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Effects of Organic Acids Injected at Different pH on Shell Mineralization of *Anodonta cygnea* (L.)

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Abstract: Freshwater mussels are among one of the most threatened faunal groups on Earth. Their vulnerability to disturbances comes from their sessile lifestyle which make them more prone to physiological stress from the changes in habitat. The maintenance of intra- and extracellular equilibrium/homeostasis under changing environmental conditions is vital for the distribution of these mussels, when imbalanced the shell calcification is compromised.

The purpose of this study was to verify the effects of several organic acids (lactic, citric and succinic acids) injected in the extrapallial fluid on the mineralization process of the shell of *Anodonta cygnea*. The impacts on the shell were evaluated by scanning electronic microscopy two weeks after injection.

Several morphological alterations were detected in the shell nacreous layer of the injected bivalve as compared to the control mussels. In general, the crystallization process was compromised in the presence of the solutions tested. The typical structure of the control nacre appears changed either in acid or basic solutions, in terms of tridimensional arrangement. Specific microstructures were observed- thickened borders of the crystals by citric acid at pH 5 and hexagonal prismatic structures with succinic acid at pH 7. The continuous exposure to these conditions may affect deeply the nacreous structure as well as the viability of these animals.

Keywords: Acidosis, Biomineralization, Shell ultrastructure, Freshwater Bivalve, Nacreous and Prismatic layer

Introduction

Freshwater bivalves are one of the most threatened animal group on the planet. Their vulnerability to disturbances in their habitat comes from their relative sessile lifestyle and changes in habitat will have physiological consequences. The water pH is a determining environmental factor in the distribution of these mussels, reductions in water pH can have a profound impact (Lopes-Lima *et al.,* 2016) since it can affect deeply the internal pH and subsequently the physiological homeostasis. Molluscs produce shells to protect and support their bodies. These skeletons are made of organomineral composites that exhibit a high degree of organization. The mineral portion is composed of calcium carbonate in the form of calcite and/or aragonite embedded on an organic matrix (Carter, 1990). According Osuna-Mascaró *et al.* (2015) the shell composition of molluscs is a mineral layer of calcium carbonate (aragonite, calcite or,

very rarely, vaterite) integrated in a small amount of organic matrix namely of proteins, polysaccharides and lipids. The proportion of organic matrix varies depending on different microstructures, being around 1% in the crossed lamellar and more than 7% in nacre, columnar prismatic (calcitic and aragonitic) and granular prismatic (>7%) (Hare and Abelson, 1964; Taylor and Layman, 1972; Checa, *et al.,* 2005; Addadi *et al.,* 2006; Harper *et al.,* 2009). Chitin is other specific organic compound present in freshwater bivalves (Osuna-Mascaró *et al.,* 2015), particularly in *Anodonta cygnea,* having a relevant framework role in the shell construction, giving cohesion and flexibility to the molluscs nacreous layer (Machado *et al.,* 1988a). The shell structural success is a consequence of several adaptations, accomplished by the combination of these biocomposites with the organic matrix which form resistant hybrid materials (Osuna-Mascaró *et al.,* 2015). The chitin layer consists of very thin fibers with a rearrangement, which appears lightly disorganized. Each fiber has a protein coat, which probably forms a complex with the chitin. The chitin-protein-complex is embedded in an additional proteinaceous matrix (Osuna-Mascaró *et al.,* 2015). On the other hand, the interface between this proteinaceous matrix and calcium carbonate can be represented by organic acid groups with high calcium affinity (Moura *et al.,* 2000; Lopes-Lima *et al.,* 2010).

The microstructure, morphology and orientation of single crystals can be defined and controlled by specific proteins of the organic matrix by cellular remodelling or by specific physicochemical condition that eventually occur on the microenvironment of shell calcification (Falini *et al.,* 1996; Belcher *et al.,* 1996; Mount *et al.,* 2004; Takeuchi *et al.,* 2008). However, these parameters and respective integrative functions are still poorly understood. Regarding the bivalves, there are some studies in marine bivalves that report the influence of environmental factors on mineralization of the shells, for example, in *Mytillus edulis* the increase in water temperature increases the proportion of aragonite/calcite in the shell (Lowenstam, 1954), while higher salinity lowers this ratio (Dodd, 1966; Eisma, 1966). In calcitic bivalves, magnesium-enriched waters are capable to cause precipitation of crystals in the form of aragonite (Checa *et al.,* 2007). The fundamental aspect to know is the active contribution levels of organic acids on shell mineralization of freshwater bivalves due to own intrinsic physical-chemical properties for combining with calcium, during precipitation mechanisms to construct calcareous layers (Lopes-Lima *et al.,* 2010).

The ultrastructure of Unionid shell is well known, being organized into three distinct layers: the outer periostracum, the intermediate aragonite prismatic layer and the inner nacreous layer. Nacre is secreted from epithelial cells of the mantle forming irregular polygonal aragonite tablets, which grow between organic sheets previously, deposited and appropriately spaced. During nacreous layer growing, there are around three consecutive layers of crystalline tablets, constructed in simultaneous (Checa, 2000; Jacob *et al.,* 2008; Lopes-Lima *et al.,* 2010).

Thus, the present study seeks to determine the contribution or the influence of different pH solutions without or with organic acids when injected in the extrapallial fluid on the habit/microstructure of the growing nacreous layer of the shell. The shell internal nacreous surface of the freshwater bivalve *Anodonta cygnea* will be analysed through scanning electron microscopy (SEM).

Materials and Methods

Adult animals of the species *Anodonta cygnea*, were collected from their natural environment in Mira Lagoon, North of Portugal and maintained in laboratory tanks with aerated and dechlorinated water for physiological stabilization. After 48 hours, 45 healthy (animals that closed their valves vigorously when disturbed) animals were selected for the study.

The bivalve sample was divided into 9 experimental groups in individual 10L tanks, with aerated and dechlorinated water and subsequently submitted to different organic acids injection. Feeding was based on microalgae diet suitable for long-term maintenance. To avoid that the animals closed the shells under these conditions, the different solutions were injected between the mantle and the shell, on the extrapallial space close to the shell. 3ml of the tested solution of organic acid at different pH were injected into the extrapallial fluid between shell and mantle every second day. The experiment endpoint corresponds to the first death that occurs after two weeks.

The control group consisted of animals kept in same conditions without any treatment. The other groups were injected with succinic acid (pH 5 and pH 7), citric acid (pH 5 and pH 7), lactic acid at pH5, and a mixture of succinic and citric acid at pH 7. We also tested the effects of strong acid and basic solutions (hydrochloric acid and sodium hydroxide). All the solutions were at a concentration of 60 mM.

Preparation of shells to SEM observation consisted on cuts with a dental drill, obtaining fragment of the shell with 1x2cm length, perpendicularly to the growth lines. To access the crystal morphology of nacreous and prismatic layers of the internal surface of the shell, the fragments were carefully brushed and rinsed with deionized water, mounted on aluminium holders and were coated with a Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment. The SEM/EDS was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M. Observations were made to all the experimental groups and on the valve directly injected.

Results

The images obtained by SEM display the effects of different pH and organic acids on the microstructure/habit of calcium carbonate crystals. All crystal forms were composed of calcium carbonate, confirmed by EDS analysis (Figure 1) with the identification of the elements carbon (C), oxygen (O) and calcium (Ca).

With regard to the shape and number of layers there were observed changes, in a general way, in all assays, as shown in Figure 2.

The image from animals without treatment (A) allowed us to visualize the standard structure and morphology of *Anodonta cygnea's* shell, presenting external hexagonal shape microstructure (habit) under 3 layers. On the contrary, this typical 3 nacreous layer structure is affected in the presence of a strong acid compound like HCl at pH 5, showing precipitation in several discontinuous layers, but still with a rhomboid-hexagonal crystal forms (B), slightly similar to the control animal. On the other hand, a different nacre appearance, presenting clusters of needle shape structures perpendicular to the surface, appear because of the action of a strong basic compound, NaOH at pH 9 (C).

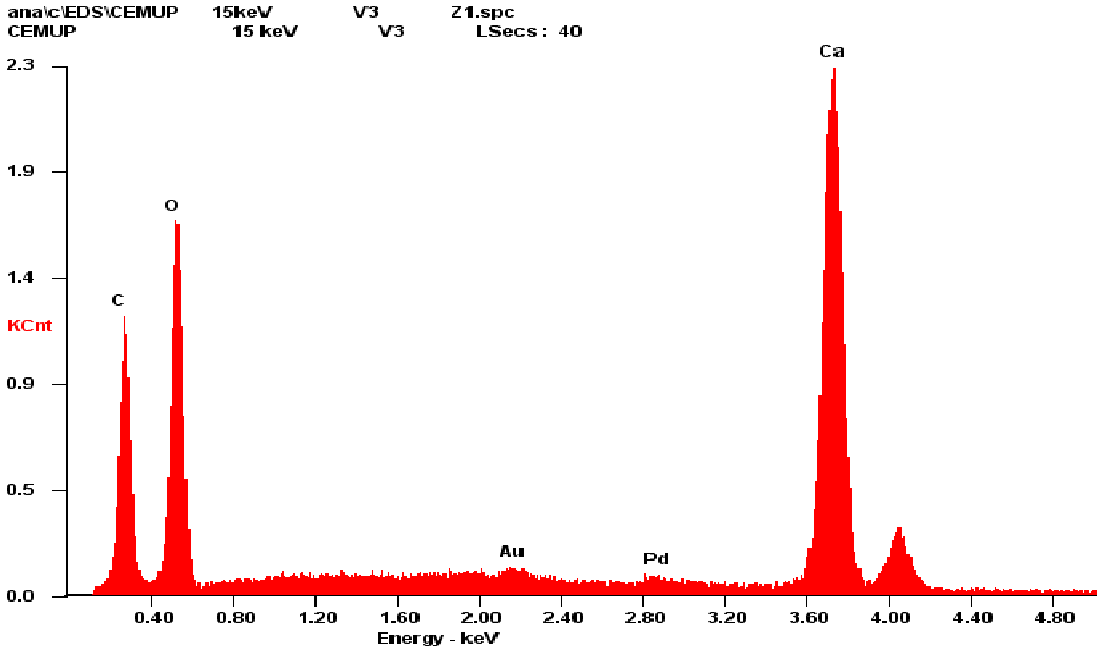

Figure 1: EDS spectrum from A. cygnea shell with identification of the main elements found.

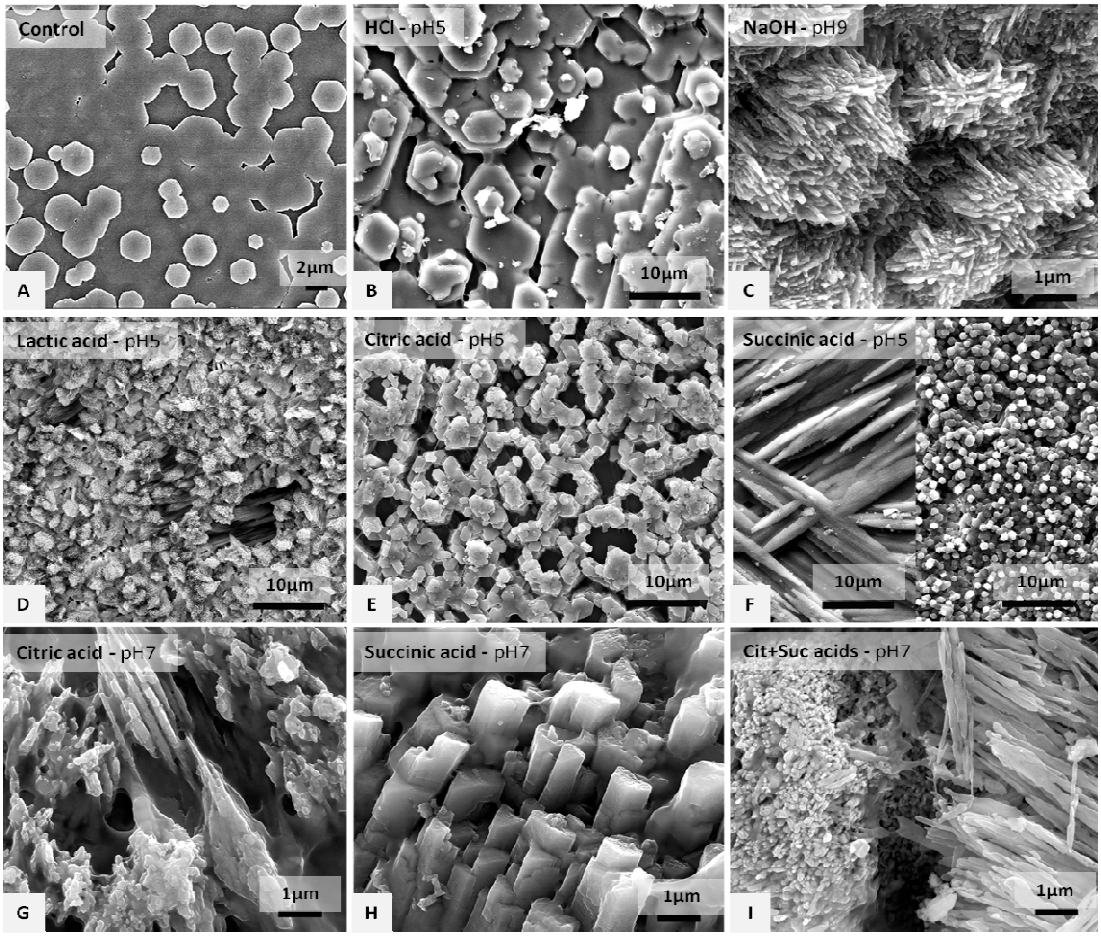

Figure 2 (A-I): This SEM observations were accomplished on the nacreous layer formed under several conditions of pH and organic acids solutions when injected in the extrapallial fluid of freshwater bivalve A. cygnea. A) control bivalve shell; B) under HCl solution; C) under NaOH solution; D, E, F) under lactic, citric and succinic solutions at pH 5; G, H, I) under citric, succinic and mixture of both solutions at pH 7.

The organic exposure experiments at pH 5 showed that the lactic acid injection presented an intense growing of numerous spicules, as prismatic structures, covered by small deformed crystal on the top (D). After treatment with citric acid solution, a thickening on the borders of the hexagonal crystals occurred around a hole (E); and the succinic acid solution induced two different effects, short thick needles and hexagonal micro-granules formation (F).

The experiments at pH 7, on the other hand, showed other deformations, the injection of citric acid leads to an amorphous disorganized precipitation, with little laminated structure (G); the succinic acid caused enormous amount of well-defined hexagonal prismatic structures (H); and the mixed solution of citric and succinic acids induced a structural malformation of disorganized calcareous prisms (I).

Discussion

Mollusks shell is a solid mixture composed of calcium carbonate and organic matrix components. These organic and inorganic elements are secreted and transported by the mantle cells and haemocytes (Coimbra *et al.,* 1988; Machado *et al.,* 1990b; Mount *et al.,* 2004) into the extrapallial fluid which is in direct contact with the inner shell surface, enable it to grow, resulting a product with singular structural properties (Lopes-Lima *et al.,* 2010). The inorganic fraction made of calcium carbonate forms two different crystals polymorphs, calcite and aragonite. Calcite crystals are present on the outer layer of the shell and aragonite crystals on the inner layer. The organic components of shell represent a small portion (0.1-5%) but will determine the nucleation and stabilization of crystals, defining the shapes and polymorphs under surrounding conditions (Fu *et al.,* 2005). Biomineralization is a continuous process throughout life of individuals and depends on numerous intrinsic and environmental factors (Checa *et al.,* 2007; de Paula and Silveira, 2009; Machado *et al.,* 2009). This mechanism is essentially a cellular process dependent on the influence of environmental factors such as temperature, pH and ionic composition of water, and also the presence of pollutants known to affect biomineralization process such as TBTO, diflubenzuron and heavy metals (Machado, Coimbra and Sá 1989; Machado *et al.,* 1990a; Lopes-Lima *et al.,* 2010, 2012). Organic compounds can influence the shape of inorganic crystals e.g. uric acid causes NaCl crystals to crystallize as octahedral instead of the usually cubes. It is possible that a similar process between the organic matter and the aragonite crystals influence the growth of the aragonite crystals (Gutmannsbauer and Hanni, 1994). There are only few studies on the effects of internal or external environmental agents on shell mineralogy of freshwater bivalves, so investigating the relations involving specific molecules and the crystal growth are an important step toward understanding the complex mechanism that is calcification. In the present study we subjected the animals to various organic compounds like citric, lactic and succinic acids under different pH conditions on the shell constitution. Specific alterations were observed comparing with the normal nacreous surface structure of the *Anodonta cygnea*'s as referenced by Lopes-Lima *et al.* (2010).

The inner shell surface of *A. cygnea* has been described by Lopes-Lima *et al.* (2010). In this study the control structure of shell is in agreement with Lopes-Lima *et al.* (2010) who have reported 3 layered structure. On the other hand, according to Machado *et al.* (1988b) severe incubation acidosis with HCl at pH3 induces intense precipitation of crystals, on the top of each other, without the previous layer being completely formed. Thus, more than 3 layered structure, indicates that an intense secretion of organic matrix occurred, with strong deposition of calcium.

In the present study, HCl solution at pH 5 injected in the shell fluid of *A. cygnea* induced minor changes more or less similar to the hexagonal structure as in the control, although with thickened borders. In contrast, other acids with organic composites at pH 5, generally display clear deformations with absence of nacreous laminar structure and present depth formations and multiple layers. It seems that, above pH 5, the physicochemical specificity inherent to the type of organic acid may causes strong and distinct effects, if compared with the HCl assay at same pH. In fact, lactic acid and succinic acid at pH 5 displays profound structural modification in the structure, but different, showing prismatic laminae or needles, respectively, while citric acid (at same pH 5), although well-deformed structure, yet can maintain a hexagonal residually habit. Strangely, with citric, succinic or a mixture of both at pH 7 it may be expected structural appearance close to the control, but occurred quite different shapes, also laminar with emphasis on succinic columnar/prismatic forms. This condition in the presence of organic acids, even at pH 7 similar to the natural physiological conditions (~ pH 7,2), reveal once more determinant influence from these biocomposites in the calcareous structure if dissolved at significant levels in the organic shell fluid of bivalve. On the other hand, in the total opposite situation at pH 9, a strong change in the structure takes place by the action of NaOH, inducing clear prismatic needles formation, instead of calcareous laminae.

The determinant influence of pH standard solutions as well as the organic acids (citric and succinic acids) with regard to how they act in the biomineralization surface, had been well demonstrated in biosynthetic membranes experiments of chitosan or cellulose incubated with various solutions of isolated composites or recombined ones (Lopes *et al.,* 2010, 2014). This biomembrane study, although *in vitro*, also revealed the distinct effect of HCl at pH 6, pH7 and NaOH at pH 9 or succinic acid at pH 6 according to the type of mentioned biosynthetic membrane.

Finally, considering all tested acids, it is possible to speculate that the little changes occurred in the nacre appearance with HCl at pH 5, can be due to the fact of *A. cygnea in vivo* conditions, to present better adaptation to the direct presence of internal H^+ changes in the fluid than that induced by the indirect action from external medium as reported by Machado *et al.* (1988b). Similar to the present study, few workers have reported that the extrapallial (shell) mantle releases significant levels of protons in autumn period through the active proton transport (Coimbra *et al.,* 1988; Machado *et al.,* 1990b). This natural acidification tendency in *A. cygnea* shell fluid during the autumn, results hexagonal crystals (Machado *et al.,* 1991). Here seems to find a similar aspect, although with visible specific differences when the HCl solution is injected *in vivo*. On the contrary, the induced acidification from the external medium (Machado *et al.,* 1988b) causes stronger effects on the nacreous layers probably due to a large extension influence in all body metabolic processes. Treatment with organic acids seems to include typical effects on nacreous layers at pH 5 and 7, possibly due to specific influence of organic compounds. Unlike HCl at pH5 (with little changes) a clear effect of NaOH at pH9 has been noticed.

Finally, the influences in all conditions, either by pH or organic compounds, may be interpreted as a direct effect on the shell, after solution injection, combined with an indirect effect through the secretory activity stimulation due to the impacts of those local agents on the mantle cells, being all process confined at the shell extrapallial compartment.

Conclusion

It can be concluded that, with *in vivo A. cygnea* assays, as compared to control, demonstrates specific variations in the case of citric and succinic acids, beyond the eventual effect of pH. The influence of these composites had already been verified by the group through *in vitro* experiments with biomembranes. These changes may lead, in a medium-long term, to significant modifications on the nacreous shell structure and further possibly affect the survival of these animals. Obviously, further studies should be conducted to evaluate more clearly different specific effects in relation to those biocomposites added to the experimental medium under specific conditions too.

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