Fertilization in the cestode *Echinococcus multilocularis* (Cyclophyllidea, Taeniidae)

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Abstract

Fertilization in the taeniid cestode *Echinococcus multilocularis* with uniflagellate spermatozoa was examined by means of transmission electron microscopy (TEM). Fertilization in this species occurs in the oviduct lumen or in the fertilization canal proximal to the ootype, where the formation of the embryonic capsule precludes sperm contact with the oocytes. Cortical granules are not present in the cytoplasm of the oocytes of this species, however, several large bodies containing granular material where frequently observed. Spermatozoa coil spirally around the oocytes and syngamy occurs by lateral fusion of oocyte and sperm plasma membranes. In the ootype, one vitellocyte associates with fertilized oocyte, forming a membranous capsule which encloses both cell types. In this stage, the spirally coiled sperm body adheres partly to the external oocyte surface, and partially enters into the perinuclear cytoplasm. The electron-dense sperm nucleus becomes progressively electron-lucent within the oocyte cytoplasm after penetration. Simultaneously with chromatin decondensation, the

elongated sperm pronucleus changes shape, forming a spherical male pronucleus, which attains the size of the female pronucleus. Cleavage begins immediately after pronuclear fusion.

Keywords: *Echinococcus multilocularis*; Taeniidae; Cestoda; fertilization; ultrastructure

Introduction

Ultrastructural aspects of fertilization in cestodes, have been rather neglected. To date, such studies have been published only for 5 cestode species (for details and references see Table 1). The same is true for the digenetic trematodes (Burton 1960, 1967; Justine and Mattei 1984; Orido 1988; for review see also Fried and Haseeb 1991) and also for the Monogenea (Justine and Mattei 1986; Kearn and Whittington 2015).

The purpose of this paper is to describe for the first time the ultrastructural aspects of fertilization in the taeniid cestode *Echinococcus multilocularis*, a parasite of medical and veterinary importance. Comparison is made with the existing data on the ultrastructure of fertilization process in other parasitic Platyhelminthes. Additionally, our study, in the framework of the PARAVAC project, constitutes a preliminary and necessary approach for a future immunohistochemical analysis for the location of the expression sites of some antigenic proteins during fertilization and their possible role in this process.

Materials and methods

Live specimens of *Echinococcus multilocularis* were isolated from the intestine of a naturally infected red fox (*Vulpes vulpes* L.) from La Roche sur Foron (France) in June 2014. Adult tapeworms were immediately rinsed with a 0.9 % NaCl solution. Later, they were fixed in cold (4 °C) 2.5 % glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in 0.1 M sodium cacodylate buffer at pH 7.4, post-fixed in cold (4 °C) 1 % osmium tetroxide with 0.9 % potassium ferricyanide [$K_3Fe(CN)_6$] in the same buffer for 1 h, rinsed in MilliQ water (Millipore Gradient A10), dehydrated in an ethanol series and propylene oxide, embedded in Spurr's resin and polymerised at 60 °C for 72 h. Ultrathin sections (60–90 nm thick) of mature segments at the level of oviduct and ootype were obtained in a Reichert-Jung Ultracut E ultramicrotome. Sections were placed on 200-µm mesh copper grids and double-stained

with uranyl acetate and lead citrate according to the Reynolds (1963). The grids were examined in a JEOL 1010 transmission electron microscope operated at 80 kV, in the "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB).

Results

In *Echinococcus multilocularis,* the uniflagellate spermatozoa surround the oocytes in a spiralling orientation (Fig. 1A-D). This occurs primarily in the fertilization canal, proximal to the ootype, but was also observed sometimes in the proximal part of the ootype (Fig. 2A-B), where the formation of the embryonic capsule precludes additional sperm contact. The sequence of the oocyte penetration by the spermatozoon can be deducted from the stages found in the proximal ootype (Fig. 1A-D). The spermatozoon, wrapping itself around the oocyte, appears to fuse with the ovum by a lateral fusion of oocyte and sperm plasma membranes along their contact lines (Fig. 1A-D).

After syngamy, the numerous cross- and oblique sections of a single sperm axoneme and its cortical microtubules were frequently observed within the oocyte cytoplasm (Figs 3 and 4A,B). As a first step initiating the fertilization, we observed a narrow end-part of the spermatozoon situated in a close spatial relationship to the oocyte surface and two centrioles with numerous microtubules of the division spindle, both situated in the adjacent peripheral layer of the oocyte (Fig. 3, left side of micrograph and Inset). In the early stage of fertilization, were observed several cross- and oblique sections of spermatozoa in the peripheral region of oocyte and a few large spherical accumulations of fine granular material in the central part of its cytoplasm (Fig. 3). However, the cortical granules, which are usually characteristic of oocytes in most animals were never observed in the cytoplasm of unfertilized or fertilized oocytes of this species. The vitellocyte (Fig. 3, see: right lower corner) associated with the adjacent oocyte, show numerous β-glycogen particles and several mitochondria in its cytoplasm. Following sperm fusion, the fertilized ova of *E. multilocularis* in the ootype surrounded by the Mehlis gland enter in contact with numerous vitelline cells and each fertilized oocyte becomes associated with a single vitellocyte; both cells are rapidly surrounded by a newly formed delicate membranous capsule (Fig. 4A,B). In this stage the cytoplasm of the fertilized ova contains numerous mitochondria, microtubule fibers of mitotic spindle, cortical microtubules of the spermatozoa and cross- or oblique sections of entire spermatozoa. The cytoplasm of associated vitellocytes contains several mitochondria, numerous β-glycogen particles and GER, while the nuclei of

vitellocytes usually contain a few electron-dense islands of condensed heterochromatin (Fig. 4A,B). The degenerating polar bodies with characteristic electron-dense, pycnotic nuclei undergoing rapid apoptosis were sometimes observed in this stage (Fig. 4A). Sometime after syngamy, in the advanced stage of fertilization, the initially very electron-dense sperm nucleus undergoes chromatin decondensation and becomes progressively electron-lucent within the oocyte cytoplasm. Simultaneously with chromatin decondensation, the elongated sperm pronucleus changes shape, forming a spherical male pronucleus, which attains the size of the female pronucleus (Fig. 5A,B). The large pronuclei contain prominent, electron-dense nucleoli and the cytoplasm surrounding them shows sometimes presence of annulate lamellae composed of several pairs of smooth membranes (Fig. 5A). Ultrastructural details of the annulate lamellae, when examined under high power magnification (Fig. 5B) show that they are composed of a few to several lamellae, each pair containing regularly spaced pores resembling those of nuclear envelope. They may occur singly or, as shown here (Fig. 5B), are stocked in groups with parallel and/or concentric arrangement in the common cytoplasm surrounding two pronuclei of *E. multilocularis.*

Discussion

The site of fertilization in *Echinococcus multilocularis* is similar to that of other cestodes, and is occurring in the distal part of the oviduct, the so-called "fertilization canal", proximal to the ootype, or in the proximal part of the ootype (Świderski 1976; Świderski and Conn 1999; Świderski *et al.* 2004; Taeleb and Ghobashy 2014). Coil (1991) reviewed literature based mainly on light microscope studies that described fertilization in cestodes and he also concluded that sperm contact the ovum in the fertilization canal proximal to the ootype where the formation of the outer embryonic capsule precludes sperm contact.

In digenean trematodes, Fried and Haseeb (1991) reviewed previous light (LM) and transmission electron microscope (TEM) studies on fertilization and concluded that the oocytes of Digenea are fertilized within the ootype or uterus and than the fertilized ovum became surrounded by vitellocytes. There are some studies based on light or electron microscopy that described fertilization in the uterus and ovary of some species. Justine and Mattei (1984) in their TEM studies on the digenean trematode *Gonapodasmius* sp. (Didymozoidae), reported that the fertilization in this species takes place in the uterus. At the moment of syngamy, the eggshell is made up of a dense

vacuolized substance that shows a perforation through which the sperm cell passes. The same authors (Justine and Mattei 1986) discovered that the fertilized female germ cells were found already in the ovary of the monogenean *Dionchus remorae*. The exact significance of these various locations where fertilization takes place is not known, but probably relates to the relative positions of ducts coming from the testes and vitelline glands, as well as to secretory products of the female reproductive ducts.

Technically, the term "ovum" refers only to a female gamete that has completed oogenetic meiosis, but has not yet completed pronuclear fusion (Conn 2000). However, in this paper we follow the general usage proposed by Burton (1960, 1967), since oogenetic meiosis generally follows fertilization in platyhelminths, while the transformations of the spermatozoon occur within the cytoplasm of the female gamete simultaneous to oogenetic meiosis**.**

The oocytes of many animal taxa contain cortical granules that are frequently associated with fertilization events such as production of a fertilization membrane (Conn 2000). No cestode studied to date possesses cortical granules (Świderski 1976; Mokhtar-Maamouri 1980; Świderski et al. 2004). However, cellular inclusions, said to be cortical granules, have recently been reported in the davaineid cestode, *Cotugnia polyacantha* by Taeleb and Ghobashy (2014). In contrast, cortical granules do occur in monogeneans (Justine and Mattei 1986), many digeneans (Orido 1988), and the majority of turbellarians (Rieger *et al*. 1991). Conn (1988) reported the coalescence of vitellocyte secretions into a transitory capsule precursor of *Mesocestoides lineatus*, and speculated that these might constitute a cortical granule/fertilization membrane analog in the cestodes. This has not yet been demonstrated experimentally, and should be studied further.

Echinococcus multilocularis is similar to most cyclophyllideans in having only a single vitellocyte associated with each oocyte during fertilization, which is involved in capsule formation, as described in other cestodes (Świderski *et al.* 1970; Świderski 1968, 1981; Młocicki *et al.* 2000). The role of vitellocytes in fertilization, if any, has not been demonstrated for any neoophoran platyhelminth, all of which have the germ cell line divided into distinct germarium and vitellarium or oocytes and vitellocytes (Świderski and Xylander 2000, Conn 2000). This well-known fact of distinct subdivision into distinct germarium and vitellarium exists in all neophoran platyhelminths. Unfortunately, the distinction between oocytes and vitellocytes has been confused by Taeleb and Ghobashy (2014) in their recent paper on the ultrastructure of

oocyte development, oogenesis and fertilization process of the davaineid cestode *Cotugnia polyacantha.* As a result of this confusion there have been serious and major misinterpretations of the process of oogenesis. For example, Figures 14-19, 22-23 and 26-29 of their paper represent vitellocytes and vitellogenesis; evidence: the vitelline material in the Neodermata is deposited only in differentiating and mature vitellocytes, but never during different stages of oogenesis or in the mature oocytes. Also in the paper by Taeleb and Ghobashy (2014) figures 4 and 5 represent most probably stages of oogenesis; Fig. 12 most probably two pronuclei and Fig. 11 beginning of pronuclear fusion. What concerns Figs. 6-8 and 10, they represent undoubtedly the early stages of embryogenesis, namely the early embryos composed of several blastomeres, surrounded by a newly formed membranous capsule. Unfortunately, all these figures have been labelled as various stages of oogenesis or fertilized oocytes. Naturally, this confusion has also resulted in misinterpretation of structures so that there may be some doubt of the presence or absence of some structures for example, cortical granules. It is beyond the scope of this discussion to correct all of the misinterpretations and questionable data of this paper on fine structure of *Cotugnia*. Other than indicate that mature oocytes are fertilized, there is no description of the *process* of syngamy. Regretfully, given the serious misinterpretations, poor fixation of examined material, lack of additional high power microphotographs, and lack of any new, valid information, it is not possible to compare our data from *Echinococcus* with that of *Cotugnia polyacantha* Fuhrmann, 1909, the correct name for the species studied by Taeleb and Ghobashy (2014).

The apparent homogeneity found in the process of fertilization in the cestodes, digeneans and monogeneans evidently contrasts with the great variety that exists in their sperm ultrastructure. The spiral coiling of spermatozoa around the oocyte, followed by fusion of the plasma membranes of the two cells, is a common feature of all cestodes studied to date (Table I), as well as for all monogeneans and digeneans reported previously (see References). The type of fertilization in *E. multilocularis* is similar to that described in *Hymenolepis diminuta* by Świderski (1976), in *Inermicapsifer madagascariensis* and *Mesocestoides lineatus* by Świderski and Conn (1999), and in *Gallegoides arfaai* by Świderski *et al.* (2004), all five of which have uniflagellate sperm. The fertilization pattern in *Proteocephalus longicollis* previously described by Świderski and Conn (1999) is similar to that of *Acanthobothrium filicolle* described by Mokhtar-Maamouri (1980), and generally resembles fertilization observed in digeneans and monogeneans. All of these have biflagellate sperm. Another difference in sperm

structure among platyhelminths is that the sperm of cestoidean (polyzoic) cestodes characteristically lack mitochondria, which are present in the sperm of digeneans and monogeneans (Ehlers 1985; Brooks 1989). Sperm of cestodarian (monozoic) cestodes are like all other non-cestode platyhelminths, in having mitochondria (for review see: Xylander 1989; Fried and Haseeb 1991; Tappenden *et al.* 1993; Kearn and Whittington 2015). The basic features of fertilization apparently are similar in all neoophoran platyhelminths, regardless of sperm morphology. This homogeneity in fertilization patterns in different groups of parasitic platyhelminths contrasts sharply with the great variety in the ultrastructure of sperm within the phylum.

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Legends

Fig. 1A-D. Diagram of the consecutive stages of fertilization in *Echinococcus multilocularis*. **A** – a preliminary stage with sperm situated near the oocyte surface, **B** –

initial stage of fusion, **C** – final stage of fusion with sperm nucleus and axoneme embedded in the oocyte cytoplasm, **D** – transformation of sperm nucleus into male pronucleus.

Abbreviations for all Figs: AL – annulate lamellae; Ax – sperm axoneme; C – capsule; Ct – centrioles; GER – granular endoplasmic reticulum; gl – glycogen in vitellocyte cytoplasm; GM – granular material; Hch – heterochromatin in vitellocyte nucleus; m – mitochondria; mf – microtubule fibers of mitotic spindle; mt – cortical microtubules of spermatozoa; N – nucleus; n – nucleolus; Od – oviduct wall; PB – polar body; Ov – ovum, fertilized oocyte; PN – pronuclei; Sp – spermatozoa; Vc – vitellocyte.

Fig. 2A and B. TEM micrographs illustrating different aspects of the early stages of fertilization process in *E. multilocularis*. **A** – A fertilized oocyte surrounded by numerous spermatozoa. Note presence of two centrioles and several elongated mitochondria in its cytoplasm and its large nucleus with a prominent, electron dense nucleolus. **B** – Cross-section trough the oviduct containing peripheral parts of two oocytes surrounded by numerous spermatozoa. Note (1) a few sections of a sperm cell penetrating into the surface zone of the ovum situated on the left side of the micrograph (2) the thick layer oviduct epithelium with granular, moderately electron-dense cytoplasm separated from the external less compact layer of circular muscles the oviduct wall.

Fig. 3. TEM micrograph illustrating several early stages of sperm penetration during fertilization process in *E. multilocularis*. Note: (1) newly fertilized ovum showing a narrow end-part of the spermatozoon in a close spatial relationship to its surface and two centrioles with numerous microtubules of the division spindle, both situated in the adjacent peripheral layer of fertilized ovum on the left side of the micrograph; (2) several cross- and oblique sections of spermatozoa in the peripheral region and four large spherical accumulations of fine granular material in the central part of the cytoplasm, (3) the vitellocyte of the early embryo situated in in the right lower corner of the micrograph with numerous particles of beta-glycogen and several mitochondria in its cytoplasm. Inset: Enlarged details of two centrioles.

Fig. 4A and B. Fertilized ova of *E. multilocularis*, each containing a fertilized oocyte and a single vitellocyte, both surrounded by a newly formed delicate embryonic capsule.

Note: (1) numerous mitochondria, microtubules of the division spindles and cross- or oblique sections of spermatozoa in the cytoplasm of the fertilized ova; and (2) several mitochondria, numerous beta-glycogen particles and vesicles in the cytoplasm of the vitellocyte, the nuclei of which usually contain electron dense of heterochromatin. The degenerating polar bodies with characteristic pycnotic nuclei undergoing rapid apoptosis were sometimes observed on micrographs as shown on Fig. 4A.

Fig. 5A and B. Ultrastructure of the pronuclei and the annulate lamellae. **A** – Stage of the two large pronuclei with electron-dense nucleoli, localized in the common cytoplasm, and surrounded by a newly formed capsule. Note presence of the annulate lamellae composed of several pairs of smooth membranes. **B** – High power magnification illustrating ultrastructural details of the annulate lamellae. As shown on this micrograph, each pair containing regularly spaced pores resembling those of nuclear envelope. They may occur singly or as shown here are stocked in groups in the cellular cytoplasm.

Figure
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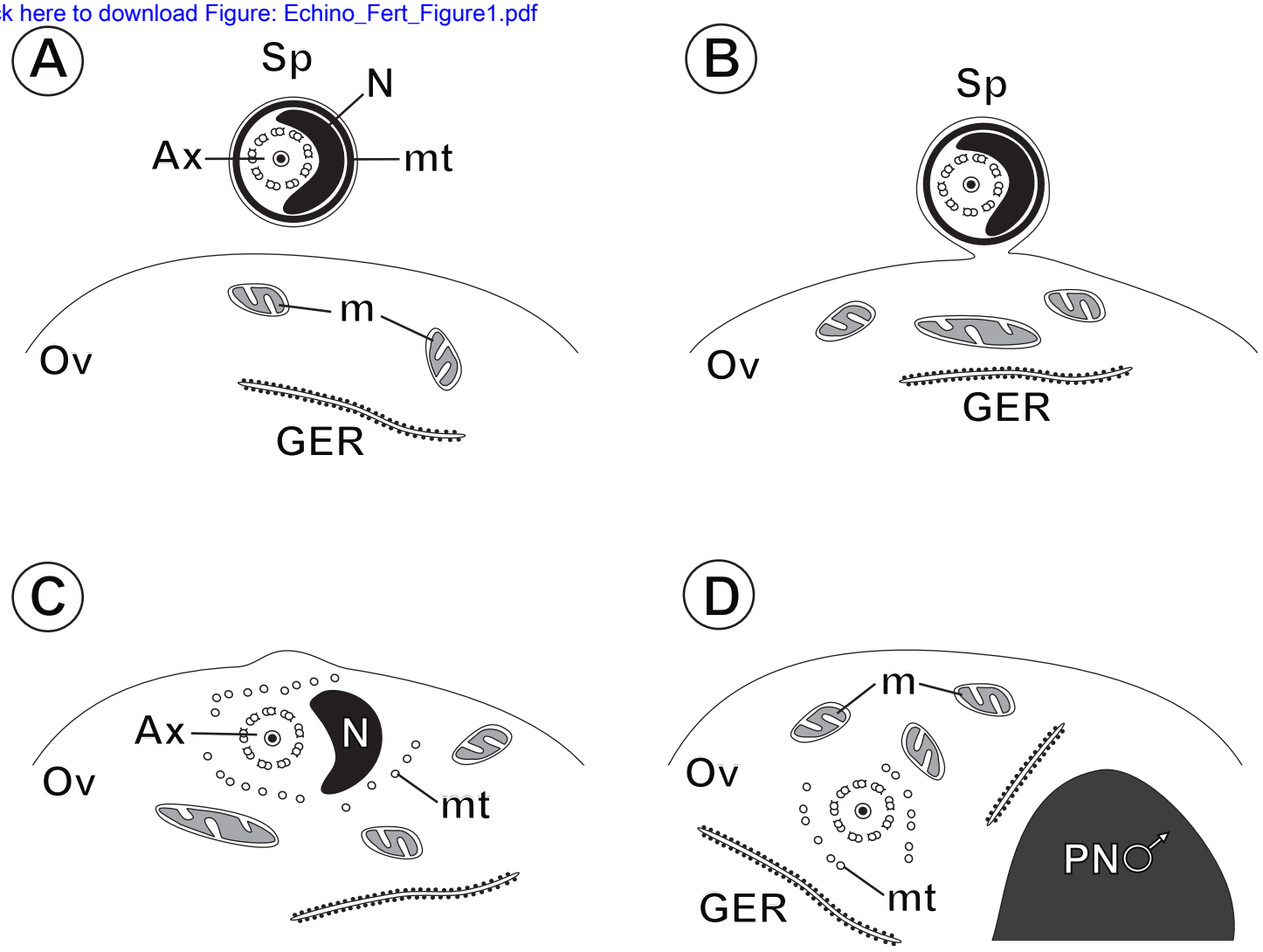

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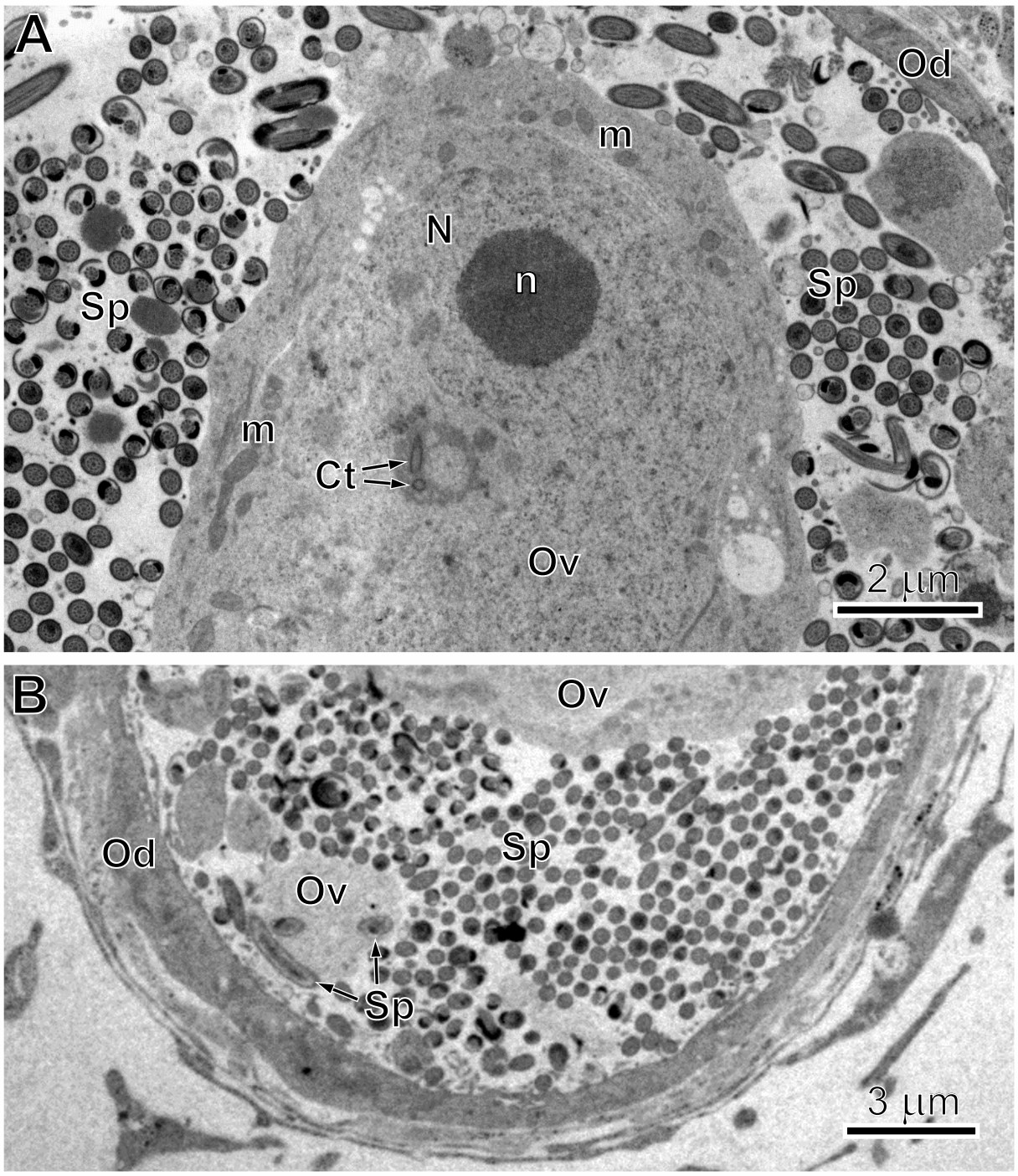

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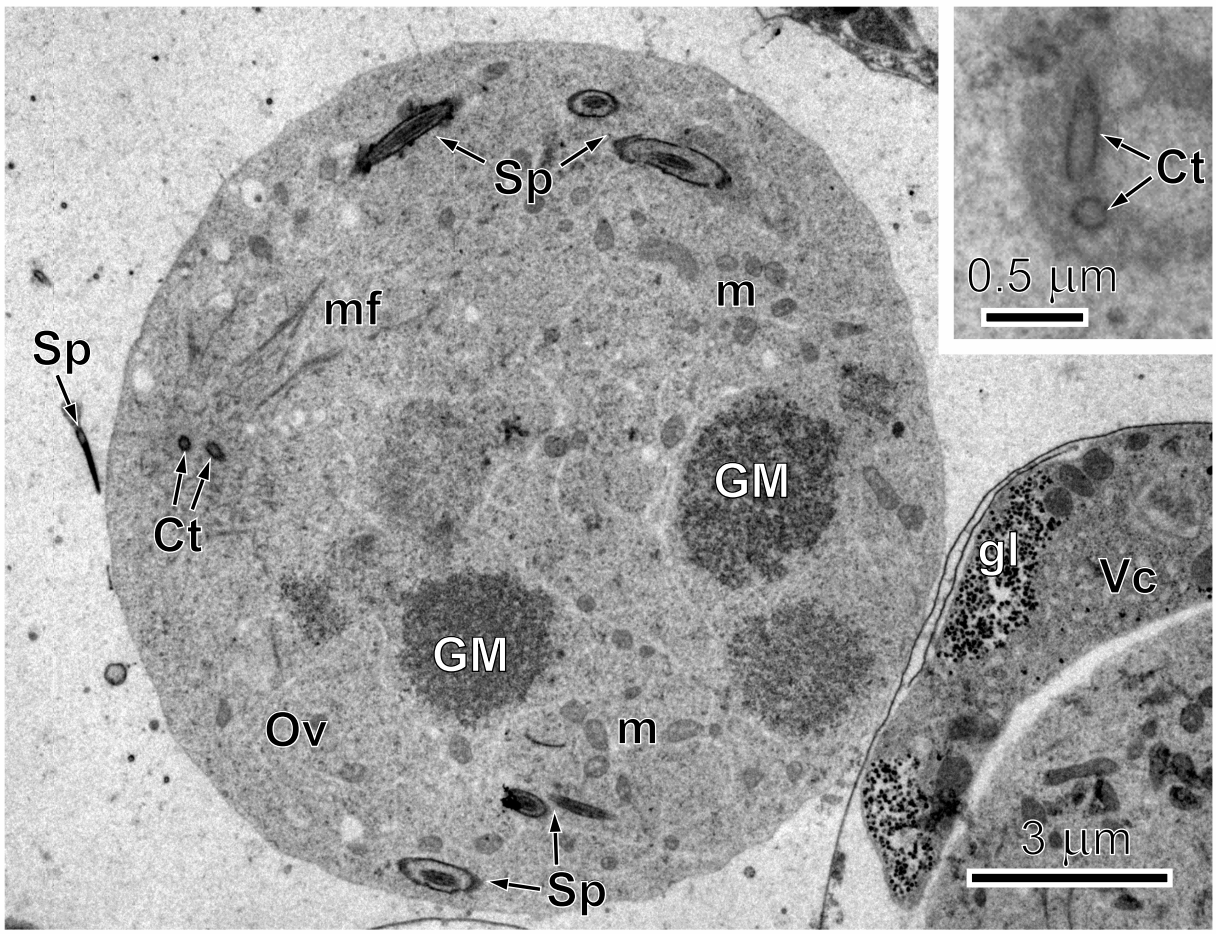

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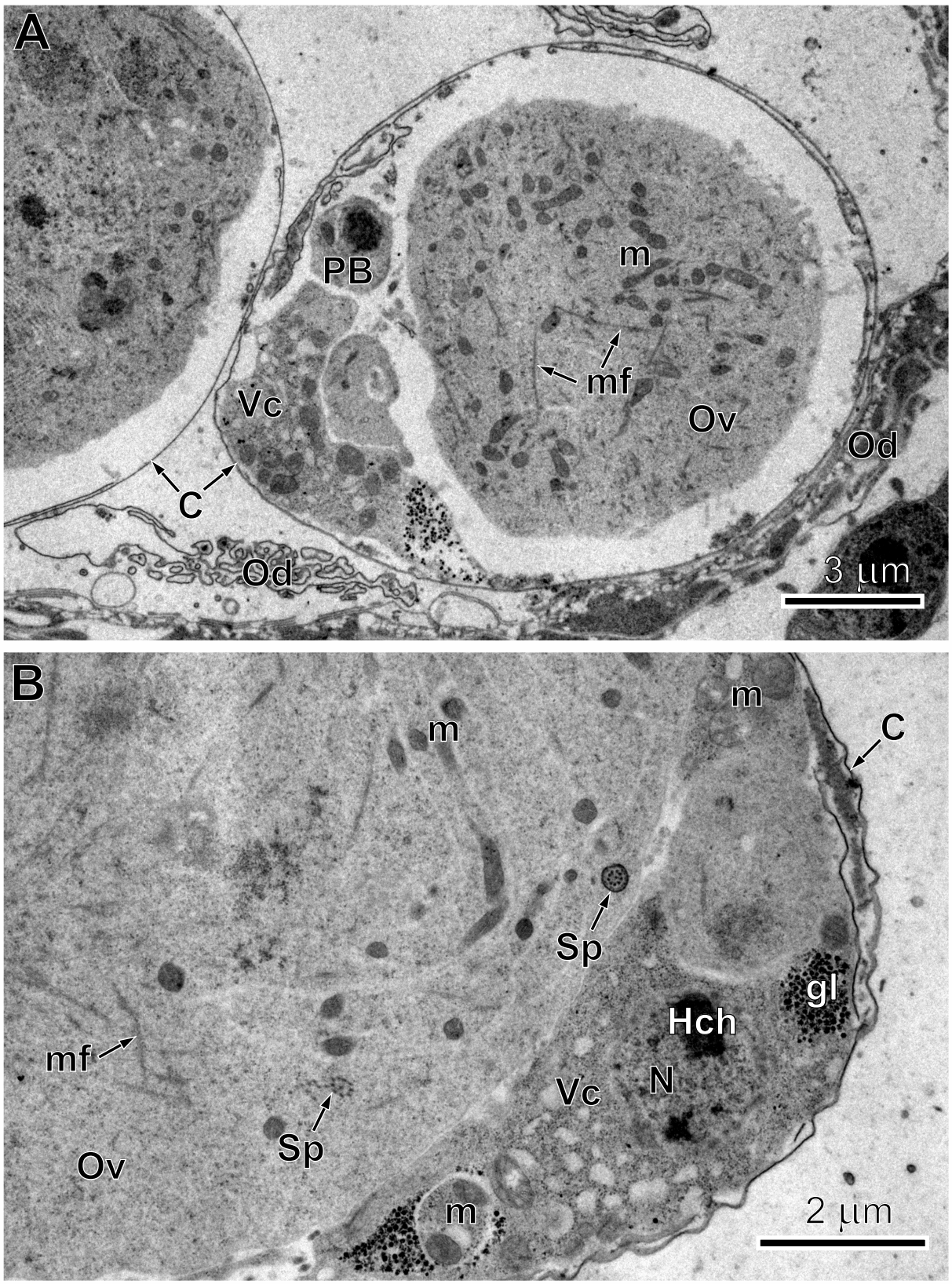

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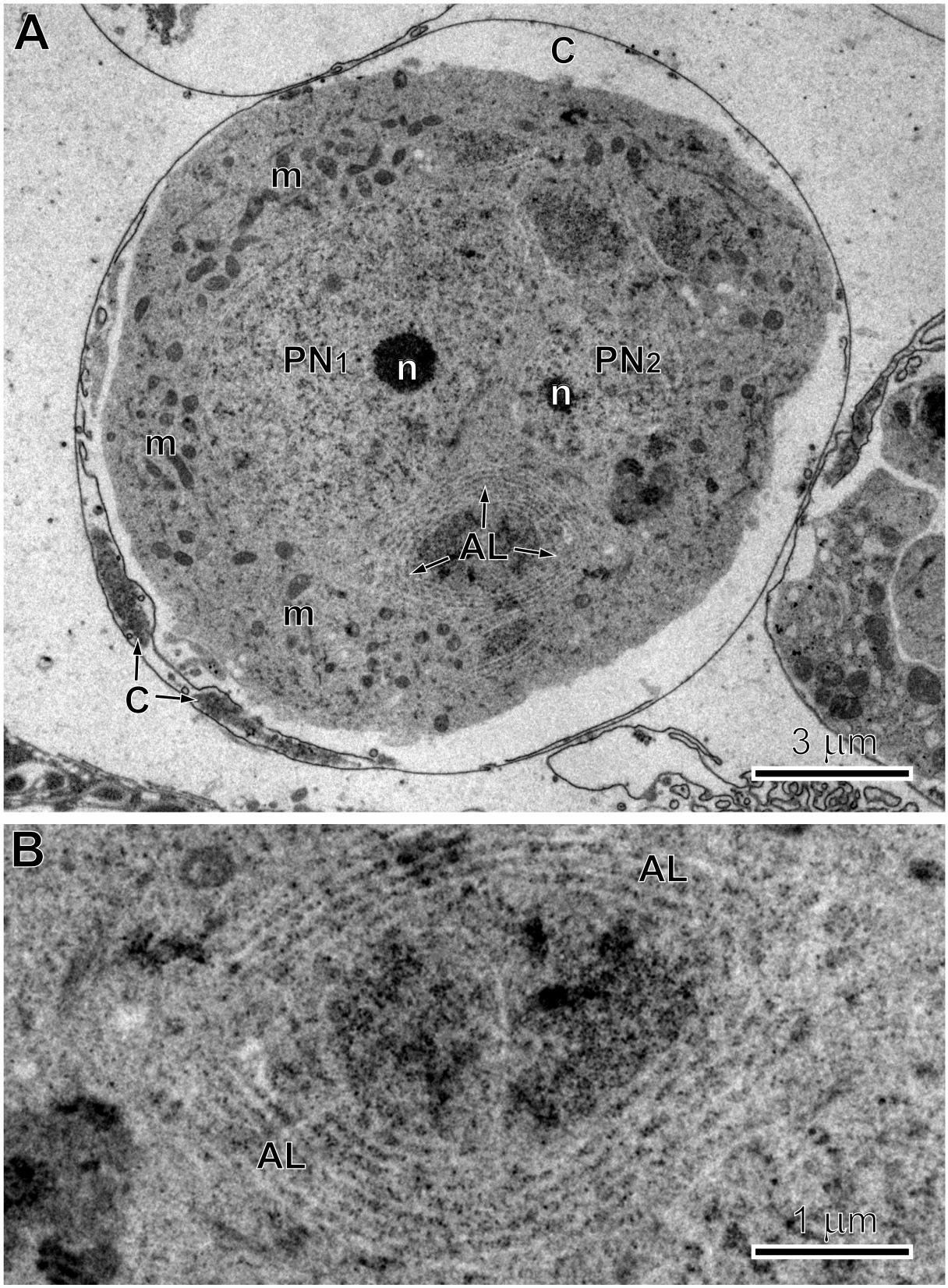

Table

Table 1. Fertilization in examined species of cestodes

Species examined	Systematic position	Number of axonemes	Reference
Acanthobothrium filicolle	Cestoda, Tetraphyllidea, Onchobothriidae	2	Mokhtar-Maamouri (1980)
Proteocephalus longicollis	Cestoda, Proteocephalidea, Proteocephalidae	2	Świderski and Conn (1999)
Mesocestoides lineatus	Cestoda, Cyclophyllidea, Mesocestoididae	1	Świderski and Conn (1999)
Gallegoides arfaai	Cestoda, Cyclophyllidea, Anoplocephalidae	1	Świderski et al. (2004)
Inermicapsifer madagascariensis	Cestoda, Cyclophyllidea, Anoplocephalidae	1	Świderski and Conn (1999)
Cotugnia polyacantha	Cestoda, Cyclophyllidea, Davaineidae	1	Taeleb and Ghobashy (2014)
Hymenolepis diminuta	Cestoda, Cyclophyllidea, Hymenolepididae	1	Świderski (1976)
Echinococcus multilocularis	Cestoda, Cyclophyllidea, Taeniidae	1	Present study