

**Spermatological characteristics of the family Glypthelminthidae (Digenea, Plagiorchioidea) inferred from the ultrastructural study of *Glypthelmins staffordi* Tubangui, 1928**

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**Abstract**

The present study describes the ultrastructural organization of the mature spermatozoon of the digenean *Glypthelmins staffordi* (Glypthelminthidae) by means of transmission electron microscopy. Live digeneans were collected from the Chinese edible frog (*Hoplobatrachus rugulosus*) in Udon Thani Province (Thailand). The ultrastructural study reveals that the mature spermatozoon of *G. staffordi* is a filiform cell, which is tapered at both extremities. It exhibits the Bakhoum et al.'s type IV of spermatozoon of digeneans characterized by the 9+'1' axonemes of trepaxonematan Platyhelminthes, the presence of the association "external ornamentation-cortical microtubules", the external ornamentation located in the posterior part of the anterior region, the arrangement of parallel cortical microtubules in two bundles and with its maximum number located in the anterior part of the sperm cell, and the presence of two mitochondria. Other

characteristics are the presence of spine-like bodies, a posterior extremity containing only the nucleus, and the presence of a large amount of glycogen granules. Results of the present study are particularly compared with the existing data in other families of the Plagiorchioidea, namely the Brachycoeliidae, the Haematoloechidae, the Omphalometridae and the Plagiorchiidae.

**Keywords:** *Glypthelmins staffordi*; Glypthelminthidae; Digenea; Sperm characters.

## 1. Introduction

The family Glypthelminthidae comprises a single genus, *Glypthelmins*, which includes numerous species, all of them parasitizing the intestine of amphibians and presenting a cosmopolitan range of distribution. The systematic position of this genus has been unclear and controversial. According to various authors, it has been placed in several families of the superfamily Plagiorchioidea, such as the Brachycoeliidae, the Macroderoididae and the Plagiorchiidae. However, some morphoanatomical characteristics, its host range and also particularities in its life-cycle suggest that it does not belong to these families (Tkach, 2008). This fact is supported by diverse molecular phylogenetic studies (Tkach et al., 2001; Olson et al., 2003; Razo-Mendivil et al., 2006). Although the molecular study of Razo-Mendivil et al. (2006) demonstrated the monophyly of the genus *Glypthelmins* and also its close relationships with the genus *Haematoloechus*, being the Glypthelminthidae and the Haematoloechidae sister clades, species of the genus *Glypthelmins* show substantial morphological and biological differences with haematoloechids (Razo-Mendivil et al., 2006; Tkach, 2008).

In this context, the ultrastructural study of the spermatozoon provides numerous characters with a potential usefulness in systematics and phylogeny. Thus, the interest of

ultrastructural sperm characters as useful criteria to interpreting relationships among Platyhelminthes have been demonstrated in diverse studies particularly in cestodes and monogeneans (Justine, 1991a, 1991b, 1998, 2001; Bâ and Marchand, 1995; Levron et al., 2010; Justine and Poddubnaya, 2018). Referring the Digenea, during last decade several works emphasized the usefulness of these ultrastructural characters (Miquel et al., 2006; Quilichini et al., 2010, 2011) and, recently, Bakhoun et al. (2017) updated all the spermatological characters and established five sperm models for the Digenea.

Concerning the Plagiorchioidea, there are ultrastructural studies on this subject for five species belonging to four families. These are *Brachycoelium salamandrae* (Brachycoeliidae), *Haematoloechus* sp. (Haematoloechidae); *Rubenstrema exasperatum* (Omphalometridae); and *Enodiotrema reductum* and *Plagiorchis elegans* (Plagiorchiidae) (Justine and Mattei, 1982; Bakhoun et al., 2011, 2013; Ndiaye et al., 2012, 2013) (details in Table 1). The aim of the present work is to provide the first complete description of the sperm characters in the family Glypthelminthidae with the study of *Glypthelmins staffordi* and to compare these with the available ultrastructural characters of the above mentioned plagiorchioideans.

## **2. Materials and methods**

### *2.1. Materials*

Live adult specimens of *Glypthelmins staffordi* Tubangui, 1928 were isolated from the Chinese edible frog *Hoplobatrachus rugulosus* (Wiegmann, 1834) collected during May 2014 by hand from a frog farm (N 17° 49.21'; E 102° 76.62'; 173 m a.s.l.) in Udon Thani Province, Thailand. The collected amphibian was immediately transported alive to the laboratory at Udon Thani Rajabhat University. The frog was anesthetized and

subsequently killed using MS222 (ethyl-4-aminobenzoate). After dissection, digeneans were isolated and fixed for transmission electron microscopy (TEM). This study was approved by the Udon Thani Rajabhat University Animal Care and Ethical Use Committee.

## *2.2. Transmission electron microscopy*

For the present TEM study, several worms were rinsed with a 0.9% NaCl solution and 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 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1% osmium tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in Milli-Q water (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in Spurr's resin and polymerized at 60 °C for 72 h. Ultrathin sections (60–90 nm thick) at the level of the seminal vesicle were obtained using a Reichert-Jung Ultracut E ultramicrotome. Sections were placed on 200-mesh copper and gold grids. Sections placed on copper grids were double-stained with uranyl acetate and lead citrate according to the Reynolds (1963) procedure. Copper grids were examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of 80 kV, in the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

## *2.3. Cytochemistry*

Sections placed on gold grids were treated according to the Thiéry (1967) test to reveal the presence of glycogen. Thus, they were treated in periodic acid (PA), thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-Q water, 24 h in TCH, rinsed in acetic solutions and Milli-Q water, 30 min

in 1% SP in the dark, and rinsed in Milli-Q water. Sections were examined in a JEOL 1010 transmission electron microscope at an accelerating voltage of 80 kV, in the CCIUTUB.

### 3. Results

The mature spermatozoon of *Glypthelmins staffordi* is a filiform cell presenting structures found in numerous digeneans. Indeed, it contains two axonemes of the 9+'1' pattern of trepaxonematan Platyhelminthes, external ornamentation of the plasma membrane, spine-like bodies, nucleus, two mitochondria, two bundles of parallel cortical microtubules, and granules of glycogen. The interpretation of numerous longitudinal and cross-sections allow us to distinguish three regions (I to III) in their spermatozoa with different ultrastructural characteristics (Figs. 1-3).

Region I (Figs. 1a-i and 3I) corresponds to the anterior region of the spermatozoon. The anterior spermatozoon extremity is characterized by the presence of the centrioles of both axonemes, which are slightly displaced longitudinally (Fig. 1a) and surrounded by a continuous submembranous layer of cortical microtubules lacking of attachment zones (Fig. 1b) or presenting only two attachment zones (Fig. 1c). This anterior area of the spermatozoon exhibits the maximum number of cortical microtubules with 32-33 microtubules (Fig. 1b, c). An external ornamentation of the plasma membrane associated with cortical microtubules appears when four attachment zones are present (Fig. 1e-g). The ornamented area also exhibits spine-like bodies (Fig. 1d-f) and the first mitochondrion (Fig. 1d, f-i). This mitochondrion is present as far as the posterior area of region I lacking of external ornamentation (Fig. 1h, i). Granules of glycogen appear in the transition toward region II (Fig. 1i).

Region II (Figs. 1j, k, 2a and 3II) corresponds to the middle region of the spermatozoon, which is mainly characterized by the presence of both axonemes, two bundles of parallel cortical microtubules, granules of glycogen and the second mitochondrion in its posterior part (Figs. 1j, k and 2a).

Region III (Figs. 2b-g and 3III) corresponds to the nuclear and posterior spermatozoon extremity. The presence of the nucleus is the main characteristic of this region (Fig. 2b-g). The transition of characters toward the posterior tip of the sperm cell is marked by (i) the disappearance of the first axoneme (Fig. 2e), (ii) the disappearance of the second mitochondrion and cortical microtubules (Fig. 2f), and finally (iii) the disappearance of the second axoneme (Fig. 2g). Therefore, the posterior spermatozoon tip is characterized by the presence of only the nucleus (Fig. 2g).

A large amount of granular material, observed along regions II and III, is confirmed as glycogen by means of the test of Thiéry (Fig. 2h).

#### **4. Discussion**

Bakhoun et al. (2017) considered two sets of sperm ultrastructural characters in the Digenea. The principal characters were used by authors to establish five different models of sperm cells in the Digenea and they include the type of axoneme, the presence/absence of a lateral expansion, the presence/absence of external ornamentation of the plasma membrane and, if present, its location and its association or not with cortical microtubules, the number of bundles of cortical microtubules, the location of the maximum number of cortical microtubules, and the number of mitochondria. Other characters, including the anterior electron-dense material, spine-like bodies, cytoplasmic ornamented buttons and the posterior character of the sperm cell, were considered as

secondary characters and they were excluded for the definition of sperm models (see Bakhoun et al., 2017).

The mature spermatozoon of *Glypthelmins staffordi*, as in all the previously studied species of the Plagiorchioidea, presents an ultrastructural organization following the sperm cell's type IV of Bakhoun et al. (2017) (see Table 1). Thus, the male gamete is characterized by the 9+'1' axonemes of the Trepaxonemata (Ehlers, 1984) as all digeneans with the exception of schistosomes and some didymozoids (Justine and Mattei, 1983; Jamieson and Justine, 2017). The presence of the association "external ornamentation-cortical microtubules" and the posterior position of this external ornamentation are other features present in the type IV of digenean spermatozoon. Most digeneans follow this pattern of association between ornamentation and microtubules, and only certain species, such as *Pronoprymna ventricosa* and the majority of hemiuroideans exhibit an external ornamentation of the plasma membrane not associated with cortical microtubules (see Quilichini et al., 2007; Ndiaye et al., 2017). Moreover, cortical microtubules are usually arranged in two bundles, at least in the principal region of the sperm cell containing the mitochondrion and/or the nucleus. The unique exception concerns hemiuroideans (for a review, see Bakhoun et al. 2017). Two possibilities are postulated by Bakhoun et al. (2017) for the location of the maximum number of cortical microtubules along the male gamete. In all the studied plagiorchioideans the maximum number of cortical microtubules is observed in the anterior part of the spermatozoon. Finally, the presence of generally two mitochondria is the last characteristic of the type IV of sperm cell. Only certain differences exist among spermatozoa of studied plagiorchioideans: (i) the presence of a single mitochondrion in the plagiorchiid *Enodiotrema reductum* and (ii) the description of two types of external ornamentation in the haematoloechid *Haematoloechus* sp. (Justine and Mattei, 1982; Ndiaye et al., 2012).

Considering secondary characters observed in this species, namely the posterior spermatozoon extremity and spine-like bodies, the available ultrastructural data in the superfamily Plagiorchioidea also reveal important similitudes. Thus, in all the studied plagiorchioideans the posterior spermatozoon character is the nucleus. Quilichini et al. (2010) have described three types of posterior spermatozoon extremities according to the transition of characters toward the posterior tip of sperm cells (the Opecoelidean, the Fasciolidean and the Cryptogonimidean types). The disappearance of cortical microtubules, then the second axoneme and finally the nucleus characterize the Fasciolidean type that exhibit male gametes of all the Plagiorchioidea analyzed to date (Justine and Mattei, 1982; Bakhoun et al., 2011, 2013; Ndiaye et al., 2012, 2013; present study). In what refers spine-like bodies, these electron-dense and submembranous elements described for the first time in the opecoelid *Opecoeloides furcatus* (Miquel et al., 2000) are also present in all the ultrastructurally studied plagiorchioideans (see Table I). Nevertheless, in the case of *Haematoloechus* sp., Justine and Mattei (1982) do not describe these structures although they are visible in their published TEM micrographs (see Fig. 10, page 328). In the Digenea sperm cells, spine-like bodies are usually present in their ornamented area, as occurs in *G. staffordi* (present study) and in the remaining studied plagiorchioideans. Only an exception exists among digeneans, namely the apocreadiid *Neoapocreadium chabaudi*, which has spine-like bodies in the non-ornamented area of the gamete (Kacem et al., 2010).

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### Conflict of interest

The authors declare that they have no conflict of interest.

### References

- Bâ, C.T., Marchand, B., 1995. Spermiogenesis, spermatozoa and phyletic affinities in the Cestoda. *Mém. Mus. Natn. Hist. Nat., Paris* 166, 87–95.
- Bakhoun, A.J.S., Bâ, C.T., Shimalov, V.V., Torres, J., Miquel, J., 2011. Spermatological characters of the digenean *Rubensstrema exasperatum* (Rudolphi, 1819) (Plagiorchioidea, Omphalometridae). *Parasitol. Res.* 108, 1283–1293.
- Bakhoun, A.J.S., Ribas, A., Eira, C., Bâ, C.T., Miquel, J., 2013. *Brachycoelium salamandrae* (Frölich, 1789) (Digenea: Brachycoeliidae): ultrastructural study of spermiogenesis and the mature spermatozoon. *Zool. Anz.* 252, 149–156.
- Bakhoun, A.J.S., Miquel, J., Ndiaye, P.I., Justine, J.-L., Falchi, A., Bâ, C.T., Marchand, B., Quilichini, Y., 2017. Advances in spermatological characters in the Digenea: review and proposal of spermatozoa models and their phylogenetic importance. *Adv. Parasitol.* 98, 111–165.
- Ehlers, U., 1984. Phylogenetisches System der Plathelminthes. *Verh. Naturwiss. Ver. Hambg. (NF)* 27, 291–294.
- Jamieson, B.G.M., Justine, J.-L., 2017. Spermatozoa, Spermatogenesis and Fertilization in *Schistosoma*. In: Jamieson, B.G.M. (Ed.), *Schistosoma: Biology, Pathology and Control*. CRC Press, Boca Raton, pp. 300–319.

- Justine, J.-L., 1991a. Phylogeny of parasitic Platyhelminthes: a critical study of synapomorphies proposed on the basis of the ultrastructure of spermiogenesis and spermatozoa. *Can. J. Zool.* 69, 1421–1440.
- Justine, J.-L., 1991b. Cladistic study in the Monogenea (Platyhelminthes), based upon a parsimony analysis of spermiogenetic and spermatozoal ultrastructural characters. *Int. J. Parasitol.* 21, 821–838.
- Justine, J.-L., 1998. Spermatozoa as phylogenetic characters for the Eucestoda. *J. Parasitol.* 84, 385–408.
- Justine, J.-L., 2001. Spermatozoa as phylogenetic characters for the Platyhelminthes. In: Littlewood, D.T.J., Bray, R.A. (Eds.), *Interrelationships of the Platyhelminthes*. Taylor and Francis, London, pp. 231–238.
- Justine, J.-L., Mattei, X., 1982. Réinvestigation de l'ultrastructure du spermatozoïde d'*Haematoloechus* (Trematoda: Haematoloechidae). *J. Ultrastruct. Res.* 81, 322–332.
- Justine, J.-L., Mattei, X., 1983. A spermatozoon with two 9 + 0 axonemes in a parasitic flatworm, *Didymozoon* (Digenea: Didymozoidae). *J. Submicrosc. Cytol.* 15, 1101–1105.
- Justine, J.-L., Poddubnaya, L.G., 2018. Spermiogenesis and spermatozoon ultrastructure in basal polyopisthocotylean monogeneans, Hexabothriidae and Chimaericolidae, and their significance for the phylogeny of the Monogenea. *Parasite* 25, 7.
- Kacem, H., Bakhoum, A.J.S., Neifar, L., Miquel, J., 2010. Spermiogenesis and spermatozoon ultrastructure of the digenean *Neoapocreadium chabaudi* (Apocreadiidae), a parasite of *Balistes capriscus* (Pisces, Teleostei). *Parasitol. Int.* 59, 358–366.

- Levron, C., Miquel, J., Oros, M., Scholz, T., 2010. Spermatozoa of tapeworms (Platyhelminthes, Eucestoda): advances in ultrastructural and phylogenetic studies. *Biol. Rev.* 85, 523–543.
- Miquel, J., Nourrisson, C., Marchand, B., 2000. Ultrastructure of spermiogenesis and the spermatozoon of *Opecoeloides furcatus* (Trematoda, Digenea, Opecoelidae), a parasite of *Mullus barbatus* (Pisces, Teleostei). *Parasitol. Res.* 86, 301–310.
- Miquel, J., Fournier-Chambrillon, C., Fournier, P., Torres, J., 2006. Spermiogenesis and spermatozoon ultrastructure of the cranial digenean *Troglotrema acutum* (Leuckart, 1842). *J. Parasitol.* 92, 441–453.
- Ndiaye, P.I., Quilichini, Y., Sène, A., Tkach, V.V., Bâ, C.T., Marchand, B., 2012. Ultrastructural study of the spermatozoon of the digenean *Enodiotrema reductum* Looss, 1901 (Platyhelminthes, Plagiorchioidea, Plagiorchiidae), parasite of the green turtle *Chelonia mydas* (Linnaeus, 1758) in Senegal. *Parasitol. Res.* 111, 859–864.
- Ndiaye, P.I., Quilichini, Y., Tkach, V.V., Greiman, S.E., Bâ, C.T., Marchand, B., 2013. Ultrastructure of the spermatozoon of the digenean *Plagiorchis elegans* (Rudolphi, 1802) (Plagiorchioidea, Plagiorchiidae). *J. Morphol.* 274, 965–972.
- Ndiaye, P.I., Quilichini, Y., Marigo, A.M., Bâ, C.T., Tkach, V.V., Marchand, B., 2017. Ultrastructural characteristics of the mature spermatozoon of the digenean *Sclerodistomum italicum* (Stossich, 1893) (Hemiuroidea, Sclerodistomidae) intestinal parasite of *Hypocanthus amia* (Teleostei, Carangidae). *Tissue Cell* 49, 15–21.
- Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A., Littlewood, D.T.J., 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *Int. J. Parasitol.* 33, 733–755.

- Quilichini, Y., Foata, J., Marchand, B., 2007. Ultrastructural study of the spermatozoon of *Pronoprymna ventricosa* (Digenea, Baccigerinae), parasite of the twaite shad *Alosa fallax* Lacepede (Pisces, Teleostei). *Parasitol. Res.* 101, 1125–1130.
- Quilichini, Y., Foata, J., Justine, J.-L., Bray, R.A., Marchand, B., 2010. Ultrastructural study of the spermatozoon of *Heterolebes maculosus* (Digenea, Opistholebetidae), a parasite of the porcupinefish *Diodon hystrix* (Pisces, Teleostei). *Parasitol. Int.* 59, 427–434.
- Quilichini, Y., Foata, J., Justine, J.-L., Bray, R.A., Marchand, B., 2011. Spermatozoon ultrastructure of *Gyliauchen* sp. (Digenea: Gyliauchenidae), an intestinal parasite of *Siganus fuscescens* (Pisces: Teleostei). *Biol. Bull.* 221, 197–205.
- Razo-Mendivil, U.J., León-Règagnon, V., Pérez-Ponce de León, G., 2006. Monophyly and systematic position of *Glypthelmins* (Digenea), based on partial *lsrDNA* sequences and morphological evidence. *Org. Divers. Evol.* 6, 308–320.
- Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17, 208–212.
- Thiéry, J.P., 1967. Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *J. Microsc.* 6, 987–1018.
- Tkach, V.V., 2008. Family Glypthelminthidae Cheng, 1959. In: Bray, R.A., Gibson, D.I., Jones, A. (Eds.), *Keys to the Trematoda*, vol. 3. CABI Publishing and The Natural History Museum, London, pp. 357–360.
- Tkach, V.V., Snyder, S.D., Świdorski, Z., 2001. On the phylogenetic relationships of some members of Macroderoididae and Ochetosomatidae (Digenea, Plagiorchioidea). *Acta Parasitol.* 46, 267–275.

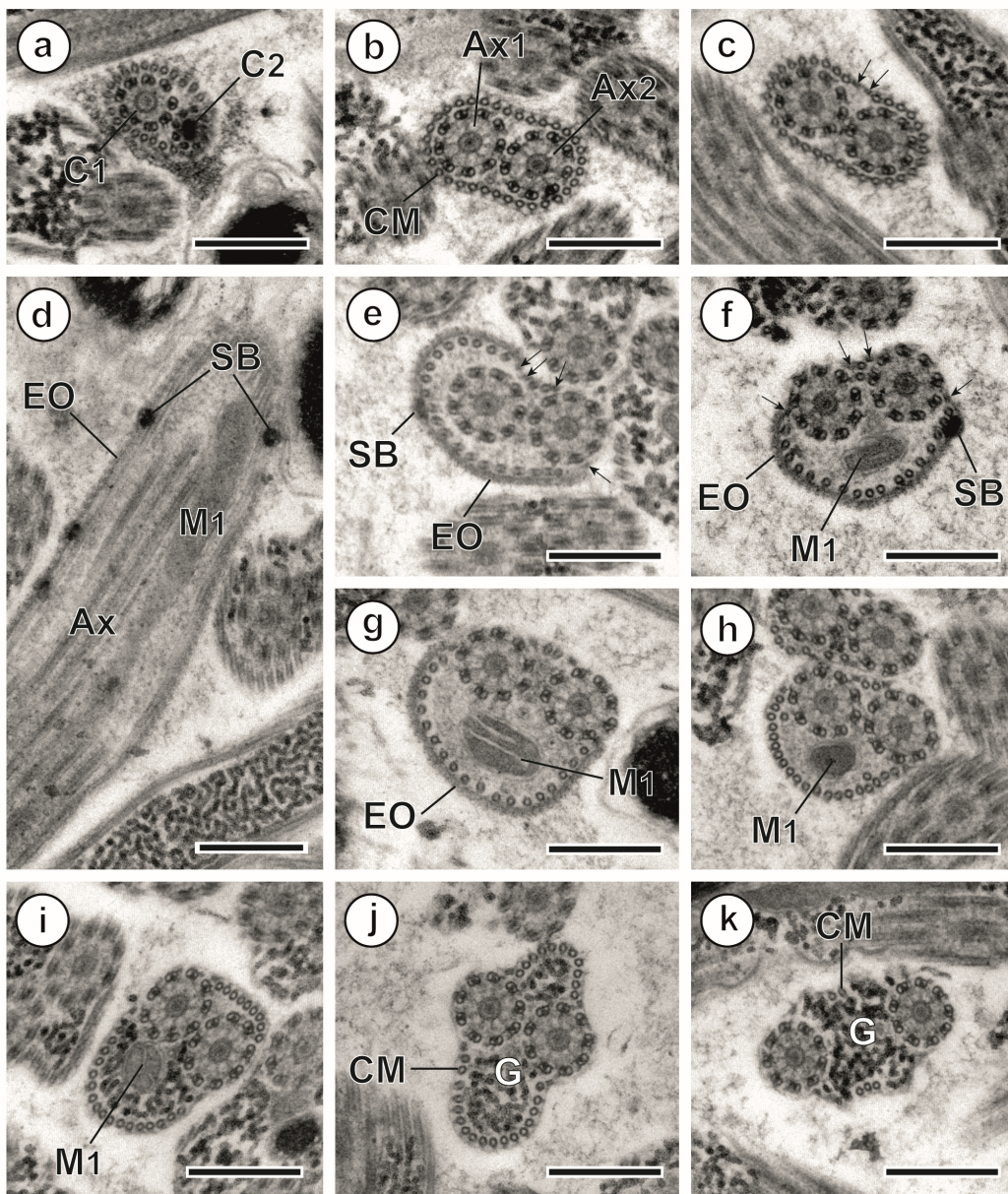
## Legends of figures

**Fig. 1.** Spermatozoon of *Glypthelmins staffordi* (regions I and II): (a–c) correlative cross-sections of the anterior spermatozoon extremity showing the two centrioles (1a), the continuous submembranous layer of cortical microtubules (1b) and the appearance of two attachment zones indicated by arrows (1c); (d) longitudinal section in the ornamented area exhibiting spine-like bodies; (e–g) cross-sections of the ornamented area showing spine-like bodies (1 e, f) and the presence of the first mitochondrion (1f, g). The presence of four attachment zones is marked by arrows (1e, f); (h, i) posterior part of region I characterized by the absence of the external ornamentation; (j, k) cross-sections illustrating anterior part of region II. Ax, axoneme; Ax1, Ax2, first and second axoneme; C1, C2, centrioles of the first and second axoneme; CM, cortical microtubules; EO, external ornamentation of the plasma membrane; G, granules of glycogen; M1, first mitochondrion; SB, spine-like bodies. Bars = 0.3  $\mu$ m.

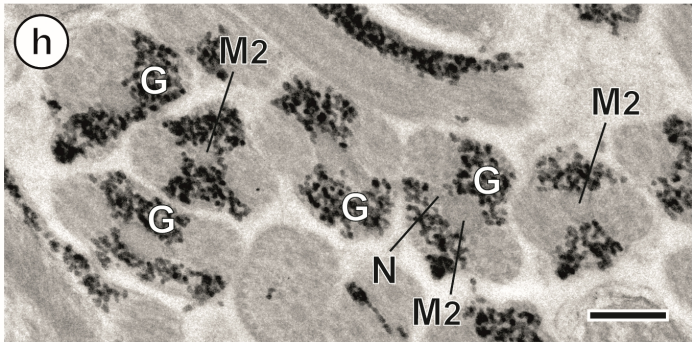
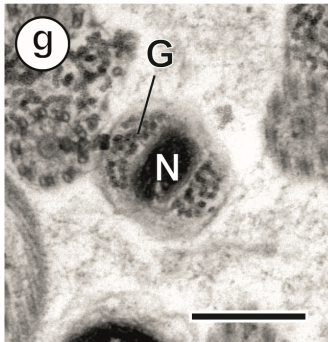
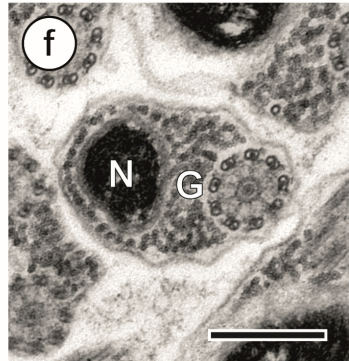
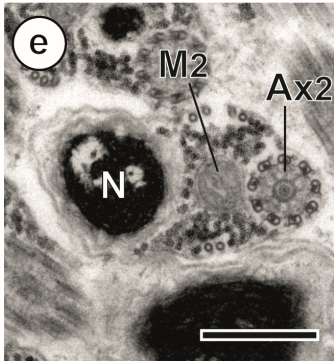
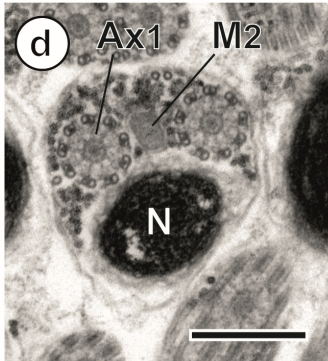
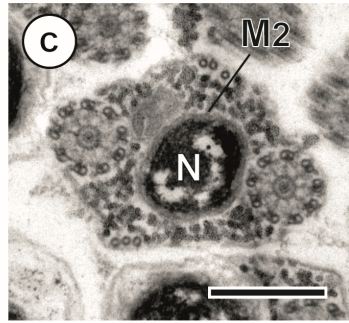
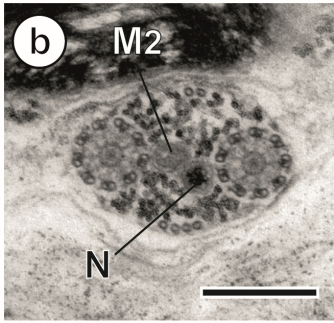
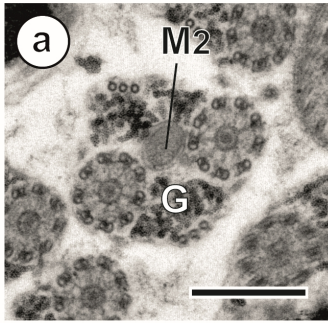
**Fig. 2.** Spermatozoon of *Glypthelmins staffordi* (regions II and III): (a) cross-section of the posterior part of region II, which exhibits the second mitochondrion; (b–g) correlative cross-sections of region III. Note the disappearance of characters (axonemes, second mitochondrion and cortical microtubules) toward the posterior tip of the spermatozoon; (h) test of Thiéry for detection of glycogen. Ax1, Ax2, first and second axoneme; G, granules of glycogen; M2, second mitochondrion; N, nucleus. Bars = 0.3  $\mu$ m.

**Fig. 3.** Schematic reconstruction of the mature spermatozoon of *Glypthelmins staffordi*. The sperm cell is organized in three different regions: region I or anterior part, region II or middle part and region III or posterior part. In order to make the diagram clearer, granules of glycogen are not shown in longitudinal sections. ASE, anterior spermatozoon extremity; Ax1, Ax2, first and second axoneme; AZ, attachment zones; C1, C2, centrioles of the first and second axoneme; CM, cortical microtubules; EO, external ornamentation

of the plasma membrane; G, granules of glycogen; M1, M2, first and second mitochondrion; N, nucleus; PM, plasma membrane; PSE, posterior spermatozoon extremity; SB, spine-like bodies.









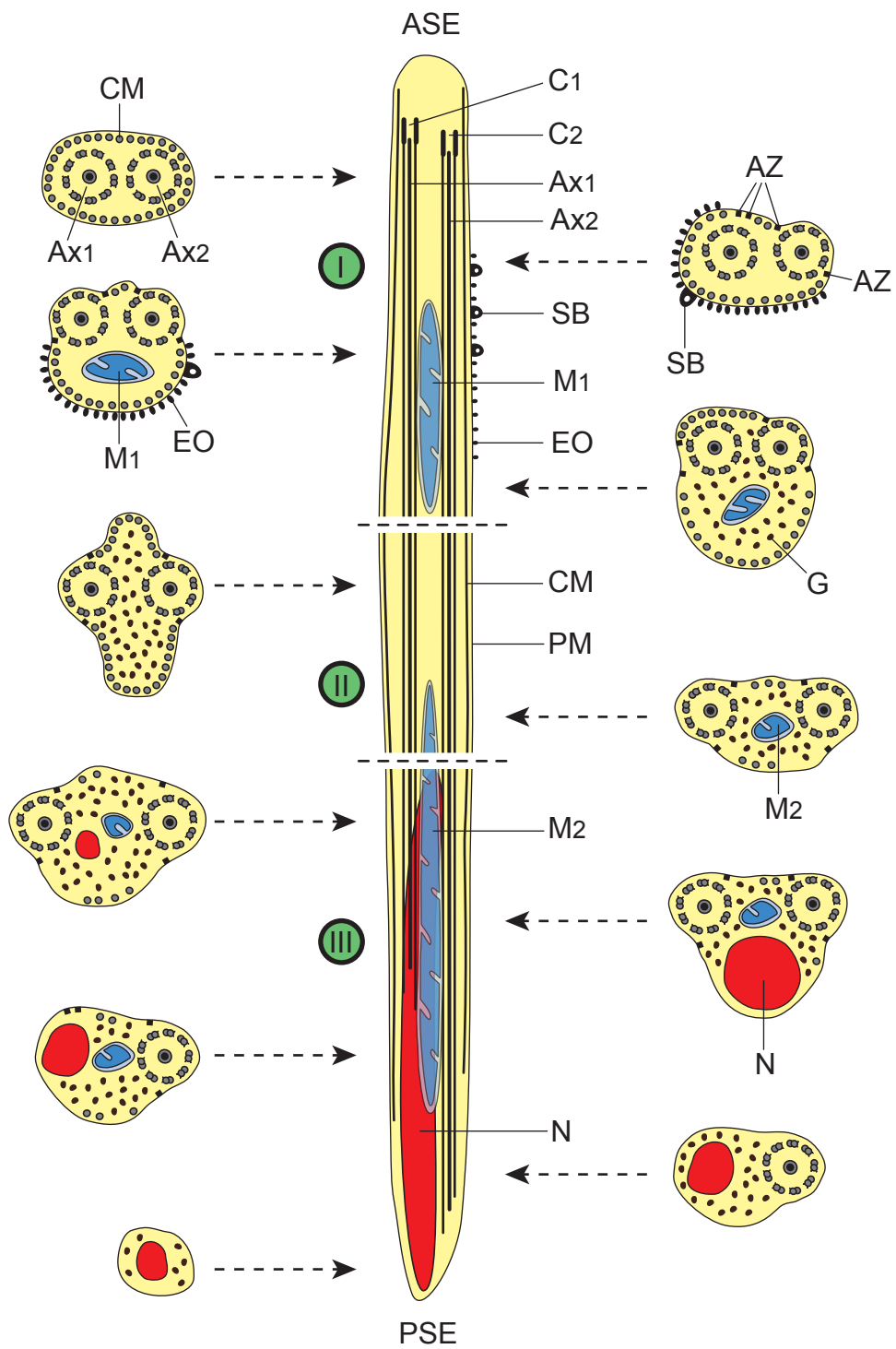


Table 1. Available data on the ultrastructure of the spermatozoon in the Plagiorchioidea.

Families and species	Spermatozoon characteristics											References
	TS	TA <sub>x</sub>	LE	EO	EO+CM	LEO	BCM	LMCM	M	SB	PSC	
BRACHYCOELIIDAE												
<i>Brachycoelium salamandrae</i>	IV	9+'1'	-	+	+	PostA	2	AntS	2	+	N	Bakhoum et al. (2013)
GLYPHTELMINTHIDAE												
<i>Glypthelmins staffordi</i>	IV	9+'1'	-	+	+	PostA	2	AntS	2	+	N	Present study
HAEMATOLOECHIDAE												
<i>Haematoloechus</i> sp.	IV	9+'1'	-	+ <sup>1</sup>	+	PostA	2	AntS	?	+ <sup>2</sup>	N	Justine and Mattei (1982)
OMPHALOMETRIDAE												
<i>Rubenstrema exasperatum</i>	IV	9+'1'	-	+	+	PostA	2	AntS	2	+	N	Bakhoum et al. (2011)
PLAGIORCHIIDAE												
<i>Enodiotrema reductum</i>	IV	9+'1'	-	+	+	PostA	2	AntS	1	+	N	Ndiaye et al. (2012)
<i>Plagiorchis elegans</i>	IV	9+'1'	-	+	+	PostA	2	AntS	2	+	N	Ndiaye et al. (2013)

AntS, anterior region of the spermatozoon; BCM, number of bundles of cortical microtubules; EO, external ornamentation of plasma membrane; EO+CM, association of external ornamentation with cortical microtubules; LE, lateral expansion; LEO, location of external ornamentation; LMCM, location of maximum number of cortical microtubules; M, number of mitochondria; N, nucleus; PostA, posterior part of the anterior region; PSC, posterior spermatozoon character, SB, spine-like bodies; TAx, type of axoneme; TS, type of spermatozoon; +/-, presence/absence of considered character; ?, unknown data.

<sup>1</sup>Authors describe two types of external ornamentation.

<sup>2</sup>Authors do not mention the presence of spine-like bodies, but they are clearly visible in the published TEM micrographs. Probably they were misinterpreted as artefacts of fixation.

## AUTHORS AGREEMENT

**Manuscript:** Spermatological characteristics of the family Glypthelminthidae (Digenea, Plagiorchioidea) inferred from the ultrastructural study of *Glypthelmins staffordi* Tubangui, 1928

**Authors:** Jordi Miquel (corresponding author), Srisupaph Poonlaphdecha and Alexis Ribas

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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