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Ultrastructure of the oncospheral envelopes in the pseudophyllidean cestode *Eubothrium salvelini* (Schrank, 1790)

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Abstract

The aim of this study is to describe the ultrastructure of oncospheral envelopes in the pseudophyllidean cestode *Eubothrium salvelini*, a parasite of salmonid fishes. Our results indicate that the eggs of *E. salvelini* differ in their ultrastructure from those of the majority of the Pseudophyllidea. The entire embryonic development, including differentiation of the mature, infective oncosphere of *E. salvelini* takes place in the uterus and not in the aquatic environment, as is common for other pseudophyllideans. Egg maturation is not simultaneous; together with mature eggs containing fully differentiated oncospheres, can be found numerous small immature, nonfertilized and nonviable abortive eggs. The normally developing eggs of *E. salvelini* are large, oval and nonoperculated. Three envelopes surround the infective hexacanths: (1) the eggshell; (2) the outer envelope originating from macromere fusion; (3) the inner envelope formed by numerous mesomeres which usually persist in the mature eggs. Our observations confirm that both the outer and the inner envelopes of *E. salvelini* eggs are cellular in origin and syncytial in nature. The typical oncospheral membrane was not observed in this species. New data on the origin and ultrastructure of oncospheral envelopes may present useful criteria for phylogenetic analysis of lower cestodes. Ontogenetic characters, such as ultrastructural aspect of morphogenesis of infective larval stages, are proposed as phylogenetic indicators in studies of cestode evolution.

Key words

Eubothrium salvelini, Pseudophyllidea, Cestoda, ultrastructure, cytochemistry, oncospheral envelopes, nonviable abortive eggs, egg apoptosis

Introduction

The pseudophyllidean cestode *Eubothrium salvelini* (Schrank, 1790) is a widely distributed parasite of salmonid fishes (Kennedy 1978a, b; Vik 1963). In lakes of the Alps, it occurs sympatrically with *E. crassum*, and both species are abundant in the lake form of brown trout, *Salmo trutta* m. *lacustris* and Arctic charr *Salvelinus alpinus* (Hanzelová *et al*. 2002), having negative effects on the growth and fitness of their fish hosts (Hoffmann *et al*. 1986, Williams and Jones 1994).

The aim of this paper is to describe the cellular organisation and ultrastructural aspects of mature eggs of *E. salvelini*, which belongs to the family Triaenophoridae Lönnber, 1889. Our preliminary results indicated that the eggs of *E. salvelini* are morphologically quite different from those of most of the families of the order Pseudophyllidea, and in particular from the eggs of the species examined by means of TEM (see Kuperman 1988; Świderski 1994, 2003; Świderski and Mackiewicz 2004). New data on this subject may present useful criteria for phylogenetic analysis of this group of "lower cestodes", because the ontogenetic characters have been proposed as phylogenetic indicators in studies of cestode evolution (Świderski 1975, 1981).

Materials and methods

Adult specimens of *Eubothrium salvelini*, were collected from the intestine of *Oncorhynchus mykiss* (Pisces, Salmonidae) from Loch Awe (Scotland, U.K.). Specimens were placed in

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cold fresh water, in order to stimulate releasing of eggs from gravid proglottids. Free eggs, sedimented at the bottom of a cultivating vessel, were collected by pipette and transferred to the fixative.

For transmission electron microscopy (TEM), the small pieces of gravid proglottids containing eggs, and the isolated eggs were both fixed in ice cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 month and then post-fixed in 1% OsO_4 for 2 h. The material was dehydrated in a graded acetone series and embedded in Spurr epoxy resin. For general topography of eggs, the semithin sections were stained with 1% toluidine blue in borax solution. The 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.

The periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) technique of Thiéry (1967) was applied in order to determine specific cytochemical localisation of glycogen at the ultrastructural level.

For scanning electron microscopy (SEM), the material was fixed using the above technique. The fixed, washed and dehydrated samples were transferred to a critical-point apparatus CPD Pelco 2, where they were dried at increasing pressure and temperature after an initial replacement of acetone with liquid CO_2. Dried eggs were mounted on stubs by carbon double tape, coated with 20 nm of gold in a sputter coater POLARON E 5100, and examined with an scanning electron microscope JEOL 6300 at 15 kV.

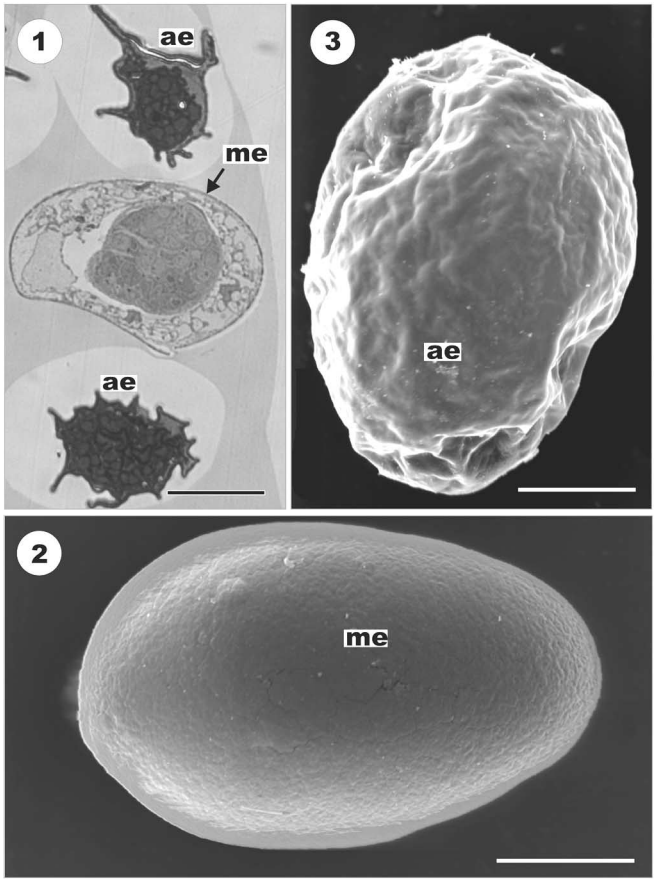


Fig. 1. Semithin section showing normally developing, mature egg of *Eubothrium salvelini* situated in the centre, surrounded by two nonviable, shrunken abortive eggs on both sides, which represent the majority of eggs in this species. Scale bar = 35 µm. **Fig. 2.** SEM micrograph of the entire normally developing mature egg of *E. salvelini*. Note the oval shape of the egg and its relatively smooth surface, with a texture of very fine wrinkles. Scale bar = 10 µm. **Fig. 3.** SEM micrograph of the nonviable shrunken egg showing an irregular outline with deep invaginations on its entire surface. Scale bar = 20 µm. **Abbreviations used in all figures:** ae – abortive eggs, B – undifferentiated blastomeres, dp – dense projections, ES – eggshell, g – glycogen, GER – granular endoplasmic reticulum, H – heterochromatin islands, I – islands of amorphous material containing small lipid droplets and myelin-like figures, IE – inner envelope, ipm – inner plasma membrane of the inner envelope, L – lipid droplets, me – mature eggs, mf – myelin-like figures, N – nucleus, n – nucleolus, OE – outer envelope, opm – inner plasma membrane of the outer envelope, r – ribosomes, v – vesicles

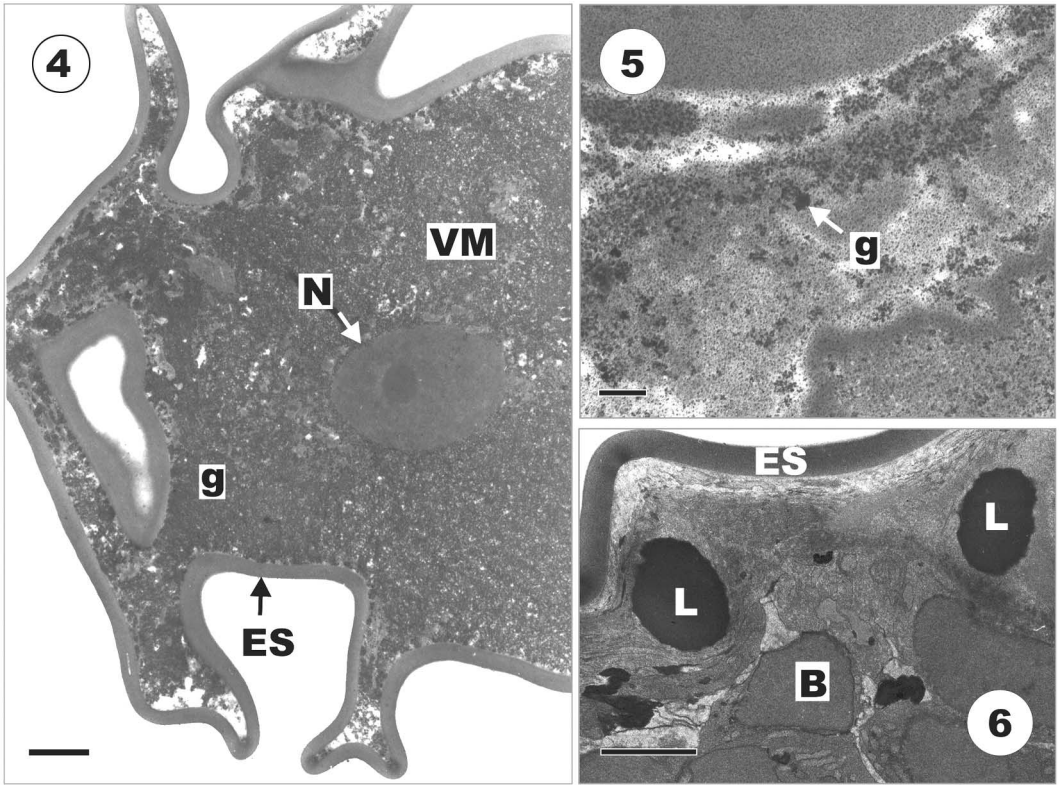
Results

The terminology employed here for describing the oncospheral envelopes of *E. salvelini* follows that previously applied in our similar studies on another pseudophyllidean, *Bothriocephalus clavibothrium*, by Świderski and Mackiewicz (2004) and a glossary of cestode embryonic and larval structures currently being prepared (Conn *et al*., in preparation). The eggshell is considered as the egg envelope, because it is formed from the shell globules of vitellocytes and not by the blastomeres of the embryo proper; in contrast, the outer envelope originating from two macromeres, and the inner envelope formed by a fusion of several mesomeres, are considered as the primary embryonic envelopes in the early preoncospheral stages of development, when the inner envelope still remains undifferentiated. At the end of embryonic development, however, the term "oncospheral envelopes" is frequently applied to the outer envelope degenerating remnants and (= together with) fully differentiated inner envelope, forming several well demarcated sublayers surrounding mature, infective hexacanths.

The entire embryonic development, including differentiation of the mature, infective oncospheres of *E. salvelini*, takes place in the uterus of the adult worm and not in the external aquatic environment as is common for a majority of other pseudophyllideans.

The egg maturation in this species is not simultaneous; mature eggs containing fully differentiated oncospheres, were always observed together with numerous small immature, nonfertilized and nonviable abortive eggs (Figs 1–6).

Among the egg samples examined by means of light (LM), scanning (SEM) and transmission (TEM) electron microscopy, a very high percentage represented much smaller



Figs 4 and 5. Cytochemical Thiery's test for polysaccharides showing distribution of the glycogen in the nonviable, irregularly-shaped abortive eggs arrested in different stages of their development. **Fig. 4.** Shows a single large nucleus surrounded by condensed accumulation of vitelline material. **Fig. 5.** Illustrates α- and β-glycogen distribution in the egg envelopes of an abortive egg. Scale bars = 2 μm. **Fig. 6.** A part of an abortive egg composed of several undifferentiated blastomeres, which in addition to deeply infolded egg capsule and compact degenerating outer and inner envelopes, show numerous large, highly osmiophilic lipid droplets always present in a thin layer of the inner envelope. Scale bar = 2 μm

shrunken, and usually irregularly-shaped nonviable eggs, the so-called "dark eggs". Some of them were composed of an oocyte surrounded by very compact vitelline material and shrunken egg capsules (Figs 1 and 3–6). This type probably represents unfertilized eggs (Figs 4 and 5). Other "dark eggs" contain numerous undifferentiated blastomeres and it appears that their development was arrested in the early stage of cleavage division (Fig. 6). In the latter type, in addition to deeply infolded eggshell and compact, degenerating layers of the outer and inner envelopes, numerous large, highly osmiophilic droplets were always present in a thin residual layer of the inner envelope (Fig. 6).

The normally developing eggs of *E. salvelini* (Figs 1, 2, 7 and 8) are large, oval and nonoperculated. Their regular surface is rather smooth, with a texture of very fine wrinkles (Fig. 2) in comparison with nonviable shrunken eggs (Fig. 3) which have a very irregular outline and many deep invaginations in their eggshells. The size of normally developing oval eggs fixed and examined by means of LM (semithin sections) and SEM, ranges between 36–40 μm in length, and 22–25 μm in width.

In the mature eggs examined by TEM (Figs 7 and 8), three envelopes surround the infective oncospheres: (1) the eggshell; (2) the outer envelope originating from macromere fusion; and (3) the inner envelope formed by numerous mesomeres. The nuclei of macromeres were present in some sections inside of the outer envelope (Fig. 7); and the nuclei of mesomeres were frequently observed in the cytoplasm of the inner envelope, where they usually persisted in the mature egg stage (Figs 9 and 10). These observations confirm that both the outer and the inner envelopes of *E. salvelini* eggs are cellular in origin and syncytial in nature. The typical oncospheral membrane characteristic of pseudophyllidean and cyclophyllidean eggs was not observed in this species.

The eggshell is a relatively thin, electron-dense external protective layer (Fig. 1). It is formed initially in the ootype as the thin vitelline capsule, and consolidated in the uterus. It originates from vitellocyte material secreted from vitelline cells in synchrony with Mehlis' gland secretions. In *E. salvelini* eggs, the external surface of the nonoperculated egg capsule is rather smooth. Its average thickness is about 0.5 μm.

The outer envelope of the mature eggs is usually a very thin, rudimentary structure (Figs 7 and 10). Some parts that still contain macromere nuclei show presence of free ribosomes, short profiles of GER, and several small spherical mitochondria. The enlarged peripheral part of the outer envelope

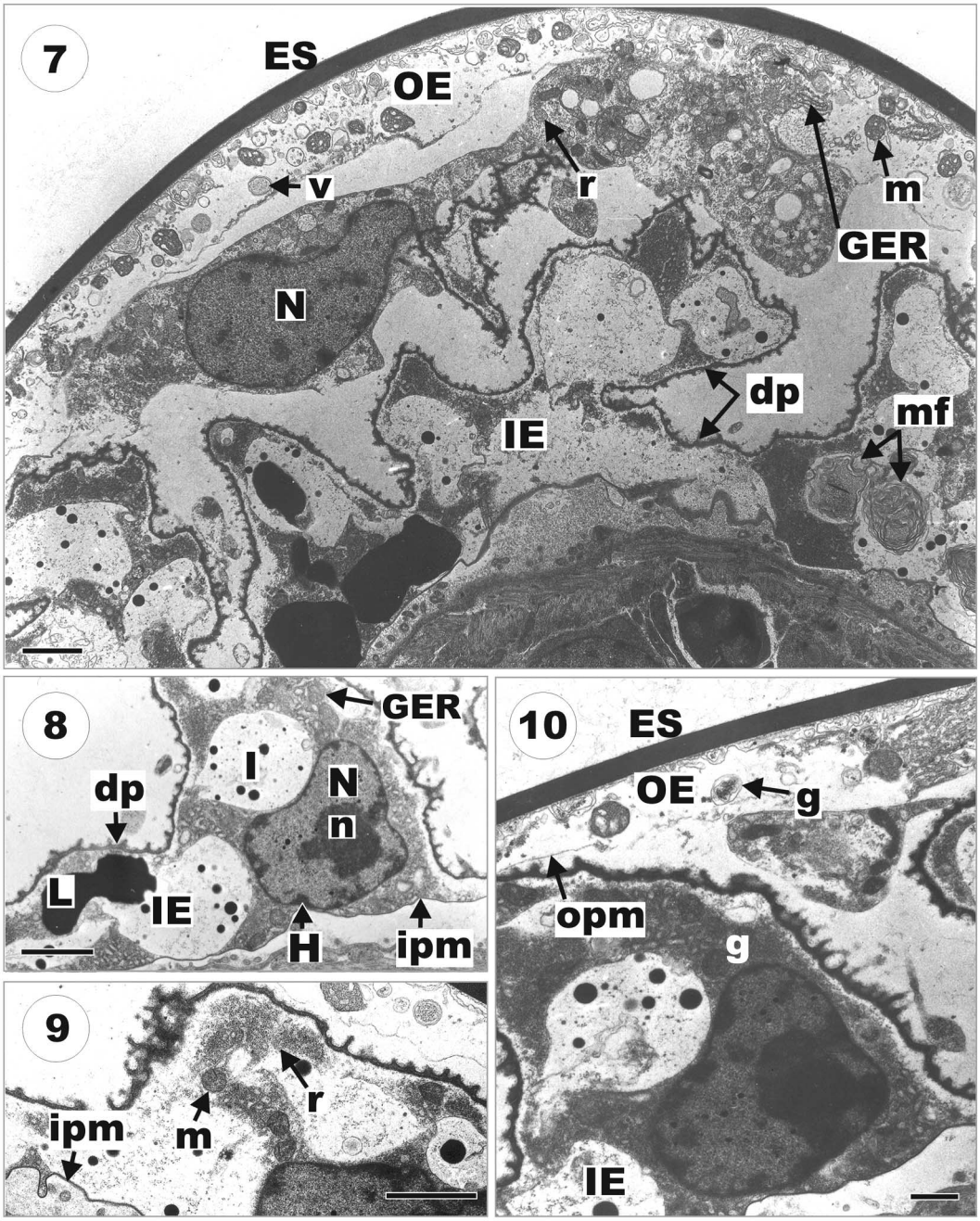


Fig. 7. Peripheral part of a mature egg of *E. salvelini* showing the outer envelope with the macromere nucleus. Note deeply infolded surface of the inner envelope covered by an electron-dense membranous structure with numerous short dense projections occasionally forming small branches or ramifications, and myelin-like figures, frequently observed in the cytoplasm of the inner envelope. Scale bar = 2 μm. **Fig. 8**. Part of the inner envelope showing characteristic ultrastructural details of macromere nucleoplasm containing dense heterochromatin islands. Note several organelles. The surface of the inner envelope is bordered by an outer electron-dense membranous structure with numerous short dense projections, while its inner surface has a very thin and delicate inner limiting plasma membrane. Note: (1) a large nucleus of the mesomere with predominant nucleolus and small heterochromatin island adjacent to the nuclear envelope; (2) granular compartments of cytoplasm rich in GER; and (3) islands of amorphous cytoplasm containing small lipid droplets and single large lipid droplets of high electron density, sometimes irregular in shape, and several myelin-like figures. Scale bar = 2 μm. **Fig. 9.** Detail of the inner envelope showing several mitochondria and ribosomes in the granular compartment and small osmiophilic lipid droplets in the amorphous regions of the inner envelope. Note the very thin inner limiting plasma membrane of this envelope. Scale bar = 2 μm. **Fig. 10.** Cytochemical test of Thiery showing glycogen distribution in the outer and inner envelopes. Note: (1) homogeneous electron-dense eggshell at the egg surface; (2) very thin and delicate inner plasma membrane of the outer envelope; (3) a large nucleus of a mesomere with dominant nucleolus and small heterochromatin island in the cytoplasm of the inner envelope; (4) several α- and β-glycogen particles in both envelopes. Scale bar = 1 μm

of the mature egg of *E. salvelini* still contains macromere nuclei. Their nucleoplasm contains several heterochromatin islands, but nucleoli were never observed. The perinuclear cytoplasm contains several cell organelles, namely spherical mitochondria, free ribosomes, short profiles of GER, Golgi complexes, and numerous vesicles of different sizes.

The inner envelope's outer surface is covered by an electron-dense membranous structure with numerous short dense projections, occasionally forming small branches or ramifications. This layer contains numerous nuclei of mesomeres that took part in the formation of this envelope and that generally persist in the mature eggs. Some of them contain prominent electron-dense nucleoli; others have large heterochromatin islands, but it usually varies in appearance with the section level. The cytoplasm of this envelope is subdivided into two very different parts: (1) the amorphous, agranular regions of very low electron density containing randomly dispersed small lipid droplets of very high electron density, sometimes reaching much larger sizes, and frequently observed myelin-like figures and (2) the compartments of granular cytoplasm very rich in free ribosomes, short profiles of GER and several mitochondria.

Discussion

The pseudophyllidean cestodes have generally complex life cycles, frequently with two intermediate hosts and one definitive host, quite often with change of environment between consecutive stages. In all cestodes, there is a clear interrelationship between functional ultrastructure of the oncospheral envelopes of infective eggs and the life cycle parameters, such as specific intermediate host and the host biotopes (Chomicz and Świderski 2004). Our results confirm previous observations that *E. salvelini* eggs complete their embryonic development in the uterus of adult worms, and gravid proglottids contain fully formed infective oncospheres (Kuperman 1978). A characteristic feature for this species is that egg maturation is not simultaneous and therefore, together with mature eggs containing fully differentiated oncospheres, there are also numerous small vacant immature nonfertilized, abortive eggs (Kuperman 1978). *E. salvelini* eggs are spontaneously released throughout the year, except for the winter months (Hanzelová *et al*. 2002). Our data support previous statements that the eggs released from gravid proglottids remain viable and infective in fresh water for 30 days at a temperature of 5–7°C (Kuperman 1978). No hatching of coracidia and no free swimming larval stage occurs in this species. For further development into the procercoid stage, the entire egg must be eaten by a freshwater copepod. Only in the intestine of the intermediate host is the eggshell ruptured into two parts, liberating the unciliated coracidium containing the hexacanth larva, which rapidly penetrates the body cavity of the crustacean. This type of life cycle may explain ultrastructural peculiarities of *E. salvelini* eggs found in the present study, such as lack of an operculum, lack of a ciliated syncytial envelope of the coracidium, and oncospheral flame cells, which are common for most other pseudophyllidean cestodes.

The eggs of *E. salvelini* are ultrastructurally quite different from those of most other pseudophyllideans thus far examined in this respect, and in particular from the well known coracidia of *Diphyllobothrium latum* and *D. dendriticum* (Grammeltvedt 1973, Kuperman 1988), *Triaenophorus nodulosus* (Timofeev and Kuperman 1967, Kuperman 1988, Korneva 1994) and bothriocephalid cestodes (Swiderski and Mokhtar 1974; Swiderski 1994, 2003; Świderski and Mackiewicz 2004; Berrada-Rkhami and Gabrion 1990). The main differences observed between the ultrastructure of eggs of *E. salvelini* and other pseudophyllideans include not only the presence or absence of the operculum and presence or absence of cilia in the syncytial coracidial envelopes, but also many additional ultrastructural differences in their hexacanths, the most evident being the absence of flame cells and therefore of the protonephridial system characteristic of a majority of previously examined coracidia.

The eggshell of *E. salvelini* is relatively thin, elastic and transparent in comparison with eggshells of other pseudophyllideans where the eggshells are very hard, much thicker and very electron-dense (Swiderski and Mokhtar 1974; Berrada-Rkhami and Gabrion 1990; Swiderski 1994, 2003; Swiderski and Mackiewicz 2004).

The much infolded outer surface of the inner envelope, covered by an electron-dense membranous structure with numerous short dense projections, may begin to swell in the intestine of the copepod intermediate host after liberation of the coracidium from the eggshell. It probably results in expanding the circumference of its initially infolded surface and in a great increase of volume of the nonciliated coracidial envelope of this species as described by Dubinina (1966). In free swimming coracidia of numerous pseudophyllideans, the permeability of the membranes of coracidia results in swelling of all layers.

An interesting homology concerns the ultrastructure of *E. salvelini* eggshell, generally resembling that observed in other pseudophyllideans, and the thick and hard outer coats or "shells" described in the mature eggs of proteocephalidean (Swiderski and Subilia 1978, Bruňanská 1999) and some species of cyclophyllidean cestodes, such as the anoplocephalids *Anoplocephaloides dentata* (see Świderski *et al*. 2001) and *Mosgovoyia ctenoides* (see Młocicki *et al*. 2005). Both types of these stiff, electron-dense structures have the same, mainly protective function; their origin, however, is completely different. Whereas the eggshells of pseudophyllideans, similar to those of trematodes, are formed in the ootypes from the shell globule material accumulated in the vitelline cells (Swiderski 1994, Świderski and Mackiewicz 2004), the outer coats or "shells" of proteocephalideans and some cyclophyllideans originate from the secretion of the uterine epithelium (Conn 1993, Conn and Forman 1993), which progressively encrusts the initially thin and delicate membranous vitelline capsule (Swiderski and Subilia 1978, Bruňanská 1999, Świderski *et al*. 2001, Młocicki *et al*. 2005).

The thickness of the capsule in the pseudophyllidean *E. salvelini*, 0.5 μm, is the same as in the proteocephalidean *Proteocephalus longicollis*, (Swiderski and Subilia 1978, Bruňanská 1999). This finding is related to the fact that the entire embryonic development of both species takes place in the uterus of the worms and there is no need for extensive protection of the embryo. The oviparous bothriocephalids, which deposit their unembryonated eggs in the external aquatic environment, require much thicker, robust and resistant eggshells for embryo protection during long periods of development in the hostile external marine environment (Swiderski and Mokhtar 1974; Swiderski 1994, 2003; Świderski and Mackiewicz 2004).

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