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1 *Echinococcus multilocularis* Leuckart, 1863 (Taeniidae): new data on sperm  
2 ultrastructure  
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**Abstract:** The present study establishes the ultrastructural organisation of the mature spermatozoon of *Echinococcus multilocularis*, which is essential for future research on the location of specific proteins involved in the sperm development in this species and also in *Echinococcus granulosus*. Thus, the ultrastructural characteristics of the sperm cell are described by means of transmission electron microscopy. The spermatozoon of *E. multilocularis* is a filiform cell, which is tapered at both extremities and lacks mitochondria. It exhibits all the characteristics of type VII spermatozoon of tapeworms, namely a single axoneme, crested bodies, spiralled cortical microtubules and nucleus, a periaxonemal sheath and intracytoplasmic walls. Other characteristics observed in the male gamete are the presence of a >900 nm long apical cone in its anterior extremity and only the axoneme in its posterior extremity. The ultrastructural characters of the spermatozoon of *E. multilocularis* are compared with those of other cestodes studied to date, with particular emphasis on representatives of the genus *Taenia*. The most interesting finding concerns the presence of two helical crested bodies in *E. multilocularis* while in the studied species of *Taenia* there is only one crested body. Future ultrastructural studies of other species of the genus *Echinococcus* would be of particular interest in order to confirm whether or not the presence of two crested bodies is a characteristic of this genus.

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**Keywords:** *Echinococcus multilocularis*, Taeniidae, Cestoda, spermatozoon, ultrastructure

## 40 41 42 43 **Introduction**

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Among taeniid cestodes, the genus *Echinococcus* includes species with great medical and veterinary importance, causing important zoonotic infections, namely cystic and alveolar echinococcosis. Within this genus, *Echinococcus multilocularis* is the zoonotic agent of human alveolar echinococcosis or alveolar hydatid disease. It is present in the northern hemisphere and its indirect life cycle includes wild canids (mainly foxes and wolfs) and also dogs as definitive hosts, harbouring the adult tapeworm, whereas some micromammals, particularly *Arvicola terrestris* and *Microtus* spp., act as intermediate hosts, harbouring the larval stage. Humans may also be infected by the metacestode of *E. multilocularis* and develop the alveolar hydatid disease.

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The usefulness of ultrastructural characters of the spermatozoon for phylogenetic

1 inference has been clearly demonstrated within Platyhelminthes, particularly in cestodes  
2 (Justine 1991a, b, 1998, 2001; Bâ and Marchand 1995; Miquel et al. 1999, 2007;  
3 Levron et al. 2010). Presently, there is a consensus concerning the necessity of  
4 integrating morphology, molecular and biological data for a better knowledge of  
5 systematics and evolution of Platyhelminthes (Hoberg et al. 1997, 1999; Littlewood et  
6 al. 1998; Olson et al. 2001; Waeschenbach et al. 2007, 2012). In the last years,  
7 numerous ultrastructural studies have been published concerning spermiogenesis and or  
8 the spermatozoon of cestodes (see Marigo 2011). Most of them refer to the order  
9 Cyclophyllidea for which more than 60 species were analysed (Marigo 2011; Yoneva et  
10 al. 2012; Bâ et al. 2014; Miquel et al. 2015). Concerning tapeworms of the family  
11 Taeniidae there are ultrastructural and spermatological studies for ten species, namely  
12 *Echinococcus granulosus* (Morseth 1969), *E. multilocularis* (Barrett and Smyth 1983;  
13 Shi et al. 1994), *Taenia crassiceps* (Willms et al. 2004; Willms and Robert 2007), *T.*  
14 *hydatigena* (Featherston 1971; Miquel et al. 2015), *T. mustelae* (Miquel et al. 2000), *T.*  
15 *parva* (Ndiaye et al. 2003), *T. pisiformis* (Tian et al. 1998a, b), *T. saginata* (Tian et al.  
16 1998a, b; Bâ et al. 2011), *T. solium* (Tian et al. 1998a, b; Willms et al. 2003) and *T.*  
17 *taeniaeformis* (Miquel et al. 2009a, b). However, studies on the spermatozoon  
18 ultrastructure of the genus *Echinococcus* have been rather neglected; the available  
19 results are limited to briefly illustrated papers or conference proceedings presenting  
20 incomplete ultrastructural data on the organisation of their sperm cells.  
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36 The aim of the present work concerns the ultrastructural study of *E.*  
37 *multilocularis* in order to draw a complete description of the spermatozoon for  
38 comparison with other cyclophyllideans, particularly taeniids. The present work  
39 constitutes the first complete study of the ultrastructural organization of the male  
40 gamete in a species of the genus *Echinococcus*. In the framework of the Paravac project,  
41 the present study on sperm ultrastructure is essential for future works with both *E.*  
42 *granulosus* and *E. multilocularis* concerning the immunohistochemical investigation of  
43 the expression sites of some antigenic proteins during sperm development.  
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## 53 **Materials and methods**

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56 Live specimens of *Echinococcus multilocularis* were isolated from the intestine of a  
57 naturally infected red fox (*Vulpes vulpes* L.) from La Roche sur Foron (France) captured  
58 in June 2014.  
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1 Adult recovered tapeworms were immediately rinsed with a 0.9 % NaCl solution.  
2 Later, they were fixed in cold (4 °C) 2.5 % glutaraldehyde in a 0.1 M sodium cacodylate  
3 buffer at pH 7.4 for a minimum of 2 h, rinsed in 0.1 M sodium cacodylate buffer at pH  
4 7.4, post-fixed in cold (4 °C) 1 % osmium tetroxide with 0.9 % potassium ferricyanide  
5 in the same buffer for 1 h, rinsed in MilliQ water (Millipore Gradient A10), dehydrated  
6 in an ethanol series and propylene oxide, embedded in Spurr's resin and polymerised at  
7 60 °C for 72 h. Ultrathin sections (60–90 nm thick) of mature segments at the level of  
8 the vas deferens were obtained in a Reichert-Jung Ultracut E ultramicrotome. Sections  
9 were placed on 200- $\mu$ m mesh copper grids and double-stained with uranyl acetate and  
10 lead citrate according to the Reynolds (1963) methodology. The grids were examined in  
11 a JEOL 1010 transmission electron microscope operated at 80 kV, in the “Centres  
12 Científics i Tecnològics” of the University of Barcelona (CCiTUB).  
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## 23 **Results**

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27 The observation of numerous ultrathin sections has enabled us to establish the main  
28 ultrastructural characteristics of the mature spermatozoon of *Echinococcus*  
29 *multilocularis* (Figs. 1, 2 and 3). The spermatozoon is a filiform cell, tapered at both  
30 extremities and lacking mitochondria. Four consecutive regions (I–IV) with differential  
31 ultrastructural features can be distinguished.  
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36 Region I (Figs. 1A–F and 3I) constitutes the anterior extremity of the  
37 spermatozoon. This region is mainly characterised by the presence of an apical cone and  
38 two crested bodies. From the anterior tip of the spermatozoon to the centriole, there is  
39 an electron-dense apical cone (Fig. 1A–C) more than 900 nm long and about 200 nm  
40 wide. The crested bodies are formed by two helical cords very close to one another that  
41 externally surround the sperm cell throughout region I (Fig. 1A–F). The maximum  
42 thickness of crested bodies is about 75 nm at the level of the centriole (Fig. 1B–D). The  
43 thickness of the crested bodies decreases progressively toward the end of region I (Fig.  
44 1F). The axoneme, of the 9+‘1’ trepaxonematan pattern (Fig. 1E), is centrally located  
45 and surrounded by a thin layer of electron lucent cytoplasm and by an electron-dense  
46 submembranous layer of cortical microtubules spirally arranged at an angle of about 45°  
47 in relation to the hypothetical longitudinal axis of the spermatozoon (Fig. 1B,C).  
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58 Region II (Figs. 1F–H, 2A and 3II) is devoid of crested body being characterised  
59 by the progressive appearance of both periaxonemal sheath and intracytoplasmic walls  
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1 (Fig. 1G–H). In fact, both periaxonemal sheath and intracytoplasmic wall are absent in  
2 the anterior part of region II (Fig. 1G). The periaxonemal sheath is a striated layer that  
3 surrounds the axoneme and the intracytoplasmic walls consist in transverse structures  
4 that connect the periaxonemal sheath with the peripheral layer of spiralled cortical  
5 microtubules.  
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9 Region III (Figs. 2B–F and 3III) corresponds to the nuclear area of the mature  
10 spermatozoon. The nucleus forms a loose spiral around the axoneme, presenting a  
11 horseshoe shape in the anterior part of region III (Fig. 2B,D,E) and an annular shape in  
12 the posterior part (Fig. 2C,F). The nucleus is localised between the rods of the  
13 periaxonemal sheath and the axoneme (Fig. 2B). In the posterior part of this region, the  
14 nucleus encircles totally the axoneme and periaxonemal sheath, while the  
15 intracytoplasmic wall disappears (Fig. 2F).  
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18 Region IV (Figs. 2G–J and 3IV) constitutes the posterior spermatozoon  
19 extremity. It is characterised by the sole presence of the axoneme surrounded by the  
20 plasma membrane (Fig. 2H). Near the posterior spermatozoon extremity, the axoneme  
21 becomes disorganised (Fig. 2I,J) and the posterior tip of the male gamete consists of  
22 electron-dense material surrounded by the plasma membrane (Fig. 2G).  
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## 31 Discussion

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36 The spermatozoon of *Echinococcus multilocularis* is a filiform cell, which lacks  
37 mitochondrion as in all the studied cestodean (polyzoic) cestodes (Justine 1998, 2001).  
38 Contrarily, spermatozoa of monozoic cestodes (Gyrocotylidea and Amphilinidea), as in  
39 all other non-cestode platyhelminths, exhibit mitochondrion (Xylander 1989). Thus, the  
40 absence of mitochondrion in the sperm cells was postulated as a synapomorphy for the  
41 Eucestoda (Justine, 1998, 2001).  
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47 According to Levron et al. (2010), four different patterns of spermatozoa were  
48 described in cyclophyllideans (IV to VII). Type IV spermatozoa can be differentiated  
49 from the remaining three due to the presence of parallel cortical microtubules. The latter  
50 are present in phyllobothriids and lecanicephalideans and also in mesocestoidids,  
51 representing a particular case within cyclophyllideans (Miquel et al. 1999, 2007). The  
52 three remaining spermatozoon types (V to VII) exhibit spiralled cortical microtubules  
53 and are observed in different families of cyclophyllideans. Type V spermatozoa are  
54 present in some anoplocephalids, in hymenolepidids and in nematotaeniids.  
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1 Spermatozoa of type VI are observed in other anoplocephalids, in catenotaeniids, in  
2 dilepidids, in dipylidiids and in gryporhynchids. Finally, type VII spermatozoa are  
3 present in some anoplocephalids, in davaineids, in metadilepidids, in paruterinids and in  
4 taeniids, and also in tetrabothriideans (see Levron et al. 2010). The ultrastructural  
5 organization of the sperm cell of *Echinococcus multilocularis* follows the type VII  
6 pattern. It corresponds to a uniflagellate spermatozoon that presents crested bodies,  
7 periaxonemal sheath and intracytoplasmic walls, spiralled cortical microtubules and  
8 nucleus spiralled around the axoneme.  
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14 The existing ultrastructural studies of spermatozoa in the family Taeniidae  
15 concern eight species of the genus *Taenia* and two of the genus *Echinococcus* (see  
16 Table 1). For *Echinococcus*, the present study is the first comprehensive work  
17 describing the complete ultrastructural organization of the sperm cell. With respect to  
18 *Taenia* species, it is interesting to remark that the published results on *T. pisiformis* are  
19 jointly presented with those of *T. saginata* and *T. solium* by Tian et al. (1998a) and the  
20 authors do not identify the species corresponding to each TEM micrograph. For the two  
21 human *Taenia* species, there are additional papers showing some aspects concerning the  
22 ultrastructural organisation of their sperm cells (Willms et al. 2003; Bâ et al. 2011).  
23 Unfortunately, there are no other papers on *T. pisiformis* and consequently this species  
24 is not included in Table 1.  
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35 Within taeniids it is crucial to perform an exhaustive comparison of sperm cell  
36 ultrastructure in species of *Echinococcus* with those of *Taenia*. To date, the  
37 ultrastructural knowledge concerning the male gamete of species of the genus  
38 *Echinococcus* is confined to a few papers or proceedings containing a reduced amount  
39 of information. For *E. granulosus*, Morseth (1969) refers the particular 9+‘1’ pattern of  
40 the axoneme, the absence of mitochondrion, and the spiralled disposition of both  
41 cortical microtubules and nucleus. Also, the published TEM micrographs show the  
42 horseshoe shape of the nucleus in cross-sections. For *E. multilocularis*, Barrett and  
43 Smyth (1983) describe the early abortion of one basal body and associated axoneme  
44 during spermiogenesis and, consequently, the presence of a single axoneme in the  
45 sperm cell. Also, Shi et al. (1994) show the presence of periaxonemal sheath in the  
46 spermatozoon of *E. multilocularis*. With respect to the genus *Taenia*, even though  
47 information on the spermatozoa ultrastructure is available for eight *Taenia* species (see  
48 Table 1), complete descriptions are available for only five of them, namely *T.*  
49 *hydatigena*, *T. mustelae*, *T. parva*, *T. saginata* and *T. taeniaeformis* (Miquel et al. 2000,  
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2009a, 2015; Ndiaye et al. 2003; Bâ et al. 2011). Considering the need to fully understand the sperm cell ultrastructural organization within taeniids, the present paper provides a full description of the ultrastructural organisation of the sperm cell of an *Echinococcus* species. The most relevant results concern the crested bodies. These structures are helical cords that externally surround the spermatozoon of most cestodes and, if present, they always characterise the anterior spermatozoon region. All of the taeniids studied to date exhibit a single crested body, whereas two crested bodies were observed in the mature spermatozoon of *E. multilocularis*. Previous ultrastructural studies on both *E. granulosus* and *E. multilocularis* sperm cells do not remark any information concerning crested bodies (Morseth 1969; Barrett and Smyth 1983; Shi et al. 1994). As for the remaining ultrastructural characters found in the sperm cell of *E. multilocularis*, they are present in the remaining studied taeniids (see Table 1), emphasising the potential importance of finding two crested bodies in *E. multilocularis* sperm cells in the present work. A complete ultrastructural study of other congener species would be of particular interest in order to confirm whether or not the presence of two crested bodies is a characteristic of the genus *Echinococcus*.

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## Legends to figures

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4 **Fig. 1** Spermatozoon of *Echinococcus multilocularis*. **A** Longitudinal section of region I.  
5 **B and C** Two details of the anterior spermatozoon extremity showing the apical cone. **D**  
6 **and E** Cross-sections of region I at the level of centriole and axoneme, respectively. **F**  
7 Transitional area between regions I and II. **G and H** Cross-sections of region II, anterior  
8 and posterior part, respectively. *AC* apical cone, *ASE* anterior spermatozoon extremity,  
9 *Ax* axoneme, *C* centriole, *CB* crested body, *CM* cortical microtubules, *IW*  
10 intracytoplasmic walls, *PS* periaxonemal sheath.  
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18 **Fig. 2** Spermatozoon of *Echinococcus multilocularis*. **A** Longitudinal section of region  
19 II. **B and C** Longitudinal sections of region III, anterior part and posterior part,  
20 respectively. **D-F** Cross-sections of region III showing the nuclear morphology evolving  
21 from horseshoe (anterior part of region, level \* in B) to annular-shape (posterior part of  
22 region, level \*\* in C). **G** Longitudinal section of region IV showing the posterior  
23 spermatozoon extremity. **H-J** Cross-sections of region IV showing the progressive  
24 disorganization of the axoneme. *Ax* axoneme, *C* centriole, *CB* crested body, *CM* cortical  
25 microtubules, *D* doublets, *IW* intracytoplasmic walls, *N* nucleus, *PS* periaxonemal  
26 sheath, *PSE* posterior spermatozoon extremity, *S* singlets.  
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36 **Fig. 3** Schematic reconstruction of the spermatozoon of *Echinococcus multilocularis*.  
37 *AC* apical cone, *ASE* anterior spermatozoon extremity, *Ax* axoneme, *C* centriole, *CB*  
38 crested body, *CM* cortical microtubules, *D* doublets, *IW* intracytoplasmic walls, *N*  
39 nucleus, *PM* plasma membrane, *PS* periaxonemal sheath, *PSE* posterior spermatozoon  
40 extremity.  
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Table 1. Spermatological characteristics of the species of the family Taeniidae.

Genus and species	Sperm type <sup>a</sup>	AC		CB		CM	N Pattern	N Shape	PS	G	IW	PSE	References
		Lenght	Width	Number	Thickness								
<i>Echinococcus</i>													
<i>E. granulosus</i>	?	?	?	?	?	40 <sup>ob</sup>	Spi	HS	?	?	?	Ax	Morseth (1969)
<i>E. multilocularis</i>	VII	>900	200	2	75	45°	Spi	HS-A	+	-	+	Ax	Present study
<i>Taenia</i>													
<i>T. crassiceps</i>	VII	?	?	1	?	45 <sup>ob</sup>	Spi	HS-A	+	-	+	Ax	Willms et al. (2004)
<i>T. hydatigena</i>	VII	2000	225	1	80	50°	Spi	HS-A	+	-	+	Ax	Miquel et al. (2015)
<i>T. mustelae</i>	VII	1900	250	1	75	45°	Spi	HS-A	+	-	+	Ax	Miquel et al. (2000)
<i>T. parva</i>	VII	>1900	225	1	60	45°	Spi	HS-A	+	-	+	Ax	Ndiaye et al. (2003)
<i>T. saginata</i>	VII	1200	230	1	50	40°	Spi	HS-A	+	-	+	N-Ax	Bâ et al. (2011)
<i>T. solium</i> <sup>c</sup>	?	?	?	?	?	Spi	Spi	HS	+	?	?	?	Willms et al. (2003)
<i>T. taeniaeformis</i>	VII	>1350	250	1	140	40°	Spi	HS-A	+	-	+	Ax	Miquel et al. (2009)

AC apical cone, Ax axoneme, CB crested body, CM angle of cortical microtubules, G granules, HS horseshoe, HS-A horseshoe to annular, IW intracytoplasmic walls, N nucleus, PS periaxonemal sheath, PSE posterior spermatozoon extremity, +/- presence/absence of considered character, ? unknown data.

All dimensions are given in nanometers.

<sup>a</sup> Type of spermatozoon according to Levron et al. (2010).

<sup>b</sup> Estimated value from TEM micrographs.

<sup>c</sup> Data obtained from spermatids.





