Acta Zoologica (Stockholm) **80:** 35–45 (January 1999)

Spermiogenesis in *Mimagoniates barberi* (Teleostei: Ostariophysi: Characidae), an oviparous, internally fertilizing fish

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Keywords:
spermiogenesis, ultrastructure, fish, *Mimagoniates*, Glandulocaudinae, internal fertilization

Accepted for publication:
21 July 1998

Abstract

Pecio, A. and Rafiński, J. 1999. Spermiogenesis in *Mimagoniates barberi* (Teleostei: Ostariophysi: Characidae), an oviparous, internally fertilizing fish. — *Acta Zoologica* (Stockholm) **80:** 35–45

The ultrastructure of spermiogenesis of *Mimagoniates barberi* (Characidae), an internally fertilizing oviparous species is described. Spermiogenesis involves conspicuous changes of the topography of the cellular constituents and modifications of their shape. The round nucleus elongates to become a very long flat plate. The long axis of the nucleus establishes the polarity of the spermatid and spermatozoon. The nucleus does not rotate and, as a consequence, the centrioles are located at the proximal tip of the spermatozoon and the flagellum runs parallel to the long axis of the nucleus. Mitochondria and most of the cytoplasm are shifted to the pole opposite the centrioles. The proximal portion of the flagellum is located in a long cytoplasmic canal which shortens during spermiogenesis and is free for most of its length in the mature spermatozoon flagellum. In the cytoplasm of elongated spermatids, close to the nuclear membrane, numerous microtubules appear which run from the centriolar pole to the mitochondrial pole forming a microtubular manchette. The microtubules persist in mature spermatozoa. It is concluded that the highly specialized structure of the spermatozoon of *M. barberi* is the result of only few developmental modifications of spermiogenesis.

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Introduction

Structure of fish spermatozoa is extremely diverse (Jamieson 1991). This diversity can be explained at least partly by the diversity of modes of reproduction in fishes. Most externally fertilized teleosts have a simple type of the spermatozoon, called aquasperm, characterized by a roundish head and a short neck region with few mitochondria (Jamieson 1991; Mattei 1991). In contrast, spermatozoa of many internally fertilized teleosts have an elongated head and other derived features. These was shown for example for groups which contain both oviparous and viviparous species like Poeciliidae and Zenarchopteridae (Jamieson and Grier 1993).

The evolution of special structural features of spermatozoa of internally fertilized fishes must have involved modifications of the process of spermiogenesis. Compared with the wealth of information on spermatozoon structure, there are relatively few studies on fish spermiogenesis. Most of these studies described either spermiogenesis in fishes with simple spermatozoa or in viviparous fishes belonging to advanced fishes of the group Acanthopterygii (Billard 1970; Mattei 1970; Mattei and Mattei 1974; Grier 1975; Brusle 1981; Billard 1983; Billard 1986; Gwo and Gwo 1993). Here we present results of an ultrastructural study of spermiogenesis in *Mimagoniates barberi* Regan 1907, a small freshwater characid fish from streams of Paraguay. This

species is oviparous but females are inseminated internally (Pecio and Rafiński 1994). This is a rare mode of reproduction in teleosts (Breder and Rosen 1966), though internal fertilization must have preceded the evolution of viviparity. *Mimagoniates barberi* belongs to Glandulocaudinae, a small group of oviparous South American characids which all practice internal insemination (Weitzman and Fink 1985; Burns *et al*., 1995). The spermatozoon of *Mimagoniates barberi* has many derived features when compared with spermatozoa of other characids with external fertilization (Pecio and Rafiński 1994). The sperm head is highly elongated, its long axis is not a continuation of the flagellar axis but runs more or less paralell to the flagellum. Live spermatozoa are flail-shaped and move with the centriolar part ahead. There is no midpiece and the mitochondria are located on the posterior tip of the head. Such spermatozoon structure seems to be unique within Ostariophysi – the largest group of extant freshwater teleosts (Jamieson 1991). Elongated spermatozoa have also been found in other glandulocaudine genera (Burns *et al*., 1995). The present study gives the first description of the spermiogenesis in this most unusual group of fishes.

Materials and Methods

Sexually active males of *M. barberi* were taken from a breeding colony kept at our Department. Testes were excised after the fishes were killed by an overdose of tricaine methanesulphonate (MS-222). Small pieces of either spermatogenic or aspermatogenic tissue of the testis were fixed in 3% glutaraldehyde in cacodylate buffer (pH = 7.4), post-fixed in 1% osmium tetroxide, dehydrated through ethanol series and embedded in Epon 812. The ultrathin sections, stained with uranyl acetate and lead citrate, were examined in a JEOL JEM-100Sx electron microscope.

For scanning electron microscopy, testes of four specimens were fixed as for TEM, dehydrated in acetone, critical-point dried, fractured, sputter-coated with gold and viewed in a JEOL JSM 35.

Results

The testis of a mature *M. barberi* is composed of two parts. In the anterior part of the testis spermatogenesis takes place, whereas its caudal portion is devoid of any spermatogenic tissue and functions as a sperm storage organ (Pecio and Rafiński, 1994). Germ cells are confined to cysts located within the seminiferous tubules. Earlier stages of spermatogenesis in *M. barberi* do not show any peculiarities; spermatogonia and spermatocytes are very similar to those described in other teleosts (Billard 1983; Selman and Wallace 1986). Spermatocytes are spheroid and their shape changes subsequently into ovoid, which in later stages of spermiogenesis become even more elongated (Fig. 1A,B). Mitochondria in secondary spermatocytes are numerous and the Golgi apparatus is close to the centriole (Fig. 2A). Clumped or slightly mottled chromatin of the spermatocyte changes into more evenly granular chromatin typical of early spermatids. The spermatids are interconnected by cytoplasmic bridges, spaces between them are partly filled with an electron dense material (Fig. 2B,C). The diameter of a chromatin granule is at this stage $\approx$40 nm. The nucleus remains more or less spherical but the cell is already polarized. A few, large mitochondria are shifted to one side of the cell, the proximal centriole is in close contact with the nuclear membrane. The flagellum is situated within a relatively short cytoplasmic canal (Fig. 2D). As the nucleus of the spermatids elongates the cytoplasmic canal also elongates (Figs 2E, 3A). The flagellum is at an angle of $\approx 45^{\circ}$ relative the long axis of the nucleus, which shows a more or less flattened surface on the side of the flagellum. Large flat cisternae develop parallel to the flattened nucleus surface. The cisternae are often associated with the Golgi apparatus and other large vesicles (Fig. 3 B-D). At this stage the spermatid shows a clear bilateral symmetry and polarity. Its long axis is defined not only by the shape of the nucleus but also by the position of the proximal centriole which is situated on the apical side of the spermatid and by the position of the mitochondria on the opposite side. Most of the cytoplasm is concentrated on the mitochondrial, posterior pole of the spermatid and on the flagellar side (Fig. 3A).

The elongation of the nucleus and the spermatid proceeds further. At this stage the nucleus is more or less tear-shaped (Fig. 4A). The nucleus has an elongated tip at the centriolar pole, while its mitochondrial tip is wide and obtuse. The chromatin is granular but the granules have enlarged to 60–70 nm in diameter. The cytoplasm is almost completely shifted to the mitochondrial pole and to the flagellar side of the spermatid (Fig. 4A). The flagellar canal elongates and reaches the mitochondrial pole (Fig. 4F).

At later stages numerous microtubules appear in the cytoplasm. They originate in two groups situated laterally to the distal centriole and continue along the centriolar-mitochondrial axis of the spermatid (Fig. 4 B-G). Closer to the centriolar pole many rows of microtubules surround the nucleus. They are also found in the cytoplasm adjacent to the flagellar canal (Fig. 4 C,D). Further in the direction of the mitochondrial pole, microtubules form a single row approaching the nuclear membrane and reach the cytoplasm of the mitochondrial tip of the spermatid (Fig. 4 E,F). The spermatids are still connected by the cytoplasmic bridges (Fig. 4G).

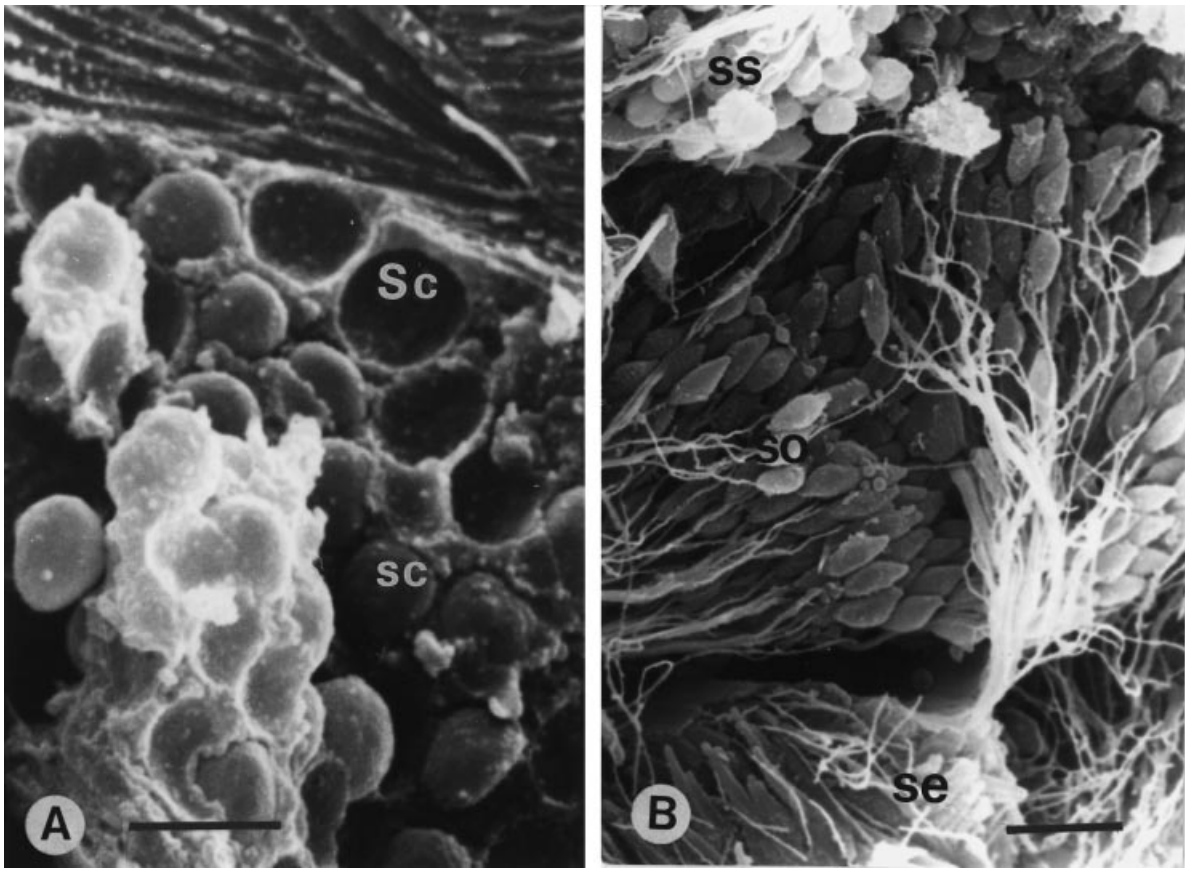


Fig. 1.—The inner surface of the spermatogenic cysts in SEM. — **A,** The primary spermatocytes (*sc*) situated in depressions of Sertoli cells (*Sc*). — **B,** Spherical (*ss*), ovoid (*so*) and strongly elongated (*se*) spermatids in separate cysts during spermiogenesis. Scale bar: 10 μm.

At the next stage, the nucleus of the spermatid elongates further and becomes compressed laterally. Elongated spermatids within the cysts have the same orientation (Fig. 5A). The centriolar tips of most spermatids are in close contact with the Sertoli cells which line the cysts (Fig. 5B). Free flagella are immersed in finely granular electron dense material (Fig. 5C). In the centriolar region the nucleus forms a flat plate with a reduced number of chromatin granules (Fig. 5D). In the rest of the nucleus chromatin granules are regularly spaced and have a diameter of 110–120 nm. At the mitochondrial pole the nucleus is also strongly compressed laterally and almost reaches the posterior tip of the spermatid.

The flagellar canal opens and becomes shorter. This process begins at the mitochondrial pole and continues in the direction of the centriolar pole (Fig. 5D). Flagella freed from the canal are often each surrounded by cytoplasm droplets (Fig. 5E). Condensation of the chromatin is not finished by the spermiation (i.e. when the cysts open and spermatozoa are shifted along tubules) and continues within the lumen of the tubules. The chromatin granules fuse together and the nucleus becomes more homogenous with only small uncondensed areas left.

The nucleus of a mature spermatozoon assumes the form of a long plate, ≈18 μm in length. Nuclear flattening is always at right angles to the plane of axonemal singlets. The nucleus is very flat and narrow at

Fig. 2.—A secondary spermatocyte and the early stages of spermiogenesis. — **A,** A secondary spermatocyte at the beginning of cell division. Chromatin mottled, Golgi apparatus (*Ga*) is close to divided centrioles (*arrows*). Scale bar: 1 μm. — **B,** Spermatids with round nuclei connected by intercellular bridges (*arrows*). Scale bar: 1 μm. — **C,** Spaces among spermatids partly filled with finely granular material. Scale bar: 0.5 μm. — **D,** Early spermatid with round nucleus and a wide zone of cytoplasm, mitochondria shifted to one pole. Proximal centriole (*pc*) close to nuclear membrane. The flagellum situated in a short cytoplasmic canal (*arrow*). Scale bar: 1 μm. — **E,** One side of the spermatid nucleus already slightly flattened with cytoplasmic cisternae (*asterisks*). Flagellum located in elongating cytoplasmic canal. Scale bar: 1 μm.

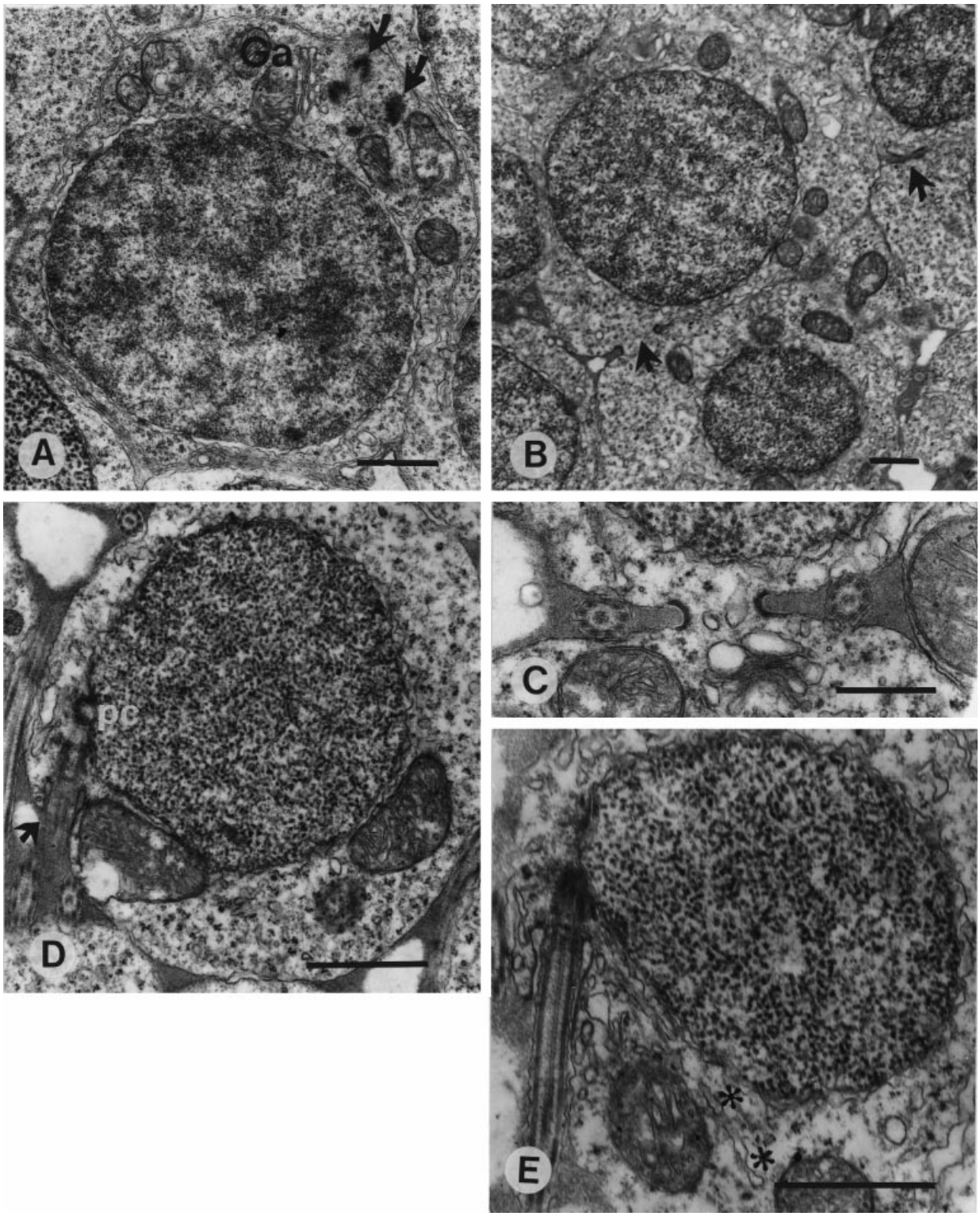


Fig. 2

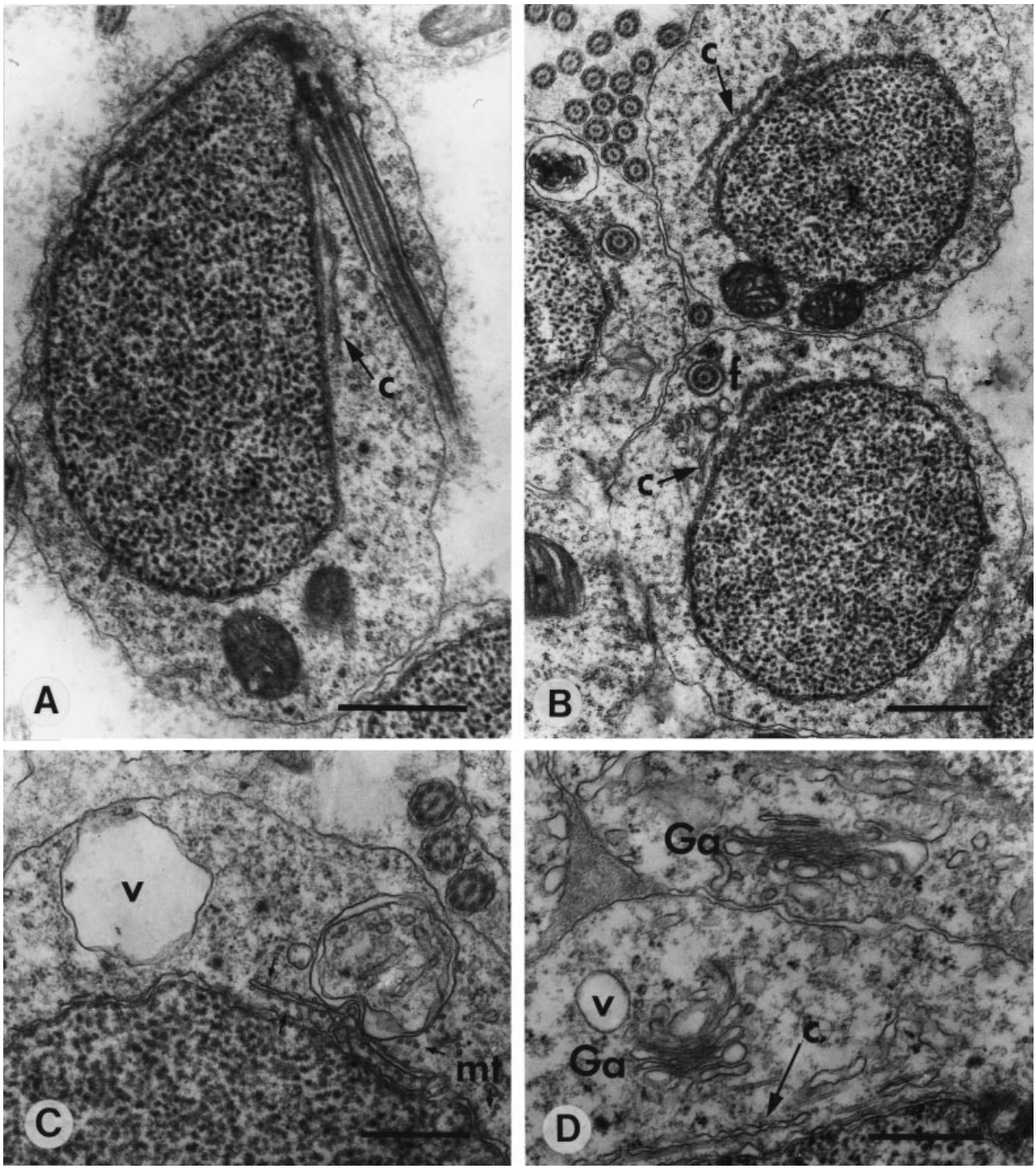


Fig. 3.—Sagittal and cross sections of the slightly elongated spermatids. — **A,** Spermatid polarized by the position of the centrioles situated at one pole and the position of mitochondria at the opposite pole. Elongated cytoplasmic canal situated at the flattened surface of spermatid nucleus. A large flat cisterna situated parallel to the flattened surface of the nucleus (*c* and *arrow*). The cytoplasm shifted to the flagellar side and mitochondrial pole. Scale bar: 1 μm. — **B,** Cross sections of elongated spermatids. Flagellum located in cytoplasmic canal (*f*). Mitochondria located in the more distal region. In both sections large flat cisternae (*c* and *arrow*) situated close to nuclear membrane on one side of nucleus. Scale bar: 1 μm. — **C,** The microtubules (*mt*) associated with a cisterna and nuclear membrane (*small arrows*), a large vesicle visible (*v*). — **D,** Flat cisternae (*arrows* and *c*), dictyosomes (*Ga*) and vesicles (*v*) situated in the cytoplasm. Scale bar: 0.5 μm.

the centriolar apex of the spermatozoon, approximately oval shaped in a cross-section in the middle and again flat and laterally compressed at the mitochondrial pole (Fig. 6A-C). Mitochondria are situated along the posterior part of the spermatozoon on one side of the nucleus and at the tip of the mitochondrial pole (Fig. 6C,D). The nucleus does not reach the very tip of the mitochondrial pole (Fig. 6D).

Numerous microtubules are found in the cytoplasm of the centriolar pole of the spermatozoon in two groups at the sides of the flagellar canal. In the middle part of the nucleus they are found in many layers on one side of the nucleus (Fig. 6B). Further, in the direction of the mitochondrial pole they are found close to the plasmalemma and mitochondria but usually they form a single layer only (Fig. 6C). A few microtubules reach the mitochondrial tip of the spermatozoon (Fig. 6D).

In a mature spermatozoon, the flagellar canal is very short (Fig. 6A,F). The spermatozoon head is ≈ 20.6 μm and the flagellum ≈ 54.0 μm in length. The centriolar pole of the head is round, whereas at the mitochondrial pole the head tapers to a short tip. The axoneme of the flagellum has a typical $9 + 2$ pattern with A tubules occluded in all doublets (Fig. 6E).

Discussion

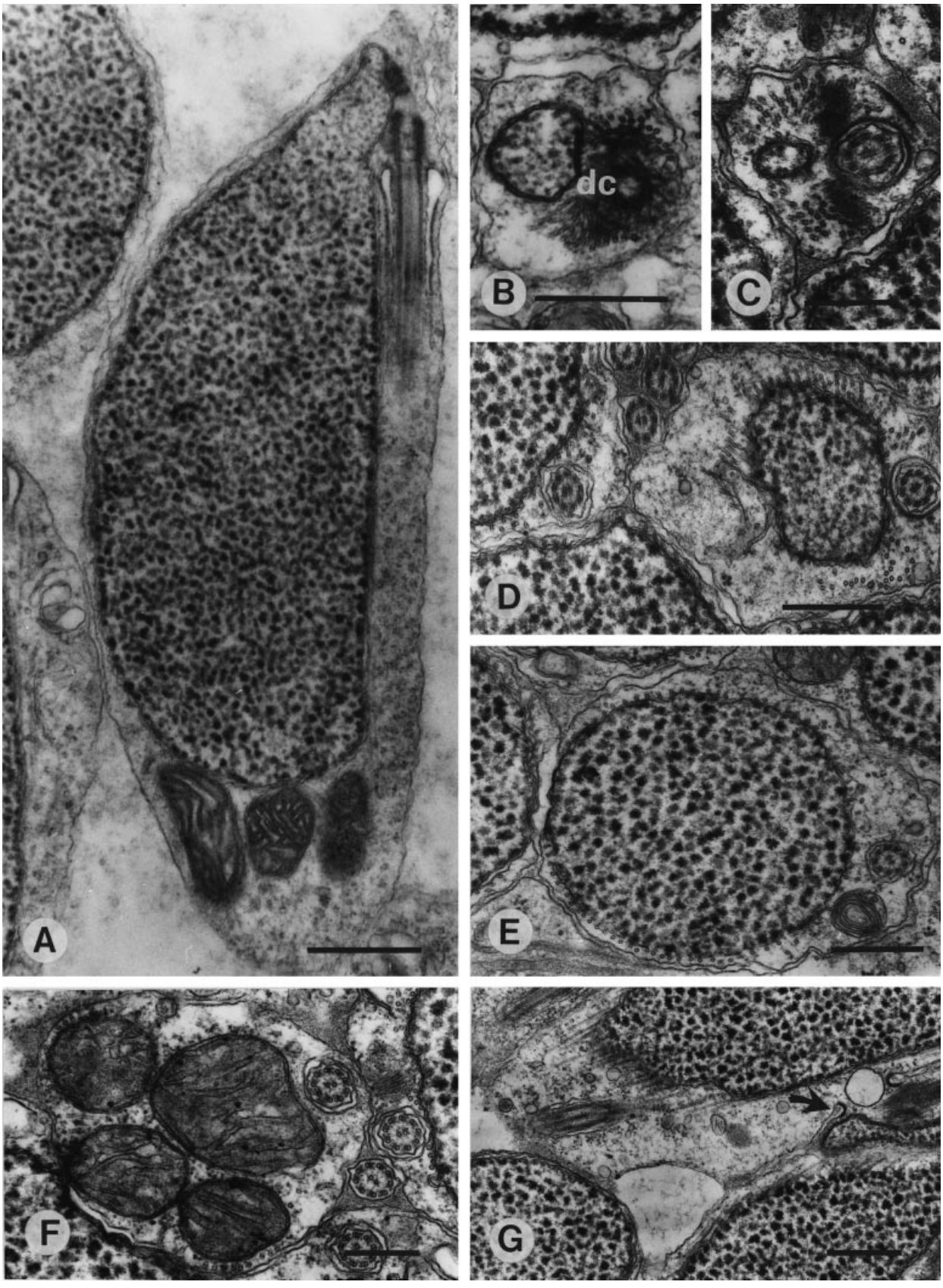
The nucleus of the spermatozoon of *M. barberi* has a form of a long flat plate. Elongation of the sperm head has been observed in many internally fertilized species, and is therefore deduced to give some advantage to the spermatozoon in the internal environment within the female (Jamieson 1991). The flattening of the *M. barberi* nuclus is at the right angles to the plane of axonemal singlets. Quite similar orientation of the axoneme and the flat nucleus has been observed in viviparous fishes belonging to the family Zenarchopteridae (Jamieson and Grier 1993). The most characteristic feature of the spermatozoon of *M. barberi* is the backward position of the head relative to the diplosome. The present study shows that in *M. barberi* the eccentric position of the diplosome in relation to the nucleus in early stages of spermatogenesis is later augmented by the backward elongation of the nucleus. In this way the proximal centriole becomes situated at the advancing, apical pole of the spermatozoon and the flagellum in live spermatozoa runs almost parallel to the long axis of the head. In several fish species the flagellum in a mature spermatozoon is asymmetrically located. In *Paracheirodon innesi*, the only characid for which the ultrastructure of the spermatozoon has been published, the head is situated slightly oblique to the flagellar axis (Jamieson 1991). Such a situation has also been described for the carp (*Cyprinus carpio*, Cyprinidae), the pike (*Esox lucius*, Esocidae) (Billard 1986), and a cichlid (*Oreochromis niloticus*) (Lou and Takahashi 1989).

In several fish species the diplosome is first situated eccentrically in relation to nucleus, later the nucleus rotates taking up its position in front of the diplosome. This type of spermiogenesis in teleosts was described as type I by Mattei (1970). Rotation of the nucleus during spermiogenesis has been found in several unrelated teleosts, for example in *Lepomis macrochirus* (Centrarchidae) (Sprando *et al.* 1988) and *Acanthopagrus schlegeli* (Sparidae) (Gwo and Gwo 1993). Even in species with elongated spermatozoon head, like Poeciliidae and Zenarchopteridae, the long axis of the sperm head is continuous with the flagellar axis (Jamieson and Grier 1993). In type II of Mattei there is no nuclear rotation during spermiogenesis and the flagellum in a mature spermatozoon is asymmetrically located. This type of spermiogenesis has been found for example in *Liza aurata* (Mugilidae) (Brusle 1981). Thus, spermiogenesis in *M. barberi* should be classified as the extreme case of type II spermiogenesis of Mattei (1970).

The spermatozoon with apically situated centrioles and backward elongated nucleus, which in general structure resembles the spermatozoon of *M. barberi*, has also been described in a scorpaenid fish *Dactylopterus volitans* (Boissin *et al.* 1968) and in a primitive bony fish *Polypterus senegalus* (Mattei 1970). The two latter species are externally fertilized. To provide functional explanation for such spermatozoon structure we have undertaken studies on movement of *M. barberi* spermatozoa.

Another characteristic and derived feature of the spermatozoon of *M. barberi* is the dislocation of mitochondria to the posterior tip of the head. In the majority of fish spermatozoa mitochondria are located in the neck region next to the diplosome beneath the nucleus. However, in

Fig. 4.—Sagittal and cross sections of the strongly elongated spermatids. — **A,** The nucleus attenuated at the centriolar pole and obtuse and round at the mitochondrial pole. The flagellar side of the nucleus is flat. Scale bar: 1 μm. — **B-F,** A sequence of cross sections of the elongated spermatids from the centiolar tip to the mitochondrial pole. Scale bar: 0.5 μm. — **B,** Two bunches of microtubules present on two sides of the distal centriole (*dc*). — **C,** The microtubules distributed in the cytoplasm around the nucleus in many rows and densly packed alongside the cytoplasmic canal. — **D,** The microtubules scattered in the cytoplasm around the nucleus. — **E,** A single row of microtubules around the nuclear membrane. — **F,** The cytoplasmic canal containing the flagellum at the level of the mitochondrial pole. A few microtubules situated mainly beneath the plasmalemma. In spaces between spermatids granular material still present. — **G,** Oblique section trough elongated spermatids still connected by the intercellular bridges (*arrow*). All microtubules parallel to the long axis of the nucleus. Scale bar: 0.5 μm.



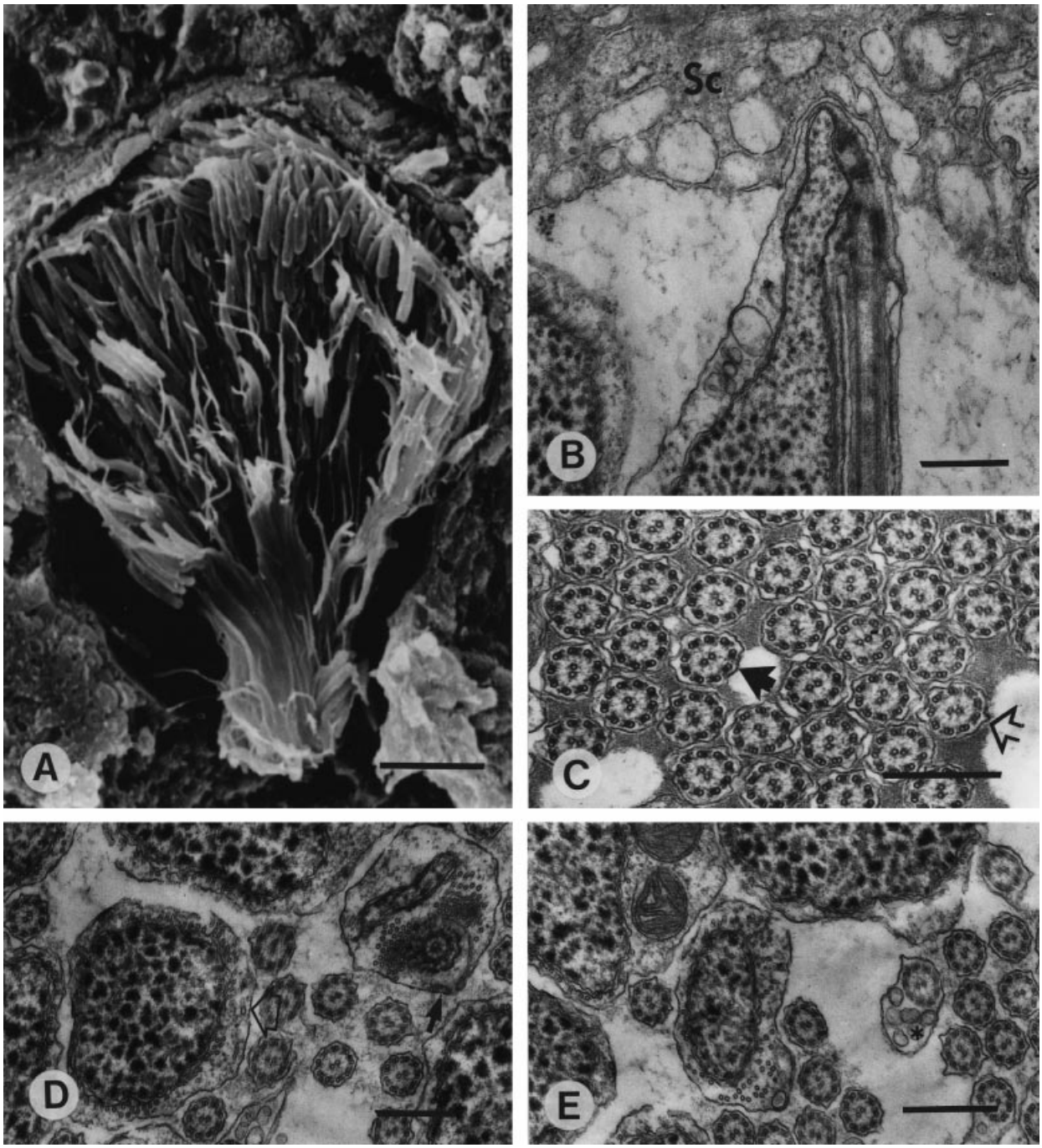


Fig. 5.—Spermatids before spermiation. — **A,** Strongly elongated spermatids organized in a parachute-like structure within a cyst before spermiation. Fractured fragment of the testis in SEM. Scale bar: 20 μm. — **B,** Strongly vacuolated cytoplasm of the Sertoli cell (*Sc*). The centriolar tip of the spermatid inserted in the depresion of Sertoli cell within a cyst. — **C,** A cross section of the bunch of flagella in a cyst immersed in a fine granular material. The axonemal A-microtubules occluded (*black arrow*) but in some not (*empty arrow*). — **D-E,** Cross sections of the elongated spermatids, the cytoplasm reduced and the laterally compressed nucleus. Flagella already emergent from the spermatid. Cytoplasmic canal present only at the very tip of the spermatid (*arrow*). The proximal part of the nucleus in a form of a flat blade. The microtubules are present in two bunches at the level of the cytoplasmic canal (D). Further towards the mitochondrial pole microtubules located around the nucleus (E). No microtubules at the flattened nuclear surface, cross section approximately at the midlength of the nucleus (D, *blank arrow*). A cytoplasmic droplet with small vesicles present on one of the flagella (*asterisk*). Scale bar: 0.5 μm.

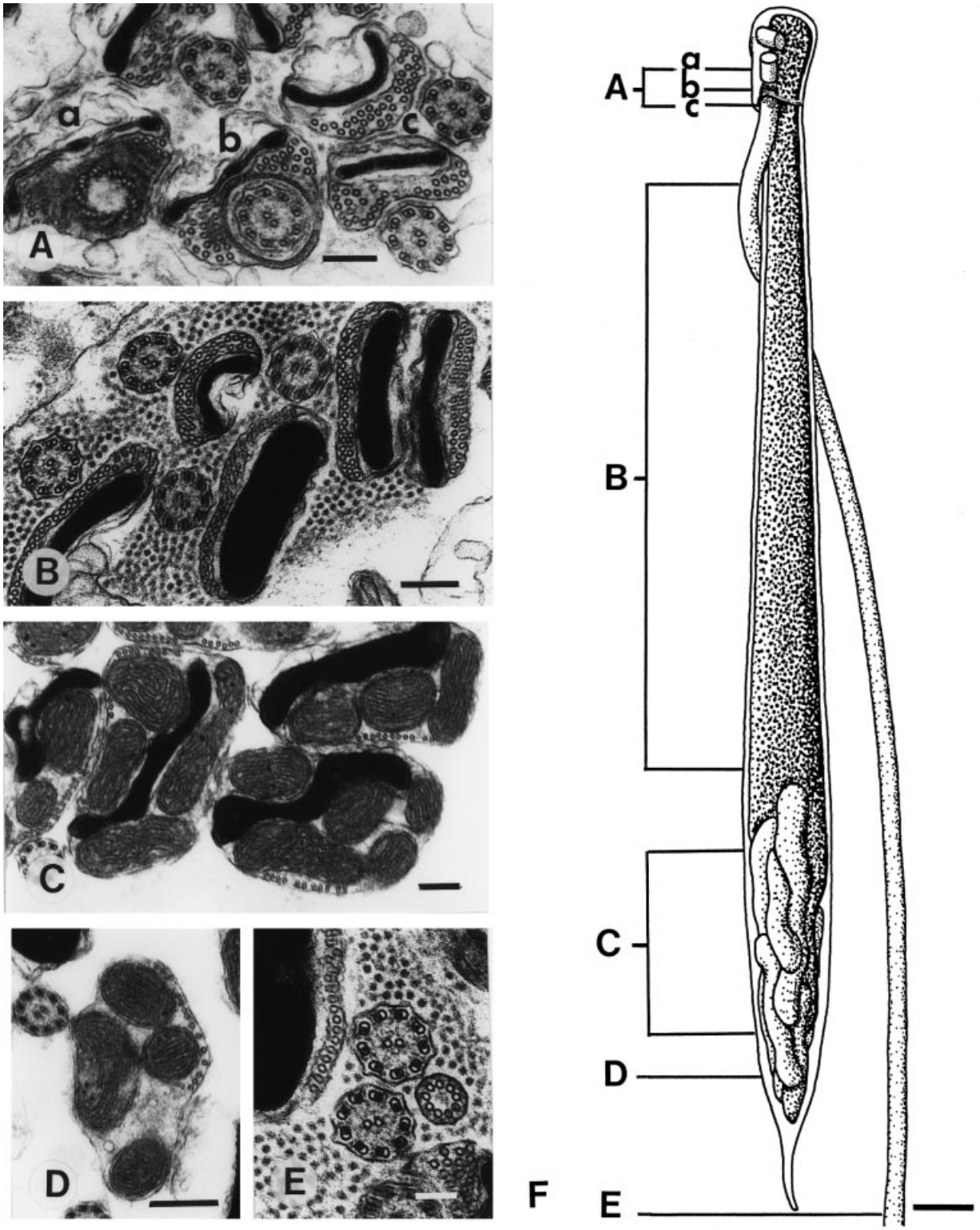


Fig. 6

several fish species mitochondria are situated excentrically. In the spermatozoon of the loach (*Misgurnus anguillicaudatus*), which together with *M. barberi* is a member of Ostariophysi, cytoplasmic components of the midpiece such as mitochondria are shifted to one side, therefore the other side of the midpiece is formed by a thin layer of cytoplasm only (Ohta *et al.* 1993). In *M. barberi*, the midpiece disapears as a result of the extreme elongation of the nucleus without rotation and the reduction of the cytoplasmic sheath on the opposite side. A location of mitochondria similar to that in spermatozoa of *M. barberi* has evolved independently in *Anguilla anguilla* (Elopomorpha) (Gibbons *et al.* 1983) and in *Blennius pholis* (Percomorpha) (Silveira *et al.* 1990).

A unique feature of the late spermatid in *M. barberi* is the presence of a single row of microtubules located close the nuclear envelope. Shifted to one side of the nucleus they are also present in a mature spermatozoon. Microtubules in association with the centriolar complex are present in spermatids of several viviparous poeciliid teleosts, these microtubules disappear by the end of the spermiogenesis (Billard 1970; Grier 1973, 1975). In poeciliids the formation of microtubules coincides with nuclear elongation and therefore one can conclude that the microtubules in these species are involved somehow in the shape change of the nucleus of spermatids. However, the presence of microtubles during fish spermiogenesis has been also reported for species with rounded, unmodified sperm heads (Yasuzumi 1971).

Circumnuclear microtubules are commonly known as the manchette and have been described in several groups of both vertebrates and invertebrates (Kessel 1970; Ferraguti and Lanzavecchia 1971; Stanley *et al.* 1972). In fishes the manchette has been found only during spermiogenesis of *Polypterus senegalus* (Mattei 1970). Since no electron micrographs for this species have so far been published, a direct comparison with *M. barberi* is difficult. The function of the microtubules in spermiogenesis of *M. barberi* is not known but quite probably the microtubules could play an active role in elongation and shaping of the sperm head. Several data indicate that this is the function of microtubules in mammalian spermiogenesis. Observations on mice bearing a mutation which affects the distribution of microtubules during spermiogenesis indicate that the microtubular manchette is involved in the shaping of the spermatid nucleus (Meistrich *et al.* 1990; Russell *et al.* 1991). This hypothesis is also supported by the finding that manchette microtubules are associated with the cytoplasmic dynein in rat spermatids (Yoshida *et al.* 1994). It suggests that the manchette may play a role in intracellular movements.

So far *M. barberi* is the only fish species in which microtubules are retained in mature spermatozoa, although recently, microtubules have also been found in the spermatozoa of another *Mimagoniates* species (J. Burns, personal communication). Microtubules form a multilayered scaffolding on the one side of the nucleus and possibly lend rigidity to a very long sperm head. This might be important both in the movement of spermatozoa within the female and during insemination. We have observed that the spermatozoa of *M. barberi* deform very quickly when mixed with aquarium water. The deformation is restricted to the distal portion of the spermatozoon where only a few microtubules are present. Since no intromittent organ is present in males of *M. barberi* the spermatozoa of this species must experience a transient osmotic stress during insemination. Unexpectedly no microtubules were found in other Glandulocaudinae genera which have even more elongated sperm heads (J.Burns, personal communication). However, unlike in *Mimagoniates* in all these species the flagellum runs in a long cytoplasmic canal along the whole length of the head which perhaps provides some support for the long head.

Acknowledgements

We thank Professor W. Kilarski (the Electron Microscopy Centre, Jagiellonian University, Kraków, Poland) and Dr F. Lahnsteiner (University of Salzburg, Austria) for access to the facilities and the staff for technical assistance. We thank also Dr J. Szymura (Jagiellonian University) for reading the manuscript and his suggestions, Dr John Burns (George Washington University, Washington, D.C.) for providing unpublished data and an anonymous reviewer for many helpful comments which improved the paper.

This research was supported by a grant IZ/BW/14/97.

Fig. 6.—A series of cross sections of mature spermatozoa from the centriolar to the mitochondrial tip (A-E). Scale bar: 0.2 μm. — **A,** The centriolar tip with strongly compressed nucleus, microtubules scattered in the cytoplasm on two sides of distal centriole (*a*), and along the cytoplasmic canal (*b*). *c* a cross section just below the opening of the cytoplasmic canal. All axonemal microtubules empty. — **B,** Cross sections through middle portion of the head of the spermatozoon. 1–2 rows of microtubules located between the nucleus membrane and plasmalemma on one side of the nucleus. — **C,** Distal portion of the spermatozoon. Mitochondria located alongside the flat nucleus. The microtubules located between plasmalemma and the mitochondria. — **D,** Tips of the spermatozoa with mitochondria and a few microtubules. — **E,** Cross section through flagella and an endpiece. All A-tubules occluded. — **F,** Schematic diagram of the spermatozoon structure. Microtubules omitted. The capital letters A-E correspond to the numbers of the micrographs to the left. Scale bar 1 μm.

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