Ultrastructural characters of the spermatozoon of the digenean *Hypocreadium caputvadum* Kacem *et al.*, 2011 (Lepocreadioidea: Lepocreadiidae), an intestinal parasite of *Balistes capriscus* in Tunisia

Caractères ultrastructurelles du spermatozoïde du digène Hypocreadium caputvadum *Kacem* et al.*, 2011 (Lepocreadioidea : Lepocreadiidae), parasite intestinal de* Balistes capriscus *en Tunisie*

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Abstract

The ultrastructural organization of the spermatozoon of the digenean *Hypocreadium caputvadum* (Lepocreadioidea: Lepocreadiidae) is described. Live digeneans were collected from *Balistes capriscus* (Teleostei: Balistidae) from the Gulf of Gabès, Tunisia (Eastern Mediterranean Sea). The mature spermatozoon of *H. caputvadum* shows several ultrastructural characters such as two axonemes of different lengths exhibiting the classical 9+'1' trepaxonematan pattern, a nucleus, two mitochondria, granules of glycogen, external ornamentation of the plasma membrane and two bundles of parallel cortical microtubules. Moreover, in the anterior extremity, the second axoneme is partly surrounded by a discontinuous and submembranous layer of electron-dense material.

Our study provides new data on the spermatozoon of *H. caputvadum* in order to improve the understanding of phylogenetic relationships in the Digenea, particularly in the superfamily Lepocreadioidea. In this context, the electron-dense material surrounding one of the axonemes in the anterior spermatozoon extremity constitutes the unique distinguishing ultrastructural character of lepocreadioideans, and it is present in spermatozoa of lepocreadiids, aephnidiogenids and gyliauchenids.

Keywords: *Hypocreadium caputvadum*; Digenea; Lepocreadioidea; Lepocreadiidae; spermatozoon; ultrastructure

Résumé

Cette étude décrit l'organisation ultrastructurale du spermatozoïde chez le digène *Hypocreadium caputvadum* (Lepocreadioidea : Lepocreadiidae). Les spécimens vivants sont prélevés de *Balistes capriscus* (Teleostei : Balistidae) provenant du Golf de Gabès, Tunisie (Est Mer Méditerranée). Le spermatozoïde mûr de *H. caputvatum* présente plusieurs caractères ultrastructuraux tels que les deux axonèmes de longueur différentes et du type 9+'1' des Trepaxonemata, un noyau, deux mitochondries, les granules de glycogène, les ornementations externes de la membrane plasmique et les deux champs de microtubules corticaux parallèles. De plus, au niveau de l'extrémité antérieure du spermatozoïde, le second axonème est entouré par un matériel dense aux électrons discontinu et sous-membranaire.

Nous apportons ainsi dans cette étude, de nouvelles données concernant le spermatozoïde de *H. caputvadum* en vue de la compréhension des relations phylogénétiques chez les digènes en général et au niveau de la superfamille Lepocreadioidea en particulier. Le matériel opaque aux électrons entourant un des deux axonèmes dans l'extrémité antérieure du spermatozoïde constitue le seul caractère ultrastructural distinctif chez les Lepocreadioidea et est présent sur le spermatozoïde des lepocreadiidés, aephnidiogenidés et gyliauchenidés.

Mots clés : *Hypocreadium caputvadum* ; Digenea ; Lepocreadioidea ; Lepocreadiidae ; spermatozoon ; ultrastructure

1. Introduction

During the last half-century, ultrastructural descriptions of spermatozoa were used as valuable tools for phylogenetic inference in the flatworms (Platyhelminthes), particularly in the tapeworms (Eucestoda) [1-10], but also in the Monogenea [4,11]. Considering digeneans, the increase of this kind of studies has motivated the analysis of spermiological data, particularly those related to the mature spermatozoon, in order to establish different types of spermatozoa according to their ultrastructural organization. In the future, the establishment of different types of spermatozoa may be useful for a better knowledge of digenean relationships [12-16]. However, few ultrastructural investigations have been carried out on the superfamily Lepocreadioidea Odhner 1905. According to Bray [17] the Lepocreadioidea include ten families, namely the Lepocreadiidae, Acanthocolpidae, Apocreadiidae, Brachycladiidae, Deropristidae, Enenteridae, Gorgocephalidae, Gyliauchenidae, Liliatrematidae and Megaperidae. Nevertheless, based on molecular analyses, Bray *et al.* [18] and more recently Bray and Cribb [19] re-organized the superfamily Lepocreadioidea. Bray *et al.* [18] found the Lepocreadioidea to be monophyletic and constituted by six well-supported groups, which are presently considered families according to Bray and Cribb [19]. These families are the Lepocreadiidae, the Aephnidiogenidae and the Lepidapedidae, which were previously considered by Bray [20] as three subfamilies of the Lepocreadiidae *s.l.*, and the Enenteridae, Gorgocephalidae and Gyliauchenidae. Moreover, according to Bray and Cribb [19] the families Acanthocolpidae, Apocreadiidae and Brachycladiidae are not closely related to the Lepocreadiidae and should be placed out of the Lepocreadioidea. With respect to the three remaining Lepocreadioidea families considered by Bray [17], namely the Deropristidae, Liliatrematidae and Megaperidae, molecular studies are still lacking. Among all these families, there are ultrastructural studies of the spermatozoon in the Aephnidiogenidae, Apocreadiidae, Deropristidae and Gyliauchenidae [14,21-24]. The present paper presents the first assessment of the ultrastructural organization of the spermatozoon of a species belonging to the family Lepocreadiidae: *Hypocreadium caputvadum*. Our results are compared with those of other digeneans, particularly lepocreadioideans.

2. Materials and methods

Live specimens of *H. caputvadum* Kacem *et al.*, 2011 [25] were collected from the intestine of grey triggerfish *Balistes capriscus* Gmelin, 1789 (Balistidae), caught in the Gulf of Gabès off Chebba (Tunisia) (34°14′N, 11°06'E). After dissection, live digeneans were routinely processed for TEM examination. Therefore, they were fixed in cold (4°C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4, postfixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4, dehydrated in an ethanol series and propylene oxide, and finally embedded in Spurr resin. Seminal vesicle was located in semi-thin sections. Later ultrathin sections were obtained using a Reichert-Jung Ultracut E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate according to Reynolds methodology [26]. Ultrathin sections were examined using a JEOL 1010 transmission electron microscope operated at an accelerating voltage of 80 kv at the CCiTUB ("Serveis Científics i Tecnològics" of the University of Barcelona, Spain).

The Thiéry technique [27] was used to locate glycogen. 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, 24h in TCH, rinsed in acetic solutions and milliQ water, 30 min in 1 % SP in the dark, and rinsed in milliQ water.

3. Results

The observation of numerous cross- and longitudinal sections allows us to distinguish three different regions from the anterior to the posterior extremities of the mature spermatozoon of *H. caputvadum*, each exhibiting distinctive ultrastructural characters (Figs 1-3). The mature spermatozoon is a long filiform cell tapered at both ends and exhibiting the usual structures found in the great majority of digeneans. Thus, it contains two axonemes, external ornamentation of the plasma membrane, nucleus, mitochondria, two parallel bundles of cortical microtubules and granules of glycogen.

Region I (Figs 1a-l,n, 3I) corresponds to the anterior extremity of the spermatozoon. The anterior part of this region is filiform, devoid of axonemes and moderately electron-dense (Figs 1a,b, 3I). The axonemes of the 9+‘1’ trepaxonematan pattern are slightly longitudinally shifted (Figs 1c, 3I). The second axoneme is partly surrounded by a discontinuous and submembranous layer of electron-dense material (Figs 1c,d, 3I). Posteriorly, cortical microtubules appear surrounding both axonemes as a continuous layer (Figs 1e, 3I). Cortical microtubules progressively become organized into two fields (Figs 1f,g, 3I). In the middle part of this region, an external ornamentation of the plasma membrane is observed in association with cortical microtubules (Figs 1h-j, 3I). In this area, the first mitochondrion appears (Figs 1i,j, 3I). In the distal area of region I, we notice the absence of external ornamentation, the reduction in the size of the mitochondrion and the appearance of granules of glycogen (Figs 1k,l, 3I). The posterior extremity of the first mitochondrion marks the transition towards the region II (Figs 1l, 3I). The glycogenic nature of this granular material was evidenced by the test of Thiéry (Fig. 1n).

Region II (Figs 1m, 2a-d, 3II) corresponds to the middle region of the spermatozoon, which is mainly characterized by the simultaneous presence of the second mitochondrion and the anterior part of the nucleus. Anterior areas of this region show two axonemes, cortical microtubules and granules of glycogen (Figs 1m, 3II). At a slightly lower level we notice the appearance of the nucleus (Figs 2a, 3II). The distal area of region II exhibits the simultaneous presence of both nucleus and the second mitochondrion (Figs 2b,c, 3II). Finally, region II ends at the posterior extremity of the second mitochondrion.

Region III (Figs 2d-i, 3III) corresponds to the posterior region of the spermatozoon, which is characterized by the presence of the posterior part of the nucleus. Consecutive cross-sections show: (i) the presence of two axonemes, nucleus, cortical microtubules and granules of glycogen (Fig. 2d), (ii) the disorganization of the first axoneme (Figs 2e,f, 3III), (iii) the disorganization of the second axoneme and disappearance of cortical microtubules (Figs 2g,h, 3III), and (iv) the nucleus distal extremity followed by the complete disappearance of doublets from the second axoneme (Figs 2h,i, 3III). The posterior spermatozoon tip exhibits doublets from the last axoneme and few granules of glycogen (Figs. 2i, 3III).

4. Discussion

The mature spermatozoon of *Hypocreadium caputvadum* shows the usual ultrastructural elements observed in most digeneans so far: two axonemes, nucleus, mitochondria, granules of glycogen, external ornamentation of the plasma membrane and two bundles of parallel cortical microtubules.

It possesses two axonemes with different lengths exhibiting the classical 9+'1' trepaxonematan pattern [28]. This is the typical structure of axonemes observed in all digeneans except for species of the genus *Schistosoma* with a special 9+'1' pattern [29] and species of *Didymozoon* with a 9+0 pattern [30,31].

Concerning the anterior spermatozoon extremity, *H. caputvadum* exhibits both axonemes; however, to our knowledge in the remaining studied species of the superfamily Lepocreadioidea, there is only one axoneme in their anterior tip as in the Gyliauchenidae *Gyliauchen* sp. and *Robphildollfusium fractum* [14,24] and the Aephnidiogenidae *Holorchis micracanthum* [23]. In the problematic families Apocreadiidae and Deropristidae there is also a single axoneme in the anterior spermatozoon extremity [21,22]. In this anterior spermatozoon extremity there is also a particular feature that consists in a discontinuous electron-dense material partially surrounding the second axoneme beneath the plasma membrane. Within the lepocreadiodeans, this electron-dense material has been described in the three analysed families, namely the Aephnidiogenidae, Gyliauchenidae and Lepocreadiidae [14,23,24,present study].

The spermatozoon of *H. caputvadum* displays external ornamentation of the plasma membrane as occurs in the remaining lepocreadioideans studied to date [14,23,24] and also in the apocreadiids and deropristids [21,22]. The role of these elements remains unknown. Nevertheless Justine and Mattei [32] hypothesized that external ornamentation participates in the fusion of the spermatozoon and ovum membranes during fertilization. In the digenean spermatozoon, the external ornamentation is present in anterior areas of the sperm cell and can present different locations. According to Quilichini *et al.* [14] there are three types of anterior spermatozoon regions depending on this character: (i) type 1 presents external ornamentation in the anterior extremity of the spermatozoon, (ii) type 2 presents external ornamentation at a more posterior level, usually in the mitochondrial region, (iii) and type 3 lacks external ornamentation. According to this classification, *H. caputvadum* is included in the second type.

The number of mitochondria in the spermatozoon of digeneans is a matter of controversy [12]. Traditionally, it was accepted that during spermiogenesis several mitochondria fuse to form a unique and long mitochondrion present in the mature spermatozoon [33,34]. Nevertheless, in order to make a logical interpretation of their observations, several authors have described more than one mitochondrion. Thus, there are descriptions of digenean spermatozoa containing one, two or three mitochondria. In the spermatozoa of the Lepocreadioidea both number and form of the mitochondrion is variable. Two mitochondria have been observed in the spermatozoon of the lepocreadiid *H. caputvadum*; the first one is located at the level of the external ornamentation of the plasma membrane and the second one is located in the area containing the nucleus. In the aephnidiogenid *H. micracanthum* Bâ *et al.* [23] described a moniliform mitochondrion that appears in the form of successive bulges, connected to each other by a mitochondrial cord, and it extends almost throughout the whole length of the spermatozoon. In the Gyliauchenidae there are two studied species namely *Gyliauchen* sp. and *R. fractum*, that exhibit one and two mitochondria respectively [14,24]. Concerning the apocreadiids and deropristids, the mature spermatozoa of both *Neoapocreadium chabaudi* and *Deropristis inflata* present two mitochondria [21,22].

The posterior tip of digenean spermatozoa is morphologically variable. Quilichini *et al.* [13] distinguished three types of posterior parts of the spermatozoon (opecoelidean type, fasciolidean type and cryptogonimidean type). These types are characterized by the sequence of characters towards the posterior spermatozoon tip. According to these authors, there is a possibility of a fourth group characterized by a different sequence: posterior extremity of the first axoneme, posterior extremity of cortical microtubules and posterior extremity of the second axoneme. This group would be represented by the Deropristidae *D. inflata* [21], the Brachylaimidae *Scaphiostomum palaearcticum* [35] and the Lecithasteridae *Aponurus laguncula* [36]. In our study the posterior spermatozoon extremity of *H. caputvadum* exhibits only a few doublets resulting from the disorganization of the second axoneme, and glycogen granules. So, *H. caputvadum* belongs to the cryptogonimidean type of Quilichini *et al.* [13]. On the other hand, taking into account several incongruences in the described posterior sperm types, several authors discussed the consideration of only the terminal character [37] instead of the sequence of characters towards the posterior spermatozoon tip. With respect to the remaining Lepocreadioidea, all the studied species exhibit the second axoneme as terminal character except *R. fractum* (Gyliauchenidae), which presents a nucleus extremity in the posterior spermatozoon tip [24].

Considering the recent reorganisation of the superfamily Lepocreadioidea [19] we summarize in Table I the most significant ultrastructural characters of the spermatozoon found in digeneans belonging to this group. We include in Table I the spermatological characters of the family Apocreadiidae (not related to lepocreadiids [19]) and also of the family Deropristidae, which lacks a molecular study confirming its status in the Lepocreadioidea. The unique distinguishing ultrastructural character present in the sperm cell is the electron-dense material surrounding one of the axonemes in the anterior spermatozoon extremity. This character is present in lepocreadiids, aephnidiogenids and gyliauchenids and it is absent in both apocreadiids and deropristids. In spite of the scarce ultrastructural studies on the superfamily, the presence of this character in the spermatozoa of these three families and its absence in apocreadiids demonstrates the utility of the sperm ultrastructure as a tool for phylogenetic inference in the Lepocreadioidea.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Figure captions

Figure 1. Prenuclear areas of the spermatozoon of *Hypocreadium caputvadum*. **(a)** Longitudinal section of region I showing the anterior spermatozoon extremity. **(b-m)** Consecutive cross-sections showing (i) the anterior spermatozoon tip (figure b), (ii) the formation of the second axoneme surrounded by an electron-dense material (figures c and d), (iii) areas exhibiting the maximum number of cortical microtubules (figures e and g), (iv) areas containing the external ornamentation of the plasma membrane (figures h-j) and the first mitochondrion (figures i-l), and (v) transitional areas before the appearance of the nucleus (figure m). **(n)** Evidence of glycogen by a positive test of Thiéry. Scale bars: 0.3 μm. Arrowhead: attachment zones; ASE: anterior spermatozoon extremity; C: centriole; CM: cortical microtubules; DM: electron-dense material; EO: external ornamentation of the plasma membrane; G: granules of glycogen; M1: first mitochondrion.

Figure 2. Nuclear area of the spermatozoon of *Hypocreadium caputvadum*. **(a-i)** Consecutive cross-sections of the nuclear area of the sperm cell showing (i) the appearance of the nucleus (figure a), (ii) the simultaneous presence of the nucleus and the second mitochondrion (figures b and c), (iii) the progressive disorganization of the first axoneme (figures d-f), (iv) the disorganization of the second axoneme and the disappearance of cortical microtubules (figures g and h), and (v) the posterior sperm tip containing only doublets and granules of glycogen (figure i). Scale bars: 0.3 μm. Ax: axoneme; CC: central core; CM: cortical microtubules; D: doublets; G: granules of glycogen; M2: second mitochondrion; N: nucleus.

Figure 3. Schematic reconstruction of the mature spermatozoon of *Hypocreadium caputvadum*. To simplify the diagram, glycogen granules are not shown in the longitudinal section. ASE: anterior spermatozoon extremity; Ax: axoneme; Ax1: first axoneme; Ax2: second axoneme; AZ: attachment zones; C: centriole; CC: central core; CM; cortical microtubules; D: doublets; DM: electron-dense material; EO: external ornamentation of the plasma membrane; G: granules of glycogen; M1: first mitochondrion; M2: second

mitochondrion; N: nucleus; PM: plasma membrane; PSE: posterior spermatozoon extremity.

Table 1. Ultrastructural characters of the spermatozoon in the Lepocreadioidea* and in the families Apocreadiidae and Deropristidae.

Families and species	Spermatozoon characters					References
	ASE	EO	SB	M	PSE	
Families included in the Lepocreadioidea*						
Aephnidiogenidae						
Holorchis micracanthum	Ax-DM	+	-	1	Ax	[23]
Gyliauchenidae						
Gyliauchen sp.	Ax-DM	+	+	1	Ax	[14]
Robphildollfusium fractum	Ax-DM	+	+	2	N	[24]
Lepocreadiidae						
Hypocreadium caputvadum	Ax-DM	+	-	2	Ax	[Present study]
Families not included in the Lepocreadioidea*						
Apocreadiidae						
Neoapocreadium chabaudi	Ax-EO-CM	+	+	2	N	[22]
Deropristidae						
Deropristis inflata	Ax	+	-	2	Ax	[21]

ASE – anterior spermatozoon extremity, Ax – axoneme, CM – cortical microtubules, DM – electron-dense material, EO – external ornamentation of the plasma membrane, M, – mitochondrion, N – nucleus, PSE – posterior spermatozoon extremity, SB – spinelike bodies, +/- – presence/absence of considered character.

*Lepocreadioidea according to Bray and Cribb [19]. Only the families studied in the molecular analysis of these authors are considered.

Figure 1

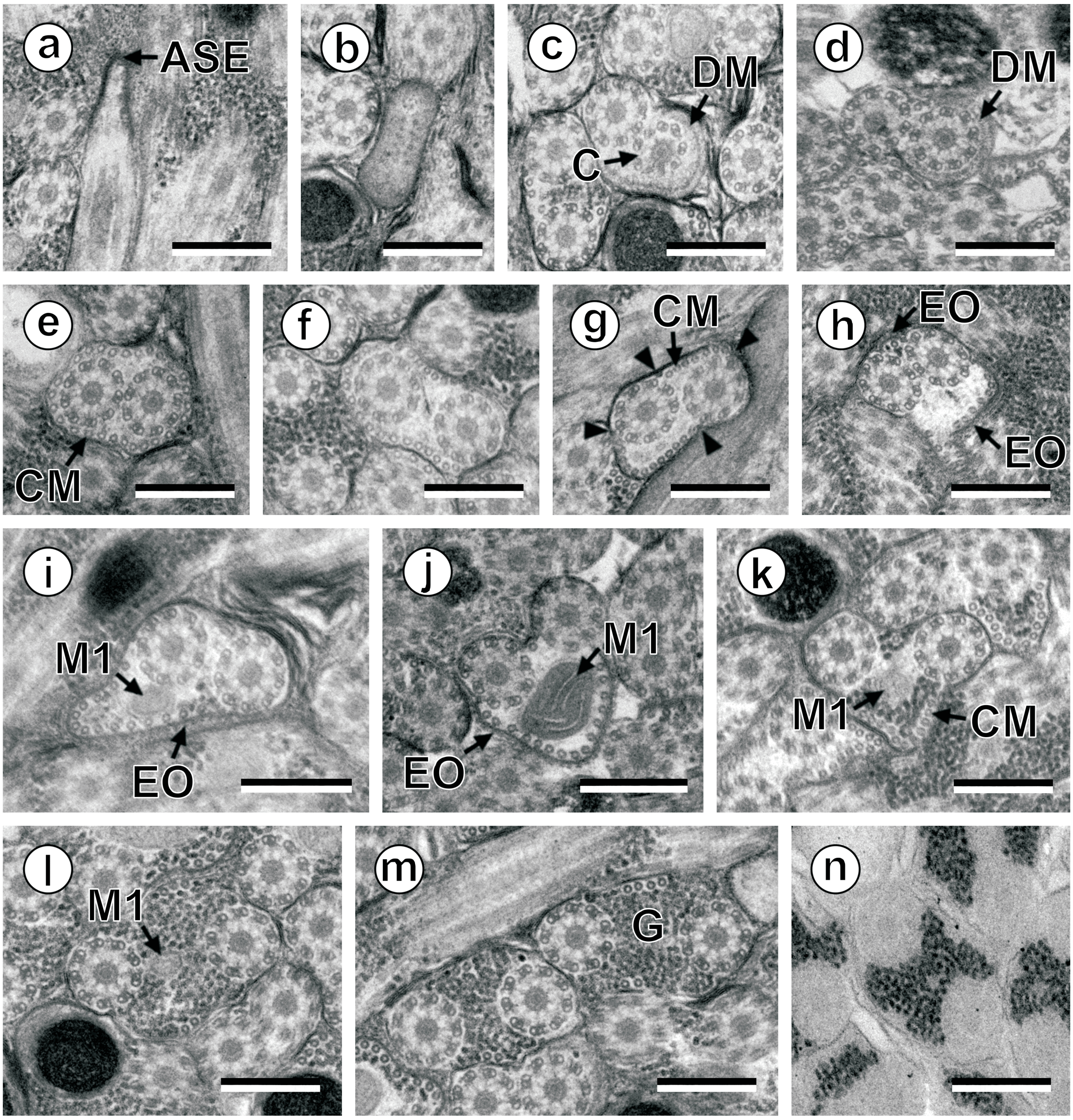

Figure 2

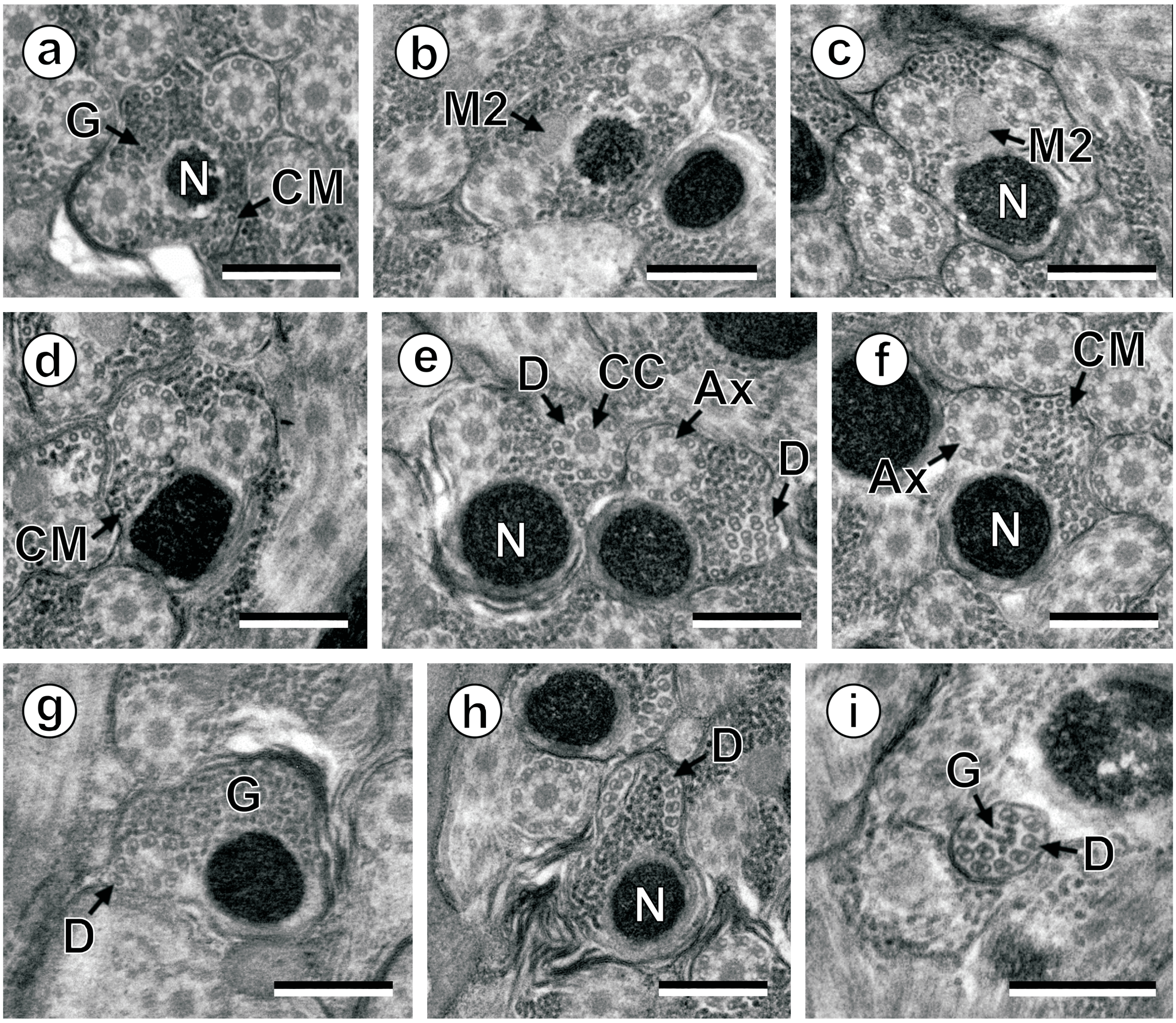

Figure 3