

## Ultrastructure of the mature spermatozoon of *Eubothrium rugosum* (Batsch, 1786) with a re-assessment of the spermatozoon ultrastructure of *Eubothrium crassum* (Bloch, 1779) (Cestoda: Bothrioccephalidea)

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### Summary

The ultrastructure of the mature spermatozoon of the bothrioccephalidean tapeworm *Eubothrium rugosum*, a parasite of the burbot, *Lota lota* (L.), was studied by transmission electron microscopy for the first time. In addition, spermatozoon ultrastructure of *Eubothrium crassum* has been re-assessed. New is the finding, that the mature spermatozoa of both species of the genus *Eubothrium* exhibit essentially the same general morphology. They are filiform cells tapering at both extremities, and they possess the two axonemes with 9+“1” pattern of Trepaxonemata, attachment zones, a nucleus, cortical microtubules (CMs), electron-dense granules, and a single crested body. Structural polymorphism of the CBs has been found within the two *Eubothrium* species for the first time. The anterior ring of electron-dense tubular structures surrounding a single axoneme marks the border between the two defined regions, region I and region II of the spermatozoon. This unique feature has only been observed in the Bothrioccephalidea. The anuclear axoneme region II of *Eubothrium* spermatozoa fluently verges into a nuclear region III. The posterior part of the spermatozoon contains one-axoneme, few CMs and a posterior extremity of the nucleus that subsequently disappears. The posterior extremity of the male gametes of the genus *Eubothrium* exhibits elements of a disorganized axoneme which characterize also spermatozoa of the family Triaenophoridae. Discussed are interspecific similarities and differences between the spermatozoa of the two *Eubothrium* species as well as between these and other Eucestoda.

Keywords: ultrastructure; spermatozoon; *Eubothrium*; Bothrioccephalidea; Cestoda

### Introduction

Ultrastructural characters of the spermatozoa are frequently used not only to highlight structural and functional features but also as a basis for phylogenetic considerations of parasitic Platyhelminthes (Neodermata) (Hoberg *et al.*, 1997, 2001; Justine, 1998, 2001, 2003; Olson *et al.*, 2001). Recent reviews on the ultrastructure of the cestode spermatozoa, based on a large number of species, provide detailed information on various sperm components and their development (Bâ & Marchand, 1995; Justine, 1998; Watson, 1999; Bruňanská, 2010).

Seven taxa of three families (Bothrioccephalidae, Echinophallidae, Triaenophoridae) within the Bothrioccephalidea have been studied so far (Świderski & Mokhtar-Maamouri, 1980; Bâ *et al.*, 2007; Levron *et al.*, 2006a, c; Šípková *et al.*, 2010; Bruňanská *et al.*, 2002; Levron *et al.*, 2005). Data available show, that the ultrastructural organization of the mature spermatozoon of *E. crassum* differs from that in other bothrioccephalideans. Therefore, the subject of the present study is to explore the fine structure of the mature spermatozoon of *Eubothrium rugosum* (Batsch, 1786), the type species of the genus. Furthermore, a re-investigation of the mature spermatozoa of *E. crassum* was made to unravel additional information of the spermatozoon ultrastructure of tapeworms of the genus *Eubothrium*.

### Materials and Methods

Specimens of *Eubothrium rugosum* (Batsch, 1786) (Cestoda: Bothrioccephalidea) were collected from burbot, *Lota*

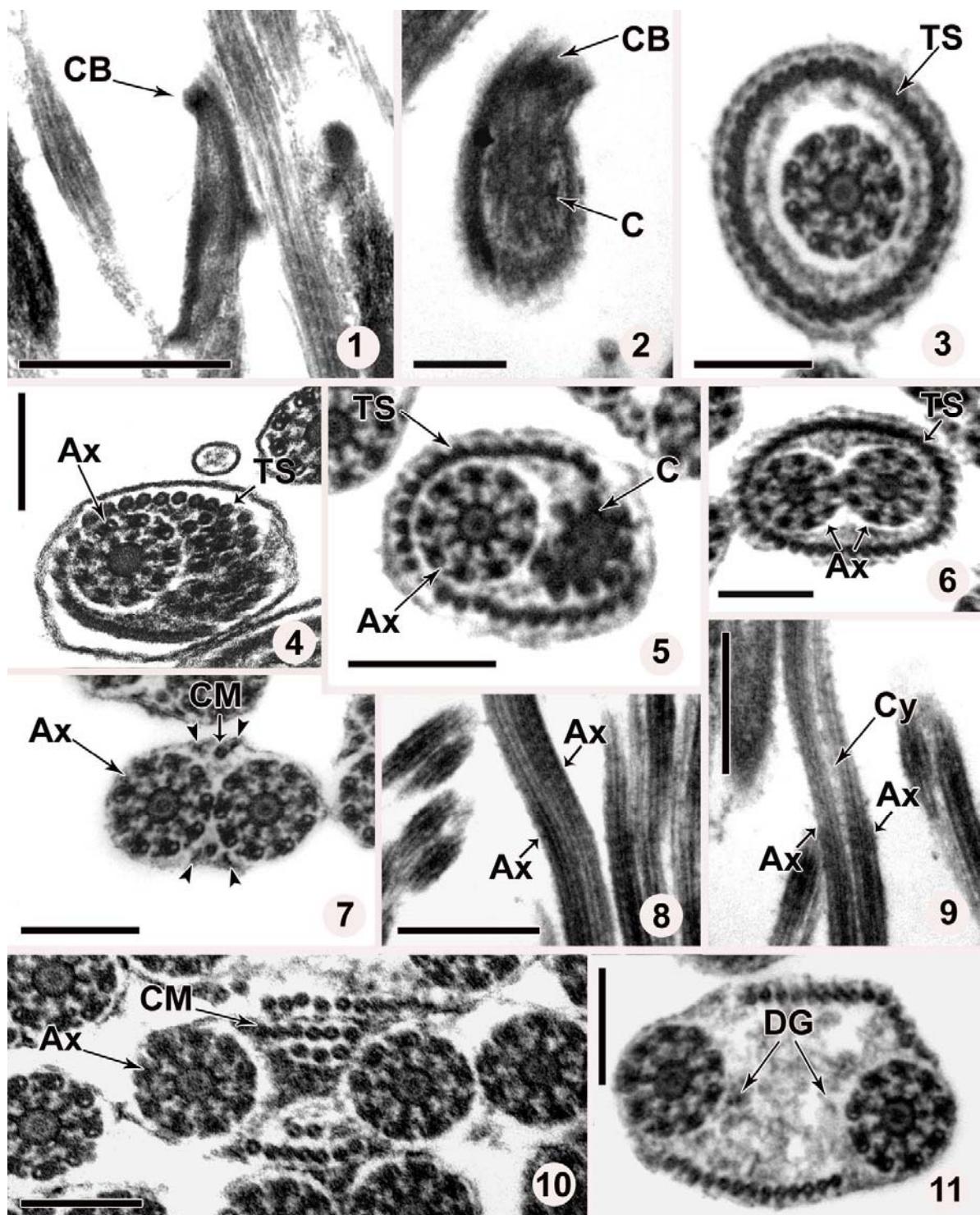


Fig. 1. Longitudinal section of the anterior extremity of the mature spermatozoon of *Eubothrium rugosum*. CB – crested body. Bar = 1  $\mu$ m; Fig. 2. Cross-section of the anterior extremity of the spermatozoon. C – centriole; CB – crested body. Bar = 250 nm; Fig. 3. Cross-section of region I with the ring of electron dense tubular structures (TS). Bar = 200 nm; Fig. 4. Cross-section of the transition zone between the regions I and II documented an increased number of tubular structures (TS) in the vicinity of the axoneme (Ax). Bar = 200 nm; Fig. 5. Serial cross-section of the transition zone between regions I and II showing the centriole of the second axoneme (C). The ring of electron dense tubular structures (TS) is disorganized. Bar = 200 nm; Fig. 6. Serial cross-section to those shown in Figs. 4 and 5, with the two axonemes (Ax) surrounded by a ring of tubular structures (TS). Bar = 200 nm; Fig. 7. Attachment zones (arrowheads) and cortical microtubules (CM) in the region II of the spermatozoon. Ax – axoneme. Bar = 200 nm; Fig. 8. Longitudinal section of region II with the two tightly packed axonemes (Ax). Bar = 1  $\mu$ m; Fig. 9. Longitudinal section of region II with the two axonemes (Ax) situated in the increased volume of the cytoplasm (Cy). Bar = 1  $\mu$ m; Fig. 10. Cross-section of the region II. Note two parallel rows of CMs arranged opposite side. Bar = 200 nm; Fig. 11 Cross-section of the posterior part of region II with the two axonemes. DG – electron-dense granules. Bar = 200 nm.

*lota* (Linnaeus, 1758), from the Rybinsk water reservoir, Yaroslavl District, Russia. Living worms were cooled in 0.9 % NaCl solution and then fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at 4°C. Mature and pregravid proglottids were separated, rinsed in the same buffer and post-fixed in 1 % OsO<sub>4</sub> at 4°C for 2h, followed by dehydration in graded alcohol series and embedding in Spurr. A series of ultrathin sections were cut using a Leica Ultracut UCT ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate. The grids were examined in a JEOL 1010 transmission electron microscope operated at 80 kV. Data concerning *E. crassum* specimens studied are detailed in Bruňanská *et al.* (2002).

## Results

Mature spermatozoa (gametes), originating from the testes and seminal vesicles of *Eubothrium rugosum* were investigated. In addition, male gametes of *E. crassum* have been re-addressed. The gametes of both *Eubothrium* species are filiform cells, tapering at both extremities. Five different characteristic regions (I – V) can be distinguished antero-posteriorly.

*Region I* (Figs. 1 – 4, 18I) corresponds to the anterior part of the spermatozoon, exhibiting one axoneme. A lateral electron-dense projections, 120 nm thick, create the helicoidal crested body of the spermatozoon (Figs. 1, 2). The

distance between the two projections of the crested body is about 600 nm (Fig. 1). In *E. rugosum*, the crested body consists of electron-dense tubular structures, surrounding the centriole (Fig. 2) and anterior part of the axoneme having the 9 + “1” trepaxonematan character. At the end of region I, electron dense tubular structures create a ring, which surrounds the central axoneme (Fig. 3) in both *Eubothrium* species. Here, the diameter of the spermatozoon is 390 nm. In the transition zone between the region I and II, the number of electron dense tubular structures markedly increases (Fig. 4).

*Region II* (Figs. 5 – 11, 18II) is characterized by the two axonemes in both *Eubothrium* species. Firstly, the centriole of the second axoneme is formed (Fig. 5). Simultaneously, the shape of the spermatozoon becomes more ovate in cross section. In *E. rugosum*, the two axonemes were only very rarely found within a closed ring of electron dense tubular structures (Fig. 6). As a rule, the ring of electron dense tubular structures is interrupted, and disappears completely when the second axoneme has appeared (Fig. 7). Both axonemes are tightly packed within a small amount of cytoplasm, containing the two cortical microtubules (CMs) situated opposed to each other (Figs. 7, 8). Four small electron-dense elements, the attachment zones, are situated on both sides of each axoneme (Fig. 7). The diameter of the spermatozoon is about 380 nm. Subsequently, the volume of cytoplasm and the number of CMs gradually increases. The cytoplasm contains electron-dense

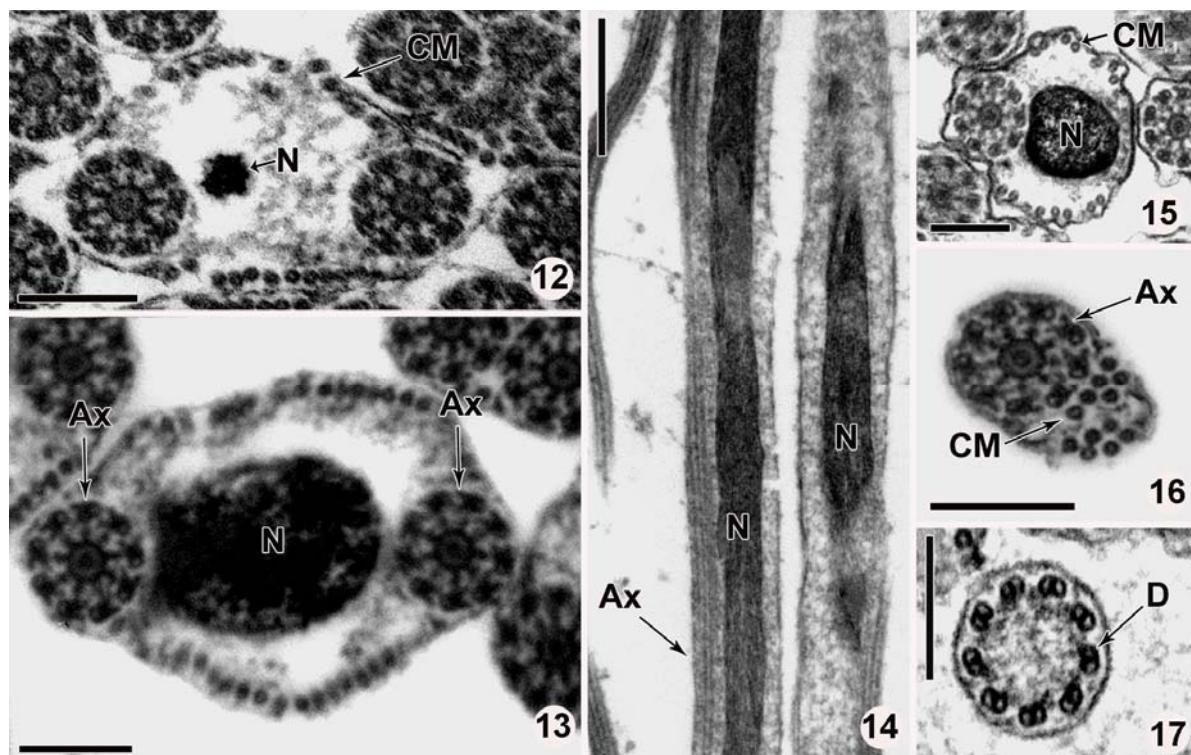


Fig. 12. Cross section of the anterior part of region III. CM – cortical microtubules, N – nucleus. Bar = 200 nm; Fig. 13. Cross-section of the central part of the region III. Ax – axoneme, N – nucleus. Bar = 200 nm; Fig. 14 Longitudinal section of region III. Ax – axoneme, N – nucleus; Bar = 1 μm; Fig. 15. Cross-section of region IV with one axoneme, nucleus (N) and a reduced number of cortical microtubules (CM). Bar = 200nm;

Fig. 16. Cross-section of the posterior part of region IV. Note the CMs arranged at the periphery of the cell, and additional CMs scattered in the cytoplasm. Ax – axoneme. Bar = 200 nm; Fig. 17. Disorganization of the axoneme into doublets (D) in the region V of the spermatozoon. Bar = 200 nm.

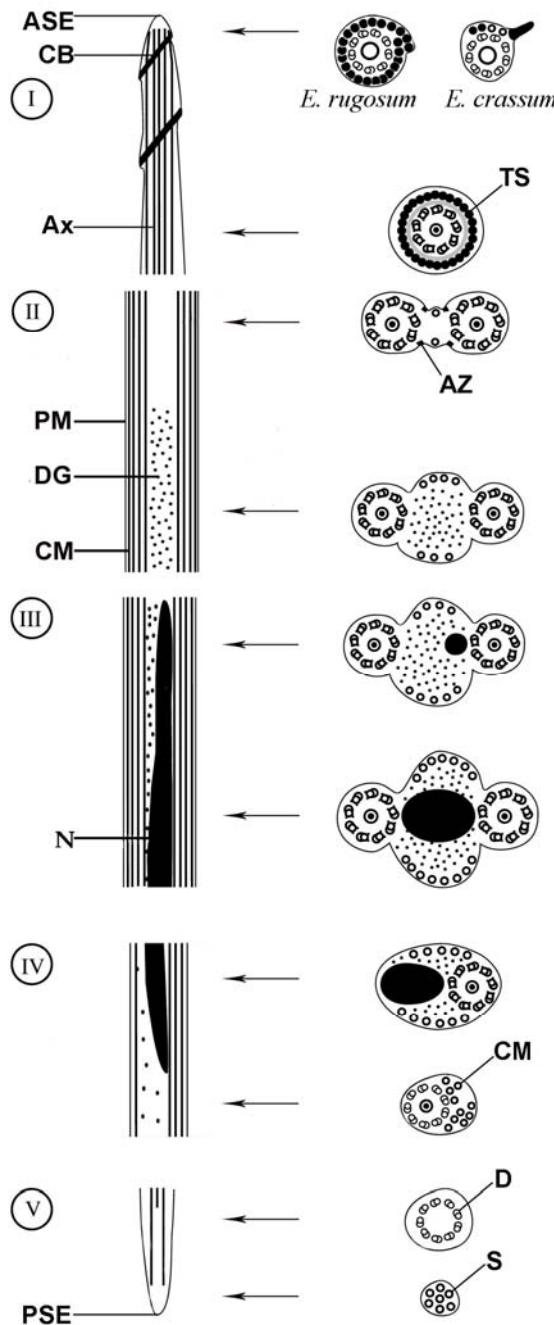


Fig. 18. Schematic reconstruction of the mature spermatozoon of *E. rugosum* and/or *E. crassum*.

Ax – axoneme, AZ – attachment zones, ASE – anterior spermatozoon extremity, CB – crested body, CM – cortical microtubules, D – doublets, DG – dense granules, N – nucleus, PM – plasma membrane, PSE – posterior spermatozoon extremity, S – singlets, TS – tubular structures.

granules (Figs. 9, 10, 11). The CMs are now arranged in two opposite rows. The CMs have a thin membrane and electron-lucent centre. Occasionally, a second short row of 3 CMs parallel with the peripheral CMs, was observed in some cross sections of region II of mature spermatozoa of *E. rugosum* (Fig. 10). The diameter of the spermatozoon is about 530 nm at the end of region II. Each row of CMs is

composed of up to 14 – 15 elements (Fig. 11).

*Region III* (Figs. 12 – 14, 18III) is the nucleated part of the spermatozoon with the two axonemes. In cross sections studied, the nucleus is ovate (oval-shaped), situated centrally between the axonemes. The anterior extremity of the nucleus is eccentrically situated (diameter small, about 60 nm) (Fig. 12). More posteriorly, the nucleus has a concentric position and its size increases progressively up to 420 nm (Figs. 13, 14). The nucleus contains strands of a fine fibrillar material. The CMs form 2 opposite rows under the plasma membrane and come into close contact with it. The cytoplasm of the spermatozoon exhibits electron-dense granules. The diametre of the cell is 860 nm.

*Region IV* (Figs. 15, 16, 18IV) contains only one axoneme, the nucleus and CMs. The diameter of the spermatozoon and its nucleus are reduced to one half when compared with the central part of the region III. Two fields of CMs are composed of some 5 elements (Fig. 15). At the end of region IV of the spermatozoon the nucleus disappears, one axoneme and some scattered CMs are situated within a small amount of cytoplasm (Fig. 16).

*Region V* (Figs. 17, 18V) is characterized by progressive disorganization of the axoneme. The central core disappears and doublets lose their arms (Fig. 17). Posteriorly, doublets become disorganized and are finally transformed into singlets. The diameter of the posterior extremity of the gamete is about 140 nm.

## Discussion

The mature spermatozoa of *Eubothrium rugosum* and *Eubothrium crassum* exhibit essentially the same general morphology. They contain the two axonemes with their attachment zones, a nucleus, an anterior ring of tubular electron-dense structures, cortical microtubules (CMs), electron-dense granules, and a single crested body (CB). The interspecific differences concern the structure of the CB.

Generally, the CB is absent in the mature spermatozoa of the evolutionary older cestodes, e.g. Gyrocotylidea and Amphilinidea (Rohde & Watson, 1986; Xylander, 1989), Caryophyllidea (Świderski & Mackiewicz, 2002; Gamil, 2008; Miquel *et al.*, 2008; Bruňanská, 2009, 2010), Spathebothriidea (Bruňanská *et al.*, 2006; Bruňanská & Poddubnaya, 2010), Diphyllobothriidea (Levron *et al.*, 2006b, 2009), Haplobothriidea (MacKinnon & Burt, 1985), or Trypanorhyncha (Miquel & Świderski, 2006; Miquel *et al.*, 2007b). The CB occurs in the more primitive two-axoneme spermatozoa of the Bothriocephalidea (Šípková *et al.*, 2010), Tetraphyllidea - Onchobothriidae (Mokhtar-Maamouri & Świderski, 1975), Tetraphyllidea - Phyllobothriidae (Mahendrasingam *et al.*, 1989), and Proteocephalidea (Bâ & Marchand, 1994; Sène *et al.*, 1997; Bruňanská *et al.*, 2003a, b, 2004a, b) as well as in the evolved single-axoneme spermatozoa (mainly members of the Cyclophyllidea, see Justine, 1998).

In both *Eubothrium* species, the CB is spiralized around the anterior part of the spermatozoon, including the cen-

triolar level, thus resembling other bothrioccephalideans (Levron *et al.*, 2005; Levron *et al.*, 2006a; Bâ *et al.*, 2007; Šípková *et al.*, 2010). The CB in *E. rugosum* consists of electron dense tubular structures, which were found also in *Triaenophorus nodulosus* and *Bothrioccephalus scorpii* (Levron *et al.*, 2005, 2006a). On the other hand, the CB of *E. crassum* is an electron-dense structure (Bruňanská *et al.*, 2002; Fig.18 in the present study) as described in bothrioccephalidean *Paraechinophallus japonicus* (Levron *et al.*, 2006c), *Parabothrioccephalus gracilis* (Šípková *et al.*, 2010), diphyllobothriidean *Duthiersia fimbriata* (Justine, 1986), in some proteocephalideans or cyclophyllideans (Bruňanská, 2010). Polymorphism of the CBs seems to exist within *Eubothrium* species.

The taxonomically and phylogenetically interesting ultrastructural features of the spermatozoa in Eucestoda include the number of axonemes, the presence/absence of the attachment zones, an anterior ring of tubular structures, arrangement of cortical microtubules (CMs), and the posterior structures.

The two axonemes of 9 + "1" pattern of the Trepaxonemata (Ehlers, 1984) characterize mature spermatozoa of both *Eubothrium* species, as well as the Bothrioccephalidea, Diphyllobothriidea, Spathobothriidea, Haplobothriidea, Trypanorhyncha, Tetraphyllidea, and Proteocephalidea (Bruňanská, 2010). The two-axoneme-structure is considered a plesiomorphic character of the Eucestoda (Justine, 1998).

The four attachment zones described in the spermatozoa of *Eubothrium* mark the point of the fusion of the two axonemes with the median cytoplasmic process during spermiogenesis (Bruňanská *et al.*, 2002; the present study).

These elements are known also in the spermatozoa of bothrioccephalideans *B. scorpii* (Levron *et al.*, 2006a), *P. japonicus* (Levron *et al.*, 2006c), diphyllobothriideans (Levron *et al.*, 2006b, 2009), trypanorhynchs *Dollfusiella spinulifera* (Miquel & Świderski, 2006), *Parachristianella trygonis* (Miquel *et al.*, 2007b) and cyclophyllidean *Joyeuxiella* (Ndiaye *et al.*, 2003). The attachment zones were detected during spermiogenesis in the spathobothriideans *Cyathoccephalus truncatus* (Bruňanská *et al.*, 2006) and *Didymobothrium rudolphii* (Bruňanská & Poddubnaya, 2010).

A ring of tubular structures marks the end of region I in *Eubothrium* species and in the mature spermatozoa of the bothrioccephalideans *T. nodulosus* (Levron *et al.*, 2005), *B. scorpii* (Levron *et al.*, 2006a), *P. japonicus* (Levron *et al.*, 2006c), and *P. gracilis* (Šípková *et al.*, 2010). Two exceptions are *B. clavibothrium* and *E. crassum* having the ring of tubular structures located in the postnuclear region (Świderski & Mokhtar-Maamouri, 1980; Bruňanská *et al.*, 2002). Re-assessment of the fine structure of the spermatozoa of *E. crassum* has shown, however, that a ring of tubular structures encircling the central axoneme at the beginning of the region V (Bruňanská *et al.*, 2002), actually should be placed at the end of region I. A ring of tubular structures may occasionally encircle the two fully formed axonemes in the spermatozoa of *E. rugosum*. This

new character has been found for the first time in the Eucestoda.

Only one or two CMs are present at the beginning of region II in *E. rugosum*. Similar pattern characterize the corresponding region of the spermatozoa of the bothrioccephalideans (Levron *et al.*, 2005, 2006a, c; Šípková *et al.*, 2010), diphyllobothriideans (Levron *et al.*, 2006b, 2009), spathobothriideans (Bruňanská *et al.*, 2006, Bruňanská & Poddubnaya, 2010) and trypanorhynchs (Miquel & Świderski, 2006; Miquel *et al.*, 2007b). This characteristic region has been described in the spermatozoon of *E. crassum*, and designated as region IV (Bruňanská *et al.*, 2002). However, re-assessment of our previous results led us to the conclusion, that ultrastructural characteristics of the spermatozoa of *E. crassum* and *E. rugosum* are congruent. Thus, the two tightly packed axonemes originally placed into region IV (Bruňanská *et al.*, 2002) actually belong to the beginning of the region II, and vice versa, the one with one axoneme and nuclear appendix originally placed into region II actually fall into region IV of the spermatozoon of *E. crassum*.

CMs form two opposite fields in the nuclear region of the spermatozoa (Justine, 1998), and also in the prenuclear two-axoneme region of the spermatozoa of the Spathobothriidea, Bothrioccephalidea, Diphyllobothriidea, Haplobothriidea, Trypanorhyncha, Tetraphyllidea, and Proteocephalidea. However, the second row of microtubules parallel with the CMs in the prenuclear two-axoneme region has been described only in the spermatozoa of the bothrioccephalidean *E. rugosum* (the present study) and spathobothriidean *Didymobothrium rudolphii* (Bruňanská & Poddubnaya, 2010).

The posterior part of the spermatozoa of both *Eubothrium* species is characterized by the following structural succession pattern: posterior extremity of the nucleus, reduced number of cortical microtubules, posterior extremity of the axoneme (Bruňanská *et al.*, 2002; the present study). This pattern was described also in bothrioccephalidean *T. nodulosus* (Levron *et al.*, 2005) and may characterise spermatozoa of the family Triaenophoridae. However, spermatozoa of the Bothrioccephalidae show remarkable variability in the posterior structure of the male gametes, relating to the sequence: posterior nucleus extremity, axoneme, CMs (Świderski & Mokhtar-Maamouri, 1980; Bâ *et al.*, 2007, Levron *et al.*, 2006). The structural heterogeneity of the posterior region of the spermatozoa within the Bothrioccephalidae seems to restrict the use of this feature as potentially interesting criterion for taxonomy or phylogeny of the Eucestoda.

The present study gives evidence that the spermatozoon of *E. rugosum* shares the basic ultrastructural and phylogenetically useful characters (the presence of two axonemes and their attachment zones, a single crested body, an anterior ring of electron-dense cortical microtubules, the organization of the two-axoneme region, and the type of the posterior part of the spermatozoon) with the spermatozoon of *E. crassum* as well as with male gametes of the Triaenophoridae representatives studied till now. Sperma-

tological data are congruent with conclusions of recent molecular studies. Both approaches support the existence of two independent lineages within the former order Pseudophyllidea: the basal Diphyllobothriidea and the more derived Bothriocephalidea (Waeschenbach *et al.*, 2007; Olson *et al.*, 2008).

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