Sperm ultrastructure in *Bathypolypus bairdii* and *B. sponsalis* (Cephalopoda: Octopoda)

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Abbreviated title: **Bathypolypus sperm ultrastructure**

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ABSTRACT

Ultrastructural comparison of the octopods *Bathypolypus bairdii* and *B. sponsalis* have been carried out by electron microscopy. Although the mature spermatozoon of *B. bairdii* is longer and thinner than that of *B. sponsalis*, its general ultrastructure is similar except for some minor differences. Their characteristic
15 acrosomes, described here for the first time, consist of a periodically banded cone surrounded by a double helix whose arrangement has been defined by a numeric expression. The plasma membrane of *sponsalis* that surrounds the acrosome has many projections filled with abundant granular cytoplasm, which appears as a star-shaped acrosome in cross section. The mitochondrial sheath contains nine to eleven mitochondria in *bairdii* but only nine in *sponsalis*. A comparison with other Octopodidae shows that the
20 nucleus of both species is the largest ever seen. Our results support the capability of sperm morphology to discriminate between species and could even discern at higher taxonomic levels.

KEY WORDS: *Bathypolypus bairdii*; *sponsalis*; spermatozoa; morphology; Octopodidae

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INTRODUCTION

Sperm morphology in general, and ultrastructure in particular, have a clear relationship with the reproductive pattern and fertilization in animals. Furthermore, it can be used as a complementary phylogenetic-taxonomic character (Tuzet, 1950). For these reasons, sperm morphology has attracted considerable interest in many different groups of organisms (Jamieson, 1991; Guidi et al., 2003; Michalik, 2007). Comparative analysis from sperm structure has proved to be useful helping to define the taxonomic position and phylogenetic relationships between many groups of gastropod and molluscs (Franzén, 1955, 1970; Giusti et al., 1990; Healy, 1988, 1990; 1995; Zhu and Yang, 2004).

To date, information about the sperm ultrastructure of octopods is scarce and mainly centered on the family Octopodidae (Galangau and Tuzet, 1968; Longo and Anderson, 1970; Healy 1989, 1993; Selmi, 1996; Jiao et al., 2005). Among the five subfamilies that compose the Octopodidae (Sweeney and Roper, 1998), the sperm ultrastructure of only two (the Octopodinae and Eledoninae) is well known and there is no information about the sperm of the Graneledoninae, Megaleledoninae and Bathypolypodinae.

Bathypolypus bairdii (Verrill, 1873) and *B. sponsalis* (P. Fischer and H. Fischer, 1892) are small, squat, short-armed octopods lacking an ink sac and notable for the relatively large size of their reproductive organs. Both inhabit a wide range of depths: *B. bairdii* 20-1545 m, *B. sponsalis* 170-1835 m (Wirz, 1958; Villanueva, 1992; Quetglas et al., 2001; Muus, 2002). The morphology of these animals is highly variable, and there is some systematic confusion. According to the review by Muus (2002), most data concerning *Bathypolypus arcticus* (Prosch, 1849) from O'Dor and Macalaster (1983) corresponded to *B. bairdii*.

B. bairdii is closely associated with water masses with temperatures ranging from 2 to 8°C. It inhabits the North Sea, from the Norwegian coast to the southern parts of the Barents Sea. It is also common on the southern slopes of the Iceland-Greenland Ridge and present in western Atlantic waters from Labrador to Miami (Muus, 2002). The south-eastern Atlantic limit seems to be the north-western Iberian waters (Pérez-Gándaras and Guerra, 1978). *B. sponsalis* is an East Atlantic species, which replaces *B. bairdii* from the Galician coast to the Cape Verde Islands and it is widespread in the Mediterranean Sea (Guerra, 1992; Muus, 2002). The aim of this work was to describe the sperm ultrastructure of *B. bairdii* and *B. sponsalis*.

MATERIAL AND METHODS

The specimens

A 68 mm mantle length (ML) mature male of *Bathypolypus bairdii* was collected by a bottom trawler in Flemish Cap at 490-520 m depth, and a 60 mm ML mature male of *Bathypolypus sponsalis* was collected by a bottom trawler off Alicante (western Mediterranean) at 400-444 m depth. Spermatophores were removed from the freshly caught specimens and fixed separately from the rest of the animal for both SEM and TEM as per the method outlined below.

The methods

For SEM, spermatophores were fixed 4 h in 2.5% glutaraldehyde in a 0.1 M Na-cacodylate buffer (pH 7.3 at 4°C) and washed for 30 min in the same buffer. The sample was then dehydrated in an ethanol series, critical point-dried in CO₂ using a Polaron E3000 and sputter-coated in a Polaron SC500 using 60% gold-palladium. Samples were then examined with a Philips XC30 SEM operating at 15 kV.

For TEM, sections of spermatophores were fixed in 3.0% glutaraldehyde in a 0.2 M sodium cacodylate buffer, pH 7.2 for 12 h at 4°C, washed in the same buffer for 4 h at 4°C and then post-fixed in buffered 2.0% osmium tetroxide for 4 h at the same temperature. After dehydration in a graded ethanol series, the fragments were embedded in Epon, sectioned by a diamond knife, double-stained with uranyl acetate and lead citrate, and observed in a JEOL 100CXII TEM operated at 80 kV.

Spermatozoa measurements were taken using the image analysis system NIS-Elements D 2.30. The following new indices have been defined in order to compare different sperm morphologies and sizes, avoiding the effect of the overall sperm size: Acrosome Length Index (Acrosome length / Head length) x 100, Nucleus Length Index (Nucleus length / Head length) x 100, Middle Piece Length Index (Middle Piece length / Head length), Neck Length Index (Neck length / Head length) x 100, Annulus Length Index (Annulus length / Middle piece length) x 100, Nucleus Width Index (Nucleus width / Middle piece length) x 100, Middle Piece Width Index (Middle piece width / Middle piece length) x 100, Annulus Width Index (Annulus width / Middle piece length) x 100 and the number of gyres per µm of the acrosome (gyres / acrosome length).

In order to describe the acrosome formula in *bairdii* and *sponsalis*, we measured the distance between gyres in ten and seven acrosomes of the cited species respectively. The distance between gyres was compared with the number of gyres using simple linear regression.

85 RESULTS

Bathypolypus bairdii

Measurements and indices are shown in Table 1 and 2, respectively. The mature spermatozoon of this species is $1130 \pm 97 \mu\text{m}$ long. It has a head (nucleus and acrosome) of $94 \pm 3.86 \mu\text{m}$, and a tail or flagellum of approximately $1036 \mu\text{m}$. The whole spermatozoon surface is surrounded by a plasma
90 membrane.

Table1. Sperm cell measurements (μm) of *Bathypolypus* species Mean, standard deviation, number of measurements.

Measures	<i>B. bairdii</i>	<i>B. sponsalis</i>
Sperm length	1130 ± 97 (n=4) ¹	940.72 ± 80 (n=3) ¹
Head length	94.62 ± 3.86 (n=4)	74.38 ± 1.58 (n=5)
Tail length	1036 ²	866 ²
Acrosome (Acr.) length	8.57 ± 0.14 (n=8)	8.17 ± 0.20 (n=7)
Acr. max width	0.43 ± 0.01 (n=10)	0.62 ± 0.01 (n=7)
Acr. min width	0.14 ± 0.01 (n=8)	0.152 ± 0.01 (n=4)
Acr. gyres separation	0.48 ± 0.02 (n=170)	0.44 ± 0.02 (n=98)
Double helix separation	0.19 ± 0.01 (n=18)	0.17 ± 0.01 (n=22)
Acr. striation separation	58.87 ± 1.59 nm (n=12)	52.77 ± 1.05 nm (n=12)
Acr. membrane–striation space	43.68 ± 3.85 nm (n=22)	107.21 ± 6.95 nm (n=23)
Acr. membrane width	11.35 ± 1.16 nm (n=18)	21.12 ± 3.05 nm (n=20)
Acrosome–nucleus space	17.21 ± 0.4 nm (n=14)	11.50 ± 1.00 nm (n=16)
Nucleus length	86.05	66.21
Nucleus width	0.45 ± 0.01 (n=5)	0.62 ± 0.01 (n=23)
Neck length	0.78	0.77
Neck width	0.51	0.51
Centriolar fossa width	0.34	0.26
Middle piece length	10.78 ± 0.47 (n=3)	9.77 ± 0.4 (n=10)
Middle piece width	0.45 ± 0.01 (n=14)	0.49 ± 0.01 (n=10)
Annulus length	1.05 ± 0.10 (n=13)	1.19 ± 0.01 (n= 3)
Annulus width	0.53 ± 0.02 (n=6)	0.57 ± 0.02 (n=11)
Principal piece diameter	0.36 ± 0.02 nm (n=18)	0.39 ± 0.03 nm (n=14)

¹ Measures obtained from broken sperm

² Sperm length – Nucleus length

The acrosome consists of a long striated cone surrounded by unbanded material that forms a double helical keel arranged in 18 gyres (Fig. 1). The equation that related the separation between gyres (y) with the number of gyres (x) is: $y = 0.5109 - 0.0038x$; $r^2=0.5112$, $p<0.0001$, $n=170$. The separation between gyres slightly decreases towards the anterior part. Nevertheless, separation between pre-gyre and post-gyre (both constituting the double helix) keeps constant. Along the double helix, the pre-gyre is bigger than the post-gyre (Fig. 1a, b, c). The basal part of the acrosome has the same diameter as the nucleus but it gradually tapers towards the anterior part (Fig. 1a, b). In longitudinal sections, the acrosome is composed of an electron opaque substance arranged periodically, that forms striations perpendicular to the long axis of the spermatozoon (Fig. 1c arrow heads). There is a constant space between the striations and the acrosomal membrane (Table 1), which is noticeable in cross sections (Fig. 1d). The plasma membrane is moderately connected with the acrosomal membrane, leaving little space to allocate the cytoplasmic substance or periacrosomal material, which has electron dense patches irregularly disposed (Fig. 1d). The junction between the acrosome and nucleus is flat, and a narrow cytoplasmic lacuna of electron lucent material between both pieces can be observed (Fig. 1c, e).

The rod-shaped nucleus is long and narrow (Table 1). The plasma membrane surrounding the nucleus is smooth and closely linked to the nuclear membrane. Different layers can be discerned in cross section: i) an outer layer or nuclear membrane with electron lucent material, which encircles the nucleus (Figs. 1f; 2a, b); ii) the nucleus or chromatin layer consisting of a very electron dense chromatin, homogeneously packed (Figs. 1e-g; 2a, b); iii) nuclear fossa made up of electron lucent material surrounded by the nucleus (Figs. 1e-g; 2a); and iv) an inner channel slightly more dense than the former that appears on the posterior part of the nucleus (Fig. 1g, arrows). The nuclear fossa extends from the anterior aspect of the centriolar fossa, almost to the tip of the nucleus, where it tapers to a point and ends blindly (Fig. 1e).

Table 2. Sperm cell indices of *Bathypolypus* species.

Species / Indices	<i>B. bairdii</i>	<i>B. sponsalis</i>
Acrosome Length Index (ALI)	9.06	10.98
Nucleus Length Index (NLI)	90.94	89.02
Neck Length Index (NeLI)	0.82	1.03
Middle Piece Length Index (MPLI)	11.39	13.14

Annulus Length Index (ALI)	9.74	12.18
Nucleus Width Index (NWI)	4.17	6.34
Middle Piece Width Index (MPWI)	4.17	5.02
Annulus Width Index (AWI)	4.92	5.83
Ratio (number gyres / μm acrossome)	2.10	2.33

The neck (Figs. 1g; 2a, d) consist of the nuclear invagination and the centriolar fossa. The centriolar fossa is connected to the nuclear fossa via a small lumen (Table 1) and contains a centriole embedded on electron lucent material (Fig. 2a). The centriole forms the axoneme that is composed of nine peripheral microtubular doublets and a central doublet, 9+2 (Fig. 2b, arrow head). The anterior part of the axoneme is surrounded by ring-shaped electron-dense material, which becomes ordered distally (Fig. 2b) into nine fibers that run parallel to the nine doublets, the coarse fibers (*sensu* Healy, 1988 and Selmi, 1996) (Fig. 2c). The coarse fibers and the axoneme constitute the axoneme-coarse fibers complex (ACF). It has a 9+9+2 structure in cross section (Figs. 2b, c; 3a, b, d, e) extending almost to the end of the tail. The coarse fibers' diameter gradually diminishes towards the tail.

The middle piece of the tail (Figs. 2c, d; 3a-c, Table 1) consists of the axoneme-coarse fibers complex, a mitochondrial sheath, and a fibrous sleeve that is bounded by the cell membrane (Figs. 2c; 3a, b, e). The mitochondrial sheath is composed of nine to eleven rounded, well-defined mitochondria. The mitochondrial matrix is electron opaque and the internal membranes are slightly discernible (Fig. 2c, arrow head). Mitochondria are elongated and run parallel to the ACF axis (Fig. 3a-c). The plasma membrane and the fibrous sleeve are folded over it selves in the most distal part of the middle piece, forming a cylindrical annulus (Fig. 3a-d). The annulus is constricted anteriorly (Fig. 3b, arrow).

The mitochondrial sheath is absent at the principal piece (Fig. 3b, c, e). A cross section (Fig. 3e) shows the 9+9+2 structure surrounded by the fibrous sleeve and the plasma membrane. The ACF is surrounded by electron lucent granular material. The principal piece's diameter at the most proximal part is 0.36 μm , but it diminishes gradually towards the end of the tail.

The end piece does not have a fibrous sheath surrounding the ACF. The coarse fibers at this level are smaller in diameter than the principal piece (Fig.3f)

Measurements and indices are shown in Table 1 and 2, respectively. The total length of the spermatozoon is $940 \pm 80 \mu\text{m}$, with a head of $74.58 \mu\text{m}$, and a tail of approximately $866 \mu\text{m}$. The acrosome is a long striated cone surrounded by a very thin banded material that forms the double helical keel arranged in 19 gyres (Fig. 4a, b). The equation that related the separation between gyres (y) with the number of gyres (x) is: $y = 0.4497 - 0.0018x$; $r^2=0.09$, $p<0.0021$, $n=98$. The distance between the striations perpendicularly arranged to the sperm axis is 52.77 nm (Fig. 4b). These are distanced 107.21 nm from the acrosomal membrane. There are many short thin striations periodically separated by 23.17 nm in the acrosomal membrane (Fig. 4b, arrow heads). In contrast with *B. bairdii*, there are deep furrows between pre and post-gyres, giving a more pronounced helix (Fig. 4b). The plasma membrane that surrounds the acrosome has many projections filled with abundant granular cytoplasm or periacrosomal material, which create a star-shaped acrosome in cross section (Fig. 4b, c). The posterior surface of the acrosome adjacent to the nuclear tip is flattened and lined with an electron opaque material (Table 1, Fig. 4b, arrow), instead of the electron lucent cytoplasmic lacunae present in *B. bairdii*. The acrosome and nucleus have the same diameter at the junction.

The long rod-shaped nucleus (Fig. 4d) is shorter and wider than *B. bairdii* (Table 1). Only three layers can be discerned under the plasma membrane in cross section: the nuclear membrane, the nucleus or chromatin layer and the nuclear fossa (Fig. 4f), although in one image we could also appreciate the inner channel (as in *bairdii*) at the nucleus anterior part (data not shown). The nuclear membrane, as well as the chromatin layer, covers the whole nucleus length (Figs. 4d-f; 5a-d). The nuclear fossa begins at the apical part of the lumen (Fig. 5a) and its anterior end is near the nucleus' anterior part (Fig. 4d, arrow).

Neck length and width of *B. sponsalis* is the same as in *B. bairdii*. However, the diameter of the centriolar fossa is smaller in *B. bairdii* (Table 1). The arrangement of the centriolar fossa changes posteriorly (Fig. 5b-d). Figure 5b shows its anterior part, near the beginning of the lumen, where only the central doublet (arrow heads) and a part of the undefined coarse fibers can be seen. A cross section through the beginning of the ACF, shows that the axoneme is surrounded by the undefined coarse fibers which constitute an electron-dense ring-shaped structure (Fig. 5c). This structure becomes ordered distally into the nine coarse fibers that run parallel to the nine doublets (Fig. 5d). The structure of the axoneme-coarse fibers complex is $9+9+2$.

A cross section of the middle piece shows that the mitochondrial sheath is formed by 9 bean-shaped mitochondria, closely disposed but not fused (Fig. 6a). Their internal membranes can be easily discerned (Fig. 6a, arrow heads). A longitudinal section shows elongated individual mitochondria running parallel to the ACF (Figs. 5a, e; 6b). The annulus is constricted anteriorly (Figs. 5e; 6b, c).

Principal and end pieces have the same characteristics as in *B. bairdii*. There is no fibrous sheath and the coarse fibers are smaller at the end piece (Fig. 6e). The transition between both pieces can be clearly observed (Fig. 6f).

TEM photography of *B. sponsalis* spermatozoa, show large amounts of electron-dense spheroids along the whole spermatophore matrix (asterisks in Figs. 4b, d; 6f), which also exist in *B. bairdii* but smaller and fewer in number (asterisks in Fig. 2a, c).

DISCUSSION

The mature spermatozoon of *B. sponsalis* resembles that of *B. bairdii* with the exception of a few differences. The separation between gyres in *B. bairdii* decreases slightly faster (see equation slopes) than in *B. sponsalis*. It is important to signal that variations in the acrosomal measurements are mainly due to different orientations in TEM images. As shown, the sperm structure is highly uniform in male octopus and the study of only one specimen catches intraspecific variation (Table 1). One of the main differences between the acrosome of *B. bairdii* and *B. sponsalis* is that the former species has less amounts of periacrosomal material than the later one. Another difference is that the cavities inside the double helix of *B. sponsalis* are deeper than in *B. bairdii* (Fig.4b).

Although the acrosome of *B. bairdii* is slightly longer than that of *B. sponsalis*, the later has one additional gyre, which means that the number of gyres per acrosome length is greater in *B. sponsalis* (Table 2). The gap between striations perpendicularly disposed to the sperm axis was larger in *B. bairdii* than in *B. sponsalis*, and the separation between the striations and acrosomal membrane was found to be more than two times larger in the former than in the later species (Table 1). A possible explanation about the striations observed at the acrosomal membrane of *B. sponsalis* would be that they were the microtubular printings caused by the contraction and posterior removal of the microtubules, whose main role would be developing the acrosome spiral. This observation agrees with those of Galangau and Tuzet

(1968), Ribes et al. (2002), Giménez-Bonafé et al. (2002), and Martínez-Soler et al. (2007) on nuclear and acrosome morphogenesis.

205 Spermatozoa of both Bathypolypodinae species here studied do not display torsion of the entire acrosome as occurs in several species of the subfamily Eledoninae (Maxwell, 1974; Selmi, 1996; Giménez-Bonafé et al., 2002). The acrosome of *Bathypolypus* is, however, a striated cone surrounded by a helical keel as in the Octopodinae (Galangau and Tuzet, 1968; Longo and Anderson, 1970; Healy, 1989; Jiao et al., 2005), but with the peculiarity of having a double helix instead of the single one present in
210 Octopodinae. The screw-shaped acrosome was considered a general characteristic of Octopodidae and it is also present in the cirrate octopod *Opisthoteuthis persephone* (Healy, 1993).

 The equidistant striations perpendicularly arranged to the long axis of the acrosome were previously observed in several species of *Octopus* (Galangau and Tuzet, 1968; Martin et al., 1970; Longo and Anderson, 1970; Jiao et al., 2005) and *Eledone* (Maxwell, 1974; Selmi, 1996). The presence of this
215 structure in two new species of Octopodidae, but members of a different subfamily, seems to indicate that, from a phylogenetic perspective, this periodic structure could be considered a peculiarity of octopod spermatozoa (Selmi, 1996). These striations were also observed by Zhu and Jang (2004) in the acrosome of the bivalve *Barbatia virescens*. However, we noticed the complete absence of striations in their illustrations.

220 The acrosome-nucleus junction of *B. sponsalis* is flattened and lined with an electron opaque material like in *Octopus bimaculatus* (Longo and Anderson, 1970). However, in *B. bairdii* an electron lucent lacuna is located in the junction as occurs in *O. vulgaris* (Galangau and Tuzet, 1968) and *O. tankahkeei* (Jiao et al., 2005). The nucleus of *sponsalis* is slightly shorter and wider than the nucleus of *bairdii*, and both are the longest ever reported in Octopodidae (Galangau and Tuzet, 1968; Longo and
225 Anderson, 1970; Martin et al., 1970; Maxwell, 1974; Selmi, 1996; Jiao et al., 2005).

 The nuclear fossa of both *Bathypolypus* species is the same structure previously observed by Galangau (1969), Longo and Anderson (1970) and Healy (1989), which was called “corps central intranucleaire”, “extra-nuclear rod” and “fibrous plug”, respectively. The nuclear fossa’s origin is almost certainly related with the centriole and its function could be related with the structural maintenance of the

nucleus, working as an axis and allowing the process of chromatin condensation all around it (Ribes et al., 2001).

Another remarkable feature is the presence of an inner cylinder inside the nuclear fossa of *B. bairdii*. This structure could be a prolongation of the centriole because it seems to be raised from the centriolar fossa and only gets up to 8-10 μm from it, but more accurate observations are needed to verify this. This feature is also present in *B. sponsalis*, but we could hardly see this feature throughout the preparations.

The electron-dense spheroids across the matrix of the spermatophores in both species, but especially in those from *B. sponsalis*, were very similar to those found in *Octopus bimaculatus* (Longo and Anderson, 1970) and *O. tankahkei* (Zhu et al., 2005). Although the nature and function of these spheroids is still not well known, it has been suggested that they could be advanced stages of lytic vesicles, product of spermatozoon maturation process during the spermatophore storage in the Needham's sac (Roura et al. submitted).

The characteristics of the tail were similar in both species, except for the number of mitochondria present at the middle piece, which was slightly greater in *B. bairdii* (11) than in *B. sponsalis* (9), although their size was smaller in *B. bairdii*.

Longo and Anderson (1970) indicated that the plasma membrane at the site of the annulus was reflected over itself, forming an intracellular space filled with electron opaque material. Based on figure 6c, we suggest that the electron opaque material is the fibrous sheath reflected, since this structure surrounds both the middle and principal piece and is tightly connected with the plasma membrane along its length.

Summing up, the sperm morphology in *Bathypolypus* is in relation to the biology of fertilization. Its internal fertilization (O'Dor and Macalaster, 1983) has given rise to the complex sperm morphology, which clearly differs from the primitive type of spermatozoon among cephalopoda (Franzén, 1955, 1970). Comparative morphology between both *Bathypolypus* species shows that the following features are valid to discern between these species: the acrosomal arrangement, the distance between acrosomal striations, the nuclear fossa's extension, the nuclear length, the nuclear width, the middle piece length and the number of mitochondria. Furthermore, some of these characters can also be used for cephalopod

phylogeny at higher levels, i.e. subfamilies: the acrosomal arrangement, the nuclear fossa's extension, the nuclear length and the number of mitochondria (Octopodinae: Galangau and Tuzet, 1968; Longo and Anderson, 1970; Healy, 1989; Jiao et al., 2005, Eledoninae: Maxwell, 1974; Selmi, 1996; Giménez-Bonafé et al., 2002, Graneledoninae: Roura et al., submitted); suborders: the nuclear length, the acrosomal striations, the acrosome arrangement, the middle piece (Cirrata: Healy, 1993); orders: very close to the primitive type (*sensu* Franzén, 1955, 1970) short head, acrosome reduced, middle piece almost absent (Vampyromorpha: Healy, 1990) and even superorders where decapodiform sperm (Franzén, 1955; Maxwell, 1975; Fields and Thompson, 1976) are closer to the primitive type than those of octopodiform. More studies are needed to check the phylogenetic validity of these characters.

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Fig. 1. *Bathypolypus bairdii* (a) Scanning electron micrograph of the acrosome showing the double helix arrangement (arrows). Bar 1000 nm. (b) Drawing of the acrosome showing the same gyres as in Fig.1a (arrows). Bar 1000 nm. (c) Transmission electron micrograph of an acrosome and nucleus (N) in longitudinal section showing periodic striations perpendicular to the long axis of the acrosome (arrowheads). Pre-gyre (g) and the enhanced post-gyre (G). Bar 500 nm. (d) Transmission electron micrograph of an acrosome in cross section, showing the acrosomal membrane (AM), plasma membrane (PM) and the periacrosomal material (PA). Bar 100 nm. (e) Transmission electron micrograph of the anterior part of the nucleus (N) and the beginning of the acrosome (A), showing the end of the nuclear fossa (arrows). Bar 1000 nm. (f) Transmission electron micrograph of the nucleus in cross section, showing three layers, the nuclear membrane (NM), the chromatin (Cr) and the nuclear fossa (NF). PM, plasma membrane. Bar 200 nm. (g) Transmission electron micrograph at different levels of the posterior part of the nucleus in cross and oblique sections, showing the inner channel (arrowheads). Cr, chromatin; Ne, neck; NF, nuclear fossa. Bar 1000 nm.

Fig. 2. *Bathypolypus bairdii* (a) Transmission electron micrograph of the neck (Ne) and middle piece (MP), showing the nuclear fossa (NF) which connects with the centriolar fossa (CF) via a small lumen (L). The chromatin (Cr) is projected on the neck forming the nuclear invagination (NI). The middle piece is marked by the beginning of the mitochondrial sheath (MS) and the fibrous sheath (FS). Asterisk show electron dense spheroid. Ax, axoneme; C, centriole; CFI coarse fibers; NM, nuclear membrane; PM, plasma membrane. Bar 300 nm. (b) Transmission electron micrograph of a cross section through the line represented in Fig. 2a, showing the centriolar fossa (CF), the coarse fibers (CFi) partially defined, the central doublet (arrowheads) and the axoneme-coarse fibers complex (ACF). Cr, chromatin; NM, nuclear membrane; PM, plasma membrane. Bar 100 nm. (c) Transmission electron micrograph of the middle piece in cross section showing the mitochondria (M), the fibrous sheath (FS), the inner mitochondrial membrane (arrowhead) and the electron dense spheroid (asterisk). CFI, coarse fibers; PM, plasma membrane. Bar 200 nm. (d) Scanning electron micrograph of the neck (Ne), middle piece (MP) and annulus (An). Note the enlarged mitochondria (arrowheads). Bar 2000 nm.

Fig. 3. *Bathypolypus bairdii* (a) Transmission electron micrograph of the middle piece (MP) and neck (Ne) in longitudinal section, showing the fibrous sheath (FS), the mitochondrial sheath (MS), the axoneme-coarse fibers complex (ACF) and the annulus (An). Bar 1000 nm. (b) Transmission electron micrograph of the annulus (An) in longitudinal section. The mitochondrial sheath (MS) finishes at a constriction (arrow) in the anterior part of the annulus. ACF, axoneme-coarse fibers complex; PP, principal piece. Bar 500 nm. (c) Scanning electron micrograph of the posterior part of the middle piece (MP) and beginning of the principal piece (PP). An, annulus; MS mitochondrial sheath. Bar 1000 nm. (d) Transmission electron micrograph of the annulus (An) in cross section. ACF, axoneme-coarse fibers complex; FS, fibrous sheath; PM, plasma membrane. Bar 200 nm. (e) Transmission electron micrograph of the cross section of the principal piece, showing the fibrous sheath (FS), the axoneme-coarse fibers complex (ACF) and the central doublet (arrowhead). PM, plasma membrane. Bar 100 nm. (f) Transmission electron micrograph of the end piece in cross section. ACF, axoneme-coarse fibers complex; PM, plasma membrane. Bar 100 nm.

Fig. 4. *Bathypolypus sponsalis* (a) Scanning electron micrograph of the acrosome and nucleus (N) forepart. PA, periacrosomal material. Bar 2000 nm. (b) Transmission electron micrograph of the acrosome-nucleus junction showing the electron-dense material (arrow) which fills the space between nucleus (N) and acrosome, and small striations along the acrosome membrane (arrowheads). Asterisk show electron dense spheroid. AM, acrosomal membrane; G, post-gyre; g, pre-gyre; NM, nuclear membrane; PA, periacrosomal material; PM, plasma membrane. Bar 500 nm. (c) Transmission electron micrograph of the acrosome in cross section showing the plasma membrane (PM) projections filled with periacrosomal material (PA). AM, Acrosomal membrane. Bar 100 nm. (d) Transmission electron micrograph of the nucleus in longitudinal section, showing the end of the nuclear fossa (arrows) near the acrosome (A), and electron dense spheroids (asterisks). Cr, chromatin layer. Bar 1000 nm. (e) Transmission electron micrograph of the nucleus at the anterior part. Cr, chromatin; NM, nuclear membrane; PM, plasma membrane. Bar 200 nm. (f) Transmission electron micrograph of the nucleus at the middle part, showing the nuclear fossa (NF). Cr, chromatin; NM, nuclear membrane; PM, plasma membrane. Bar 200 nm.

Fig. 5. *Bathypolypus sponsalis* (a) Transmission electron micrograph longitudinal section through the posterior part of the nucleus, showing the connection between the nuclear fossa (NF) and the centriolar fossa (CF) via a small lumen (L). ACF, axoneme-coarse fibers complex; Cr, chromatin; NM, nuclear membrane; MS, mitochondrial sheath; PM, plasma membrane. Bar 1000 nm. Cross lines represent cross sections at the beginning of the centriolar fossa (b), at the beginning of the ACF (c), and almost at the end of the neck (d). (b) Transmission electron micrograph at the beginning of the centriolar fossa, where the central microtubules are present (arrowheads) and the coarse fibers (CFi) begin to develop. Cr, chromatin; NM, nuclear membrane; PM, plasma membrane. Bar 200 nm. (c) Transmission electron micrograph at the beginning of the axoneme-coarse fibers complex (ACF) showing the undefined coarse fibers (CFi). CF, centriolar fossa; Cr, chromatin; NM, nuclear membrane; PM, plasma membrane. Bar 200 nm. (d) Transmission electron micrograph at the end of the centriolar fossa (CF), showing the nine coarse fibers (CFi) well defined. Cr, chromatin; NM, nuclear membrane; PM: plasma membrane. Bar 200 nm. (e) Transmission electron micrograph of the Neck (Ne), middle piece (MP), annulus (An) and principal piece (PP). Bar 2000 nm.

Fig. 6. *Bathypolypus sponsalis* (a) Transmission electron micrograph of the middle piece in cross section, showing the mitochondrial sheath (MS), the axoneme-coarse fibers complex (ACF), the fibrous sheath (FS) and the mitochondrial inner membranes (arrowheads). Bar 100 nm. (b) Transmission electron micrograph of a longitudinal section through the posterior part of the middle piece (MP), the annulus (An) and the principal piece (PP). ACF, axoneme-coarse fibers complex; FS, fibrous sheath; MS, mitochondrial sheath. Bar 200 nm. (c) Transmission electron micrograph of a cross section through the annulus (An). Inset: annulus wall composed by the plasma membrane (PM) and the fibrous sheath (FS). The axoneme-coarse fibers complex (ACF) is also surrounded by the PM and the FS. Bar 200 nm. (d) Transmission electron micrograph of a cross section at the principal piece showing the fibrous sheath (FS) and the plasma membrane (PM) surrounding the axoneme-coarse fibers complex (ACF). Bar 100 nm. (e) Transmission electron micrograph of the end piece in cross section showing the plasma membrane (PM) surrounding the axoneme-coarse fibers complex (ACF). Bar 100 nm. (f) Transmission electron micrograph of the transition zone (arrow) between the principal piece (PP) and the end piece (EP), where the fibrous sheath (FS) disappears. PM, plasma membrane. Asterisk show the electron-dense spheroid. Bar 500 nm.