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Embryonic Development in the Intrauterine Eggs of *Orientocreadium batrachoides* Tubangui, 1931 (Trematoda, Digenea)

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Abstract: Intrauterine embryonic development of the eggs of *Orientocreadium batrachoides* was examined by means of transmission electron microscopy. The eggs exhibited all of the consecutive stages of embryonic development. The proximal uterus usually contained unembryonated eggs, each was composed of a fertilized oocyte and few vitelline cells; these vitelline cells were fused forming the vitelline syncytium. The distal regions of the uterus were filled with eggs containing fully developed embryos. The electron dense egg shell was very thick and consisted of a tanned protein.The operculum was formed after shell formation. The eggs underwent cleavage divisions ending by the formation of the early embryo composed of several blastomeres of different sizes. Three types of blastomereswere present: macromeres, mesomeres and micromeres. Simultaneously, several micromeres exhibited clear signs of degeneration and apoptosis. The results of the present study were compared with those previously reported for other parasitic Platyhelminthes.

Key words: Intrauterine Eggs · *Orientocreadium* · Digenea · Ultrastructure

INTRODUCTION

Ultrastructural features of the intrauterine embryonic development of digenetic trematode eggs were scarce, no more than a few species up to date. Studies of the internal trematode egg structure using transmission electron microscopy (TEM) have been impeded by technical difficulties in getting the egg contents well fixed and infiltrated with embedding media, with problems in cutting the thick and hard eggshells [1]. Besides TEM studies on eggs of *Aspidogaster limacoides* [2, 3] and cestode one [4-6] studies on digenetic trematode eggs have concerned mainly with parasites of medical or veterinary importance as species of *Fasciola* [7] *Schistosoma* [8-10] the blood fluke *Sanguinicola* [11]. *Opisthorchis* [12] and recently, the digeneans *Maritrema feliui*[1] and *Brandesia turgida* [13].

The aim of the present TEM study was to describe, for the first time in Egypt, the ultrastructure of the embryonic development stages in the intrauterine eggs of *Orientocreadium batrachoides* Tubangui, 1931 (Trematoda, Digenea) caught from *Clariasgariepinus* and compare the results with those described for eggs of other digenetic trematodes.

MATERIALS AND METHODS

Adult specimens of *Orientocreadium batrachoides* were collected from the intestine of *Clarias gariepinus* (Family: Clariidae) caught from the River Nile at the region of Kaluobia Governorate during spring months of 2012 and treated for transmission electron microscopy. Live worms were rinsed in 0.9% NaCl solution, fixed with 2% gluteraldehyde in 0.1M phosphate buffer, pH 7.3, for 2 h at 4°C. Following a buffer wash, tissues were postfixed in 1% osmium tetroxide in 0.1M phosphate buffer at pH 7.3 for 1 h, ultrathin sections were cut using a diamond knife of an LKB 4800 Ultratome, mounted on uncoated copper grids and double stained with alcoholic uranyl acetate and aqueous lead citrate for 20 min. Ultrathin sections were viewed in a Joel 1200 EX2-transmission electron microscope at 80 kV.

RESULTS

The eggs of *Orientocreadium batrachoides* measured 28-30µm long and 14-17 µm wide; they were elongated, oval and operculate. They can be classified as oligolecithal due to the presence of few vitellocytes

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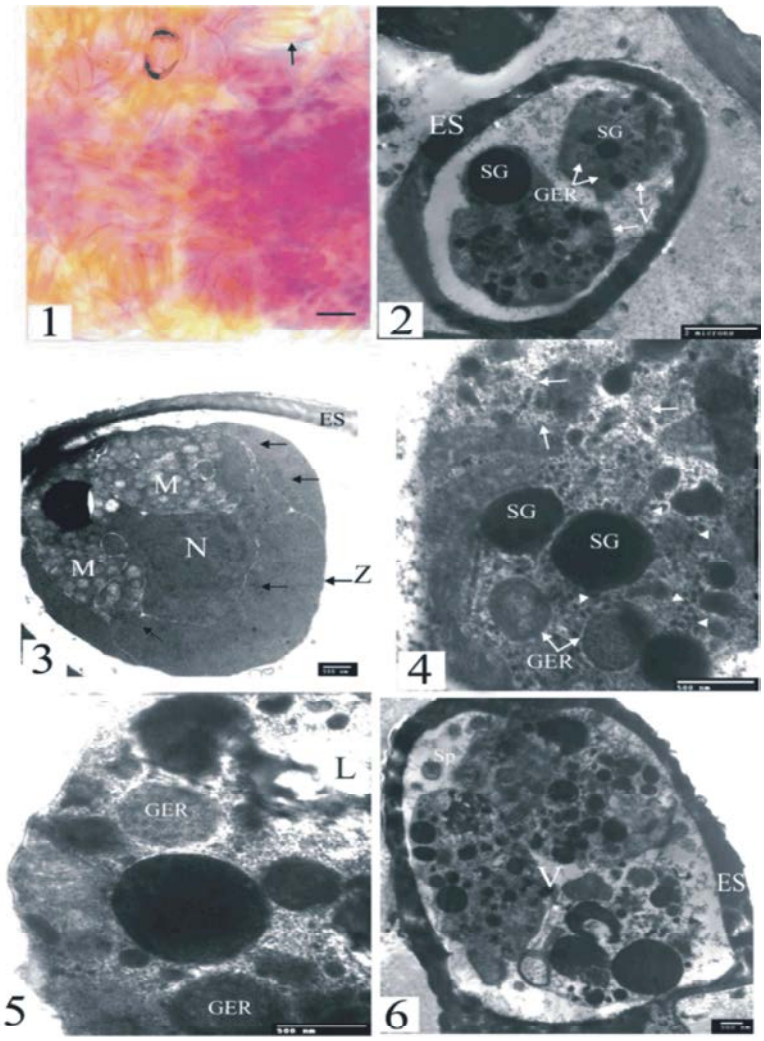

Fig. 1: A semithin section of several elongated and oval eggs of *Orientocreadium batrachoides* with operculum(Arrow). *Scale bar= 40 µ*

Fig. 2: Proximal part of the uterus of *O. batrachoides* with unembryonated eggs (Fertilized oocyte) with the appearance of vitellocytes (v) surrounded by a thick egg shell(ES). Note a compact granular cytoplasm of the vitellocyte rich in free ribosomes, granular endoplasmic reticulum(GER) and several shell globules (SG).*Scale bar= 2 µ*

Fig. 3: Proximal part of the uterus of *O. batrachoides* showing fertilized ovum with early embryo (Zygote). (Z) surrounded with egg shell (ES).Note large nucleus(N) surrounded by several mitochondria(M) and granular endoplasmic reticulum(arrows). *Scale bar =500nm*

Figs. 4: 5 High magnification of the cytoplasm of vitellocytes showing several dense balls of granular endoplasmic reticulum (GER) embedded in high accumulation of rosette-glycogen particles (Arrowheads) and single-glycogen particles (Arrows).Note shell globules(SG) and lipid droplets(L). *Scale bar= 500nm*

Fig. 6: Proximal part of the uterus of *O. batrachoides* with unembryonated egg composed of few vitellocytes (V) surrounded with egg shell (ES). Note mitochondria (M),lipid droplet (L) and presence of traces of sperm axoneme. (SP).*Scale bar= 500nm*

accompanying each fertilized oocyte during egg formation (Figs. 1, 2, 6). In the present study, the eggs exhibited all of the consecutive stages of embryonic development; whereas the proximal regions of the uterine lumen usually contained unembryonated eggs or eggs with early embryos (Figs. 2, 3, 13) the posterior or distal regions of the uterus were filled with eggs containing fully developed ones (Figs. 8, 16).

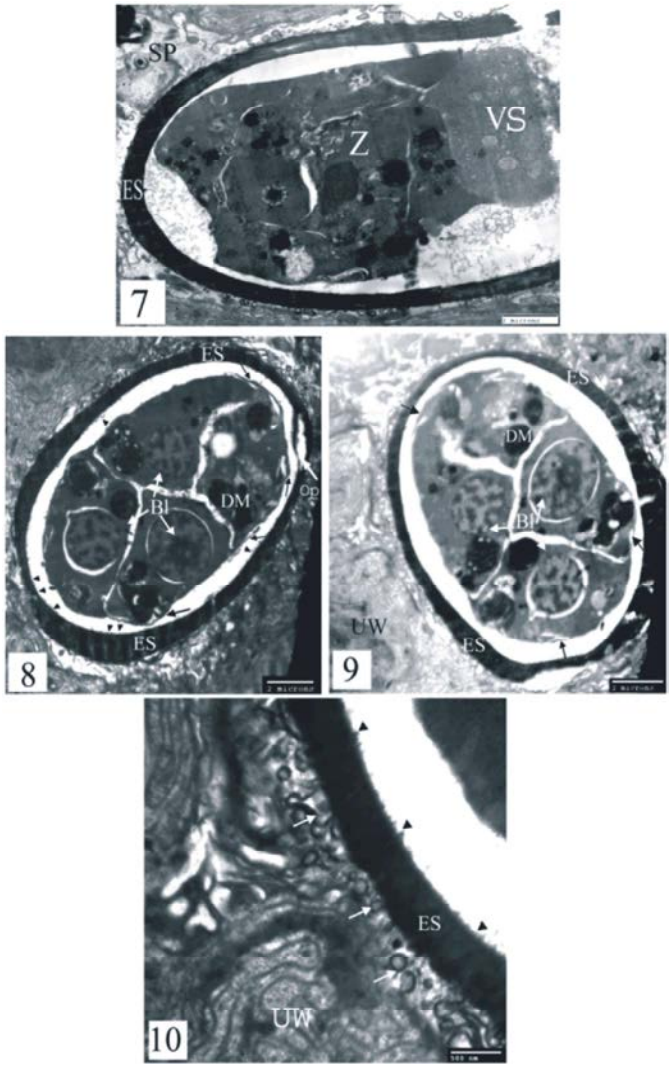

Fig. 7: Proximal part of the uterus of *O. batrachoides* with newly formed embryo composed of vitelline syncytium(VS) and zygote (Z) undergoing mitotic divisions and surrounded with egg shell (ES). Note axoneme of sperm(SP) around the egg shell. *Scale bar =2 µ*

Fig. 8: Distal part of the uterus of *O. batrachoides* showing embryonated egg containing an early embryo composed of several blastomeres (Bl) of different sizes and surrounded with egg shell(ES). Note the processes developed from blastomeres (Arrows) and the internally located tinny dark depositions on the eggshell (Arrowheads) in addition to degenerated micromeres (DM). Note also the appearance of the operculum (OP). *Scale bar =2 µ*

Fig. 9: Distal part of the uterus of *O. batrachoides* showing embryonated egg containing an early embryo composed of several blastomeres (Bl) of different sizes surrounded with egg shell (ES). Note the processes developed from blastomeres (Arrows), degenerated micromeres (DM) and vesicles of different sizes from the uterine wall(UW).*Scale bar = 2µ*

Fig. 10: A high magnification of the egg shell (ES) showing internally located tinny dark depositions on the eggshell (Arrowheads) and surrounded with several vesicles (Arrows) of different sizes from the uterine wall (UW). *Scale bar =500nm*

Unembryonated egg was composed of a fertilized oocyte (Ovum) and few vitelline cells. The fertilized oocyte was formed of a large nucleus containing an electron-dense nucleolus, surrounded by a thick layer of granular cytoplasm rich in free ribosomes, numerous mitochondria and cisternae of granular endoplasmic

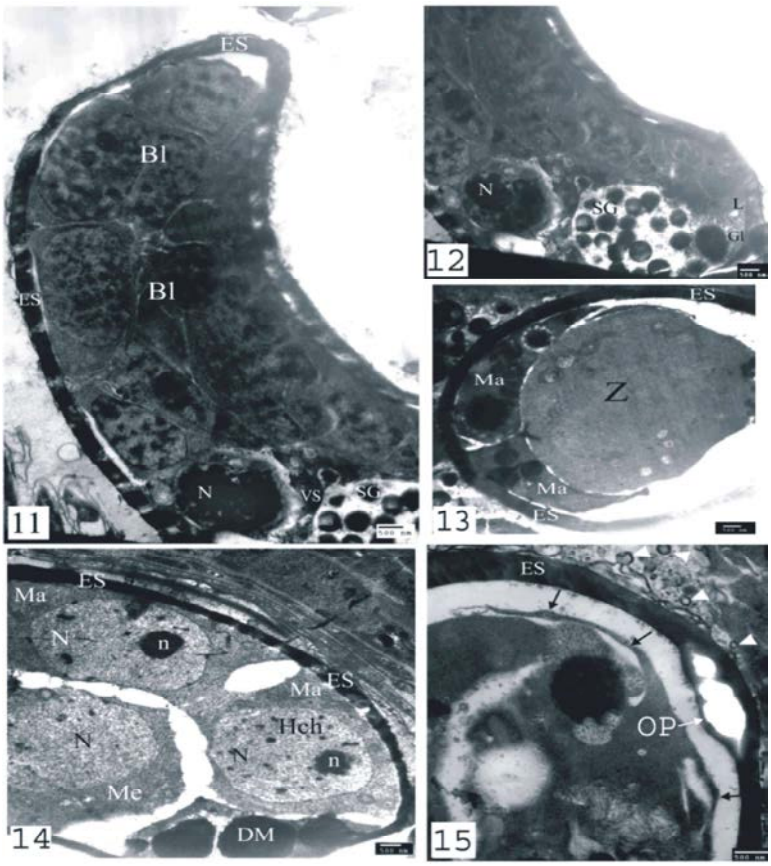

Fig. 11: Distal part of the uterus of *O. batrachoides* showing embryonated egg with several blastomeres(Bl) surrounded with egg shell (ES).Note degenerated vitelline nucleus (N) and shell globules (SG) of vitelline syncytium (VS). *Scale bar = 500nm*

Fig. 12: Distal part of the uterus of *O. batrachoides* showing the remnants of vitelline syncytium represented by glycogen (Gl), lipids (L), shell globules (SG) and degenerated vitelline nucleus (N). *Scale bar = 500nm*

Fig. 13: Embryonated egg showing zygote (Z) with mitotic division surrounded with egg shell (ES). Note two large macromeres (Ma) taking a crescent-shape form with nucleus positioned at one pole of the egg. *Scale bar =500nm*

Fig. 14: Embryonated egg with two macromeres(Ma) containing large (N) prominent nucleolus (n) and heterochromatin (Hch). Note the appearance of mesomeres (Me) and degenerated micromeres (DM) surrounded with egg shell. (ES)*Scale bar =500nm*

Fig. 15: High magnification of advanced stage of the developed embryo showing the egg shell (ES) with the operculum (OP). Note the processes developed from blastomeres (Arrows) and several vesicles of different sizes from the uterine wall (Arrowheads). *Scale bar =500nm*

reticulum (GER) (Fig. 3). The cytoplasm of the vitellocytes usually contained spherical balls of concentrically arranged cisternae of GER bodies and surrounded by heavy accumulations of α-glycogen rosettes and β-glycogen particles (Figs. 4-6). The vitelline cells were fused forming the vitelline syncytium situated directly beneath the newly-formed egg shell (Fig.7). At an early stage of embryonic development,the vitelline syncytium disappeared and was no longer visible in eggs containing several blastomeres (Fig. 11). Remnants of the vitelline material were represented mainly by glycogen, lipid droplets and a great number of shell globules of different size and electron-density, in addition to degenerating vitellocytes nuclei situated beneath the embryonic envelope and clearly separated from it (Figs. 11, 12).

The newly-formed electron dense egg shell of *O. batrachoides* was very thick which varied slightly in thickness in different regions (Figs. 2, 7-9) and consisted

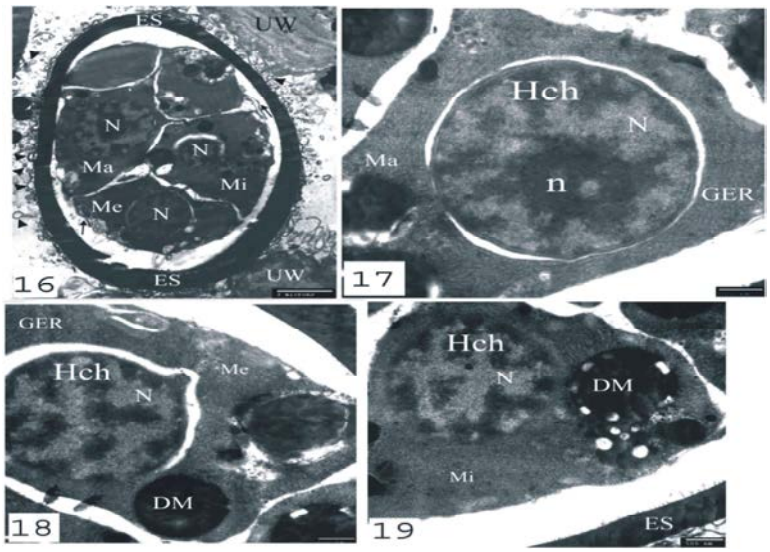

Fig. 16: Embryonated egg showing the macromere (Ma), mesomere (Me) and micromere (Mi) surrounded with egg shell (ES). Noet nucleus (N) and the processes developed from blastomeres (Arrows) and several vesicles (Arrowheads) of different sizes from the uterine wall (UW). *Scale bar =2 µ*

Fig. 17: High magnification of a macromere (Ma) showing large nucleus (N) with nucleolus (n) and heterochromatin (Hch) surrounded with granular endoplasmic reticulum(GER). *Scale bar =500nm*

Fig. 18: High magnification of a mesomere (Me) showing large nucleus (N) surroundedw ithheterochroma tin (Hch). Note the presence of a degenerated micromere (DM). *Scale bar =500nm*

Fig. 19: High magnification of a micromere (Mi) showing small nucleus (N)with heterochromatin (Hch). Note also the presence of a degenerated micromere (DM). *Scale bar =500nm*

of a tanned protein. With egg development, the egg shell progressively thickened with the appearance of the operculum after shell formation at the narrower pole of the egg shell surface (Figs. 8, 15), whereas the opposite pole is covered by a continuous thick and solid layer of electron-dense shell. It was to be noted that several vesicles of different sizes from the uterine wall were oriented externally along the egg in close association with the egg shell surface, in addition to internally located tinny dark depositions on the egg shell; such structures persisted till the configuration of developed embryos (Figs. 8-10, 15, 16).

Eggs underwent cleavage divisions which terminated by the formation of the early embryo composed of several blastomeres of different sizes (Figs. 11, 16). The increasing numbers of blastomeres observed in the advanced stages of embryonic development provided additional evidence for the presence of cleavage divisions. Three types of blastomeres were present: macromeres, mesomeres and micromeres (Figs. 8, 9, 17-19) their localization within the embryo and the ultrastructural characteristics of their nucleus and cytoplasm progressively changed during their differentiation.

The first cleavage division of the zygote produced two large macromeres (Figs. 13, 14). Their nuclei remained for some time positioned at one pole of the egg. They were localized just beneath the egg shell, flattened taking a crescent-shape form, usually near the surface of the early embryo. The macromeres moved progressively to the central part of the embryo, they exhibited a large spherical nucleus containing a prominent, electron dense nucleolus, with islands of heterochromatin which were more or less randomly dispersed in the nucleoplasm and surrounded by GER bodies (Figs. 16, 17).

The mesomeres appeared to be large cells in terms of the volume of their nucleus and cytoplasm (Figs. 14, 16, 18). Their large nucleus contained numerous, irregular, dense islands of heterochromatin. During advanced stages of the embryonic development of *O. batrachoides*, the size of the embryo, which was composed of a growing number of blastomeres, increased rapidly. Evident cytoplasmic processes of blastomeres were present (Figs. 8, 9, 15) which fused forming a common syncytial layer of embryonic envelope situated beneath the egg shell.

The following cleavage divisions frequently took place simultaneously and were accompanied by the early degeneration or apoptosis of some micromeres (Figs. 8, 9,14). Their thin layer of cytoplasm surrounded the spherical nucleus which was of a pycnotic type with highly condensed karyoplasm containing large heterochromatin islands at its periphery (Fig. 19) in addition to large electron-dense regions of focal cytoplasmic degradation.

DISCUSSION

The ultrastructural aspects of the intrauterine eggs in *O. batrachoides* were similar to a great extent to those described previously for several trematode and even cestode species.

The oligolecithal type of *O. batrachoides* eggs resembled those of the digeneans *Maritrema feliui* and *Brandesia turgid* [1, 13] and species belonging to some cestode orders [14]. This type of eggs contain a much smaller number of vitellocytes per fertilized ovum in comparison with the polylecithal eggs of *Aspidogaster limacoides* [2] and species of other cestode orders [6, 15, 16]. The chemical nature of the nutritive reserves accumulated in the vitellocytes do not differ in either the oligolecithal or polylecithal type; such reserves were represented exclusively of cytoplasmic and nuclear glycogen seen as α-glycogen rosettes and β-glycogen particles and/or numerous lipid droplets and shell globules [1,6, 17, 18]. In *O. batrachoides*, as described previously in other digeneans [18] mature vitelline cells were very rich in glycogen as α-glycogen rosettes and β-glycogen particles, lipid droplets which represented nutritive reserves for the developing embryo and shell globule clustered playing an important role in egg shell formation.

In *B. turgida* eggs, both nutritive and protective functions of the vitelline cells and embryonic envelopes were taken over by the uterus;this observation let Świderski *et al*. [13] to conclude that the two functions of vitellocytes may be much intensified or reduced to some or to a great extent in trematodes and cestodes with entirely intrauterine, partially intrauterine or entirely free embryonic development taking place in the external aquatic environment during their life cycles.

The close association of a fertilized ovum with vitelline cells in *O. batrachoides* and the initiation of egg shell formation, which took place in the proximal region of the ootype, were in agreement with those reported previously in *A. Limacoides* [2] digeneans and lower cestodes [10, 15] but in contrast to the cestodes *Diplocotyle olrikii* and *Didymobothrium rudolphii* where egg shell formation takes place in the proximal region of the uterus [4, 6].

The GER bodies frequently observed in egg-enclosed vitellocytes of *O. batrachoides* greatly resembled those described in *A. limacoides* [2] and the caryophyllidean *Wenyonia virilis* [19] where there are two functions for these bodies: they may be involved in the synthesis of glycoproteins and/or in the formation of foci of cytoplasmic degradation.

Degeneration of vitellocytes in *O. batrachoides* started at the early embryo stage appearing in the form of their fusion into a common vitelline syncytium; this was situated beneath the embryonic envelope, surrounding the developing embryo, but clearly separated from the syncytial cytoplasm of the embryonic envelope itself. Further, they underwent autolysis, during which their cell organelles and inclusions become reabsorbed by the embryo. Such observations were previously recorded in *A. limacoides* [2, 3].

The newly-formed eggshell of *O. batrachoides* consists of tanned protein, as in most trematodes[2]. In trematodes, eggshell formation results from the combined action of Mehlis' gland secretion and shell globules from the vitellocytes as proved by Świderski *et al*. [1]. It has been assumed that Mehlis' gland secretion is responsible for the release of shell globules from the vitellocytes; in addition, it forms a primary membrane which later becomes reinforced by the coalescence of vitelline cell globules during the passage of newly formed eggs through the ootype [7, 20, 21]. The secretion of Mehlis' gland also serves to lubricate both the ootype and the luminal surface of the uterine wall and takes part in shell tanning and/or hardening as the eggs pass through the uterus [2].

In *O. batrachoides*, several vesicles of different sizes from the uterine wall were oriented externally along the egg in close association with the eggshell surface. In the cestode *D. olrikii*, numerous vesicles liberated from the ootype wall are accumulated outside of the nascent egg shell in addition to vesicular material on the inside of the newly formed shell [4]. According toWells and Cordingley [22] studying the mechanism of new egg shell formation in *S. mansoni*, the egg shell precursor enzymes were synthesized by the vitelline cells and stored in the vesicles as well as in the membrane-bound shell globule clusters. They added that the contents of these vesicles trigger the tanning process. Poddubnaya *et al*. [4] added that similar ootype vesicles are active at the initial stages of egg shell formation in *D. olrikii* by providing the

enzymes required for deposition and subsequent coalescence of the shell globules. In *O. batrachoides*, internally located tinny dark depositions on the eggshell were also recorded; such structures werenot shown previously and they may be secretory adding also in the tanning process.

In *O. batrachoides*, the operculum differentiates in the later stages of egg shell formation from a continuous layer of eggshell material, similar to the results reported previously in *A. limacoides*s [2].

In *D. rudolphii*,Świderski*et al.*[6] recorded the progressive degeneration and autolysis of the vitellocytes after the completion of their important functions of eggshell formation and the nutrition of the embryo. During this process,the autolysis of degenerating micromeres and the released nutritive reserves in the form of cellular debris and chemical components become reabsorbed by the embryo; these materials represent a source of nutritive resources for the developing embryo [1]. Such degeneration or apoptosis, of both vitellocytes and some of the micromeres, also takes place during both early and more advanced stages of embryonic development and blastomere differentiation.

In *O. batrachoides*, evident cytoplasmic processes developed from the blastomeres; these processes fused forming a common syncytial layer of embryonic envelope situated beneath the egg shell. Similar processes were recorded only from the macromeres in the microphallid trematode *M. feliui* [1] and after their fusion they form a common syncytial layer of the outer envelope situated just beneath the egg shell. In *A. limacoides* [2] as in all other aspidogastreans examined [23] only a characteristic single-layered embryonic envelopein a close contact with the egg shell has been reported and described. On the other hand, two embryonic envelopes were reported for the majority of digeneans and eucestodes examined previously [10, 16, 24]. Moreover, these embryonic envelopes undergo further differentiation into secondary envelopes (Egg-envelopes), which surround the fully developed larva of both groups [10, 15, 16].

Degeneration of some micromeres started at the early embryo stage in *O. batrachoides*. In the eggs of some trematodes and cestodes [8-10, 15, 19], the first sign of micromeres degeneration and transformation is the appearance of regions of focal cytoplasmic degradation or different types of lysosomes.As previously reported by Świderski *et al*. [1] the degeneration of numerous micromeres results in a reduction in the number of embryonic cells. Such process is a common feature for both lower and higher cestodes and in trematodes [8-10]. The pycnotic nuclei and dense bodies recorded in the present study may represent the remnants of the autolysed nuclei and cytoplasm of micromeres undergoing apoptosis, as also described previously in *M. feliui* [1].

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