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Spermatological characteristics of the Trypanorhyncha inferred from new ultrastructural data on species of Tentaculariidae, Eutetrarhynchidae, and Progrillotiidae

*Caractéristiques spermatologiques des Trypanorhyncha inférées de nouvelles données ultrastructurales d'espèces de Tentaculariidae, Eutetrarhynchidae et Progrillotiidae*Jordi Miquel ^{a,b,*}, Zdzisław Świdorski ^{c,d}^a Laboratori de Parasitologia, Departament de Microbiologia i Parasitologia Sanitàries, Facultat de Farmàcia, Universitat de Barcelona, Avenue Joan XXIII, s/n, 08028 Barcelona, Spain^b Institut de Recerca de la Biodiversitat (IRBIO), Facultat de Biologia, Universitat de Barcelona, Avenue Diagonal, 645, 08028 Barcelona, Spain^c W. Stefański Institute of Parasitology, Polish Academy of Sciences, Warsaw, Poland^d Department of General Biology and Parasitology, Warsaw Medical University, Warsaw, Poland

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ABSTRACT

The present study focuses on the ultrastructural characteristics of both spermiogenesis and the spermatozoon in the order Trypanorhyncha. New ultrastructural data are presented for two species of the unexplored superfamily Tentacularioidea, *Nybelinia queenslandensis*, and *Kotorella pronosoma*. The present study also provides supplementary data on the superfamily Eutetrarhynchoidea, with the analysis of spermiogenesis and spermatozoon of two progrillotiids, *Progrillotia dasyatidis* and *Pro. pastinacae*, and new ultrastructural data concerning spermiogenesis in the eutetrarhynchids *Dollfusiella spinulifera* and *Parachristianella trygonis*. Spermiogenesis in trypanorhynchids follows the Bâ and Marchand's type I and the ultrastructural organisation of the mature spermatozoon corresponds to the Levron et al.'s type I. The most remarkable characters concerns the number of electron-dense plates constituting the intercentriolar body during spermiogenesis and in the variability of the arc-like row of thick cortical microtubules present in the anterior areas of the spermatozoon because of its variability according to the species.

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R É S U M É

Cette étude porte sur les caractéristiques ultrastructurales de la spermiogenèse et du spermatozoïde dans l'ordre Trypanorhyncha. Des nouvelles données sont présentées pour deux espèces, *Nybelinia queenslandensis* et *Kotorella pronosoma*, de la super-famille précédemment non explorée Tentacularioidea. Cette étude fournit aussi des informations supplémentaires sur la super-famille Eutetrarhynchoidea, avec une analyse de la spermiogenèse et du spermatozoïde de deux Progrillotiidae, *Progrillotia dasyatidis* et *Pro. pastinacae*, et de nouvelles données ultrastructurales concernant la spermiogenèse chez les Eutetrarhynchidae *Dollfusiella spinulifera* et *Parachristianella trygonis*. La

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spermiogénèse chez les Trypanorhynques suit le Type I de Bâ et Marchand et l'organisation ultrastructurale du spermatozoïde correspond au type I de Levron et al. Les caractères les plus remarquables concernent le nombre de plaques denses aux électrons qui constituent le corps intercentriolaire pendant la spermiogénèse et la variabilité de la rangée en arc de microtubules corticaux épais présents dans les régions antérieures du spermatozoïde, à cause de la variabilité parmi les espèces.

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1. Introduction

Trypanorhynchs are polyzoic cestodes, readily recognised by their rhyncheal apparatus, and are common metazoan parasites of marine fish. Adult worms parasitize elasmobranch fishes while their larval stages occur in a variety of teleosts, elasmobranchs and marine invertebrates (crustaceans, cephalopods and bivalves), including zooplankton [1]. Trypanorhynchs possess a scolex with two or four bothria [2] and also a tentacular apparatus consisting of four retractile tentacles armed with hooks that are attached to four bulbs [3]. This rhyncheal apparatus is unique within the cestodes, and provides a strong synapomorphy that supports the monophyly of this order [4].

This order has been considered to be one of the most chaotic and confusing tapeworm groups, but recent work has shed considerable light on their systematics [5]. Morphological evidence strongly supports the monophyly of trypanorhynchs [1,5]. Although Waeschenbach et al. [6] presented molecular data that support the monophyly of the Trypanorhyncha, other molecular studies have suggested that this order is paraphyletic and consists of two well-supported clades [4,7–9]. The first clade groups together the superfamilies Eutetrarhynchoidea and Tentacularioidea while the second clade groups together the Gymnorhynchoidea, the Lacistorhynchoidea and the Otobothrioidea. Recently, Olson et al. [10], in a combined analysis of molecular and morphological data, identified two clades with each one occurring in the two principal clades of the elasmobranch definitive hosts (rays or sharks) and they proposed the suborders Trypanobatoida and Trypanoselachoida for these two major clades according to the primarily hosts parasitized, rays and sharks, respectively. The Trypanobatoida contains the Tentacularioidea (including the eutetrarhynchoidea) and the Trypanoselachoida contains the Lacistorhynchoidea, the Otobothrioidea and the Gymnorhynchoidea.

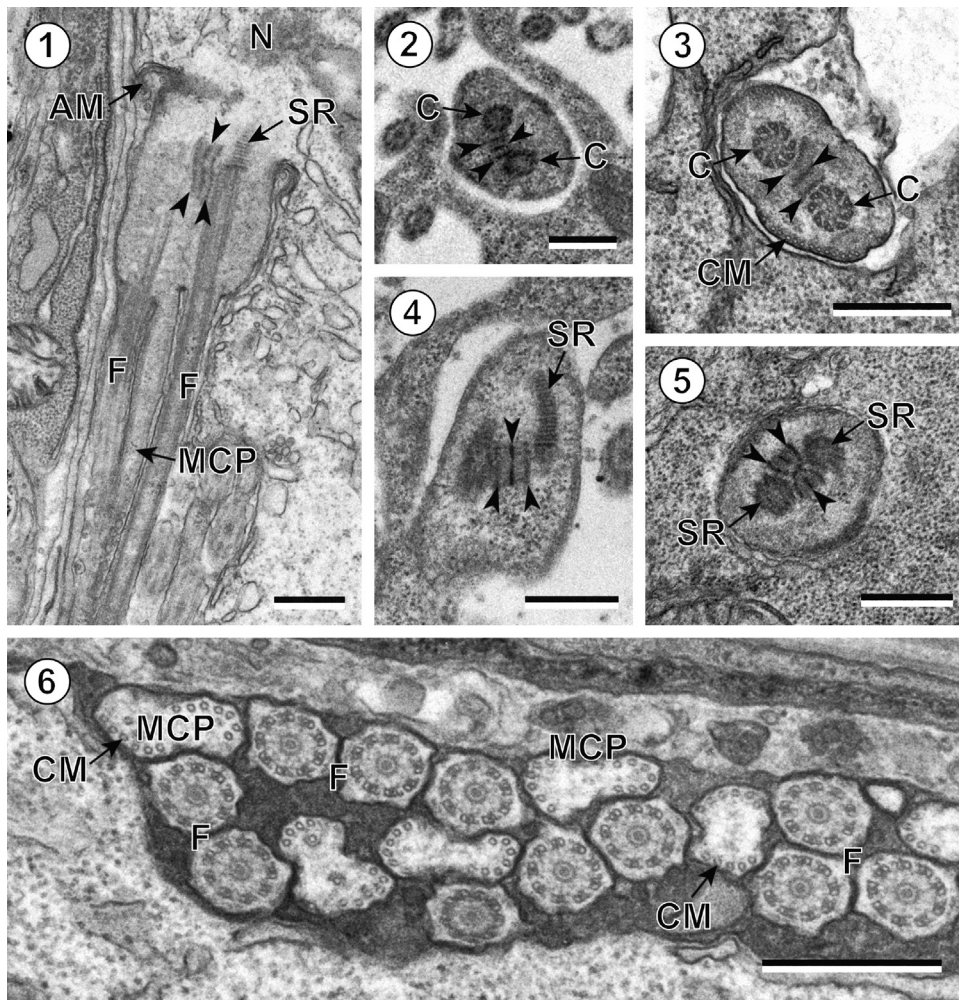
The ultrastructural studies on cestode spermatozoa have proved useful in interpreting their phylogenetic relationships within the Platyhelminthes and could, therefore, provide useful morphological indicators of the phylogeny and/or classification of trypanorhynchs [11–19]. However, in the Trypanorhyncha, studies of spermiogenesis and/or spermatozoa are limited to species belonging to three of the five superfamilies, namely Gymnorhynchoidea, Lacistorhynchoidea and Eutetrarhynchoidea. These are *Grillotia erinaceus* and *Lacistorhynchus tenuis* [20–22] (Lacistorhynchoidea), *Aporhynchus menezesi* [23] (Gymnorhynchoidea), and *Dollfusiiella spinulifera* and *Parachristianella trygonis* [24,25] (Eutetrarhynchoidea).

The aim of this present study is to analyse, for the first time, the spermatological patterns of two species of the unexplored superfamily Tentacularioidea, *Nybelinia queenslandensis* and *Kotorella pronosoma*. The present study also provides supplementary data on the superfamily Eutetrarhynchoidea, with the analysis of spermiogenesis and mature spermatozoon of two progrillotiids, *Progrillotia dasyatidis* and *Progrillotia pastinacae*, and new ultrastructural data concerning spermiogenesis in the eutetrarhynchoidea *Dollfusiiella spinulifera* and *Parachristianella trygonis*. These new ultrastructural observations are focussed in the number of plates, constituting the intercentriolar body during spermiogenesis and in the variability of the arc-like row of thick cortical microtubules present in the anterior areas of the spermatozoon. In fact, these two characters are showed as the most interesting in this order because of its variability according to the species.

2. Materials and methods

Live adult specimens of *N. queenslandensis* and *K. pronosoma* were collected by Prof. Ian Beveridge, University of Melbourne, from *Carcharhinus melanopterus* and *Himantura granulata*, respectively, caught off Lizard Island (Queensland, Australia). *D. spinulifera* was provided by Prof. Malcolm K. Jones from *Rhinobatos typus* collected on the reef flats at Heron Island (Queensland, Australia). *Pro. dasyatidis* and *Pro. pastinacae* were collected from *Dasyatis tortonesei* and *Dasyatis pastinaca*, respectively, caught off Sidi Mansour and Zarzis (Gulf of Gabès, Tunisia). Finally, *P. trygonis* was collected from *Dasyatis pastinaca* caught off Sidi Mansour (Gulf of Gabès, Tunisia). All the three species isolated from Tunisian elasmobranchs were collected in collaboration with Dr Lassad Neifar (University of Sfax).

After dissection, the mature proglottids from these cestodes were routinely processed for transmission electron microscopy examination. Thus, 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, post-fixed in cold (4 °C) 1% osmium tetroxide (OsO₄) with 0.9% potassium ferricyanide [K₃Fe(CN)₆] in the same buffer for 1 h, rinsed in milliQ water, dehydrated in an ethanol series and propylene oxide, and finally, embedded in Spurr's resin. Ultrathin sections (50–60 nm thick) were obtained using a Reichert–Jung Ultracut E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate. The ultrathin sections were examined using a JEOL 1010 TEM operated at an accelerating voltage of 80 kV.



Figs. 1–6. Spermiogenesis in trypanorhynch. (1) Zone of differentiation of *Parachristianella trygonis* before the proximo-distal fusion of the flagella (F) with the median cytoplasmic process (MCP). Note the three electron-dense bands of the intercentriolar body (arrowheads). AM: arched membranes; N: nucleus; SR: striated rootlets. (2–5) Cross-sections showing the number of electron-dense plates of the intercentriolar body (arrowheads) in *Nybelinia queenslandensis* (Fig. 2), *Dollfusiella spinulifera* (Fig. 3), *Kotorella pronosoma* (Fig. 4) and *Progrillotia dasyatidis* (Fig. 5). C: centrioles; CM: cortical microtubules; SR: striated rootlets. (6) Cross-sections of flagella (F) and median cytoplasmic processes (MCP) before the proximo-distal fusion in *Dollfusiella spinulifera*. CM: cortical microtubules. Scale bars = 0.5 μm .

3. Results

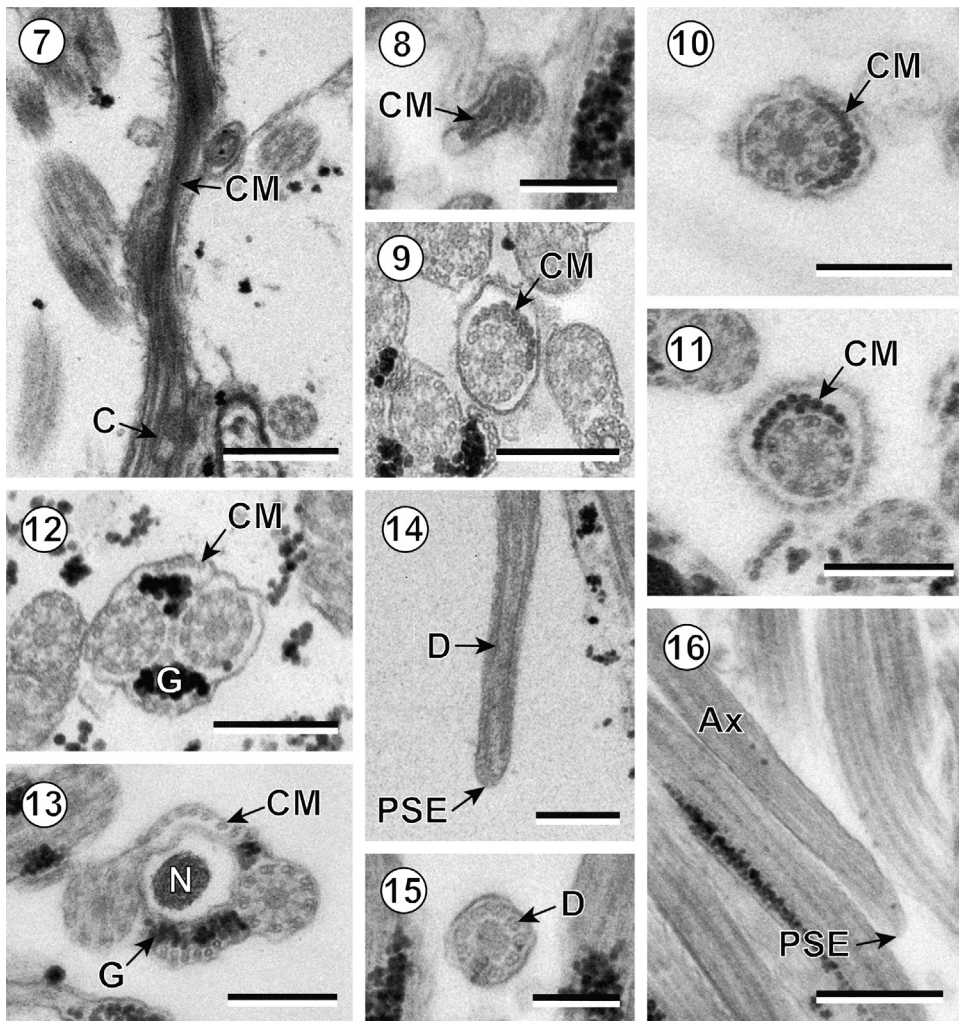
3.1. Spermiogenesis

Spermiogenesis in all the species studied begins with the formation of a differentiation zone in each spermatid. This area is a conical-shaped region bordered by cortical microtubules and delimited at its base by a ring of arching membranes. It also has two centrioles associated with striated rootlets and separated by an intercentriolar body. The intercentriolar body consists of three electron-dense plates separated by electron-lucent ones in all the species studied (*K. pronosoma*, *N. queenslandensis*, *D. spinulifera*, *P. trygonis*, *Pro. dasyatidis* and *Pro. pastinacae*) ((Figs. 1–5)). The two centrioles develop into two orthogonal flagella with respect to a developing median cytoplasmic process. The cortical microtubules lengthen along this growing

median cytoplasmic process (Fig. 6). The nucleus initiates its elongation and the two flagella undergo a flagellar rotation and become parallel to the median cytoplasmic process (Fig. 6). The flagellar rotation is followed by the proximo-distal fusion of the two flagella with the median cytoplasmic process. Finally, the ring of arching membranes is tightened and the young spermatozoon is detached from the residual cytoplasm.

3.2. Spermatozoon

The spermatozoon of trypanorhynch, as in other cestodes, is a long filiform cell, tapered at both ends, which lacks mitochondria. Its cytoplasm contains: (1) two axonemes of different lengths with the 9 + '1' pattern of trepaxonematan Platyhelminthes, (2) an arched row of thick and parallel cortical microtubules near the anterior



Figs. 7–16. Mature spermatozoon in trypanorhynchs. (7) Longitudinal section near the anterior extremity of the spermatozoon of *Kotorella pronosoma*. C: centriole; CM: cortical microtubules. (8) Cross-section of the anterior extremity of the spermatozoon of *Progrillotia pastinacae*. CM: cortical microtubules. (9–11) Cross-sections of anterior areas of the spermatozoon (region I) of *Kotorella pronosoma* (Fig. 9), *Progrillotia pastinacae* (Fig. 10) and *Nybelinia queenslandensis* (Fig. 11) showing the arc-like row of thick cortical microtubules (CM). (12) Cross-section of region II of the spermatozoon of *Kotorella pronosoma* showing the two fields of thin cortical microtubules (CM) and granules of glycogen (G). (13) Cross-section of nuclear area (region III) of the spermatozoon of *Progrillotia pastinacae* showing the parallel disposition of the nucleus (N). CM: cortical microtubules; G: granules of glycogen. (14–16) Cross- and longitudinal sections of region IV of the spermatozoon of *Kotorella pronosoma* (Fig. 14) and *Progrillotia pastinacae* (Figs. 15–16) showing the posterior spermatozoon extremity (PSE) and the disorganization of the axoneme (Ax) into doublets (D). Scale bars = 0.5 μm (Figs. 7 and 16), 0.3 μm (Figs. 9–14), 0.2 μm (Figs. 8 and 15).

extremity, (3) two rows of thin and parallel cortical microtubules, (4) a parallel nucleus, and (5) glycogen.

According to these ultrastructural characteristics, the mature spermatozoon of trypanorhynchs can be subdivided into four arbitrary regions characterized by different ultrastructural features.

Region I (Figs. 7–11) constitutes the anterior extremity of the sperm cell and contains a single axoneme. The anterior tip of the spermatozoon exhibits only a few microtubules. When the axoneme appears, it is surrounded by a submembrane arc of thick and parallel cortical microtubules. In some species, several isolated

cortical microtubules are located between this arc-like layer and the axoneme (Table 1).

Region II (Fig. 12) is the pre-nuclear area containing two axonemes. In this region, the parallel cortical microtubules are thin and arranged in two fields.

Region III (Fig. 13) is the nuclear area of the spermatozoon. The nucleus is elongated and is disposed between the two axonemes. At the end of this region, the cortical microtubules disappear.

Region IV (Figs. 14–16) is the post-nuclear area of the male gamete and constitutes the posterior extremity of the spermatozoon that terminates with a single axoneme.

Table 1
Ultrastructural characters of spermiogenesis and the spermatozoon in the trypanorhynch.

Superfamilies, families and species	Spermiogenesis		Spermatozoon										Reference
	Type ^a	IB	Type ^b	ASE	Ax	CB	ArcCM (n)	M	CM	N	G	PSE	
Tentacularioidea													
Tentaculariidae													
<i>Kotorella pronosoma</i>	I	3	I	CM	2	–	+(10+3)	–	0°	0°	+	1Ax	[Present study]
<i>Nybelinia queenslandensis</i>	I	3	I	?	2	–	+(10+1)	–	0°	0°	+	1Ax	[Present study]
Gymnorhynchoidea													
Aporhynchidae													
<i>Aporhynchus menezesi</i>	I	5	I	CM	2	–	+(7)	–	0°	0°	+	1Ax	[23]
Lacistorhynchoidea													
Lacistorhynchidae													
<i>Grillotia erinaceus</i>	I	7	I	?	2	–	?	–	0°	0°	+	?	[20]
<i>Lacisthynchus tenuis</i>	I	5	I	?	2	–	?	–	0°	0°	+	?	[21,22]
Eutetrarhynchoidea													
Eutetrarhynchidae													
<i>Dollfusella spinulifera</i>	I	3	I	CM	2	–	+(10)	–	0°	0°	+	1Ax	[24, present study]
<i>Parachristianella trygonis</i>	I	3	I	CM	2	–	+(10)	–	0°	0°	+	1Ax	[25, present study]
Progrillotiidae													
<i>Progrillotia dasytidis</i>	I	3	I	?	2	–	+(?)	–	0°	0°	+	1Ax	[Present study]
<i>Progrillotia pastinacae</i>	I	?	I	CM	2	–	+(10+2)	–	0°	0°	+	1Ax	[Present study]

ArcCM: arc-like row of cortical microtubules; ASE: anterior spermatozoon extremity; Ax: axoneme; CB: crested bodies; CM: cortical microtubules; G: granules of glycogen; IB: intercentriolar body; M: mitochondrion; n: maximum number of microtubules in the arc-like row of cortical microtubules; N: nucleus; PSE: posterior spermatozoon extremity; +/-: presence/absence of considered character; ?: unknown data.

^a Type of spermiogenesis is according to Bâ and Marchand [14].

^b Type of spermatozoon is according to Levron et al. [19]

4. Discussion

4.1. Spermiogenesis

Spermiogenesis in trypanorhynch is of type I of Bâ and Marchand [7]. This pattern is characterized by the growth of two orthogonal flagella with respect to a developing median cytoplasmic process. The two flagella undergo a flagellar rotation and become parallel to the median cytoplasmic process. Later, they fuse in a proximo-distal pattern and produce a spermatozoon with two axonemes.

The zone of differentiation is a conical-shaped region that contains two centrioles associated to two striated rootlets and exhibits an intercentriolar body between them. The intercentriolar body consists of a variable number of electron-dense plates according to the species. In our study we present new data on six species, namely *D. spinulifera*, *P. trygonis*, *Pro. dasytidis* and *Pro. pastinacae* (Eutetrarhynchoidea), and *K. pronosoma* and *N. queenslandensis* (Tentacularioidea). In all of these species except for *Pro. pastinacae*, the intercentriolar body consists of three electron-dense plates. These are the species belonging to the Tentacularioidea and Eutetrarhynchoidea (Table 1). In previous studies, Świdorski [22] describes seven electron-dense plates in *Lacisthynchus tenuis*, and McKerr [20] and Marigo et al. [23] describe five electron-dense plates in *Grillotia erinaceus* and *Aporhynchus menezesi*, respectively. These three species are included in two other superfamilies, namely the Gymnorhynchoidea and the Lacistorhynchoidea (Table 1).

4.2. Spermatozoon

The spermatozoon of trypanorhynch, as in other cestodes, is a long filiform cell, tapered at both ends, which lacks mitochondria. Its cytoplasm contains: (1) two axonemes of different lengths with the 9+1' trepaxonemat pattern [26], (2) an arched row of thick, parallel cortical microtubules near the anterior extremity, (3) two rows of thin, parallel cortical microtubules, (4) an elongated nucleus parallel to axonemes, and (5) glycogen in the form of α -glycogen rosettes and/or β -glycogen particles. Thus, the anterior and posterior extremities of the spermatozoon exhibit cortical microtubules and a single axoneme, respectively (Fig. 17).

In fact, the ultrastructural organization of the mature spermatozoa in trypanorhynch follows the type I of Levron et al. [19]. This pattern is also present in the orders Spathebothriidea, Diphyllbothriidea and Haplobothriidea and it is characterized by the presence of two axonemes and by the parallel disposition of both cortical microtubules and nucleus. The type I spermatozoon also lacks a crested body, a periaxonemal sheath and intracytoplasmic walls [19].

Unlike the majority of cestodes, trypanorhynch spermatozoa lack a crested body or bodies [23–25, present study], and, consequently, the postulated synapomorphy of crested body or bodies for the Eucestoda is questionable [16,17,19]. There are other eucestode orders that also lack these anterior helical structures, e.g. the Caryophyllidea, the Spathebothriidea and the Diphyllbothriidea [27–38].

The arc-like row of cortical microtubules has been observed in the anterior part of the spermatozoon of

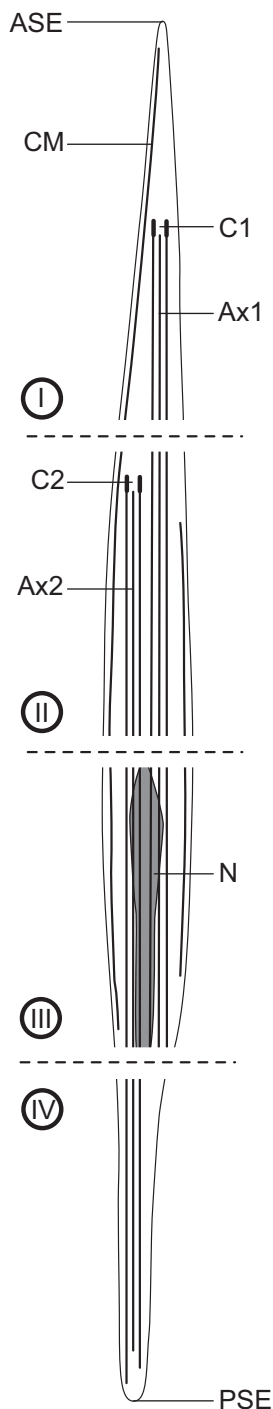


Fig. 17. Schematic drawing of the mature spermatozoon of trypanorhynch species. ASE: anterior spermatozoon extremity; Ax1: first axoneme; Ax2: second axoneme; C1: centriole of the first axoneme; C2: centriole of the second axoneme; CM: cortical microtubules; N: nucleus; PSE: posterior spermatozoon extremity.

numerous eucestodes, but there is variability in their aspect and number of elements. In trypanorhynchs the number of cortical microtubules forming this arc-like layer varies from seven to ten microtubules. Thus, *K. pronosoma*,

N. queenslandensis, *D. spinulifera*, *P. trygonis* and *Pro. pastinacae* possess an arc-like row consisting of ten microtubules [24,25, present study], whereas according to Marigo *et al.* [23], in *A. menezesi* the maximum number is seven (Table 1). Anterior spermatozoon extremities showing a similar arrangement of cortical microtubules describing an arc-like layer are found in caryophyllideans [30,31,34,36,37], spathebothriideans [28,33], the former pseudophyllideans (Diphyllobothriidea and Bothriocephalidea) [29,32,39–43], the diphyllideans [44,45], the tetraphyllideans [46–50], the proteocephalideans [51–54], and in the mesocestoidid cyclophyllideans [55,56]. However, an arc-like layer consisting of thick cortical microtubules, as described in the trypanorhynchs, occurs only in the former pseudophyllideans, in the tetraphyllideans, in the proteocephalideans and in the mesocestoidids [29,32,39–42,46–56].

5. Concluding remarks

The results presented both here and in the previous studies indicate insignificant ultrastructural differences between the spermatological characters of the seven species studied and offer no additional support for the hypothesis, suggested by some authors that the trypanorhynchs are a polyphyletic group. However, these slight differences of sperm ultrastructure are coincident with the view of these authors [4,7–10]. The most remarkable differences between these species are (i) the number of electron-dense plates constituting the intercentriolar body (5 to 7 in the Gymnorhynchoidea and the Lacistorhynchoidea versus 3 in the Tentacularioidea and the Eutetrarhynchoidea) and (ii) the number of microtubules that form the arc-layer of thick cortical microtubules present in the anterior spermatozoon extremity (around 7 in the Gymnorhynchoidea and the Lacistorhynchoidea versus around 10 in the Tentacularioidea and the Eutetrarhynchoidea).

Disclosure of interest

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

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