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The sperm ultrastructure of *Merluccius merluccius* (Teleostei, Gadiformes): phylogenetic considerations

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Abstract

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The anacrosomal aquasperm of the gadiform *Merluccius merluccius* is ultrastructurally similar to the advanced type II spermatozoa (perciform-type sperm) typically found in most Perciformes. The perciform-type spermatozoon is characterized by the lateral insertion of the flagellum and the location of the centrioles outside the nuclear fossa. Apart from these characteristics, the spermatozoon of *M. merluccius* is remarkable because of the mutually parallel arrangement of the centrioles, a rare feature among fishes, which is considered an apomorphic condition for animal sperm cells. Within the superorder Paracanthopterygii, which contains a large diversity of sperm patterns resulting from a high number of apomorphies, a perciform-type sperm is present only in the order Gadiformes. The significance of the presence of perciform-type spermatozoa in the three investigated gadiform families is discussed in a phylogenetic context.

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Introduction

Since the advent of electron microscopy, metazoan comparative spermatology has contributed considerably to a better understanding of the phylogenetic relationships between animals. In fishes in particular, a preliminary overview by Mattei (1970) laid the foundations for the comparative study of sperm ultrastructure from an evolutionary viewpoint. Two further reviews (Jamieson 1991; Mattei 1991) made evident the usefulness of spermatozoal ultrastructure in the investigation of phylogenetic relationships in various fish taxa. However, ichthyologists have paid little attention to the comparative analysis of spermatozoal patterns for systematic purposes.

Mattei (1970) classified the simple anacrosomal aquasperm of teleosteans into two principal sperm types. In the apomorphic type II spermatozoon, the so-called perciform type, the centrioles remain outside the nuclear fossa and the flagellum inserts eccentrically into the sperm head. This sperm morphology is the most widely distributed among the perciforms, although it is not exclusive to them as it is also found in the Gadiformes (Jamieson 1991; Mattei 1991; Lahnsteiner *et al.* 1994). Within the gadiforms, sperm ultrastructure has been studied previously in only three species from three different families, *Laemonema laureysi* Poll (Moridae) (Mattei 1991), *Merluccius polli* Cadenat (Merlucciidae) (Mattei 1991), and *Lota lota* (L.) (Gadidae) (Lahnsteiner *et al.* 1994), therefore the sperm morphology in this teleost order remains very poorly known. The asymmetrical emplacement of the flagellum in the spermatozoa of *L. laureysi* and *M. polli* (perciform-type arrangement) was considered by Mattei (1991) to be a character sufficiently sound to recognize within the Gadiformes a clear phylogenetic affinity between the families Moridae and Merlucciidae, justifying their grouping into the same suborder (Gadoidei). Later, Lahnsteiner *et al.* (1994) showed a similar synapomorphic insertion of the sperm flagellum lateral to the nucleus in the burbot, *Lota lota* (Gadidae).

In this paper we make a comparative study of the spermatozoal ultrastructure of the European hake, *Merluccius merluccius* (L.). Our aim is to enlarge the current knowledge of specific characters of gadiform spermatozoa to contribute to the construction of phylogenetic arrangements in the Paracanthopterygii, a phylogenetically ambiguous teleostean

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group in which the sperm structure exhibits a great diversity of forms.

Materials and Methods

Adult male *Merluccius merluccius* were collected by trawl in the Gulf of Cádiz (southern Iberian Peninsula) in March, 2000 during the research cruise ARSA 0300 of the Spanish Oceanographic Institute (IEO). Samples of semen and small fragments of testes (~1 mm^3 in size) were fixed for 3–4 h in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate buffer (pH 7.2) containing 10% sucrose. Following two 60-min washes in cacodylate buffer, the samples were post-fixed for 1 h at 4 °C in cacodylate-buffered osmium tetroxide, and rinsed several times in the buffer. After the double fixation, the sperm suspension was treated either for scanning (SEM) or transmission (TEM) electron microscopy. For SEM, spermatozoa were attached to coverslips previously coated with 0.1% poly L-lysine. Following dehydration in an ascending series of acetones, the samples were critical-point-dried, sputter-coated with gold, and examined in a Jeol JSM 820 electron microscope. For TEM, either tissue blocks or sperm pellets obtained by centrifugation at 2500 *g* for 10 min, were dehydrated through a series of acetones and embedded in Spurr's epoxy resin. Thin sections (~80 nm thick) were doubly stained with uranyl acetate and lead citrate and examined in a Jeol JEM 1200 EX electron microscope.

Results

In the acrosome-less spermatozoon of *Merluccius merluccius* the rounded head is followed by an elongate midpiece and the tail (Fig. 1A,B). The head and the midpiece together measure about 3.5 µm in length and 1.75 µm in width. The head contains the nucleus, which shows a homogeneous electron-dense chromatin, lacks nuclear pores and is ovoid in shape, somewhat flattened at its apical surface (Fig. 1C,D). The midpiece is conical, tapering at the distal end (Fig. 1A–C). On SEM the flagellum, which measures about 30 µm in length, displays a smooth cylindrical shape throughout its length; no lateral fins are observed (Fig. 1A).

The two centrioles (~250 nm long and ~180 nm wide) are parallel and located in one plane perpendicular to the longitudinal axis of the sperm cell (Figs 1E,F and 2). They are ~450 nm apart (centre-to-centre distance) and show the conventional 9 + 0 configuration. Centrally, at the base of the nucleus, the nuclear envelope forms two shallow invaginations in coincidence with the location of the centrioles, but these remain outside the nuclear depressions (Fig. 1E). The flagellar apparatus is attached to the nucleus by means of a lateral plate made of an electron-dense material that keeps the lateral surface of the axonemal basal body connected to the nuclear envelope (Fig. 1G). Therefore, the axoneme is orientated parallel to the basal surface of the nucleus, and the insertion of the flagellar apparatus in the spermatozoon is asymmetrical (Fig. 2). The proximal centriole is also linked to the nuclear surface by granular material (Fig. 1E). In the midpiece, an array of microtubules is occasionally seen projecting from the proximal centriole (Fig. 1F). Nine radial fibres project from the basal body triplets (Fig. 3A,B) and contact the plasma membrane at the caudal part of the centriole (Figs 1E and 3A). At this point, the plasma membrane forms a deep, narrow invagination (cytoplasmic canal) between the flagellum and the cytoplasmic collar in the midpiece (Figs 1C,F and 3A,C). The cytoplasmic canal surrounds the proximal segment of the flagellum for ~2 µm. Throughout the cytoplasmic canal the plasma membrane is thickened with a dense layer on its inner side (Fig. 3A,C,D,F,G). The spermatozoon of *M. merluccius* retains a considerable amount of cytoplasm in the elongate midpiece, which contains numerous electron-clear vesicles, several round or ovoid mitochondria and smooth tubular membrane cisternae (Figs 1C,D,F, 2, and 3C,E,F). The mitochondria measure ~0.5 µm in diameter, and show irregular cristae and a moderately electron-dense matrix (Figs 1C,D and 3E,F). It is difficult to determine their exact number from electron micrographs; up to five mitochondrial profiles can be seen in cross-sections of the midpiece close to the nucleus, and more than 10 mitochondrial units appear to be present throughout the midpiece.

The axoneme has the usual 9 + 2 arrangement (Fig. 3F,G) except at its most proximal end, adjacent to the basal body, where the pair of central microtubules is absent (Fig. 3A,D). At this level the peripheral doublets are attached to the flagellar membrane by means of Y-shaped bridges (Fig. 3D). The flagellum lacks intratubular differentiations and lateral extensions of the membrane (lateral fins or ribbons).

Discussion

As in the overwhelming majority of neopterygian fishes, *Merluccius merluccius* possesses typical uniflagellate anacrosomal aquasperm. These are regarded as a physiological adaptation to the external fertilization of thick-shelled eggs endowed

Fig. 1—Electron micrographs of *Merluccius merluccius* spermatozoa. —**A**, **B**, SEM micrographs of spermatozoa showing the head, midpiece and tail; spermatozoa 1 and 2 stand with the side of insertion of the flagellum orientated up and down, respectively. —**C**–**G**, TEM micrographs of sagittal (**C**, **G**), oblique (**D**), transverse (**E**), and frontal (**F**) sections of the spermatozoon. Abbreviations: ax, axoneme; cc, cytoplasmic canal; dc, distal centriole (basal body); fl, flagellum; h, head; lp, lateral plate connecting the basal body to the nuclear envelope at the base of the nucleus; m, mitochondria; mp, midpiece; mt, microtubules projecting from the proximal centriole; n, nucleus; pc, proximal centriole; rf, radial fibres attaching the basal body to the plasma membrane at the proximal end of the cytoplasmic canal; sc, smooth membrane cisternae; v, midpiece vesicles; arrowheads, opening of cytoplasmic canal.

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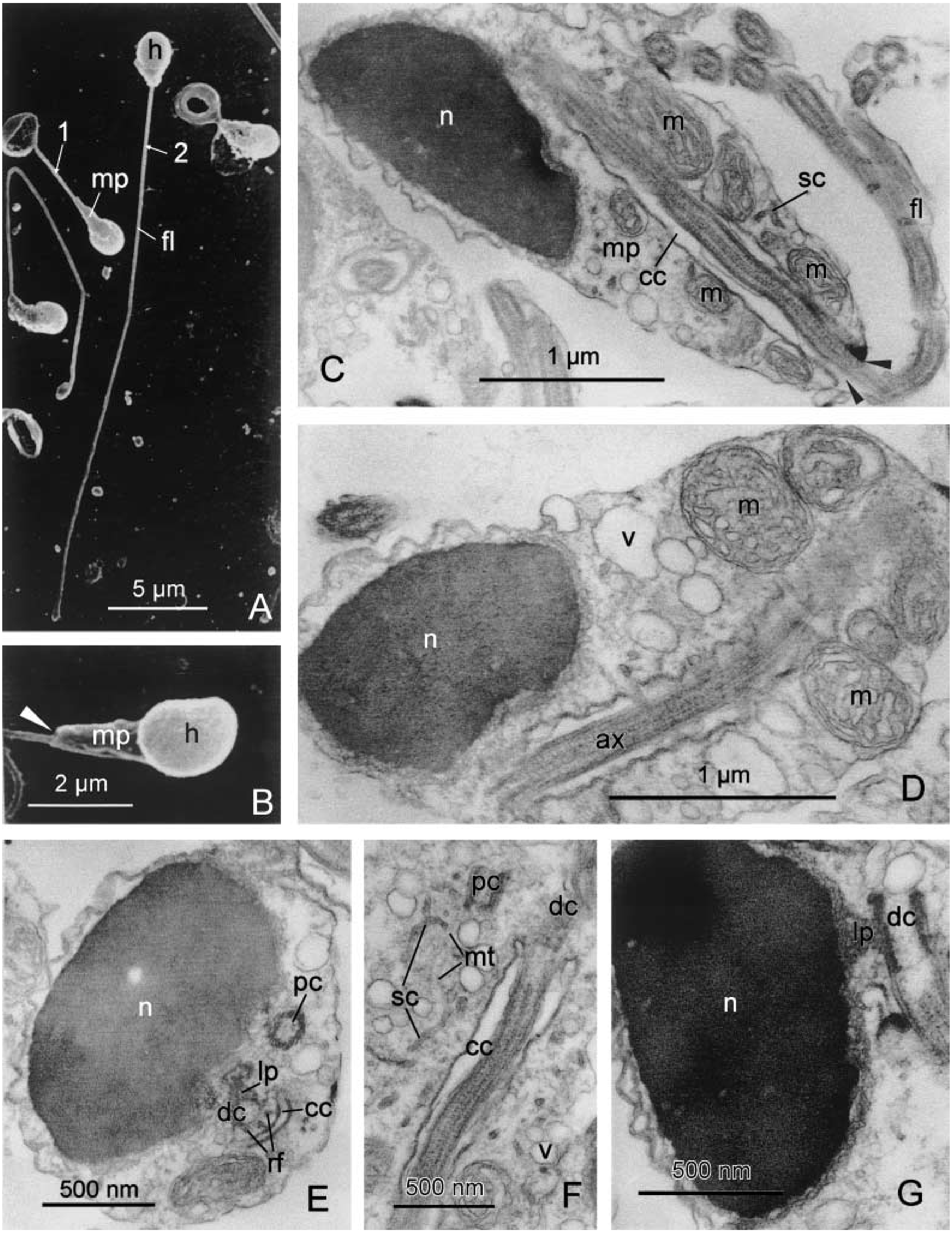
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with a micropyle (Jamieson 1991). Although very simple in morphology, the anacrosomal aquasperm of the Teleostei can adopt a wide range of structural variations that, at least in some taxa, prove valuable in taxonomy (Baccetti *et al.* 1984; Jamieson 1991; Mattei 1991). Thus, a single sperm model cannot represent the large diversity of forms found in the whole group (Mattei 1970; Billard 1986; Koch and Lambert 1990). Mattei (1970) classified the simple acrosome-less spermatozoa (the plesiosperm of Jamieson 1991) of the Teleostei into two distinct general categories. Unlike the type I sperm, the presumed apomorphic type II sperm is characterized largely by the tangential insertion of the flagellum in relation to the nuclear base. As this sperm configuration is by far the most common among the Perciformes (Mattei 1991), it is usually termed the perciform-type teleostean sperm, though a similar type of spermatozoon is also present in the Gadiformes. Confirming previous observations on three gadiform species (Mattei 1991; Lahnsteiner *et al.* 1994), the spermatozoon of *M. merluccius* shows a lateral insertion of the flagellum in relation to the nucleus, and a location of the centrioles external to the nuclear invaginations. As in the other gadiforms studied so far (Mattei 1991; Lahnsteiner *et al.* 1994), intratubular differentiations – inner septa located in the A microtubules of doublets 1, 2, 5 and 6, originally described as a specific perciform-type sperm feature (Mattei *et al.* 1979) – are not found in *M. merluccius*. However, their presence does not appear to be an essential character of identity for the perciform-type spermatozoon because they are similarly lacking in many perciforms possessing such a sperm model (Gwo *et al.* 1994; Hara and Okiyama 1998; Abascal *et al.* 2002). Other features shared by all the known gadiform spermatozoa are the elongate

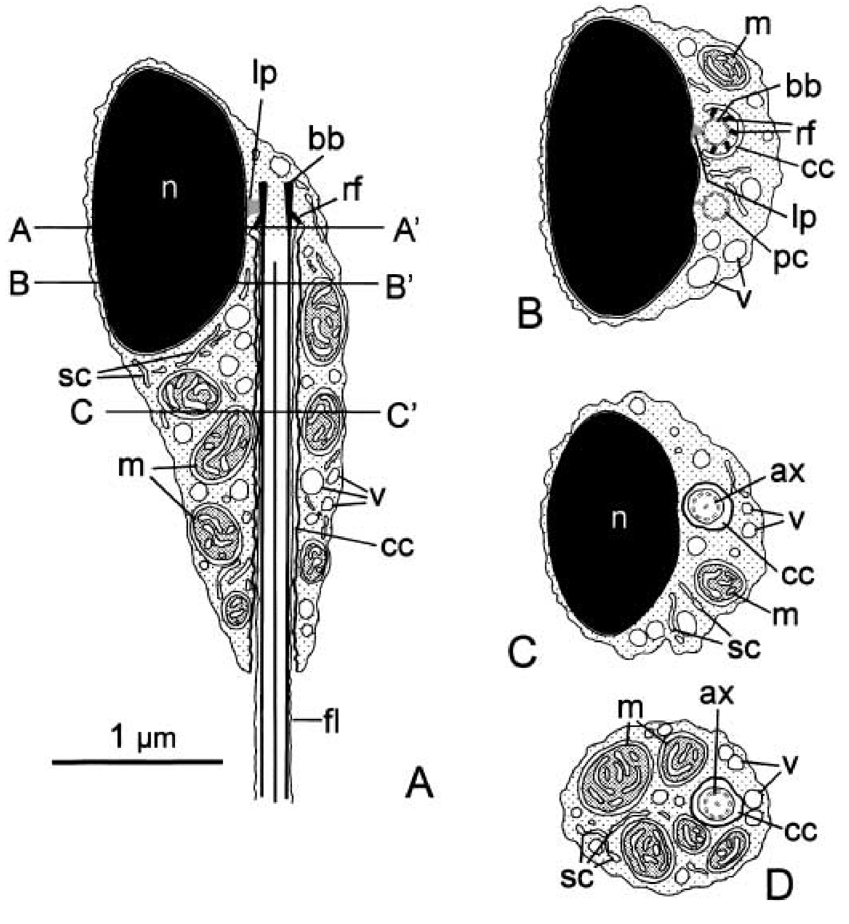

Fig. 2—Schematic drawing of the sperm of *Merluccius merluccius*. —**A**, Sagittal section at the level of the distal centriole (basal body). —**B**–**D**, Consecutive cross-sections through the head [at the level of the centrioles (approximately A–A′) and axoneme (B–B′)], and through the midpiece (C–C′) (all front views). Abbreviations: ax, axoneme; bb, basal body (distal centriole); cc, cytoplasmic canal; fl, flagellum; lp, lateral plate; m, mitochondria; n, nucleus; pc, proximal centriole; rf, radial fibres; sc, smooth membrane cisternae; v, midpiece vesicles.

Fig. 3—TEM micrographs of sections at various levels of *Merluccius merluccius* spermatozoa, including the nuclear and midpiece regions. —**A**, **C**, sagittal sections. —**B**, **D**–**G**, transverse sections. Abbreviations: ax, axoneme; cc, cytoplasmic canal; dc, distal centriole (basal body); lp, lateral plate; m, mitochondria; n, nucleus; rf, radial fibres; sc, smooth membrane cisternae; v, midpiece vesicles; arrows, Y-shaped bridges at the proximal region of the axoneme; asterisk, anterior part of the axoneme devoid of central microtubules; double arrowheads, dense layer underlying the cytoplasmic canal membrane.

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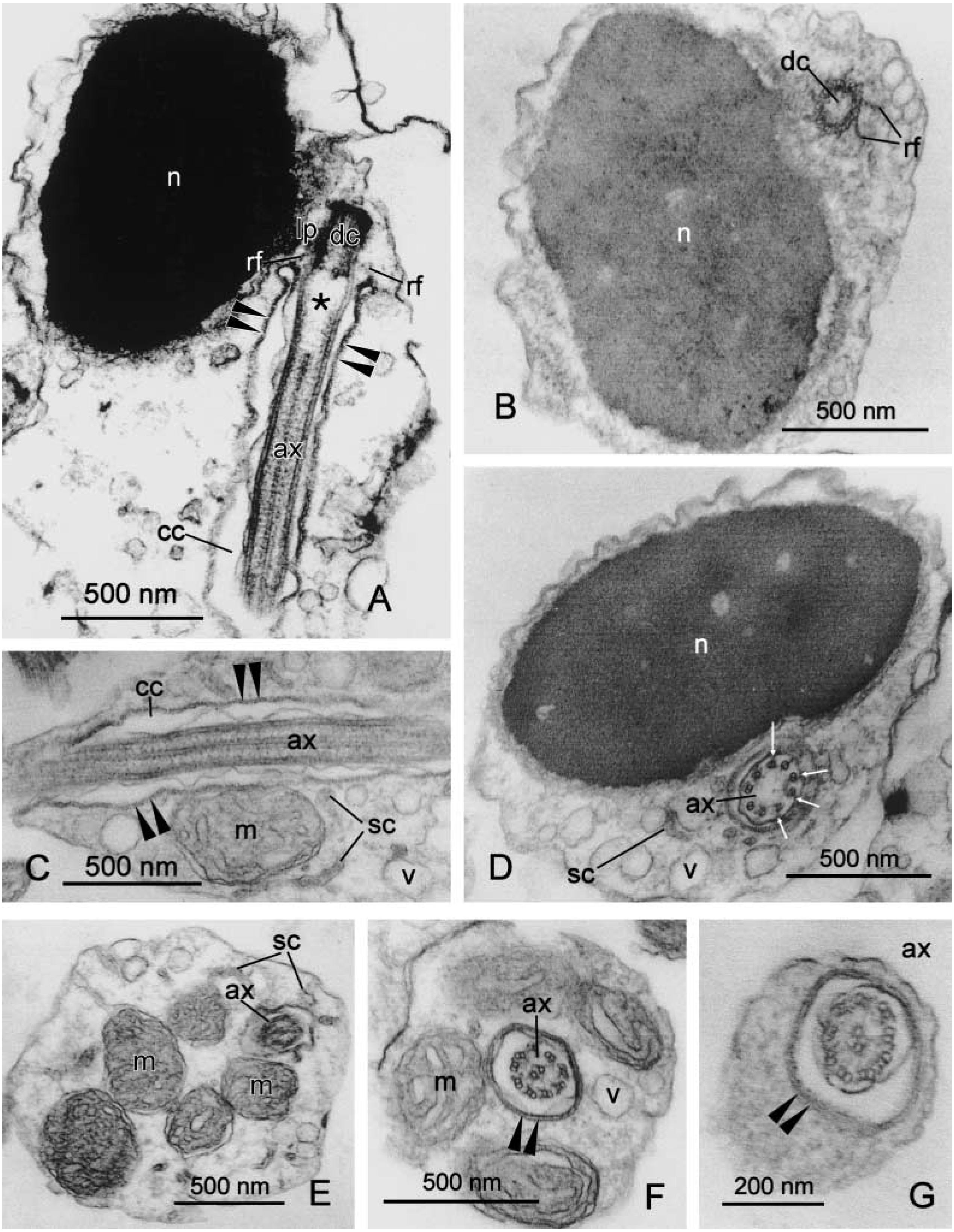
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midpiece and deep cytoplasmic canal, and the absence of lateral fins or ribbons in the flagellum. Although these characteristics suggest a relatively high homogeneity in the sperm ground plan of the Gadiformes – pending confirmation in other families of the group – which is the only known paracanthopterygian order possessing a typical perciform-type sperm, the occurrence of a variety of dissimilarities in a number of characters defines distinct interspecific differences which will be discussed subsequently. Whether such differences bear taxonomic implications at the family level will only be known when information is available for many more species.

A striking feature of the spermatozoon of *M. merluccius* is the mutually parallel arrangement of the proximal and distal centrioles, an apomorphic condition that seems unique among the few investigated gadiforms. Thus, in the burbot, *Lota lota* (Gadidae), the sperm centrioles are arranged at right angles (Lahnsteiner *et al.* 1994). On the other hand, Mattei (1991; Fig. 10) depicts sperm models for the families Merlucciidae and Moridae as having mutually perpendicular centrioles. Another line drawing illustrating the sperm of *Merluccius polli* shows, however, an arrangement of the proximal centriole at about 45° relative to the basal body (Mattei 1991; Fig. 15). Quite rare in fishes, a parallel orientation of the sperm centrioles has been observed in the agnathan *Lampetra*, the osteoglossomorphs *Pantodon* and *Gymnarchus* (Jamieson 1991), and the pomacentrid perciform *Chromis chromis* (Lahnsteiner and Patzner 1997), whereas the most frequent perpendicular arrangement (the one normally found in somatic cells) is considered plesiomorphic in animal sperm cells (Jamieson 1991). Radial fibres (satellite rays) have not been reported for other gadiforms, but are clearly seen radiating from the distal centriole and attaching it to the plasma membrane at the proximal end of the cytoplasmic canal in *M. merluccius*. These structures, probably plesiomorphic, are not of general application in neopterygian phylogeny, because they are seen in many taxa from the most primitive neopterygians – the garpike, *Lepisosteus osseus* (L.) (Afzelius 1978) and the bowfin, *Amia calva* L. (Afzelius and Mims 1995) – to the advanced order Atheriniformes, where satellite rays are particularly well developed (Jamieson 1991). In the spermatozoon of *A. calva* the centrioles remain at a considerable distance from the nucleus and close to the cell surface; consequently, the associated plasma membrane is not drawn towards the nucleus and into the sperm midpiece, and therefore a cytoplasmic canal does not form in this species. Other structures associated with the basal body and axoneme, such as the lateral plate and the Y-shaped bridges, are of widespread occurrence in teleosteans, in both spermatozoa type I and type II (Eiras-Stofella *et al.* 1993; Gwo *et al.* 1993, 1994; Gwo 1995; Lahnsteiner and Patzner 1997; Hara and Okiyama 1998; Abascal *et al.* 2002), hence these elements are of limited use in phylogenetic analyses.

A common source of interspecific variation in the sperm structure in fishes is the number of mitochondria contained in the midpiece (Baccetti *et al.* 1984; Mattei 1991), and this holds true for the gadiforms. While several mitochondria are found in the spermatozoa of *Laemonema laureysi*, *Merluccius polli* (Mattei 1991) and *M. merluccius* (this study), the sperm of *Lota lota* differs from these in that the midpiece bears a single horseshoe-shaped mitochondrion (Lahnsteiner *et al.* 1994). The duration of sperm motility has been related to the size of the midpiece (Billard *et al.* 1995). Obviously, the size of the midpiece depends to some extent on the number of mitochondria stored by the sperm cell, which also determines the depth of the post-nuclear canal (Baccetti *et al.* 1984). In agreement with this observation, the sperm cytoplasmic canal in *Lota lota* is shallower than it is in *M. merluccius*.

The superorder Paracanthopterygii, believed to be the sister group of the Acanthopterygii, is an ill-defined group whose monophyly is dubious (Lauder and Liem 1983; Nelson 1994). Certainly, the high diversity of sperm morphologies found among its different orders (Jamieson 1991; Mattei 1991), with the occurrence of many auto-apomorphies, is of little help in clarifying possible interrelationships. Rather, this wide variety would support the suspicion that the currently accepted classifications of the Paracanthopterygii may include unrelated groups (Lauder and Liem 1983; Nelson 1994). As stated above, among the paracanthopterygians a perciform-type sperm is known only for the order Gadiformes (Mattei 1991; Lahnsteiner *et al.* 1994; present study). Although the number of species investigated to date is very small, a relatively wide distribution of a perciform-type sperm throughout the order appears unequivocal, because this type of spermatozoon has been found in the three families examined (Gadidae, Merlucciidae and Moridae) – the family Zoarcidae is here excluded from the order Gadiforms following Nelson (1994) [The family Zoarcidae, in which the sperm ultrastructure has been described for internally fertilizing species *Zoarces elongatus* (Koya *et al.* 1993) and *Macrozoarces americanus* (Yao *et al.* 1995), has been classified by some authors into the order Gadiformes (Lauder and Liem 1983). These species show a modified sperm adapted to internal fertilization (Jamieson 1991)]. If the perciform-type spermatozoon of the gadiforms is homologous to the derived type II sperm described for perciforms by Mattei (1970), then this spermatozoal pattern should be envisaged as a plesiomorphy for the Paracanthopterygii–Acanthopterygii assemblage (a supposition that seems highly unlikely), and the Gadiformes would be closer to the acanthopterygians than the remaining paracanthopterygians are. If, in contrast, the type I sperm, which is present in a large number of acanthopterygian families including several families of perciforms, is considered the plesiomorphic condition for the Paracanthopterygii–Acanthopterygii assemblage, a perciform-type spermatozoon would have evolved independently in both taxa. Then, the considerable structural similarity between the spermatozoa of the gadiforms and many perciforms (those possessing a

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type II sperm) would be the result of convergent evolution, and both sperm models would be polyphyletic. This hypothesis appears to be more plausible, but further comparative research is needed.

If the monophyly of the Paracanthopterygii is assumed, the ancestral (presumably simple) spermatozoal pattern must have undergone a great radiation which led to an extensive variety of sperm morphologies. These include, apart from the gadiform sperm pattern described herein, simple type I spermatozoa (Antennariidae and Chaunacidae), biflagellate sperm (Batrachoidiformes and Gobiesociformes – this latter order is excluded from the Paracanthopterygii by some authors (Nelson 1994) – and spermatozoa with well developed midpiece and nucleus (*Neoceratias*) (Jamieson 1991; Mattei 1991). Such a rich sperm diversity renders it difficult to elaborate congruent phylogenetic schemes based on spermatozoal features alone. In our view, a thorough comparative investigation of the sperm ultrastructure in the Paracanthyopterygii might be of great help in systematics. Nevertheless, as a result of the paucity of studies on the spermatozoal ultrastructure in this teleostean superorder, there are many gaps that should be filled prior to an effective application of the comparative spermatology to the phylogeny of the group.

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