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Culture of the gastropod *Pomacea dolioides* (Reeve, 1856): effects of calcium on growth, survival and shell regeneration

Rafaela Fernanda Batista Ferreira[1], Fernando Jose Zara[2], Bruno Sampaio Sant'Anna[1]*

[1]Universidade Federal do Amazonas – Instituto de Ciências Exatas e Tecnologia, Programa de Pós-Graduação em Ciência e Tecnologia para Recursos Amazônicos – Itacoatiara (AM), Brazil.

[2]Universidade Estadual Paulista – Departamento de Biologia, Laboratório de Morfologia de Invertebrados – Jaboticabal (SP), Brazil.

*Corresponding author: santannabs@ufam.edu.br

ABSTRACT

Gastropods of the genus *Pomacea* are exploited for food in different parts of the world. *Pomacea dolioides* has recently received attention on its stocking density and meat characteristics, remaining a gap on the effects of calcium in its cultivation. The present study evaluated the influence of calcium dissolved in water on the culture of the gastropod *P. dolioides* considering the growth, weight, survival, ultrastructure, and shell regeneration time. Juveniles were distributed into six treatments with different concentrations of Ca^{2+}. The calcium dissolved in water was essential, as gastropod did not survive for more than 40 days without calcium. Also it showed greater growth and fattening with 60 mg·L^{-1} of $CaSO_4$ or more, in addition to a thicker shell with two layers of crystals. The calcium carbonate content in the shells was significantly higher in the treatment with 80 mg·L^{-1} of $CaSO_4$. The regeneration time did not differ between treatments. Based on these results, it was concluded that the calcium dissolved in the water influences the culture of the gastropod *P. dolioides* in relation to the length, inorganic and organic weight and calcium of the snail, and 80 mg·L^{-1} of $CaSO_4$ is the ideal concentration to culture species.

Keywords: *Pomacea*; Growth; Regeneration; Aquaculture.

Cultivo do gastrópode *Pomacea dolioides* (Reeve, 1856): efeitos do cálcio no crescimento, sobrevivência e regeneração da concha

RESUMO

Gastrópodes do gênero *Pomacea* são explorados para alimentação em diferentes partes do mundo. *Pomacea dolioides* recentemente tem recebido atenção sobre sua densidade de estoque e características da carne, permanecendo uma lacuna sobre os efeitos do cálcio no seu cultivo. O presente estudo avaliou a influência do cálcio dissolvido na água no cultivo do gastrópode *P. dolioides* considerando crescimento, peso, sobrevivência, ultraestrutura e tempo de regeneração na concha. Juvenis foram distribuídos em seis tratamentos com diferentes concentrações de Ca^{2+}. O cálcio dissolvido na água foi essencial. Gastrópodes não sobreviveram mais de 40 dias sem cálcio. Também, apresentaram maior crescimento e engorda com 60 mg·L^{-1} de $CaSO_4$ ou mais, em adição a uma concha mais grossa com duas camadas de cristais. O carbonato de cálcio contido na concha foi significantemente maior no tratamento com 80 mg·L^{-1} de $CaSO_4$. O tempo de regeneração não diferiu entre os tratamentos. Com base nestes resultados, pôde-se concluir que o cálcio dissolvido na água influencia no cultivo do gastrópode *P. dolioides* em relação a comprimento, peso inorgânico e orgânico e cálcio do gastrópode, e 80 mg·L^{-1} de $CaSO_4$ é a concentração ideal para cultivar essa espécie.

Palavras-chave: *Pomacea*; Crescimento; Regeneração; Aquicultura.

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INTRODUCTION

Calcium ions are critical in the biology of mollusks for the distribution, survival, and adaptation of gastropods to the environment and play a key role in shell formation, growth, oviposition, and resistance to attack by predators (Nduku & Harrison, 1976; Ohta & Saeki, 2020; Thomas et al., 1974; Tunholi et al., 2011). Furthermore, calcium has been described as one of the factors that influence freshwater gastropod populations (Glass & Darby, 2009; Thomas et al., 1974; Watson & Ormerod, 2004).

Calcium carbonate is the main component of the shell (Limeira Jr., 2023; Meldrum, 2003). Calcium used by the mantle for shell formation originates from absorbed and secreted calcium by the digestive system or through direct absorption by the body from the environment (Morrison & Cochrane, 2008; Yang et al., 2016). Calcium carbonate crystals increase throughout the animal's life by crystallization, which depends on intrinsic and environmental factors (De Paula & Silveira, 2009). The aragonite and calcite crystals are arranged in layers to form the shell structure, together with the nacre (Dauphin et al., 2014; De Paula & Silveira, 2009; De Paula et al., 2010). This structure is then externally covered with non-calcified periosteum (De Paula & Silveira, 2009).

The shell of mollusks is a rigid external structure that supports and protects soft tissue from predators, creates resistance against water pressure from the medium (Li et al., 2016; Silva & Debacher, 2010), and regenerates damaged areas (Kádár, 2008; Liu et al., 2013). These protective exoskeletons can break when exposed to external environmental factors such as desiccation, exposure to ultraviolet light, bacteria (Trinkler et al., 2010; Trinkler et al., 2011), and predators (Cadée, 2011). Once broken, the mollusk becomes more susceptible to infection and predation (Yang et al., 2016). In this regard, rapid regeneration is critical for the resistance to stress and survival of these animals (Liu et al., 2017; Yang et al., 2016).

In recent years, studies on the relationship between calcium and the development of mollusks have increased notably. Ebanks et al. (2010) characterized the mechanism of calcium and carbonate acquisition needed to calcify the shell of embryos. In environments where calcium concentrations are less than 20 $mg{\cdot}L^{-1}$, mollusks have exhibited increased cutaneous respiration and decreased motility (Dalesman et al., 2011). Magalhães et al. (2011) observed that calcium from the shell moves to the hemolymph in *Biomphalaria glabrata* (Say, 1818) exposed to different $CaCO_3$ solutions and concluded that calcium is moved frequently depending on the amount available in the environment. Bukowski and Auld (2014) found that the predatory effect *versus* shell thickness in *Acute physa* (Draparnaud, 1805) increased with the availability of calcium, leading to the development of heavier and thicker shells. In *Pomacea paludosa*, calcium percentage, crushing resistance, and weight are affected by different calcium concentrations (Glass & Darby, 2009), similarly *Pomacea flagellata* (Say, 1829) cultured in elevated calcium concentrations (500 $mg{\cdot}L^{-1}$), which showed better growth and built stronger shells (Jesús-Navarrete et al., 2023).

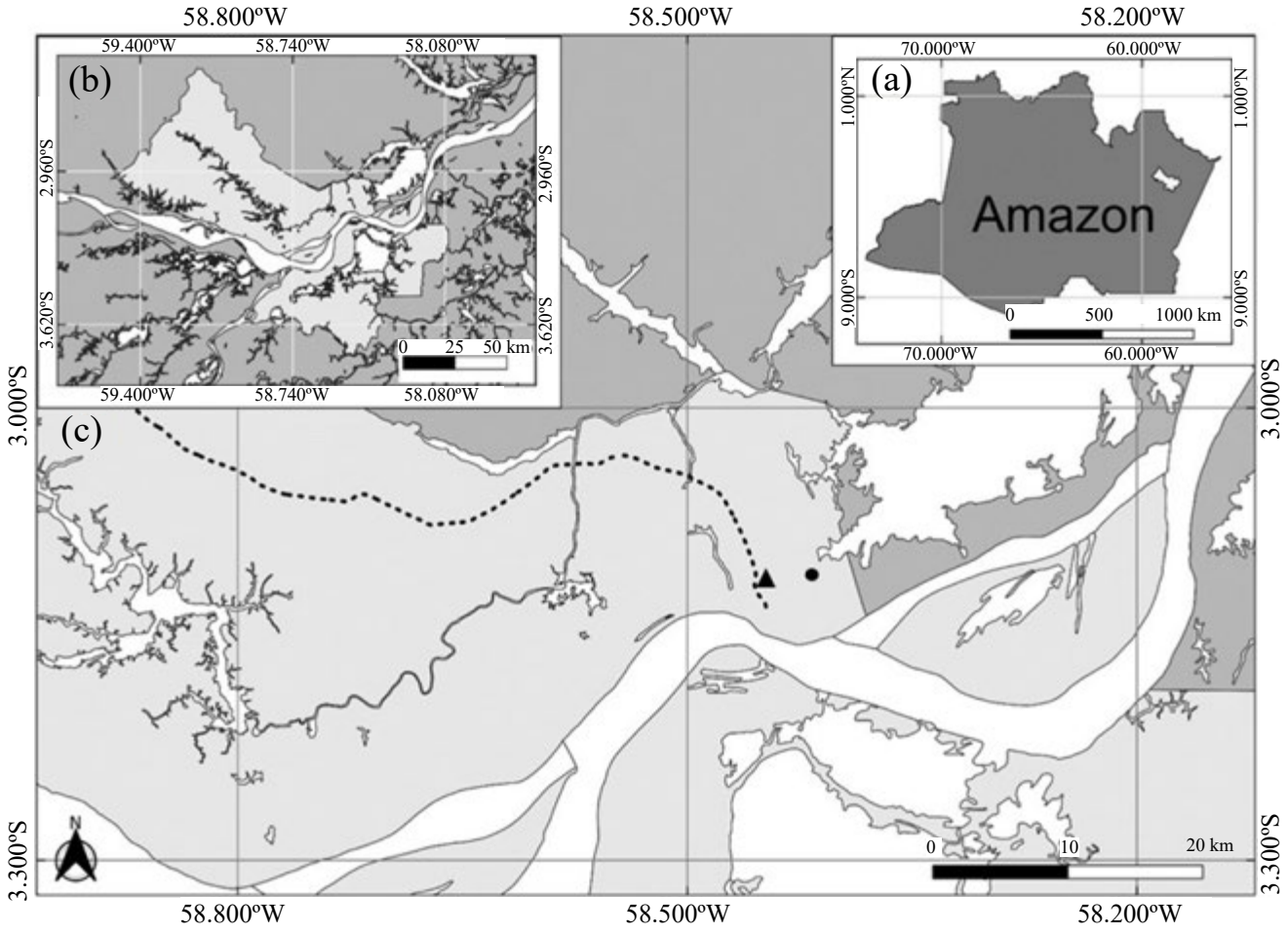
With the growth of aquaculture, gastropods of the genus *Pomacea* are interesting species for sustainable cultivation, being exploited for food around the world. Recently, scientific studies were conducted on the imposex, oviposition substrate, reproductive morphology of the gastropod *Pomacea dolioides* (Reeve, 1856) (Fonseca et al., 2017; Melo et al., 2017; Paschoal & Oliveira, 2017). Investigations on the stock density (Pires-Júnior et al., 2019) meat yield and sensorial evaluation were developed (Dantas & Sant'Anna, 2021). Despite that, no studies have been conducted on the relationship between calcium and the development of the species. The present study evaluated the influence of calcium concentration dissolved in water on the growth, weight, survival, ultrastructure, and regeneration time of shells of the gastropod *P. dolioides*.

MATERIAL AND METHODS

Effect of calcium concentration on development

The experiments were conducted from February to December 2019. To investigate the effect of calcium concentration on the development of *P. dolioides*, five egg masses were carefully collected at Lagoa da Poranga (03°07'11.4"S, 58°27'13.0"W) in the municipality of Itacoatiara, Amazonas, Brazil (Fig. 1), and placed in perforated support floating in a plastic box (31 × 25 × 12.9 cm) with 5 L of water and continuous aeration, until hatching of juveniles. After hatching, the juveniles were grown for seven days and fed with lettuce *ad libitum*. Subsequently, 540 juveniles measuring in mean 2.75 ± 0.22 mm in shell length and 0.0039 ± 0.0009 g were randomly selected for the experiment.

The water used in the experiment was a mixture of base salts made with 300 L of deionized water, 36 g of magnesium sulfate, 57.6 g of sodium bicarbonate, and 2.4 g of potassium chloride, according to Glass and Darby (2009). The experiment was performed for 60 days, with six treatments for the calcium sulfate concentrations in the water of gastropods: 0, 20, 40, 60, 80, and 100 $mg{\cdot}L^{-1}$ of calcium sulfate dihydrate (knowing that 1 $mg{\cdot}L^{-1}$ of calcium sulfate dihydrate corresponds to 0.233 $mg{\cdot}L^{-1}$ of calcium). For each treatment, three replicas were used



●: Mamud Amed: collection of gastropods; ▲: Poranga Lake: collection of egg masses.

Figure 1. Geographic location of sampling sites in the municipality of Itacoatiara, Amazonas, Brazil. (a) Amazon; (b) Itacoatiara; (c) sampling locations.

composed of three boxes (46 × 26 × 17 cm) with a 20 L water capacity, and 30 gastropods were placed in each of the 18 boxes.

The experiment was conducted in controlled conditions with a 12 h day/night cycle and at room temperature. Every week, water variables such as temperature (27.73°C ± 0.47) and pH (8.16 ± 0.23) were measured with a Hanna pH/ORP/ISE HI98185 meter, as well as nitrite (0.82 ± 0.99 $mg \cdot L^{-1}$) and ammonia (0.05 ± 0.04 $mg \cdot L^{-1}$) with colorimetric tests. In all replicas, aerators were placed to maintain the water oxygenated, and every two days, 25% of the water was siphoned to remove excreta and food remains. The water removed was replaced with water in the concentration of the respective treatment.

On the first day of the experiment, the shell length of the juveniles was measured (distance from siphonal canal to apex) using stereomicroscope with camera attached to a computer with Motic software, precision = 0.01 mm, and weighed in analytical scale = 0.0001 mg. Then, the juveniles were transferred to each plastic box mentioned above with 15 L of water and fed daily with hydroponic frisee lettuce for the first 20 days, followed by fish feed containing 34% crude protein with 10% of biomass per day. The amount of feed was adjusted every five days according to biometrics and mortality data (Pires-Junior et al., 2019). On the last day of the experiment, the gastropods were weighed on analytical scale (0.0001 mg) and measured with caliper (0.05 mm).

The specimen samples were deposited in the Malacological Collection of the Instituto Oswaldo Cruz (#11434) and identified by Dr. Silvana Carvalho Thiengo. This study was developed in accordance with Brazilian laws, as determined by the biodiversity authorization and information system (Sistema de Autorização e Informação em Biodiversidade #7158399/63335) linked to the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio).

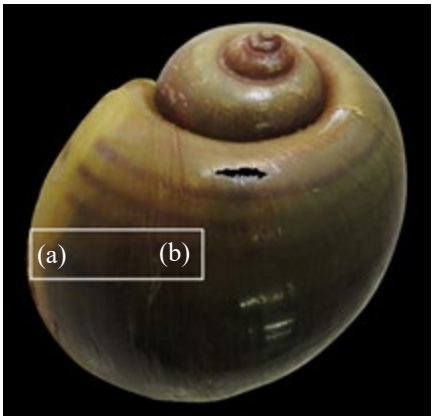
Determining the calcium concentration

The calcium concentration was determined in the gastropod (soft tissues) and the shell separately. To determine the calcium concentration in the whole gastropod, four gastropods from each replica were used, totaling three samples composed of four gastropods for each treatment. After 60 days, the gastropods were anesthetized for 1 h in 50% magnesium chloride solution to prevent the loss of hemolymph when exposed to ethanol and then preserved in 70% ethanol. The preserved gastropods were rinsed with deionized water, and the soft parts were separated from the shell. The shells were discarded, and the whole animals were dried in an oven at 90°C for 24 h. Subsequently, the dried gastropods were weighed on analytical scale (0.0001 g) and heated in muffle furnace at 500°C for 1.5 h. The ash was weighed on analytical scale (0.0001 g), and calcium (g) was determined by multiplying the ash weight by 0.40 (Brodersen & Madsen, 2003). The percentage of calcium was calculated by dividing the amount of calcium by the total dry weight of the tissue and multiplied by 100 (Glass & Darby, 2009).

To determine the calcium concentration in the shells, three shells were selected from each replica, totaling nine samples per treatment, followed by the technique described by Soído et al. (2009). The shells were dried at room temperature for four days, weighed, and calcined in muffle furnace at 450°C for 48 h. The ashes were weighed and diluted in 50 mL of concentrated nitric acid. The solution was kept in a digester for 6 hours, and 2 mL of hydrogen peroxide (H_2O_2) was added to clarify the resulting solution. The sample was diluted 100 times in distilled water, and five aliquots of 25 mL were used to determine calcium using ethylenediaminetetraacetic acid (EDTA), according to Baird et al. (2017). The mass of calcium carbonate was calculated using the volume of EDTA discarded in the titration process and expressed in mg of $CaCO_3$ g^{-1} of ash (Soído et al., 2009).

Characterizing the microstructure of shells

The shells were structurally characterized using three shells of each treatment described above. First, the shells were cut using stainless steel scissors. A perpendicular cut was made to the growth line of the shell measuring 0.5 × 1.5 cm to visualize the growth line and the previously calcified layers (Fig. 2). Then, they were submitted to the UNESP Laboratory of Electron Microscopy at Universidade Estadual Paulista "Júlio de Mesquita Filho" for analysis. The samples were sputter-coated with gold (5 nm) using a Denton Vacuum Desck II Sputter Coater. The shells of each treatment were photographed with the ZEISS EVO scanning electron microscope with electron beam ranging between 10–15 kV. The thickness of the photographed shells was measured (µm) using ImageJ software calibrated by the scale of each micrography obtained from the equipment.



a: growth area of the outer lip of the shell; *b*: calcified area around the body.

Figure 2. Illustration of the cut made in the shells of gastropods *Pomacea dolioides* for scanning electron microscopy analysis. Scale bar: 0.5 cm.

Effect of calcium on shell regeneration

Samples of *P. dolioides* juveniles were collected manually and using dip nets in the neighborhood of Mamud Amed (03°06'40.7"S, 058°25'28.5"W), municipality of Itacoatiara, Amazonas, Brazil (Fig. 1). The gastropods were carefully placed in plastic boxes with water and aeration and transported to the Laboratory of Zoology of the Instituto de Ciências Exatas e Tecnologia at Universidade Federal do Amazonas, where they were kept in a circular 310 L polyvinyl chloride (PVC) tank with a closed recirculation system with 180 L/h water renewal and external biological filter. They were fed the same fish feed containing 34% crude protein under natural lighting for two weeks before the experiment.

The shell morphology of 180 juveniles of *P. dolioides* was carefully inspected, and only individuals with perfect shells were used in the experiment. Then, shell length of the gastropods was measured using a caliper (0.05 mm). Mean shell length of the gastropods was 24.05 ± 0.62 mm.

A triangular cut (Fig. 3a) with an 11 mm base in the outer lip of the shell and 8 mm sides was made toward and around the shell body of 90 snails in the first experimental group (Yang et al., 2016). In the second group, a rectangular incision (Fig. 4a) measuring 4 × 8 mm was made from the outer lip of the shell opening towards the back of the body (Liu et al., 2017) using surgical scissors. Both cuts had the same area (32 mm^2). Each group of 90 snails was divided into six treatments with 15 individuals each to induce regeneration. After the cuts, 15 gastropods were randomly selected for each triplicate treatment (same calcium concentration treatments used in the previous experiment). These were marked with enamel of varying colors

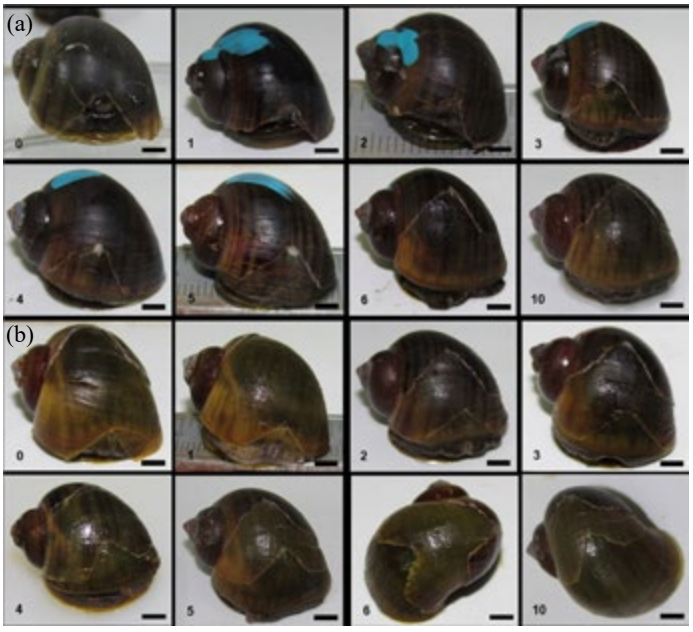


Figure 3. Images of shell regeneration of *Pomacea dolioides*, triangular incision. (a) First incision. (b) Second incision. Scale bar: 0.5 cm.

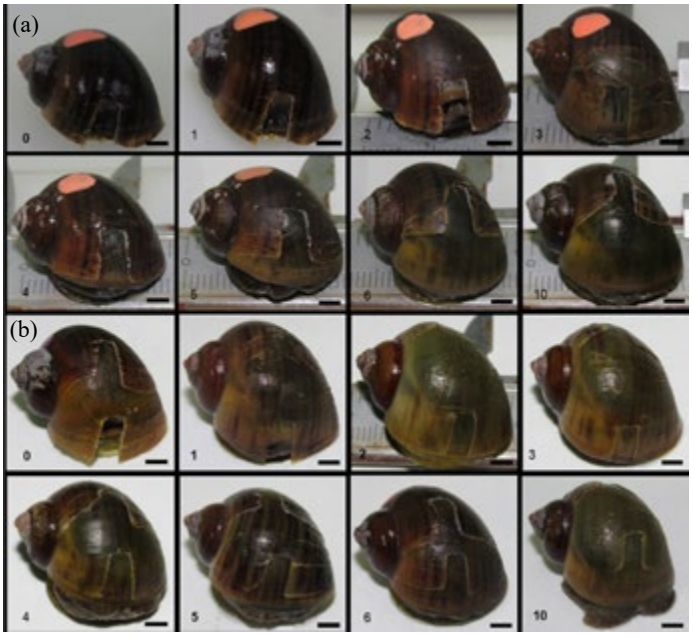


Figure 4. Images of shell regeneration of *Pomacea dolioides*, rectangular incision. (a) First incision. (b) Second incision. Scale bar: 0.5 cm.

to monitor each individual and transferred to plastic boxes with 15 L of water, different calcium concentrations, and the same fish feed. Daily photographic records of the regenerated shells were obtained using a Canon Powershot SX530 HS digital camera.

The gastropods were kept and fed in the same way as in the previous experiment. The experiment was conducted in natural conditions with a 12 h day/night cycle and at room temperature for 10 days. After complete regeneration of the sectioned part of the 15 snails in each treatment, a second incision was made, and the gastropods were kept at the same concentration described above until complete regeneration of the shell (Figs. 3b and 4b).

Data analysis

Biomass gain (BG) was evaluated using Eq. 1:

$$BG = (Pf \times Nf) - (Pi \times Ni) \quad (1)$$

Where: Pf: final weight; Nf: final number of gastropods; Ni: initial number of gastropods; Pi: initial weight.

For growth analysis, the specific growth rate (SGR) was calculated using Eq. 2:

$$SGR = (\text{Log } Pf - \text{Log } Pi) \times 100/T \quad (2)$$

Where: Pf: final weight; Pi: initial weight; T: elapsed time.

Absolute weight gain (AWG) was calculated using Eq. 3:

$$GPB = Pf - Pi \quad (3)$$

Where: Pf: final weight; Pi: initial weight.

Percentage weight gain (PWG) was calculated using Eq. 4:

$$GPP = Pf - Pi \times 100/Pi \quad (4)$$

Where: Pf: final weight; Pi: initial weight.

Survival rate (SR) was evaluated with Eq. 5:

$$TS = (Nf \times 100)/Ni \quad (5)$$

Where: NF: number of final gastropods; Ni: number of initial gastropods (Pires-Junior et al., 2019).

Data on biomass gain, specific growth rate, absolute weight gain, and percentage weight gain of juveniles were subjected to the Shapiro-Wilk's normality test. As the data were normal, simple variance analysis was conducted, supplemented with the Tukey's test. The χ^2 test and contingency table were used to analyze the proportion of individuals who survived at the end of the experiment among treatments. The size and weight of juveniles in relation to cultivation time were determined using covariance analysis and the F-test.

The percentages of calcium in the whole animals of each treatment were transformed into sine arc to meet the

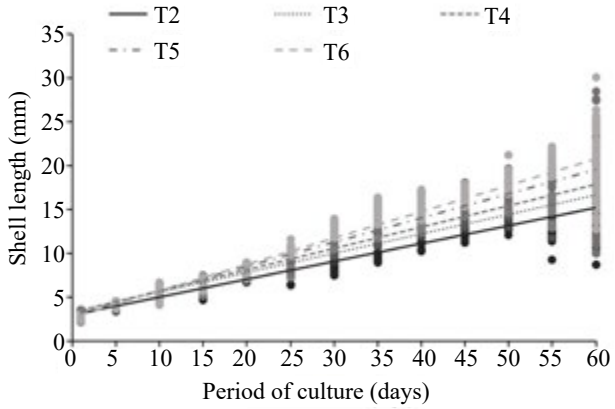
assumptions of analysis of variance (ANOVA). Subsequently, calcium in whole gastropods was compared among treatments using one-way variance analysis and supplemented with the Tukey's test. The calcium concentration and thickness of the shells were compared using the Kruskal-Wallis' test, supplemented by the Dunn's test.

The survival data of shell regeneration were transformed into sine arc to meet the ANOVA assumptions. Factorial ANOVA was used to compare the survival rate and regeneration time of the shells among calcium concentrations (six treatments), cut type (triangular or square), and inductions (first and second). The significance level adopted for all analyses was $p < 0.05$.

RESULTS

The survival rate of juveniles over the 60 days of the experiment showed significant difference among the treatments ($\chi^2 = 21.846$; GL = 4; $p = 0.0002$), with survival of 84% in the treatment with higher calcium concentration (100 mg·L^{-1}) and of 0% in the treatment with no calcium (0 mg·L^{-1}), in which juveniles did not survive for more than 40 days. Treatments with higher calcium concentrations (80 and 100 mg·L^{-1}) had significantly higher values for biomass gain (F = 10.1445; DF = 4; $p = 0.001514$) and absolute weight gain (F = 37.399; DF = 4; $p < 0.0001$) (Fig. 5). The specific growth rate (F = 9.25; DF = 4; $p = 0.002154$) and percentage weight gain (F = 8.6735; GL = 4; $p = 0.002737$) were higher from 60 mg·L^{-1} of calcium (Fig. 5).

Figure 6 shows the comparison between the straight lines of the relationship shell length and cultivation days, in which treatment 6 presented significant difference among the tested calcium concentrations (ANCOVA, F = 79.782; DF = 4;



T: treatment.

Figure 6. Relationship between experiment time (days) and shell length (mm) of *Pomacea dolioides* in different calcium concentrations: T2: 20 mg·L^{-1}, T3: 40 mg·L^{-1}, T4: 60 mg·L^{-1}, T5: 80 mg·L^{-1}, and T6: 100 mg·L^{-1} of calcium sulfate dihydrate.

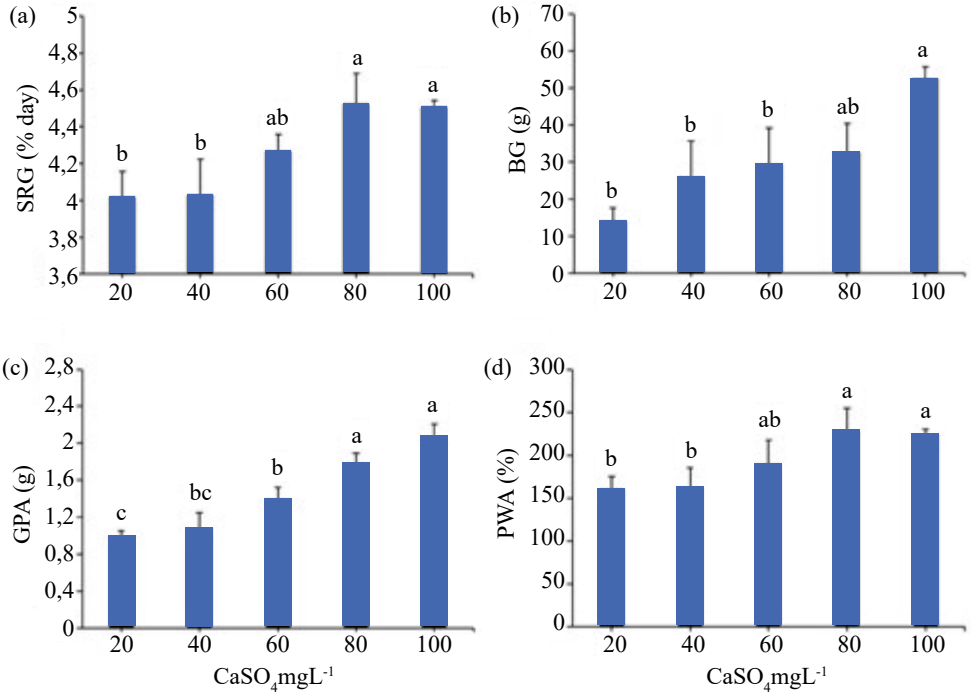
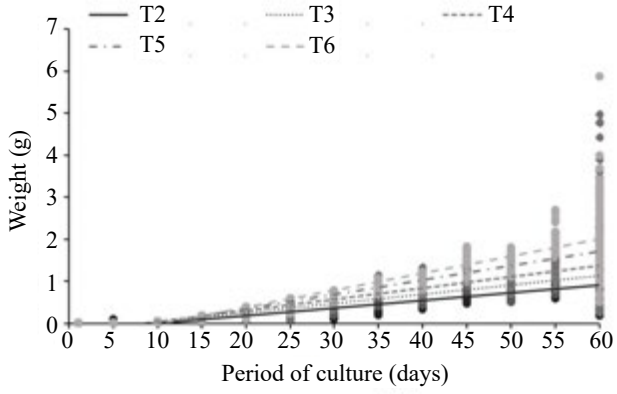


Figure 5. Development parameters of *Pomacea dolioides* subjected to five concentrations of calcium for 60 days. (a) Specific growth rate (SGR). (b) Biomass gain (BG). (c) Absolute weight gain (AWG). (d) Percentage weight gain (PWG).

$p < 0.0001$) with greater growth of gastropods. The slope of the straight lines for the relationship between cultivation days and weight gain differed significantly among treatments (ANCOVA, F = 52.148; DF = 4; $p < 0.0001$). Treatment 6 also provided greater weight gain for *P. dolioides* (Fig. 7).

The calcium percentage in whole snails differed among the treatments (F = 26.26; DF = 4; $p = 0.000028$). Calcium was significantly higher in snails treated with 60, 80, and 100 mg·L^{-1} of $CaSO_4$ $2H_2O$ (20.59 ± 1.2%) compared to snails treated with 20 and 40 mg·L^{-1} (15.82 ± 0.82%) (Fig. 8). The calcium carbonate concentration in the shells differed among the treatments (H = 23.35539; GL = 4; p = 0.0001), with greater $CaCO_3$ content in gastropods subjected to a concentration of 80 mg·L^{-1} of $CaSO_4$. Statistically, however, the concentration of 80 mg·L^{-1} did not differ from the concentration of 40 and 60 mg·L^{-1} for calcium carbonate in the shells. Gastropods submitted to the concentration of 80 mg·L^{-1} also presented greater shell weight and ash (Table 1).

Under scanning electron microscopy, the shells of *P. dolioides* exhibited periostracum with periostracal hairs in rows near the shell opening (Figs. 9a and 9b). Directly underneath, there was a crossed-lamellar layer with plate-shaped crystals arranged in columns (Figs. 9c and 9d) and a pearly layer, in which each lamella consists of long aragonite needles arranged in the same direction (Figs. 9c and 9e). No significant difference was observed in shell thickness for the different $CaSO_4$ concentration tested (H = 8.866667, GL = 4, $p = 0.0645$). However, a trend was observed, in which the highest calcium concentrations (60, 80, and 100 mg·L^{-1} $CaSO_4$) showed greater thickness (Figs. 10 and 11). Moreover, in the three highest concentrations, the crossed-lamellar layer was thicker and more evident (Figs. 11c, 11d and 11e).



T: treatment.

Figure 7. Relationship between experiment time (days) and weight gain (g) of *Pomacea dolioides* subjected to different calcium concentrations: T2: 20 mg·L^{-1}, T3: 40 mg·L^{-1}, T4: 60 mg·L^{-1}, T5: 80 mg L^{-1}, and T6: 100 mg·L^{-1} of calcium sulfate dihydrate.

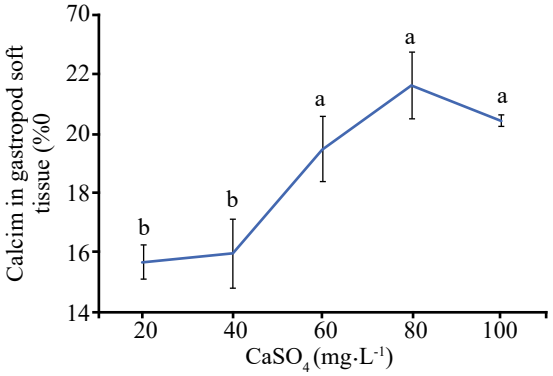


Figure 8. Percentage of calcium in gastropods subjected to different concentrations of calcium sulfate.

The shell regeneration of *P. dolioides* juveniles began on the first day with the appearance of a thin film of the periostracum. This repair gradually advanced from the apex of the triangle and rectangle to the lower margin of the cut shell. Once the gap was covered, a scar was formed between the old and regenerated shell (Figs. 3 and 4). The mean survival rate of gastropods was 97.5 ± 7.41%, with no significant difference between the factors calcium concentrations, cut type (triangular and rectangular), and induction ($F_{5,48} = 0.79273$; $p = 0.56022$) (Fig. 12). However, survival was significant between the two types of cut and induction ($F_{1,48} = 5.433$; $p = 0.02400$), and the gastropods showed

Table 1. Shell weight, ash, and calcium carbonate mass values of *Pomacea dolioides*. Mass of calcium carbonate in the shells followed by different letters differed significantly (Kruskal Wallis' test).

Calcium sulphate (mg·L^{-1})	Shell weight (g)	Ash weight (g)	Mass (mg of $CaCO_3$ g^{-1} of ash)
20	0.5158	0.4374	73.68 ± 1,02 ^{c}
40	1.2508	1.1567	84.63 ± 0.39 ^{abc}
60	1.7232	1.5778	86.53 ± 0.57 ^{ab}
80	3.0453	2.7625	89.06 ± 0.30 ^{a}
100	2.6747	2.418	82.54 ± 0.23 ^{bc}

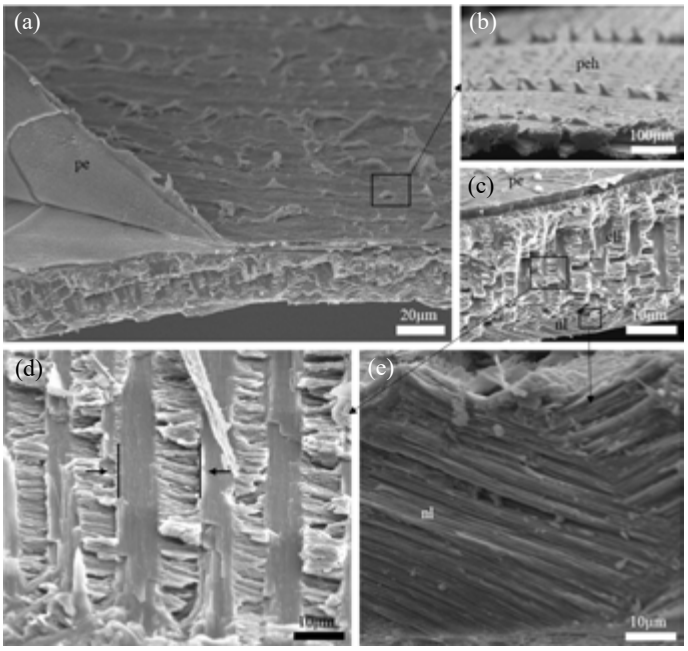


Figure 9. Scanning electron micrographs of cross-section of *Pomacea dolioides* shell. (a) Cross-section showing the periostracus (pe). (b) Periostracus, detailed view of the periostracal hairs (peh). (c) Section showing the crossed-lamellar layer (cll) and nacreous layer (nl). (d) Detail of crossed-lamellar layer of the first order (setae) and (e) view of nacreous layer (nl).

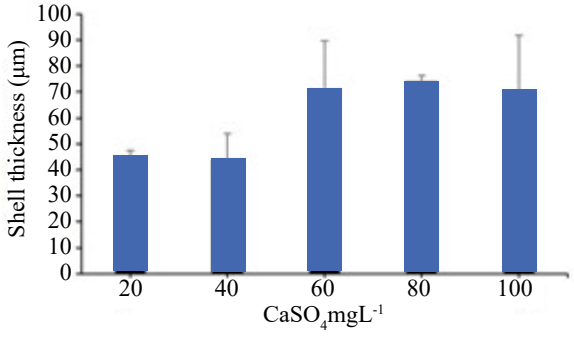


Figure 10. Shell thickness (µm) of *Pomacea dolioides* subjected to different concentrations of calcium sulfate.

a higher survival rate with the rectangular incision in the second induction (Fig. 13).

The mean shell regeneration time for individuals subjected to different calcium sulphate concentrations (0, 20, 40, 60, 80, and 100 mg·L^{-1}), two types of cuts (triangular and rectangular), and two inductions were 3 ± 1.2 days (Figs. 3 and 4), with no significant difference between the factors described ($F_{5,318}$ = 0.542; p = 0.744672) (Fig. 14). In general, the different calcium

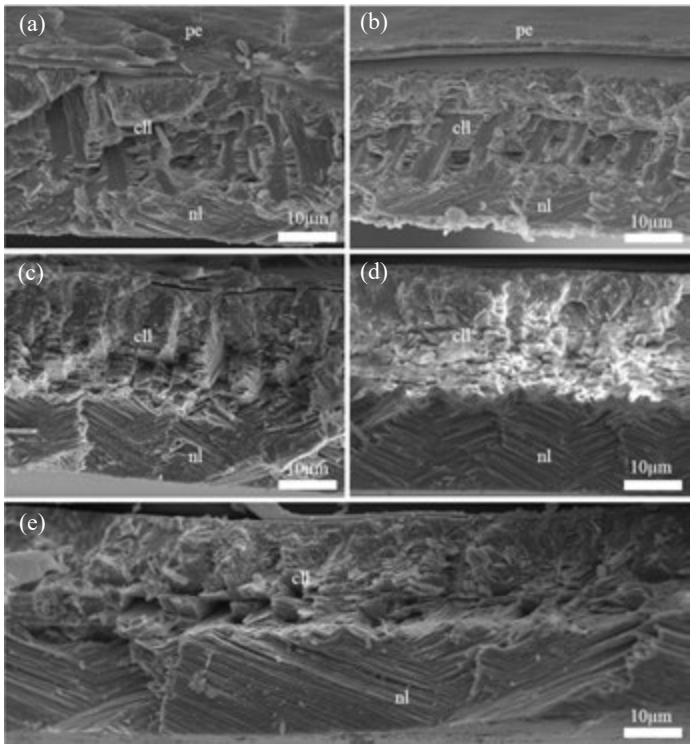


Figure 11. Scanning electron micrographs of cross-section of *Pomacea dolioides* shell for different treatments, showing a thickening of the nacreous layer as the calcium concentration increases. (a) View of crossed-lamellar layer (cll) and nacreous layer (nl) of gastropod shell treated with 20 mg/$CaSO_4$. (b) Detailed view of crossed-lamellar layer (cll) and nacreous layer (nl) of gastropod shell treated with 40 mg/$CaSO_4$. (c) Crossed-lamellar layer (cll) and nacreous layer (nl) of gastropod shell treated with 60 mg/$CaSO_4$. (d) View of crossed-lamellar layer (cll) and nacreous layer (nl) of gastropod shell treated with 80 mg/$CaSO_4$. (e) Crossed-lamellar layer (cll) and nacreous layer (nl) of gastropod shell treated with 100 mg/$CaSO_4$.

concentrations and the type of cut (triangular and rectangular) did not influence the survival rate and regeneration time of the shells in the gastropod *P. dolioides* in the first induction.

DISCUSSION

The survival of the Amazon gastropod *P. dolioides* subjected to different calcium concentrations differed from the survival rate observed by Glass and Darby (2009) for *P. paludosa*, which was 100% for all treatments, including at the lowest calcium sulfate concentration of 10 mg·L^{-1}. In the present study, the survival of juveniles was between 0 and 84% for treatments of 0 and 100 mg·L^{-1} of $CaSO_4$, respectively. The 100% mortality for snails

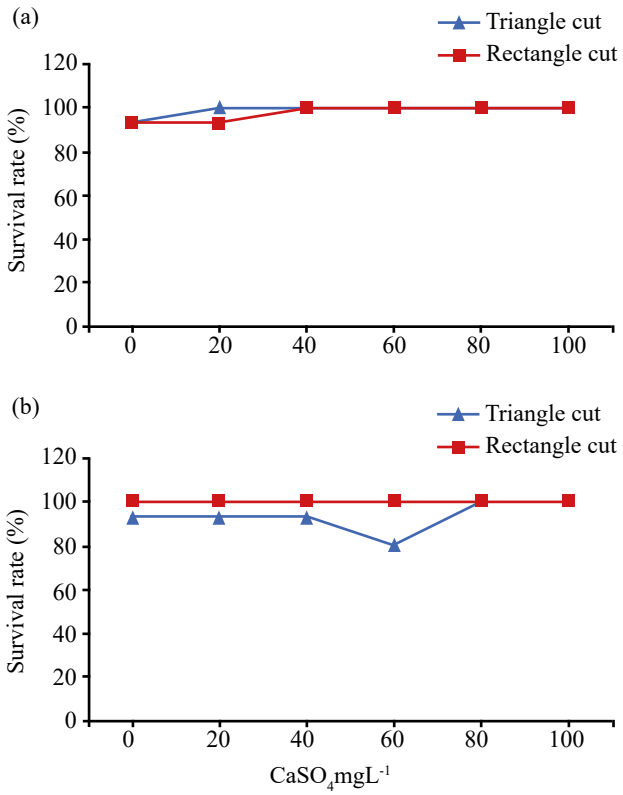


Figure 12. Survival rate of the gastropod *Pomacea dolioides* with regard to $CaSO_4$ concentrations, cut type (triangular and rectangular), and two inductions. (a) First induction. (b) Second induction.

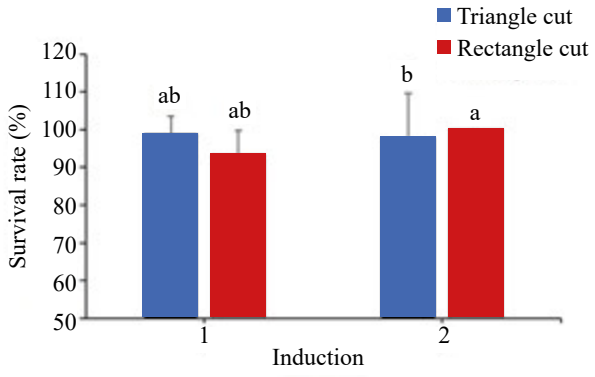


Figure 13. Survival rate of the gastropod *Pomacea dolioides* with regard to cut type (triangular and rectangular) and two inductions. Different letters indicate significant differences according to Tukey's test after factor analysis.

in the absence of calcium was also reported by Madsen (1987) in the gastropod *Bulinus truncatus* (Audouin, 1827) in the first four weeks of the experiment. According to Nduku and Harrison (1976), cultivating snails in low calcium concentrations causes stress in the animals due to the low calcium level available for

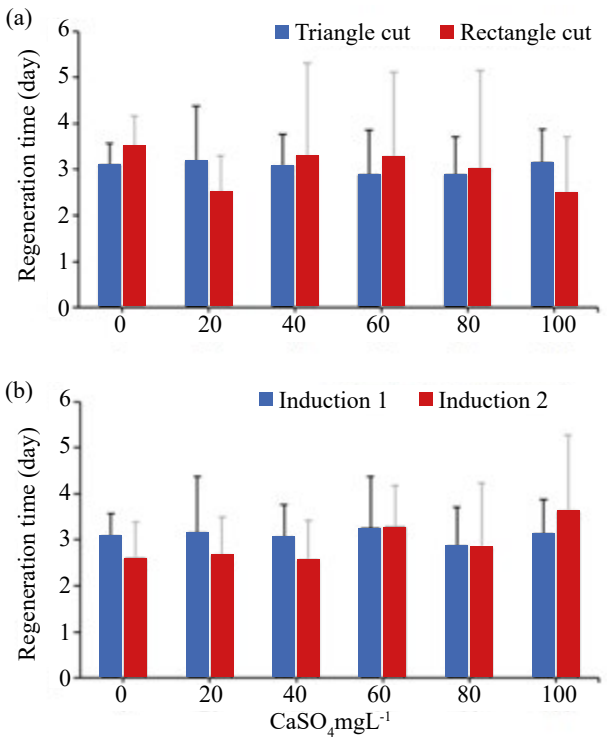


Figure 14. Regeneration time of the gastropod *Pomacea dolioides* according to calcium sulfate concentrations. (a) Cut type. (b) Induction number.

normal physiological processes and shell construction. Also, according to these authors, at the calcium concentration of 2 $mg \cdot L^{-1}$, the gastropods are already under stress.

Lower specific growth and weight gain rates in P. *dolioides* were associated with lower calcium sulfate concentrations: 20 and 40 $mg \cdot L^{-1}$ and 20, 40, and 60 $mg \cdot L^{-1}$, respectively, similarly for *P. flagellate*, whose growth was more at higher calcium concentrations of 500 $mg \cdot L^{-1}$ (Jesús-Navarrete et al., 2023). According to Thomas et al. (1974), absolute and specific growth rates tend to increase when calcium concentrations increase, also resulting in an increase in weight percentage. Furthermore, Glass and Darby (2009) found significant differences in the growth of *P. paludosa* for the three highest concentrations (40, 80, and 100 $mg \cdot L^{-1}$) and the two lowest concentrations (10 and 20 $mg \cdot L^{-1}$). Brodersen and Madsen (2003) and Madsen (1987) observed that shell diameter and weight (inorganic and organic) increase with the calcium concentration in other gastropod species. In this regard, the rate of calcium absorption from the external environment is significantly related to the growth rate of the shell (Thomas et al., 1974). Glass and Darby (2009) suggested that at least part of gastropod shells originates from the calcium in water. The remaining calcium is absorbed by gastropods from the food.

The increased $CaSO_4$ concentration in water had a significant effect on the calcium content in gastropods (soft tissue), with an increasing calcium concentration in the gastropod as the calcium in water increased. Thus, our results were not consistent with those of Glass and Darby (2009), for which the calcium percentage in whole gastropods did not differ among treatments. This may indicate physiological differences in calcium absorption within the genus since Glass and Darby (2009) used a similar sample design when investigating *P. paudosa*. However, the percentages of Ca^{2+} observed in the present study were similar to those of gastropods treated with water from the environment in the study of Glass and Darby (2009).

The results for calcium carbonate in the shells of *P. dolioides* followed the same pattern found for growth rates, weight gain, and calcium percentage in whole animals, with significant effects for the highest $CaSO_4$ concentrations. The mass of calcium carbonate in the shells was greater in gastropods subjected to 80 $mg \cdot L^{-1}$ of calcium sulphate, suggesting that *P. doliodes* absorbs enough calcium for its processes when inserted in an environment in which the calcium concentration is higher than needed. In contrast, this study confirmed that in environments with very low calcium concentrations, gastropods absorb calcium dissolved in water, but the growth rates are low, and the shells become thin (Brodersen & Madsen, 2003; Madsen, 1987; Thomas et al., 1974).

The mass of calcium carbonate in shells of *P. dolioides* was lower than the mass found by Soído et al. (2009) in the gastropod *Bradybaena similaris* (Ferussac, 1821) and by Darwin and Padmavathi (2018) in the gastropods *Telescopium telescopium* (Linnaeus, 1758) and *Pirenella cingulata* (Gmelin, 1791), with 874.24 ± 56.617, 621 ± 11.37, and 420 ± 6.8 mg of $CaCO_3$ g^{-1}, respectively. These differences may be related to shell thickness, hardness, habitat, and the type of $CaCO_3$ crystal structure, which vary according to the species. Compared with the hard shell of many gastropods, the shell of *Pomacea* is thin, fragile, and easily damaged in its environment (Yang et al., 2016).

The microstructure of *P. dolioides* shells was consistent with data from Estebenet et al. (2006) for *P. canaliculata* with respect to the spiral-shaped periostracal protrusions. Studies conducted by Meenakshi et al. (1975) revealed that aragonite is the mineral component of the shell of *P. paludosa*. The crystal structure of the shell of *Pomacea lineata* (Spix, 1827) also contained a biological matter essentially consisting of $CaCO_3$, with a predominance of aragonite (De Paula & Silveira, 2009; De Paula et al., 2010). The crossed-lamellar layer of the shell of *P. dolioides* is similar to *P. lineata*, with plate-shaped crystals that accumulate in columns (De Paula & Silveira, 2009; De Paula et al., 2010).

Our results indicated that there is a relationship between calcium dissolved in water and the development of the gastropod *P. dolioides* regarding length, inorganic and organic weight, and calcium of the shell and the whole snail. Therefore, it is important to consider the environment in which the animal is inserted when acquiring Ca^{2+} ions (Ebanks et al., 2010).

The survival rates of the gastropod *P. dolioides* subjected to different calcium concentrations in the shell regeneration process were slightly higher than the survival rates recorded by Liu et al. (2017) in juveniles of *P. canaliculata*, which were 92.31 and 96.15%, indicating that *P. dolioides* can survive at least two consecutive shell injuries. Moreover, our results showed that the species could regenerate its shell quickly and cover the incision in two to six days. According to Yang et al. (2016), removing a piece of the shell's outer lip causes the animal to retract with the shell opening closed by the operculum, which rapidly induces shell regeneration. This onset of regeneration is the result of a nonspecific immune response related to wound healing (Kádár et al., 2008; Liu et al., 2013). The appearance of a thin regenerated shell initially as calcium crystals to seal the gap as soon as possible was reported by Yang et al. (2016) on the first day after induction in *P. canaliculata*.

The repair time of the shells of *P. dolioides* was lower than the time described for other mollusks, considering that the time required to regenerate damaged shells varies between the species and habitats of the mollusks (Kádár, 2008; Kádár et al., 2008; Liu et al., 2013). Furthermore, juvenile snails have a greater capacity to regenerate injured shells than adults (Liu et al., 2017). The regeneration of the blue mussel *Mytilus edulis* (Linnaeus, 1758), for example, occurred 29 days after injury (Hüning et al., 2016). Moreover, Liu et al. (2018) reported a repair time of 29 days for *Hyriopsis cumingii*. Studies conducted with *P. canaliculata* reported a regeneration time in juveniles of five to seven days (Liu et al., 2017; Yang et al., 2016), while shell regeneration in adults occurred after 10 days (Liu et al., 2017). These shell repair times were observed at the first incision.

It is important to note that the shell of *P. dolioides* continued to grow after regeneration of the damaged area in both inductions, although growth was slower in the second induction. Liu et al. (2017) also observed that initial regeneration and multiple regenerations did not decrease the regeneration capacity of the shell. These same authors reported that multiple inductions of shell regeneration in *P. canaliculata* significantly increased the regenerated area. According to Yang et al. (2016), shell growth occurs in the distal edge, in which tiny layers are added to the underlying layers. As a result, the shell grows more in length than

in thickness (Marin & Luquet, 2004). Furthermore, the crossed-lamellar structure in the shell of *P. dolioides* exhibits the property of rapid growth, and its repair processes are faster than those of the nacre when the shell is damaged (Liang et al., 2010).

In the present study, calcium concentrations did not influence the survival rate and regeneration time of the shells in the gastropod *P. dolioides* in the first induction. These results may be related to the calcium reserve that the gastropods already had, as the juveniles were collected in the environment and subjected to different calcium concentrations in the laboratory. In addition, the hardness and crystal structure of the shell may have influenced regeneration since the shells of *Pomacea* are thin and fragile when compared to the hard shells of other gastropods (Yang et al., 2016). *Pomacea dolioides* exhibits a high survival rate and average time of three days to regenerate the shells.

Although the calcium concentration in the water did not interfere with shell regeneration of the gastropod *P. dolioides*, studies have reported that different calcium concentrations affect the growth rate, thickness, and the organic and inorganic weight of shells (Brodersen & Madsen, 2003; Jesús-Navarrete et al., 2023; Madsen, 1987; Thomas et al., 1974). Li et al. (2016) studied the participation of hemocytes in the formation of calcium carbonate crystals in shell regeneration of the oyster *Pinctada fucata* (Goud, 1850) and observed crystal structures in hemocytes composed of calcium, carbon, oxygen, phosphorus, and silicon that were similar to those of the $CaCO_3$ crystal in the surrounding environment. Thus, the calcium concentration dissolved in the aquatic environment is important for shell regeneration.

In addition to calcium, characteristics such as temperature, pH, water quality, and food availability influence the distribution of mollusks, growth, and shell structure (Martin et al., 2001; Silva & Debacher, 2010; Suzuki & Nagasawa, 2013). Furthermore, the water exchange management during the experiment maintained the ammonia and nitrite concentrations at acceptable conditions for cultivation, with no risk of harm to the development of gastropods due to these variables, so there was no need for monitoring. Thus, the variables for water quality described in the present study agree with the reference values for *Pomacea* cultivation (Pierre et al., 2017; Posch et al., 2012; Rodríguez & Carranza, 2007).

For the present study, we can conclude that the calcium concentration did not influence the shell regeneration process of the gastropod *P. dolioides*, suggesting that the species used its calcium reserve to regenerate the injured areas in a relatively short time. Consequently, this rapid regeneration can prevent infections and predations and increase their chances of survival and establishment in the environment.

CONCLUSION

In the present study, calcium concentrations did not influence the survival rate and regeneration time of the shells in the gastropod *P. dolioides* in the first induction, and regeneration was faster than other congeneric gastropods (three days). Calcium dissolved in water was essential for the culture of *P. dolioides*, since, in the absence of calcium, the gastropods did not survive for more than 40 days, showing better development from 60 $mg \cdot L^{-1}$ of calcium. The results presented for the mass of calcium carbonate in the shells of *P. dolioides* followed the same pattern found for growth rates, weight gain, and percentage of calcium in whole animals, with significant gains for the highest concentrations of calcium in the water (60, 80 and 100 $mg \cdot L^{-1}$). The concentration of 80 $mg \cdot L^{-1}$ of calcium sulfate showed better results, being the ideal concentration for the cultivation of *P. dolioides*.

CONFLICT OF INTEREST

Nothing to declare

DATA AVAILABILITY STATEMENT

All data were generated or analyzed in this study.

AUTHORS' CONTRIBUTIONS

Investigation: Ferreira, R.F.B.; **Methodology:** Ferreira, R.F.B.; **Formal Analysis:** Ferreira, R.F.B., Zara, F.J.; **Writing – original draft:** Ferreira, R.F.B., Zara, F.J., Sant'Anna, B.S.; **Conceptualization:** Sant'Anna, B.S.; **Data curation:** Sant'Anna, B.S.; **Writing – review & edition:** Sant'Anna, B.S.; **Final approval:** Sant'Anna, B.S.

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REFERENCES

Baird, R., Rice, E., & Eaton, A. (2017). Standard methods for the examination of water and wastewaters. In C. E. W. Rice, A. D. Eaton, & American Water Works Association (Eds.), *Water Environment Federation, American Public Health Association* (pp. 71-90).

Brodersen, J., & Madsen, H. (2003). The effect of calcium concentration on the crushing resistance, weight and size of *Biomphalaria sudanica* (Gastropoda: Planorbidae). *Hydrobiologia,* 490, 181-186. https://doi.org/10.1023/A:1023495326473

Bukowski, S. J., & Auld, J. R. (2014). The effects of calcium in mediating the inducible morphological defenses of a freshwater snail, *Physa acuta*. *Aquatic Ecology,* 48(1), 85-90. https://doi.org/10.1007/s10452-013-9468-6

Cadée, G. C. (2011). *Hydrobia* as 'Jonah in the whale': Shell repair after passing through the digestive tract of shelducks alive. *Palaios,* 26(4), 245-249. https://doi.org/10.2110/palo.2010.p10-095r

Dalesman, S., Braun, M. H., & Lukowiak, K. (2011). Low environmental calcium blocks long-term memory formation in a freshwater pulmonate snail. *Neurobiology of Learning and Memory,* 95(4), 393-403. https://doi.org/10.1016/j.nlm.2010.11.017

Dantas, E. P. F., & Sant'Anna, B. S. (2021). The edible apple snail (*Pomacea dolioides* (Reeve, 1856)): Meat yield and sensorial evaluation. *International Food Research Journal,* 28(5), 953-959. https://doi.org/10.47836/ifrj.28.5.08

Darwin, C. H., & Padmavathi, P. (2018). Preliminary assessment of calcium in six molluscan shells of Tamilnadu coast, India. *Ecology, Environment and Conservation,* 24, 302-305. https://doi.org/10.13140/RG.2.2.30050.79048

Dauphin, Y., Cuif, J. P., Castillo-Michel, H., Chevallard, C., Farre, B., & Meibom, A. (2014). Unusual micrometric calcite–aragonite interface in the abalone shell *Haliotis* (Mollusca, Gastropoda). *Microscopy and Microanalysis,* 20(1), 276-284. https://doi.org/10.1017/S1431927613013718

De Paula, S. M., & Silveira, M. (2009). Studies on molluscan shells: Contributions from microscopic and analytical methods. *Micron*, 40(7), 669-690. https://doi.org/10.1016/j.micron.2009.05.006

De Paula, S. M., Huila, M. F. G., Araki, K., & Toma, H. E. (2010). Confocal Raman and electronic microscopy studies on the topotactic conversion of calcium carbonate from *Pomacea lineata* shells into hydroxyapatite bioceramic materials in phosphate media. *Micron*, 41(8), 983-989. https://doi.org/10.1016/j.micron.2010.06.014

Ebanks, S. C., O'Donnell, M. J., & Grosell, M. (2010). Characterization of mechanisms for Ca^{2+} and HCO_3^-/CO_3^{2-} acquisition for shell formation in embryos of the freshwater common pond snail *Lymnaea stagnalis*. *Journal of Experimental Biology*, 213(23), 4092-4098. https://doi.org/10.1242/jeb.045088

Estebenet, A. L., Martín, P. R., & Burela, S. (2006). Conchological variation in *Pomacea canaliculata* and other South American Ampullariidae (Caenogastropoda, Architaenioglossa). *Biocell*, 30(2), 329-335.

Fonseca, A. M., Hattori, G. Y., Costa, M. B., & Sant'Anna, B. S. (2017). Imposex in two apple snails of the Amazon. In B. S. Sant'Anna & G. Y. Hattori (Eds.), *Amazonian Apple Snails* (pp. 47-65). Nova Science Publishers.

Glass, N. H., & Darby, P. C. (2009). The effect of calcium and pH on Florida apple snail, *Pomacea paludosa* (Gastropoda: Ampullariidae), shell growth and crush weight. *Aquatic Ecology*, 43, 1085-1093. https://doi.org/10.1007/s10452-008-9226-3

Hüning, A. K., Lange, S. M., Ramesh, K., Jacob, D. E., Jackson, D. J., Panknin, U., Gutowska, M. A., Philipp, E. E. R., Rosenstiel, P., Lucassen, M., & Melzner, F. (2016). A shell regeneration assay to identify biomineralization candidate genes in mytilid mussels. *Marine Genomics*, 27, 57-67. https://doi.org/10.1016/j.margen.2016.03.011

Jesús-Navarrete, A., Sanchez, D. J. C. A., & Ortíz-Hernádez, C. (2023). Growth and shell hardness of the apple snail *Pomacea flagellata* Say, 1829, reared at three calcium concentrations. *PeerJ*, 11, e14840. https://doi.org/10.7717/peerj.14840

Kádár, E. (2008). Haemocyte response associated with induction of shell regeneration in the deep-sea vent mussel *Bathymodiolus azoricus* (Bivalvia: Mytilidae). *Journal of Experimental Marine Biology and Ecology*, 362(2), 71-78. https://doi.org/10.1016/j.jembe.2008.05.014

Kádár, E., Tschuschke, I. G., & Checa, A. (2008). Post-capture hyperbaric simulations to study the mechanism of shell regeneration of the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* (Bivalvia: Mytilidae). *Journal of Experimental Marine Biology and Ecology*, 364(2), 80-90. https://doi.org/10.1016/j.jembe.2008.07.028

Li, S., Liu, Y., Liu, C., Huang, J., Zheng, G., Xie, L., & Zhang, R. (2016). Hemocytes participate in calcium carbonate crystal formation, transportation and shell regeneration in the pearl oyster *Pinctada fucata*. *Fish & Shellfish Immunology*, 51, 263-270. https://doi.org/10.1016/j.fsi.2016.02.027

Liang, Y., Zhao, J., & Wu, C. (2010). The micro/nanostructure characteristics and the mechanical properties of *Hemifusus tuba* conch shell. *Journal of Bionic Engineering*, 7, 307-313. https://doi.org/10.1016/S1672-6529(10)60261-2

Limeira Jr., S. C. M., Rodrigues, S. C., & Ghilardi, R. P. (2023). Characterization of the cross-lamellar structure of *Olivancillaria urceus* (Gastropoda: Olividae) and its dissolution pattern. *Micron*, 166, 103416. https://doi.org/10.1016/j.micron.2023.103416

Liu, A. X., Jin, C., Li, H., Bai, Z., & Li, J. (2018). Morphological structure of shell and expression patterns of five matrix protein genes during the shell regeneration process in *Hyriopsis cumingii*. *Aquaculture and Fisheries*, 3(6), 225-231. https://doi.org/10.1016/j.aaf.2018.09.005

Liu, L. L., Zhao, S., Yang, J. E., Zhang, N. Q., Zhao, H., Wu, Z., He, T. M. Y., & Guo, J. (2017). Regeneration of excised shell by the invasive apple snail *Pomacea canaliculata*. *Marine and Freshwater Behaviour and Physiology*, 50(1), 17-29. https://doi.org/10.1080/10236244.2016.1261455

Liu, Y., Bai, Z., Li, Q., Zhao, Y., & Li, J. (2013). Healing and regeneration of the freshwater pearl mussel *Hyriopsis cumingii* Lea after donating mantle saibos. *Aquaculture*, *392-395*, 34-43. https://doi.org/10.1016/j.aquaculture.2013.01.035

Madsen, H. (1987). Effect of calcium concentration on growth and egg laying of *Helisoma duryi*, *Biomphalaria alexandrina*, *B. camerunensis* and *Bulinus truncatus* (Gastropoda: Planorbidae). *Journal of Applied Ecology*, 24(3), 823-836. https://doi.org/10.2307/2403983

Magalhães, A. C. S., Pinheiro, J., & Mello-Silva, C. C. (2011). A mobilização do cálcio em *Biomphalaria glabrata* exposta a diferentes quantidades de carbonato de cálcio. *Revista de Patologia Tropical*, 40(1), 46-55. https://doi.org/10.5216/rpt.v40i1.13916

Marin, F., & Luquet, G. (2004). Molluscan shell proteins. *Comptes Rendus Palevol*, 3(6-7), 469-492. https://doi.org/10.1016/j.crpv.2004.07.009

Martin, P. R., Estebenet, A. L., & Cazzaniga, N. J. (2001). Factors affecting the distribution of *Pomacea canaliculata* (Gastropoda: Ampullariidae) along its southernmost natural limit. *Malacologia*, 43(1), 13-23.

Meldrum, F. C. (2003). Calcium carbonate in biomineralisation and biomimetic chemistry. *International Materials Reviews*, 48(3), 187-224. https://doi.org/10.1179/095066003225005836

Melo, I. B., Hattori, G. Y., & Sant'Anna, B. S. (2017). Reproduction and substrate selection for oviposition of the gastropod *Pomacea dolioides* (Reeve, 1856). In B. S. Sant'Anna & G. Y. Hattori (Eds.), *Amazonian apple snails* (pp. 89-107). Nova Science Publishers.

Morrison, A. E., & Cochrane, E. E. (2008). Investigating shellfish deposition and landscape history at the Natia Beach site, Fiji. *Journal of Archaeological Science,* 35(8), 2387-2399. https://doi.org/10.1016/j.jas.2008.03.013

Nduku, W. K., & Harrison, A. D. (1976). Calcium as a limiting factor in the biology of *Biomphalaria pfeifferi* (Krauss), (Gastropoda: Planorbidae). *Hydrobiologia,* 49, 43-170. https://doi.org/10.1007/BF00772685

Ohta, T., & Saeki, I. (2020). Comparisons of calcium sources between arboreal and ground-dwelling land snails: Implication from strontium isotope analyses. *Journal of Zoology,* 311(2), 137-144. https://doi.org/10.1111/jzo.12767

Paschoal, L. R. P., & Oliveira, L. J. F. (2017). Histology and histochemistry of the testes in two Amazonian apple snails. In B. S. Sant'Anna & G. Y. Hattori (Eds.), *Amazonian apple snails* (pp. 127-144). Nova Science Publishers.

Pierre, S. M., Quintana-Ascencio, P. F., Boughton, E. H., & Jenkins, D. G. (2017). Dispersal and local environment affect the spread of an invasive apple snail (*Pomacea maculata*) in Florida, USA. *Biological Invasions*, 19, 2647-2661. https://doi.org/10.1007/s10530-017-1474-5

Pires-Júnior, A. N., Hattori, G. Y., & Sant'Anna, B. S. (2019). Effect of stock density of cultured Amazon apple snail *Pomacea dolioides* (Gastropoda: Ampullariidae) in Brazil. *Brazilian Journal of Animal Science*, 48, 1-8. https://doi.org/10.1590/rbz4820180053

Posch, H., Garr, A. L., Pierce, R., & Davis, M. (2012). The effect of stocking density on the reproductive output of hatchery-reared Florida apple snails, *Pomacea paludosa*. *Aquaculture*, 360-361, 37-40. https://doi.org/10.1016/j.aquaculture.2012.07.007

Rodríguez, F. V. I., & Carranza, M. M. (2007). Validación del cultivo semi-intensivo de caracol Tote (*Pomacea flagellata*), en el trópico húmedo. *AquaTIC*, (27), 16-30.

Silva, D., & Debacher, N. A. (2010). Caracterização físico-química e microestrutural de conchas de moluscos bivalves provenientes de cultivos da região litorânea da ilha de Santa Catarina. *Química Nova*, 33(5), 1053-1058. https://doi.org/10.1590/S0100-40422010000500009

Soído, C., Vasconcellos, M. C., Diniz, A. G., & Pinheiro, J. (2009). An improvement of calcium determination technique in the shell of molluscs. *Brazilian Archives of Biology and Technology*, 52(1), 93-98. https://doi.org/10.1590/S1516-89132009000100012

Suzuki, M., & Nagasawa, H. (2013). Mollusk shell structures and their formation mechanism. Canadian Journal of Zoology, 91(6), 349-366. https://doi.org/10.1139/cjz-2012-0333

Thomas, J. D., Benjamin, M., Lough, A., & Aram, R. H. (1974). The effects of calcium in the external environment on the growth and natality rates of *Biomphalaria glabrata* (Say). *Journal of Animal Ecology*, 43(3), 839-860. https://doi.org/10.2307/3539

Trinkler, N., Jean-François, B., Frédéric, M., Maylis, L., Jolivet, A., Philippe, C., & Christine, P. (2011). Mineral phase in shell repair of Manila clam *Venerupis philippinarum* affected by brown ring disease. *Diseases of Aquatic Organisms*, 93, 149-162. https://doi.org/10.3354/dao02288

Trinkler, N., Sinquin, G., Querne, J., & Paillard, C. (2010). Resistance to brown ring disease in the Manila clam, *Ruditapes philippinarum*: A study of selected stocks showing a recovery process by shell repair. *Journal of Invertebrate Pathology*, 104(1), 8-16. https://doi.org/10.1016/j.jip.2009.12.007

Tunholi, V. M., Lustrino, D., Tunholi-Alves, V. M., Garcia, J. S., Mello-Silva, C. C. C., Maldonado, J. R. A., & Rodrigues, M. (2011). Influence of *Echinostoma paraensei* (Lie and Basch, 1967) infection on the calcium content in *Biomphalaria glabrata* (Say, 1818). *Experimental Parasitology*, 129(3), 266-269. https://doi.org/10.1016/j.exppara.2011.07.016

Watson, A. M., & Ormerod, S. J. (2004). The distribution of three uncommon freshwater gastropods in the drainage ditches of British grazing marshes. *Biological Conservation*, 118(4), 455-466. https://doi.org/10.1016/j.biocon.2003.09.021

Yang, S., Ni, L., Zhao, L., Yang, J., Liu, Q., Zhang, J., He, Z., & Peng, S. (2016). Repair process and enzymatic activity associated with induction of shell regeneration in the invasive species. *Molluscan Research*, 36(3), 207-212. https://doi.org/10.1080/13235818.2015.1128603