

Ultrastructure and cytochemistry of the mature spermatozoon of *Khawia armeniaca* (Cholodkovsky, 1915) (Caryophyllidea: Lytocestidae), a parasite of *Capoeta capoeta sevangi* (De Filippi, 1865) (Teleostei, Cyprinidae)

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Summary

The mature spermatozoon of *Khawia armeniaca*, a monozoic caryophyllidean parasite of tempral fish *Capoeta capoeta sevangi* (De Filippi, 1865) from the Lake Sevan, Armenia, has been studied using transmission electron microscopy and cytochemical technique of Thiéry (1967) for the first time. The mature spermatozoon of *K. armeniaca* consists of a single axoneme with the 9+‘1’ trepaxonematan structure, cortical microtubules and nucleus which are situated parallel to the longitudinal axis of the spermatozoon, and a moderately electron-dense cytoplasm with glycogen particles. The cortical microtubules are arranged in one continuous semi-circle beneath the plasma membrane in Region II and anterior part of Region III of the mature spermatozoon. The two opposite rows of cortical microtubules are observed in the remaining nuclear and at the beginning of the postnuclear part (Regions III, IV) of the male gamete. The number of cortical microtubules is remarkably variable in the spermatozoa of various *Khawia* species. *K. armeniaca* exhibits the highest number of cortical microtubules in comparison with *K. sinensis* and *K. rossittensis*. Glycogen was detected in the cytoplasm of prenuclear (II), nuclear (III) and postnuclear (IV) regions with different ultrastructural organization of the mature spermatozoon of *K. armeniaca*. Variations of sperm ultrastructural characters within caryophyllideans and other cestodes are discussed.

Keywords: Cestoda; Caryophyllidea; *Khawia armeniaca*; spermatozoon; ultrastructure

Introduction

Mature spermatozoa of the Eucestoda show a high variability and provide important characters useful for phylogenetic studies (Justine, 1998, 2001; Levron *et al.*, 2010). In general, they contain one or two axonemes, cortical microtubules, a nucleus, and various granules in the cytoplasm (Justine, 2003). Male gametes of the order Caryophyllidea are of special interest, as phylogenetic relationships within this group and its interrelationships with other tapeworms remain unclear (Waeschenbach *et al.*, 2012; Caira & Littlewood, 2013).

The Caryophyllidea is considered to comprise 42 genera and 122 valid species arranged in four families (Scholz & Oros, 2017). Up-to-date, sperm characters of 12 caryophyllidean taxa of 3 families have been described (see Table 1). Of these taxa, 1 species (*Breviscolex orientalis*) belongs to the family Capingentidae, 5 species (*Archigetes sieboldi*, *Caryophyllaeus laticeps*, *Glaridacris catostomi*, *Hunturella nodulosa*, *Wenyonia virilis*) belong to the family Caryophyllaeidae, and 6 species (*Atractolytocestus huronensis*, *Caryophyllaeides fennica*, *Khawia sinensis*, *Khawia rossittensis*, *Lytocestus indicus*, *Monobothrioides chalmersius*) belong to the family Lytocestidae (see Yoneva *et al.*, 2011; Bruňanská *et al.*,

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2019; Bruňanská & Kostič, 2012; Świderski & Mackiewicz, 2002; Yoneva *et al.*, 2012a; Gamil, 2008; Bruňanská *et al.*, 2011; Matoušková *et al.*, 2018, Bruňanská, 2009; Matoušková *et al.*, 2019; Yoneva *et al.*, 2012b; Arafa & Hamada, 2004). It is of note that one of the most speciose genera of caryophyllidean tapeworms is *Khawia* which includes 7 valid species (Scholz *et al.* 2011). Whereas complete spermatological data are available for two *Khawia* species (see Table 1), ultrastructural study of the third species, *Khawia armeniaca*, has been focused exclusively on spermiogenesis (Bruňanská & Poddubnaya, 2006).

Therefore, the present study aims to provide missing ultrastructural characters of the mature spermatozoa of *K. armeniaca* which are necessary for better understanding of the male gametes morphology within the genus *Khawia*, the order Caryophyllidea, and the Eucestoda.

Materials and Methods

Adult specimens of *Khawia armeniaca* (Cholodkovski, 1915) were collected from the intestine of *Capoeta capoeta sevangi* (De Filippi, 1865) (Pisces: Cyprinidae), from Lake Sevan, Armenia.

Transmission electron microscopy

The tapeworms were cut into small pieces, fixed immediately in ice cold 3 % glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 3 – 8 h, followed by three changes of cacodylate buffer and postfixed in 1 % OsO₄ for 1 h. The material was dehydrated in a graded alcohol series, acetone, and embedded in Araldite. The ultrathin sections (90 nm) were cut on diamond knife using a Leica Ultracut UCT ultramicrotome, placed on copper grids, and

double-stained with uranyl acetate (30 min.) and lead citrate (20 min.). The grids were examined in a JEOL 1010 transmission electron microscope operated at 80 kV.

Cytochemistry

Cytochemical technique of Thiéry (1967) with periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) was used for visualisation of glycogen in the mature spermatozoa. Ultrathin sections were placed on gold grids, treated in 1 % PA (20 – 25 min.), washed in distilled water, processed with 1 % TSC (40 min.), washed in 10 % acetic acid and distilled water, treated in 1 % SP (30 min.) and finally washed in distilled water. The grids were observed in JEOL 1010 transmission electron microscope.

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

A large number of cross- and longitudinal sections of the mature spermatozoa from vasa deferentia of *Khawia armeniaca* have been investigated in the present study.

Vas deferens

The vas deferens is situated between vasa eferentia and the ejaculatory duct of the cirrus pouch. The wall of convoluted vas deferens is formed by a thin syncytial epithelial layer, the luminal

Table 1. Variation of the maximum number of cortical microtubules (CM) in the spermatozoa of the Caryophyllidea.

Families	Genus Species	CM	References
Capingentidae	<i>Breviscolex orientalis</i>	15	Yoneva <i>et al.</i> , 2011
Caryophyllaeidae	<i>Archigetes sieboldi</i>	11	Bruňanská <i>et al.</i> , 2019
	<i>Caryophyllaeus laticeps</i>	15	Bruňanská & Kostič, 2012
	<i>Glaridacris catostomi</i>		Świderski & Mackiewicz, 2002
	<i>Hunturella nodulosa</i>	10	Yoneva <i>et al.</i> , 2012a
	<i>Wenyonia virilis</i>	30	Gamil, 2008
Lytocestidae	<i>Atractolytocestus huronensis</i>		Bruňanská <i>et al.</i> , 2011
	<i>Caryophyllaeides fennica</i>	19	Matoušková <i>et al.</i> , 2018
	<i>Khawia armeniaca</i>	35	present study
	<i>Khawia rossittensis</i>	22	Matoušková <i>et al.</i> , 2019
	<i>Khawia sinensis</i>	15	Bruňanská, 2009
	<i>Lytocestus indicus</i>	25	Yoneva <i>et al.</i> , 2012b
	<i>Monobothrioides chalmersius</i>	40	Arafa & Hamada, 2004

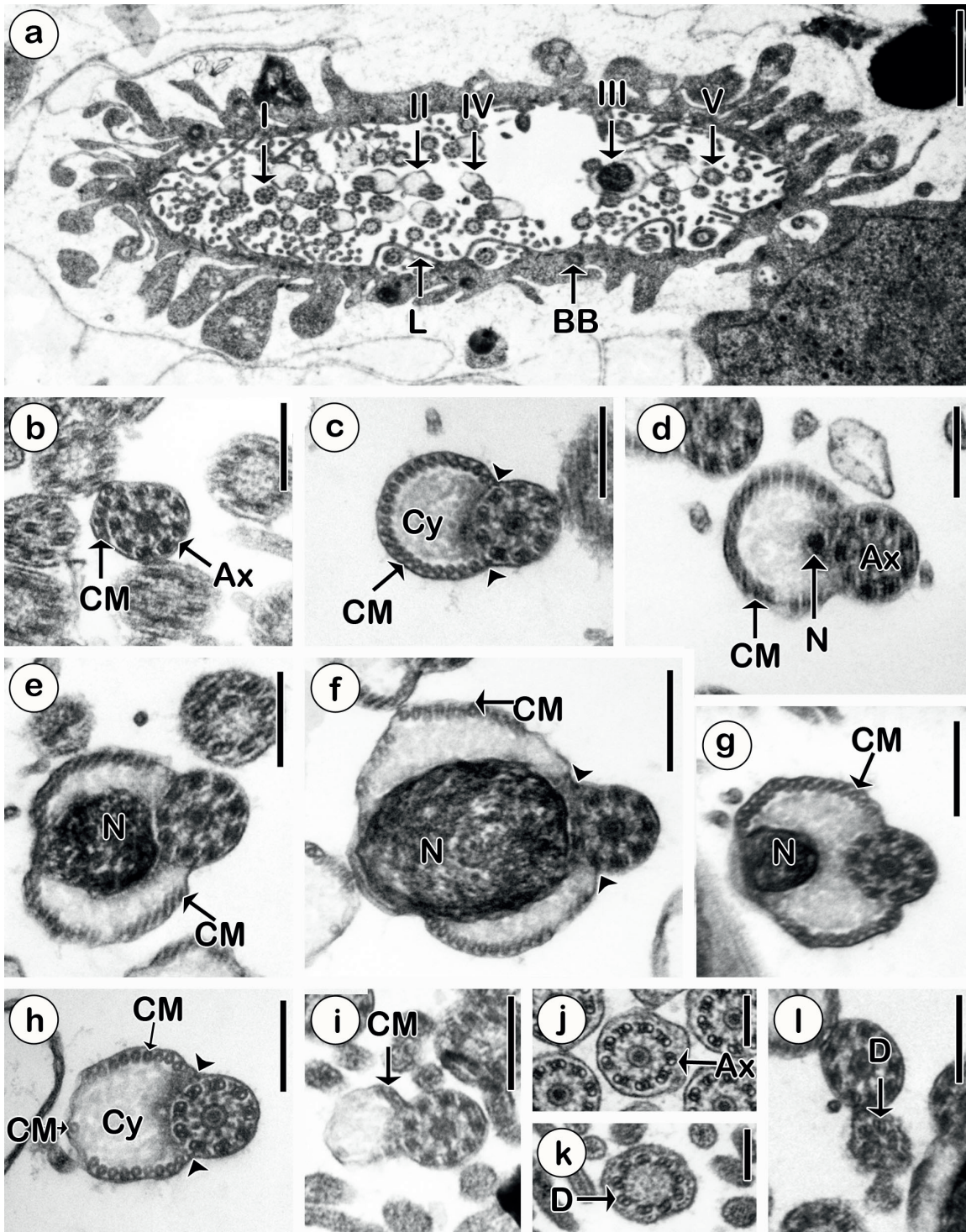


Fig. 1. Cross sections of the mature spermatozoon of *K. armeniaca*: (a) vas deferens with five different regions of the spermatozoon (I-V); (b) region I presents one axoneme and six cortical microtubules; (c) region II shows cortical microtubules which are arranged in a semi-circle under the plasma membrane; (d-g) region III or nucleated region: small diameter of anterior part of the nucleus (d), which gradually increases (e), reaches a maximum in the middle part (f), and diminishes again more posteriorly (g); (h-j) region IV shows cortical microtubules which are arranged in two opposite rows (note one single microtubule in the middle between the two opposite rows) (h), rapidly reduced volume of cytoplasm and number of cortical microtubules (i, j); (k-l) region V with one axoneme which lost its central structure (k) and undergoes disorganization into doublets (l). Ax, axoneme; Arrowheads, attachment zones; BB, basal body of the cilium; CM, cortical microtubules; D, doublets; L, lamellae; N, nucleus. Scale bars: a = 1000 nm, b-h, l = 200 nm, i = 250 nm, j, k = 125 nm.

surface of which is lined by numerous sinuous lamellae and/or rare cilia (measuring up to 3.5 μm) (Fig. 1a).

Mature spermatozoon

Male gametes are long, filiform cells, tapered at both extremities, with one incorporated axoneme of the 9+1' trepaxonematan structure, cortical microtubules and a nucleus which are situated parallel to the longitudinal axis of the spermatozoon, and granules of glycogen. Five different regions (I – V) with specific ultrastructural organisation can be recognized (Figs. 1, 2, 3).

Region I (Figs. 1a, b; 3I) corresponds to the anterior extremity of the spermatozoon. It contains one axoneme which is surrounded by a semiarc of up to 5 cortical microtubules (CM) located under the plasma membrane. The diameter of the spermatozoon is about 300 nm.

Region II (Figs. 1a, c; 2a, d; 3II) exhibits an increase of both the volume of cytoplasm and the number of CM. The CM are arranged in one continuous row composed of up to 23 elements (Fig. 1c). Granules of glycogen (Figs. 2a, d), can be detected in the cytoplasm using Thiéry method (1967). One pair of attachment zones illustrates the points of fusion of the free flagellum with the median cytoplasmic process during spermiogenesis (Figs. 1c; 3II). The diameter of the spermatozoon reaches up to 460 nm.

Region III (Figs. 1a, d, e, f, g; 2b, 3III) corresponds to the middle part of the mature spermatozoon, containing the nucleus. The diameter of the nucleus is about 40 nm at the beginning (Fig. 1d), gradually enlarges (Fig. 1e) up to 500 nm in its middle region (Fig. 1f). Posteriorly, the nucleus diameter diminishes again, having about 160 nm near its posterior extremity (Fig. 1g). The CM are arranged in a semicircle at first (Fig. 1d). Subsequently, an enlarged nucleus is approaching the plasma membrane of the spermatozoon,

thus dividing originally continuous semicircle of CM in two opposite parts (Fig. 1e, f, g), each consisting of 12 – 15 elements. One pair of attachment zones can be recognized (Fig. 1f). Based on examination of cross sections, the maximum number of CM in the spermatozoon of *K. armeniaca* is 35 elements, which may occur in the principal nucleated region of the male gamete. In addition, the cytoplasm of the spermatozoon contains scattered granules of glycogen (Fig. 2b). The diameter of the mature spermatozoon in region III is ranging from 480 nm (anterior and posterior parts) to 720 nm (middle part).

Region IV (Figs. 1a, i, j, 2c, 3IV) is the postnuclear part of the spermatozoon. The CM are arranged at first in two opposite rows, each having 9 elements, and one single microtubule is situated in the middle between the two opposite rows (Fig. 1h). More posteriorly, a strong reduction of the cytoplasm volume, and only a few CM (up to 4) are observed (Fig. 1i). Here, the diameter of the spermatozoon is diminished to 380 nm. At the end of Region IV only one axoneme is present (Fig. 1j).

Region V (Figs. 1a, k, l, 3V), or the posterior part of the spermatozoon, contains only one axoneme which undergoes major disorganization: the central core unit disappears at first (Fig. 1k), and is followed by further disorganization of peripheral doublets (Figs. 1l, 3V). The very posterior extremity of the *K. armeniaca* spermatozoon is around 140 nm in diameter.

Discussion

This study provides an evidence that ultrastructural architecture of the mature spermatozoon of *Khawia armeniaca* principally resembles that of other lytocestiids and caryophyllideans. Their male gametes exhibit one axoneme; cortical microtubules and nucleus

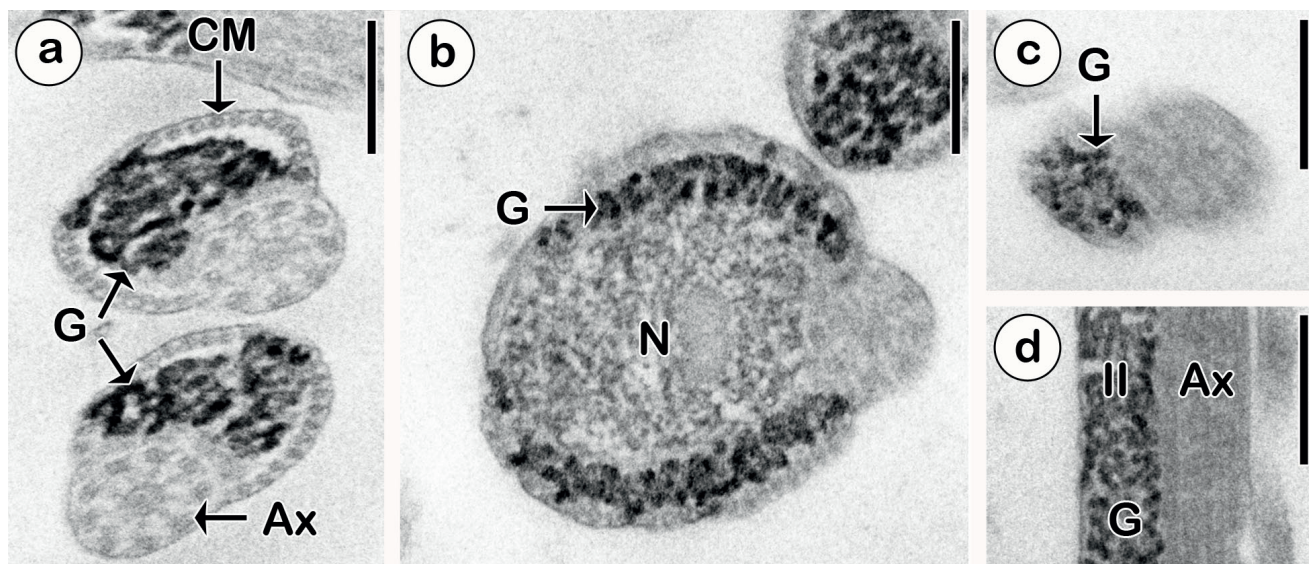


Fig. 2. Granules of glycogen in the cytoplasm of the mature spermatozoon of *K. armeniaca* after application of Thiéry method (1967): (a) cross section of region II; (b) cross section of region III; (c) cross section of region IV; (d) longitudinal section of region II. Ax, axoneme; CM, cortical microtubules; G, glycogen; N, nucleus. Scale bars: a-c = 200 nm, d = 250 nm.

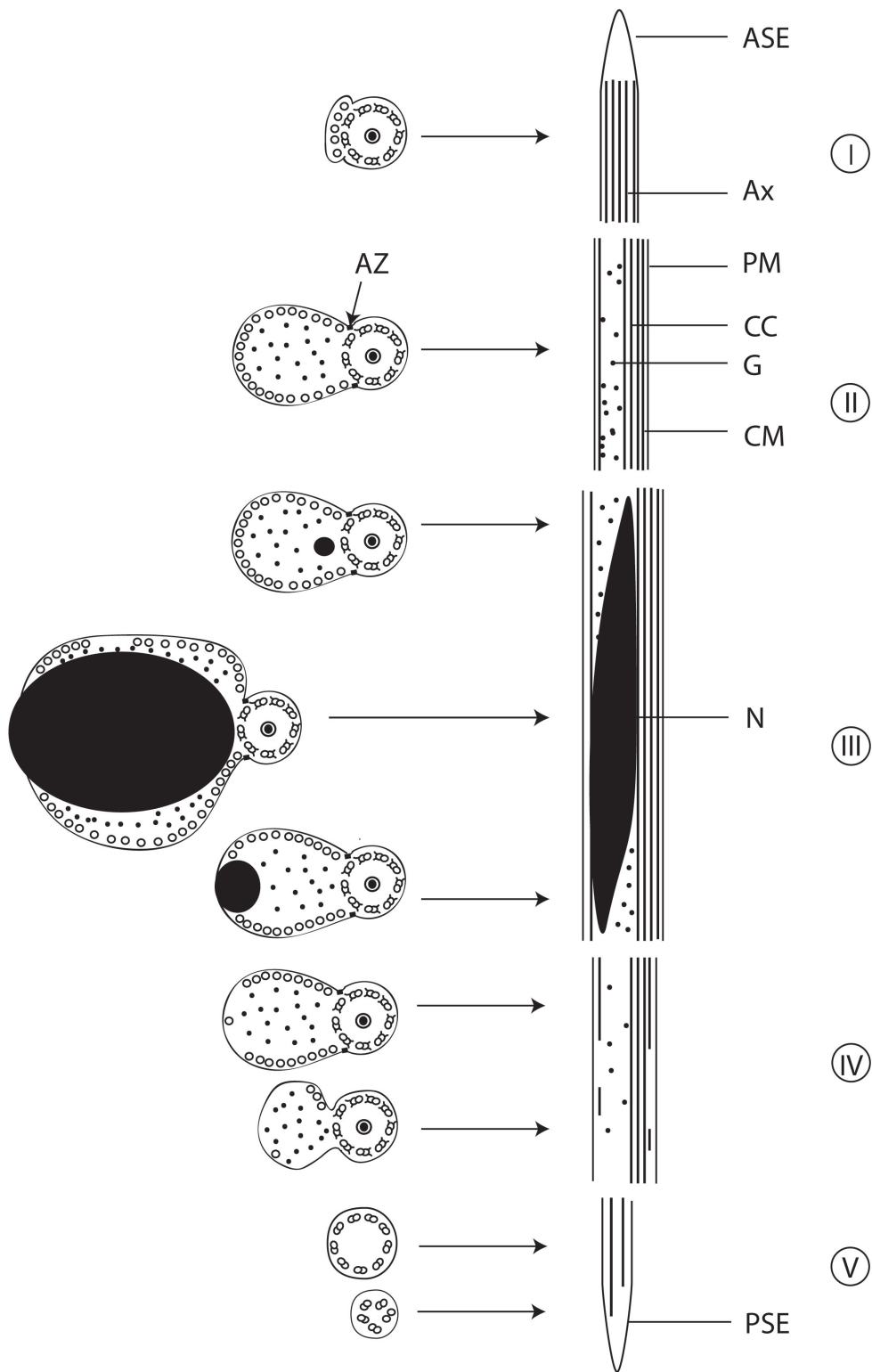


Fig. 3. Schematic reconstruction of the mature spermatozoon of *K. armeniaca*. I–V, five different regions of the mature spermatozoon; ASE, anterior spermatozoon extremity; Ax, axoneme; AZ, attachment zones; CC, central core; CM, cortical microtubules; G, glycogen; N, nucleus; PM, plasma membrane; PSE, posterior spermatozoon extremity.

which are situated parallel to the long axis of the spermatozoon (Bruňanská, 2010; Levron *et al.*, 2010). Caryophyllidean tape-worms have the spermatozoon with 9+‘1’ axonemal structure which is typical for the Trepaxonemata (Ehlers, 1984). Their axoneme is composed of the central core unit interconnected with nine peripheral doublet microtubules which are associated with inner and outer dynein arms. Traditionally, it was believed that the central core unit contains the central electron-dense core, electron-lucent intermediate area, and peripheral electron-dense cortical sheath. In contrast, recent electron tomography observations have revealed the presence of the two tubular structures in the central axonemal electron-dense core of two lytocestiid caryophyllideans, *Caryophyllaeides fennica* and *Khawia rossittensis* (Matoušková *et al.*, 2018, 2019). Future electron tomography studies are necessary to elucidate the structure of the central electron-dense core of the axonemes in the spermatozoa of *K. armeniaca*.

The parallel alignment of CM in a longitudinal axis occurs in most Eucestoda (except for the Cyclophylloidea and Tetrabothriidea, which have spiralled CM), Digenea and Monogenea (Justine, 2001; Bruňanská 2010; Bakhom *et al.* 2017). Based on the number of axonemes (one or two), the parallel/spiralled pattern of CM, the parallel/spiralled pattern of the nucleus, and some other ultrastructural characters, seven types of the male gametes have been proposed for the Eucestoda (Levron *et al.*, 2010). In cross sections, CM are distributed either into two fields located between the two axonemes (Types I and II) or in one field (Types III–VII). Type III spermatozoon exhibits ultrastructural organisation, which is specific exclusively for the Caryophyllidea. According classification of Levron *et al.* (2010) it includes one field of CM loosely scattered under the plasma membrane of the nucleated region of the spermatozoon in cross sections. In contrast, the present study reveals dual arrangement of CM, i.g. (i) in one field or a semicircle, and (ii) in two fields, or two opposite rows in regions III and IV of the spermatozoa in *K. armeniaca*. Whereas most caryophyllideans have the CM loosely scattered under the plasma membrane (Gamil, 2008; Bruňanská, 2009; Yoneva *et al.*, 2011, 2012a, b; Bruňanská & Kostič, 2012; Matoušková *et al.*, 2018; Bruňanská *et al.*, 2019), the CM in *K. armeniaca* are tightly packed. A continuous field of tightly packed CM has been reported also in the spermatozoa of lytocestiid *Monobothrioides chalmersius* by Arafa and Hamada (2004). On the other hand, two opposite rows of CM have been reported in region II of the spermatozoa of lytocestiid *Khawia rossittensis* by Matoušková *et al.* (2019) and caryophyllaeid *Glaridacris catostomi* by Świderski and Mackiewicz (2002). However, up to date, the simultaneous occurrence of both types of arrangement of CM has never been reported in the spermatozoa of caryophyllidean species. Here, it is worth to note that CM are distributed into two fields situated between the two axonemes in the gyrocotylidean and amphilinidean cestodes (Xylander 1989, Bruňanská *et al.* 2012) and in most digeneans (Bakhom *et al.* 2017). However, the latter taxa differs from that of any Eucestoda by the presence of a mitochondrion in the spermatozoon.

The location of the maximum number of CM would be an interesting ultrastructural criterion, especially in connection with their presumed role in spermatozoan movements. The maximum number of CM in the spermatozoa within the order Caryophyllidea is not constant (Tab. 1). It occurs in the anterior parts of the spermatozoa most frequently, and varies between 10 and 30 in the Caryophyllaeidae, or 15 – 40 in the Lytocestidae. At the intraspecific level of *Khawia*, the highest maximum number of the CM (35) has been reported in *K. armeniaca* (present study), whereas *K. rossittensis* exhibits maximum 22 CM (Matoušková *et al.*, 2019) and *K. sinensis* only 15 elements (Bruňanská, 2009).

The occurrence of AZ in the mature spermatozoa of cestodes is not rare, as these structures represent the points of fusion of the free flagellum/flagella with the median cytoplasmic protrusion during spermiogenesis. The AZ were detected in the spermatozoa with one and/or two axonemes. The one-axoneme spermatozoa exhibit one pair of AZ. Within the Caryophyllidea, the AZ are situated in regions II, III or IV of the mature spermatozoa (Yoneva *et al.*, 2012b; Matoušková *et al.*, 2018, 2019; Bruňanská *et al.*, 2019; present study). The two pairs of AZ have been reported in the two-axonemes spermatozoa of the Amphilinidea (Bruňanská *et al.*, 2012a), Spathebothriidea (Bruňanská *et al.*, 2006; Bruňanská & Poddubnaya, 2010), Bothriocephalidea (Levron *et al.*, 2006a, b; Marigo *et al.*, 2012), Diphyllbothriidea (Levron *et al.*, 2006c, 2009, 2013; Bruňanská *et al.*, 2012b), and Trypanorhyncha (Miquel & Świderski, 2006; Miquel *et al.*, 2007; Marigo *et al.*, 2011). Glycogen is the major carbohydrate storage form in animals and represents the major energy store of the spermatozoa in the Eucestoda (Euzet *et al.*, 1981). The presence of glycogen was detected using Thiéry method (1967) in the spermatozoa of the Caryophyllidea (Bruňanská, 2009; Yoneva *et al.*, 2011; Bruňanská & Kostič, 2012; Matoušková *et al.*, 2018; Bruňanská *et al.*, 2019; present study), and in male gametes of other cestodes, e.g. Diphyllbothriidea, Bothriocephalidea, Haplobothriidea, Diphyllidea, Trypanorhyncha, Tetracyphylloidea, Proteocephalidea, and Tetrabothriidea (Levron *et al.*, 2010). In these cestodes, glycogen is localised in the sperm cytoplasm and it was never found in the sperm axonemes.

The spermatozoa travel on their route from the site of the origin towards the cirrus pouch through various canals of the male reproductive system, including vas deferens. The basic structure of the vasa deferentia of *K. armeniaca* resembles that in caryophyllidean *Atractolytocestus huronensis* (Bruňanská *et al.*, 2011), gyrocotylideans or amphilinideans (Rohde & Watson, 1986; Xylander, 1989). Interestingly, vasa deferentia of other Caryophyllidea, Spathebothriidea, Diphyllbothriidea or Proteocephalidea have no cilia (Davydov *et al.*, 1994; Korneva & Davydov, 2001; Poddubnaya, 2002; Poddubnaya *et al.*, 2005).

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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