



**Spermiogenesis and spermatozoon ultrastructure of the diphylloidean cestode *Echinobothrium euterpes* (Neifar, Tyler and Euzet 2001) Tyler 2006, a parasite of the common guitarfish *Rhinobatos rhinobatos***

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Prof. Mehlhorn  
Editor, Parasitology Research

Dear Dr. Mehlhorn,

We hereby submit the manuscript “Spermiogenesis and spermatozoon ultrastructure of the diphyllidean cestode *Echinobothrium euterpes* (Neifar, Tyler and Euzet 2001) Tyler 2006, a parasite of the common guitarfish *Rhinobatos rhinobatos*” for publication in Parasitology Research.

Yours sincerely,

Jordi Miquel

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7 **Spermiogenesis and spermatozoon ultrastructure of the diphyllidean cestode**  
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9 **common guitarfish Rhinobatos rhinobatos**  
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## Abstract

Spermiogenesis and the ultrastructural characters of the spermatozoon of Echinobothrium euterpes are described by means of transmission electron microscopy, including cytochemical analysis for glycogen. Materials were obtained from a common guitarfish Rhinobatos rhinobatos caught in the Gulf of Gabès (Tunisia). Spermiogenesis in E. euterpes is characterized by the orthogonal development of two unequal flagella followed by the flagellar rotation and the proximodistal fusion of these flagella with the median cytoplasmic process. The most interesting pattern characterizing the diphyllidean cestodes is the presence of a triangular body constituted by fines and dense granules without visible striation and assimilated at the striated rootlets. This pattern, only related in the Diphyllidea cestodes may be a synapomorphy of this order. Spermiogenesis is also characterized by the presence of a very short flagellum (around 1  $\mu\text{m}$  long), observed in all the stages of spermiogenesis. This type of flagellum has never been commented in the diphyllidean cestodes and should be considered as an evolved character in this group. In the latest stage of spermiogenesis, this short axoneme probably degenerates. Thus, the mature spermatozoon of E. euterpes possesses only one axoneme of 9+“1” trepaxonematan pattern. It also exhibits a single helical electron-dense crested body, a spiralled nucleus, few parallel cortical microtubules, and  $\alpha$ -glycogen granules. Similitudes and differences between spermatozoa of diphyllideans are discussed.

**Keywords:** Echinobothrium euterpes, Diphyllidea, Cestoda, spermiogenesis, spermatozoon, ultrastructure

## Introduction

The phylogeny of the order of Diphyllidea has been controversial for a long time. Since recognition and validation of this group by various authors, there has been a divergence of opinion regarding the affinities of this order (Ivanov 1999; Tyler 2006). The justification for maintaining the Diphyllidea in an order range was strengthened (reinforced) by Caira et al. (1999, 2001) and Ivanov et al. (1999), who demonstrated the monophyly of the order. In the most recent keys to the cestodes (Khalil et al. 1994), the order Diphyllidea was recognized as comprising three families: Echinobothriidae, Ditrachybothriidiidae and Macrobothriidiidae. This taxonomic status of the order was fairly stable, and was accepted by most, if not all, cestode systematists worldwide (Hoberg et al. 1997, 1999, 2001; Mariaux 1998; Caira et al. 1999; Olson et al. 2001). However, more recently, Tyler (2006) proposes in a monograph study that the order Diphyllidea only includes two genera and 36 species classified into two families

(Echinobothriidae and Ditrachybothridiidae). Posteriorly, with the description of six additional species namely Echinobothrium diamanti (Ivanov and Lipshitz 2006), Echinobothrium sinensis described as belonging to the genus Macrobothridium (Li and Wang 2007), Echinobothrium minutamicum (Twohig et al. 2008), Echinobothrium nataliae, Echinobothrium reginae and Echinobothrium vojtai (Kutcha and Cairn 2010) the order currently count 42 valid species.

Echinobothrium euterpes described by Neifar et al. (2006) was firstly assigned to the genus Macrobothridium, which they considered as a valid genus. Latter, Tyler (2006), based on a morphologic and phylogenetic analysis considers the genus Macrobothridium as synonymous of Echinobothrium, and all the species were transferred into the later genus.

To date, only four species belonging to the genus Echinobothrium are ultrastructurally studied from a spermatological point of view. These are Echinobothrium affine, Echinobothrium harfordi, Echinobothrium typus and Echinobothrium brachysoma (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). In the present paper, the ultrastructure of spermiogenesis and the spermatozoon of Echinobothrium euterpes have been studied by means of TEM in order to obtain more information on this genus, to resolve some spermatological gaps on the Diphyllidea (Levron et al. 2010) and to increase the spermiological general database of Eucestoda.

### Materials and methods

Live specimens of Echinobothrium euterpes were collected from the spiral intestine of the common guitarfish Rhinobatos rhinobatos caught in the Gulf of Gabès (Tunisia). The living cestodes were placed in a 0.9% NaCl solution. After dissection, different portions of mature proglottides containing testes and seminal ducts were normally processed for transmission electron microscope 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 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 7.2, dehydrated in an ethanol series and propylene oxide, and finally embedded in Spurr's resin. 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 (Reynolds 1963). Ultrathin sections were examined using a JEOL 1010 TEM operated at an accelerating voltage of 80 kV.

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5 For proving the presence of glycogen particles, the Thiéry's technique (Thiéry 1967) was used.  
6 Gold grids were treated in periodic acid, thiocarbohydrazide and silver proteinate (PA-TCH-SP)  
7 as follows: 30 min in 10% of PA, rinsed in distilled water, 24 hr in TCH, rinsed in acetic  
8 solutions and distilled water, 30 min in 1% SP in the dark, and rinsed in distilled water.  
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## 13 **Results**

### 14 Spermogenesis (Figures 1 to 3)

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16 In *E. euterpes*, spermogenesis starts with the formation of a zone of differentiation at the  
17 periphery of each spermatid. This differentiation zone is a conical area containing nucleus and  
18 two centrioles and delimited at its base by a ring of arched membranes (Fig. 1a). In cross-  
19 section, the centrioles appear as formed by nine triplets (Fig. 1b) and are situated in a  
20 cytoplasmic protrusion bordered by few cortical microtubules (Fig. 1b). Each centriole  
21 supports a dense mass that is interpreted as homologous to a striated rootlet and both are  
22 separated by an intercentriolar body (Fig. 1c). The later is formed by three electron dense  
23 plates (Fig. 1c). The centrioles elongate and give rise to two unequal free flagella which grow  
24 orthogonal to a cytoplasmic extension and later rotate (Fig. 1d) and become parallel with the  
25 cytoplasmic process (Fig. 1e,f). The short flagellum presents more or less an equal longer in all  
26 the viewed spermatids and never exceeds 1µm (Fig. 1e). Cross-section in the proximal region  
27 of the differentiation zone shows that the cortical microtubules are organized in two opposite  
28 fields of six to eight microtubules in both sides to the median cytoplasmic process (Fig. 2a).  
29 More posteriorly, in the lower part with one flagellum, this number decreases to one or two  
30 (Fig. 2b). The fusion of these three processes occurs in the so-called proximodistal fusion. In a  
31 later stage, the nucleus enlarges, moves across the ring of arched membranes, and initiates its  
32 migration along the spermatid body between the two axonemes (Fig 2c). At this stage the short  
33 flagellum is shown near the ring of arched membranes (Fig 2c). After the migration of nucleus,  
34 the short axoneme takes down and is localised more distally in the spermatid (Fig. 2d). In  
35 advanced stages of spermogenesis, an apical cone and the single helical crested body appear at  
36 the basis of the spermatid (Fig. 2e). Spermogenesis finishes with the detachment of the  
37 spermatozoon from the residual cytoplasm as a result of the narrowing of the ring of arched  
38 membranes (Fig 2e).  
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### 58 Spermatozoon (Figures 4 to 7)

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3 The mature spermatozoon of *E. euterpes* is a filiform cell, tapered at both extremities, and  
4 lacks mitochondrion. From the anterior to posterior extremity, it is possible to distinguish four  
5 different regions (I-IV) with distinct ultrastructural characters and without any discontinuity.  
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10 Region I (Fig. 4a-g) corresponds to the anterior part of the gamete. The anterior tip consists of  
11 an electron-dense apical cone that is long and electron-dense (Fig. 4a) and is surrounded by the  
12 single crested body which begins its helical course more or less at the level of the anterior  
13 spermatozoon extremity and attains the axoneme (Fig. 4a,b). The anterior spermatozoon  
14 extremity is electron-lucent (Fig. 4b). In cross-section, this apical cone appears as an  
15 assemblage of some electron-dense tubular structures (Fig. 4c) coiled by the crested body (Fig.  
16 4c). Later, appears the centriole (Fig. 4d,e) which announces the beginning of the axoneme  
17 (Fig. 4f). The axoneme of the 9+1 trepaxonematan pattern is centrally positioned. This  
18 region has a particularity to possess few parallel cortical microtubules only present in a short  
19 area (Fig. 4f). In fact, this is the only part of the male gamete that contains cortical  
20 microtubules. Region I finishes with the disappearance of the crested body (Fig. 4g).  
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32 Region II (Fig. 5a-d) represents the area of the spermatozoon located between the crested body  
33 and the nuclear areas. In the areas of this region the axoneme is only surrounded by the plasma  
34 membrane (Fig. 5a,b). Later granules of glycogen granules become gradually visible in the  
35 middle part of this region and are uniformly distributed around the axoneme (Fig. 5a,c,d).  
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41 Region III (Figs. 5d-f, 6a) corresponds to the nuclear region of the spermatozoon. The nucleus  
42 is spiralled around the single axoneme (Fig. 5d). In cross-sections, it appears horseshoe in form  
43 and partially encircles the axoneme (Fig. 5e). The granules of glycogen are still present and are  
44 isolated in the opposite side without nucleus. The Thiéry's test has permitted to determine that  
45 this electron-dense granular material is glycogen ( $\beta$ -glycogen type) (Fig. 5f). At the end of this  
46 region, the dimension of the nuclear spire decreases and the nucleus progressively disappears  
47 (Fig. 6a).  
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55 Region IV (Fig. 6a-e) corresponds to the postnuclear area of the spermatozoon containing only  
56 the axoneme coiled by granules of glycogen (Fig. 6a). In the posterior end, these granules  
57 decreased in number and form a thin layer roughly the axoneme (Fig. 6b). The axoneme  
58 becomes progressively disorganized towards the distal end of this region (Fig. 6c-e): firstly the  
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3 peripheral doublets become disorganized (Fig. 6c), break apart into singlets encircling the  
4 central core (Fig. 6d); afterwards the central core disappears. The granules of glycogen reach  
5 up the vicinity of the posterior tip of the spermatozoon (Fig. 6e).  
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## 10 Discussion

### 11 Spermogenesis

12 Spermogenesis in E. euterpes involves a differentiation zone with a symmetric median  
13 cytoplasmic process with very few peripheral microtubules. It exhibits also three electron-  
14 dense plates that constitute the intercentriolar body separating two centrioles which support  
15 two dense masses corresponding to the typical striated roots. Each centriole grows and forms  
16 a free flagellum which rotates and later fuses with the median cytoplasmic process. This  
17 pattern is assimilated to the type I spermogenesis of Bâ and Marchand (1995) and is registered  
18 in most of the Diphyllidea (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui  
19 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) with the exception of E. harfordi in  
20 which the flagellar bud do not participate at the spermogenesis process (Mokhtar-Maamouri  
21 and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri  
22 1986/88). Subsequently, E. harfordi possesses the type II spermogenesis contrary to the other  
23 Diphyllidea.  
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37 The type I spermogenesis in E. typus, E. brachysoma, E. affine (Mokhtar-Maamouri and  
38 Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88)  
39 and E. euterpes is globally similar but offers some particularities. All the studied diphyllideans  
40 exhibit dense masses at the place of the striated rootlets. This structure shows a granular aspect  
41 and has a triangular form. Dense masses are only reported in diphyllidean species and might  
42 be capable to play the same function that the striated rootlets. Thus, this character only found  
43 in Diphyllidea could be considered as a synapomorphy for this order.  
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51 The intercentriolar body in the Diphyllidea comprises several parallel disk-shaped plates of  
52 different electron density. As occurs in E. euterpes in the present study, three electron-dense  
53 plates form the intercentriolar body also in E. typus and in E. brachysoma. (Azzouz-Draoui  
54 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). Contrary, E. harfordi possesses five  
55 electron-dense plates (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985;  
56 Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and in E. affine the number of plates  
57 constituting the intercentriolar body is undefined and composed by multiple electron-dense  
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3 plates, approximately 10 (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985;  
4 Azzouz-Draoui and Mokhtar-Maamouri 1986/88). The heterogeneity of this character within  
5 species belonging to the same order is not sufficient for indicate a possible polyphyly of this  
6 order.  
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11 The most interesting feature evidenced in the present study is the presence in the differentiation  
12 zone of two type of flagellum: a well developed flagellum and a short flagellum. The later  
13 growths, but never exceed 1  $\mu\text{m}$ . Very similar pattern is viewed in E. harfordi, in which the  
14 differentiation zone shows two centrioles that give rise to a well developed flagellum and a  
15 flagellar bud that persists but later disappears after the migration of the nucleus (Mokhtar-  
16 Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-  
17 Maamouri 1986/88). However, this flagellar bud not participates at the spermiogenesis process,  
18 whereas in E. euterpes, the short flagellum contributes to the process of spermiogenesis and  
19 subsists after the nuclear migration.  
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30 According to Justine (1998) the general diagram of spermiogenesis described in the  
31 Diphyllidea contains two unequal flagella; one flagella as shorter than the other and thus E.  
32 harfordi could be simply considered as an extreme case in which the shorter flagellum is  
33 particularly reduced. This reduction of the axoneme should be an evolved character and then  
34 the spermiogenesis pattern in E. harfordi (Mokhtar-Maamouri and Azzouz-Draoui 1984;  
35 Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and E. euterpes could  
36 be considered more evolved than the remaining diphyllideans.  
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44 Contrary to that viewed in other Eucestoda, in the Diphyllidea the distribution of cortical  
45 microtubules in the spermatid cells is curious. In E. brachysoma as in E. typus (Azzouz-Draoui  
46 1985) very few microtubules are described only in the early stages of spermiogenesis, while in  
47 E. harfordi microtubules were reported as lacking or difficult to distinguish (Mokhtar-  
48 Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-  
49 Maamouri 1986/88). In contradiction with these three species, E. affine (Mokhtar-Maamouri  
50 and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri  
51 1986/88) possesses peripheral microtubules arranged in two fields along the median  
52 cytoplasmic expansion and E. euterpes also presents cortical microtubules in all the  
53 spermiogenesis stages, but they are restricted in the proximal part of the differentiation zone.  
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3 This lack and/or paucity of cortical microtubules, is only related in Diphyllidea and may be an  
4 important pattern restricted only in this group.  
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9 The great differences observed between these five species belonging to a same genus, indicate  
10 that further observation is needed for validate o refute the heterogeneity of the ultrastructural  
11 characters of the spermiogenesis of this group. Additionally, spermiogenesis in E. harfordi and  
12 E. euterpes resembles that of the tetraphyllidean Phyllobothriidae Phyllobothrium lactuca (Sène  
13 et al. 1999). The later shows the Bâ and Marchand's type I spermiogenesis and presents both a  
14 long and a short flagella as occurs in E. euterpes, although all the remaining phyllobothriids  
15 present the type II (Mokhtar-Maamouri 1979; Euzet et al. 1981; MacKinnon and Burt 1984).  
16 Indeed, it is an intermediated character because this pattern may be considered as comprising  
17 between type I and II spermiogenesis but it is more similar to the type II in which one  
18 flagellum degenerate o is not formed. The type II spermiogenesis is described in cestodes  
19 belonging to the Tetraphyllidea-Phyllobothriidae (Mokhtar-Maamouri 1979; Euzet et al. 1981;  
20 MacKinnon and Burt 1984), Caryophyllidea (Świdorski and Mackiewicz 2002; Arafa and  
21 Hamada 2004; Bruňanská and Poddubnaya 2006; Gamil 2008; Miquel et al. 2008; Bruňanská  
22 2009; Yoneva et al. 2011) and Mesocestoididae (Miquel et al. 1999, 2007a) and is basically  
23 characterised by the formation of two centrioles but only one of them gives a flagellum. The  
24 single flagellum growth orthogonally, rotates and fuses with the cytoplasmic extension.  
25 Finally, the previous differentiation zone with one flagellum and one flagellar bud produces a  
26 mature sperm cell with only one axoneme.  
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#### 42 Spermatozoon

43 The mature spermatozoon of the five Diphyllidea examined until now shows three different  
44 patterns (types I, II and IV) according to Levron et al. (2010). The type I spermatozoon  
45 exhibited by the species of Spathebothriidea (Bruňanská et al. 2006; Bruňanská and  
46 Poddubnaya 2010) Diphyllbothriidea (Levron et al. 2006a, 2009), Haplobothriidea  
47 (MacKinnon and Burt 1985), Trypanorhyncha (Miquel and Świdorski 2006; Miquel et al.  
48 2007b; Marigo et al. 2011) and Diphyllidea (Mokhtar-Maamouri and Azzouz-Draoui 1984;  
49 Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) is characterized by the  
50 presence of two axonemes, parallel cortical microtubules and parallel nucleus, and by the  
51 lacking of crested body. The type II spermatozoon reported in Botriocephalidea (Świdorski and  
52 Mokhtar-Maamouri 1980; Bruňanská et al. 2002; Levron et al. 2005, 2006b, 2006c; Bâ et al.  
53 2007; Šípková et al. 2010, 2011), Tetraphyllidea-Onchobothriidae (Mokhtar-Maamouri and  
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3 Świdarski 1975; Mokhtar-Maamouri 1982; Quilichini et al. 2007), Proteocephalidea  
4 (Świdarski and Eklu-Natey 1978; Sène et al. 1997; Bruňanská et al. 2003a, 2003b, 2004a,  
5 2004b) and Diphyllidea (Azzouz-Draoui 1985) is characterized by the presence of two  
6 axonemes, helical crested body, parallel nucleus and parallel cortical microtubules. The type  
7 IV spermatozoon, characterized by the presence of one axoneme, helical crested body, parallel  
8 nucleus and parallel cortical microtubules, is described in some Tetrephyllidea-  
9 Phyllobothriidae (Mokhtar-Maamouri 1979; MacKinnon and Burt 1984), in the  
10 Mesocestoididae cyclophyllideans (Miquel et al. 1999, 2007a) and in certain Diphyllidea  
11 (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and  
12 Mokhtar-Maamouri 1986/88 and present study). According to this classification of the  
13 spermatozoon of eucestodes (Levron et al. 2010) there are some problematic spermatozoa  
14 difficult to place. It is the case of Sandonella sandoni (Bâ and Marchand 1984), a  
15 Proteocephalidea which presents only one axoneme, or the commented case of Phyllobothium  
16 lactuca (Sène et al. 1999) or Trilocularia acanthiaevulgaris (Mahendrasingam et al. 1984), two  
17 Phyllobothriidae which present two axonemes. These particular patterns observed in these  
18 species could be considered as evolved characters.  
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33 Considering diphyllidean species, in E. affine, E. brachysoma, and E. typus (Mokhtar-  
34 Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-  
35 Maamouri 1986/88) an apparently plesiomorphic pattern of spermiogenesis engenders a  
36 spermatozoon with two axonemes that are longitudinally well displaced, and make that the  
37 zone with two axoneme become quasi inexistent. A single helical crested body is also  
38 described in the anterior part of spermatozoa of E. brachysoma and E. typus, therefore the male  
39 gamete of this two species is most similar to the type II of Levron et al. (2010). In contrast, no  
40 crested body is related in E. affine (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-  
41 Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) which is included in the type I  
42 spermatozoon. The type IV is related in E. harfordi (Mokhtar-Maamouri and Azzouz-Draoui  
43 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and in our study  
44 in E. euterpes. The mature spermatozoon contains only a single axoneme, a helical crested  
45 body and nucleus.  
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58 In E. euterpes, although in the final stage of spermiogenesis the short flagellum was evidenced,  
59 the observation of numerous sections from different specimens permits us to affirm that a  
60 possible overlapping region of the two axonemes is absent in the mature spermatozoon of and,

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3 thus we conclude that one of the axonemes (the short one) degenerate at the end of  
4 spermiogenesis as in E. harfordi (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-  
5 Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88).  
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10 The most interesting components of all the mature spermatozoon of Eucestoda include the  
11 presence of cortical microtubules. However, in the Diphyllidea, to date, cortical microtubules  
12 are only related in the mature spermatozoon of E. affine (Mokhtar-Maamouri and Azzouz-  
13 Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). In E.  
14 euterpes, a short and reduced in number bundle of parallel cortical microtubules are observed  
15 only in the first part of the spermatozoon, precisely in the region with crested body.  
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23 In the review of sperm ultrastructure, Justine (1998) supposed that the absence of microtubules  
24 in diphyllidean was been a problem of fixation and judged more prudent to consider that  
25 microtubules are present in all species. However, the present study of E. euterpes confirms that  
26 cortical microtubules lack or are strongly reduced in the diphyllidean cestodes (Mokhtar-  
27 Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-  
28 Maamouri 1986/88 and present study).  
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35 There is a spiralled horseshoe-shaped nucleus encircling the axoneme in E. euterpes as occurs  
36 in other diphyllideans such as E. harfordi, E. typus and E. brachysoma (Mokhtar-Maamouri  
37 and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri  
38 1986/88). This pattern also makes additional difficulties to class these sperm cells into the  
39 classification of Levron et al. (2010), because the type I and II spermatozoon present a parallel  
40 nucleus. Thus, from the five species studied, only the mature spermatozoon of E. affine  
41 (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and  
42 Mokhtar-Maamouri 1986/88) corresponds to the description of Levron et al. (2010) with a  
43 parallel nucleus.  
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53 The ultrastructure of the posterior area of the spermatozoon in E. euterpes shows only the  
54 axoneme surrounded by the plasma membrane with some granules of glycogen. The distal tip  
55 of the spermatozoon of E. euterpes is characterized by the transformation of doublets into  
56 singlets previously to the disappearance of the central core unit. This schema is not in  
57 agreement with those found in the previously studied diphyllideans (Mokhtar-Maamouri and  
58 Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88).  
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3 The general schema of the posterior typ includes firstly the disappearance of the central core,  
4 and more posteriorly the transformation of the peripheral doublets into singlets (Mokhtar-  
5 Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-  
6 Maamouri 1986/88). The  $\beta$ -glycogen granules are present in all the studied diphyllideans  
7 (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and  
8 Mokhtar-Maamouri 1986/88 and present study) and only E. affine present both types,  $\alpha$  and  $\beta$ -  
9 glycogen (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui  
10 and Mokhtar-Maamouri 1986/88) (see Table 1). The presence of granules of glycogen at the  
11 bottom of the sperm cell in E. euterpes and this pronounced presence in all the Diphyllidea  
12 (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and  
13 Mokhtar-Maamouri 1986/88) would permit to think that they should compensate the absence  
14 of cortical microtubules.  
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## 26 Conclusion

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28 The most important character in the spermatozoon of cestodes named “number of axonemes” is  
29 problematic in the Diphyllidea. The number of axonemes in the sperm cell is one of the most  
30 interesting phylogenetical component concerning spermatological characters. During  
31 spermiogenesis E. harfordi possesses a flagellum and a flagellar bud, E. euterpes, E. typus and  
32 E. brachysoma exhibits both short and long flagella, while in E. affine the short flagellum likes  
33 to more developed than in the other species. In the mature spermatozoon, the number of  
34 axonemes becomes unclear.  
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42 In the Diphyllidea the spermiogenesis is globally coherent between species and presents more  
43 or less the same characteristics, but the pattern of the spermatozoon is so variable that is urgent  
44 to perform additional studies in order to resolve this problem and elucidate the real design of  
45 the sperm cell.  
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51 In our opinion, the previous studies of diphyllideans were not complete or were wrongly  
52 interpreted. At this time spermatological studies has not so developed than to date, and we  
53 think that the spermatozoon described in previous diphyllideans should be contain only one  
54 axoneme after the degeneration of one of them in final stages of spermiogenesis. This  
55 hypothesis should explain the spiralled form of the nucleus and the infrequency and/or absence  
56 of the sections with two axonemes.  
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## Legends to figures

**Fig.1** Spermiogenesis in Echinobothrium euterpes. **a** Zone of differentiation in the initial stage of spermiogenesis showing the presence of two centrioles (C). AM arched membranes, N nucleus. Bar 1  $\mu$ m. **b** Cross-section of the proximal part of the spermatid showing the nine triplets of centrioles (C). CM cortical microtubules. Bar 0.5  $\mu$ m. **c** Cross-section of a proximal

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3 area of the spermatid showing the three electron-dense plates forming the intercentriolar body  
4 and both dense masses (DM). Bar 0.5  $\mu\text{m}$ . **d** Longitudinal section of a zone of differentiation  
5 during the flagellar rotation. AM arched membranes, DM dense mass, F flagellum, IB  
6 intercentriolar body. Bar 1  $\mu\text{m}$ . **e** Longitudinal section of a zone of differentiation after the  
7 flagellar rotation showing the aspect of the short flagellum (F2). AM arched membranes, DM  
8 dense mass, F1 first flagellum, MCP median cytoplasmic process, N nucleus. Bar 0.5  $\mu\text{m}$ . **f**  
9 Another longitudinal section of the zone of differentiation after the flagellar rotation showing  
10 the difference of length between both flagella. AM arched membranes, DM dense mass, F1  
11 first flagellum, F2 second flagellum, MCP median cytoplasmic process. Bar 0.5  $\mu\text{m}$ .

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21 **Fig. 2** Spermiogenesis in Echinobothrium euterpes. **a** Cross-section of the spermatid showing  
22 both flagella (F) and the number of cortical microtubules (CM) in the proximal part of the  
23 median cytoplasmic expansion (MCP). C centriole. Bar 0.5  $\mu\text{m}$ . **b** Cross-sections of spermatids  
24 showing the decreasing of the number cortical microtubules (CM) at a distal level of the  
25 spermatid containing the first flagellum (F1). MCP median cytoplasmic process. Bar 0.5  $\mu\text{m}$ . **c**  
26 Longitudinal section of a zone of differentiation after the proximodistal fusion of axonemes  
27 (Ax1 and Ax2) showing the migration of the nucleus (N). (Note the position of the short  
28 axoneme). AM arched membranes. Bar 1  $\mu\text{m}$ . **d** Longitudinal section of the spermatid after the  
29 penetration of the nucleus, showing the downward position of the short axoneme (Ax2). AM  
30 arched membranes, Ax1 first axoneme. Bar 1  $\mu\text{m}$ . **e** Longitudinal section of a spermatid in a  
31 final stage of spermiogenesis showing the appearance of the apical cone (AC) and the crested  
32 body (CB). Note the constriction of the ring of arched membranes. Bar 1  $\mu\text{m}$ .

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44 **Fig. 3** Diagram showing the main stages of spermiogenesis in Echinobothrium euterpes.

45 AC apical cone, AM arched membranes, Ax1 axoneme 1, Ax2 axoneme 2, C1 centriole 1, C2  
46 centriole 2, CB crested body, CM cortical microtubules, DM dense mass, F1 flagellum 1, F2  
47 flagellum 2, IB intercentriolar body, MCP median cytoplasmic process, N nucleus.

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53 **Fig. 4** Mature spermatozoon of Echinobothrium euterpes. **a** Longitudinal section of the apical  
54 cone (AC) surrounded by the crested body (CB). ASE anterior spermatozoon extremity, Ax  
55 axoneme, C centriole Bar 1  $\mu\text{m}$ . **b** Detail of the anterior spermatozoon extremity (ASE). AC  
56 apical cone, CB crested body. Bar 0.5  $\mu\text{m}$ . **c-f** Consecutive cross-sections from the anterior  
57 spermatozoon extremity to the appearance of the axoneme. Note the presence of some cortical  
58 microtubules (CM) in the axoneme area. Ax axoneme, C centriole, CB crested body, CM  
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3 cortical microtubules. Bar 0.5  $\mu\text{m}$ . **g** Longitudinal section showing the transition area between  
4 regions I and II. Note the end of the crested body (arrowhead). Ax axoneme, CB crested body.  
5 Bar 1  $\mu\text{m}$ .  
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10 **Fig. 5** Mature spermatozoon of Echinobothrium euterpes. **a** Another longitudinal section of the  
11 transition area between region I and II showing the appearance of granules of glycogen (G) in  
12 the Region II (arrowhead). CB crested body. Bar 1  $\mu\text{m}$ . **b** Cross-sections of the Region II  
13 before the appearance of granules of glycogen. Ax axoneme, PM plasma membrane. Bar  
14 0,5 $\mu\text{m}$ . **c** Several cross-sections of the glycogen area (G). Bar 0,5 $\mu\text{m}$ . **d** Longitudinal section of  
15 the transition zone between regions II and III (nuclear region). G granules of glycogen, N  
16 nucleus. Bar 1  $\mu\text{m}$ . **e** Cross-section of the nuclear region showing the nucleus (N) in a horse-  
17 shoe shape. Bar 1  $\mu\text{m}$ . **f** Several regions of spermatozoon showing the presence of glycogen  
18 (G) evidenced by the method of Thiéry. Bar 0,5 $\mu\text{m}$ .  
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28 **Fig. 6** Mature spermatozoon of Echinobothrium euterpes. **a** Longitudinal section of the  
29 transition zone between regions III and IV. N nucleus. Note the end of the nucleus (arrowhead).  
30 Bar 1  $\mu\text{m}$ . **b** Cross-section of the postnuclear area showing the decreasing of the granules of  
31 glycogen (G). Bar 0,5 $\mu\text{m}$ . **c,d** Consecutive cross-sections of the posterior area of the  
32 spermatozoon showing the disorganisation of the axoneme. D doublets, S singlets. Bar 0,5 $\mu\text{m}$ .  
33 **e** Longitudinal section of the posterior area of the spermatozoon. Note the presence of the  
34 granules of glycogen in this zone. G granules of glycogen, PSE posterior spermatozoon  
35 extremity. Bar 0,5 $\mu\text{m}$ .  
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44 **Fig. 7** Schematic reconstruction of the mature spermatozoon of Echinobothrium euterpes. To  
45 simplify the diagram, the granules of glycogen are not shown in the longitudinal section.  
46 AC apical cone, ASE anterior spermatozoon extremity, Ax axoneme, C centriole, CB crested  
47 body, CM cortical microtubules, G glycogen, N nucleus, PM plasma membrane, PSE posterior  
48 spermatozoon extremity, S singlets.  
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Species References	Spermiogenesis							Spermatozoon								
	Type	FR	PF	IB	DM	CM	Ax	Type	ASE	AC	CB	CM	Ax	N	G	PSE
<u>Echinobothrium typus</u> Azzouz-Draoui (1985)	I	+	+	3	+	+	2	II	Ax	?	+	-	2	S	β	Ax
<u>Echinobothrium brachysoma</u> Azzouz-Draoui (1985)	I	+	+	3	+	?	2	II	Ax	?	+	-	2	S	β	Ax
<u>Echinobothrium affine</u> Mokhtar-Maamouri and Azzouz-Draoui (1984) Azzouz –Draoui (1985) Azzouz-Draoui and Mokhtar-Maamouri (1986/88)	I	+	+	≈10	+	+	2	I	CM	?	-	+	2	P	α, β	Ax
<u>Echinobothrium harfordi</u> Mokhtar-Maamouri and Azzouz-Draoui (1984) Azzouz –Draoui (1985) Azzouz-Draoui and Mokhtar-Maamouri (1986/88)	II	+	+	5	+	+	1+1B	IV	Ax	?	+	-	1	S	β	Ax
<u>Echinobothrium euterpes</u> present study	I	+	+	3	+	+	2	IV	CM	+	+	+	1	S	β	Ax

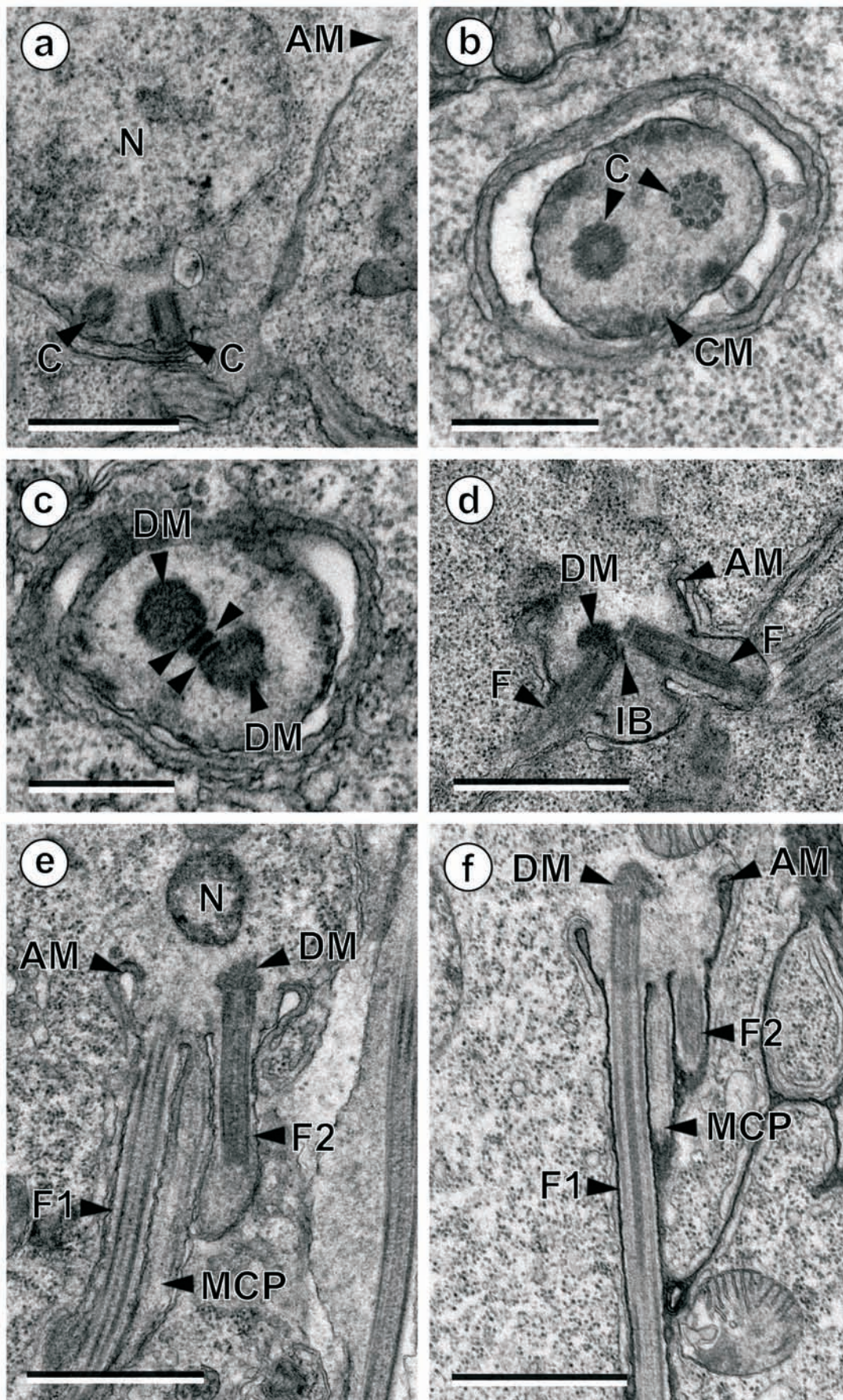
Table 1: Spermatological characters in the diphyllidean cestodes.

Spermiogenesis characters: Ax, number of axonemes; CM, cortical microtubules; DM, dense mass; FR, flagellar rotation; IB, number of plates of intercentriolar body; PF, proximodistal fusion.

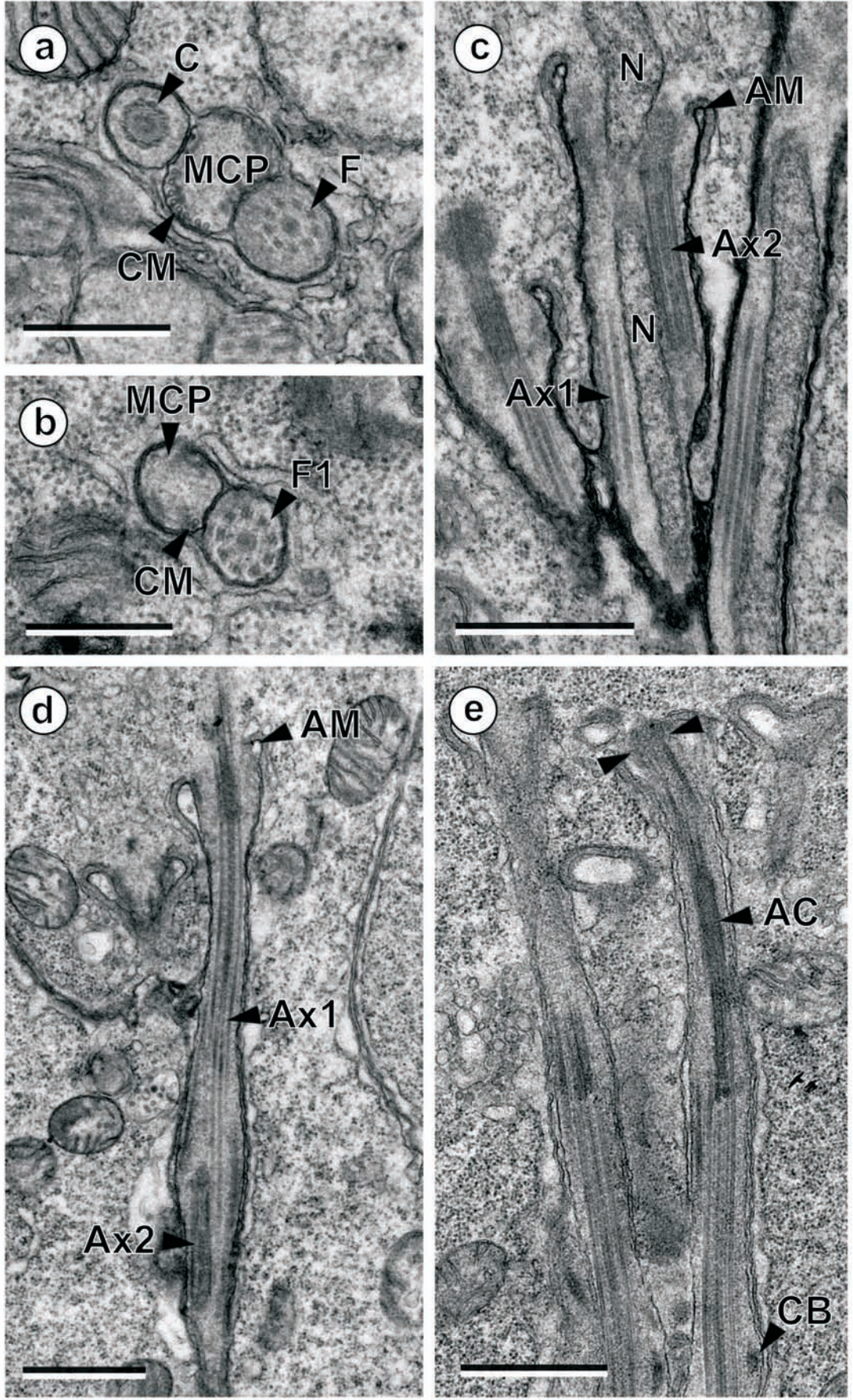
Spermatozoon characters: AC, apical cone; ASE, anterior spermatozoon extremity; Ax, axoneme; Ax, number of axonemes; CB, crested body; CM, cortical microtubules; G: type of glycogen; N, nucleus: S, spiralled; P, parallel; PSE, posterior spermatozoon extremity; +/-: presence/absence of character. ?, unknown data or required to be confirmed.

Spermiogenesis types are considered according Bâ and Marchand (1995).

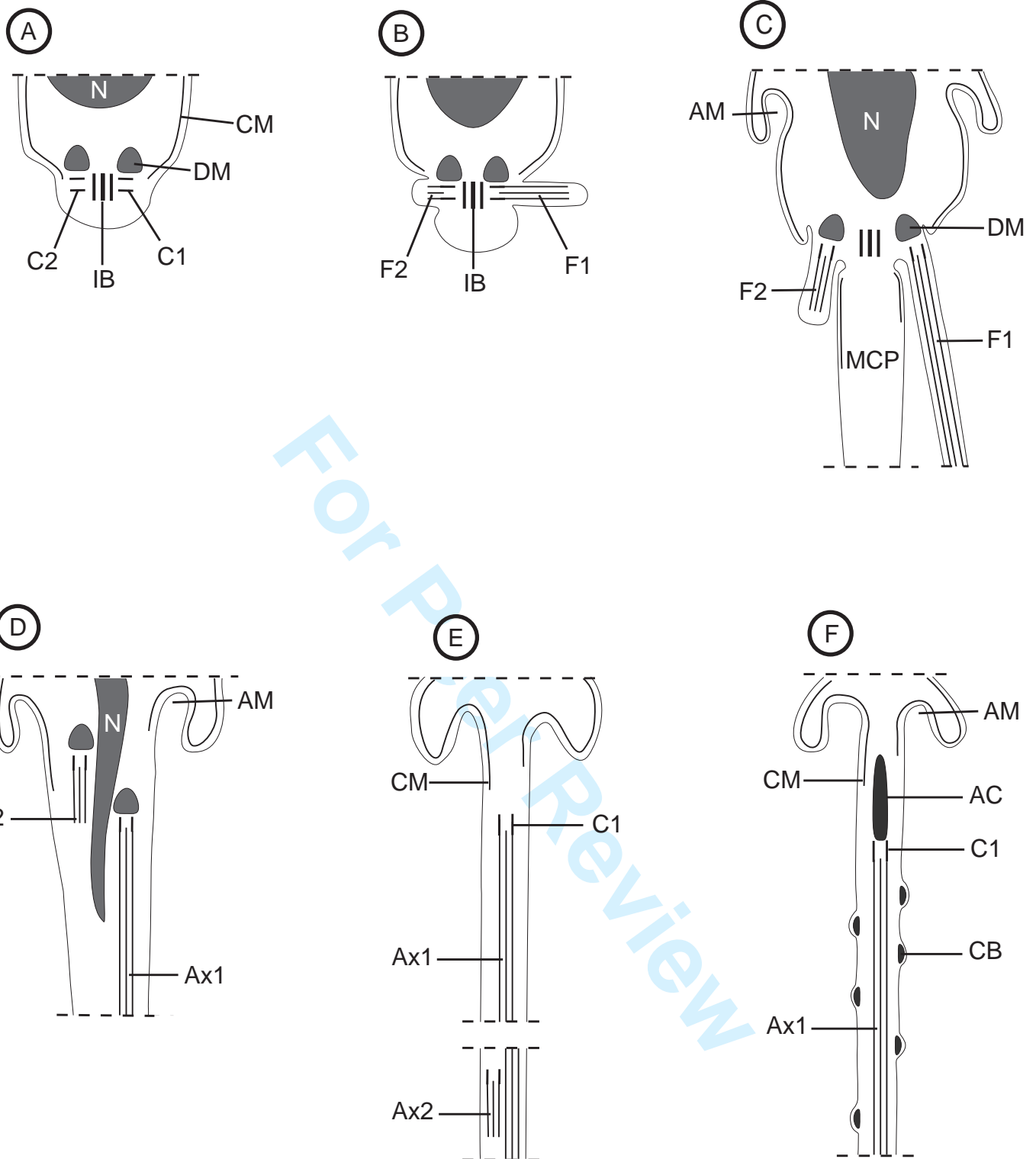
Spermatozoa types are considered according Levron et al. (2010).



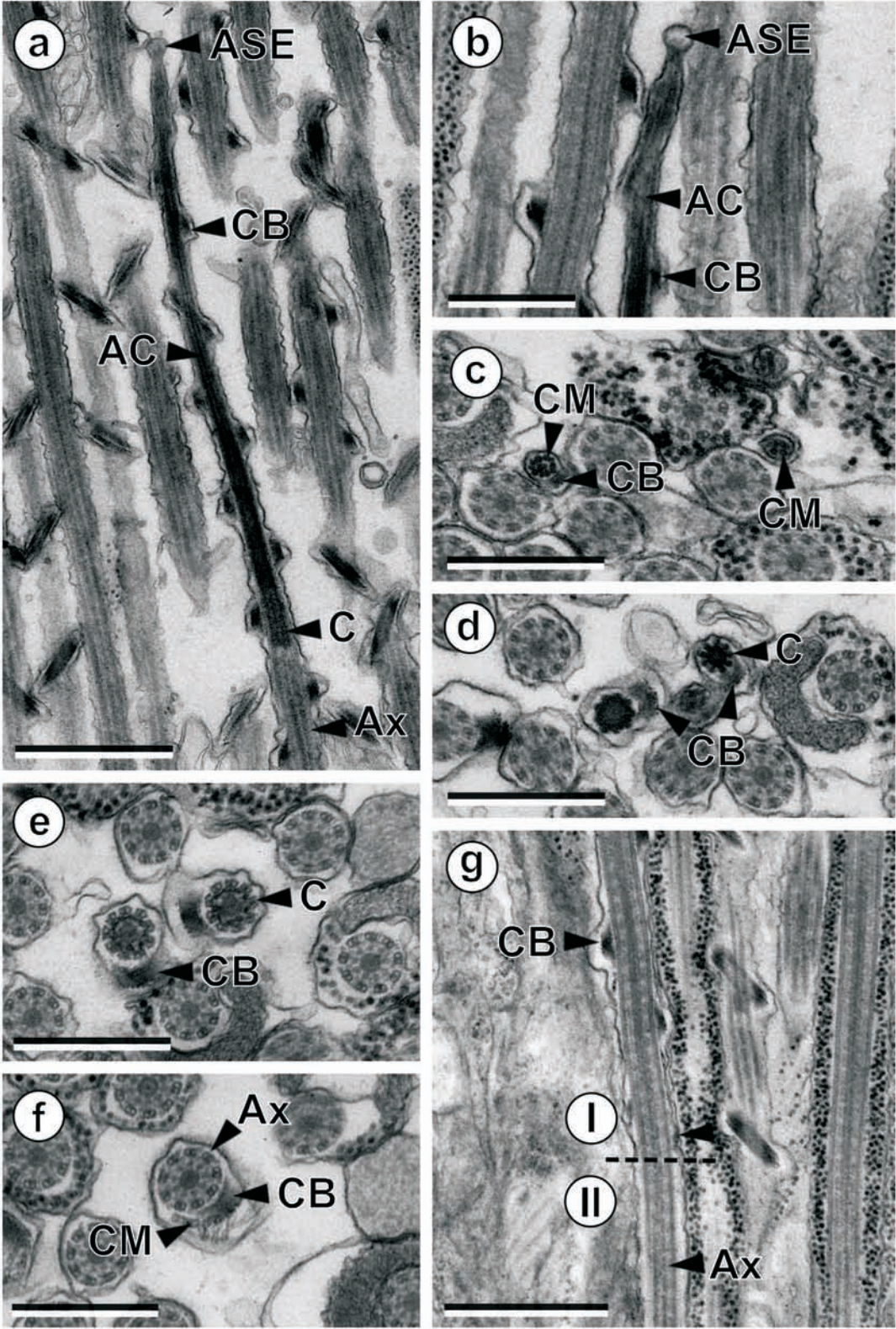
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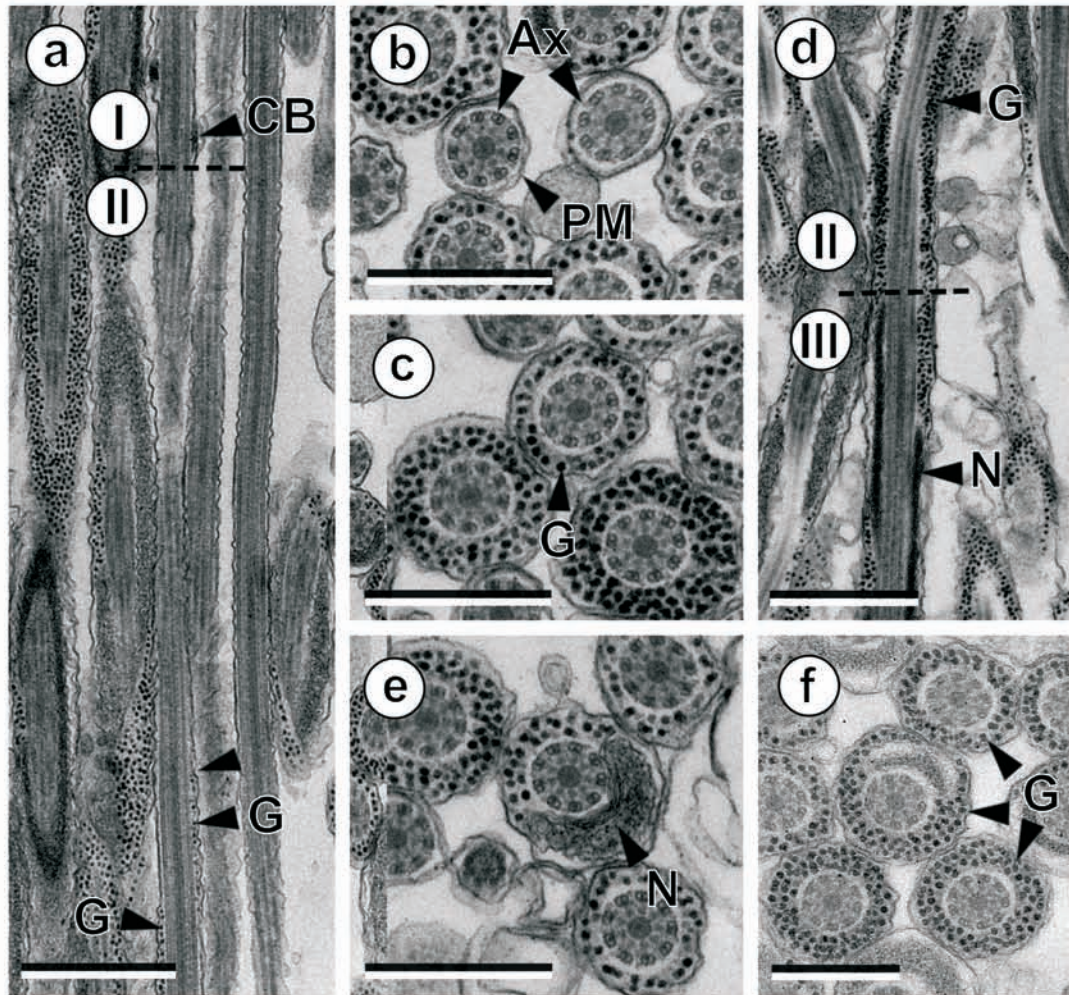




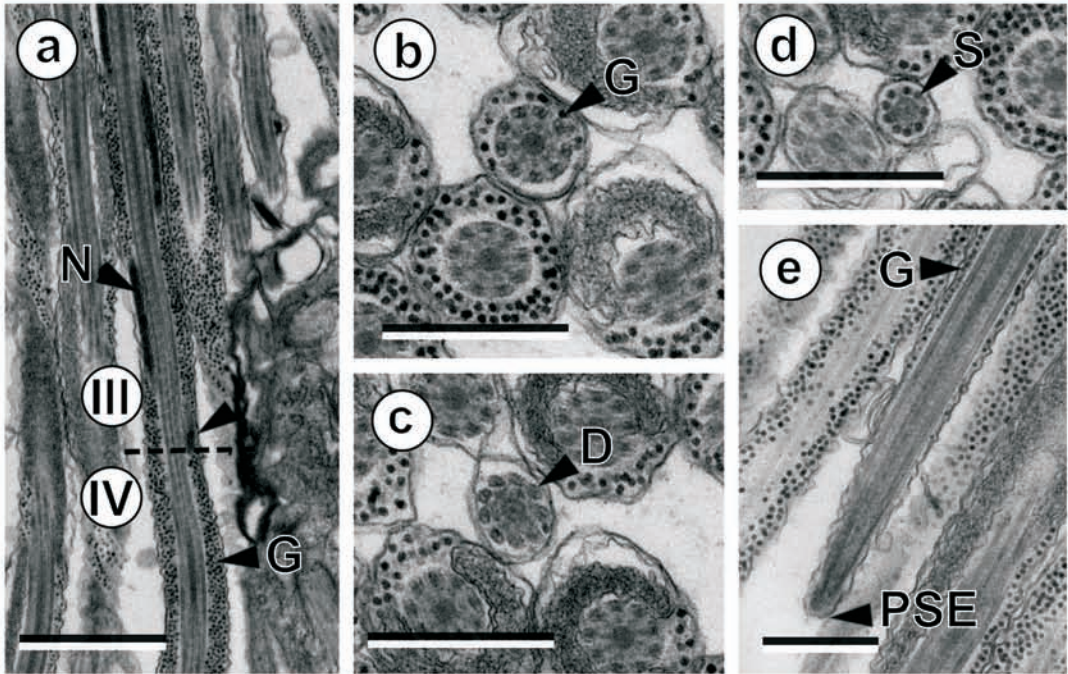


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