Sperm cell ultrastructure of the haplosplanchnid *Haplosplanchnus caudatus* (Platyhelminthes: Digenea) – an intestinal parasite of *Mugil cephalus*, and its potential phylogenetic application

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Abstract

The ultrastructural characters of mature spermatozoon of the digenean *Haplosplanchnus caudatus* were described by means of transmission electron microscopy (TEM). Live parasites were collected from the digestive tract of flathead grey mullet *Mugil cephalus* (Teleostei: Mugilidae) caught off the Gulf of Gabes in La Chebba (Tunisia). The present study provides the first ultrastructural data on a digenean belonging to the superfamily Haplosplanchnoidea. The spermatozoon of *H. caudatus* is a filiform cell which exhibits: (i) two axonemes with the 9+'1'pattern of trepaxonematan Platyhelminthes having about the same length, (ii) an external ornamentation of the plasma membrane associated with cortical microtubules and located in the anterior part of the sperm cell, (iii) spine-like bodies, (iv) two mitochondria containing large mitochondrial matrix granules, (v) a nucleus, and (vi) granules of glycogen irregularly distributed. Moreover, the spermatozoon of *H. caudatus* displays the Quilichini et al.'s cryptogonimid type of posterior extremity. The mitochondrial matrix granules are described for the first time in a digenean sperm cell.

Keywords: *Haplosplanchnus caudatus*; Haplosplanchnidae; Digenea; ultrastructure; sperm characters

1. Introduction

The Haplosplanchnidae is considered as one of the smaller families of digenetic trematodes and the sole family in the suborder Haplosplanchnata (Olson et al., 2003), including species parasitizing a wide range of marine, estuarine, and freshwater fishes (Huston et al., 2017; Madhavi, 2005; Nahhas et al., 1997). Haplosplanchnidae includes four

subfamilies, namely, the Haplosplanchninae, the Haplosplanchnoidinae, the Hymenocottinae, and the Schikhobalotrematinae. *Haplosplanchnus caudatus* is a member of the subfamily Haplosplanchninae (Madhavi, 2005).

Basing on a molecular study, Olson et al. (2003) considered the systematic position of Haplosplanchnidae to be problematic and far from being satisfactorily resolved due to the instability of its placement, depending on data set and analytic approach. In addition, the study performed by Olson et al. (2003) revealed that the Haplosplanchnoidea is not grouped with any other taxon but diverges immediately before the Echinostomata and is well separated from the Haploporidae. However, it was not considered as a distinct group at higher levels before. La Rue (1957) placed the Haplosplanchnidae in the superfamily Echinostomatoidea, but Brooks et al. (1985) included it with the Haploporidae and Megaperidae in their new order Haploporiformes. Due to many inconsistencies and the absence of a robustness in the existing classification of Haplosplanchnidae, ultrastructural studies of species belonging to this family are of great importance to bring additional information, complementing hence molecular analyses and morphological data for a better understanding of the relationships within this family of digeneans.

Euzet et al. (1981) indicated for the first time that all detailed data on comparative characters in ultrastructure of cestode spermatozoa represent very useful criteria for precise analysis of the phylogenetic interrelationships among cestodes and other parasitic Platyhelminthes. The results of other recent and related studies as well as those of our work have strongly proved this statement, and stressed that information about spermatozoon ultrastructure is of crucial importance for better understanding of the phylogeny of parasitic flatworms (Bakhoum et al., 2017; Justine, 1991a, 1991b, 1995, 1998, 2001; Levron et al., 2010; Quilichini et al., 2010a, 2011).

In this paper, we present for the first time observations of the ultrastructural organization of the mature *Haplosplanchnus caudatus* spermatozoon, contributing hence in the filling of gaps in the knowledge of sperm ultrastructure in the superfamily Haplosplanchnoidea. These data can also be useful as a source of significant characters to elucidate phylogenetic relationships within parasitic Platyhelminthes.

2. Materials and methods

Adult specimens of *Haplosplanchnus caudatus* were gathered live from the digestive tract of flathead grey mullet *Mugil cephalus* Linnaeus, 1758 (Teleostei, Mugilidae) captured in the Mediterranean Sea, off La Chebba (34°14′N, 11°06'E) (Tunisia) in November 2015 and December 2016.

Immediately after their extraction, adult worms were rinsed with a NaCl solution (9‰) and fixed for a minimum of 2 h in cold (4 °C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4, and subsequently rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4. They were then postfixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in MilliQ water (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in Spurr resin, and polymerized at 60 °C for 72 h. Ultrathin (60-90 nm thick) sections were obtained using a Reichert-Jung Ultracut-E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate following Reynolds (1963). All stained grids were studied with a JEOL 1010 transmission electron microscope operated at 80 kV, in the 'Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)'.

The Thiéry (1967) technique was used for cytochemical detection glycogen at ultrastructural level. Gold grids were treated in periodic acid, thiocarbohydrazide and silver

proteinate (PA-TCH-SP) as follows: 30 min in 10% PA, rinsed in MilliQ water, 24 h in TCH, rinsed in acetic solutions and MilliQ water, 30 min in 1% SP in the dark, and rinsed in MilliQ water.

3. Results

Observation of numerous ultrathin sections from the seminal vesicle of *Haplosplanchnus caudatus* enables us to distinguish three different regions (I–III) from the anterior to the posterior extremities of the male gamete (Figs. 1-3). The mature spermatozoon of *H. caudatus* exhibits two axonemes of the 9+'1' trepaxonematan pattern, external ornamentation of the plasma membrane, spine-like bodies, nucleus, two mitochondria, two bundles of parallel cortical microtubules, and glycogen granules.

Region I (Figs. 1a-e and 3I) corresponds to the anterior region of the spermatozoon. Cross-sections through the anterior tip show axonemal microtubules accompanied by the anterior extremity of the first mitochondrion and external ornamentation of the plasma associated with cortical microtubules (Fig. 1a, b). At a slightly distal level, the centrioles of both axonemes appear and the first mitochondrion increases its size (Fig. 1c). The mitochondrion contains dense granules (white arrows) deposited in the matrix with granule diameters ranging from about 20 to 50 nm. Moreover, the spermatozoon has an extramembranous ornamentation associated with cortical microtubules on its ventral side (mitochondrial) (Fig. 1a-e). In addition, the posterior part of this region exhibits spine-like bodies (Figs. 1e and 3I).

Region II (Figs. 1f-h and 3II) represents the middle region of the mature spermatozoon. It is a transitional area before the nuclear region which is mainly characterized by the disappearance of the external ornamentation of the plasma membrane and the spine-like

bodies. The anterior area of region II is mainly characterized by the presence of the two axonemes, a single bundle of cortical microtubules, and the distal part of the first mitochondrion (Figs. 1f and 3II). The middle part of this region is characterized by the simultaneous presence of both axonemes and the distribution of cortical microtubules into two fields (Figs. 1g and 3II). In the distal part of region II the second mitochondrion appears (Fig. 1h). It contains granules deposited in the matrix as reported in the first mitochondrion (Figs. 1h and 3II). Besides, a large amount of granular material has also been observed in this posterior part.

Region III (Figs. 1i-p, 2a,b and 3II) corresponds to the nuclear and posterior spermatozoon region. Initially, the nucleus is located between the two axonemes (Fig. 1i) and it progressively displaces to the dorsal side of the sperm cell (Fig. 1j-l). In the anterior part of this region both nucleus and second mitochondrion are simultaneously presented with the maximum number of cortical microtubules (about 19) (Fig. 1i). Towards the posterior part of region III, the transition of characters is as follows: (i) disorganization of the first axoneme (Fig. 1k-o), (ii) disappearance of the second mitochondrion (Fig. 2l), (iii) disorganization of the second axoneme (Fig. 1m-o), and (iv) disappearance of the nucleus (Fig. 1p). The posterior tip of the spermatozoon is characterized by the presence of both axonemal doublets and singlets (Figs. 1p and 4III).

The glycogenic nature of the electron-dense granules observed along the sperm cell was evidenced by applying Thiéry's test (Fig. 2b).

4. Discussion

The ultrastructural study of the spermatozoon of *H. caudatus* reveals a general character pattern found in the majority of digeneans. Thus, it presents two axonemes with the 9+'1'

trepaxonematan pattern (Ehlers, 1984), parallel cortical microtubules arranged in two bundles, external ornamentation of the plasma membrane, spine-like bodies, nucleus, mitochondria, and glycogen granules. However, there are several specific features mainly located in the anterior extremity of the spermatozoon. In fact, the great morphological variety observed in the anterior part of digenean spermatozoa might be a suitable criterion for taxonomy and/or phylogenetic considerations.

The anterior extremity of the male gamete of *H. caudatus* is characterized by the simultaneous presence of two 9+'1' axonemes, parallel cortical microtubules, external ornamentation of the plasma membrane, and the first mitochondrion. In general, the mature spermatozoa of digeneans have two axonemes of unequal length being more or less longitudinally displaced one against the other (Bakhoum et al., 2017). In contrast, the two axonemes in *H. caudatus* have about the same length. The centrioles of both axonemes appear simultaneously in the anterior spermatozoon extremity, and the two axonemes disorganize at the same level in the nuclear posterior extremity. To our knowledge a similar organization has been found only in the lecithasterid *Aponurus laguncula* (Quilichini et al., 2010b). The posterior sperm extremity in *H. caudatus* shows the disorganization of both axonemes. Quilichini et al. (2010a) analysed the great variability in posterior extremities of digenean sperm cells and established three types of posterior parts of the spermatozoon (opecoelidean type, fasciolidean type and cryptogonimidean type). These authors considered the transition of principal characters (cortical microtubules, nucleus and second axoneme) towards the posterior tip of the sperm cell. Thus, the posterior spermatozoon extremity of *H. caudatus* corresponds to the cryptogonimidean type of Quilichini et al. (2010a) characterized by the following sequence: disappearance of cortical microtubules, then disappearance of the nucleus, and finally, the disorganization and disappearance of the axoneme. However, in *H. caudatus*, the posterior character includes doublets of both axonemes.

The cortical microtubules are described in the sperm cells of most digeneans studied so far (Bakhoum et al., 2017). Three aspects of these tubular structures underlying the plasma membrane are considered as interesting ultrastructural criteria used for phylogenetic inference: the presence or absence of cortical microtubules, the location of their maximum number, and the number of bundles. In *H. caudatus*, the cortical microtubules are arranged into two fields and present with the maximum number in the anterior extremity of nucleus.

In Digenea the number of cortical microtubules varies according to the species from zero in *Didymozoon* sp. (Justine and Mattei, 1983) to 73 in *Diplodiscus subclavatus* (Bakhoum et al., 2011). The location of the maximum number of these tubular structures is also variable depending on the species. Quilichini et al. (2007) proposed that the spermatozoon of digenean parasites could be divided into two groups based on the location of the maximum number of cortical microtubules along the spermatozoon: the first group with the maximum number of these elements in the anterior part and the second one in a middle or a more posterior part of the spermatozoon. The spermatozoon of *H. caudatus* belongs to the second type, also existing in other digeneans, particularly the Hemiuroidea, the Lepocreadioidea, and the Opecoeloidea (see Bakhoum et al., 2017). The cortical microtubules are arranged into two fields in most digeneans as occurs in *H. caudatus*. Yet, species belonging to the Hemiuroidea superfamily do not follow this pattern. Thus, lecithasterids, hemiurids, and sclerodistomids present a single field of cortical microtubules placed on the ventral side of the gamete (Ndiaye et al., 2014, 2017; Quilichini et al., 2010b) while sperm cells of some didymozoids (*Didymocystis* and *Didymozoon* species) are devoid of cortical microtubules (Justine and Mattei, 1983; Pamplona-Basilio et al., 2001).

In *H. caudatus,* as in numerous digeneans, the cortical microtubules are associated with the external ornamentation (see Bakhoum et al., 2017) while in other digeneans ornamentation is not associated with cortical microtubules (Dione et al., 2016; Ndiaye et al.,

2017; Quilichini et al., 2007, 2010b). This pattern with or without cortical microtubule association as well as the location of the external ornamentation along the spermatozoon are considered valuable criteria used for phylogenetic analysis within the Digenea. They are also of particular interest for the establishment of spermatozoa models (Bakhoum et al., 2017; Quilichini et al., 2011). In this context, Quilichini et al. (2011) suggested that digenean spermatozoa could be divided into three types based on the presence/absence and location of the external ornamentation: (i) type 1 presents an external ornamentation in the anterior extremity of the spermatozoon; (ii) type 2 presents an external ornamentation at a more posterior level; and (iii) type 3 lacks external ornamentation. *H. caudatus* follows the Quilichini et al.'s type 1 spermatozoon. This type is particularly present in species belonging to the Bucephaloidea, Echinostomatoidea, Microscaphidioidea, Paramphistomoidea, Pronocephaloidea, and Hemiuroidea with some exceptions (see Bakhoum et al., 2017).

The digenean spermatozoa have mitochondria and the variability of this character has recently been analysed. Traditionally, authors considered the presence of a single mitochondrion in the mature spermatozoa as a result of the fusion of several mitochondria during spermiogenesis (see Burton, 1972). However, posteriorly different authors interpreted the ultrastructural organization of sperm cells with more than one mitochondrion (see Bakhoum et al., 2017 for a review). *H. caudatus* has two mitochondria in their spermatozoa and it is interesting to note the presence of numerous large granules of different sizes in the matrix of both mitochondria. To our knowledge, this is the first time that such a granular material has been observed in mitochondria of a platyhelminth sperm cell. The mitochondrial matrix granules were described from numerous mammalian cells and their number and their sizes vary greatly (see Fawcett, 1969). According to this author, they are involved in the transport of ions and, thus, in the regulation of internal ionic mitochondrial environment. In this sense, e.g., Kawahara et al. (2009) described the accumulation of calcium in the

osteoclasts mitochondrial granules of rat, and suggested it to play a role in the bone resorption. This accumulation prevents the increase in calcium concentrations in osteoclasts cytoplasm. The mitochondrial matrix granules have recently been identified as RNA granules similar to the cytoplasmic RNA granules. The mitochondrial RNA granules contain proteins involved in RNA metabolism. It was demonstrated that they represent centres for posttranscriptional RNA processing and the biogenesis of mitochondrial ribosomes (see Antonicka et al., 2013; Antonicka and Shoubridge, 2015; Jourdain et al., 2013). Unfortunately, at this stage we can only report very briefly the presence of these unique mitochondrial granules in the spermatozoa of *H. caudatus* and illustrate their ultrastructure. Therefore, an attempt for elucidation of their function will obviously require a separate molecular study.

Competing interests

The authors declare that they have no conflict of interest.

Acknowledgements

The authors wish to thank the staff of the "Centres Científics i Tecnològics" of the University of Barcelona (CCTiUB) for their assistance in the preparation of samples. JM is a member of the 2017-SGR-1008 research group.

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Legends

Fig. 1. Mature spermatozoon of *Haplosplanchnus caudatus*. (a) Cross-section of the anterior spermatozoon extremity showing the ornamented area of the sperm cell and the presence of the first mitochondrion. (b-d) Consecutive cross-sections of region I showing the progressive appearance of both axonemes. (e) Cross-section of the posterior part of region I with spine-like bodies. (f-h) Cross-sections of region II: anterior part with posterior extremity of the first mitochondrion, middle part and posterior part with the anterior extremity of the second axoneme. (i-k) Anterior part of nuclear region showing the simultaneous presence of second mitochondrion and nucleus. (l-o) Consecutive cross-sections of the region III showing the disorganization of axonemes. (p) Posterior sperm tip containing only some axonemal singlets and doublets. White arrows indicate the mitochondrial matrix granules. ASE, anterior spermatozoon extremity; Ax1, first axoneme; Ax2, second axoneme; C1, centriole of the first axoneme; C2, centriole of the second axoneme; CC2, central core of the second axoneme; CM, cortical microtubules; D1, doublets of the first axoneme; D2, doublets of the second axoneme; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M1, first mitochondrion; M2, second mitochondrion; N, nucleus; S, singlets; SB, spine-like bodies. Scale bars: 300 nm.

Fig. 2. Mature spermatozoon of *Haplosplanchnus caudatus*. (a) Cross-sections of region III showing the mitochondrial matrix granules in the second mitochondrion. (b) Cytochemical test of Thiéry evidencing glycogen at ultrastructural level. G, granules of glycogen; MMG, mitochondrial matrix granules; N, nucleus. Scale bars: 200 nm.

Fig. 3. Schematic reconstruction of the mature spermatozoon of *Haplosplanchnus caudatus*. The sperm cell is organized in three different regions: region I or anterior part, region II or middle part and region III or posterior part. In order to make the diagram clearer, granules of

glycogen are not shown in longitudinal sections. ASE, anterior spermatozoon extremity; Ax1 and Ax2, first and second axoneme; C1 and C2, centriole of the first and the second axoneme; CM, cortical microtubules; D1, doublets of the first axoneme; D2, doublets of the second axoneme; EO, external ornamentation of the plasma membrane; G, granules of glycogen; MMG, mitochondrial matrix granules; M1 and M2, first and second mitochondrion; N, nucleus,; PM, plasma membrane; PSE, posterior spermatozoon extremity; S, singlets; SB, spine-like bodies.

Figure 1

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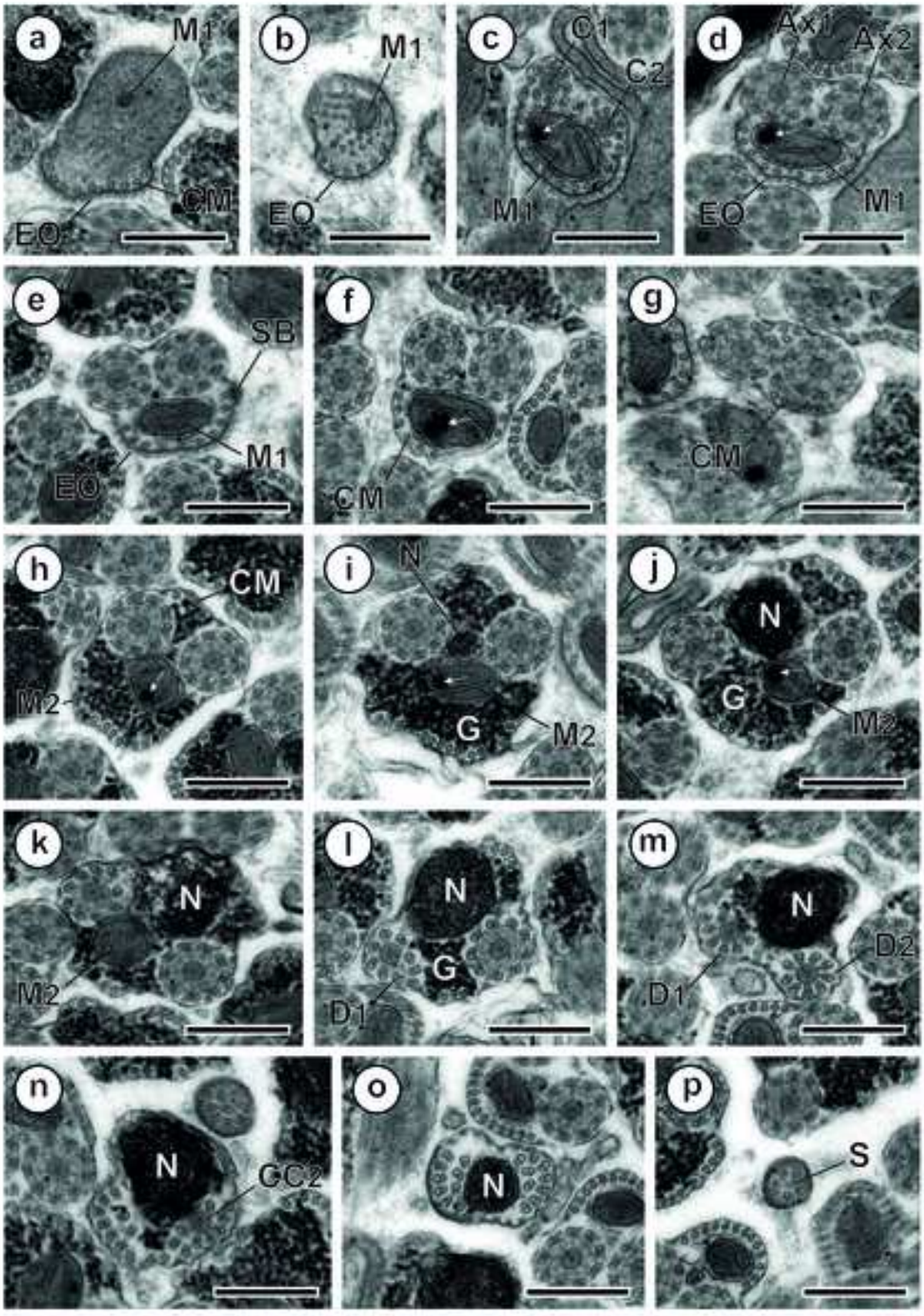

Figure 2

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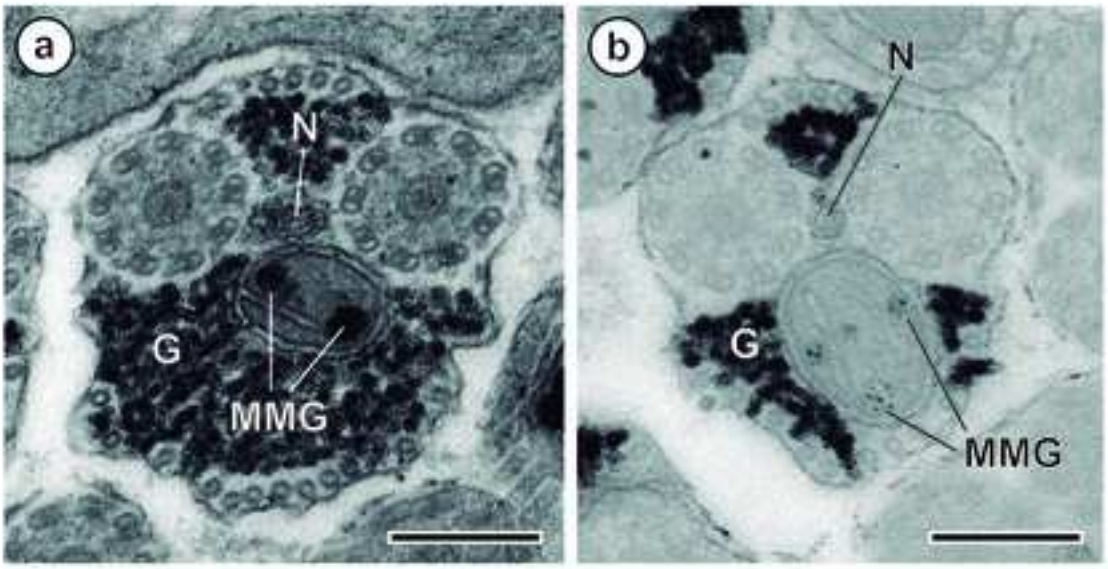

Figure 3