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3 **Sperm characters of the digenean *Nephrotrema truncatum* (Troglotrematidae): a kidney**  
4 **parasite of *Crocidura russula* (Soricidae) and their phylogenetic significance**  
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## Abstract

Spermatological characteristics of the troglotrematid digenean *Nephrotrema truncatum*, a parasite of *Crocidura russula*, have been investigated by means of transmission electron microscopy. The ultrastructural study reveals that the mature spermatozoon of *N. truncatum* exhibits many ultrastructural characters previously described in most gorgoderoideans. These are two axonemes of the 9+'1' trepaxonematan pattern, four attachment zones, a lateral expansion, an external ornamentation of the plasma membrane associated with spine-like bodies and cortical microtubules, and in the posterior part of the anterior spermatozoon region, two bundles of parallel cortical microtubules with the maximum number located in the anterior part of the spermatozoon, a nucleus, two mitochondria, and granules of glycogen. The obtained results are compared with those of other digeneans, particularly the Gorgoderoidea. The sperm cells gorgoderoideans are of type IV characterised by a 9+'1' pattern of axonemes, the presence of an external ornamentation associated with cortical microtubules and located in posterior area of the anterior extremity, the presence of two bundles of cortical microtubules, the maximum number of cortical microtubules located in the anterior region of the spermatozoon, and the presence of generally two mitochondria. However, dicrocoeliids and troglotrematids have spermatozoa with ornamentation of the plasma membrane and lateral expansions.

**Keywords:** Digenea, Gorgoderoidea, Spermatozoon, Ultrastructure

## Introduction

The Gorgoderoidea is one of the largest superfamilies in the Digenea, which includes 12 families and 123 genera (Littlewood et al. 2015). The systematics of gorgoderoideans is controversial and recently several families have been moved in or out (Curran et al. 2006; Bray and Blair 2008;

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3 Pérez-Ponce de León et al. 2011; Henerek and Literák 2013; Littlewood et al. 2015). This is the  
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5 case of the Brachycoelidae and Collyriclidae, considered by Bray and Blair (2008) in the  
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7 Gorgoderoidea, but recently moved to Plagiorchiodea and Microphalloidea, respectively (Pérez-  
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9 Ponce de León et al. 2011; Henerek and Literák 2013). Contrary to this, the Allocreadiidae  
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11 family is included in the Gorgoderoidea (Curran et al. 2006). Among the 12 families of  
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13 gorgoderoideans is the Troglotrematidae, which includes cosmopolitan species of parasites in  
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15 different organs and tissues of mammals and seabirds. The systematic status of the members of  
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17 this family is controversial (for a review, see Blair et al. 2008). According to these authors, the  
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19 Troglotrematidae has 10 genera. Among them, is the genus *Nephrotrema* and its type species *N.*  
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21 *truncatum* (Leuckart, 1842), a frequently recorded parasite of the kidney of small insectivorous  
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23 mammals in Europe and objective of the ultrastructural study of the spermatozoon carried out in  
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25 the present work.  
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31 The usefulness of ultrastructural characters of spermiogenesis and/or the spermatozoon in the  
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33 interpretation of relationships among parasitic platyhelminthes has been demonstrated for many  
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35 years (for a review, see Justine 1991 and Levron et al. 2010). In the last decade, the potential of  
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37 this source of characters for phylogenetic purposes in the Digenea has been analysed by various  
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39 authors (Quilichini, Foata et al. 2007; Quilichini et al. 2010a, 2011; Bakhoum et al. 2017). To  
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41 date, there are more or less complete spermatological studies in a large number of digeneans  
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43 belonging to 18 superfamilies and more than 50 families (Justine 1995; Bakhoum et al. 2017;  
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45 Kacem and Miquel 2018; Ndiaye et al. 2018). In the Gorgoderoidea published works on the  
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47 ultrastructure of the spermatozoon involve the Acanthocolpidae, Allocreadiidae, Dicrocoeliidae,  
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49 Paragonimidae and Troglotrematidae families (Robinson and Halton 1982; Orido 1988; Cifrian et  
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51 al. 1993; Agostini et al. 2005; Miquel et al. 2006; Quilichini, Foata, Orsini et al. 2007; Bakhoum,  
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53 Quilichini, Justine et al. 2015; Bakhoum, Quilichini, Miquel et al. 2015). According to the above-  
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3 mentioned systematic reorganisations (Pérez-Ponce de León et al. 2011; Henerek and Literák  
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5 2013), the ultrastructural studies of spermatozoa of *Brachycoelium salamandrae*  
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7 (Brachycoeliidae) and *Collyricloides massanae* (Collyriclidae) (see Bakhoum et al. 2013, 2014)  
8  
9 have not been considered for comparative purposes in the Gorgoderoidea.  
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12 The aim of this study is to describe the sperm characters and their organisation in the  
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14 spermatozoon of *N. truncatum*. It represents the second study of this family, the sperm of  
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16 *Troglostrema acutum* being the other troglotrematid described to date (Miquel et al. 2006).  
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18 Additionally, our results on the ultrastructure of the spermatozoon are compared with those of  
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20 other digeneans, in particular troglotrematids and other gorgoderoideans, to highlight the  
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22 potential of sperm characters in phylogeny.  
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## 28 **Materials and methods**

### 31 ***Materials***

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34 Live adult specimens of *Nephrotrema truncatum* were collected from the kidneys of a naturally  
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36 infected *Crocidura russula* (Hermann, 1780) (Soricidae) accidentally trapped in the Natural  
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38 Reserve of Py (Pyrenean Mountains, France) in October 2017.  
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### 44 ***Transmission electron microscopy (TEM)***

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46 For TEM, several live adult worms were immediately rinsed with a 0.9% NaCl solution and fixed  
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48 in cold (4 °C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum  
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50 of 2 h, rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1% osmium  
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52 tetroxide with 0.9% potassium ferricyanide in the same buffer for 1 h, rinsed in Milli-Q water  
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54 (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in  
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3 Spurr's resin and polymerised at 60 °C for 72 h. Ultrathin sections (60–90 nm thick) at the level  
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5 of the seminal vesicle were obtained using a Reichert-Jung Ultracut E ultramicrotome. Sections  
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7 were placed on 200-mesh copper and gold grids. Sections placed on copper grids were double-  
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9 stained with uranyl acetate and lead citrate according to the Reynolds (1963) procedure. Copper  
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11 grids were examined in a JEOL 1010 transmission electron microscope operated at an  
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13 accelerating voltage of 80 kV, in the 'Centres Científics i Tecnològics' of the University of  
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15 Barcelona (CCiTUB).  
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### 21 ***Cytochemistry***

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23 Sections placed on gold grids were treated according to the Thiéry (1967) test to reveal the  
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25 presence of glycogen. Thus, they were treated in periodic acid (PA), thiocarbohydrazide (TCH)  
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27 and silver proteinate (SP) as follows: 30 min in 10% PA, rinsed in Milli-Q water, 24 h in TCH,  
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29 rinsed in acetic solutions and Milli-Q water, 30 min in 1% SP in the dark and rinsed in Milli-Q  
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31 water. Sections were examined in a JEOL 1010 transmission electron microscope in the  
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### 40 **Results**

41  
42 The observation of numerous ultrathin sections at the level of seminal vesicle allowed us to  
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44 distinguish three regions in the mature spermatozoon of *N. truncatum* (Figures 1-4). These  
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46 regions exhibit different ultrastructural characteristics and organisation.  
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### 51 ***Region I***

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53 Region I corresponds to the anterior extremity of the spermatozoon (Figures 1a-l, 2g and 4I). The  
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55 anterior spermatozoon tip is sharp and lacks axonemes (Figure 1a). The two axonemes of the  
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3 9+'1' pattern appear simultaneously and they are surrounded by a continuous and submembranous  
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5 layer of cortical microtubules (Figure 1b-e). This is the area of the sperm cell that has the  
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7 maximum number of cortical microtubules (about 37) (Figure 1e). Two and four attachment  
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9 zones appear progressively (Figure 1e-g). Different characteristics can be observed in areas with  
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11 the four attachment zones: (i) cortical microtubules distributed in two bundles (Figures 1g, j-l and  
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13 4I), (ii) appearance of an external ornamentation of the plasma membrane (Figures 1g-l and 4I),  
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15 (iii) presence of spine-like bodies (Figures 1i-k and 4I), (iv) presence of a reduced lateral  
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17 expansion (Figures 1g, h and 4I), and (v) appearance of the first mitochondrion in the posterior  
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19 part of region I (Figures 1i-l and 4I). Granules of glycogen irregularly distributed are also  
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21 observed along this region (Figures 1f-l and 2g).  
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### 28 ***Region II***

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30 Region II is the middle region of the spermatozoon (Figures 2a-g and 4II). It is characterised by  
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32 the disappearance of the external ornamentation of the plasma membrane and the spine-like  
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34 bodies. The anterior part of this region presents the posterior extremity of the first mitochondrion  
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36 (Figures 2a, b and 4II) while the posterior part exhibits the anterior extremity of the second  
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38 mitochondrion (Figures 2e, f and 4II). The inter-mitochondrial area has only the two axonemes,  
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40 cortical microtubules and granules of glycogen (Figure 2c, d, g).  
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### 47 ***Region III***

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49 Region III corresponds to the posterior region of the spermatozoon (Figures 2g, 3a-i and 4III). It  
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51 is mainly characterised by the presence of the nucleus. In the anterior part of region III, the  
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53 second mitochondrion and the nucleus are both present (Figures 3a-c and 4III). The nucleus  
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55 increases progressively its size and the number of cortical microtubules are reduced (from 3 or 4  
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3 + 6 to 2 + 2 or 3) (Figure 3a-c). Posteriorly, the second mitochondrion disappears and the first  
4 axoneme is disorganized (Figure 3 d, e). Transition toward the posterior spermatozoon tip shows  
5 the termination of cortical microtubules, the disorganisation and disappearance of the second  
6 axoneme and the posterior extremity of the nucleus (Figure 3f-i). Several singlets from the  
7 second axoneme reach the posterior tip of the spermatozoon (Figure 3i).

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14 The granular material observed in all the regions has been determined as glycogen by means  
15 of the test of Thiéry (Figure 2g).

## 16 17 18 19 20 21 **Discussion**

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23 The mature spermatozoon of *N. truncatum* exhibits many ultrastructural characters previously  
24 described in most gorgoderoideans and also in other digeneans. These are two axonemes of the  
25 9+'1' trepaxonematan pattern (Ehlers 1984), a nucleus, two mitochondria, two bundles of parallel  
26 cortical microtubules, four attachment zones, a lateral expansion, an external ornamentation of  
27 the plasma membrane, spine-like bodies and granules of glycogen (see Table 1).

### 28 29 30 31 32 33 ***Anterior spermatozoon extremity***

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38 The anterior extremity of the spermatozoon of *N. truncatum* has two axonemes surrounded by a  
39 continuous and submembranous layer of cortical microtubules. This arrangement has been  
40 described previously in other gorgoderoideans, namely *Stephanostomoides tenuis*,  
41 *Brachylecithum eliomydis* and *Troglorema acutum* (Miquel et al. 2006; Bakhoun, Quilichini,  
42 Justine et al. 2015; Bakhoun, Quilichini, Miquel et al. 2015). However, variability in the anterior  
43 extremity is evident in the Gorgoderoidea. Thus, other species have only one of the axonemes  
44 accompanied or not by cortical microtubules (see Table 1).

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3 material. This dense material has been found in numerous species belonging to the  
4  
5 Aephnidiogenidae, Atractotrematidae, Cryptogonimidae, Gyliuchenidae, Monorchiidae or  
6  
7 Opescoelidae (see Bakhoun et al. 2017). However, all the studied gorgoderoideans lack this  
8  
9 character.  
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#### 14 ***Lateral expansion, external ornamentation and spine-like bodies***

15  
16 Bakhoun et al. (2017) have considered diverse morphological types of cytoplasmic expansions  
17  
18 associated with cortical microtubules under the general term of lateral expansions. All these  
19  
20 expansions are located in the anterior area of the spermatozoon and they are usually associated  
21  
22 with other characters such as the external ornamentation of the plasma membrane and spine-like  
23  
24 bodies. In the Gorgoderoidea only *T. acutum* exhibits a lateral expansion with a hook-shaped  
25  
26 morphology (Miquel et al. 2006), whereas the remaining studied species lack a lateral expansion.  
27  
28 Nevertheless, the mature spermatozoa of the dicrocoeliid *Dicrocoelium hospes* seem to have a  
29  
30 reduced expansion (Agostini et al. 2005). In the present study we have also observed a reduced  
31  
32 cytoplasmic expansion in *N. truncatum*.  
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38 An external ornamentation of the plasma membrane has been described in the mature  
39  
40 spermatozoon of numerous digeneans and various authors analysed the potential of this character  
41  
42 for phylogenetic inference (Quilichini, Foata et al. 2007; Quilichini et al. 2011; Bakhoun et al.  
43  
44 2017). Three types of anterior extremities according to the presence/absence of this character and,  
45  
46 if present, its location have been considered by these authors: ornamentation (i) present in the  
47  
48 anterior extremity, (ii) present in a more posterior area or (iii) absent. In the Gorgoderoidea, all  
49  
50 the studied species have ornamentation of the plasma membrane located in posterior areas of the  
51  
52 anterior region, except in the dicrocoeliids *Corrigia vitta* and *Dicrocoelium dendriticum*, which  
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2  
3 lack ornamentation in their sperm cells (Robinson and Halton 1982; Cifrian et al. 1993).  
4

5 Concerning the external ornamentation of the plasma membrane there is another important  
6  
7 criterion useful for phylogenetic purposes and useful to establish sperm types in the Digenea: its  
8  
9 association or not with cortical microtubules (Bakhoum et al. 2017). In the case of the  
10  
11 Gorgoderoidea, all the studied species have external ornamentation associated with cortical  
12  
13 microtubules with the exception of the above-mentioned dicrocoeliids that lack ornamentation  
14  
15 (Robinson and Halton 1982; Cifrian et al. 1993).  
16  
17

18  
19 Spine-like bodies were originally described as prominent submembranous electron-dense  
20  
21 structures that contain a sort of vesicle (Miquel et al. 2000). Among the Gorgoderoidea, as occurs  
22  
23 in *N. truncatum*, the spermatozoon of the majority of studied species have spine-like bodies (see  
24  
25 Table 1). There are only two dicrocoeliids (*C. vitta* and *D. dendriticum*) that lack these structures  
26  
27 (Robinson and Halton 1982; Cifrian et al. 1993). According to various authors, these structures  
28  
29 could have been misinterpreted as artefacts of fixation in older papers (see Bakhoum et al. 2017).  
30  
31 For example, in the paragonimid *Paragonimus ohirai* (Orido 1988) and in the haematoloechid  
32  
33 *Haematoloechus* sp. (Justine and Mattei 1982) spine-like bodies are clearly present but authors  
34  
35 omitted these in the respective descriptions of sperm cells. In fact, since its original description,  
36  
37 spine-like bodies have been usually found in numerous digeneans. We think that the absence of  
38  
39 these ultrastructural elements in certain species should be considered with caution, particularly in  
40  
41 those belonging to groups containing a large number of records. This would be the case of  
42  
43 gorgoderoideans with the two above-mentioned dicrocoeliids lacking spine-like bodies  
44  
45 (Robinson and Halton 1982; Cifrian et al. 1993).  
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### 51 52 53 ***Cortical microtubules***

54  
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56 The presence of parallel cortical microtubules has been described in digenean spermatozoa  
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3 except in the genus *Didymozoon* (Justine and Mattei 1983). In all the digeneans in which these  
4  
5 structures were detected, the existing variability in the male gamete refers to the number,  
6  
7 disposition and location of the maximum number. As occur in *N. truncatum* and other  
8  
9 gorgoderoideans, in digeneans cortical microtubules are arranged into two bundles, at least in  
10  
11 principal regions of the sperm cell containing the nucleus and/or mitochondria. Only in certain  
12  
13 hemiuroideans (Hemiuridae, Lecithasteridae and Sclerodistomidae) these ultrastructural elements  
14  
15 are grouped in a single bundle (for a review, see Bakhoum et al. 2017).  
16  
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18

19 The location of the maximum number of cortical microtubules is another interesting  
20  
21 character. According to Bakhoum et al. (2017), all the species of the Gorgoderoidea have the  
22  
23 maximum number of cortical microtubules in the anterior region of the spermatozoon (see Table  
24  
25 1). The maximum number of microtubules is quite homogeneous among species of this  
26  
27 superfamily, being between 30 and 44 microtubules. This is a range usually found between  
28  
29 digenean spermatozoa. However, as above-mentioned, it is interesting to note the absence of  
30  
31 cortical microtubules in *Didymozoon* sp. and also their reduced number (5 to 10 microtubules) in  
32  
33 certain hemiuroideans. Other species, e.g. *Pronoprymna ventricosa*, *Adlardia novaecaledoniae* or  
34  
35 *Aphallus tubarium* (Quilichini, Foata et al. 2007; Quilichini et al. 2009; Foata et al. 2012) also  
36  
37 exhibit a reduced number (around 10 microtubules). By contrast, *Scaphiostomum palaearticum*  
38  
39 (Ndiaye et al. 2002) and other species with a large lateral expansion, such as *Diplodiscus*  
40  
41 *subclavatus* (Bakhoum et al. 2011) or *D. ampicurus* (Diagne unpublished data) have a higher  
42  
43 number (65, 75 and 63 microtubules, respectively).  
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### 51 ***Mitochondria***

52  
53 In the Digenea, the number and morphology of the mitochondrion/a vary according to the  
54  
55 species. The number of mitochondria varies between 1 and 3 (for a review, see Bakhoum et al.  
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2  
3 2017). In the Gorgoderoidea, all the species analysed until now have two mitochondria except the  
4  
5 microcoeliids *C. vitta* and *D. dendriticum* (Robinson and Halton 1982; Cifrian et al. 1993).

6  
7 Concerning their morphology, various authors have described the presence of moniliform  
8  
9 mitochondria constituted by several mitochondrial bulges joined by a mitochondrial cord. This is  
10  
11 the case of *Holorchis micracanthum* (Aephnidiogenidae), *Timoniella imbutiforme*  
12  
13 (Cryptogonimidae), *Opechona bacillaris* (Lepocreadiidae), *Macvicaria obovata* (Opecoelidae)  
14  
15 and *Enodiotrema reductum* (Plagiorchiidae) (Bâ et al. 2011; Ndiaye et al. 2012, 2015; Kacem et  
16  
17 al. 2017; Kacem, Quilichini et al. 2017). In the Gorgoderoidea only *Stephanostomoides tenuis*  
18  
19 (Acanthocolpidae) has this type of mitochondrion (Bakhoum, Quilichini, Justine et al., 2015).  
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### 26 ***Posterior spermatozoon extremity***

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28 The posterior spermatozoon extremity is another region with a great variability of characteristics.  
29  
30 Considering the sequence of disappearance of principal characters (nucleus, axoneme and cortical  
31  
32 microtubules) in the posterior extremity of the male gamete, and after comparison of 19 species  
33  
34 of Digenea belonging to 12 families, Quilichini et al. (2010a) distinguish three types of posterior  
35  
36 spermatozoon extremities. These are: type 1 (opecoelidean type) characterised by the sequence  
37  
38 ‘axoneme, nucleus and cortical microtubules’; type 2 (fasciolidean type) with the sequence  
39  
40 ‘cortical microtubules, axoneme and nucleus’ and type 3 (cryptogonimidean type) with the  
41  
42 sequence ‘cortical microtubules, nucleus and axoneme’. Based on the posterior mitochondrion  
43  
44 and axonemes of the spermatozoon of *Aponurus laguncula* described by (Quilichini et al.,  
45  
46 2010b), Bakhoum et al. (2017), recommend that only the terminal character of the spermatozoon  
47  
48 should be used. Thus, three types of posterior spermatozoon extremities have been established: (i)  
49  
50 a posterior spermatozoon extremity containing only cortical microtubules, (ii) a posterior  
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52 spermatozoon extremity containing only the nucleus, and (iii) a posterior spermatozoon extremity  
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3 containing only one axoneme. The posterior extremity of the mature spermatozoon of *N.*  
4  
5 *truncatum* as in the other troglotrematid (*T. acutum* -Miquel et al. 2006) follow the  
6  
7 cryptogonimidean type. The remaining studied families of gorgoderoideans, the allocreadiids,  
8  
9 paragonimids and dicrocoeliids (except *D. hospes* -Agostini et al. 2005) also seem to follow the  
10  
11 cryptogonimidean pattern, while acanthocolpids differ from them and exhibit the fasciolidean  
12  
13 type of posterior extremity. This variability within gorgoderoideans is clearly reflected in Table 1  
14  
15 at the level of the posterior spermatozoon character: axoneme or nucleus.  
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### 21 ***Sperm model for the Gorgoderoidea***

22 Bakhoum et al. (2017) have defined five spermatological models in the Digenea based on the  
23  
24 principal spermatological characters described in the Digenea. These principal characters concern  
25  
26 axonemes, the external ornamentation of the plasma membrane, the lateral expansion, cortical  
27  
28 microtubules and the mitochondrion. The mature spermatozoon of species of the Gorgoderoidea  
29  
30 is of type IV characterised by a 9+'1' pattern of axonemes, the presence of an external  
31  
32 ornamentation associated with cortical microtubules and located in posterior area of the anterior  
33  
34 extremity, the presence of two bundles of cortical microtubules, the maximum number of cortical  
35  
36 microtubules located in the anterior region of the spermatozoon, and the presence of generally  
37  
38 two mitochondria. There are, however, certain discrepancies concerning the Dicrocoeliidae and  
39  
40 Troglotrematidae concerning ornamentation of the plasma membrane and lateral expansions.  
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### 14 15 16 17 **Disclosure statements**

18  
19 No potential conflict of interest was reported by the authors.  
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### 23 Captions of figures

26 **Figure 1.** Region I of the spermatozoon of *Nephrotrema truncatum*. (a) longitudinal section of  
27 the anterior extremity of the spermatozoon, (b-d) cross-sections showing the progressive  
28 appearance of axonemes. Note the presence of a continuous and submembranous layer of cortical  
29 microtubules, (e, f) cross-sections showing the presence of two attachment zones (arrows), (g, h)  
30 cross-sections of anterior area of the ornamented region. Arrows indicate the presence of four  
31 attachment zones, (i) longitudinal section of the posterior area of the ornamented region showing  
32 the simultaneous presence of external ornamentation, spine-like bodies and the first  
33 mitochondrion, (j-l) cross-sections of posterior area of the ornamented region. ASE: anterior  
34 spermatozoon extremity, Ax1: first axoneme, C1 and C2: centrioles of the first and second  
35 axonemes, CM: cortical microtubules, EO: external ornamentation of the plasma membrane, G:  
36 granules of glycogen, LE: lateral expansion, M1: first mitochondrion, SB: spine-like bodies, scale  
37 bars: 0.3  $\mu\text{m}$ .

40 **Figure 2.** Region II and test of Thiéry of the spermatozoon of *Nephrotrema truncatum*. (a-f)  
41 consecutive cross-sections from the anterior area of region II with posterior extremity of the first

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3 mitochondrion to the posterior area containing the anterior extremity of the second  
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5 mitochondrion, (g) positive test of Thiéry for glycogen in regions I, II and III (R-I, R-II and R-  
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7 III). CM: cortical microtubules, G: granules of glycogen, M1 and M2: first and second  
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9 mitochondrion, N: nucleus, scale bars: 0.3  $\mu\text{m}$ .

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12 **Figure 3.** Region III of the spermatozoon of *Nephrotrema truncatum*. (a-c) cross-sections of  
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14 areas with simultaneous presence of nucleus and second mitochondrion, (d, e) cross-sections  
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16 showing the interruption of the second mitochondrion, the disorganisation of the first axoneme  
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18 and the progressive reduction of cortical microtubules, (f) longitudinal section showing the  
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20 posterior spermatozoon extremity, (g, h) cross-sections showing the disorganisation of the second  
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22 axoneme, (i) cross-section at the level of posterior tip with some singlets. Ax2: second axoneme,  
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24 CC1 and CC2: central core of the first and second axoneme, CM: cortical microtubules, G:  
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26 granules of glycogen, M2: second mitochondrion, N: nucleus, PSE: posterior spermatozoon  
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28 extremity, S2: singlets of the second axoneme, scale bars: 0.3  $\mu\text{m}$ .

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33 **Figure 4.** Schematic reconstruction of the spermatozoon of *Nephrotrema truncatum*. ASE:  
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35 anterior spermatozoon extremity, Ax1 and Ax2: first and second axoneme, AZ: attachment zones,  
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37 C1 and C2: centrioles of the first and second axoneme, CC1 and CC2: central core of the first and  
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39 second axoneme, CM: cortical microtubules, EO: external ornamentation of the plasma  
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41 membrane, G: granules of glycogen, LE: lateral expansion, M1 and M2: first and second  
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43 mitochondrion, N: nucleus, PM: plasma membrane, PSE: posterior spermatozoon extremity, S2:  
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45 singlets of the second axoneme, SB: spine-like bodies.  
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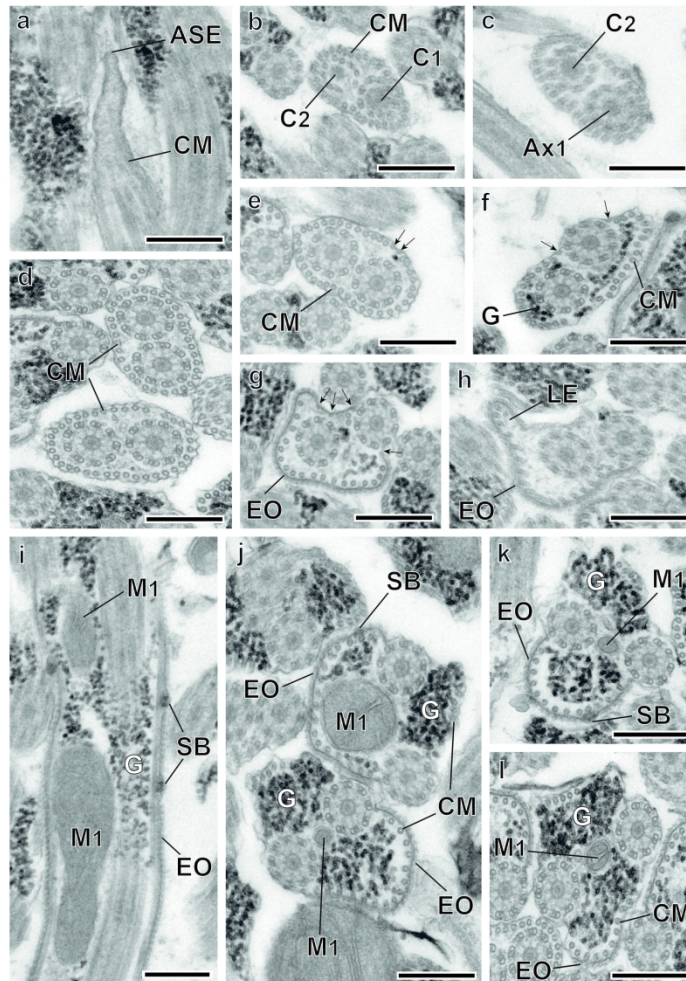


Figure 1. Region I of the spermatozoon of *Nephrotrema truncatum*. (a) longitudinal section of the anterior extremity of the spermatozoon, (b-d) cross-sections showing the progressive appearance of axonemes. Note the presence of a continuous and submembranous layer of cortical microtubules, (e, f) cross-sections showing the presence of two attachment zones (arrows), (g, h) cross-sections of anterior area of the ornamented region. Arrows indicate the presence of four attachment zones, (i) longitudinal section of the posterior area of the ornamented region showing the simultaneous presence of external ornamentation, spine-like bodies and the first mitochondrion, (j-l) cross-sections of posterior area of the ornamented region. ASE: anterior spermatozoon extremity, Ax1: first axoneme, C1 and C2: centrioles of the first and second axonemes, CM: cortical microtubules, EO: external ornamentation of the plasma membrane, G: granules of glycogen, LE: lateral expansion, M1: first mitochondrion, SB: spine-like bodies, scale bars: 0.3  $\mu$ m.

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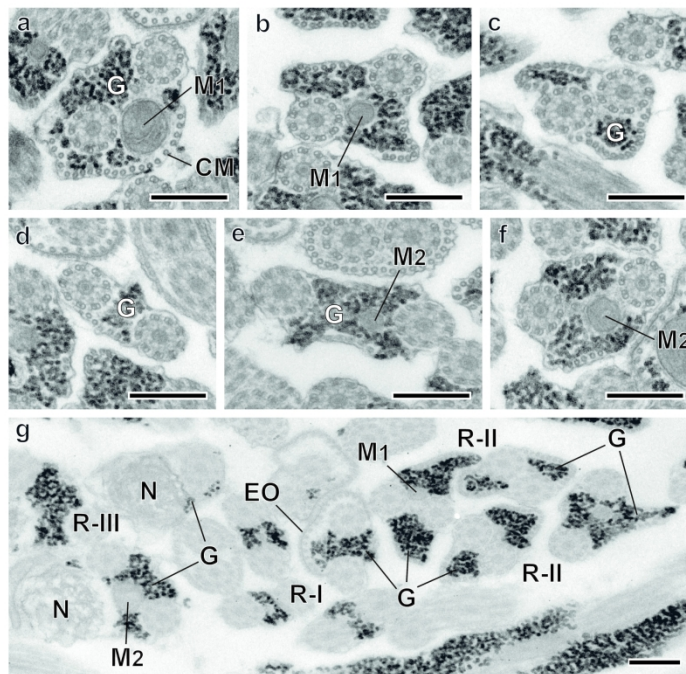


Figure 2. Region II and test of Thiéry of the spermatozoon of *Nephrotrema truncatum*. (a-f) consecutive cross-sections from the anterior area of region II with posterior extremity of the first mitochondrion to the posterior area containing the anterior extremity of the second mitochondrion, (g) positive test of Thiéry for glycogen in regions I, II and III (R-I, R-II and R-III). CM: cortical microtubules, G: granules of glycogen, M1 and M2: first and second mitochondrion, N: nucleus, scale bars: 0.3  $\mu\text{m}$ .

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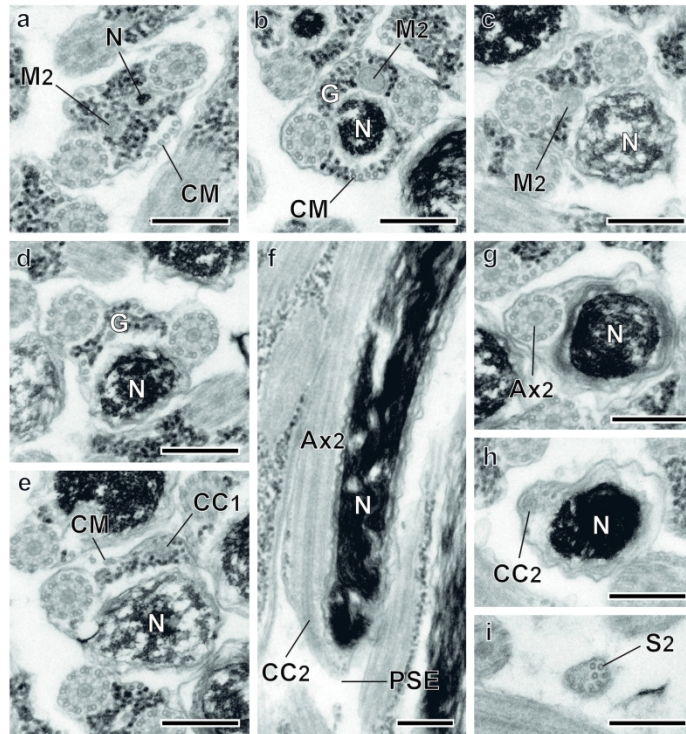
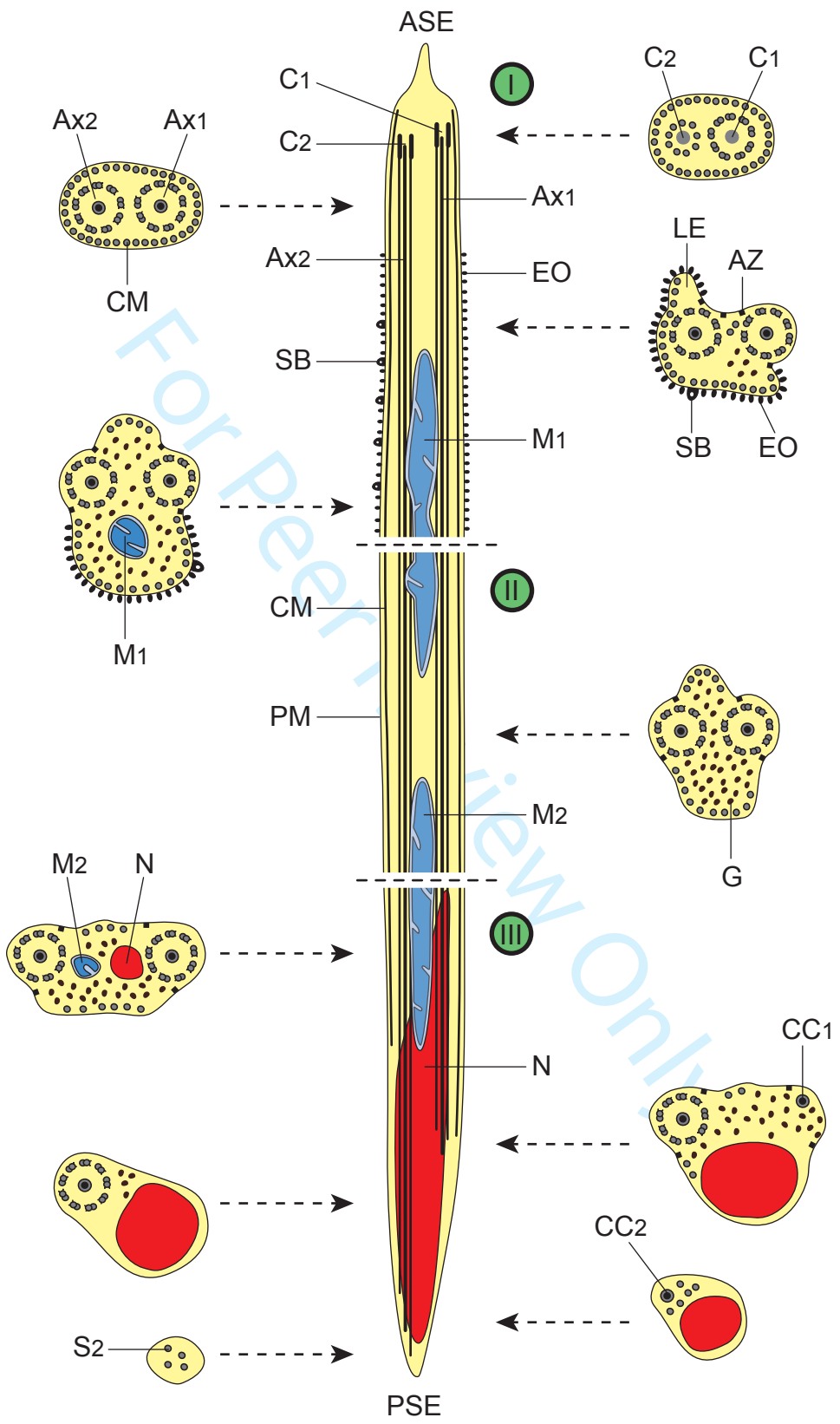


Figure 3. Region III of the spermatozoon of *Nephrotrema truncatum*. (a-c) cross-sections of areas with simultaneous presence of nucleus and second mitochondrion, (d, e) cross-sections showing the interruption of the second mitochondrion, the disorganisation of the first axoneme and the progressive reduction of cortical microtubules, (f) longitudinal section showing the posterior spermatozoon extremity, (g, h) cross-sections showing the disorganisation of the second axoneme, (i) cross-section at the level of posterior tip with some singlets. Ax2: second axoneme, CC1 and CC2: central core of the first and second axoneme, CM: cortical microtubules, G: granules of glycogen, M2: second mitochondrion, N: nucleus, PSE: posterior spermatozoon extremity, S2, singlets of the second axoneme, scale bars: 0.3  $\mu\text{m}$ .

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**Table 1.** Ultrastructural characteristics of the spermatozoon in the Gorgoderoidea

Families/Species	ASE	LE	EO	EO+CM	LEO	MCM	LMCM	M	SB	PSC	References
<b>Acanthocolpidae</b>											
<i>Stephanostomoides tenuis</i>	2Ax-CM	–	+	+	PostA	31	AntS	2*	+	N	Bakhoum, Quilichini, Justine et al. (2015)
<i>Stephanostomum murielae</i>	1Ax-CM	–	+	+	PostA	30	AntS	2	+	N	Bakhoum, Quilichini, Justine et al. (2015)
<b>Allocreadiidae</b>											
<i>Crepidostomum metoecus</i>	1Ax	–	+	+	PostA	32	AntS	2	+	Ax	Quilichini, Foata, Orsini et al. (2007)
<b>Dicrocoeliidae</b>											
<i>Brachylecithum eliomydis</i>	2Ax-CM?	–	+	+	PostA	44	AntS	2	+	Ax	Bakhoum, Quilichini, Miquel et al. (2015)
<i>Corrigia vitta</i>	?	–	–	NA	NA		AntS	1?	–	Ax?	Robinson and Halton (1982)
<i>Dicrocoelium dendriticum</i>	?	–	–	NA	NA	44	AntS	1	–	?	Cifrian et al. (1993)
<i>Dicrocoelium hospes</i>	1Ax	+(red.)	+	+	PostA	36	AntS	2	+	N	Agostini et al. (2005)
<b>Paragonimidae</b>											
<i>Paragonimus ohirai</i>	1Ax-CM?	–	+	+	PostA	32	AntS	2?	+	Ax?	Orido (1988)
<b>Troglorematidae</b>											
<i>Nephrotrema truncatum</i>	2Ax-CM	+(red.)	+	+	PostA	37	AntS	2	+	Ax	Present study
<i>Troglorema acutum</i>	2Ax-CM?	+	+	+	PostA	34	AntS	2	+	Ax	Miquel et al. (2006)

AntS: anterior part of the spermatozoon, ASE: anterior spermatozoon extremity, Ax: axoneme, BCM: number of bundles of cortical microtubules, CM: cortical microtubules, EO: external ornamentation of the plasma membrane, EO+CM: association external ornamentation and cortical microtubules, LE: lateral expansion, LEO: location of the 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, PostA: posterior part of the anterior region, PSC: posterior spermatozoon character, red.: reduced, SB: spine-like bodies, +/-: presence/absence of the considered character, ?: doubtful or unknown data, \*one mitochondrion is moniliform.