**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**

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Prof. Mehlhorn
Editor, Parasitology Research

Dear Dr. Mehlhorn,

We hereby submit the manuscript “Spermiogenesis and spermatozoon ultrastructure of the diphyllobothriidean 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
Adji Mama Marigo\textsuperscript{1,2}, Catarina Eira\textsuperscript{3,4}, Cheikh Tidiane Ba\textsuperscript{5}, Jordi Miquel\textsuperscript{1,2*}

Spermiogenesis and spermatozoon ultrastructure of the diphylloid cestode \textit{Echinobothrium euterpes} (Neifar, Tyler and Euzet 2001) Tyler 2006, a parasite of the common guitarfish \textit{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 µm 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” trepanematan pattern. It also exhibits a single helical electron-dense crested body, a spiralled nucleus, few parallel cortical microtubules, and α-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, Ditrachybothriidae and Macrobothriidae. 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 Caira 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 Diphylidea (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.
For proving the presence of glycogen particles, the Thiéry’s technique (Thiéry 1967) was used. Gold grids were treated in periodic acid, thiocarbohydrazide and silver proteinate (PA-TCH-SP) as follows: 30 min in 10% of PA, rinsed in distilled water, 24 hr in TCH, rinsed in acetic solutions and distilled water, 30 min in 1% SP in the dark, and rinsed in distilled water.

**Results**

**Spermiogenesis (Figures 1 to 3)**

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

**Spermatozoon (Figures 4 to 7)**
The mature spermatozoon of *E. euterpes* is a filiform cell, tapered at both extremities, and lacks mitochondrion. From the anterior to posterior extremity, it is possible to distinguish four different regions (I-IV) with distinct ultrastructural characters and without any discontinuity.

Region I (Fig. 4a-g) corresponds to the anterior part of the gamete. The anterior tip consists of an electron-dense apical cone that is long and electron-dense (Fig. 4a) and is surrounded by the single crested body which begins its helical course more or less at the level of the anterior spermatozoon extremity and attains the axoneme (Fig. 4a,b). The anterior spermatozoon extremity is electron-lucent (Fig. 4b). In cross-section, this apical cone appears as an assemblage of some electron-dense tubular structures (Fig. 4c) coiled by the crested body (Fig. 4c). Later, appears the centriole (Fig. 4d,e) which announces the beginning of the axoneme (Fig. 4f). The axoneme of the 9+"1" trepaxonematan pattern is centrally positioned. This region has a particularity to possess few parallel cortical microtubules only present in a short area (Fig. 4f). In fact, this is the only part of the male gamete that contains cortical microtubules. Region I finishes with the disappearance of the crested body (Fig. 4g).

Region II (Fig. 5a-d) represents the area of the spermatozoon located between the crested body and the nuclear areas. In the areas of this region the axoneme is only surrounded by the plasma membrane (Fig. 5a,b). Later granules of glycogen granules become gradually visible in the middle part of this region and are uniformly distributed around the axoneme (Fig. 5a,c,d).

Region III (Figs. 5d-f, 6a) corresponds to the nuclear region of the spermatozoon. The nucleus is spiralled around the single axoneme (Fig. 5d). In cross-sections, it appears horseshoe in form and partially encircles the axoneme (Fig. 5e). The granules of glycogen are still present and are isolated in the opposite side without nucleus. The Thiéry’s test has permitted to determine that this electron-dense granular material is glycogen (β-glycogen type) (Fig. 5f). At the end of this region, the dimension of the nuclear spire decreases and the nucleus progressively disappears (Fig. 6a).

Region IV (Fig. 6a-e) corresponds to the postnuclear area of the spermatozoon containing only the axoneme coiled by granules of glycogen (Fig. 6a). In the posterior end, these granules decreased in number and form a thin layer roughly the axoneme (Fig. 6b). The axoneme becomes progressively disorganized towards the distal end of this region (Fig. 6c-e): firstly the
peripheral doublets become disorganized (Fig. 6c), break apart into singlets encircling the central core (Fig. 6d); afterwards the central core disappears. The granules of glycogen reach up the vicinity of the posterior tip of the spermatozoon (Fig. 6e).

**Discussion**

Spermiogenesis

Spermiogenesis in *E. euterpes* involves a differentiation zone with a symmetric median cytoplasmic process with very few peripheral microtubules. It exhibits also three electron-dense plates that constitute the intercentriolar body separating two centrioles which support two dense masses corresponding to the typical striated roots. Each centriole grows and forms a free flagellum which rotates and later fuses with the median cytoplasmic process. This pattern is assimilated to the type I spermiogenesis of Bá and Marchand (1995) and is registered in most of the Diphyllidea (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) with the exception of *E. harfordi* in which the flagellar bud do not participate at the spermiogenesis process (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). Subsequently, *E. harfordi* possesses the type II spermiogenesis contrary to the other Diphyllidea.

The type I spermiogenesis in *E. typus, E. brachysoma, E. affine* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and *E. euterpes* is globally similar but offers some particularities. All the studied diphylideans exhibit dense masses at the place of the striated rootlets. This structure shows a granular aspect and has a triangular form. Dense masses are only reported in diphylidean species and might be capable to play the same function that the striated rootlets. Thus, this character only found in Diphyllidea could be considered as a synapamorphy for this order.

The intercentriolar body in the Diphyllidea comprises several parallel disk-shaped plates of different electron density. As occurs in *E. euterpes* in the present study, three electron-dense plates form the intercentriolar body also in *E. typus* and in *E. brachysoma* (Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). Contrary, *E. harfordi* possesses five electron-dense plates (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and in *E. affine* the number of plates constituting the intercentriolar body is undefined and composed by multiple electron-dense.
plates, approximately 10 (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). The heterogeneity of this character within species belonging to the same order is not sufficient for indicate a possible polyphyly of this order.

The most interesting feature evidenced in the present study is the presence in the differentiation zone of two type of flagellum: a well developed flagellum and a short flagellum. The later growths, but never exceed 1 μm. Very similar pattern is viewed in *E. harfordi*, in which the differentiation zone shows two centrioles that give rise to a well developed flagellum and a flagellar bud that persists but later disappears after the migration of the nucleus (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). However, this flagellar bud not participates at the spermiogenesis process, whereas in *E. euterpes*, the short flagellum contributes to the process of spermiogenesis and subsists after the nuclear migration.

According to Justine (1998) the general diagram of spermiogenesis described in the Diphyllidea contains two unequal flagella; one flagella as shorter than the other and thus *E. harfordi* could be simply considered as an extreme case in which the shorter flagellum is particularly reduced. This reduction of the axoneme should be an evolved character and then the spermiogenesis pattern in *E. harfordi* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and *E. euterpes* could be considered more evolved than the remaining diphyllideans.

Contrary to that viewed in other Eucestoda, in the Diphyllidea the distribution of cortical microtubules in the spermatid cells is curious. In *E. brachysoma* as in *E. typus* (Azzouz-Draoui 1985) very few microtubules are described only in the early stages of spermiogenesis, while in *E. harfordi* microtubules were reported as lacking or difficult to distinguish (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). In contradiction with these three species, *E. affine* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) possesses peripheral microtubules arranged in two fields along the median cytoplasmic expansion and *E. euterpes* also presents cortical microtubules in all the spermiogenesis stages, but they are restricted in the proximal part of the differentiation zone.
This lack and/or paucity of cortical microtubules, is only related in Diphyllidea and may be an important pattern restricted only in this group.

The great differences observed between these five species belonging to a same genus, indicate that further observation is needed for validate or refute the heterogeneity of the ultrastructural characters of the spermiogenesis of this group. Additionally, spermiogenesis in *E. harfordi* and *E. euterpes* resembles that of the tetraphyllidean Phyllobothriidae *Phyllobothrium lactuca* (Sène et al. 1999). The later shows the Bâ and Marchand’s type I spermiogenesis and presents both a long and a short flagella as occurs in *E. euterpes*, although all the remaining phyllobothiids present the type II (Mokhtar-Maamouri 1979; Euzet et al. 1981; MacKinnon and Burt 1984). Indeed, it is an intermediated character because this pattern may be considered as comprising between type I and II spermiogenesis but it is more similar to the type II in which one flagellum degenerate o is not formed. The type II spermiogenesis is described in cestodes belonging to the Tetraphyllidea-Phyllobothriidae (Mokhtar-Maamouri 1979; Euzet et al. 1981; MacKinnon and Burt 1984), Caryophyllidea (Świderski and Mackiewicz 2002; Arafa and Hamada 2004; Bruňanská and Poddubnaya 2006; Gamil 2008; Miquel et al. 2008; Bruňanská 2009; Yoneva et al. 2011) and Mesocestoididae (Miquel et al. 1999, 2007a) and is basically characterised by the formation of two centrioles but only one of them gives a flagellum. The single flagellum growth orthogonally, rotates and fuses with the cytoplasmic extension. Finally, the previous differentiation zone with one flagellum and one flagellar bud produces a mature sperm cell with only one axoneme.

**Spermatozoon**

The mature spermatozoon of the five Diphyllidea examined until now shows three different patterns (types I, II and IV) according to Levron et al. (2010). The type I spermatozoon exhibited by the species of Spathebothriidea (Bruňanská et al. 2006; Bruňanská and Poddubnaya 2010) Diphyllobotheidae (Levron et al. 2006a, 2009), Haplobothriidea (MacKinnon and Burt 1985), Trypanorhyncha (Miquel and Świderski 2006; Miquel et al. 2007b; Marigo et al. 2011) and Diphylleida (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) is characterized by the presence of two axonemes, parallel cortical microtubules and parallel nucleus, and by the lacking of crested body. The type II spermatozoon reported in Botriocephalidea (Świderski and Mokhtar-Maamouri 1980; Bruňanská et al. 2002; Levron et al. 2005, 2006b, 2006c; Bâ et al. 2007; Šípková et al. 2010, 2011), Tetraphyllidea-Onchobothriidae (Mokhtar-Maamouri and
Świderski 1975; Mokhtar-Maamouri 1982; Quilichini et al. 2007), Proteocephalidea (Świderski and Eklu-Natey 1978; Sène et al. 1997; Bruňanská et al. 2003a, 2003b, 2004a, 2004b) and Diphyllidea (Azzouz-Draoui 1985) is characterized by the presence of two axonemes, helical crested body, parallel nucleus and parallel cortical microtubules. The type IV spermatozoon, characterized by the presence of one axoneme, helical crested body, parallel nucleus and parallel cortical microtubules, is described in some Tetraphyllidea-Phyllobothriidae (Mokhtar-Maamouri 1979; MacKinnon and Burt 1984), in the Mesocestoididae cyclophyllideans (Miquel et al. 1999, 2007a) and in certain Diphyllidea (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88 and present study). According to this classification of the spermatozoon of eucestodes (Levron et al. 2010) there are some problematic spermatozoa difficult to place. It is the case of Sandonella sandoni (Bâ and Marchand 1984), a Proteocephalidea which presents only one axoneme, or the commented case of Phyllobothium lactuca (Sène et al. 1999) or Trilocularia acanthiae vulgaris (Mahendrasingam et al. 1984), two Phyllobothriidae which present two axonemes. These particular patterns observed in these species could be considered as evolved characters.

Considering diphyllidean species, in E. affine, E. brachysoma, and E. typus (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) an apparently plesiomorphic pattern of spermiogenesis engenders a spermatozoon with two axonemes that are longitudinally well displaced, and make that the zone with two axoneme become quasi inexistent. A single helical crested body is also described in the anterior part of spermatozoa of E. brachysoma and E. typus, therefore the male gamete of this two species is most similar to the type II of Levron et al. (2010). In contrast, no crested body is related in E. affine (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) which is included in the type I spermatozoon. The type IV is related in E. harfordi (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) and in our study in E. euterpes. The mature spermatozoon contains only a single axoneme, a helical crested body and nucleus.

In E. euterpes, although in the final stage of spermiogenesis the short flagellum was evidenced, the observation of numerous sections from different specimens permits us to affirm that a possible overlapping region of the two axonemes is absent in the mature spermatozoon of and,
thus we conclude that one of the axonemes (the short one) degenerate at the end of spermiogenesis as in *E. harfordi* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88).

The most interesting components of all the mature spermatozoon of Eucestoda include the presence of cortical microtubules. However, in the Diphyllidea, to date, cortical microtubules are only related in the mature spermatozoon of *E. affine* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). In *E. euterpes*, a short and reduced in number bundle of parallel cortical microtubules are observed only in the first part of the spermatozoon, precisely in the region with crested body.

In the review of sperm ultrastructure, Justine (1998) supposed that the absence of microtubules in diphyllidean was been a problem of fixation and judged more prudent to consider that microtubules are present in all species. However, the present study of *E. euterpes* confirms that cortical microtubules lack or are strongly reduced in the diphyllidean cestodes (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88 and present study).

There is a spiralled horseshoe-shaped nucleus encircling the axoneme in *E. euterpes* as occurs in other diphyllideans such as *E. harfordi*, *E. typus* and *E. brachysoma* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). This pattern also makes additional difficulties to class these sperm cells into the classification of Levron et al. (2010), because the type I and II spermatozoon present a parallel nucleus. Thus, from the five species studied, only the mature spermatozoon of *E. affine* (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) corresponds to the description of Levron et al. (2010) with a parallel nucleus.

The ultrastructure of the posterior area of the spermatozoon in *E. euterpes* shows only the axoneme surrounded by the plasma membrane with some granules of glycogen. The distal tip of the spermatozoon of *E. euterpes* is characterized by the transformation of doublets into singlets previously to the disappearance of the central core unit. This schema is not in agreement with those found in the previously studied diphyllideans (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88).
The general schema of the posterior typ includes firstly the disappearance of the central core, and more posteriorly the transformation of the peripheral doublets into singlets (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88). The β-glycogen granules are present in all the studied diphylleideans (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88 and present study) and only E. affine present both types, α and β-glycogen (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) (see Table 1). The presence of granules of glycogen at the bottom of the sperm cell in E. euterpes and this pronounced presence in all the Diphylleida (Mokhtar-Maamouri and Azzouz-Draoui 1984; Azzouz-Draoui 1985; Azzouz-Draoui and Mokhtar-Maamouri 1986/88) would permit to think that they should compensate the absence of cortical microtubules.

Conclusion

The most important character in the spermatozoon of cestodes named “number of axonemes” is problematic in the Diphylleida. The number of axonemes in the sperm cell is one of the most interesting phylogenetical component concerning spermatological characters. During spermiogenesis E. harfordi possesses a flagellum and a flagellar bud, E. euterpes, E. typus and E. brachysoma exhibits both short and long flagella, while in E. affine the short flagellum likes to more developed than in the other species. In the mature spermatozoon, the number of axonemes becomes unclear.

In the Diphylleida the spermiogenesis is globally coherent between species and presents more or less the same characteristics, but the pattern of the spermatozoon is so variable that is urgent to perform additional studies in order to resolve this problem and elucidate the real design of the sperm cell.

In our opinion, the previous studies of diphylleideans were not complete or were wrongly interpreted. At this time spermatological studies has not so developed than to date, and we think that the spermatozoon described in previous diphylleideans should be contain only one axoneme after the degeneration of one of them in final stages of spermiogenesis. This hypothesis should explain the spiralled form of the nucleus and the infrequency and/or absence of the sections with two axonemes.
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References


Sène A, Bâ CT, Marchand B (1997) Ultrastructure of spermigenesis and the spermatozoon of Nomimoscolex sp. (Cestoda, Proteocephalidea) intestinal parasite of Clarotes laticeps


**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 µm. b Cross-section of the proximal part of the spermatid showing the nine triplets of centrioles (C). CM cortical microtubules. Bar 0.5 µm. c Cross-section of a proximal
area of the spermatid showing the three electron-dense plates forming the intercentriolar body and both dense masses (DM). Bar 0.5 µm. **d** Longitudinal section of a zone of differentiation during the flagellar rotation. AM arched membranes, DM dense mass, F flagellum, IB intercentriolar body. Bar 1 µm. **e** Longitudinal section of a zone of differentiation after the flagellar rotation showing the aspect of the short flagellum (F2). AM arched membranes, DM dense mass, F1 first flagellum, MCP median cytoplasmic process, N nucleus. Bar 0.5 µm. **f** Another longitudinal section of the zone of differentiation after the flagellar rotation showing the difference of length between both flagella. AM arched membranes, DM dense mass, F1 first flagellum, F2 second flagellum, MCP median cytoplasmic process. Bar 0.5 µm.

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

**Fig. 3** Diagram showing the main stages of spermiogenesis in *Echinobothrium euterpes*. AC apical cone, AM arched membranes, Ax1 axoneme 1, Ax2 axoneme 2, C1 centriole 1, C2 centriole 2, CB crested body, CM cortical microtubules, DM dense mass, F1 flagellum 1, F2 flagellum 2, IB intercentriolar body, MCP median cytoplasmic process, N nucleus.

**Fig. 4** Mature spermatozoon of *Echinobothrium euterpes*. **a** Longitudinal section of the apical cone (AC) surrounded by the crested body (CB). ASE anterior spermatozoon extremity, Ax axoneme, C centriole Bar 1 µm. **b** Detail of the anterior spermatozoon extremity (ASE). AC apical cone, CB crested body. Bar 0.5 µm. **c-f** Consecutive cross-sections from the anterior spermatozoon extremity to the appearance of the axoneme. Note the presence of some cortical microtubules (CM) in the axoneme area. Ax axoneme, C centriole, CB crested body, CM
cortical microtubules. Bar 0.5 µm. g Longitudinal section showing the transition area between regions I and II. Note the end of the crested body (arrowhead). Ax axoneme, CB crested body. Bar 1 µm.

**Fig. 5** Mature spermatozoon of *Echinobothrium euterpes*. a Another longitudinal section of the transition area between region I and II showing the appearance of granules of glycogen (G) in the Region II (arrowhead). CB crested body. Bar 1 µm. b Cross-sections of the Region II before the appearance of granules of glycogen. Ax axoneme, PM plasma membrane. Bar 0.5µm. c Several cross-sections of the glycogen area (G). Bar 0.5µm. d Longitudinal section of the transition zone between regions II and III (nuclear region). G granules of glycogen, N nucleus. Bar 1 µm. e Cross-section of the nuclear region showing the nucleus (N) in a horse-shoe shape. Bar 1 µm. f Several regions of spermatozoon showing the presence of glycogen (G) evidenced by the method of Thiéry. Bar 0.5µm.

**Fig. 6** Mature spermatozoon of *Echinobothrium euterpes*. a Longitudinal section of the transition zone between regions III and IV. N nucleus. Note the end of the nucleus (arrowhead). Bar 1 µm. b Cross-section of the postnuclear area showing the decreasing of the granules of glycogen (G). Bar 0.5µm. c,d Consecutive cross-sections of the posterior area of the spermatozoon showing the disorganisation of the axoneme. D doublets, S singlets. Bar 0.5µm. e Longitudinal section of the posterior area of the spermatozoon. Note the presence of the granules of glycogen in this zone. G granules of glycogen, PSE posterior spermatozoon extremity. Bar 0.5µm.

**Fig. 7** Schematic reconstruction of the mature spermatozoon of *Echinobothrium euterpes*. To simplify the diagram, the granules of glycogen are not shown in the longitudinal section.

AC apical cone, ASE anterior spermatozoon extremity, Ax axoneme, C centriole, CB crested body, CM cortical microtubules, G glycogen, N nucleus, PM plasma membrane, PSE posterior spermatozoon extremity, S singlets.
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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.
Spermatozoos characters: AC, apical cone; ASE, anterior spermatozoos 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 spermatozoos extremity; +/-: presence/absence of character. ?, unknown data or required to be confirmed.
Spermiogenesis types are considered according Bâ and Marchand (1995).
Spermatozoos types are considered according Levron et al. (2010).
Images a, b, c, d, and e show various cellular structures labeled with the following:

- **MCP** (Membrane-Covered Particle)
- **CM** (Cytoplasmic Membrane)
- **F** (filament)
- **F1** (filament 1)
- **AM** (Axoneme)
- **Ax1** (Axoneme 1)
- **Ax2** (Axoneme 2)
- **N** (Nucleus)
- **AC** (Axoneme Core)
- **CB** (Cilia Base)

Scale bars indicate the scale of the images.