

Sperm ultrastructure of *Helicometra epinepheli* (Platyhelminthes, Digenea, Opecoelidae), parasite of *Epinephelus fasciatus* (Pisces, Teleostei)

Y. Quilichini¹, J. Foata¹, J-L. Justine^{2,3}, R.A. Bray⁴ and B. Marchand¹

¹CNRS UMR 6134, University of Corsica, "Parasites and Mediterranean Ecosystems" Laboratory, Corte, France, ²UMR 7138 Systématique, Adaptation, Evolution, Muséum National d'Histoire Naturelle, Paris, France, ³Aquarium des Lagons, Nouméa, Nouvelle-Calédonie and ⁴Department of Zoology, Natural History Museum, London, UK

Summary. This paper describes the ultrastructure of the mature spermatozoon of the digenean *Helicometra epinepheli*. The ultrastructural elements observed are: two axonemes with a 9+“1” pattern of Trepaxonemata, four attachment zones, a nucleus, two mitochondria, external ornamentation of the plasma membrane, spine-like bodies and cortical microtubules. A particularity of this spermatozoon is the presence of an apical cone. However, the spermatozoon presents the general pattern of the Opecoelidae and thus differs from *Helicometra fasciata* (which appears as an exception in this family) by several ultrastructural features: the presence of external ornamentation and spine-like bodies and the arrangement of cortical microtubules around the first mitochondrion in the region of the ornamentation.

Each ultrastructural feature is discussed and compared with the literature to highlight characters which could be useful for phylogeny.

Key words: Spermatozoon, *Helicometra epinepheli*, Digenea, Platyhelminthes, TEM

Introduction

For many years, the spermatological study of the Platyhelminthes has been used in phylogeny and contributed to our knowledge of the relationships between taxa of this group (Ehlers, 1984, 1986; Justine, 1991, 1995, 1997, 1998; Bâ and Marchand, 1995;

Levron et al., 2010).

Several groups of Platyhelminthes have recently been the subject of detailed phylogenetic analyses based on spermatogenesis, e.g., the Eucestoda (Levron et al., 2010). However, the great number of families and genera and the low number of species studied so far (less than 1%) does not enable us to perform such a study for the Digenea.

In this paper we reconstitute the spermatozoon of the digenean *Helicometra epinepheli* Yamaguti, 1934. To date the spermatozoa of five Opecoelidae have been studied: *Helicometra fasciata* (Levron et al., 2003), *Nicolla testiobliquum* (Quilichini et al., 2007a), *Nicolla wisniewskii* (Quilichini et al., 2007c), *Opecoeloides furcatus* (Miquel et al., 2000) and *Poracanthium furcatum* (Levron et al., 2004b). We have chosen a *Helicometra* species because the spermatozoon of *H. fasciata* presents significant ultrastructural differences to other Opecoelidae: i.e., the absence of external ornamentation of the plasma membrane, the absence of spine-like bodies and the absence of the organisation of cortical microtubules in a semi-circle around the first mitochondrion. The aim of this study is to compare the spermatozoon of *H. epinepheli* with the other digenean spermatozoa and more especially with the Opecoelidae in order to highlight criteria interesting for phylogeny.

Materials and methods

Helicometra epinepheli Yamaguti, 1934 has been recorded from three species of groupers off New Caledonia and its synonymy has been discussed (Justine et al., 2010). Adult specimens were collected live from

the intestine of naturally infected blacktip grouper *Epinephelus fasciatus* (Forsskål, 1775) (Pisces, Teleostei) caught off Nouméa, New Caledonia (South West Pacific Ocean).

Worms were removed from their hosts, fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, rinsed in 0.1 M sodium cacodylate buffer at pH 7.2, post-fixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ethanol and propylene oxide, embedded in Spurr (Spurr, 1969) and polymerised at 60°C for 24 h.

Ultra-thin sections (60-90 nm) of the seminal vesicle were cut on an ultramicrotome (Power tome PC, RMC Boeckeler®). Sections were placed on 300 and 200-mesh copper grids and stained with uranyl acetate and lead citrate (Reynolds 1963).

Sections were examined on a Hitachi H-600 transmission electron microscope, operating at an accelerating voltage of 75 kV, in the "Service d'Étude et de Recherche en Microscopie Électronique" of the University of Corsica (Corte, France).

Results

Numerous observations of cross and longitudinal sections of the spermatozoon of *Helicometra epinepheli* showed different ultrastructural characters: an apical cone, 2 axonemes with a 9+“1” pattern, 4 attachment zones, cortical microtubules, external ornamentation of the plasma membrane, spine-like bodies, 2 mitochondria and a nucleus. These ultrastructural features enable us to reconstitute this spermatozoon. Four regions (regions I-IV) have been considered.

Region I (Figs. 1, 4): anterior region of the spermatozoon

The anterior extremity of the spermatozoon is characterised by an apical cone (Fig. 1a, b). In cross section an electron-dense material in the centre of the cone is observed (Fig. 1b).

The shift of the second axoneme is observed in figure 1c. Indeed, the first axoneme is completely formed with nine doublets of microtubules with arms, a central core and filaments connecting the peripheral doublets with the central core, whereas the second axoneme presents only seven doublets and two singlets of microtubules without arms, filaments or a central core. Moreover the anterior extremity of the second axoneme has a central position in the spermatozoon. At this level the spermatozoon presents a lateral expansion characterised by a thicker plasma membrane.

Figure 1d shows the middle part of this region. At this level the two axonemes are completely formed and possess a 9+“1” pattern. The four attachment zones are present and four cortical microtubules are counted. This number increases to the posterior end of this region: 8 (Fig. 1e) and 9 (Fig. 1f). The anterior extremity of the first mitochondrion is observed in the Figure 1f.

Region II (Figs. 2a-e, 4): in this region the two axonemes, their four attachment zones and the first mitochondrion are still present

The characteristics of this region are the presence of external ornamentation (Fig. 2a-e) and spine-like bodies (Fig. 2c,d) and the number and the disposition of the cortical microtubules (Fig. 2a,c,e).

The position of the mitochondrion enables us to distinguish the ventral side (mitochondrial side) of the spermatozoon (Fig. 2a).

In this region the number of cortical microtubules increases from the anterior part to the posterior part: 12 (Fig. 2a) and 13 (Fig. 2c,e). This number increases only in the dorsal side (from 1 to 2) and is constant (11) in the ventral side. Moreover, the microtubules observed on the ventral side are disposed in a semi-circle around the mitochondrion.

The external ornamentation of the plasma membrane and the spine-like bodies are located on the ventral side of the spermatozoon.

The posterior part of this region is characterised by the posterior extremity of the first mitochondrion (Fig. 2e).

Region III (Figs. 2f-h, 4): this region is characterised by the two axonemes, the four attachment zones, and cortical microtubules

The first mitochondrion, the external ornamentation and the spine-like bodies are absent in this region.

The cortical microtubules are arranged as two fields located between the two axonemes and their number increases from the anterior to the posterior part of this region: 13 (Fig. 2f); 14 (Fig. 2g) and 17 (Fig. 2h).

Region IV (Figs. 3, 4): this region corresponds to the posterior part of the spermatozoon

The anterior part of this region is characterised by the anterior extremity of the second mitochondrion (Fig. 3a) and of the nucleus (Fig. 3b) associated, respectively, with 17 and 18 cortical microtubules. We observe then the widening of the nucleus (Fig. 3c).

Figure 3d shows 21 cortical microtubules and the posterior extremity of the first axoneme characterised by the presence of the central core located between two attachment zones.

The middle part of this region presents one axoneme, the second mitochondrion, a wide nucleus and 23 cortical microtubules (Fig. 3e).

The posterior extremities of the second mitochondrion and of the second axoneme are located at about the same level and are associated with 26 cortical microtubules (Fig. 3f).

Distally, the posterior extremity of the nucleus is surrounded by 27 cortical microtubules (Fig. 3g). This is the maximum number of cortical microtubules in the

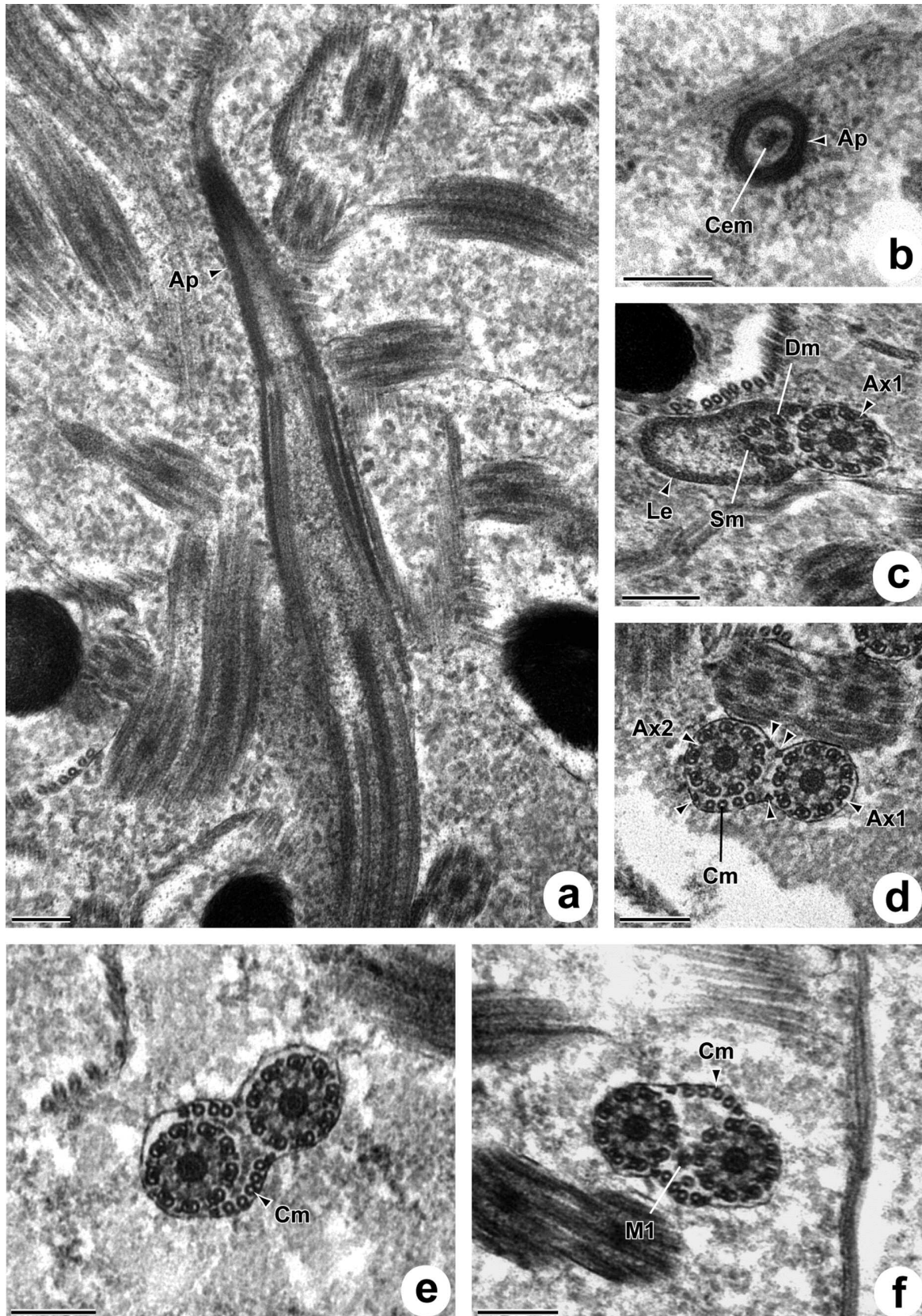
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Fig. 1. Cross and longitudinal sections of region I of the spermatozoon of *Helicometra epinepheli*. **a.** Longitudinal section of the anterior extremity of the spermatozoon showing the apical cone. **b.** Cross section of the apical cone showing the central electron-dense material. **c.** Anterior extremity of the completely formed first axoneme, shift of the second characterised by only singlets and doublets of microtubules and lateral expansion. **d.** Cross section showing 2 axonemes completely formed, 4 attachment zones and 4 cortical microtubules. **e.** 8 cortical microtubules. **f.** 9 cortical microtubules and anterior extremity of the first mitochondrion. Arrowheads indicate the attachment zones. Ap, apical cone; Ax1, first axoneme; Ax2, second axoneme; Cem, central electron-dense material; Cm, cortical microtubule; Dm, doublet of microtubules; M1, first mitochondrion; Sm, singlet of microtubule. Bar: 0.2 μm .

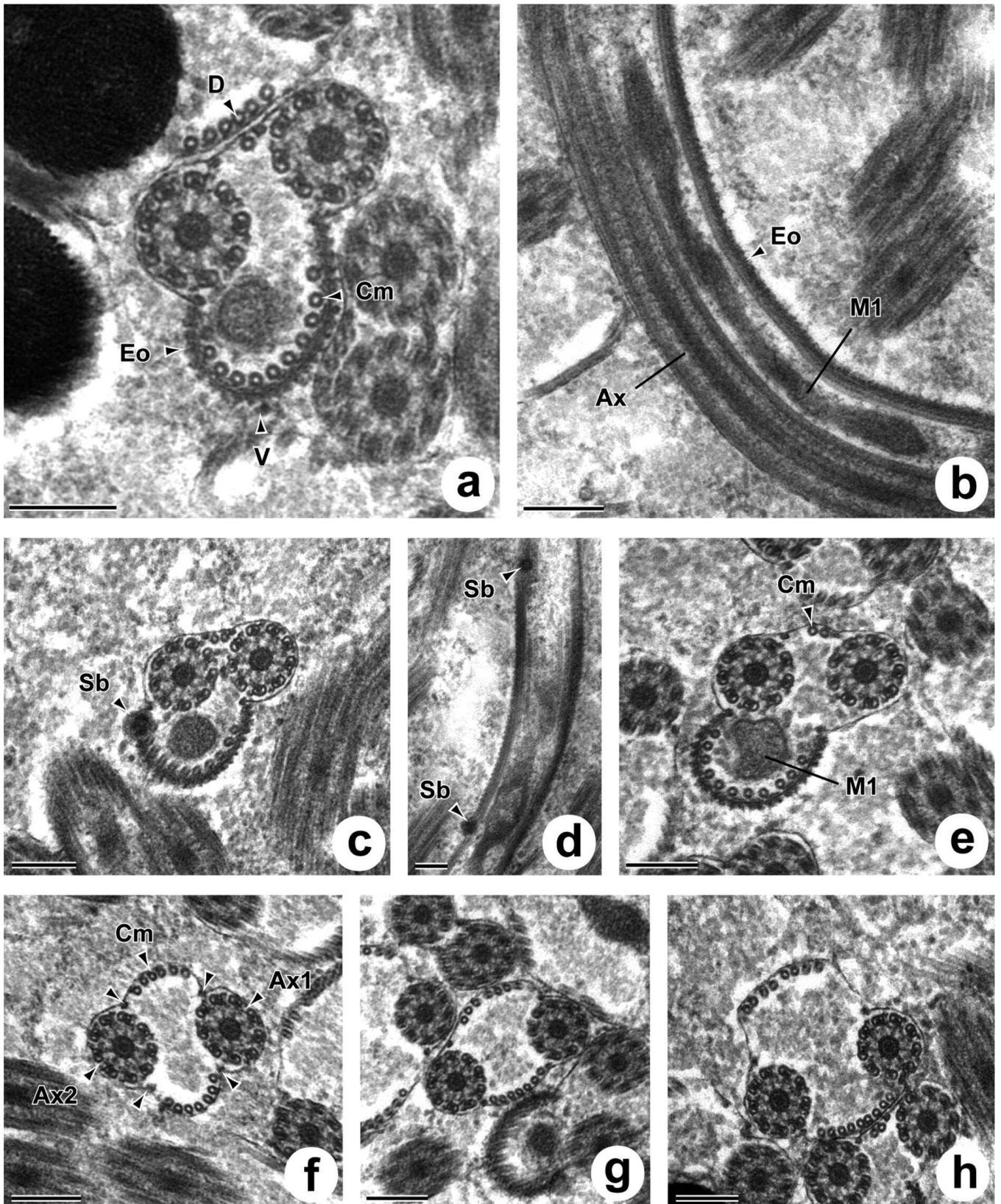


Fig. 2. Cross and longitudinal sections of region II (a-e) and of region III (f-h) of the spermatozoon of *Helicometra epinepheli*. **a.** Cross section showing the dorsal and the ventral (mitochondrial) side, 12 cortical microtubules and external ornamentation. **b.** Longitudinal section showing one axoneme, the first mitochondrion and the external ornamentation. **c.** Cross section showing a spine-like body and 13 cortical microtubules. **d.** Longitudinal section showing spine-like bodies. **e.** Posterior extremity of the first mitochondrion and 13 cortical microtubules. **f.** Cross section showing the 2 axonemes, the 4 attachment zones and 13 cortical microtubules. **g.** 14 cortical microtubules. **h.** 17 cortical microtubules. Arrowheads indicate the attachment zones. Ax, axoneme; Ax1, first axoneme; Ax2, second axoneme; Cm, cortical microtubule; D, dorsal side; Eo, external ornamentation; M1, first mitochondrion; Sb, spine like body; V, ventral side. Bar: 0.2 μm .

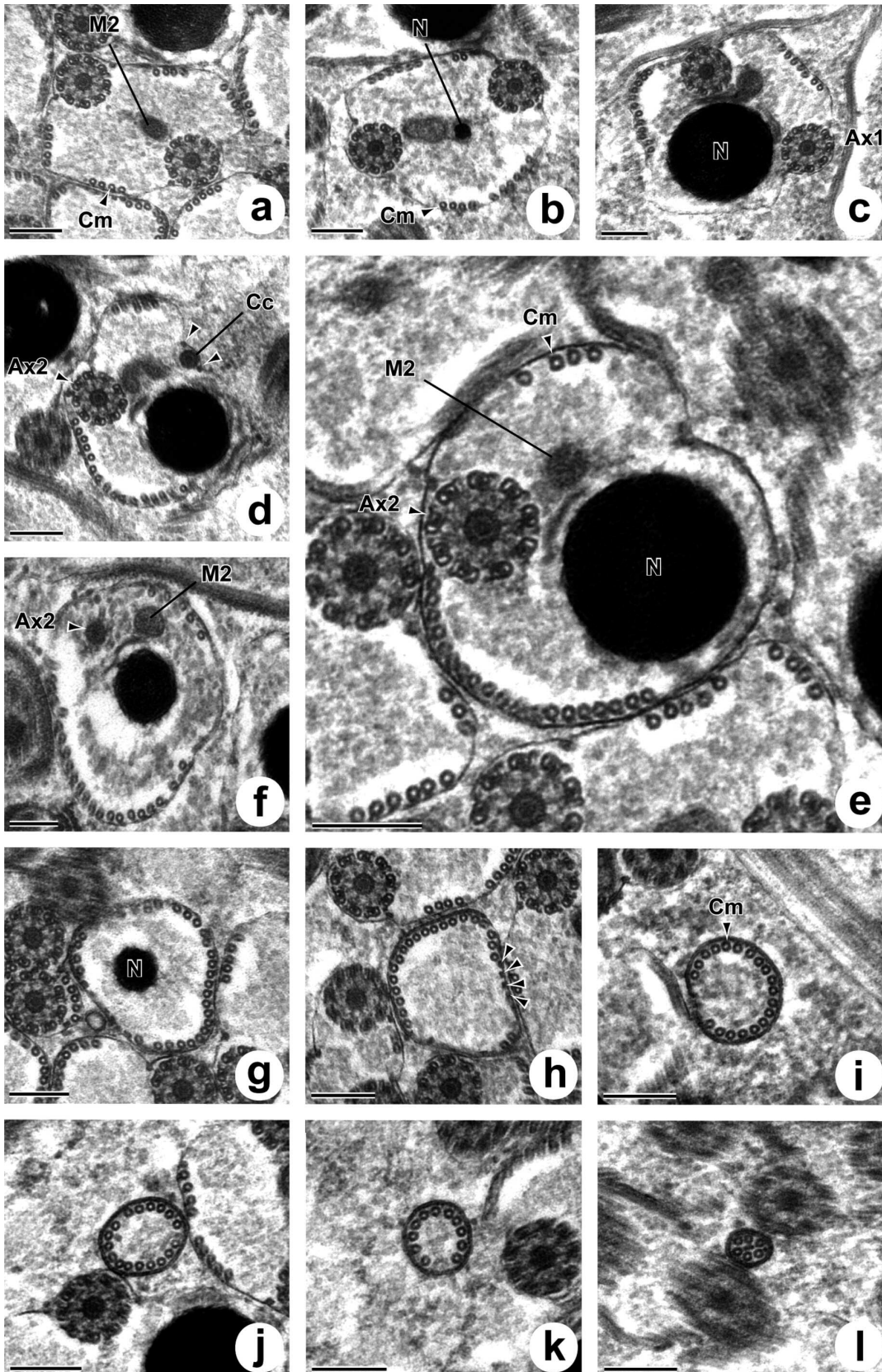
Sperm of *Helicometra epinepheli*

Fig. 3. Cross sections of region IV of the spermatozoon of *Helicometra epinepheli*. **a.** Anterior extremity of the second mitochondrion and 17 cortical microtubules. **b.** Anterior extremity of the nucleus and 18 cortical microtubules. **c.** Widening of the nucleus. **d.** Posterior extremity of the first axoneme characterised by a central core and 21 cortical microtubules. **e.** The second axoneme, a nucleus, the second mitochondrion and 23 cortical microtubules. **f.** Posterior extremities of the second axoneme and of the second mitochondrion and 26 cortical microtubules. **g.** Posterior extremity of the nucleus and 27 cortical microtubules. **h.** 22 cortical microtubules and attachment zones. **i.** 19 cortical microtubules. **j.** 17 cortical microtubules. **k.** 11 cortical microtubules. **l.** 6 cortical microtubules. Arrowheads indicate the attachment zones. Ax1, first axoneme; Ax2, second axoneme; Cc, central core; Cm, cortical microtubule; M2, second mitochondrion; N, nucleus. Bar: 0.2 μ m.

Table 1. Some ultrastructural characteristics of digenean spermatozoa.

Families/Digenean species	Le	Eo	Sb	M	Cmsc	Pp type	References
Allocreadiidae							
<i>Crepidostomum metoecus</i>	+	+	+	2		3	(Quilichini et al. 2007d)
Apocreadiidae							
<i>Neoapocreadium chabaudi</i>	-	+	+	2			(Kacem et al. 2010)
Brachylaimidae							
<i>Brachylaimus aequans</i>	-	-	-	1			(Zdarska et al. 1991)
<i>Scaphiostomum palaearticum</i>	+	+	-	1			(Ndiaye et al. 2002)
Bucephalidae							
<i>Prosorhynchoides borealis</i> (as <i>Bucephaloides gracilescens</i>)	-	+	-	1			(Erwin & Halton 1983; Bartoli et al. 2006)
<i>Pseudorhipidocotyle elpichthys</i>	-	+	-	1			(Tang et al. 1998)
Cryptogonimidae							
<i>Adlardia novaecaledoniae</i> (as <i>Siphoderina elongata</i>)	-	+	+	2	11	3	(Miller et al. 2009; Quilichini et al. 2009)
<i>Anisocoelium capitellatum</i>	-	+	+	3	8	3	(Ternengo et al. 2009)
<i>Stemmatostoma pearsoni</i>	-	+	-	2	8	3	(Jamieson & Daddow 1982)
Deropristidae							
<i>Deropristis inflata</i>	-	+	-	2			(Foata et al. 2007)
Dicrocoeliidae							
<i>Dicrocoelium chinensis</i>	-	-	-	1			(Tang 1996)
<i>Dicrocoelium dendriticum</i>	-	-	-	1			(Cifrian et al. 1993)
<i>Dicrocoelium hospes</i>	+	+	+	2		2	(Agostini et al. 2005)
<i>Corrigia vitta</i>	-	-	-	1			(Robinson & Halton 1982)
Didymozoidae							
<i>Didymozoon</i> sp.	-	-	-	1			(Justine & Mattei 1983)
<i>Gonapodasmius</i> sp.	-	+	-	1			(Justine & Mattei 1982a; Justine & Mattei 1984b)
<i>Didymocystis wedli</i>	-	-	-	1			(Pamplona-Basilio et al. 2001)
Echinostomatidae							
<i>Echinostoma caproni</i>	+	+	+	1			(Iomini & Justine 1997)
Fasciolidae							
<i>Fasciola hepatica</i>	+	+	+	2		2	(Stitt & Fairweather 1990; et al. 2003a)
<i>Fasciola gigantica</i>	+	+	+	1		2	(Ndiaye et al. 2004)
Felodistomidae							
<i>Proctoeces maculatus</i>	-	+	-	1			(Justine 1995)
<i>Pronoprymna ventricosa</i>	-	+	-	1		3	(Quilichini et al. 2007b)
Gastrothylacidae							
<i>Carmyerius endopapillatus</i>	+	+	+	1		2	(Seck et al. 2008a)
Haematoloechidae							
<i>Haematoloechus medioplexus</i>	+	+	-	1			(Justine & Mattei 1982b)
Haploporidae							
<i>Saccocoelioides godoyi</i>	-	+	-	1			(Baptista-Farias et al. 2001)
Heterophyidae							
<i>Cryptocotyle lingua</i>	-	-	-	1			(Rees 1979)
Lecithasteridae							
<i>Aponurus laguncula</i>	-	+	-	1			(Quilichini et al. 2010a)
Lecithodendriidae (sensu lato)							
<i>Ganeo tigrinum</i>	-	-	-	1			(Sharma & Rai 1995)
Monorchidae							
<i>Monorchis parvus</i>	-	+	-	2			(Levron et al. 2004c)
Microphallidae							
<i>Maritrema linguilla</i>	-	-	-	2			(Hendow & James 1988)
Notocotylidae							
<i>Notocotylus neyrari</i>	-	+	+	2		3	(Ndiaye et al. 2003b)
Opecoelidae							
<i>Helicometra epinepheli</i>	+	+	+	2	11	1	Present study
<i>Helicometra fasciata</i>	+	-	-	1		1	(Levron et al. 2003)
<i>Nicolla testibliquum</i>	-	+	+	2	15	1	(Quilichini et al. 2007a)
<i>Nicolla wisniewskii</i>	-	+	+	2	15	1	(Quilichini et al. 2007c)
<i>Opecoeloides furcatus</i>	-	+	+	1	12	1	(Miquel et al. 2000)
<i>Poracanthium furcatum</i>	+	+	+	2	12	1	(Levron et al. 2004b)
Opistholebetidae							
<i>Heterolebes maculosus</i>	-	+	+	2	11	1	(Quilichini et al. 2010b)
Paragonimidae							
<i>Paragonimus miyazakii</i>	-	-	-	>1			(Sato et al. 1967)
<i>Paragonimus ohirai</i>	-	+	+	2			(Orido 1988)
Paramphistomidae							
<i>Cotylophoron cotylophorum</i>	+	+	+	1			(Seck et al. 2008b)
<i>Paramphistomum microbothrium</i>	+	+	+	1			(Seck et al. 2007)
Phaneropsolidae							
<i>Postorchigenes gymnesicus</i>	-	+	-	2		3	(Gracenea et al. 1997)
Troglotrematidae							
<i>Troglotrema acutum</i>	-	+	+	2		3	(Miquel et al. 2006)
Zoogonidae							
<i>Diphterostomum brusinae</i>	-	+	-	1		2	(Levron et al. 2004a)

+/-, presence/absence of considered character; empty fields indicate a lack of information concerning the considered criterion; Cms, number of cortical microtubules disposed in semi-circle; Eo, external ornamentation; Le, lateral expansion; M, number of mitochondrion; Pp type, posterior part type of the spermatozoon according to (Quilichini et al. 2010b); Sb, spine-like bodies.

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entire spermatozoon. Then, this number decreases progressively to the posterior extremity of the spermatozoon: 22 (Fig. 3h), 19 (Fig. 3i), 17 (Fig. 3j), 11 (Fig. 3k) and 6 (Fig. 3l). This decrease is associated with the narrowing of the spermatozoon.

We can also notice that the attachment zones remain visible in the posterior part of the spermatozoon (Fig. 3h).

Discussion

The anterior extremity of the spermatozoon

The anterior extremity of the spermatozoon of *Helicometra epinepheli* forms a cone-like structure. This cone is delimited by a wide electron dense wall. To our knowledge it is the first time that such a structure has been described in a digenean spermatozoon. Indeed, a fine undulated expansion or a flexible shape have already been observed in the Opcoelidae (*Nicolla wisniewskii* (Quilichini et al., 2007c)) or in other families of digenean (Dicrocoeliidae: *Dicrocoelium hospes* (Agostini et al., 2005); Opistholebetidae: *Heterolebes maculosus* (Quilichini et al., 2010b)) but never with this aspect of rigidity. This structure can be compared with the apical cone found in different groups of Cestoda: i.e. in some Proteocephalidea (*Nomimoscolex sp.* (Sène et al., 1997) and *Sandonella sandoni* (Bâ and Marchand, 1994)) and especially in Cyclophyllidea. Recently, the character "apical cone" (presence or absence) has been used in a phylogenetic study (Levron et al., 2010), but the definition of apical cone need to be clarified in this groups because the same term defines different structures.

The anterior part of the spermatozoon also presents a lateral expansion with an electron dense wall. This structure is probably the prolongation of the apical cone. Lateral expansions have already been observed in digenean spermatozoa (Table 1). Within the Opcoelidae studied so far, only *Helicometra fasciata* (Levron et al., 2003) and *Poracanthium furcatum* (Levron et al., 2004b) possess such structures.

The axonemes

The spermatozoon of *H. epinepheli* possesses two axonemes with a 9+1 pattern of Trepaxonemata and confirms the homogeneity of the Digenea concerning this criterion. Indeed, in all the literature on digenean spermatology, only *Didymozoon sp.* possesses two axonemes with a 9+0 pattern (Justine and Mattei, 1983, 1984a).

The attachment zones, corresponding to the fusion lines of the flagella with the median cytoplasmic process during spermiogenesis, are observed in the entire spermatozoon except at its anterior and posterior extremities. Moreover, it is interesting to notice that these elements remain visible, and not associated with the axonemes, in the posterior part of the spermatozoon.

We could not determine exactly the length of the axonemes but we noticed a small shift between them. This shift is also found in the majority of digenean spermatozoa.

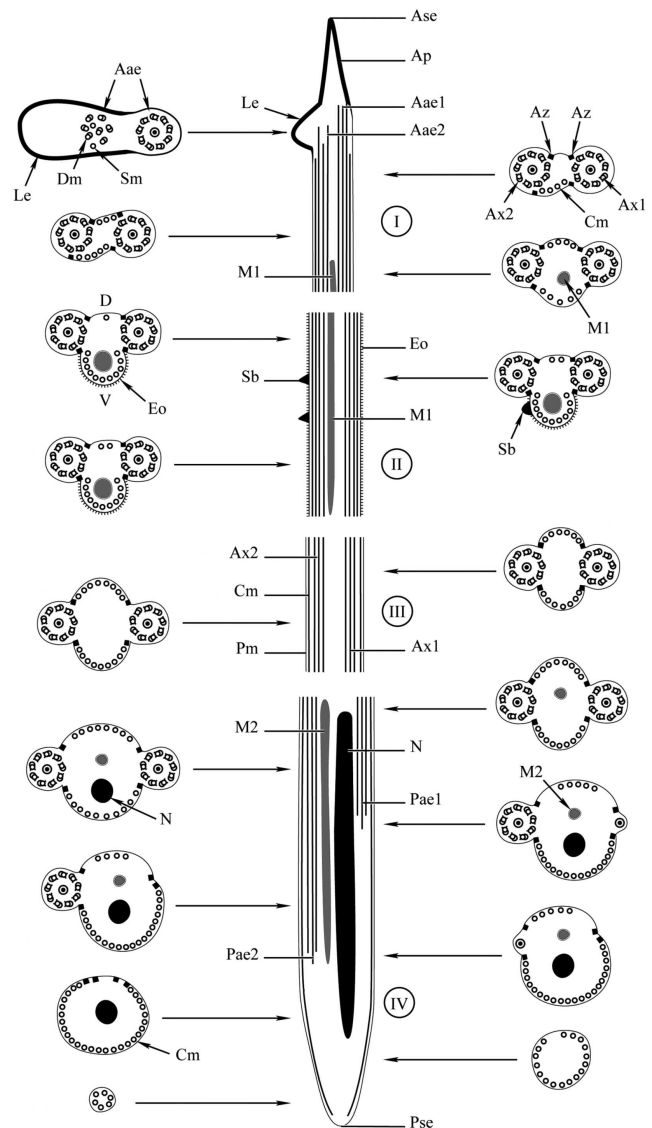


Fig. 4. Diagram showing the ultrastructural organisation of the mature spermatozoon of *Helicometra epinepheli*. (I) Region I. (II) Region II. (III) Region III. (IV) Region IV. Aae, axoneme anterior extremity; Aae1, anterior extremity of the first axoneme; Aae2, anterior extremity of the second axoneme; Ap, apical cone; Ase, anterior spermatozoon extremity; Ax1, first axoneme; Ax2, second axoneme; Az, attachment zones; Cm, cortical microtubule; D, dorsal side; Dm, doublet of microtubules; Eo, external ornamentation; Le, lateral expansion; M1, first mitochondrion; M2, second mitochondrion; N, nucleus; Pae1, posterior extremity of the first axoneme; Pae2, posterior extremity of the second axoneme; Pm, plasma membrane; Pse, posterior spermatozoon extremity; Sb, spine-like body; Sm, singlet of microtubule; V, ventral side.

The external ornamentation and spine-like bodies

The external ornamentation of the plasma membrane and spine-like bodies have been observed in the second region of the spermatozoon of *H. epinepheli*. These elements have been described in several digenean spermatozoa (Table 1).

The presence or absence of this element is the principal difference between the two species of *Helicometra* studied so far. In *Helicometra fasciata* (Levron et al., 2003) this element has not been described whereas in *H. epinepheli* and in the other Opecoelidae (Miquel et al., 2000; Levron et al., 2004b; Quilichini et al., 2007a,c) this element is present.

Moreover we notice that in all the spermatozoa of Opecoelidae this ornamentation is on the ventral side (mitochondrial side). This observation has also been made in the *Cryptogonimidae* (Jamieson and Daddow, 1982; Quilichini et al., 2009; Ternengo et al., 2009) and in the *Opistholebetidae* (Quilichini et al., 2010b).

The spine-like bodies observed on the spermatozoon of *H. epinepheli* are always associated with the external ornamentation as in other spermatozoa of digeneans excepted in *Neopocreadium chabaudi* (Kacem et al., 2010). Indeed, this apocreadiid has a spermatozoon with 3 spine-like bodies at the same level and without association with the external ornamentation of the plasma membrane. It appears that the number and the disposition of these elements varie from one species to another, and it is not unlikely that in certain species these very small elements were not clearly observed by the authors.

The mitochondria

The presence or absence of mitochondrion is a spermatological character commonly used in phylogeny of the Platyhelminthes (Ehlers, 1986; Rohde, 1990; Justine, 1991; Levron et al., 2010). Concerning the digenean, all the species studied possess at least one mitochondrion. But this number can vary from 1 to 3 (Table 1). This variability can be observed within the same family. Indeed, within the *Opecoelidae*, *Helicometra epinepheli* possesses 2 mitochondria, like *Nicolla testiobliquum* (Quilichini et al. 2007a), *Nicolla wisniewskii* (Quilichini et al., 2007c) and *Poracanthium furcatum* (Levron et al., 2004b), whereas *Helicometra fasciata* (Levron et al., 2003) and *Opecoeloides furcatus* (Miquel et al., 2000) possess only one mitochondrion.

The cortical microtubules

The observation of a great number of cross-sections of the spermatozoon of *Helicometra epinepheli* confirms to us that the organisation of the cortical microtubules is well defined.

Their arrangement in region II is characterised by 11 elements in the ventral side associated with the external

ornamentation of the plasma membrane. These microtubules are arranged as a semi-circle around the first mitochondrion. Such organisation has been found in three families of digeneans (*Cryptogonimidae*, *Opecoelidae*, *Opistholebetidae*) (Table 1) but with differing numbers of cortical microtubules: 8 in *Anisocoelium capitellatum* (Ternengo et al., 2009) and *Stemmatostoma pearsoni* (Jamieson and Daddow, 1982), 11 in *Adlardia novaecaledonia* (as *Siphoderina elongata*) (Miller et al., 2009; Quilichini et al., 2009) and *Heterolebes maculosus* (Quilichini et al., 2010b), 12 in *Opecoeloides furcatus* (Miquel et al., 2000) and *Poracanthium furcatum* (Levron et al., 2004b), 15 in *Nicolla testiobliquum* (Quilichini et al., 2007a) and *Nicolla wisniewskii* (Quilichini et al., 2007c).

The posterior part of the spermatozoon

Recently Quilichini et al. (2010b) have proposed a classification of the digenean spermatozoa according to the sequence of their posterior part (Table 1). They have distinguished 3 principal types: Type 1 or opecoelidean type (posterior extremity of the second axoneme - posterior nucleus extremity - cortical microtubules); Type 2 or fasciolidean type (posterior extremity of the second axoneme - posterior extremity of the nucleus); Type 3 or cryptogonimidean type (posterior extremity of the nucleus - posterior extremity of the second axoneme). This classification does not exclude the existence of a fourth group which could be defined in later studies.

In the spermatozoon of *Helicometra epinepheli*, the posterior extremities of the second axoneme and of the second mitochondrion are located at the same level. Nevertheless, the presences of the nucleus extremity and of cortical microtubules enables us to classify this species within Type 1 and confirms the homogeneity of the opecoelid spermatozoa using this criterion.

Conclusion

The spermatozoon of *Helicometra epinepheli* enables us to:

- confirm the homogeneity of the Trepaxonemata concerning the structure of axonemes with a 9+“1” pattern
- confirm the homogeneity of the digeneans concerning the presence of two axonemes and one or more mitochondria
- differentiate *Helicometra fasciata* from the other Opecoelidae by the absence of external ornamentation, spine-like bodies and cortical microtubules disposed in semi-circle around the first mitochondrion. This also confirms that *H. epinepheli* is not synonymous with *H. fasciata* (Justine et al., 2010).
- observe for the first time an apical cone in a digenean.

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