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An ultrastructural study of the egg wall surrounding the miracidium of the digenean *Brandesia turgida* (Brandes, 1888) (Plagiorchiida: Pleurogenidae), with the description of a unique cocoon-like envelope

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ABSTRACT

The intrauterine eggs of the pleurogenid trematode *Brandesia turgida* (Brandes, 1888), exhibiting advanced stages of miracidial differentiation and fully formed, ciliated miracidia, were examined by means of transmission electron microscopy (TEM). Each embryonated egg is composed of a mature miracidium surrounded by a four-layered egg wall: (1) an outer, anucleate layer external to the eggshell, which forms a thick cocoon; (2) the operculate egg-shell; (3) a small remnant of the compact, granular cytoplasm of the outer embryonic envelope (*sensu stricto*); and (4) a relatively distinct cellular remnant of the inner embryonic envelope. Layers enveloping the egg apparently play an important role in the protection, metabolism and storage of nutritive reserves for the developing miracidium. The outer, anucleate layer, or cocoon, situated externally to the eggshell and composed of a transparent, electron-lucent substance with numerous dense, osmiophilic islands attached to its peripheral membrane, has never previously been seen in TEM studies of the eggs of parasitic platyhelminths. The origin, formation, functional ultrastructure and chemical composition of this peculiar layer remain enigmatic, although its function appears to be protective. The thick, electron-dense eggshell resembles that of other trematodes, exhibiting a characteristic fissure zone around the operculum. The very small, indistinct remnants of the outer embryonic envelope appear in the form of a very thin, compact, granular cytoplasm closely attached to the inner surface of the eggshell. Conversely, the inner embryonic envelope is frequently apparent at one or both poles of the developed egg as a syncytial envelope formed by the fusion of mesomeres. This envelope, even in eggs containing a fully formed miracidium, still has the features of a metabolically active layer with an energy storage capability. Lysosome-like structures observed in some eggs may be involved in the autolysis of the embryonic envelopes.

1. Introduction

Little information is available on the ultrastructure of egg envelopes, including the eggshell, in digenean trematodes examined by means of transmission electron microscopy (TEM). There are several scanning electron microscopical (SEM) studies on the surface structure of trematode eggs, but TEM studies have been impeded by serious technical difficulties in getting the egg contents well fixed and infiltrated with embedding media, and also with problems in cutting the thick, hard eggshells. Existing TEM studies have usually involved parasites of medical or veterinary importance, namely *Schistosoma mansoni*, *S. haematobium* (see Świderski et al., 1980; Eklu-Natey et al., 1982a,b; Świderski, 1984, 1985, 1988, 1994a,b), *S. mattheei* (see Świderski, 1986), *S. japonicum* (see Jones et al., 2008) and *Opisthorchis viverrini* (see Khampoosa et al., 2011).

Brandesia turgida (Brandes, 1888) (Plagiorchiida: Pleurogenidae) is a parasite of frogs in the Palaearctic region. For many years, pleurogenids (as pleurogenines), mainly from amphibians, were included with the lecithodendriines, mainly from bats, within the family Lecithodendriidae, as the groups had a similar morphology, especially in terms of the short gut-caeca and

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virgulate cercariae, and a wide use of insects as intermediate hosts (Yamaguti, 1975; Lotz and Font, 2008). However, more recently, the Pleurogenidae has been accepted as a distinct family (Bray, 2008; Lotz and Font, 2008) within the Microphalloidea based mainly on molecular data. Pleurogenids usually occur as intestinal parasites (sometimes in the hepatic system and elsewhere) of amphibians (occasionally reptiles), but *B. turgida* is very unusual in that it lives within crypts in the intestinal wall. It also has an unusual morphology, as the small ventral sucker is close to the posterior end of the worm and the large uterus occupies most of the body (Oshmarin and Oshmarin, 1989; Sey, 1992).

The aim of the present study was to describe the ultrastructure of the egg envelopes of *B. turgida* from the marsh frog *Pelophylax ridibundus* (Pallas, 1771), previously known as *Rana ridibunda*, in Europe. The ultrastructure of the egg-enclosed, differentiating and fully formed, ciliated miracidium of this species will be a subject of a separate paper.

2. Materials and methods

Adult specimens of *Brandesia turgida* were obtained from crypts in the intestinal wall of naturally infected frogs, *Pelophylax ridibundus*, collected near the Rybinsk Reservoir on the Volga River, Russia. Live digeneans were rinsed in 0.9% NaCl solution, fixed in cold (~4 °C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 10 days, washed overnight in 0.1 M sodium cacodylate buffer at pH 7.4, postfixed in cold (~4 °C) 1% OsO_4 in the same buffer for 1 h, dehydrated in a graded series of ethanol and propylene oxide, and embedded in a mixture of Araldite and Epon. Ultrathin sections (~40–60 nm) were cut on a Leica Ultracut UCT ultramicrotome, collected on copper grids and double stained with uranyl acetate and lead citrate. Sections were examined in JEOL 1011 transmission electron microscope operating at 80 kV.

3. Results

The intrauterine eggs (Fig. 1A and B) of mature *Brandesia turgida*, exhibiting advanced stages of miracidial differentiation and containing fully formed, ciliated miracidia, were examined by means of TEM. Each embryonated egg is composed of a developing or fully developed miracidium surrounded by a four-layered egg wall: (1) an outer anucleate layer situated externally to the eggshell and forming a thick cocoon; (2) the operculate eggshell; (3) a very thin remnant of the compact, granular cytoplasm of the outer embryonic envelope (*sensu stricto*); and (4) a relatively distinct cellular remnant of the inner embryonic envelope.

Even a very low power TEM micrograph (Fig. 1A), illustrating the general topography of several fully developed eggs of *B. turgida* in the distal part of the uterus, clearly shows that the shell of each egg is surrounded by an outer anucleate layer, which appears to be characteristic for this species. This layer forms a thick cocoon composed of a transparent, electron-lucent substance with numerous small, electron dense islands irregularly dispersed around its peripheral membrane (Fig. 4G). Even at early stages of development, degenerating eggs undergoing apoptosis (Fig. 1B) and composed of the cellular debris of the nuclei and cytoplasm of several disintegrating blastomeres already exhibit the presence of a very thin layer of this external cocoon. In such eggs, numerous small electron-dense islands close to the peripheral membrane can be seen in the cocoon surrounding the operculate eggshell (Fig. 1B), providing direct evidence that cocoon formation occurs very early in egg development.

Observations made on eggs containing an immature miracidium, but at relatively advanced stages of differentiation (Fig. 2A and B), were helpful in providing a better understanding of the origin and formation of both outer embryonic envelope and inner egg envelopes. At such stages of development, fusion of the much flattened, elongate and reduced in size nuclei of the macromeres, situated directly beneath the eggshell, results in the formation of the syncytial outer envelope (Fig. 2A and B). Prior to their fusion, these macromere nuclei exhibit numerous dense islands of heterochromatin closely adjacent to their nuclear envelope (Fig. 2A and B). At a somewhat later stage, the large, spherical nuclei of the mesomeres (Fig. 2A and B), situated beneath the outer envelope, also undergo a progressive fusion, which results in the inner envelope. Of the other blastomeres of the embryo proper (Fig. 2A and B), a great majority differentiate into various miracidial cells and structures, but among them are degenerating micromeres with compact, electron-dense nuclei (Figs. 2A and 4E).

In those eggs containing a fully formed, ciliated larva, the miracidial apical papillae are usually directed towards the opercular pole of the eggshell (Fig. 3A and B). A high power TEM micrograph (Fig. 3B), showing details of the three egg envelopes, the shell and operculum, and the apical region of the ciliated miracidium, illustrates a very close contact between the operculum and small discs or flattened islands of a peripheral, electron dense material situated close to the surface of the cocoon. The

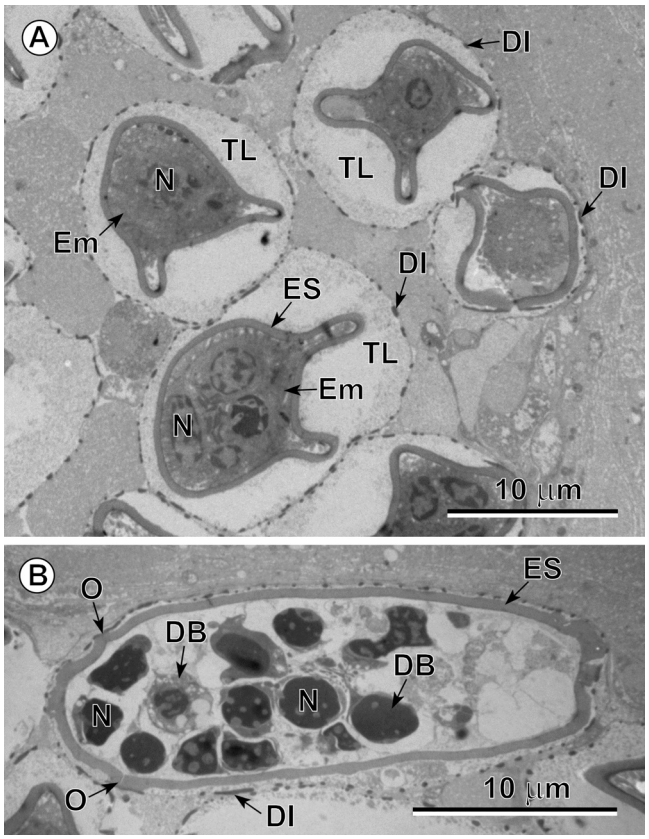

Fig. 1. (A) Low power TEM micrograph illustrating the general topography of several mature eggs of *Brandesia turgida* in the distal part of the uterus. *Note*: (1) an outer anucleate layer, situated externally to the eggshell and forming a thick cocoon composed of a transparent, electron-lucent substance; and (2) numerous small, electron dense islands irregularly dispersed around the egg surface and attached to its peripheral membrane. (B) Degenerating egg undergoing apoptosis. *Note*: (1) several degenerating blastomeres and nuclei; (2) a very thin layer of the external electron-lucent cocoon with peripheral islands of electron dense material; and (3) a formed operculum on the left side of the eggshell. DB, degenerating blastomeres; DI, dense islands of electron dense material at peripheral membrane of external, electron-lucent cocoon; Em, differentiating embryos; ES, eggshell; N, nucleus; O, operculum; TL, transparent layer of external electron-lucent cocoon.