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Study of Embryonic Shell Formation in *Anodonta cygnea:* A New Revised Functional Approach

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Abstract: Morphological studies based on specific cytochemical staining were carried out in young embryos and larvae of the freshwater bivalve *Anodonta cygnea* and analyzed by light microscopy. X-ray analyses were also accomplished and compared with previous x-ray diffraction results in order to understand the early shell formation. Supported by the present morphological cytochemical results we can speculate a new functional hypothesis for the early shell formation, during the first stages of the embryo development in *Anodonta cygnea.* Thus, instead of the classic theory concerning the "shell gland" (on its secretion restrict meaning), the embryos' body present some specific dorsal ectodermal cells, around the invagination pore, which differentiates and migrates to the shell formation region followed by cellular expansion. Further cell disintegration will occur in this region, releasing protein-like β-keratin and chitin material (secreted together) and forming later the organic cuticle, under a similar mechanism usually described for the nails structure. Simultaneously, a calcareous layer is developed on the inner face of the cuticle by deposition of crystalline calcium carbonate on its biomineralizing organic matrix, mainly under the aragonite form. Eventually the early shell calcium supply will be processed across the vitelline membrane, moving from the external rich calcium compartment of the mother gills on a gradient dependence. This larval shell structure and elementary properties will be maintained until juvenile shell starts to be constructed by the mantle epithelia outside the mother gills.

Keywords: *Anodonta cygnea*, Embryonic shell, Embryos, Gastrula stage, Light microscopy

Introduction

The embryonic shell formation on the bivalve molluscs has been well described by several authors through a classic hypothesis which began with Leydig (1850) more than 160 years ago, and was gradual and slightly developed by others with different histological interpretations (Lillie, 1895; Roule, 1894; Dawydoff, 1928; Franc, 1960; Grassé *et al*., 1970; Wood, 1974; Kniprath, 1981; Eyster and Morse, 1984). According to these authors there is an initial heteromorphic cellular division which originates a morula and then a blastula with differentiated blastomeres. Lankester (1873) and Ganin

(1873) proposed that an invagination in the dorsal face of blastula epithelium appears early during the embryonic development period, which is denominated “shell gland”, but distinct from other invaginations which in turn will form the endodermis. This concept of a specific and effective shell gland has also been accepted in other works without large functional differences (Franc, 1960; Grassé *et al*., 1970; Wood, 1974; Calloway and Tumer, 1978), in general to explain that the shell is strictly a product of a glandular segregation of all shell components (organic and mineral fractions). Even more, they hypothesized that, while highly prismatic type cells of the shell gland secrete the organic material (organic cuticula), flat type cells secrete the calcium (calcareous layer) (Arey, 1924; Giusti *et al*., 1975; Kniprath, 1981). According to them, both fractions are segregated unpaired at the beginning and gradually recover the embryonic larvae, only becoming divided into two distinct valves afterwards. In a review concerning this subject, Kniprath (1981) introduced a new concept based on different histological observations to explain the larval shell formation but functionally with a similar approach. So, instead of a “shell gland” for the invagination, she denominated it as a “shell field” (SF) to the external region of invagination. Later, Eyster and Morse (1984) corroborate this concept but adding the idea of a “shell field invagination” (SFI), still with probable and partial participation on the shell material secretion.

The main purpose of this study is to reexamine the classic and the more contemporaneous hypothesis for the embryonic shell formation in freshwater bivalve of *Anodonta cygnea* usually collected from the Mira Lagoon (Aveiro). Thus, supported on morphological and cytochemical observations by light microscopy and also a technical revision of published data by X-Ray analyses (Castilho *et al*., 1989), we propose to analyze and clarify the effective existence and mechanism of a specific shell gland, on its own restricted sense, or eventually the existence of an alternative process during the early phases of larval shell development.

Materials and Methods

Adult gravid animals of *Anodonta cygnea* were collected during the early autumn, with some water and sediments, from Mira Lagoon in North of Portugal, and kept in aerated and decholorinated water at room temperature for a few days.

For morphological studies, non-stained eggs and larvae (glochidium) were observed *in vivo.* Very young embryos (blastula/gastrula stages) were removed from the mother gills and immediately fixed in ethanolic Bouin’s solution (Panreac, Barcelona, Spain) and stored in 70% ethanol at room temperature for no more than 2 months. The sections were dehydrated in an ascending series of ethanol solutions, cleared in xylene (Merck, Darmstadt, Germany) and embedded in paraffin (Merck). The paraffin blocks were sectioned (5 μm thick) using a Leitz 1512 microtome, and the sections were mounted on glass slides. After deparaffinization in xylene and rehydration in a descending series of ethanol solutions, the sections were stained with haematoxylin (Merck) and eosin (Sheehan and Hrapchak, 1980). From these serial sections, cytochemical studies were also made by periodic-acid Schiff (PAS), glycogen enzymatic hydrolysis (salivary amylase) and saturated Congo Red solution. For the specification of

acid mucopolysaccharides (MPS) with sulphate groups, alcian blue stain (pH 2.6) was used (Wagner and Shapiro, 1957) as well as a staining procedure combining alcian blue, salivary amylase and PAS (Vialli, 1953). Characterization of the specific nature of the sulphate groups was made using the paraldehyde fuchsin (PAF) stain after pre-oxidation with perfomic acid (Toennies and Homiller, 1942). Cupric sulphate-dithiooxamide reaction (McGee-Russell, 1955) was performed to detect Ca^{++} deposits.

Lastly, the sections were mounted under a coverslip with DPX (BDH Laboratory Supplies, Poole, England). Observations were made with a Leitz Aristoplan compound microscope. Photographs of representative sections were taken with a digital camera (Olympus model C5050Z, Germany), and the images were assembled and labelled using Photoshop 5.0 (Adobe Systems, San Jose, CA, USA).

The calcium level in the embryos at different stages was determined qualitatively by energy dispersive microanalysis (EDS) with JEOL JSM-35C scanning electron microscope operated at 25 kv. The embryos were air dried and mounted on SEM specimen stubs with conductive silver paint and coated with gold.

In order to confirm published results, the same method used by Castilho *et al.* (1989) for the X-ray diffraction analysis was again carried out in the larval shells of *A. cygnea*, being previously cleaned by bacterial decomposition and using Debye-Sherrer camera of 11.4 cm diameter and a Philips power supply PW 1130/00 operated at 40 kv and 20 mA (RadiationCuKα).

Results

Morphological observations in embryonic stages

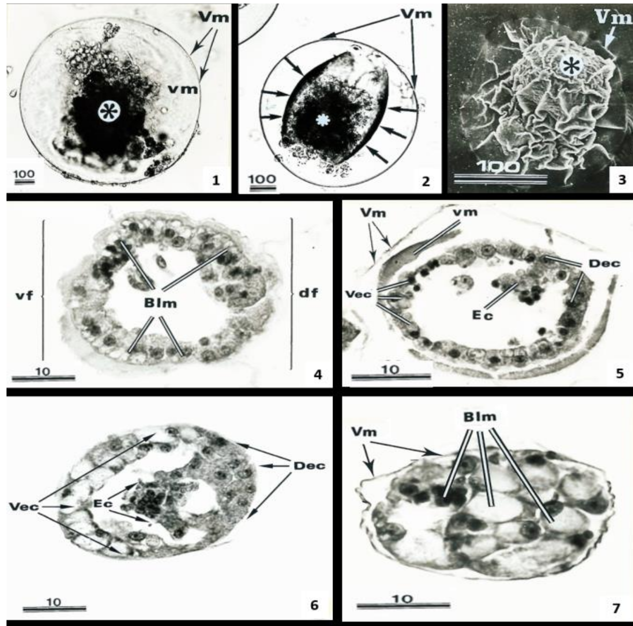
Egg extemporary preparations were observed *in vivo* (Figs. 1, 2) and after dehydration (Fig. 3). Eggs extracted from the mother gills of *A. cygnea* were observed *in vivo* and measured around 0.8 mm in diameter. The external aspect of the dehydrated eggs at Scanning electron microscopy (SEM) (Fig. 3) showed clearly the existence of a distinct, consistent and flexible vitelline membrane protecting the egg. The early embryo presents a vitelline membrane, a vitelline mass and a cellular arrangement on the morula phase, resulting from the first segmentation stages. Histological sections were prepared from egg serial cuts, using specific cytochemical techniques. The first stage is characterized by a cellular dense arrangement resulting from first segmentations corresponding to the morula, followed by the initial blastula stages, slightly oval and with its narrower region pointed to the dorsal face (Figs. 1, 4 and Diag. 1a).

During the second stage of embryo (middle blastula) the blastomeres present in general the same size and shape, constituted by prismatic or columnar cells, where the cytoplasm is acidophilic while the central nucleus is basophilic. The vitelline mass is basophilic as well (Fig. 4; Diag 1b).

In the end of blastula, third embryo stage, the ventral region blastomeres change their morphology becoming gradually flat, with their eosinophilic area more reduced and the nucleus becomes peripheral (Fig. 8; Diag. 1c). The blastomeres of the dorsal region become slightly round in shape, keeping their acidophilic characteristics. At this stage a

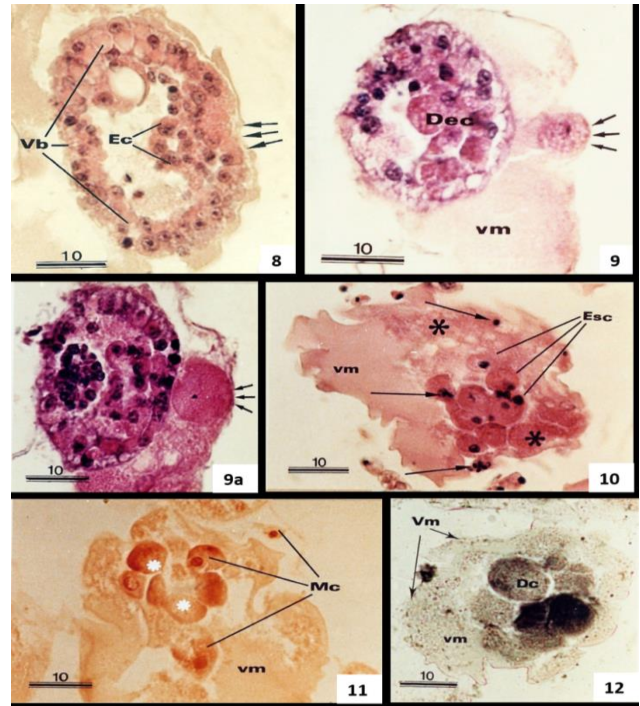
simultaneous dorsal invagination of smaller cells begins. These cells are still acidophilic (Fig. 5, Diag. 1d).

During the fourth embryo stage, the ectodermal cells of the ventral region present



Figs. 1-7: Morphological and hystochemical observations of very young *A. cygnea* embryos at different stages and with different cytochemical staining. **Figure 1** – Light microscopy (LM) observation of the egg *in vivo*. Vitelline membrane (Vm); cellular body (*); vitelline mass (vm). **Figure 2** – LM of the egg *in vivo*. Calcified valves (arrows); cellular body (*); Vitelline membrane (Vm). **Figure 3** – Scanning Electron Microscopy (SEM) observation of a dehydrated egg. Vitelline membrane (Vm); cellular body (*). **Figure 4** – LM of an egg section at the blastula stage, stained with hemalum-eosin, showing already the dorsal and ventral regions where the cells are mainly round and prismatic, respectively. Blastomeres (Blm); ventral face (vf); dorsal face (df). **Figure 5** – Beginning of the gastrula stained with hemalum-eosin observed by LM. Dorsal ectodermal cells (Dec); ventral ectodermal cells (Vec); vitelline mass (vm); vitelline membrane (Vm); endodermal cells (Ec) invagination. **Figure 6** – Gastrula observed by LM and stained with hemalum-eosin. Dorsal ectodermal cells with a round shape (Dec); ventral ectodermal cells with a flat shape (Vec); endodermal cells (Ec) invagination. **Figure 7** – A LM magnification of ventral region blastomeres stained with hemalum-eosin, showing enlarged blastomeres (Blm) with peripheral nucleus and an apparent empty cytoplasmic aspect due to little eosinophilic staining. Vitelline membrane (Vm).

an eccentric nucleus and a usually uncolored cytoplasm with small portions of basophilic nature. Near the invagination, dorsal ectodermal cells continue to be eosinophilic with a nearly central nucleus. They multiply



Figs. 8–12: Light microscopy (LM) observations of very young embryos of *A. cygnea* at different stages and with different cytochemical staining. **Figure 8**– Beginning of the gastrula stained with hemalum-eosin. Dorsal ectodermal cells with a round shape (Ec); ventral ectodermal region with few lateral cells transforming into a flat cells (Vb). **Figure 9 and 9a -** LM of cross section of a very young embryo (gastrula stage), on the dorsal region, stained with hemalumen-eosin. Dorsal ectodermal cells (Dec); Eosinophilic cell displaced by migration followed by expansion (arrows); Vitelline mass (vm). **Figure 10**– Cross section of a very young embryo (gastrula stage) stained with hemalumen-eosin showing ectodermal cells, migrated from dorsal region, in autolysis. Eosinophilic cells (Esc); Disintegration of nuclear structures (arrows); Vitelline mass (vm); Free eosinophilic cytoplasmic mass (*). **Figure 11** – Cross section of a very young embryo (gastrula stage) stained with Congo Red showing ectodermal cells, migrated from dorsal region, in autolysis. Migrated cells (Mc); Vitelline mass (vm); Free cytoplasmic mass (*). **Figure 12** – Cross section of a very young embryo (gastrula stage) stained with Cu sulphate dithiooxamide showing disintegrated cells. Vitelline mass (vm); Calcium deposit on disintegrated cells (Dc); Vitelline membrane (Vm).

and increase in volume becoming denser ectodermal cells (Figs. 6, 7; Diag. 1e). The invagination opening suffers a strong narrowing while the endodermal cells proliferate in the interior of the blastula (Fig. 6; Diag. 1e) in order to initiate the gastrula phase in the embryo.

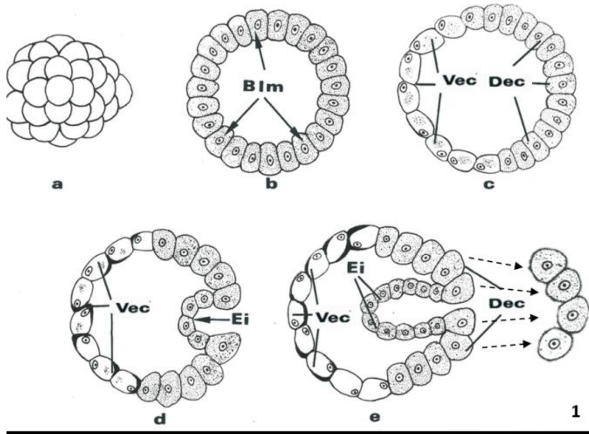
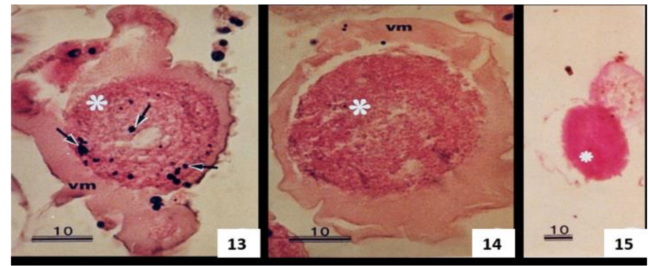


Diagram 1: Gastrula formation: a) Oval-shaped morula; b) Blastula with prismatic, eosinophilic blastomeres and central nucleus (Blm); c) Dorsal prismatic cells (Dec), flat ventral cells, little eosinophilic and peripheral nucleus (Vec); d) Endodermal invagination (Ei) in the dorsal face; e) Dorsal ectodermal cells which will move out and disintegrate giving rise to free eosinophilic cytoplasmic masses (Dec).

Hystochemical observations

Cuticle formation

The beginning of cuticle formation occurs at the embryo gastrula stage from the larger eosinophilic cells which gradually separate from the dorsal ectodermal follicle (Fig. 9; Diag. 1e, 2a) around the gastrula opening. These cells increase in volume and enter a gradual autolysis process which leads to the disappearance of the cellular membrane and contour, nucleus and nucleolus (Figs 9-12, Diag 2b, 2c). Simultaneously with the process of cellular degeneration, the fusion of eosinophilic cell cytoplasm takes place (Figs. 10-12; Diag 2b, 2c). This process leads to the formation of a large eosinophilic mass, dense and homogeneous (Figs. 13-15; Diag. 2c, 2d) with a nearly oval form and few nuclear residues.



Figs: 13-15 – Light microscopy (LM) observations of young embryos of *A. cygnea* at different stages and with different cytochemical staining. **Figure 13** - Longitudinal section of the free cytoplasmatic mass ("shell") stained with hemalumen-eosin. Vitelline mass (vm); Desintegration of nuclear structures (arrows); Free eosinophilic mass (*). **Figure 14** - Longitudinal section of the free cytoplasmatic mass ("shell"), without nuclear structures, stained with hemalumen-eosin. Vitelline mass (vm); Free eosinophilic cytoplasmic mass (*). **Figure 15 –** Longitudinal section of the cuticle, resulting from the great concentration of the free cytoplasmic mass (*), with a staining procedure combining alcian blue, salivary amylase and PAS Cuticle.

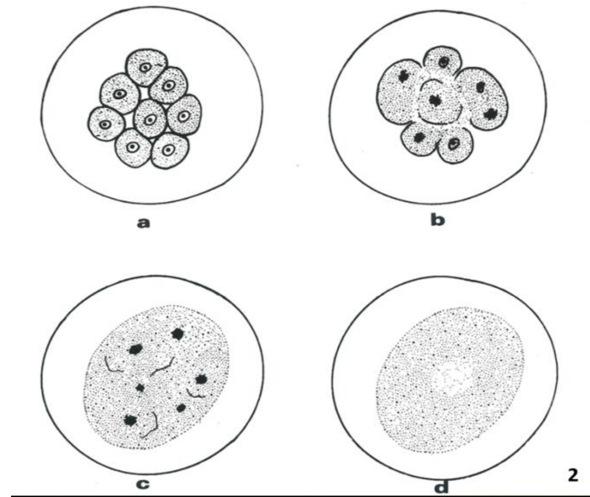


Diagram 2: Cuticle formation: a) Dorsal ectodermal cells that suffered migration; b) Cell structures autolysis; c) Cytoplasmic mass formation with nuclear structures; d) Cytoplasmic masses devoid of nuclear structures.

Ectodermal Cells

Based on the cytochemical techniques (Figs. 4-15) it was possible to create the Table 1 which presents the different results concerning several embryonic components of early

Table 1 – Different cytochemical results on the very young embryos (gastrula) of *A. cygnea*. *The positive reactions to several tests disappear during embryonic development. The "shell" word means free cytoplasmic mass (organic compounds of early shell). He-Eo - haematoxylin and eosin; PAS - Periodic-Acid Schiff; PAF - paraldeydefuchsin.

Material / Methods	Ventral Cells *	Dorsal Cells	Vitelline Mass	Desintegrated Cells	"Shell Matrix"
He-Eo	+ (Eo)	++ (Eo)	+ - (He)	++ (Eo)	++ (Eo)
PAS	+	++	+	++	++
Amylase + PAS	+ (PAS)	++ (PAS)	+ (PAS)	++ (PAS)	++ (PAS)
Congo Red	-	+	-	+	+
Alcian Blue	-	-	+	-	-
Alcian Blue + PAS	+ (PAS)	++ (PAS)	+ (A. Blue)	++ (PAS)	++ (PAS)
Alcian Blue + Amylase + PAS	+ (PAS)	++ (PAS)	+ (A. Blue)	++ (PAS)	++ (PAS)
PAF (pre-ox.)	-	-	-	-	-
Cu Sulphate-Dithiooxamide	-	-	-	+	+

embryos (blastula) to later (gastrula) stages with reports of all cytochemical reactions obtained in the cytoplasm, nucleus and organic shell matrix. In summary, the Congo Red test was positive for eosinophilic cells and free cytoplasmic mass. PAS test was positive for basophilic vitelline and free cytoplasmic masses and strongly positive for eosinophilic cells. The Congo Red and PAS tests were negative for ventral ectodermal cells. In embryonic larvae from eggs, positive reactions in the valves and in the cuticles, isolated by treatment with KOH 0.5 M, were obtained with the Congo Red test and the Schulze's reaction, respectively.

Early shell calcification

Small quantities of calcium were detected inside the embryos through EDS microanalysis during the different embryonic stages. The values obtained, expressed in impulses (I/50 sec), were proportional to the different developmental stages, being on average: 120 I/50 sec., 262 I/50 sec., 320 I/50., and 12740 I/50 sec. The last one was obtained from a middle embryonic stage of the shell development scale (Figs. 13, 14). The x-ray diffraction analysis of larval shells

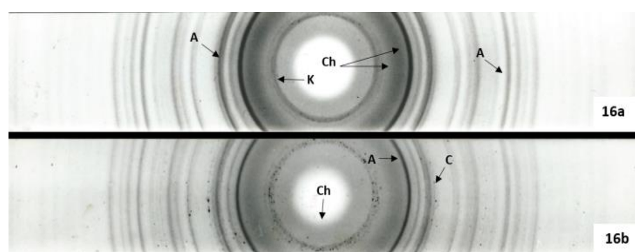


Fig. 16: (a) X-ray diffraction analysis of a larval shell with sample rotation (b) X-ray diffraction analysis without sample rotation. Aragonite (A) (1.2Å, 1.7 Å, 1.8 Å, 1.9 Å, 2.1 Å, 2.3 Å, 2.7 Å, 3.4 Å, 4.23 Å); Calcite (C) (3.0 Å), Chitin band (Ch) (4.4 Å -5.4 and 10.5 Å); Protein-like β-keratin (K) (6.6 Å). All observations are in agreement with the data of Castilho *et al.* (1989).

(glochidium), as already reported by Castilho *et al.* (1989), showed the presence of some reflections with reticular distances: 1.2Å; 1.7 Å; 1.8 Å; 1.9 Å; 2.1 Å; 2.3 Å; 2.7 Å; 3.0 Å; 3.4 Å; 4.2 Å; 4.4-5.4 Å and 6.7Å (Figs. 16a, 16b, similar to those from Castilho *et al.*, 1989). According to these authors, these reflections indicate the combined presence of chitin and aragonite under crystalline organization and protein-like β-keratin in the larval shell cuticle.

Discussion

The first studies on the ontogeny of shell formation were accomplished by Leydig (1850) suggesting that the earliest embryonic shell is a simple gland secretion occurred at the beginning of the ectoderm cell division for the gastrula formation. Since then, the most usual term to designate the secretory organ of the molluscan shell has been "shell gland" (or glande coquillière) and according to Lankester (1873) and Ganin (1873), it results from the invagination of the dorsal epithelium during the embryo development. Other authors followed using the shell gland term within the restrict sense, as a secretory structure correlated with the first invaginated epithelium (Roule, 1894; Lillie, 1895; Dawydoff, 1928; Franc, 1960; Wood, 1974). However, Blochmann (1883), Schmidt (1895) and later Kniprath (1981) understood that the entire epithelium concerned with shell secretion, before and after evagination are involved on the embryo shell formation. Based on this observation, they suggest that the term shell gland restricted to the invaginated stage is not adequate to the shell development. Hence, they adopted other hypothesis, in a broader sense, suggesting that cells related to

the invagination and evagination, processes should be directly involved in the early shell and thus it should be called "shell field epithelium" (SFI). Moreover, the formation of the larval shell is initiated by a specialized group of ectodermal cells, outmost cells of the SFI (Weiss *et al*., 2002; Schönitzer and Weiss, 2007) which secretes the organic matrix (periostracum).

Although, the shell secretory epithelium is not yet well understood as a classic restrict gland, according to Eyster and Morse (1984), it is still ambiguous due to clear lack of embryogenic data. However, regarding the general literature, there is one detail in common to a large species of mollusks that is the correlation of the beginning of larval shell with the invagination process in the dorsal ectodermic region, but still with relevant questions related to shell field region. So, the main questions still remain: (i) Does the first shell organic matrix come from the invagination inner cells (like reported by Eyster and Morse (1984) in *Spissula solidissima*) ?; (ii) in this species, what is the function for the invagination outer cells?; (iii) what is the specific function for cells of invagination and evagination in other species?; (iv) Is the secretory mechanism originated exclusively from the internal cells of invagination pore or from the external cell of the pore, as indicated by Eyster (1983) for *Aeolidia papillosa*?; and (v) Is the organic material secreted from highly prismatic cells in the shell field while the calcium is secreted from flat cells according to Kniprath (1981)?

To clarify these doubts, it is necessary to go on with deep morphological, ultrastructural and cytochemical observations in order to explain in detail the embryology of the larval shell in bivalves. Enzymatic studies are an additional and relevant way to give specific information to understand the cells activity on the shell field and thus the origin of specific materials that make the shell matrix and calcium carbonate minerals.

Accordingly, in the analyses of the temporal and spatial activity of the alkaline phosphatase (AP) enzymes in the shell tissues in *Biomphalaria glabrata* (Marxen *et al*., 2003) and in *Haliotis turbeculata* (Gaume *et al*., 2011), it was found that AP activities reach its maximum before early shell formation. These results confirm a role of AP in the initiation of mineralization but not in mineralization itself, as previously observed in vertebrates (Genge *et al*., 1988). These results suggested that AP could help in matrix protein maturation by acting like a phosphoprotein phosphatase (Lau *et al*., 1985; Marxen *et al*., 2003; Gaume *et al*., 2011). Furthermore, according to Marxen *et al.* (2003), the peroxidase activity was present from the start of the periostracum production in *Biomphalaria glabrata,* while acid phosphatase found in considerable amounts in the embryonic shell-forming tissue suggested that acid phosphatase (ACP) might participate in the alteration of the calcifying matrix, prior to mineral deposition. Relatively to the carbonic anhydrase activity, Gaume *et al*. (2011) indicated its presence from the early stages, occurring in a succession of activation and inhibition events throughout the larval development of *Haliotis tuberculata,* suggesting a clear physiologic control of embryonic shell calcification.

Additional and complementary studies based on a genetic marker method to control the temporal and spatial variations in the

calcifying activity, over mollusk early shell development, are also a powerful and appropriate technique. Supported on this method, Gaume *et al*. (2011) detected a strong increase in the level of calcitonin gene-related molecules in post-larvae, suggesting that endocrine control takes place just after metamorphosis in *Haliotis tuberculata*.

Contrarily to what happens in most of Lamellibranchia (Dawydoff, 1928) the egg of *Anodonta cygnea* is surrounded by a spherical envelope, the morphology of which is well individualized as a vitelline membrane. As it has already been described by several researchers (Roule, 1894; Lillie, 1895; Dawydoff, 1928; Franc, 1960; Grassé *et al*., 1970; Wood, 1974), the shell formation of the glochidium larva is wholly accomplished inside the egg. More recently, this was emphasized by the ultrastructural study of embryonic shell of *A. cygnea* by Castilho *et al.* (1989) and also with present morphological study. One fundamental aspect that should be well explored concerns the physiological role of this vitelline membrane, mainly the ionic calcium transport or diffusion since the eggs are allocated in a very rich calcium compartment of mother gills. So, it seems obvious to speculate that this calcium would moves towards the inside of the eggs according the internal-external electrochemical gradient and subsequent calcium absorption/deposition mechanisms by the early shell matrix. On the other hand, based on the present morphological and cytochemical observations related to the cells from the blastula and gastrula phases and "shell matrix" or "primitive shell" structure, our study suggests a very different hypothesis from the usual "shell gland" or even the segregation from the "shell field" cells. Thus, it concerns on a very specific histofunctional development and physiological behavior mainly from dorsal ectodermal cells which are located in the "shell field" around the invagination pore. In fact, we observed that the cells on this region, proliferate and migrate from the ectodermal region across vitelline mass towards the external part of embryonic body and gradually disintegrate. Then, the resulting free cytoplasmatic mass fuses and occurs a simultaneous and gradual calcification and subsequent shell formation. To explain these alternative phenomena we can support on the known mechanism about the autolysis role of acid phosphatase present in the ectodermal cells during the organic matrix preparation of the hard tissue (embryonic shell cuticle) development which Marxen *et al*. (2003) already indicated. It is known that acid phosphatases are lysosomal enzymes, which mainly participate in the degradation/dephosphorylation of proteins (Blair *et al*., 1986; Hermann, 1987). Accordingly, embryos of *Biomphalaria glabrata* show strong ACP activity, associated with the degradation of yolk particles. High activity of ACP in the cytosol of single cells in the embryonic shell forming tissue is indicative of the autolysis process (Heryanto *et al*., 1997*).* The death of isolated cells has been reported as an important process during normal early development (Hirata and Hall, 2000). Curiously, this cell autolysis phenomenon is a similar process to that described for keratinization (Bragulla and Homberger, 2009). So, both processes differ greatly from any glandular process or other studies based on the secretion from the ectodermal cell in shell field. Keratins and

keratin filaments are involved in other functions than mechanical, such as cell signaling, transport, compartmentalization, polarity, shape and cell differentiation, structural integrity and mechanical resilience (Coulombe and Omary, 2002; Gu and Coulombe, 2007; Oshima, 2007; Vaidya and Kanojia, 2007; Magin *et al.*, 2007; Bragulla and Homberger, 2009).

So, in this context, a very particular function for keratin is defined as certain filament-forming proteins with specific physicochemical properties which are usually extracted from the cornified layer of the epidermis (Bragulla and Homberger, 2009). Then, the cells of the keratinizing and cornifying epidermis and of its derivatives, e.g. hair and nail, die in a programmed cell death and become the corneocytes in the superficial stratum corneum. According to the same authors (Bragulla and Homberger, 2009), the keratinocytes are pushed towards the surface of the epidermis by the newly produced cells in the basal layer and go through various stages of maturation (i.e. keratinization only or keratinization and cornification). So, the process of cornification involves the programmed death of the keratinocytes (Houben *et al.*, 2007). This epidermal programmed cell death is different from the programmed cell death in apoptosis because the extra- and intracellular signaling cascade, that is characteristic of apoptosis, is not activated (Lippens *et al.*, 2005; Denecker *et al.*, 2008). Apoptosis can take place at any stage of cell differentiation but the process of cornification can start only after a cell has already gone through a certain differentiation. Hence, cornification is not a type of apoptosis (Denecker *et al.*, 2008) but a distinct process with specific mechanism and function. In summary, the hard-keratinization consist of the differentiation processes producing cytoskeleton of the hard-keratinized tissue which is designated by 'cornification' occurred in the epithelia of hairs, nails, horn and hoofs of mammals showing hard, nearly stiff properties of these structures (Bragulla and Homberger, 2009).

So, supported by the present cytochemical observations we can speculate a keratinization/cornification process, similar to that occurring in hair or nails, attending to the eosinophilic characteristics and the positivity to Congo Red shown by SF cells during the dorsal cell proliferation, migration and disintegration as well as in the free cytoplasmatic mass ("shell matrix"). These similar staining reactions suggest a very direct relationship among these components and also a coherent sequence for the larval shell development. The positive reaction with the Congo Red gave evidence for the presence of protein-like keratin in these components. According to Lillie (1954), the keratin reveals an acidophilic reaction which is in accordance with our results. However, according to Lehninger (1982), the negative reaction to the PAF with pre-oxidation suggests a weak existence of cysteine (mainly present in β-keratin structure) since the sulphate groups is absent. On the contrary, a possible presence of this organic compound β-keratin was also supported by X-ray diffraction analysis (Castilho *et al.*, 1989) and confirmed now by the present results, which shows a reflection with a typical reticular distance of β-keratin (6.6 Å) (Lehnninger, 1982). Curiously, the β-keratins are frequently referred as a specific product in hard-cornified tissues of sauropsid skin (reptiles and birds) and resemble with the keratin filament-associated proteins

(KFAPs) of mammals ('matrix proteins') in both molecular weight and sulfur content (Fraser *et al*., 1972). Hence, the so-called β-keratins of birds and reptiles resemble more closely to mammalian KFAPs and not α-keratins (Fraser *et al*., 1972). According to our combined data it is possible to speculate the presence of a modified KFAPs proteins which are adapted under fibrils structure (Castilho *et al*., 1989) in the early shell cuticle of *A. cygnea.*

A chitinization process possibly is also an usual phenomenon occurring from the dorsal cells in order to prepare the early shell in *A. cygnea* embryos. Recently, it has been demonstrated that chitin fulfills various structural tasks in the formation of larval shells of the bivalve mollusk *Mytilus galloprovincialis* (Weiss and Schonitzer, 2006) and *Anodonta cygnea* larval shell (Castilho *et al*., 1989). Weiss *et al.* (2006) and Schönitzer and Weiss (2007) showed that the shell forming tissue of larval *Mytilus galloprovincialis* and *Atrina rigida* has a homologous gene for chitin synthase which is expressed even in early embryonic stages and presenting a functional role of cytoskeletal forces in the precisely controlled mineral deposition process of mollusk shell biogenesis. According to them, the chitin can induce hierarchical patterns into chitin mineral-composites such as prismatic, nacre, and crossed-lamellar shell types. Similar to the report of Lowenstam (1981), the present data suggests that chitin formation during the larval development may involve the regulatory targets of "biologically controlled" mineralization. This leads to a novel understanding of the role of the epithelial cells based on a programmed monitoring of the dynamics between the secretion of shell precursor components and its interfaces with the developing epithelial cells.

In fact, the present data on the dorsal region of *A. cygnea* embryo showed disintegrating cells and free cytoplasmatic mass ("shell") with a strong reaction to the PAS, by combined staining with alcian blue, salivary amylase and PAS. These reactions together suggest a clear relationship among the same or similar compounds along different embryonic stages. In the early embryonic stages, the ventral cells have identical reactions which rapidly disappear during cell differentiation. The results indicate the presence of neutral mucopolysaccharides in all the above mentioned dorsal elements. These eosinophilic compounds seem to be a structural polysaccharide, called chitin. Such hypothesis is in agreement with positive response to Schulze's reaction observed in isolated cuticles (shell matrix). According to Lillie (1954), scleroproteins and glucides (saccharides) may combine themselves forming a block structure. Rudall (1963) adds that this association may happen through a co-valent combination. So, this seems to be in agreement with the existence of a shell cuticle in *A. cygnea* composed mainly by chitin and keratin fibrils. Moreover, the present revised results are according to the X-ray diffraction analysis of Castilho *et al.* (1989) providing evidence for the presence of chitin mucopolysaccharide and protein-like β-keratin. Thus, a typical reflection of chitin (4.7Å), (Rudall, 1963), is within the obtained band reflection of 4.4-5.4Å. According to Rudall (1963), this band is not well defined when an association of chitin with a protein-like β-keratin (6.6 Å band) occurs, which is also in agreement with Castilho *et al.* (1989).

Matoltsy (1962) pointed out that, in insect cuticles, chitin and proteins are usually secreted together by the same cells with a subsequent sclerotization (darkening and hardening). Thus, our data highlights that the embryonic shell cuticle (matrix) of *A. cygnea* is formed by secretion of chitin and β-keratin like in the same dorsal cells which migrate and disintegrate on the shell region.

As far as calcification process is concerned, our revised data is in accordance with the X-ray diffraction analysis of Castilho *et al*. (1989) showing that in the larval stages $CaCO_3$ deposition forms calcite crystals (3.0Å), probably vaterite (4.2Å), but mainly aragonite crystals (1.2 Å, 1.7 Å, 1.8 Å, 1.9 Å, 2.1 Å, 2.3 Å, 2.7 Å and 3.4 Å). On the other hand, the Cu sulphate dithiooxamide reaction shows clearly that the $CaCO_3$ deposition occurs during young embryonic stages, in the free cytoplasmatic mass. By microanalysis (EDS) of the embryos in several stages of development, a gradual increase in internal calcium levels was found to be proportional to valve size. This data is in accordance with Gaume *et al.* (2011) who indicated an increased calcium carbonate contents in the embryonic shell based on the alizarin staining, where the carbonic anhydrase is present to catalyze the reversible hydration of carbon dioxide and so the acceleration of bicarbonate ions formation for $CaCO_3$ precipitation (Gaume *et al*., 2011; Coimbra *et al*., 1988; Machado *et al*., 1990).

According to some authors (Crofts, 1937; Jablonski and Lutz, 1980; Jackson *et al*., 2007; Kniprath, 1981; Marin *et al*., 2007; Gaume *et al*., 2011), the synthesis of organic components and CO_3 for an embryo shell formation will begin early. In our opinion, these results suggest that the calcium moves from external reserves (gill), through vitelline membrane, into the inner of the fertilized egg and further $CaCO_3$ deposition occurs in the free cytoplasmatic mass where specific biomineralizing compounds are present to combine with it. Thus, it appears that the classic hypothesis which indicates organic matrix and calcium secretion directly from the "shell gland" seems increasingly less obvious while the present physiological approach, clearly contradictory, reveal a more functional and adequate hypothesis for the embryonic shell formation understanding. This discrepancy, in particular concerning the calcium kinetic movement during the embryos developmental stages should be further investigated with detailed ultrastructural and physiological studies for a clear explanation of the vitelline membrane behaviour concerning the ionic calcium movements.

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