with two elongate mitochondria localized to one side. Mitochondria are also located posterior to the nucleus forming a mitochondrial region. The single flagellum, which lacks fins, is lateral to the nucleus and initially contained within the greatly elongate cytoplasmic canal before exiting the canal at its posterior terminus. The spermatozoon of *C. riisei* exhibits several characters typical of "introsperm," such as an elongate nucleus and midpiece (mitochondrial region). The nuclear chromatin in the spermatozoon remains "flocculent" and is never as condensed as that seen in many characid sperm. Differences in spermiogenesis between externally fertilizing and inseminating characids are discussed.

Foram analisadas a espermiogênese e ultraestrutura dos espermatozóides de dois caracídeos com modos de fertilização distintos: fertilização externa em *Hemigrammus erythrozonus* e inseminação em *Corynopoma riisei*. A espermiogênese em *H. erythrozonus* é caracterizada pelo desenvolvimento lateral do flagelo, rotação nuclear, formação de uma fossa nuclear rasa, condensação de cromatina por eliminação da área elétron-lúcida na região periférica do núcleo e renovação da membrana nuclear. Membrana multilamelada e corpos multivesiculares foram observados durante a eliminação do excesso de citoplasma. O espermatozóide exibe os caracteres típicos do "aquaespermatozóide," com uma cabeça esférica que contem um núcleo esférico com cromatina muito condensada, várias mitocôndrias pequenas localizadas na base do núcleo e dentro de um colar citoplasmático, extedendo-se em uma bainha citoplasmática longa que rodeia a parte anterior do único flagelo, que está contido dentro de um canal citoplasmático. O flagelo carece de aletas. Os centríolos proximais e distais são quase paralelos, com as partes anteriores dos dois localizadas dentro de fossas nucleares pouco profundas. A espermiogênese em *C. riisei* é caracterizada pelo alongamento nuclear ao longo do flagelo, a formação de um canal citoplasmático longo, deslocamento e alongamento das mitocôndrias e uma condensação uniforme da cromatina por todo o núcleo por meio do aumento do diâmetro dos grânulos de cromatina. O espermatozóide tem um núcleo alongado com duas mitocôndrias alongadas dispostas em um lado. Algumas mitocôndrias localizam-se posteriormente ao núcleo formando uma região mitocondrial. O único flagelo, que carece de aletas,

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é lateral ao núcleo, posicionado anteriormente dentro de um canal citoplasmático muito alongado. O espermatozóide de *C. riisei* exibe vários caracteres típicos de "introespermatozóides" tais como um núcleo alongado e parte média alongada (região mitocondrial). A cromatina nuclear no espermatozóide permenece floculenta e nunca está tão condensada como nos espermatozóides de muitos outros caracídeos. Se discutem as diferenças na espermiogênese entre caracídeos com fertilização externa e os inseminadores.

Key words: Nuclear rotation, Aquasperm, Introsperm, Characids.

Introduction

In fishes, the initial stages of spermatogenesis, which include the mitotic and meiotic divisions, exhibit limited variability among species. This contrasts sharply with the later stage of spermiogenesis when spermatids differentiate into the spermatozoa that are adapted to the particular fertilization mode of a given species. The greatest diversity of spermatozoa is observed within the Teleostei, the largest vertebrate group comprised of nearly 27,000 extant species (Nelson, 2006). Teleost reproductive modes vary from oviparous to viviparous, and from external to internal fertilization (Breder & Rosen, 1966). External fertilization is the dominant method of fertilization in nearly 97% of teleost species. In externally fertilizing species, spermatozoa are released into seawater or freshwater, which may occasionally be highly acidic, or into viscous secretions (*e.g.* in Blennidae) (Lahnsteiner *et al.*, 1990). Fusion of egg and sperm (fertilization) occurs soon after release from the gonads once spermatozoa enter the micropyles of the eggs. Spermatozoa of externally fertilizing species, referred to as "aquasperm," generally possess spherical to ovoid heads containing spherical to ovoid nuclei, several small mitochondria located immediately posterior to the nucleus and within a cytoplasmic collar, and one or two flagella whose anterior portions are contained within a cytoplasmic canal of variable length (Jamieson, 1991).

Insemination, where sperm are introduced into the ovary, or internal fertilization, where sperm and egg actually fuse within the ovary, have been documented in about 3% of teleost species (Breder & Rosen, 1966; Koya *et al*., 2002; Burns & Weitzman, 2005). In males, adaptations for sperm transfer to the female have involved transformation of fins or other body regions into intromittent organs (Meisner, 2005; Burns & Weitzman, 2006), as well as modifications of both testis and sperm morphology (Burns *et al*., 1995, 2000). Spermatozoa of inseminating species, referred to as "introsperm," often become relatively complex with elongate nuclei and midpiece regions (Jamieson, 1991). Modifications of aquasperm into complex introsperm appear to have evolved independently in at least 24 teleostean families and even within congeneric species (*e.g.* Poeciliidae) (Reznick *et al*., 2002; Burns & Weitzman, 2005). Another modification related to the habits of insemination and internal fertilization is the production of sperm packets, both unencapsulated, referred to as spermatozeugmata, and encapsulated, called spermatophores (Grier & Parenti, 1994; Pecio *et al*., 2005). The anatomy and physiology of female reproductive systems have also been modified to permit the survival of introduced sperm for variable time periods, thus delaying fertilization and allowing for sperm competition among different males (Birkhead & Moller, 1997). All of these factors have probably influenced to some degree the evolution of modified sperm that are differentiated during the process of spermiogenesis.

In the family Characidae, insemination appears to have evolved independently at least three times: in members of the tribe Compsurini of the subfamily Cheirodontinae (Burns *et al*., 1997); in the subfamily Glandulocaudinae and in the subfamily Stevardiinae (Nelson, 1964; Burns e*t al*., 1995; Weitzman *et al*., 2005). The species of the latter two subfamilies had previously all been included in the Glandulocaudinae, but a recent re-analysis of several morphological characters led to their being separated into the Glandulocaudinae (comprised of the genera *Glandulocauda*, *Mimagoniates*, and *Lophiobrycon*) and the Stevardiinae, containing all of the other genera previously included within the Glandulocaudinae (Weitzman *et al*., 2005).

Data on spermiogenesis in externally fertilizing species of the Characidae are very limited, having been described only for *Brycon cephalus* (Romagosa *et al*., 1999), *Bryconops affinis* (Andrade *et al*., 2001) and species in the genera *Salminus* and *Brycon* (Veríssimo-Silveira *et al.*, 2006). However, some information on spermiogenesis is available for several other families belonging to the order Characiformes, including Erythrynidae (Quagio-Grassiotto *et al*., 2001), Curimatidae (Quagio-Grassiotto *et al*., 2003), Anostomidae (Pecio, 2003) and Alestidae (Shahin, 2006).

For inseminating characids, data on spermiogenesis is available only for the glandulocaudine *Mimagoniates barberi* (Pecio & Rafiński, 1999). Descriptions of the ultrastructure of sperm, however, are available for the following characids: subfamily Stevardiinae, *Diapoma speculiferum*, *Diapoma* sp., *Corynopoma riisei*, and *Pseudocorynopoma doriae* (Burns & Weitzman, 2005; Burns *et al*., 1998) and *Scopaeocharax rhinodus*, *Tyttocharax cochui* and *T. tambopatensis* (Pecio *et al*., 2005); subfamily Glandulocaudinae, *Mimagoniates barberi* and *M. microlepis* (Pecio & Rafiński, 1994, 1999; Burns *et al*., 1998); subfamily Cheirodontinae, *Macropsobrycon uruguayanae* (Burns *et al*., 1998), and two species currently *incertae sedis*, *Bryconadenos tanaothoros* (Weitzman *et al*., 2005) and *Brittanichthys axelrodi* (Javonillo *et al*., 2007). Sperm morphology in these inseminating species can show marked variations, for example, in the orientation of the centrioles, degree of nuclear elongation, presence of accessory micro-

tubules, or reduction of the cytoplasmic collar (Burns *et al*., 1998; Pecio *et al*., 2005).

The main purpose of the present study is to provide information on spermiogenesis in the externally fertilizing *Hemigrammus erythrozonus* (*incertae sedis*) and the inseminating *Corynopoma riisei* (subfamily Stevardiinae). This analysis will demonstrate the differences between the differentiation of an aquasperm and that of an introsperm in two species of Characidae. The data presented for *C. riisei* is the first study on spermiogenesis in the subfamily Stevardiinae. This information will contribute to the growing database of ultrastructural characters that are being used in hypothesizing phylogenetic relationships among stevardiine and other characiform species (Weitzman & Menezes, 1998).

Materials and Methods

Four males of *Hemigrammus erythrozonus*, from a population originally in Guyana, were obtained from a local aquarist shop and kept in an aquarium (80x45x40cm) for several months on a natural photoperiod prior to this study. Fish were fed daily *ad libitum* with small crustaceans and *Tubifex* worms. Four mature males of SL 21-27 mm were killed by immersion in a 1% solution of tricaine methasulphonate (MS 222). The gonads were removed, cut into small fragments, and fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4) overnight. The material was postfixed in 1% osmium tetroxide in the same buffer, dehydrated in alcohol and embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate and lead citrate and were later examined under a JEOL JEM-100Sx transmission electron microscope. After dehydration, one small testis sample was dried in LADD CPD, then fractured and sputter-coated with gold and viewed in a JEOL JSM scanning electron microscope.

The specimen of *Corynopoma riisei* used for SEM was from the collection of the National Museum of Natural History, Smithsonian Institution (Washington, DC, USA), USNM 219619, SL 43 mm. This specimen had initially been fixed in 10% formalin and later transferred to 70% ethanol. A small sample of this testis was rinsed in tap water and postfixed in 1% osmium tetroxide in phosphate buffer for 2 hrs. After dehydration the testis was processed in the same manner as the sample of *H. erythrozonus*.

The testes of *C. riisei* used for TEM were from two aquarium specimens derived from stock originally collected by SHW in 1977 from the río Manzanares, between Cumaná and Cumanacoa, Sucre, Venezuela, SL 34.3 and 32.8 mm. After killing the specimens by immersion in a lethal dose of MS 222, the testes were removed and small pieces fixed in modified Karnovsky's fixative (Ito & Karnovsky, 1968), then rinsed in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer for 30 min. Tissues were then rinsed in phosphate buffer, dehydrated in an ethanol series, infiltrated and embedded in Araldite 502. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM-100Sx transmission electron microscope.

Results

Spermiogenesis in *Hemigrammus erythrozonus*. The early spermatids of *H. erythrozonus* are spherical and possess nuclei with heterogeneous chromatin surrounded by a wide zone of cytoplasm (Fig. 1A). The centrioles are arranged nearly perpendicular one to another and located lateral to the spherical nucleus. The distal centriole (= basal body) extends to the flagellar axoneme, whose anterior portion is located within a cytoplasmic canal. During flagellar extension the mitochondria are located at the base of nucleus and possess a dark matrix (Fig. 1B). At this time, the orientation of the centrioles changes to a nearly parallel arrangement, while small fossae appear in the nucleus to accommodate the tips of both centrioles (Fig.1C). Before nuclear rotation chromatin is slightly mottled (Fig.1B) or finely granular (Fig. 1C). During the next stage of cell differentiation the nucleus rotates over the centriolar complex. This process is relatively synchronous given that within the same cyst, spermatids both before and after rotation can be seen (Fig.1D). After nuclear rotation, the head, midpiece and flagellum are arranged in a linear fashion (Fig.1E). The slight asymmetry of some nuclei relative to the long axis of the proximal centriole may be due to incomplete rotation. Most mitochondria are located at the side of the nucleus which does not rotate, forming an asymmetric midpiece.

During all of the above stages it was possible to observe the elimination of multivesicular bodies and lamellated membrane from the cytoplasm surrounding the nucleus (Fig.1D) and multilamellated membrane from the area of the cytoplasmic collar. In addition, nuclear chromatin became progressively more condensed in the spermatids within the cysts (Fig.1F), and this even continued after release of the cells into the lumen of the sperm ducts.

The nuclei of spermatozoa within the lumen of the sperm ducts possess highly condensed chromatin along with a more electron-lucent area at the periphery of the nucleus, which appears to be subsequently eliminated by the renewal of the nuclear membrane on the border of highly condensed chromatin (Fig. 2A). A similar electron-lucent area is also observed within the midpiece of spermatozoa. The nucleus of the spermatozoon of *H. erythrozonus* is roughly spherical and approximately 2 μm in diameter (Figs. 2B-C). The chromatin is highly condensed and two shallow fossae contain the tips of each centriole. In frontally sectioned spermatozoa (Figs. 2B; Fig. 3A) the distal centriole (= basal body) is oriented at an angle of 146 degrees relative to the proximal centriole. Posterior to the nucleus, several mitochondria and vesicles are present in the cytoplasmic collar. The spherical to ovoid mitochondria, which have an electron-dense matrix, are distributed in irregular arrays (Fig. 2C) with their numbers decreasing more posteriorly (Figs. 2D-F). Posterior to the mitochondrial region on one side and just below the nucleus on the other, a thin cytoplasmic sleeve surrounds the anterior part of the flagellum (Figs. 2G-H; Fig. 3B). The axoneme of the flagellum forms the typical 9 + 2 pattern of microtubules, with

no intratubular differentiation (Fig. 2I). The single flagellum lacks fins.

Spermiogenesis in *Corynopoma riisei*. As seen on a fractured surface with SEM, cysts containing spermatogonia, spermatocytes and spermatids at the beginning of spermiogenesis in *C. riisei* (Fig. 4A) are very similar to those observed in *H. erythrozonus*, with all cells completely filling the cysts and randomly distributed within them. The spermatids in more advanced stages of spermiogenesis, on the other hand, show significant elongation of their heads, which are oriented toward one pole of the cyst, with the flagella closely packed at the opposite pole (Fig. 4B). The arrangement of these cells resembles a "bouquet of flowers".

During the preliminary phase of flagellum formation, early spermatids are round with both centrioles in nearly perpendicular arrangement located tangential to the nucleus. The proximal centriole, part of which is located in a shallow fossa, has electron-dense material (intercentriolar lamellated body) on one side (Fig. 5A). At this point a slight degree of nuclear rotation may occur, as evidenced by the thin nuclear area directly anterior to the centriolar complex (Fig. 5B, asterisk). However, no further rotation is evident. Instead, the nucleus elongates lateral and posterior to the centriolar complex. During spermatid elongation the part of the nucleus located above the proximal centriole forms the anterior tip of the spermatid. The chromatin at this stage is finely granular (Fig. 5B). The spherical to ovoid mitochondria are located in the cytoplasm mainly on the flagellar side of the cell and below the nucleus (Fig. 5C).

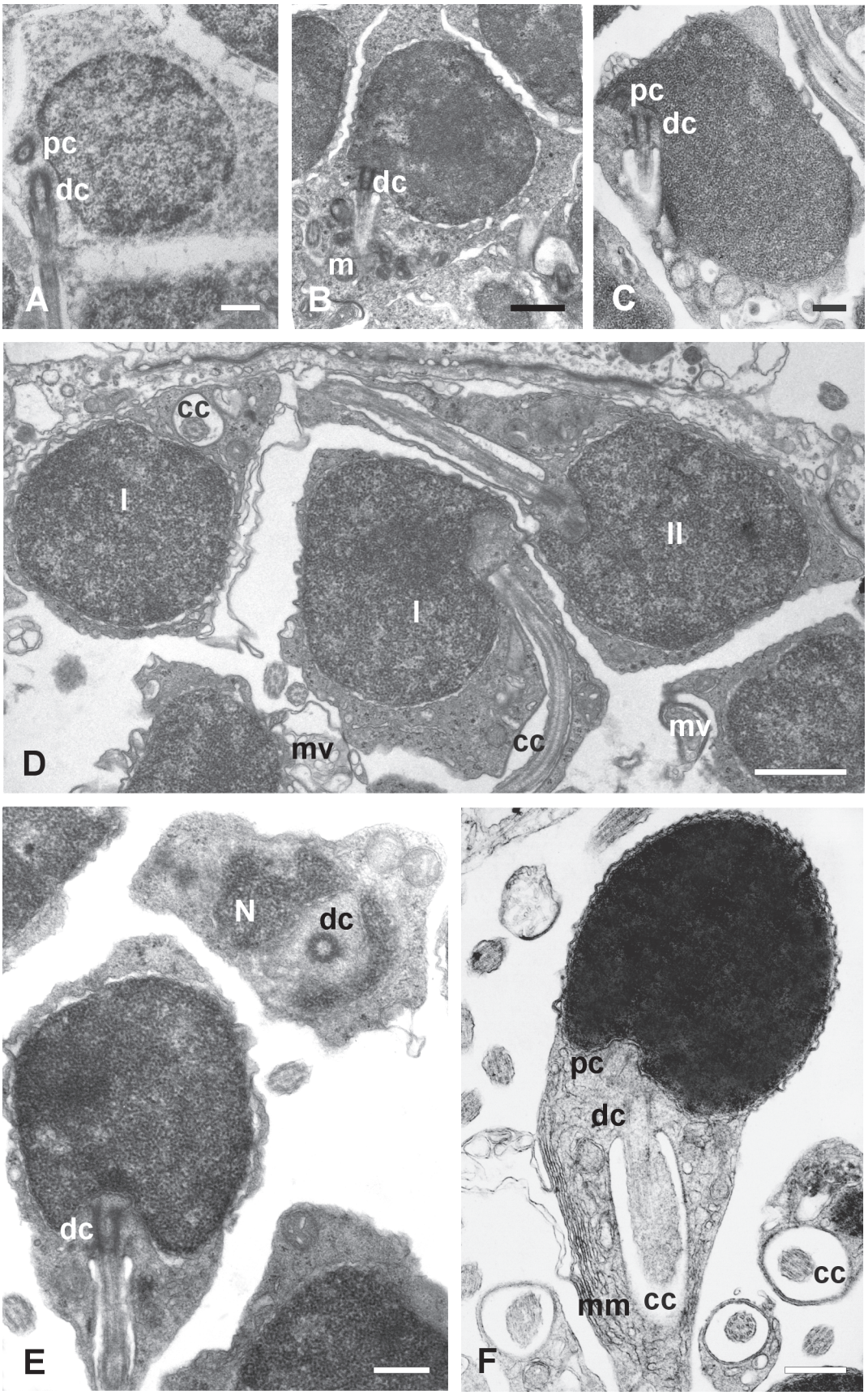
During the next stage the nucleus changes shape, becoming thinner and slightly flattened at the centriolar pole and thicker and more ovoid (in transverse section) posterior to the centrioles (Fig. 6A-B). The number of mitochondria located alongside the anterior part of the nucleus is reduced to two, whereas in the posterior part of the nucleus multiple mitochondria are distributed around the nucleus (Fig. 6C-D). At this time the single flagellum also elongates. The anterior portion of the flagellum in located within an elongate cytoplasmic canal which runs the entire length of the spermatid (Fig. 6A, C-F). At later stages the nucleus of the spermatid elongates further with the chromatin condensing into larger "granules." The anterior part of the nucleus is flattened in the plane of the proximal centriole with a depression containing the distal centriole and proximal part of the flagellum (Fig. 7A). Mitochondria are located on the concave side of the nucleus and on either side of the flagellum (Fig. 7B). Posterior to the nucleus 4 -7 mitochondria are found, thus forming a mitochondrial region (Fig. 7C).

The spermatozoon of *C. riisei* is approximately 8.4 μm long and 1.5 μm wide (Fig. 8). All structures seen in the final stages of spermiogenesis are also present in the spermatozoon, where the incompletely condensed chromatin remains "flocculent". Along the nucleus elongate mitochondria are localized to one side, whereas posterior to the nucleus mitochondria are slightly larger and wider (Fig. 8A, E-F). All mitochondria possess multiple lamellar cristae, and glycogen rosettes can be seen among some of them (Fig. 8A). The shape of the nucleus changes from very flat at the anterior part to wider and more rounded toward the posterior part (Figs. 8B-E). The single flagellum is contained within the cytoplasmic canal along the entire nuclear and mitochondrial regions of the cell, extending beyond the mitochondrial region before exiting the end of the collar (Figs. 8D-H; Fig. 9). The flagellar axoneme consists of the typical 9 + 2 microtubule doublets, with no intratubular differentiation. The flagellum lacks fins.

Discussion

The introsperm seen in *C. riisei* probably evolved from an aquasperm similar to that seen in *H. erythrozonus* as an adaptation to the reproductive habit of insemination. The modifications to an aquasperm may have involved an inhibition of most of the process of nuclear rotation common to many aquasperm. Thus, instead of a symmetrical aquasperm where the flagellum is perpendicular to the nucleus as in *H. erythrozonus*, the new introsperm would have the flagellum running parallel to an elongate nucleus as in *C. riisei*. Most of the process of nuclear rotation may have been inhibited as well in all inseminating species analyzed thus far in the subfamily Glandulocaudinae (*Mimagoniates barberi, M. microlepis*) (Pecio & Rafiński, 1994, 1999; Burns *et al*., 1998), subfamily Stevardiinae (*Diapoma speculiferum, Diapoma* sp., *Pseudocorynopoma doriae, Scopaeocharax rhinodus, Tyttocharax tambopatensis* and *T. cochui*) (Burns *et al*., 1998; Pecio *et al*., 2005), and two species currently *incertae sedis*, *Bryconadenos tanaothoros* (Weitzman *et al*., 2005) and *Brittanichthys axelrodi* (Javonillo *et al*., 2007). In all of these species, spermatozoa have mitochondria located alongside and/or posterior to the elongate nuclei, centrioles located to one side near the anterior end of the nucleus, and a single flagellum running alongside the nucleus within a cytoplasmic canal of variable length (Pecio & Rafiński, 1994, 1999; Burns *et al*., 1998; Pecio *et al*., 2005; Weitzman *et al*., 2005;

Fig. 1. (next page) Early stages of spermiogenesis in *Hemigrammus erythrozonus*. **A -** Early spermatid with centrioles and forming flagellum located oblique to the nucleus. Scale bar 0.5 μm. **B -** Spermatid with mottled chromatin before nuclear rotation. Scale bar 1 μm. **C -** Spermatid before nuclear rotation with finely granular chromatin and mitochondria located on one pole of the nucleus. Scale bar 0.5 μm. **D** - Spermatids within spermatocysts before (I) and after nuclear rotation (II) with clumping chromatin. Scale bar 1 μm **E** - Spermatids after nuclear rotation with asymmetrical nuclei in sagittally and transversely sectioned cells. Scale bar 0.5 μm **F** - Late spermatid with highly condensed chromatin and multilamellated body alongside the cytoplasmic sleeve. Scale bar 0.5 μm. cc – cytoplasmic canal, dc – distal centriole, pc – proximal centriole, m – mitochondrion, mm – multilamellated body, mv – multivesicular body, N-nucleus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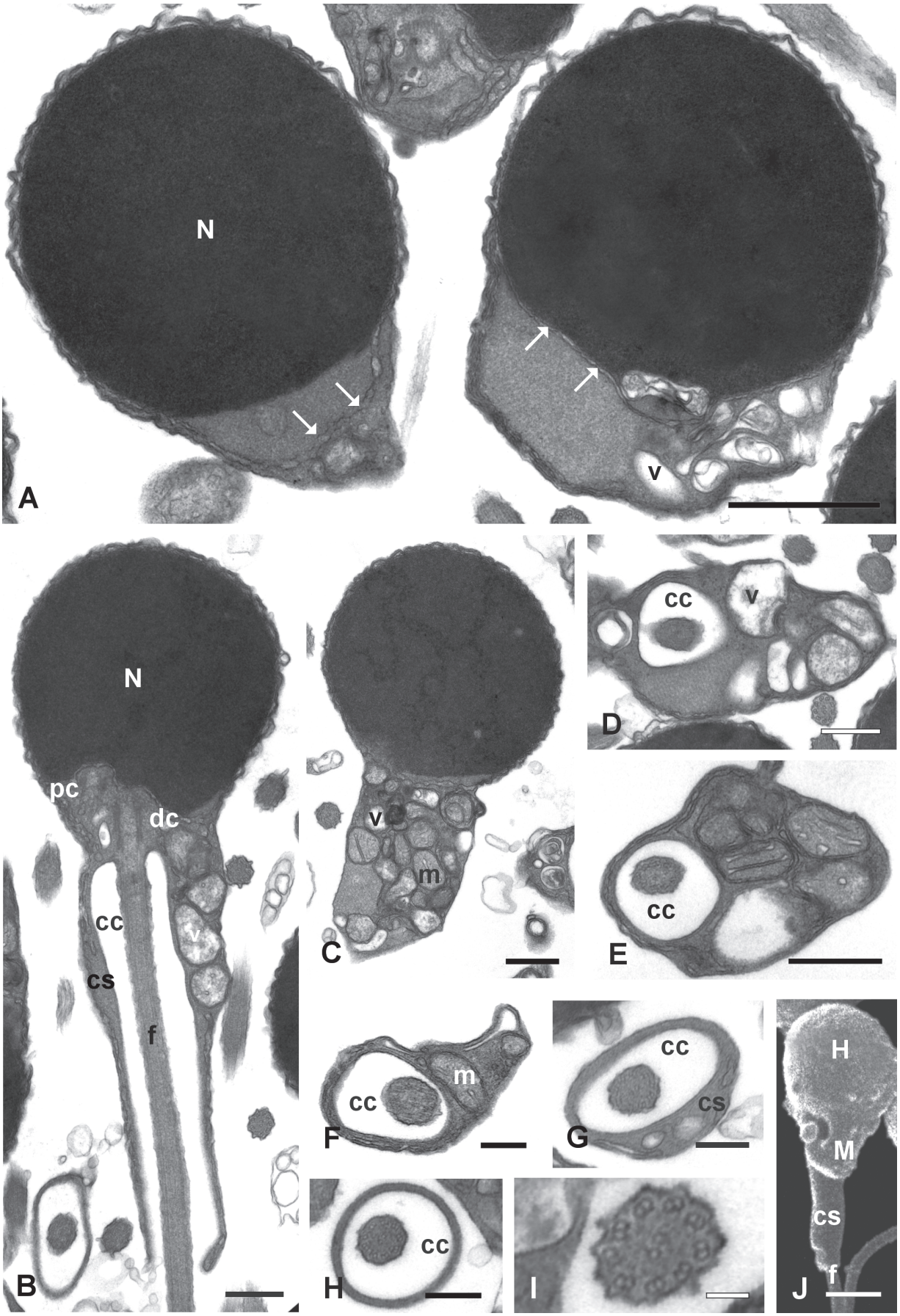
Javonillo *et al*., 2007). The nuclear rotation observed in *H. erythrozonus* is typical of all externally fertilizing characiforms analyzed to date with the exception of *Acestrorhynchus falcatus* (Matos *et al*., 2000). The presence or absence of nuclear rotation has been described in the Teleostei by Mattei (1970), who classified two types of aquasperm. In Type I the nucleus rotates during spermiogenesis and as a result the flagellar axis is perpendicular to the base of the nucleus, whereas in type II there is no rotation and the flagellum remains parallel to the base of the nucleus. Mattei (1970) reported that spermiogenesis without nuclear rotation is more typical of aquasperm in more derived teleostean taxa, such as species in the order Perciformes. The only inseminating characid studied to date whose spermatozoon shows evidence of complete nuclear rotation is *Macropsobrycon uruguayanae* of the subfamily Cheirodontinae (Burns *et al*., 1998). The spermatozoon of *C. riisei* may actually represent a highly modified type I sperm that underwent only the beginning of nuclear rotation. The type II aquasperm described by Mattei (1970) has both centrioles located outside of any nuclear fossae. The location of at least part of the centrioles of *C. riisei* within nuclear fossae further supports its spermatozoon as being a modified type I sperm.

During spermiogenesis in *H. erythrozonus*, the chromatin changes from having a finely granular or mottled appearance throughout the entire volume of the nucleus to being homogenously condensed and highly electron-dense in one area of the nucleus and more electron-lucent in a peripheral area, particularly in spermatozoa present in the lumen. In species of Curimatidae and in *H. malabaricus* (Erythrinidae) (Quagio-Grassiotto *et al*., 2001, 2003), all externally fertilizing, nuclear condensation is very similar to that observed in *H. erythrozonus*. However, the elimination of the electron-lucent area seen in *H. erythrozonus* during the final stage of condensation was only observed in *Chilodus punctatus* (Anostomidae) (Pecio, 2003). Nuclear condensation involving the gradual enlargement of chromatin granules described here for *C. riisei* is also typical for the other inseminating stevardiines, *S. rhinodus* and *Tyttocharax* spp. (personal observations), as well as the inseminating glandulocaudine, *M. barberi* (Pecio & Rafiński, 1999; Pecio *et al*., 2005). The chromatin in the spermatozoon of most inseminating characids studied to date is highly condensed (Pecio & Rafiński, 1994, 1999; Burns *et al*., 1998; Pecio *et al*., 2005). In *C. riisei*, however, the chromatin of the spermatozoon has a flocculent appearance more similar to that seen in another stevardiine, *Pseudocorynopoma doriae* (Burns *et al*., 1998; Burns & Weitzman, 2005). A similar type of chromatin condensation was also described in the externally fertilizing *Salminus brasiliensis*, *Brycon microlepis* and *B. orbignyanus*, primitive representatives of Characidae (Verissimo-Silveira, 2006). The variation in chromatin condensation seen among characids suggests the presence of different types of protamines associated with the DNA (Saperas *et al*., 1993).

During spermiogenesis in the species of the present study, the nuclei assume very different shapes, resulting in sperm heads that are markedly different. The spherical shape of the nucleus in *H. erythrozonus* is a character typical not only of the aquasperm of all characiforms studied to date, but also of aquasperm of many other teleostean fishes (Jamieson, 1991). The slightly asymmetric shape of the nucleus, along with eccentrically located shallow nuclear fossae containing the tips of the nearly parallel centrioles, was also described in another characid species, *Paracheirodon innesi* (Jamieson, 1991). A nearly parallel arrangement of the centrioles was found in the inseminating genera, *Scopaeocharax* and *Tyttocharax*, whereas in other inseminating genera, such as *Mimagoniates, Diapoma* and *Pseudocorynopoma*, the centrioles are arranged perpendicular to one another as seen in *C. riisei*. In all of the above inseminating species, the centrioles are located near the anterior tip of the cell, lateral to the elongate nucleus (Burns *et al*., 1998; Pecio *et al*., 2005).

Elongate nuclei have been described in most inseminating characids studied to date (Pecio & Rafiński, 1994, 1999; Burns *et al*., 1995, 1997, 1998, 2000; Azevedo *et al*., 2000; Burns & Weitzman, 2005, 2006; Weitzman *et al*., 2005; Javonillo *et al*., 2007), as well in many other internally fertilizing teleosts (Jamieson, 1991). However, inseminating species that produce spermatozoa more similar to aquasperm include *Planaltina* spp. (Burns *et al*., 1995; Menezes *et al*., 2003), *Kolpotocheirodon theloura* (Burns *et al*., 1997; Malabarba & Weitzman, 2000), *Attonitus ephimeros* and *A. irisae* (Weitzman *et al*., 2005), *Monotocheirodon pearsoni* (Burns & Weitzman, 2006) and *Knodus* sp. (Burns & Weitzman, 2005) among the Characidae and *Ameca splendens, Aetonobius toweri, Characoden lateralis* and *Xenotoca eiseni* among the Goodeidae (Jamieson, 1991).

Fig. 2. (next page) **A-I**. Spermatozoa in lumen of sperm ducts of *H. erythrozonus* with TEM. **A -** Heads of spermatozoa with nuclei containing highly condensed chromatin as well as electron-lucent areas both within and behind nucleus. Arrows show nuclear membrane. Scale bar 1 μm. **B** - Frontally sectioned spermatozoon with slightly asymmetrical nucleus containing small fossae for tips of both centrioles that are oriented at an angle of 146 degrees. Scale bar 1 μm. **C -** Laterally sectioned head and midpiece with mitochondria located mainly to one side and beneath nucleus within cytoplasmic collar. Several vesicles (v) are visible among several mitochondria. Scale bar 0.5 μm. **D-H -** Transversely-sectioned midpiece with flagellum within cytoplasmic canal. Mitochondria are located mainly on one side of the midpiece, progressively reduced in number posteriorly in the cytoplasmic collar (**D**-**F**) and absent in its distal portion (**G** and **H**). Scale bar 0.5 μm for **D-F**, and scale bar 0.25 μm for **G-H**. **I** - Free flagellum with typical 9+2 axoneme with all microtubules electron-lucent. Scale bar 0.1 μm. **J -** Spermatozoon in *H. erythrozonus* with SEM. Scale bar 1 μm . cc – cytoplasmic canal, cs – cytoplasmic sleeve, dc – distal centriole, f – flagellum, H – head, m – mitochondrion, M – midpiece, N – nucleus, pc – proximal centriole, v – vesicles.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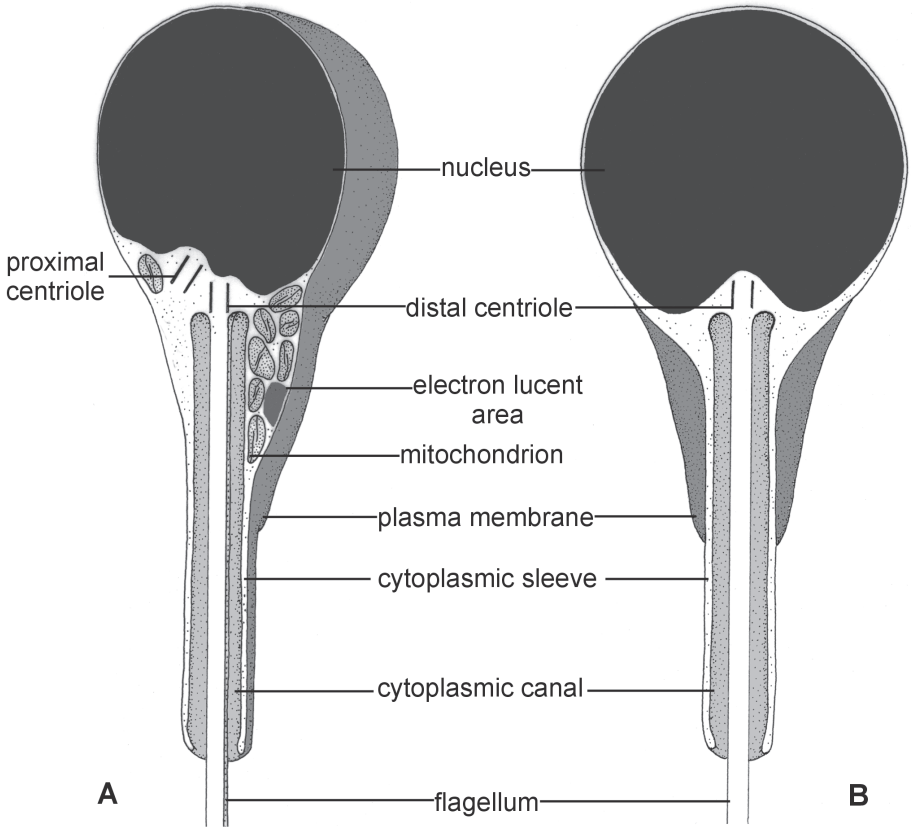

Fig. 3. Drawings of frontally (A) and sagitally (B) sectioned spermatozoon in *H. erythrozonus*.

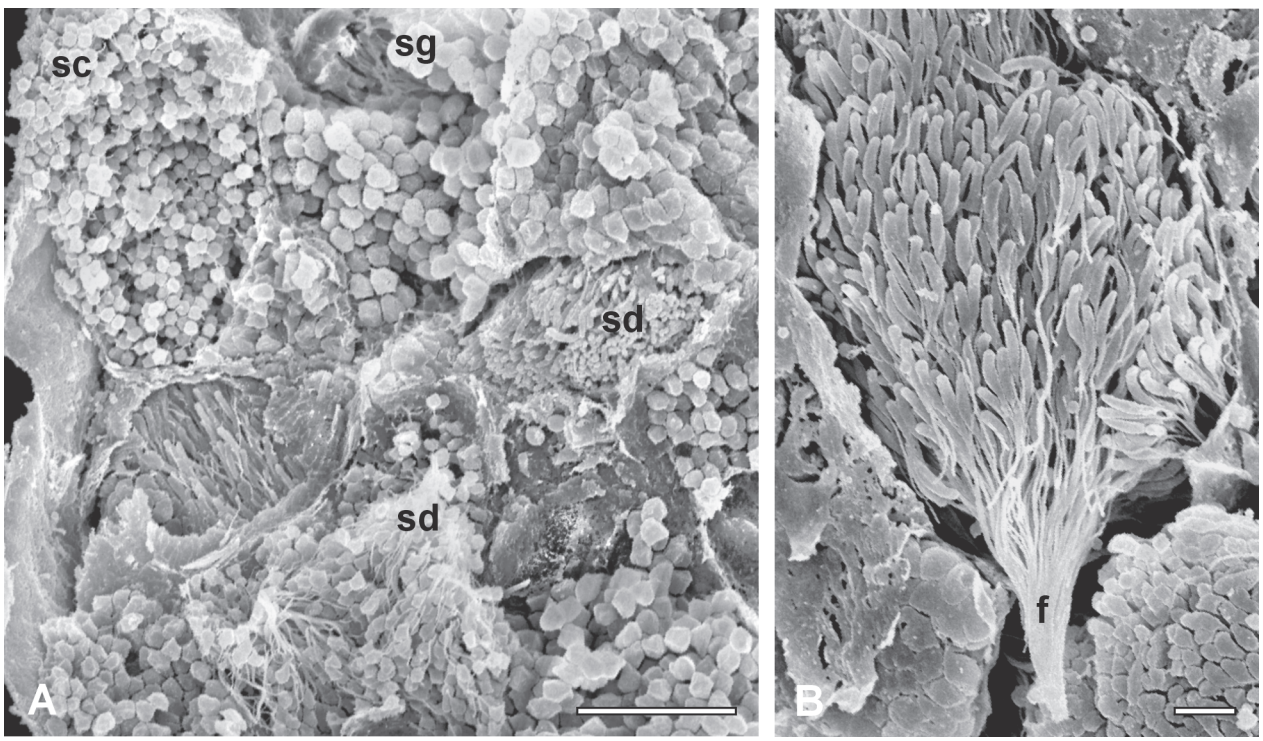

Fig. 4. Fractured part of testis in *Corynopoma riisei* with SEM. **A** - Spermatocysts containing cells in different stages of spermatogenesis; Scale bar 25 μm. **B** - Arrangement of late spermatids within spermatocyst before spermiation. Scale bar 50 μm. f – flagellum, sg-spermatogonia; sc-spermatocytes; sd-spermatids.

Nuclear elongation during spermiogenesis to produce a final sperm cell that is long and thin has been described as an adaptation associated with insemination in many teleost families (Jamieson, 1989, 1991; Mattei 1991; Burns *et al*., 1998; Jamieson & Grier 1993; Burns & Weitzman; 2005). This streamlined shape may allow for more efficient movement of sperm cells through viscous fluids and narrow pathways in the ovary (Fawcett, 1970; Gardiner, 1978; Jamieson, 1991), as well as aid in the coalescence of spermatozoa into sperm packets. Formation of sperm clumps would result in greater densities of cells being transferred to the female, thus increasing the effectiveness of sperm transfer (Ginzburg, 1968). Histological sections of testes of *C. riisei* have shown sperm cells to be arranged into flowing patterns, although distinct spermatozeugmata could not be identified (Burns *et al*., 1995). However, Kutaygil (1959) made direct observations on living specimens and described the transfer of sperm "heaps" to the female. *C. riisei* may indeed produce sperm clumps that are embedded in a mucoid material, which can be seen in histological sections stained with the periodic acid/Schiff reagent (personal observations). Production of a type of spermatozeugma in this species is also supported by the distinct clumping of cells seen in Fig. 4. Inseminating characids known to produce distinct spermatozeugmata include the glandulocaudine genera *Glandulocauda*, *Mimagoniates* and *Lophiobrycon* (Pecio & Rafiński, 1994, 1999, 2001; Burns *et al*., 1995; personal observation), the stevardiine genera *Scopaeocharax*, *Tyttocharax* and *Xenurobrycon* (Burns *et al*., 1995; Burns & Weitzman, 2005; Pecio *et al*., 2005) and two species currently *incertae sedis*, *Bryconadenos tanaothoros* (Weitzman *et al*., 2005) and *Brittanichthys axelrodi* (Javonillo *et al*., 2007).

Elongation of the nucleus in *C. riisei* proceeds without the aid of accessory microtubles. This contrasts with the situation in *M. barberi* where accessory microtubules, which are present around the nucleus during spermiogenesis when nuclear elongation occurs, persist in the mature spermatozoon as single row located at one side of the nucleus (Pecio & Rafiński, 1999). Accessory microtubules are also present in mature spermatozoa of the stevardiine genera *Scopaeocharax* and *Tyttocharax* (Pecio *et al*., 2005), the cheirodontine *Macropsobrycon uruguayanae* (Burns & Weitzman, 2005) and the *incertae sedis* species *Bryconadenos tanaothoros* (Weitzman *et al*., 2005), although data on spermiogenesis are lacking. The appearance of accessory microtubules during

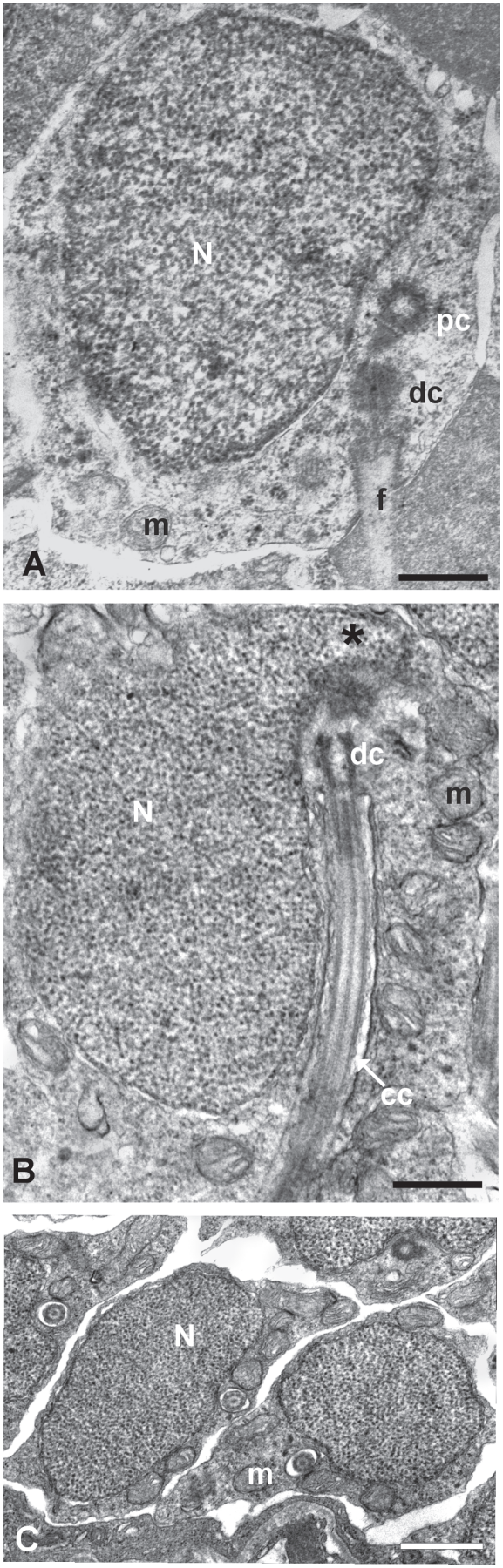

Fig. 5. (next column) Sagittal and transverse sections of early spermatids in *C. riisei*. **A -** Spermatid with finely granular chromatin at the beginning of flagellum formation. Scale bar 0.5 μm. **B -** Sagittal section through spermatid at the beginning of nuclear elongation. Scale bar 0.5 μm. **C -** Transversely sectioned spermatids during nuclear elongation along with displacement of mitochondria. Scale bar 1 μm. cc – cytoplasmic canal, dc – distal centriole, f – flagellum, m – mitochondrion, N – nucleus, pc – proximal centriole, asterisk – portion of nucleus immediately anterior to centriolar complex.

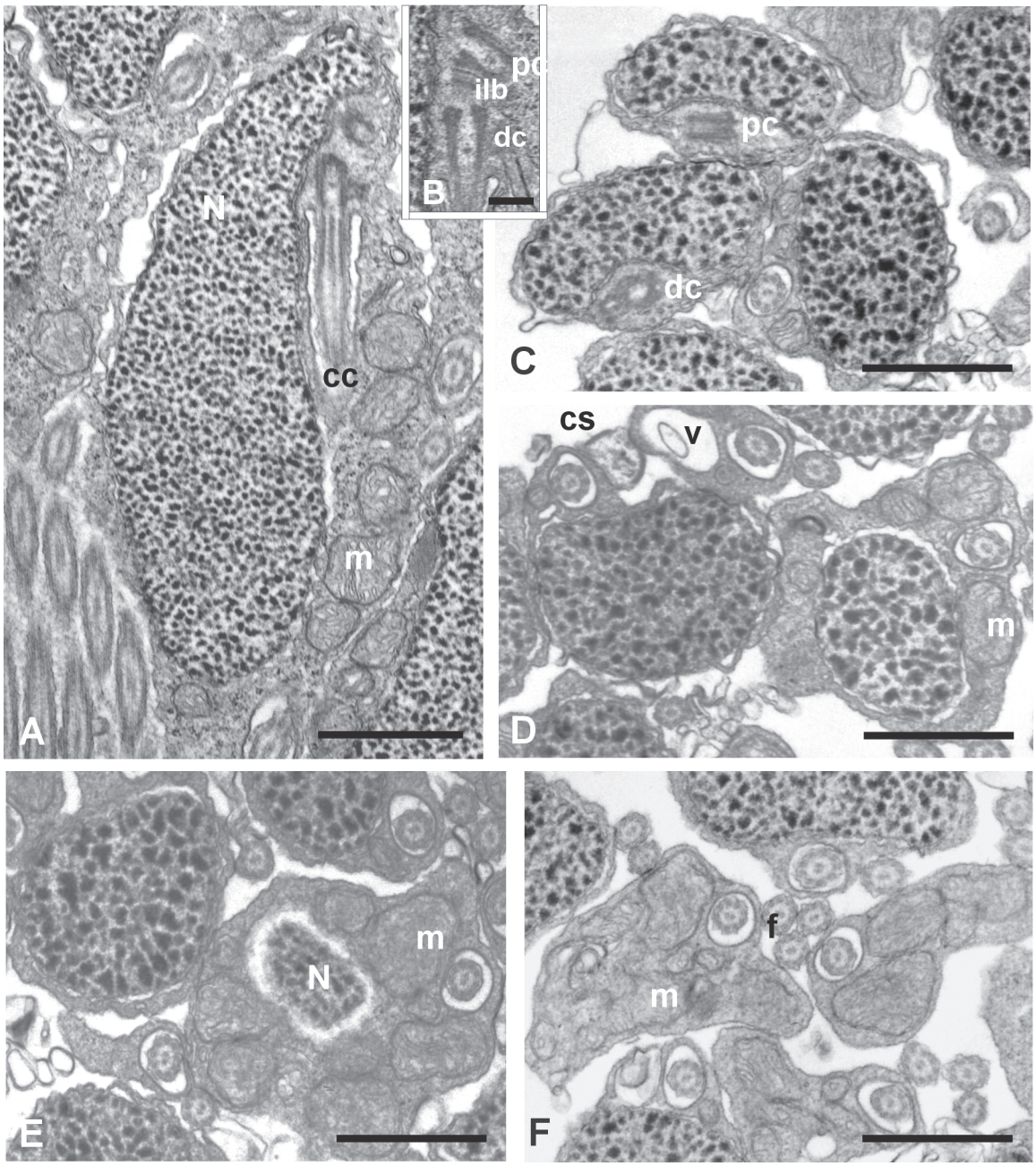

Fig. 6. Intermediate, elongated spermatids. **A -** Longitudinally sectioned spermatid with elongate nucleus containing highly granular chromatin and flagellum running within cytoplasmic canal. Scale bar 0.5 μm. **B - inset:** Arrangement of proximal and distal centrioles with intercentriolar lamellated body. Scale bar 0.25 μm. **C -** Transverse sections of tip of spermatids containing proximal and distal centrioles and anterior part of flagellum. Scale bar 0.5 μm **D -** Transverse sections of the middle portion of nucleus with mitochondria located around the nucleus. **E -** Posterior portion of nucleus showing numerous mitochondria, and flagellum contained within cytoplasmic canal. **F -** Flagellum within the cytoplasmic canal of the mitochondrial region. Scale bar 0.5 μm. cc – cytoplasmic canal, cs – cytoplasmic sleeve, dc – distal centriole, f – flagellum, ilb – intercentriolar lamellated body, m – mitochondrion, N – nucleus, pc – proximal centriole, v – vesicles.

spermiogenesis, coincident with nuclear elongation, was described in the viviparous poeciliids, *Gambusia affinis, Poecilia latipinna* and *P. reticulata*, where they appear during nuclear rotation (Billard, 1970; Grier, 1973, 1975). However, in the viviparous *Anableps anableps* of the Anablepidae, the sister group to Poeciliidae, during spermiogenesis no accessory microtubules are present when spermatid elongation occurs, in spite of the mature spermatozoon having a structure similar to that seen in poeciliids (personal observation).

An elongate cytoplasmic collar, which appears during spermatid formation in *C. riisei*, persists in the spermatozoon, with the flagellum running the entire length of the head

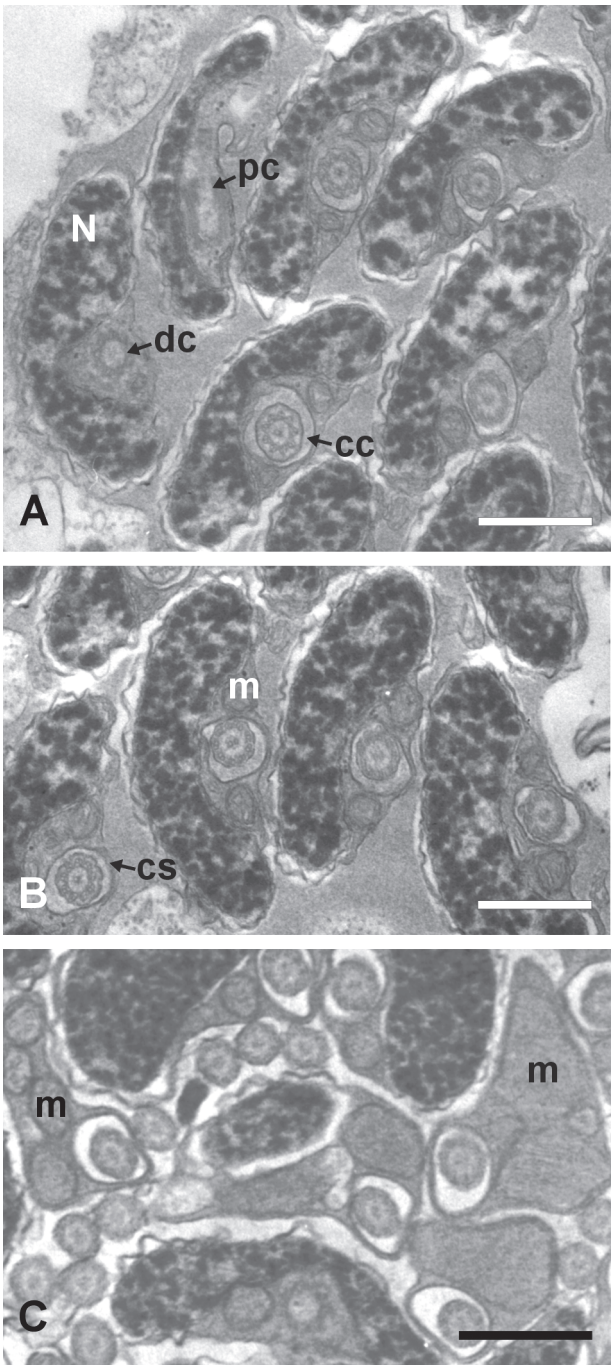

Fig. 7. Sections of greatly elongated and flattened spermatids with nuclei containing flocculent chromatin condensed into large "granules." **A -** Transverse sections through spermatids near the centriolar pole showing proximal (pc) and distal centrioles (dc). **B -** Transverse sections of nuclear region showing flagellum located within cytoplasmic canal between two elongate mitochondria. **C -** Transverse sections of mitochondrial region where flagellum runs within the cytoplasmic canal. Scale bar for all figures 0.5 µm. cc – cytoplasmic canal, dc – distal centriole, cs –cytoplasmic sleeve, m – mitochondrion, N – nucleus, pc – proximal centriole.

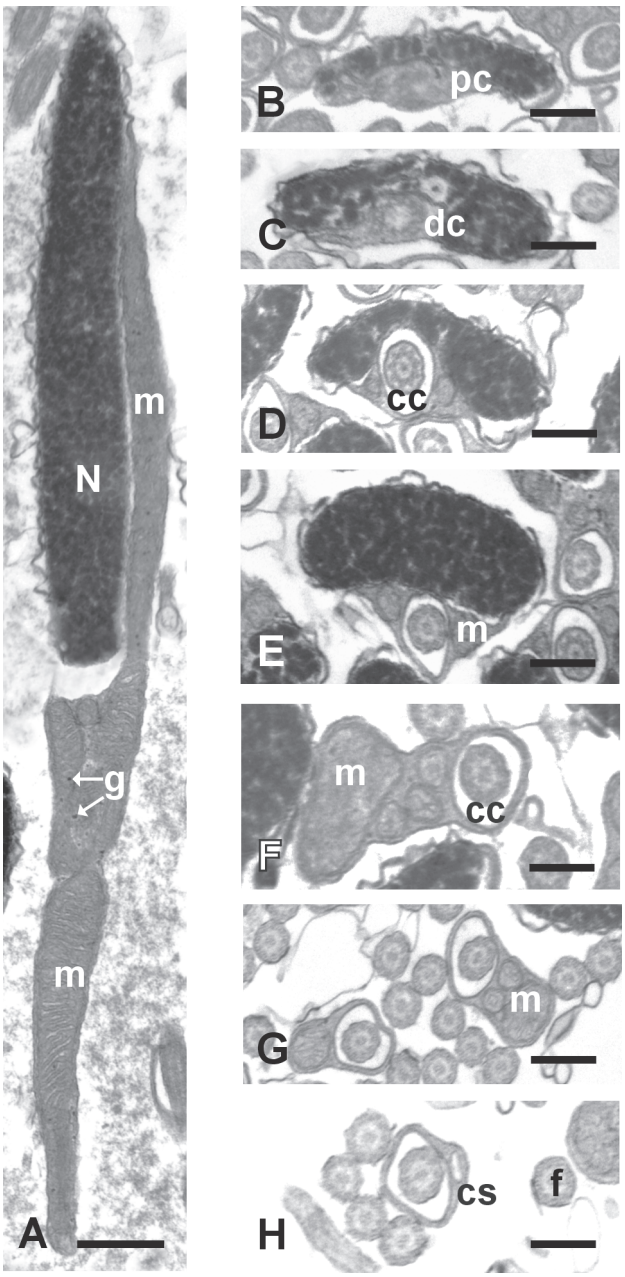

Fig. 8. Mature spermatozoon in *C. riisei*. **A -** Longitudinally sectioned spermatozoon with mitochondria running alongside nucleus and forming the mitochondrial region; glycogen rosettes (g) are present among mitochondria. **B-E**. Progressively more posterior transverse sections of spermatozoa. **B-**Tip of nuclear region containing the proximal centriole (pc). **C-**Region of distal centriole (dc). **D -** Anterior part of flagellum within cytoplasmic canal (cc) in the concavity of the nucleus. **E -** Cytoplasmic canal between two mitochondria (m). **F-H**. Transverse sections of mitochodrial region showing flagellum within the cytoplasmic canal (cc), surrouded by cytoplasmic sleeve (cs) and free. Scale bar for all figures 0.5 µm. f – flagellum, N-nucleus.

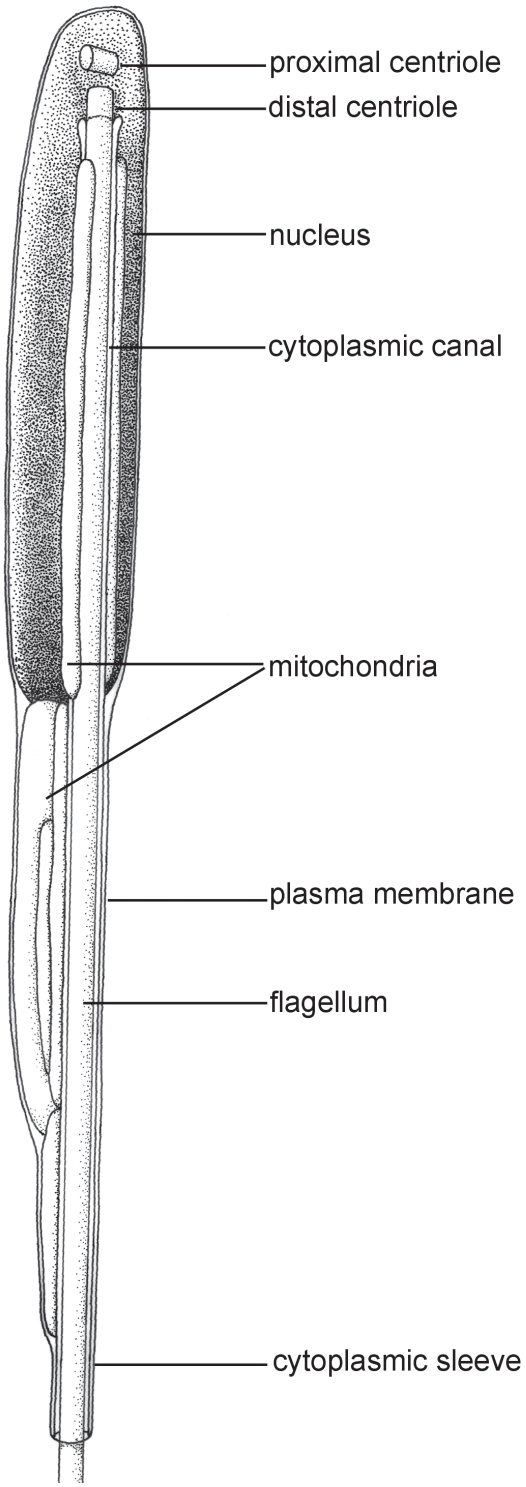

Fig. 9. Drawing of spermatozoon in *C. riisei.*

within the canal. An elongate collar is retained in the spermatozoon in the characid genera *Diapoma* and *Pseudocorynpoma* (Burns *et al*., 1998), and *Brittanichthys* (Javonillo *et al*., 2007), whereas in *Mimagoniates* (Pecio & Rafiński, 1994, 1999; personal observations) and in *S. rhinodus* and *T. tambopatensis* (Pecio *et al*., 2005) most of the cytoplasmic collar degenerates during the final stage of spermiogenesis, and in the spermatozoon the collar is limited to the very anterior part of the cell.

Our results demonstrate marked differences in the process of spermiogenesis between an externally and an inseminating species of Characidae. Differences also exist for spermiogenesis among inseminating characid species, such as those belonging to the subfamilies Stevardiinae and Glandulocaudinae. One of the main goals of this study has been to uncover ultrastructural characters that may prove useful for future phylogenetic analyses of these fishes.

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