

First spermatological data on the digenean genus *Ityogonimus* derived from the description of sperm characters of *Ityogonimus ocreatus* (Brachylaimidae: Ityogoniminae)

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ABSTRACT

The present study describes the ultrastructural organization of the spermatozoa of the brachylaimid digenean *Ityogonimus ocreatus* (Ityogoniminae) by means of transmission electron microscopy (TEM). Live digeneans were collected from the digestive tract of an Iberian mole *Talpa occidentalis* (Eulipotyphla, Talpidae) captured accidentally during a vole pest control campaign in Priesca (Asturias, Spain). The TEM study reveals that the *I. ocreatus* sperm are filiform, tapered at both extremities, and have two 9+1' trepaxonematan axonemes, external ornamentation of the plasma membrane associated with cortical microtubules, spine-like bodies, two bundles of parallel cortical microtubules and one mitochondrion overlapping the anterior part of the nucleus. The external ornamentation of the plasma membrane is located in the posterior part of the anterior region. The maximum number of parallel cortical microtubules (45) is located in the anterior part of the sperm cells. Our results are compared with the available data on the family Brachylaimidae, especially on the other Ityogoniminae studied to date (*Scaphiostomum palaearticum*).

1. Introduction

The digenean *Ityogonimus ocreatus* belongs to the family Brachylaimidae, which comprises two subfamilies, the Brachylaiminae and the Ityogoniminae (Pojmańska, 2002). The subfamily Ityogoniminae includes exclusively the genera *Ityogonimus* and *Scaphiostomum*. Representatives of the genus *Ityogonimus* are parasites of talpids and this genus currently has three recognized species: *I. lorum* and *I. ocreatus* parasites of *Talpa* spp. in the Palaearctic region (Milazzo et al., 2002; Ribas and Casanova, 2006; Adalid et al., 2018), and *I. scalopi*, a parasite of *Scapanus aquaticus* inhabiting part of the Nearctic region (Turner and McKeever, 1980). The species *I. ocreatus* has been reported as a parasite of the European mole *Talpa europaea*, the Iberian mole *T. occidentalis*, and the Italian mole *T. romana* (see Milazzo et al., 2002; Adalid et al., 2018).

The study of sperm characters has been demonstrated as a useful tool

to analyse the phylogenetic relationships in different groups of Platyhelminthes such as monogeneans and cestodes (Justine, 1991a, b, 1998, 2001; Bâ and Marchand, 1995; Levron et al., 2010; Justine and Poddubnaya, 2018). In the Digenea, many studies have been carried out to assess the phylogenetic usefulness of their spermatological ultrastructural characteristics (Quilichini et al., 2007, 2010, 2011; Bakhomou et al., 2017a; Kacem et al., 2020). To our knowledge, the ultrastructural study of the spermatozoa in the Digenea involves more than 140 species. In some groups, such as the bucephalids, hemiurids or opecoelids, the number of studied species is quite important and the analyses of the observed ultrastructural characteristics provided useful data for possible phylogenetic inferences (see Ndiaye et al., 2018; Bâ et al., 2020a; Kacem et al., 2020). This is not the case of brachylaimids, which have been poorly studied. Within the family Brachylaimidae, to date, only the studies of *Brachylaima mascomai* (Brachylaiminae) and *Scaphiostomum palaearticum* (Ityogoniminae) are extensive enough to extract the most

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interesting ultrastructural features of the spermatozoa and their organization (González-Moreno, 2002; Ndiaye et al., 2002). Considering the subfamily Brachylaiminae, studies on *Brachylaima aequans* (Žďárská et al., 1991) and *Postharmostomum gallinum* (Chen and Lu, 1994) present partial descriptions of the ultrastructure of the sperm cells.

The aim of the present study is to describe the ultrastructural organization of the sperm characters of *I. ocreatus* (Brachylaimidae, Ityogonimidae). This study will increase the ultrastructural spermatological database on the Digenea and contribute to a better understanding of the phylogenetic relationships within the Brachylaimidae.

2. Materials and methods

2.1. Specimens

Live specimens of *I. ocreatus* were recovered from the intestine of a naturally infected Iberian mole *Talpa occidentalis* trapped accidentally in July 2016 during a vole pest control campaign in Priesca (Asturias, Spain).

2.2. Transmission electron microscopy

Adult flukes 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 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 copper and gold grids (200 µm mesh size). Sections placed on copper grids were double-stained with uranyl acetate and lead citrate according to the Reynolds procedure (Reynolds, 1963). Stained ultrathin sections were examined in a JEOL 1010 transmission electron microscope operated at an accelerating voltage of 80 kV, in the "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB).

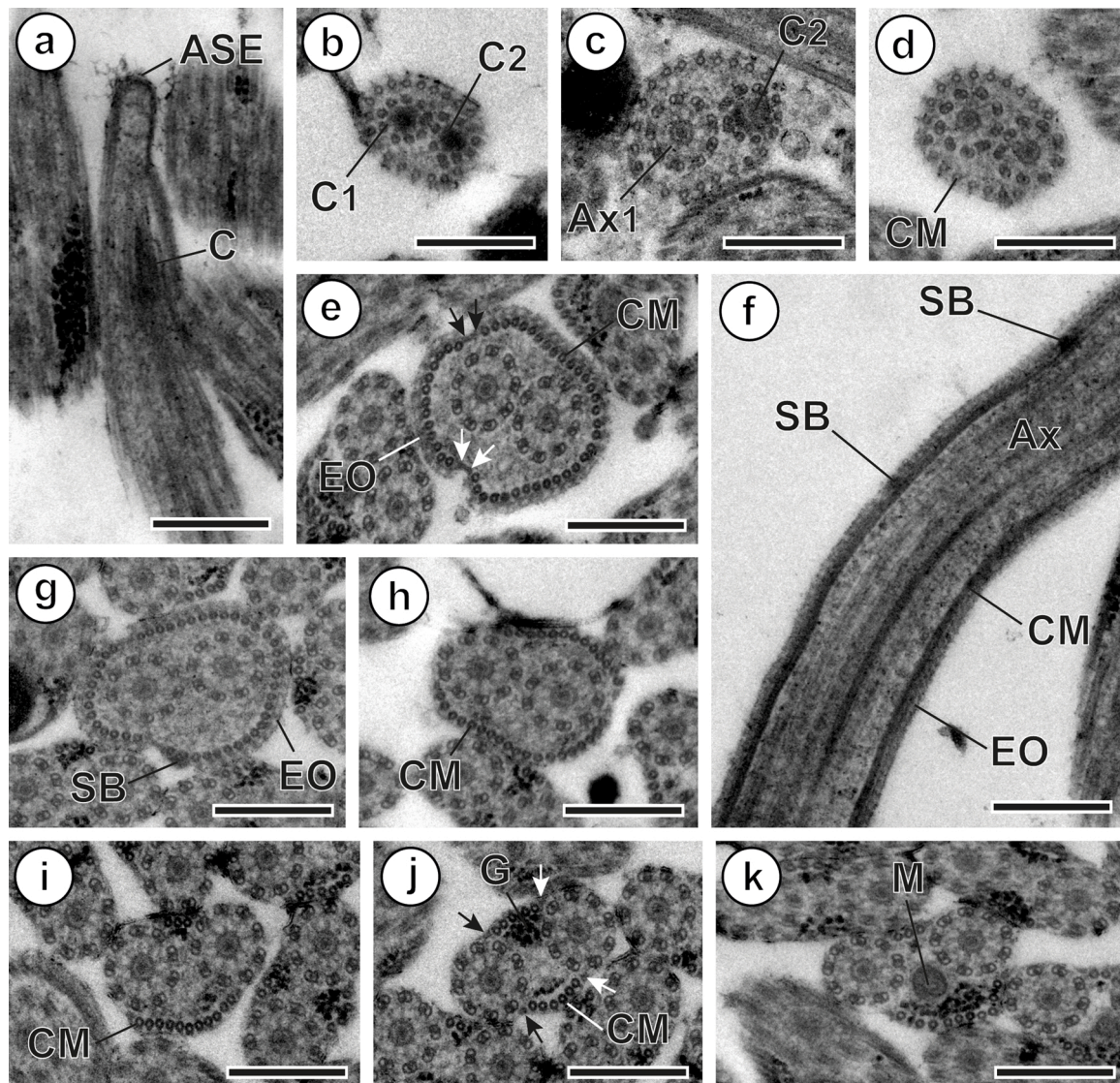


Fig. 1. Spermatozoa of *I. ocreatus*: Regions I (a–g) and II (h–k). (a) longitudinal section of the anterior spermatozoon extremity, (b–d) cross-sections of the non-ornamented area of region I, (e–g) longitudinal and cross-sections of the ornamented area of region I, (h–j) cross-sections of the anterior part of region II, (k) cross-section of the posterior part of region II showing the mitochondrion. Arrows: attachment zones, ASE: anterior spermatozoon extremity, Ax: axoneme, Ax1: first axoneme, C: centriole, C1 and C2: centrioles of the first and second axoneme, CM: cortical microtubules, EO: external ornamentation of the plasma membrane, G: granules of glycogen, M: mitochondrion, SB: spine-like bodies, scale bars: 0.3 µm.

2.3. Cytochemistry

Sections placed on gold grids were treated according to the Thiéry test (Thiéry, 1967) to reveal the ultrastructural localization of glycogen. Thus, sections were treated in periodic acid (PA), thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10 % PA, rinsed in Milli-Q water, 30 – 45 min in 0.2 % TCH in 20 % acetic acid, rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark, and rinsed in Milli-Q water. Ultrathin sections were also examined in a JEOL 1010 transmission electron microscope in the CCIUTUB.

3. Results

The interpretation of several cross- and longitudinal sections of the spermatozoa of *I. ocreatus* enables us to describe their ultrastructural organization in three regions, from the anterior to posterior sperm extremities (Figs. 1–3). The *I. ocreatus* sperm are filiform and exhibit the following characteristics: two axonemes of the 9+1' pattern of trepaxonematan Platyhelminthes, two bundles of parallel cortical microtubules, one mitochondrion, nucleus, external ornamentation of the

plasma membrane, spine-like bodies and large amount of glycogen granules.

3.1. Region I

Region I (Figs. 1a-g and 3 I) corresponds to the sperm anterior region. The anterior tip is filiform (Fig. 1a). The two centrioles appear at the same level, and both centrioles and the anterior portion of the axonemes are surrounded by a discontinuous and submembranous layer of 18–20 cortical microtubules (Figs. 1b-d and 3 I). The posterior part of region I is mainly characterized by an external ornamentation of the plasma membrane associated with cortical microtubules and by the presence of spine-like bodies (Figs. 1e-g and 3 I). This ornamented area has the maximum number of cortical microtubules (around 45), which are organized in two bundles (Fig. 1e, g).

3.2. Region II

Region II (Figs. 1h-k and 3 II) corresponds to the middle zone of the sperm cells, which are characterized by the absence of external

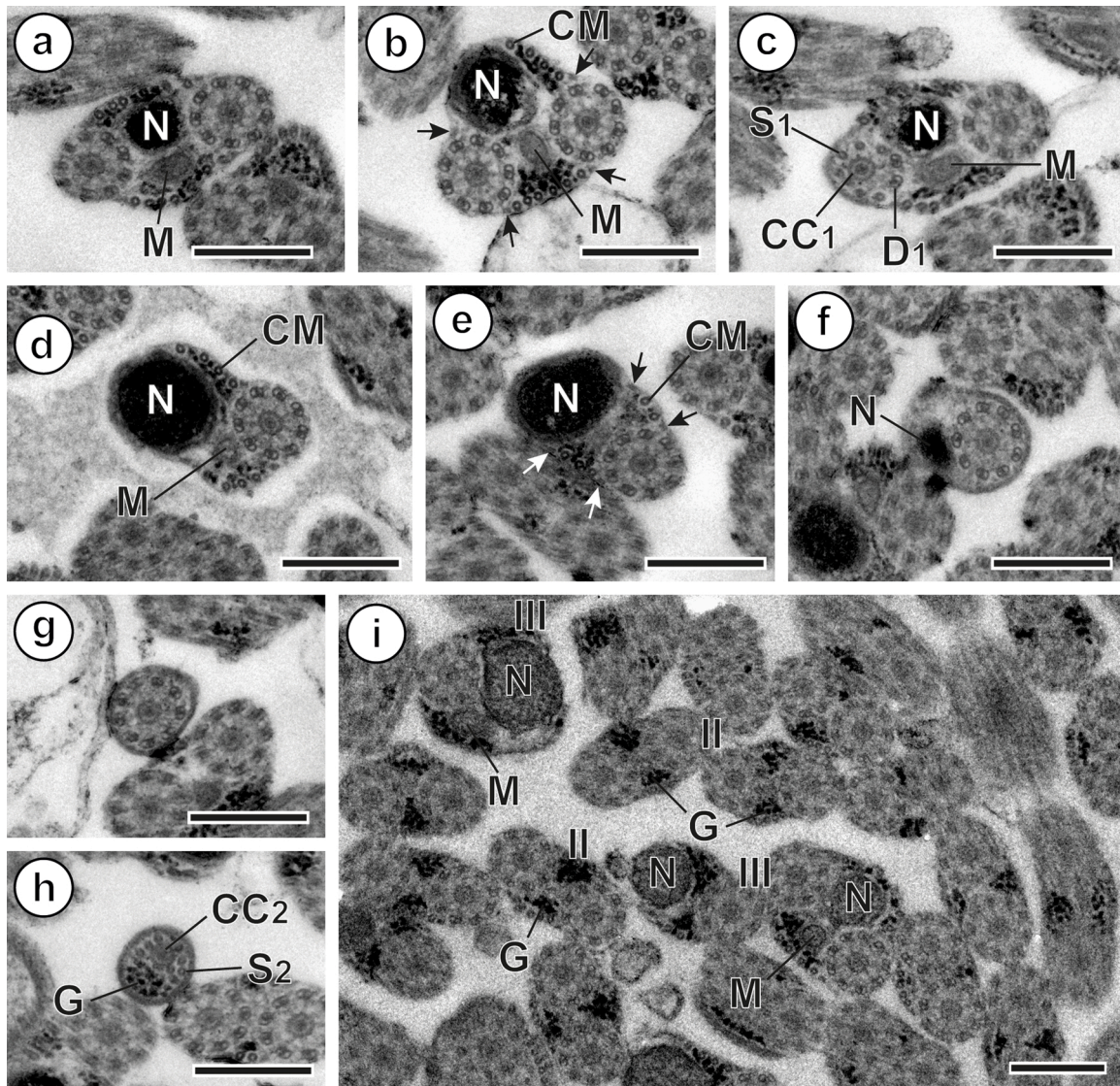


Fig. 2. Spermatozoa of *I. ocreatus*: Region III (a-h) and test of Thiéry for glycogen (i). (a-h) correlative cross-sections showing the transition of characters towards the posterior spermatozoon extremity, (i) positive reaction for glycogen after the Thiéry test. Arrows: attachment zones, II: sperm region II, III: sperm region III, CC1 and CC2: central core of the first and second axoneme, CM: cortical microtubules, D1: doublets of the first axoneme, G: granules of glycogen, M: mitochondrion, N: nucleus, S1 and S2: singlets of the first and second axoneme, scale bars: 0.3 μ m.

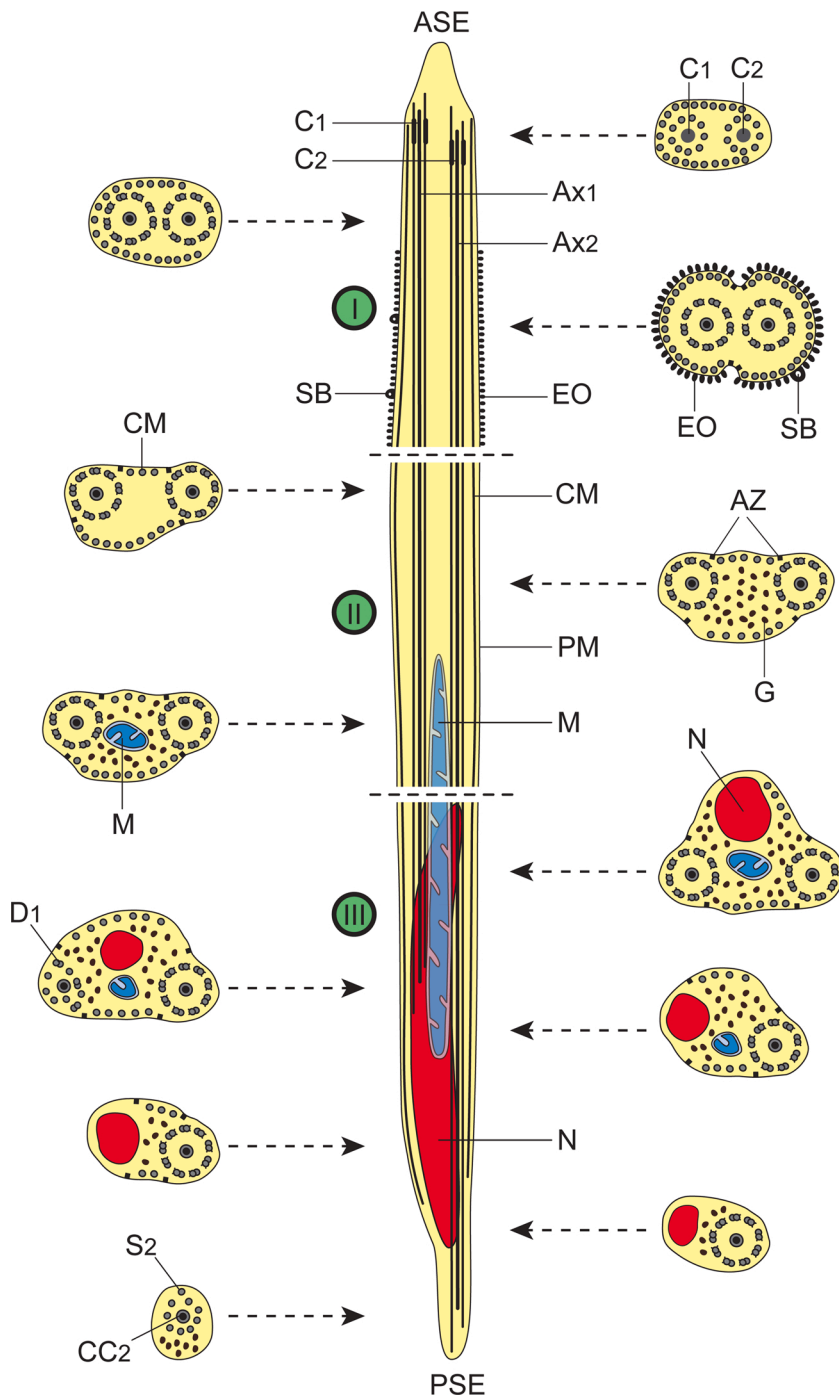


Fig. 3. Schematic reconstruction of the spermatozoon of *I. ocreatus*. The sperm cells are organized in three different regions: region I or anterior part, region II or middle part and region III or posterior part. In order to make the diagram clearer, granules of glycogen are not shown in longitudinal sections. ASE: anterior spermatozoon extremity, Ax1 and Ax2: first and second axoneme, AZ: attachment zones, C1 and C2: centriole of the first and the second axoneme, CC2: central core of the second axoneme, CM: cortical microtubules, D1: doublets of the first axoneme, EO: external ornamentation of the plasma membrane, G: granules of glycogen, M: mitochondrion, N: nucleus, PM: plasma membrane, PSE: posterior spermatozoon extremity, S2: singlets of the second axoneme, SB: spine-like bodies.

ornamentation of the plasma membrane (Figs. 1h-j and 3 II) and by the appearance of the mitochondrion in the posterior part of this region (Figs. 1k and 3 II). Other characteristics of region II are the reduction in the number of cortical microtubules and the appearance of granules of glycogen.

3.3. Region III

Region III (Figs. 2a-h and 3 III) corresponds to the sperm posterior region. This region is characterized by the presence of the nucleus and the posterior extremity of the mitochondrion (Figs. 2a-d and 3 III). Glycogen granules are also present in this region. The transition of characters towards the posterior extremity of the spermatozoa is as

follows: (i) disorganization and disappearance of the first axoneme (Fig. 2c, d), (ii) posterior extremity of the mitochondrion (Fig. 2d, e), (iii) disappearance of cortical microtubules (Fig. 2f), and (iv) posterior extremity of the nucleus (Fig. 2f, g). Thus, the posterior tip of the sperm cells exhibits the progressive disorganization of the second axoneme (Fig. 2g, h).

3.4. Cytochemical test of Thiéry

The granules of glycogen are irregularly distributed along regions II and III of the spermatozoa and they were evidenced by the cytochemical test of Thiéry (Fig. 2i).

4. Discussion

The spermatozoa of *I. ocreatus* have the characters usually found in most digeneans: two 9+1' axonemes, external ornamentation of the plasma membrane, parallel cortical microtubules, mitochondrion and nucleus.

4.1. Axonemes

The *I. ocreatus* sperm have two axonemes showing the 9+1' pattern, typical of the Trepaxonemata. This pattern is observed in the majority of digeneans and consists in nine peripheral doublets with dynein arms and a highly structured central element (Ehlers, 1984). There are two other types of axonemes in the digenean sperm cells. One type is the 9+1' special pattern of schistosomes that consists of a single diffuse central element and nine peripheral doublets devoid of dynein arms (see Jamieson and Justine, 2017). The other one is the 9+0 pattern typical of *Didymozoon* species that consists of nine doublets without the outer dynein arms and lacking the central structure (Justine and Mattei, 1983).

Recently, Matoušková et al. (2018, 2019) have analysed the structure of the central core of the 9+1' axonemes of two caryophyllidean cestodes by means of electron tomography analysis. These authors found two helicoidal tubular structures in the central electron-dense core of the axonemes of *Caryophyllaeides fennica* and *Khawia rossittensis*.

4.2. External ornamentation of the plasma membrane

Two groups of digeneans can be distinguished according to the location of the external ornamentation in their spermatozoa. In the first group, the ornamentation appears in the proximal part of the spermatozoa. In the second one, the external ornamentation is located in a more distal position of the spermatozoa (Quilichini et al., 2007). According to Quilichini et al. (2011) a third group can be added, including species without ornamentation. The spermatozoa of *I. ocreatus* belong to the first group, which is in agreement with findings described in *S. palaearticum*, the other Ityogoniminae studied until now (Ndiaye et al., 2002). Moreover, as in most digeneans, the external ornamentation is associated with cortical microtubules (see Bakhoun et al., 2017a). In fact, only hemiuroideans (see Kacem et al., 2020) and *Pronoprymna ventricosa* (Faustulidae) (Quilichini et al., 2007) have external ornamentation not associated with cortical microtubules.

4.3. Cortical microtubules

Three characteristics involving cortical microtubules are important in phylogenetic inference: their presence or absence, the location of their maximum number and the number of bundles (Bakhoun et al., 2017a). Cortical microtubules are present in the sperm cells of most digeneans, except for *Didymocystis wedli* (Pamplona-Basilio et al., 2001) and *Didymozoon* sp. (Justine and Mattei, 1983), which have no microtubules. Moreover, when present, these tubular elements can be organized in one or two bundles. The majority of digeneans have two bundles as in the Ityogoniminae representatives *I. ocreatus* (present study) and *S. palaearticum* (Ndiaye et al., 2002) and also in the Brachylaiminae (Žďárská et al., 1991; Chen and Lu, 1994; González-Moreno, 2002). However, other digeneans such as the hemiurids, sclerodistomids and lecithasterids exhibit only a single bundle (see Kacem et al., 2020).

Two groups of digeneans can be distinguished according to the position of the maximum number of cortical microtubules (Bakhoun et al., 2017a). One group has the maximum number of cortical microtubules in the middle part of the gametes and the other group has the maximum number of cortical microtubules in the anterior part of the spermatozoa. *I. ocreatus* belongs to the second group, with a maximum number of 45 peripheral microtubules.

4.4. Spine-like bodies

Considering the subfamily Ityogoniminae, this study showed that the sperm cells of *I. ocreatus* have spine-like bodies. Contrarily, these structures are absent in the male gametes of *S. palaearticum* (Ndiaye et al., 2002), the other representative of the subfamily Ityogoniminae. In the remaining studied brachylaimids belonging to the subfamily Brachylaiminae, spine-like bodies were not reported (Žďárská et al., 1991; Chen and Lu, 1994; González-Moreno, 2002 -see Table 1). Spine-like bodies were described for the first time in *Opecoeloides furcatus* and the authors describe these elements as more or less triangular electron-dense prominences containing a submembranous spherical vesicle (Miquel et al., 2000). Since then, spine-like bodies were found in the spermatozoa of numerous digeneans (see Bakhoun et al., 2017a).

4.5. Mitochondrion

Digeneans have one, two or three mitochondria in their spermatozoa (for a review see Bakhoun et al., 2017a). They result from the fusion of several mitochondria in the spermatid during spermiogenesis (Burton, 1972). The species *I. ocreatus* has one mitochondrion located in regions II and III of the male gametes. The morphology of the mitochondrion is filiform as in the other brachylaimids studied to date (Chen and Lu, 1994; González-Moreno, 2002; Ndiaye et al., 2002) as well as in most digeneans (see Bakhoun et al., 2017a). However, some authors found other types of mitochondrion, such as the moniliform mitochondria, consisting in several mitochondrial bulges joined by a mitochondrial cord. This is the case of the acanthocolpid *Stephanostomoides tenuis* (Bakhoun et al., 2015), the sclerodistomoidid *Sclerodistomoides pacificus* (Bâ et al., 2020b), aepnidiogenids of the genus *Holorchis* (Bâ et al., 2011; Kacem and Miquel, 2020a), the leprocreadiids *Opechona bacillaris* and *Prodistomum polonii* (Ndiaye et al., 2015; Kacem and Miquel, 2020b), the opecoelids *Allopodocotyle pedicellata* and *Macvicaria obovata* (Bakhoun et al., 2017b; Kacem et al., 2017a), the cryptogonimids *Aphallus tubarium* and *Timoniella imbutiforme* (Foata et al., 2012; Kacem et al., 2017b) or the plagiorchiid *Enodiotrema reductum* (Ndiaye et al., 2012). Furthermore, yet another type of mitochondrion was recently described in the opecoelid *Allopodocotyle tunisiensis* (Kacem et al., 2019), consisting of a filiform mitochondrion with a posterior U-shaped extremity.

4.6. Posterior spermatozoon extremity

The posterior part of the spermatozoa of *I. ocreatus* are characterized by the following succession of disappearance of principal structures towards the posterior extremity of the gametes: 'first axoneme - mitochondrion - cortical microtubules - nucleus - second axoneme'. This sequence differs from that described for *S. palaearticum*, which revealed the disappearance of the posterior extremity of the mitochondrion, followed by the first axoneme, the nucleus and finally, cortical microtubules and/or the second axoneme (Ndiaye et al., 2002). In fact, Quilichini et al. (2010) established three types of posterior spermatozoon extremities according to this criterion. However, due to several inconsistencies in some digeneans, the consideration of the terminal or last character seems to be more suitable when comparing different families (see Bakhoun et al., 2017a). Thus, the terminal character in *I. ocreatus* is the posterior extremity of the second axoneme, whereas in *S. palaearticum* the terminal character is the second axoneme or cortical microtubules (Ndiaye et al., 2002). Concerning the Brachylaiminae, both *B. mascomai* and *P. gallinum* seem to have the second axoneme as the terminal character in the spermatozoa (Chen and Lu, 1994; González-Moreno, 2002 -see Table 1).

5. Conclusion

The spermatozoa of representatives of the subfamily Ityogoniminae

Table 1

Available data on the ultrastructure of the spermatozoon in the Brachylaimidae.

Subfamilies and species	Spermatozoon characters													References
	TS	ASC	TAx	LE	EO	EO + CM	LEO	BCM	MCM	LMCM	M	SB	PSC	
BRACHYLAIMINAE														
<i>Brachylaima aequans</i>	?	1Ax?	9+'1'	?	?	?	?	2	22?	?	1?	?	?	Žďárská et al. (1991)
<i>Brachylaima mascomai</i>	IV?	1Ax + CM?	9+'1'	-	-	NA	NA	2	20?	AntS?	1	-	Ax?	González-Moreno (2002)
<i>Postharmostomum gallinum</i>	?	2Ax + CM?	9+'1'	?	?	?	?	2	?	?	1	?	Ax?	Chen and Lu (1994)
ITYOGONIMINAE														
<i>Ityogonimus ocreatus</i>	V?	2Ax + CM	9+'1'	-	+	+	AntA	2	45	AntS	1	+	Ax	Present study
<i>Scaphiostomum palaearticum</i>	V?	1Ax?	9+'1'	-	+	+	AntA	2	69?	AntS	1	-	Ax/ CM?	Ndiaye et al. (2002)

AntA: anterior part of the anterior sperm region, AntS: anterior sperm region, ASC: anterior sperm character, Ax: axoneme, BCM: number of bundles of cortical microtubules, CM: 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, MCM: maximum number of cortical microtubules, N: nucleus, NA: not applicable, PSC: posterior sperm character, SB: spine-like bodies, TAx: axoneme type, TS: sperm type, +/-: presence/absence of considered character, ? : doubtful or unknown data.

seem to follow the type V proposed by Bakhoun et al. (2017a). This model is characterized by the presence of two 9+'1' axonemes, a lateral expansion, the association 'external ornamentation - cortical microtubules', external ornamentation located in the anterior part of the proximal region, two bundles of parallel cortical microtubules, the maximum number of cortical microtubules located in the anterior part, and one mitochondrion. However, spermatozoa of both *S. palaearticum* and *I. ocreatus* lack lateral expansions. As emphasized by Bakhoun et al. (2017a), the available ultrastructural data of the spermatozoa in digeneans are congruent with molecular analyses that support the basal dichotomy of the Digenea with two lineages, the Diplostomida and the Plagiorchida (see Olson et al., 2003; Pérez-Ponce de León and Hernández-Mena, 2019). The Diplostomida includes the superfamilies Diplostomoidea, Brachylaimoidea and Schistosomatoidea. In the latter, several species of schistosomes have been analysed and all of them exhibit the type I of spermatozoa, which is mainly characterized by the 9+'1' special pattern of axonemes (see Jamieson and Justine, 2017). In the Plagiorchida, the spermatozoa follow the remaining models (types II to V) (see Bakhoun et al., 2017a). In contrast with schistosomes, the existing data on the Brachylaimoidea indicate a model V spermatozoon. However, considering the current status of knowledge, further ultrastructural studies of spermatozoa are needed in diplostomoideans, particularly in the Leucochloridiidae as a related family of brachylaimids, to carefully assess the possible models of sperm cells in the different taxa of the Diplostomida.

Ethical animal disclosure

This study was carried out in strict accordance with the recommendations of the guidelines of animal welfare in experimental science and the European Union legislation (Directive 86/609/EEC).

CRedit authorship contribution statement

Roser Adalid: Field work, TEM study, Drafting the manuscript. **Jordi Torres:** Approval of the manuscript. **Màrius Vicent Fuentes:** Approval of the manuscript. **Jordi Miquel:** Field work, TEM study, Analysis of results, Review the manuscript, Approval of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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