A comparative study of the ultrastructural characteristics of the mature spermatozoa of two fellodistomids *Tergestia clonacantha* and *T. laticollis* and contribution to the phylogenetic knowledge of the Gymnophalloidea

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Abstract – The ultrastructure of the mature spermatozoa of *Tergestia clonacantha* and *T. laticollis* collected from the digestive tracts of fishes from New Caledonia is described using transmission electron microscopy and compared to that of related species. The spermatozoa of the two species exhibit the general pattern described in most digeneans, namely two axonemes with the 9 + “1” pattern of the Trepaxonemata, nucleus, mitochondrion, cortical microtubules, an external ornamentation of the plasma membrane, spine-like bodies and granules of glycogen. The spermatozoa of *T. clonacantha* and *T. laticollis* show the same ultrastructural model with some speciﬁcities in each case, particularly in the disposition of the structures in the posterior extremities of the spermatozoon. This study conﬁrms that ultrastructural characters of the mature spermatozoon are useful tools for the phylogenetic analysis of the Digenea.

Key words: *Tergestia clonacantha*, *T. laticollis*, Ultrastructure, Spermatozoon, Gymnophalloidea, Fellodistomidae.

Introduction

It is now recognized that the ultrastructure of mature spermatozoa constitutes a useful complement to molecular, morphological and biological data in the understanding of the phylogeny of the Platyhelminthes. Studies of sperm structure in the poorly known orders and additional comparative studies are expected to provide new information for elucidation of phylogenetic relationships [2, 7, 11, 17, 25, 28]. Characters of special interest are: (1) presence or absence of mitochondria in the mature spermatozoon and their number, (2) the type of spermiogenesis, (3) presence or absence of the intercentriolar body during spermiogenesis, (4) the striated roots, (5) the periaxonemal sheath, (6) apical electron dense material in the spermatozoon, (7) the apical cone, (8) the ultrastructure of the anterior or posterior extremities, (9) the form and disposition of the nucleus, (10) the number and distribution of cortical microtubules, (11) the spine-like bodies, (12) the external ornamentation of the plasma membrane, (13) the disposition

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of the glycogen granules, and (14) the expansion of the plasma membrane.

The subclass Digenea is one of the largest groups of internal metazoan parasites with more than 18,000 recognized species belonging to 150 families [9]. Spermatological data are available for more than 100 species. The available data are not equally distributed among all the taxonomic levels of Digenea. Some families have never been studied, while some genera have already been the subject of several studies. Among these genera, we considered: the Aphanidiogenidae Holorchis [3, 12], the Bucephalidae Prosorhynchus [18, 26], the Dicrocoeliidae Dicrocoelium [5, 10], the Fasciolidae Fasciola [21, 22], the Hemimuridae Lecithochirium [23], the Lepocreadiidae Biamium [30], the Notocotylidae Notocotylus [20, 24], the Opecoelidae Allocotyle [6, 15], Helicometra [16, 29] and Nicolla [27, 28], the Opisthorchiidae Opisthorchis [19, 32], and the Zoogonidae Lecithostaphylus [4, 14].

In this study, we describe the ultrastructural features of the mature spermatozoa of Tergestia clonacantha and T. laticollis. Only the fellodostomid Tergestia acaenochephala [13] has been studied among the five families of Gymnophalloidea. The aim of this work was to add spermatological data for the Fellodostomidae and the Gymnophalloidea. Moreover, we aimed to confirm the stability of ultrastructural sperm characters at a low taxonomic level (species) to emphasize their usefulness to discriminate higher taxonomic groups (orders or superfamilies).

### Material and methods

Live adult specimens of Tergestia clonacantha Manter, 1963 and T. laticollis (Rudolphi, 1819) Stossich, 1899 were collected from the digestive tracts of Gymnophallidea, respectively, off Nouméa (New Caledonia). Specimens were rinsed with 0.9% NaCl solution and fixed in cold (4 °C) 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2, postfixed in cold (4 °C) 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ethanol and propylene oxide, embedded in Spurr’s resin and finally polymerized at 60 °C for 24 h. Ultrathin sections (60–90 nm thick) were cut on an ultramicrotome (Power tone PC, RMC Boeckeler) with diamond knife and placed on copper and gold grids. Copper grids were double stained with uranyl acetate and lead citrate. To reveal the presence of glycogen, golds grids were stained according to the method of Thiéry [31] with periodic acid (PA), thiocarbohydrazide (TCH) and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-Q water, 2 h in TCH, rinsed in acetic solution and Milli-Q, 30 min in 1% SP in the dark and rinsed in Milli-Q water. Then, copper and gold grids were examined using a Hitachi H-7650 electron microscope operated at 80 kV in the “Service d’Étude et de Recherche en Microscopie Électronique” of the University of Corsica (Corte, France).

### Results

The observation of numerous transverse sections in the seminal vesicle of the spermatozoa of Tergestia clonacantha and T. laticollis under the transmission electron microscope permitted us to distinguish four regions (I–IV) from the anterior to the posterior parts of mature spermatozoa of these species. The spermatozoa exhibit the main ultrastructural characteristics generally described in digeneans, namely two axonemes with the 9 + “1” pattern of the Trepaxonemata, nucleus, mitochondria, cortical microtubules, an external ornamentation of the plasma membrane, spine-like bodies, and granules of glycogen. However, the spermatozoa of T. clonacantha and T. laticollis present some peculiarities that we describe below.

**Region I** (Figs. 1A–1C, 3A–3F and 5I). This region corresponds to the anterior extremity of the mature spermatozoon characterized by the presence of a first axoneme in T. clonacantha (Fig. 1A) as in T. laticollis (Figs. 3A–3C). Thus, cortical microtubules and a second axoneme appear progressively (Figs. 1C and 3E–3F). There are only a few cortical microtubules in the anterior extremity of the spermatozoon. On the micrographs we counted 4 microtubules in T. clonacantha (Fig. 1C) and 6 in T. laticollis (Fig. 3F).

**Region II** (Figs. 1D–1H, 3G–3K and 5I). In addition to the structures described in the posterior extremity of the region I of the spermatozoon, namely the two axonemes and cortical microtubules, we distinguish the presence of an external ornamentation of the plasma membrane (Figs 1D–1H and 3G–3K), spine-like bodies (Figs. 1H and 3K), and a significant increase in the number of cortical microtubules. This number increases from 5 microtubules in the anterior part to 36 in the posterior part of this region in T. clonacantha (Figs. 1D–1G) and from 6 to 36 in the case of T. laticollis (Figs. 3F–3K). In the two species, cross-sections of the spermatozoon at the posterior level of this region show the maximum number of cortical microtubules arranged in a continuous layer interrupted by attachment zones under the plasma membrane (Figs 1G and 3K). The external ornamentation of the plasma membrane is described on one side of the sections only in this region II (Figs. 1D–1H, 3G–3K and 5I).

**Region III** (Figs. 1I–1N, 3L–3Q and 5II). This region is characterized by the disappearance of spine-like bodies and the external ornamentation of the plasma membrane, the presence of the first mitochondrion (Figs. 1I–1L and 3L–3N) and the disappearance of one of the axonemes in the posterior part of this region (Figs 1N and 3P–3Q). The number of cortical microtubules also decreases progressively, from 36 to 9 in T. clonacantha (Figs. 1M–1N) and from 36 to 13 in T. laticollis (Fig. 3Q).

**Region IV** (Figs. 2A–2E, 4A–4H, 5IV). This posterior region of the spermatozoa exhibits some differences between the two species. In T. clonacantha, it is characterized by the appearance of the second mitochondrion in addition to the axoneme and cortical microtubules (Fig. 2A). The nucleus appears posterior to the anterior end of the mitochondrion (Fig. 2B). The second mitochondrion disappears at the posterior extremity of the spermatozoon (Fig. 2C), then the cortical microtubules, then the axoneme (Figs. 2D–2E). Thus, the cross-section of the posterior extremity of the spermatozoon shows the presence of the nucleus only (Fig. 2E).

In the case of T. laticollis, cross-sections in the anterior part of this region exhibit the appearance of the nucleus in addition
to the second axoneme and cortical microtubules (Figs. 4A–4B), then the second mitochondrion (Fig. 4C). Cross-sections toward the posterior extremity show a progressive disappearance of cortical microtubules, then the second mitochondrion, and finally the axoneme (Figs. 4D–4F).

The posterior extremity of the spermatozoon exhibits the presence of the nucleus only (Figs. 4G–4H).

Use of Thiéry’s method demonstrated the presence of glycogen granules in the mature spermatozoa of *Tergestia clonacantha* and *T. laticollis* (Figs. 2F–2G and 4I–4J).
Discussion

The ultrastructure of the mature spermatozoon of *Tergestia clonacantha* and *T. laticollis* shows the general features described in the spermatozoon of most digeneans, namely: two axonemes with the 9+“1” pattern of the Trepaxonemata, a nucleus, a mitochondrion or two, cortical microtubules, external ornamentation of the plasma membrane, spine-like bodies, and granules of glycogen. However, the mature spermatozoa of *T. clonacantha* and *T. laticollis* exhibit some peculiarities and give additional spermatological data for a better understanding of the relationships between the Gymnophalloidea and other digenean superfamilies. As is the case in many species of the superfamilies Gorgoderoida, Lepocreadioidea, Opecoeloidea and Plagiorchioidea [7] with spermatozoa of type III or IV, we also found two mitochondria in the spermatozoa of *T. clonacantha* and *T. laticollis*. These are located in the posterior part of the mature spermatozoon in *T. clonacantha* and *T. laticollis*, in contrast to *T. acanthocephala* [13] which has the first mitochondrion in the anterior part of the spermatozoon. In addition, the mature spermatozoon of *T. acanthocephala* was described as similar to type IV proposed by Bakhoum et al. [7] but spermatozoa of *T. clonacantha* and *T. laticollis* (present study) exhibit type III. Thus, the assignation of type IV for spermatozoa of Gymnophalloidea in Bakhoum et al. [7] should be revised. This demonstrates the need for more studies in this superfamily to elucidate ultrastructural characteristics of the spermatozoa and their relationships. The ultrastructural model of the mature spermatozoon described in *T. clonacantha* and *T. laticollis* by the present study highlights some differences between them, mainly in the posterior region of the spermatozoa. The posterior extremity of the spermatozoon is characterized in the two species by the presence of the nucleus only, as in *T. acanthocephala* [13]. Nevertheless, in the case of *T. clonacantha*, before this extremity, we described successively the appearance of the second mitochondrion, then the nucleus, then the disappearance of cortical microtubules, then the extremity of the second axoneme, and finally the posterior extremity of the nucleus. In parallel and successively, in *T. laticollis*, the nucleus appears, then the second
Figure 3. (A–Q) Transmission electron micrographs of regions I, II and III of the mature spermatozoon of *Tergestia laticollis*. Scale bars = 0.2 μm. (A–F) Sections in the anterior extremity of the spermatozoon. (A–B). Longitudinal (A) and cross-sections (B) in the anterior extremity of the spermatozoon with only the centriole of axoneme 1 (Aae1). (C) Cross-section in the anterior extremity of the spermatozoon with only one axoneme (A × 1). (D) Cross-section in the anterior extremity of the spermatozoon with axoneme 1 and three cortical microtubules. (E–F) Cross-section in region I of the spermatozoon with axoneme 1 and the centriole of axoneme 2 (Aae2) (E), then the two axonemes (A × 1 and A × 2) (F). (G–K) Cross-sections in region II of the mature spermatozoon showing the two axonemes, an increasing number of cortical microtubules up to 36 in (K), and the external ornamentation of the plasma membrane (Eo) and spine-like body (Sb) (K). (L–Q) Cross-sections in region III of the mature spermatozoon showing only two axonemes and a row of 26 cortical microtubules (L), 25 (M), the appearance of the first mitochondrion (M1) (N). Then, the disappearance of the first mitochondrion (O), disappearance of axoneme 1 (Pae1) and the disorganization of axoneme 1 (P). (Q) Cross-section in the extremity of region III showing only a row of 13 cortical microtubules and axoneme 2.
mitochondrion, then the end of cortical microtubules, then the posterior extremity of the second axoneme, and finally the posterior extremity of the nucleus. Comparative ultrastructural studies of the mature spermatozoon have been performed in several species belonging to the same genus, within the Digenea. Overall, the authors found a similar general pattern. Table 1 shows that the same spermatozoon type (according to Bakhoum et al. [7]) has usually been found for species of the same genus: type III for *Holorchis micracanthum* [3] and *H. pycnoporus* [12]; type V for *Prosorhynchus aculeatus* and [18] *P. longisaccatus* [26]; type V for the Diplodiscidae *Diplodiscus amphicrinus* [10] and *D. subclavatus* [5]; type V for *Fasciola gigantica* [22] and *F. hepatica* [21]; type II for *Lecithochirium microstomum* [23] and *L. musculus* [23]; type III for the Lepocreadiidae *Bianium arabicum* [30] and *B. plicitum* [30]; type IV for the Opisthorchiidae *Opisthorchis felineus* [32] and *O. viverrini* [19]; type IV for the Opecoelidae *Allopodocotyle pedicellata* [6] and *A. tunisiensis* [15], and type III for the Opecoelidae *Nicolla testiobliquum* [27] and *N. wisniewskii* [28]. Nevertheless, some differences have been observed between spermatozoa of species belonging to the same genus like the Notocotylidae *Notocotylus neyrai* [20] (undefined type) and *N. noyeri* [24] (type IV) concerning the location of the

Figure 4. (A–H) Transmission electron micrographs of the posterior region (or region IV) of the mature spermatozoon of *Tergestia laticollis*. Scale bars = 0.2 μm. Cross-sections in region IV of mature spermatozoon of *Tergestia laticollis* showing the appearance of the nucleus (A–B), then the second mitochondrion (M2) (C). (D–E) Cross-sections showing progressively the disappearance firstly of the second mitochondrion and cortical microtubules. (F) Cross-section showing the disorganization of axoneme 2 (Pae2). (G) Cross-section in the posterior extremity of the spermatozoon exhibiting only the nucleus and a few granules of glycogen. (I–J). Transmission electron micrograph showing many sections of the mature spermatozoon of *Tergestia laticollis* with granules of glycogen (G) highlighted by the Thiéry method. Scale bars = 0.5 μm.
Figure 5. Schematic reconstruction of the mature spermatozoa of *Tergestia clonacantha* and *T. laticollis*. Aae1 = anterior extremity of the first axoneme, Aae2 = anterior extremity of the second axoneme, Ase = anterior spermatozoon extremity, A × 1 = first axoneme, A × 2 = second axoneme, Az = attachment zone, Cm = cortical microtubules, Eo = external ornamentation, 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.
Table 1. Comparative data on the ultrastructure of the mature spermatozoon in some digeneans of the same genus and their origins.

| Families and species | Spermatozoon characters | Spermatozoon type | Reference | Host and host origin |
|----------------------|-------------------------|--------------------|-----------|----------------------|
| **Principal characters** | **Secondary characters** |                     |           |                      |
|                       | Tax Le Eo Eo + Cm LEO BCn LMCn M Adn Sh Cob Psc |                     |           |                      |
| Aepnelidogenidae      |                         |                     |           |                      |
| *Notocotylus noyeri*   | PostA 2 MedS 1 + − − − Ax III |                     | [3]       |                      |
| *Notocotylus neyrai*   | PostA 2 MedS 2 + − − − Ax III |                     | [12]      |                      |
| Notocotylidae         |                         |                     |           |                      |
| *Bianium plicitum*     | AntA 2 AntS 1 − + + − Ax V |                     | [18]      | Conger conger (Spain) |
| *Tergestia laticollis* | AntA 2 AntS 1 − + + − Ax V |                     | [26]      | Epinephelus maculatus (New Caledonia) |
| Tergestidae           |                         |                     |           |                      |
| *Dicrocoelium dendriticum* | PostA 2 AntS 2 − + + − N IV |                     | [1]       | Bos indicus (Senegal) |
| Dicrocoelidae         |                         |                     |           |                      |
| *Bianium arabicum*     | AntA 2 AntS 1 − + + − N V |                     | [22]      | Bos indicus (Senegal) |
| Dicrocoeliidae        |                         |                     |           |                      |
| *Holorchis acanthocephala* | PostA 2 AntS 2 − + + − N IV |                     | [13]      | Belone belone gracilis (Tunisia) |
| Diplococidae          |                         |                     |           |                      |
| *Hemirhamphus far*     | AntA 2 AntS 1 − + + − N V |                     | [21]      | Rattus rattus (France) |
| Diplocodiscidae       |                         |                     |           |                      |
| *Fasciola gigantica*   | AntA 2 AntS 1 − + + − N V |                     | [23]      | Hemirhamphus far (New Caledonia) |
| *Fasciola hepatica*    | AntA 2 AntS 1 − + + − N V |                     | [23]      | Lagocephalus sceleratus (New Caledonia) |
| Felledostomidae       |                         |                     |           |                      |
| *Tergestia clionacantha* | MedS 2 − + + − N III |                     | Present | Present study (New Caledonia) |
| *Tergestia laticeps*   | MedS 2 − + + − N III |                     | Present | Present study (New Caledonia) |
| Hemiuridae            |                         |                     |           |                      |
| *Lechicytridium microstomum* | AntA 2 AntS 1 − + + − N V |                     | [24]      | Micratris aragista (Spain) |
| *Lechicytridium musculus* | AntA 2 AntS 1 − + + − N V |                     | [24]      | Micratris aragista (Spain) |
| Lepocreadiidae        |                         |                     |           |                      |
| *Bianium plicitum*     | AntA 2 AntS 1 − + + − N V |                     | [26]      | Lagocephalus laevigatus (Senegal) |
| *Bianium plicitum*     | AntA 2 AntS 1 − + + − N V |                     | [26]      |                      |
| Notocotylidae         |                         |                     |           |                      |
| *Notocotylus neyrai*   | AntA 2 AntS 2 − + + − Ax Undefined |                     | [20]      | Micratris aragista (Spain) |
| *Notocotylus noyeri*   | AntA 2 AntS 2 − + + − Ax IV |                     | [24]      | Micratris aragista (Spain) |
| Opecocoelidae         |                         |                     |           |                      |
| *Allopodocotyle pedicellata* | PostA 2 AntS 2 + + + − Cm IV |                     | [6]       | Speratus aurata (Tunisia) |
| *Allopodocotyle tunisiensis* | PostA 2 AntS 2 + + + − Cm IV |                     | [15]      | Solea aegyptiaca (Tunisia) |
| *Helicometra epinepheli* | PostA 2 MedS 2 + + + − Cm IV |                     | [29]      | Epinephelus fasciatus (New Caledonia) |
| *Helicometra fasciata* | MedS 2 + + + − Cm IV |                     | II       |                      |
| *Nicola testisobilguum* | PostA 2 MedS 2 − + + − Cm IV |                     | [16]      | Labrus merula (France) |
| *Nicola testisobilguum* | PostA 2 MedS 2 − + + − Cm IV |                     | [16]      | Labrus merula (France) |
| (Continued on next page) | | | | |
Conflict of interest

The Editor-in-Chief of Parasite is one of the authors of this manuscript. COPE (Committee on Publication Ethics, http://publicationethics.org), to which Parasite adheres, advises special treatment in these cases. In this case, the peer-review process was handled by an Invited Editor, Jérôme Depaquit.

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Table 1. (Continued)

| Families and species | Spermatozoan characters | Spermatozoan type | Reference | Host and host origin |
|----------------------|-------------------------|-------------------|-----------|---------------------|
|                      | Principal characters    | Secondary characters |          |                     |
| Opisthorchididae      |                         |                   |           |                     |
| Opisthorchis felineus | 9 + “1” - + +          | PostA 2 AntS 2    | IV 32     | Mesocricetus auratus (Unknown origin) |
|                      |                         |                   |           |                     |
| Opisthorchis viverrini| 9 + “1” - + +          | PostA 2 AntS 2    | IV 19     | Mesocricetus auratus (Thailand) |
| Zoogonidae            |                         |                   |           |                     |
| Lecithostaphylus parexocoeti | 9 + “1” - + +   | AntA 2 AntS 2   | Undefined 4 | Cheilopogon pinnatifidus (Senegal) |
|                       |                         |                   |           | Belone belone gracilis (Tunisia) |

Adm, anterior electron-dense material; AntA, anterior part of the anterior region; AntS, anterior region of the spermatozoon; Ax, axoneme; Bcm, number of bundles of cortical microtubules; Cm, cortical microtubules; Cob, cytoplasmic ornamented buttons; D, cortical microtubules; Eo, external ornamentation of the plasma membrane; Eo + Cm, association of external ornamentation with cortical microtubules; L, lateral expansion; LEO, location of the external ornamentation; LM/cm, location of maximum number of cortical microtubules; M, number of mitochondria; MedS, median part of the spermatozoon; N, nucleus; PostA, posterior to the anterior region; Psc, posterior spermatozoon character; Sb, spine-like bodies; Tax, type of axoneme; +/-, presence/absence of considered character; ?, doubtful or unknown data.
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