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Spermiogenesis and sperm ultrastructure of two species of *Duthiersia*, parasites of monitors, with a review of spermatological characters in the Diphyllobothriidea (Cestoda)

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ABSTRACT

The present study compares the spermiogenesis of two species of the diphyllobothriidean cestodes (family Scyphocephalidae), *Duthiersia expansa* and *Duthiersia fimbriata*, parasites of Asian and African monitors (*Varanus* spp.), and establishes the ultrastructural organization of the spermatozoon of *D. fimbriata*. The spermatids of both species are similar and exhibit a differentiation zone containing two centrioles and electron-dense material in the apical region. The centrioles are separated from one another by an intercentriolar body and each is associated with a striated root. During the early stage of spermiogenesis, the centrioles form two flagella that grow orthogonally to the median cytoplasmic process. The orthogonal development is followed by a flagellar rotation and proximodistal fusion with median cytoplasmic process. The mature spermatozoon of *D. fimbriata* is characterized by the presence of two axonemes presenting the type 9 + “1” trepaxonematan pattern, parallel cortical microtubules and a nucleus. The most interesting finding is confirmation of the absence of a crested body in the anterior extremity of the gamete, which seems to be a typical feature of all diphyllobothriideans. This observation contradicts the previous information on *D. fimbriata* provided by Justine (1986), who reported the crested body for the species. The pattern of spermiogenesis of scyphocephalids, represented by two species of *Duthiersia*, is similar to that in members of the Diphyllobothriidae. The existing data on species of all three families of the Diphyllobothriidea are reviewed and it is shown that the main characters of the sperm cell typical of these cestodes resemble those of other basal groups of tapeworms, which represents another support of their ancestral position.

1. Introduction

Diphyllobothriideans, which were previously assigned to the order Pseudophyllidea Carus, 1863 (see Kuchta et al., 2008), are well known parasites of mammals and birds, with some species of *Diphyllobothrium* (broad fish tapeworm) and *Spirometra* (causative agents of sparganosis) parasitic in man (Muller, 2001; Scholz et al., 2009). However, the order also includes species parasitizing reptiles (monitor lizards and snakes), which are poorly known (Kuchta et al., 2008). These cestodes are placed in three genera, *Bothridium* Blainville, 1824, *Duthiersia* Perrier, 1873 and *Scyphocephalus* Riggenbach, 1898. Some authors (Freze, 1974; Kuchta et al., 2008) recognize the family Scyphocephalidae to accommodate these three genera, whereas others, such as Schmidt (1986) and Bray et al. (1994), placed them in the Diphyllobothriidae. These cestodes from reptilian hosts are characterized by the presence of the scolex of unique morphology with strongly modified bothria (Bray et al., 1994) and molecular data indicate that they represent a sister group to the Diphyllobothriidae (Brabec et al., 2006).

Spermiogenesis and sperm ultrastructure have been studied in several species of diphyllobothriidean cestodes, but almost all belonged to the only one of the three recognized families, Diphyllobothriidae (for review see Levron et al., 2010, in press). Recently, Bruňanská et al. (2012) have studied the ultrastructure of the mature spermatozoon of a species of monotypic family Cephalochlamydidae, *Cephalochlamys namaquensis* (Cohn, 1906), a parasite of clawed frogs (Xenopidae), which may represent the most basal extant taxon of the Diphyllobothriidea (Brabec et al., 2006).

The only ultrastructural data on species of the Scyphocephalidae were provided by Justine (1986), who described the spermatozoon of *Duthiersia fimbriata* from *Varanus niloticus* L. in Senegal. The author reported the presence of a crested body (“corps en crête”), but the existence of this structure is questioned by the fact that

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it has never been observed in any other diphyllobothriideans or cestodes of closely related groups, *i.e.*, Haplobothriidea, Caryophyllidea and Spathebothriidea (see Bruňanská and Poddubnaya, 2010; Levron et al., 2010; Yoneva et al., 2011, 2012a,b; Bruňanská and Kostič, 2012). Another discrepancy between the data provided by Justine (1986) and those presented in recent papers (Levron et al., 2006a, 2009, in press; Bruňanská et al., 2012) represents the fact that only one type of cortical microtubules was reported in *Diphyllobothrium latum* (L.), *Ligula intestinalis* (L.) and *Schistocephalus solidus* (Müller, 1779), whereas *D. fimbriata* allegedly possesses two types (Justine, 1986).

Unusual spermatological characters of *D. fimbriata*, which differ from those of other members of the order, did not allow for generalization of the evolutionary patterns of sperm development in this cestode order (see Fig. 1 in Levron et al., 2010). Recently, specimens of two species of *Duthersia* from monitors in Africa and Asia, respectively, were obtained and fixed for ultrastructural observations, which made it possible to compare their spermiogenesis and to reinvestigate sperm ultrastructure of these cestodes, which seem to represent an ancient lineage of diphyllobothriideans. The new data were also compared with those available on the other species of the order with the aim of defining spermatological characteristics typical of this newly established cestode order. This information might facilitate its better circumscription and the assessment of its phylogenetic position among basal "true" tapeworms (Eucestoda).

2. Materials and methods

D. fimbriata (Diesing, 1854) was found in *Varanus exanthematicus* (Reptilia: Varanidae) imported to the Czech Republic from Ghana, Africa and dissected on 4 January 2012 by R.K. (field code B9). *Duthiersia expansa* Perrier, 1873 was found in *Varanus salvator* collected in the West Mountains, Thang Hoa Province, Vietnam and dissected in Vietnam on 5 October 2010 by R.K. and T.S. (field code VNH 85b). Voucher specimens of both species are deposited in the collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic (IPCAS Nos. C-269 and C-630).

Tapeworms were rinsed in 0.9% NaCl solution and fixed with 1.5% glutaraldehyde and 1.5% paraformaldehyde solutions in 0.1 M Hepes at pH 7.4. After washing with 0.1 M Hepes at pH 7.4, they were post-fixed in cold (4 °C) 1% OsO_4 in the same buffer for 1 h, dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. Ultrathin sections (60–90 nm in thickness) were cut on a Leica Ultracut UCT ultramicrotome, placed on copper grids and double stained with uranyl acetate and lead citrate according to Reynolds (1963). Ultrathin sections were examined under a JEOL 1010 transmission electron microscope at 80 kV in the Laboratory of Electron Microscopy, Institute of Parasitology, České Budějovice.

3. Results

3.1. Spermiogenesis

Spermiogenesis of *D. expansa* and *D. fimbriata* is illustrated in Figs. 1A–J, 2A–I and 3A–E. It proceeds in the testes where spermatids are grouped in rosettes connected to the central cytophore. Each spermatid contains a nucleus and numerous mitochondria (Figs. 1A and 2A). At the beginning of spermiogenesis, a small cytoplasmic protrusion, the zone of differentiation, appears at the periphery of spermatids (Fig. 1A). The differentiation zone, bordered by cortical microtubules, is characterized by the presence of two centrioles interconnected by an intercentriolar body (Figs. 1B, 2B and 3A). In addition, each centriole is associated with a striated root oriented perpendicularly to its long axis (Figs. 1C and 2C, D). At this stage, an accumulation of electron-dense material is present in the apical region of the zone of differentiation (Figs. 1C, 2C and 3B). In both *Duthiersia* species, the intercentriolar body exhibits five electron-dense layers. The electron-dense layers are of different thickness: a thick central layer and two thinner external ones on both sides. Additionally, they are separated by four electron-lucent layers (Figs. 1D, 2B and 3A).

Initially, the centrioles are oriented in the same plane and give rise to free flagella, which grow outwards (Figs. 1E, 2D and 3B). Both flagella rotate and become parallel to the median cytoplasmic process (Figs. 1G, H, 2E, G and 3C, D). Afterwards, the three structures fuse proximodistally. The fusion occurs at the level of four attachment zones, which can be observed inside the median cytoplasmic process (Figs. 1F, 2F and 3D). After the proximodistal fusion, the nucleus elongates and migrates along the spermatid body (Figs. 1I and 2H). In sections, the spermatid contains two axonemes and a nucleus (Figs. 1J, 2I and 3E). At the final stage of spermiogenesis, the young spermatozoon is detached from the residual cytoplasm.

3.2. Spermatozoon

Mature spermatozoa of *D. fimbriata* from vas deferens were observed. The ultrastructural organization of the spermatozoon is illustrated in Figs. 4A–N and 5I–V. The male gamete is a filiform cell, which is tapered at both extremities and lacks mitochondria. From the anterior to the posterior extremities of the gamete, it is possible to distinguish five regions with different ultrastructural characteristics.

Region I (Figs. 4A–C and 5I) constitutes the anterior part of the spermatozoon. The centriole of the first axoneme characterizes the beginning of this region. At this level the centriole is lined with three cortical microtubules. Later, it gives rise to the first axoneme, which comprises nine sets of doublets of microtubules arranged in a cylinder around a central unit and therefore presents the typical 9 + "1" trepaxonematan axonemal pattern. The axoneme is partially bordered by a few parallel cortical microtubules.

Region II (Figs. 4D–H and 5II) constitutes the prenuclear area of the spermatozoon. It is characterized by the presence of two axonemes and parallel cortical microtubules. The anterior area of the region is marked by the appearance of several tubular elements, preceding the formation of the second axoneme. More posteriorly, the second axoneme emerges. Cross sections through the portion with the two axonemes shows that they are situated very close to each other, being separated by only one and two microtubules on each sides. Later, the number of cortical microtubules, distributed in two opposite rows of 2 and 7 microtubules, increases progressively. At this level, the volume of the cytoplasm between the two axonemes also increases.

Region III (Figs. 4I–L and 5III) represents the nuclear part of the spermatozoon. It possesses two axonemes and the nucleus. This region starts when a nucleus with small diameter appears. At the anterior portion of this region, its proximal part is eccentrically situated between the axonemes. Posteriorly, it becomes situated centrally inside the sperm cell and enlarges gradually towards the middle area of region III. The nucleus is electron-dense and contains fibrillar patches of chromatin. At the posterior area of region III, its diameter decreases and one of the axonemes loses its central element.

Region IV (Figs. 4M and 5IV) is characterized by the presence of only one axoneme and the nucleus. A few cortical microtubules are still present. Cross section shows a decrease of the width of the gamete.

Region V (Figs. 4N and 5V) constitutes the posterior extremity of the male gamete. The last axoneme disappears and the posterior part of the sperm cell contains only the distal part of the nucleus.