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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.

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,b, 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, Bakhoum 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

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

Families and species	Spermatozoon characteristics											References
	TS	TAx	LE	EO	EO + CM	LEO	BCM	LMCM	M	SB	PSC	
BRACHYCOELIIDAE												
Brachycoelium salamandrae	IV	9+'1'	–	+	+	PostA	2	AntS	2	+	N	Bakhoum et al. (2013)
GLYPTHELMINTHIDAE												
Glypthelmins staffordi	IV	9+'1'	–	+	+	PostA	2	AntS	2	+	N	Present study
HAEMATOLOECHIDAE												
Haematoloechus sp.	IV	9+'1'	–	+[1]	+	PostA	2	AntS	?	+[2]	N	Justine and Mattei (1982)
OMPHALOMETRIDAE												
Rubenstrema exasperatum	IV	9+'1'	–	+	+	PostA	2	AntS	2	+	N	Bakhoum et al. (2011)
PLAGIORCHIIDAE												
Enodiotrema reductum	IV	9+'1'	–	+	+	PostA	2	AntS	1	+	N	Ndiaye et al. (2012)
Plagiorchis elegans	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.

[1] Authors describe two types of external ornamentation.

[2] 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.

Brachycoelium salamandrae (Brachycoeliidae), *Haematoloechus* sp. (Haematoloechidae); *Rubenstrema exasperatum* (Omphalometridae); and *Enodiotrema reductum* and *Plagiorchis elegans* (Plagiorchiidae) (Justine and Mattei, 1982; Bakhoum 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 CCiTUB.

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 3 I) 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, 2 a and 3 II) 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,