



OPEN

Ca²⁺ addition facilitates the shell repair with eggs production of *Pomacea canaliculata* through biomineralization and food intaking regulation

Yingtong Chen^{1,2,3,4}, Fucheng Yao^{1,2,3,4}, Jiaen Zhang^{1,2,3,4}✉, Chunxia Zhang^{1,2,3,4}, Zhong Qin^{1,2,3,4} & Jing Guo⁵

Pomacea canaliculata was by far one of the most harmful invasive organisms in the world, causing serious harm to aquatic crops and ecosystem. Calcium carbonate is a common component of aquatic environment, which is important for the growth of *Pomacea canaliculata*. Therefore, the objective of this study was to investigate the response characteristics of *P. canaliculata* suffered shell breakage to the addition of calcium carbonate in water environment. In this experiment, we explored the effects of calcium carbonate addition on the *P. canaliculata* shell repair rate, food intake, egg production, shell strength, and calcium content through breaking the snails shell and the addition of calcium carbonate treatment. The results showed that snail broken-shell repaired mostly within 21 days. The snails experienced a significant increase in shell repair rates during earlier days of the treatment, especially for female snails. Food intake of snails exhibited different patterns when their shells were broken and calcium carbonate was added. Shell breakage treatment combined with calcium carbonate addition significantly increased the diameter of snail eggs compared with the control and the calcium carbonate addition treatment without shell-broken snail group. There was no significant difference in shell strength or calcium content of male snails between the treatments. The study suggests that *P. canaliculata* exhibits a sex-dependent response pattern when subjected to shell damage and calcium carbonate addition. The findings can provide some references to better understand the invasion mechanism and survival strategy of the *P. canaliculata*.

Pomacea canaliculata is a globally invasive snail species. This species was introduced to China from Argentina in the 1980s for economic purposes as a food source. However, these snails were subsequently abandoned in natural aquatic environments and rice fields due to their unpalatability and their role as carriers of the pathogenic parasite *Angiostrongylus cantonensis*. This situation resulted in significant agricultural losses in southern China, particularly in rice production¹. The remarkable adaptability of *P. canaliculata* populations to their surroundings enables them to proliferate within aquatic ecosystems, posing a substantial threat to local biodiversity².

Currently, snail control primarily relies on chemical methods, which unfortunately result in significant ecological harm and the snail's pesticide resistance due to prolonged use of chemicals. In recent years, notable progress has been made in the biological management of *P. canaliculata*, taking advantage of its natural predators, a strategy well-suited for China with its prevalent biodiversity. The majority of these predators target the snails by breaking their shells and consuming the soft tissues within the shells. Some researchers observed that the size of the mouth in *Trachemys scripta elegans* correlates directly with the size of the snails they prey upon. Certain predators are restricted to prey on smaller snails due to limitations in their biting power and size^{3,4}.

¹Guangdong Provincial Key Laboratory of Eco-Circular Agriculture, South China Agricultural University, Guangzhou 510642, China. ²Department of Ecology, College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China. ³Guangdong Engineering Research Center for Modern Eco-Agriculture and Circular Agriculture, Guangzhou 510642, China. ⁴Key Laboratory of Agro-Environment in the Tropics, Ministry of Agriculture and Rural Affairs, Guangzhou 510642, China. ⁵Henry Fok School of Biology and Agriculture, Shaoguan University, Shaoguan 512005, China. ✉email: jeanzh@scau.edu.cn

Consequently, the strength of the snail's shell emerges as a pivotal factor influencing the success of predation by its natural enemies.

The shell is the result of biomineralization, composed primarily of minerals and organic molecules, with calcium carbonate constituting 90% of its composition^{5,6}. Biomineralization is a meticulously orchestrated process in which the mantle assumes a crucial role⁷. The mantle of mollusks promotes calcium accumulation during shell formation, overseeing neurosecretion to regulate shell growth by secreting an organic matrix that aids in calcium carbonate deposition, a pivotal factor in shell formation^{8,9}. Microscopic examinations by researchers revealed that the molluscan mantle undergoes a series of metamorphic stages. Notably, the juvenile phase emerges as a critical period for mantle development in mollusk growth. Furthermore, observations showed enhanced mitochondrial and endoplasmic reticulum development in the epithelial cells at the mantle's periphery, with the central region characterized by increased cytoplasm and fewer organelles. This suggests that the reparative capacity of the mantle's edge surpasses that of the central region, with repair rates potentially influenced by environmental conditions^{10,11}.

Shell repair rate serves as a vital metric for evaluating the snail's proficiency in shell restoration¹². Measuring the shell repair rate allows us to discern the speed and effectiveness of shell restoration within various treatment groups¹³. Feeding rate, quantifying the snails' food consumption, serves as an indicator of their activity and growth status^{14,15}. Comparative analysis of feeding rates among distinct treatment groups facilitates an examination of the feeding patterns and food utilization capabilities of *P. canaliculata* subjected to shell breakage stress.

Egg diameter serves as a key metric for evaluating the reproductive capacity of *P. canaliculata*^{16,17}. Measuring egg diameter provides insights into the reproductive strategies and fertility of snails subjected to shell breakage stress in various treatment groups¹⁸. Shell strength and calcium content serve as indicators for assessing shell quality and calcium metabolism in snails¹⁹. Comparative analysis of shell strength and calcium content among distinct treatment groups enables us to understand changes in shell quality and calcium metabolism in snails exposed to shell breakage stress^{20,21}.

Environmental factors exert a profound influence on mollusks' biomineralization process, especially concerning shell repair. Biomineralization is a meticulously regulated process, characterized by intricate interactions among diverse internal factors²². Calcium ions play a pivotal role in biomineralization and hold particular significance in mollusk shell repair. Environmental calcium ion concentrations directly influence the accessibility of calcium ions to mollusks, its elevation enhances the mollusks' capacity to absorb and utilize calcium ions, thereby expediting shell repair and formation²³.

Temperature, pH, nutrient availability, salinity, environmental pollution, and oxidative stress are factors that exert influence on the biomineralization process^{24–29}. The cumulative impact of environmental factors on mollusks' biomineralization process is intricate and substantial. Favorable environmental conditions can facilitate the seamless advancement of biomineralization, whereas adverse conditions may hinder shell repair and growth, consequently impacting mollusks' survival and reproductive abilities^{30–36}.

In this study, we devised four distinct experimental treatments to explore the adaptive strategies of *P. canaliculata* when subjected to shell breakage stress. We conducted tests on the shell repair rate, food intake, egg diameter, shell strength, and calcium content of the snails. Calcium carbonate, a pivotal contributor to the shell formation process of *P. canaliculata*, was intentionally selected as a variable. Thus, by manipulating the addition of calcium carbonate and the snail shell breakage treatment, we aimed to gain a more comprehensive understanding of snail adaptation and response to shell repair under stressful conditions. Furthermore, monitoring changes in calcium content in *P. canaliculata* can elucidate the regulatory mechanisms of biochemical substances necessary for the shell repair process. Examining the relationships between these indicators can assist us in unveiling the ecological adaptability of *P. canaliculata* in response to shell breakage stress and in substantiating the presence of their resilient survival mechanisms and regulatory processes.

Materials and methods

Experimental materials

We gathered all tested *P. canaliculata* individuals from irrigation ditches on a farm situated at South China Agricultural University in Guangzhou, Guangdong Province, China (23° 14' N, 113° 38' E). The snails were then reared in plastic aquaria measuring 45 cm × 35 cm × 35 cm, submerged to water with an approximate depth of 20 cm. They were provided with daily feedings of fresh lettuce within a climate-controlled indoor balcony.

Experimental Performance

Following several days of rearing, we randomly selected healthy adult *P. canaliculata* of comparable size (shell height 25 ± 5 mm, weight 5.3 ± 0.5 g). After differentiating males and females, we marked their shells using a waterproof pen. We utilized twelve plastic aquariums, each filled with 15 cm of water and containing 10 male and 10 female snails. We employed analytical grade (AR) calcite powder as the source of calcium carbonate, adding 15 g per day. The treatments comprised a shell-broken snail group with calcium carbonate addition, a shell-broken snail group without calcium carbonate addition, a calcium carbonate addition treatment without shell-broken snail group, and a blank control snail group without shell-broken treatment and calcium carbonate addition. We installed screens on the water surface to prevent the escape of *P. canaliculata* and promptly removed any deceased snails. We provided an ample daily supply of lettuce as the primary food source for the snails. We replaced water in plastic aquariums using tap water weekly, retaining approximately 3/4 of the original volume to maintain water clarity. We measured the water temperature daily at 16:00 using a thermostat positioned approximately 5 cm below the water surface with an accuracy of 0.1 °C. Eggs produced on the same day were promptly collected. The experiment spanned 21 days, during which the snails were fed once daily at 16:00, and

any remaining lettuce was removed before the subsequent feeding. The experimental treatments and groupings are shown in Table 1.

Shell strength measurement

Dissecting scissors and a scalpel were employed to delicately separate the snail's shell from its flesh without causing damage. We utilized an Edbaure pressure testing machine equipped with a digital display push-pull meter, possessing an accuracy of 0.1 N, to ascertain the shell's compressive capacity. For testing, the shell was positioned flat on the platform with the operculum facing downward. Subsequently, the machine applied pressure to the shell until it fractured, at which point the operation was terminated. The numerical value displayed at this juncture represented the shell's strength.

Shell repair observation and calculation

Photographs of each shell-broken snail were taken at the time points of 0, 2nd, 4th, 6th, 8th, 11th, and 21st day. Subsequently, the width of the clipped shell and height of the shell repair was quantified using Vernier calipers, after which the snails were returned to their respective aquariums. The shell repair area and repair rate are calculated according to the following equations:

$$\begin{aligned} \text{Area of repair (AR)} &= W \times H \\ \text{Repair rate (\%)} &= 100\% \times (\text{ARl} - \text{ARf})/\text{Act}; \end{aligned}$$

“W” indicates the width of the clipped shell; “H” indicates the height of the shell repair; “ARl” indicates the area of shell repair for the latter measurement; “ARf” indicates the shell repair area of the former measurement; “Act” denotes the area of the shell cutted off at the beginning of the test.

Measurement of snail eggs

We collected fresh egg masses daily, recorded their weight and quantity, and individually stored each egg mass in a dedicated container. After the experiment concluded, we immersed the egg masses in a 2% NaOH solution for 1.5 h. We measured the diameter (in millimeters) of each egg by randomly selecting 10 eggs and assessing their dimensions with a Vernier caliper (Model: DL91150).

Measurement of food intake

Before feeding, excess water on the surface of lettuce was removed. Feeding amounts were recorded on days 2, 3, 8, 12, and 19 following the experimental treatment. Fresh lettuce, pre-weighed, was provided to each experimental group. After 24 h, the remain lettuce was collected, drained, and weighed again to calculate the snails' food intake. The food intake for each group was determined in relation to the survival count.

$$\begin{aligned} \text{Total food intake (g)} &= \text{Total weight of the lettuce fed (g)} - \text{Total weight of the remain lettuce next day (g)}; \\ \text{Food intake (g)} &= \text{Total food intake (g)} / \text{Number of snail survivors on the day}. \end{aligned}$$

Measurement of calcium and protein content

We assessed calcium content and protein concentration using kits from Nanjing Jiancheng Bioengineering Institute. Calcium content was determined using the methyl thymol blue method, while total protein concentrations (TP) were determined via the Bicinchoninic Acid (BCA) method^{37,38}. The procedures were conducted as follows: (1) preparation of the test solution: accurately weighed mantle tissue (100 mg) was mixed with physiological saline at a ratio of weight (g):volume (mL)=1:9. The mixture was homogenized under ice-water bath conditions at 2500 rpm and centrifuged for 10 min. The resulting supernatant was retained for testing or further dilution. (2) Calcium and protein content determination: followed the kit's specified procedures.

$$\text{The calcium content (mmol/gprot)} = \frac{A(\text{Treatment}) - A(\text{Blank})}{A(\text{Standard}) - A(\text{Blank})} \times C(\text{Standard}) / \text{TP}$$

$$\text{TP (g/L)} = \frac{A(\text{Treatment}) - A(\text{Blank})}{A(\text{Standard}) - A(\text{Blank})} \times C(\text{Standard}) \times N$$

A(Treatment): tested data for each treatment group. A(Blank): tested data for blank group. C(Standard): standard concentration, 1 mmol/L. TP: total protein content. N: pre-test dilution times of a sample.

Number	Group	Treatment
1	SC	A shell-broken snail group with calcium carbonate addition
2	S	A shell-broken snail group without calcium carbonate addition
3	C	Calcium carbonate addition treatment without shell-broken snail group
4	CK	Blank control snail group without shell-broken treatment and calcium carbonate addition

Table 1. Experimental grouping and treatment.

Statistical analysis

Statistical analyses were conducted using SPSS version 20.0. Normality analysis was performed on male and female snails within the same treatment and at the same time. To assess the impact of calcium carbonate and shell breakage on shell repair in *P. canaliculata*, we employed repeated-measures ANOVA and Duncan's multiple comparisons to analyze the repair rates, food intake, shell strength and calcium content across various treatments. Additionally, we analyzed the differences in shell repair rates, shell strength and calcium content between male and female snails under the same treatments at the end of the experiment using t-tests. Graphs were created using Origin 8.0. Significance was set at $P < 0.05$, or at $P < 0.01$.

Result

Shell repair rates of *P. canaliculata* in different treatments

The snail shell repair rate exhibited an initial increase followed by a gradual decline, reaching its lowest point at day 21 under both treatments (Fig. 1). Notably, only in the fourth day, we observed significant differences in female snails between the two treatments, with the SC snail group showing significantly higher values than the S group ($P < 0.05$). In the S group, both males and females displayed an average repair area of $1.0 \pm 0.02 \text{ mm}^2$ per day. On the day 2, this rate was 14.2% for males and 16.8% for females, followed by an upward trend in the early period, peaking at 17.6% for males and 16.6% for females on day 6. Subsequently, a significant decrease occurred until the end of the experiment ($P < 0.05$), with rates on the final day at 1.4% for males and merely 0.6% for females. In the meantime, the SC group displayed a pattern of increase followed by decrease in both males and females, with identical average repair area of $0.9 \pm 0.02 \text{ mm}^2$ per day. In this group, the rate significantly increased ($P < 0.05$) from the 2nd to the 4th day, with 9.6% for males and 13.9% for females on the second day, and peaking at 20.9% and 18.6% on the fourth day, respectively. The repair rates exhibited a sharp decrease on the final day, at 1.2% for males and 1.4% for females ($P < 0.01$). Repeated-measures ANOVA results indicated no significant effect of calcium carbonate addition on the rate of shell repair ($F_{1,8} = 0.018$, $P > 0.05$).

Food intake of *P. canaliculata* in different treatments

The food intake of the *P. canaliculata* substantially exhibited a "first increasing, then decreasing" trend over time in C and CK groups (Fig. 2). In the CK group, the feeding rate significantly increased on day 8 but decreased from day 8 to the end of the experiment. Similarly, the C group displayed a significant increase in food intake from the day 3 to day 12, followed by a subsequent decrease. In S group, food intake showed a change trend of "first increasing, then keeping stable, finally decreasing" with the highest cumulative feeding amount. In the SC group, food intake kept a relative stable level, while it reached a peak with amount of 1.59 g on day 8. Notably, there were no significant differences in food intake between the CK and C groups during the first two days of the experiment. Repeated-measures ANOVA indicated a significant impact of the interaction between calcium carbonate addition and shell breakage on the food intake of snails ($F_{1,8} = 7.453$, $P < 0.05$).

Egg production of the *P. canaliculata* in different treatments

Throughout the experiment period, the CK snail group produced 2 egg masses, each containing an average of 25.0 ± 4.0 eggs; the C group also produced 2 egg masses, with an average of 79.5 ± 40.5 eggs per mass; the S group yielded 4 egg masses, averaging 62.5 ± 12.6 eggs per mass, while the SC group produced 6 egg masses, each containing an average of 65.83 ± 24.4 eggs. The average egg diameters were $1.94 \pm 0.03 \text{ mm}$, $1.85 \pm 0.06 \text{ mm}$, $2.23 \pm 0.04 \text{ mm}$, and $2.60 \pm 0.02 \text{ mm}$ in the CK group, C group, S group, and SC group, respectively (Fig. 3). Notably, the egg diameter in the SC group was significantly larger than that in the CK and C groups. Repeated-measures ANOVA revealed a highly significant change in egg diameter due to shell breakage ($F_{1,16} = 164.574$, $P < 0.001$), indicating that the snails with damaged shells produced larger eggs in diameter than those with unbroken shells. Additionally, the input of calcium carbonate led to a significant difference in egg diameter ($F_{1,16} = 7.801$, $P < 0.05$).

Shell strength of *P. canaliculata* in different treatments

A partially significant variation in snail shell strength was observed among the female groups (Fig. 4). The range of shell strength for male snails across all four treatments was from 57 to 75 N. Female snails, on the other hand, exhibited the highest shell strength of 80 N in the C group, which was significantly greater than the S group (53 N) and the SC group (56 N). Interestingly, there were no significant differences in shell strength between male and female snails within the same treatment. Repeated-measures ANOVA indicated the absence of significant differences in shell strength related to calcium carbonate addition ($F_{1,40} = 0.081$, $P > 0.05$), shell breakage treatment ($F_{1,40} = 1.286$, $P > 0.05$), and gender ($F_{1,40} = 0.037$, $P > 0.05$).

Calcium content of *P. canaliculata* in different treatments

The total protein concentration of snails under all the treatments was determined using the BCA method (Table 2). Calcium content in the mantle under each treatment is presented in Fig. 5. In the CK group, male snails had a mantle calcium content of $0.94 \pm 0.35 \text{ mmol/g prot}$, while female snails had $0.64 \pm 0.10 \text{ mmol/g prot}$. Male snails in the C group exhibited the lowest calcium content at $0.52 \pm 0.15 \text{ mmol/g prot}$. Notably, female snails in the SC treatment had significantly higher mantle calcium content than those in the other three treatment groups after 21 days of treatment. Repeated-measures ANOVA indicated no significant differences in calcium content caused by gender ($F_{1,40} = 0.004$, $P > 0.05$), shell breakage ($F_{1,40} = 0.776$, $P > 0.05$), or calcium carbonate addition ($F_{1,40} = 0.132$, $P > 0.05$).

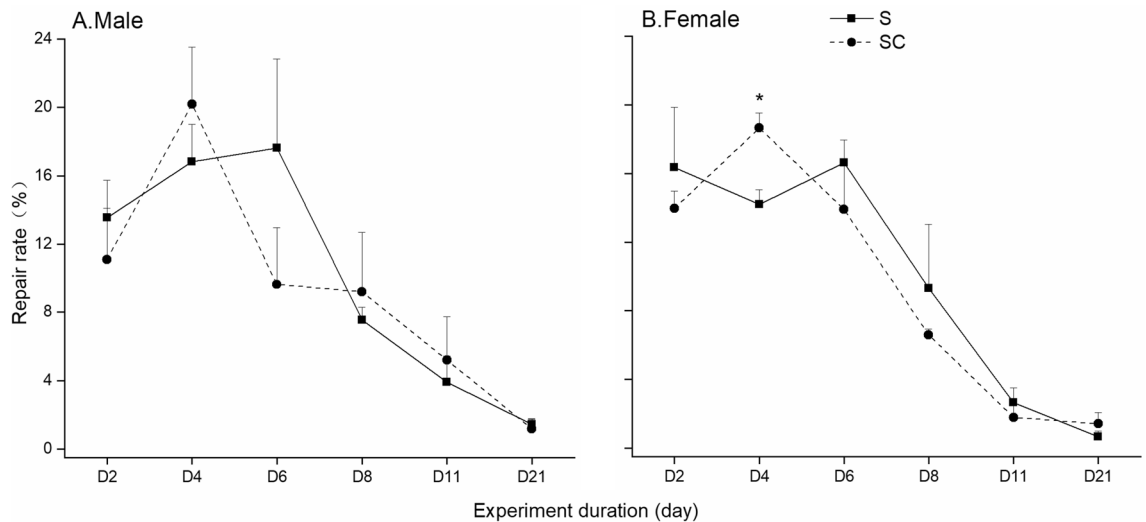


Figure 1. Shell repair rate of *P. canaliculata* in different treatments with different genders. SC and S refer to shell-broken snail group with calcium carbonate addition, and shell-broken snail group without calcium carbonate addition, C and CK refer to calcium carbonate addition treatment without shell-broken snail group and blank control snail group without shell-broken treatment and calcium carbonate addition, respectively. *Indicates significant differences between treatments at the same time.

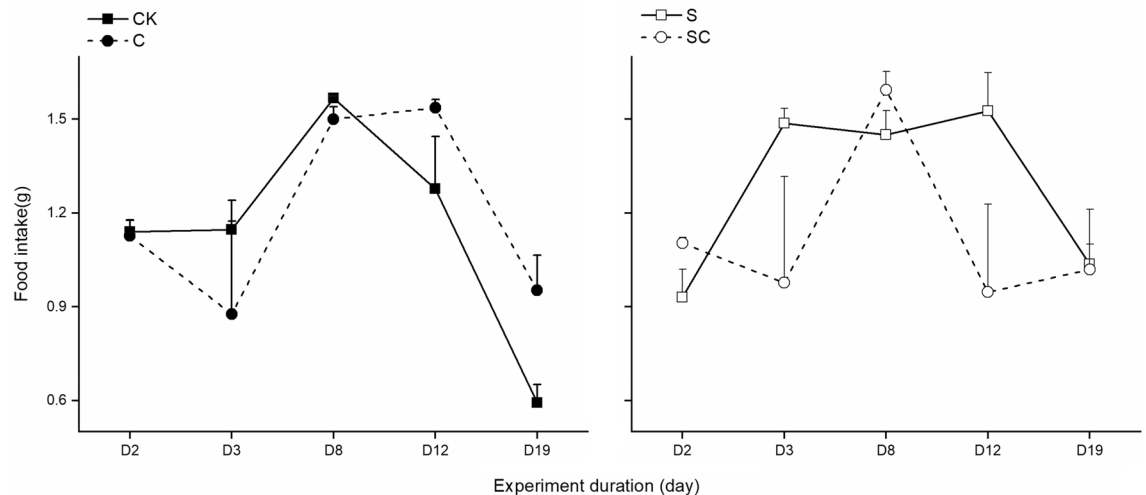


Figure 2. Food intake of *P. canaliculata* under different treatments. SC and S refer to shell-broken snail group with calcium carbonate addition, and shell-broken snail group without calcium carbonate addition, C and CK refer to calcium carbonate addition treatment without shell-broken snail group and blank control snail group without shell-broken treatment and calcium carbonate addition.

Discussion

As depicted in Fig. 6, the broken-shell repair process and egg production of *P. canaliculata* are affected and regulated by several important factors, such as food and calcium supplies and other environmental conditions. In our study, the addition of calcium carbonate initially resulted in an increase in the snail shell repair rate, followed by a subsequent decrease. This trend can be attributed to the initial boost in calcium availability, facilitating the shell repair process. However, the repair process may reach a saturation point over time, limiting further improvement in the repair rate³⁹. Furthermore, excessive calcium carbonate supplementation could adversely affect the snails by disrupting their acid–base balance or inducing toxic effects, ultimately reducing the shell repair rate⁴⁰.

In the present study, we observed that shell-broken snails without calcium carbonate addition treatment had a higher level of food intake than those with calcium carbonate addition treatment. The variations in the food intake of snails subjected to both shell damage and calcium carbonate addition may be attributed to several reasons. Firstly, it is likely that the shell damage induced stress responses in the snails, changing their usual feeding behavior⁴¹. In order to repair the broken shell, the snails needed to eat more lettuce to supply calcium

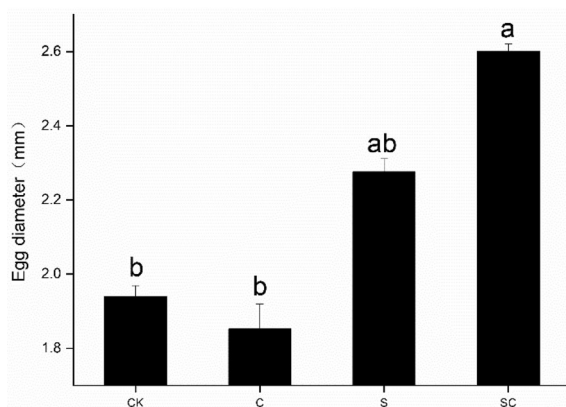


Figure 3. Egg diameter of *P. canaliculata* under different treatments. SC and S refer to shell-broken snail group with calcium carbonate addition, and shell-broken snail group without calcium carbonate addition, C and CK refer to calcium carbonate addition treatment without shell-broken snail group and blank control snail group without shell-broken treatment and calcium carbonate addition. Different lowercase letters in the graph indicate significant differences between different treatments.

and offset the calcium deficiency when no additional calcium was input. Meanwhile, the shell-broken female snails had higher calcium content in the calcium carbonate addition treatment than that in the treatment without calcium carbonate addition, further verifying that the injured snails can directly intake calcium in the water, which may subsequently reduce their feeding amount. Secondly, the addition of calcium carbonate could lead to alterations in water chemistry, including an increase in pH, which may also affect snails feeding⁴².

In the snail group with both shell breakage and calcium carbonate addition treatment, a significant increase in egg diameter was observed. The combination of shell breakage and calcium addition can influence the physiological processes of *P. canaliculata*, impacting egg development and growth. Shell breakage likely initiates a cascade of responses within the organism, including the activation of cell proliferation and differentiation, particularly during egg development. Calcium, being a pivotal component of snail shells, plays a crucial role; with a sufficient calcium supply, snails can utilize calcium more effectively for egg shell development and reinforcement, resulting in larger egg diameters^{43–46}. The augmentation in egg size may serve as an adaptive response, making it more challenging for predators to prey on the larger eggs, as observed in other species like *Diatraea saccharalis*, *Pomacentridae*, and *Trematomus bernacchii*^{47–49}. Nonetheless, further investigations are required to unveil the related mechanisms governing this phenomenon. When exposed to the stress of shell damage, *P. canaliculata* likely undergoes a series of physiological and biochemical responses, including the activation of specific biochemical pathways to cope with external pressures^{50–52}. These responses can lead to alterations in body fluid composition, subsequently affecting egg development and size. Additionally, the input of calcium carbonate serves as an additional source of calcium, facilitating the process of egg calcification¹⁹. Tahara *et al.* conducted experiments comparing body fluid composition and egg size across different environments⁵³. Further research

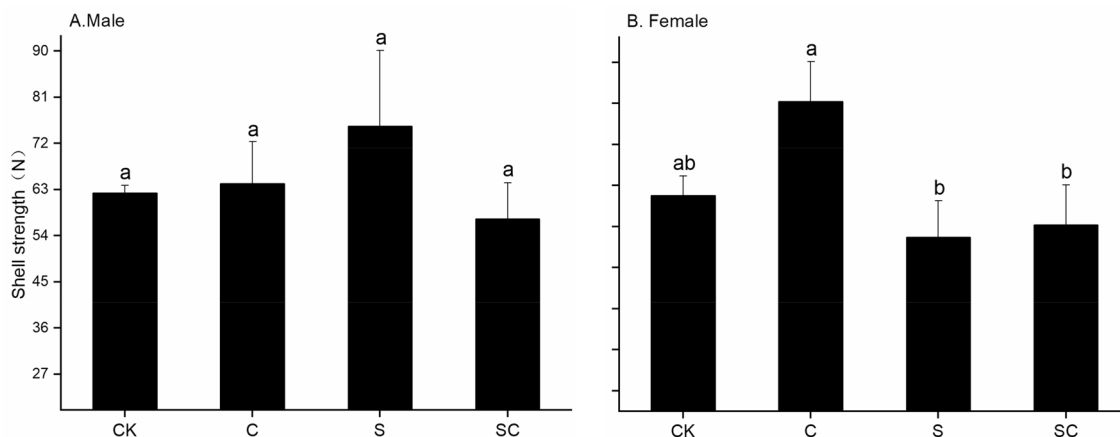


Figure 4. Shell strength of different sexes of *P. canaliculata* under different treatments. SC and S refer to shell-broken snail group with calcium carbonate addition, and shell-broken snail group without calcium carbonate addition, C and CK refer to calcium carbonate addition treatment without shell-broken snail group and blank control snail group without shell-broken treatment and calcium carbonate addition. Different lowercase letters in the graphs indicate significant differences under the same sex between different treatments.

Sex	Sample size	CK	C	S	SC
Male	6	2.81 ± 0.54	8.73 ± 5.54	2.33 ± 0.58	2.62 ± 0.53
Female	6	2.70 ± 0.46	1.85 ± 0.21	6.83 ± 4.66	1.85 ± 0.29

Table 2. Total protein content (TP) of *P. canaliculata* mantle under different treatments (g/L).

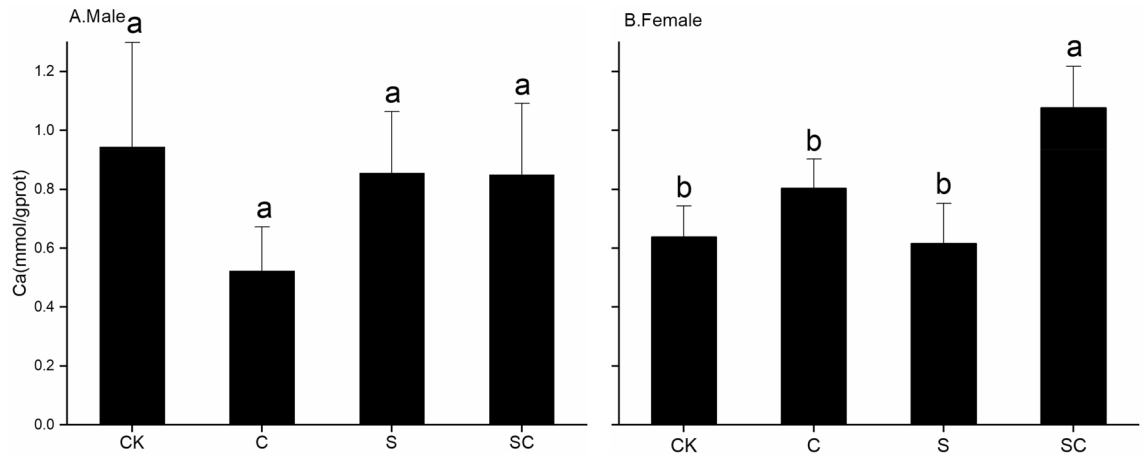


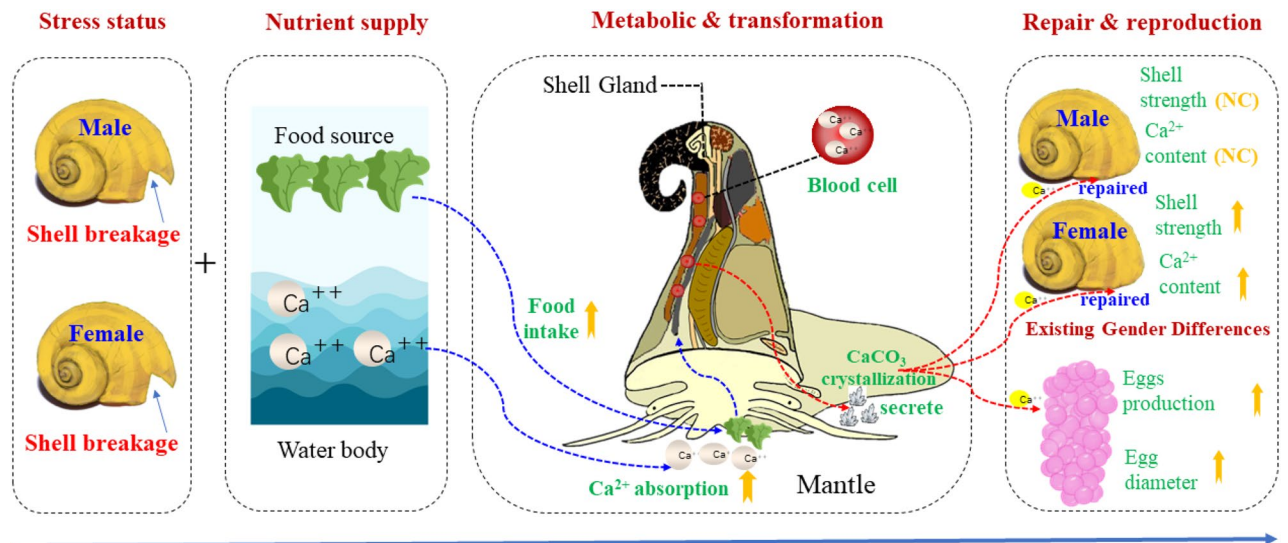
Figure 5. Calcium content of *P. canaliculata* under different treatments. Different lowercase letters indicate significant differences under the same sex between different treatments. SC and S refer to shell-broken snail group with calcium carbonate addition, and shell-broken snail group without calcium carbonate addition, C and CK refer to calcium carbonate addition treatment without shell-broken snail group and blank control snail group without shell-broken treatment and calcium carbonate addition.

is essential to comprehensively investigate changes in the body fluid composition of injured *P. canaliculata* and to identify the signaling molecules involved in regulating egg size.

Among female snails, shell strength exhibited significant differences among the most treatments. The snail group without broken shells but treated with calcium carbonate addition had significantly highest shell strength, the reason would be that the supplementation of calcium carbonate contributes to increase calcium content and shell density of snails, thereby fortifying shell strength⁵⁴. The control group, having undergone no specific treatment, demonstrated intermediate shell strength. Shell damage treatments likely influenced the integrity and structure of snail shells, resulting in reduced shell strength, but the shell-broken snail group with calcium carbonate addition showed an increasing trend in shell strength, relative to the shell-broken snail group without calcium carbonate addition, which may also indicate the contribution of calcium carbonate addition. Conversely, there were no significant differences in shell strength and calcium content among male snails across the various treatments. This variance between female and male snails might be attributed to the pronounced influence of hormonal factors and reproductive activities on snail shell strength and calcium content.

The shell repair capacity of mollusks has a direct bearing on their susceptibility to pests and diseases. In laboratory rearing conditions, all individuals of *Buccinum undatum* completely restored their damaged shells within 30 days⁵⁵. *Pinctada fucata* exhibited significant shell repair within 5–20 days following shell edge damage⁵⁶. The mantle, vital for mollusk biomineralization, is comprised of epithelial cells capable of secreting essential inorganic ions such as Ca^{2+} and CO_3^{2-} . It plays a pivotal role in snail shell regeneration⁵⁷. The mantle is supposed to promptly allocate resources and energy for repairing damaged shells and tissues when breakage occurs. Snails initiate the growth of new shells as early as 1 day after shell breakage, often completely covering the damaged area within 5–10 days. The repair rate is notably high in the early stages but gradually diminishes as shell repair progresses. Notably, during the experiment, the addition of calcium to the water environment led to a more pronounced increase in the beginning of shell repair process, likely due to the facilitation of the biomineralization process through calcium intake by snails, thereby improving the repair rate, especially for female snails.

After suffered shell injuries, gastropods usually better allocate energy and safeguard themselves from environmental threats to minimize further harm and potential mortality when their delicate soft tissues become exposed⁵⁸. The repair process is intricately governed by biomolecules, and in the case of oysters, shell matrix proteins (SMPs) play a pivotal role in regulating CaCO_3 crystallization in vitro. However, the composition of the newly repaired shell differs from that of the original one¹⁷. Mollusks invest substantial energy and resources in shell repair, affecting their overall metabolic functioning during the post-injury period⁵⁹. Spawning represents a critical stage in snail life history, with snails strategically allocating energy based on environmental nutrient content to enhance population stability and expansion⁶⁰. In the present study, we observed a close relationship between Ca^{2+} addition and egg diameter, with females producing more offspring in response to shell breakage to ensure population growth. However, our study solely investigate the calcium content of snails under one-time shell breakage stress with calcium carbonate addition and subsequent repair process in laboratory. Therefore,



Ca²⁺ involved repair process of the broken shell and egg production of the snail

Figure 6. Ca²⁺ involved repair process of the broken shell and egg production of *P. canaliculata*. The blue dashed curved lines indicate the input process of materials. The red dashed curved lines indicate the output process of materials. The up arrows near the parameters mean the increase of values. The “NC” near the parameters means no change.

further research is warranted to elucidate the effects of the frequent shell breakage and repair process on snail population development, growth, and invasion in fields. In addition, exploring related mechanisms, such as gene expression, is imperative for a comprehensive understanding of this phenomenon.

Conclusion

This study showed that when *P. canaliculata* facing with shell damage, the addition of calcium carbonate in water increased the shell repair rate of female snails in the earlier period, and then the rate gradually decreased. Food intake of *P. canaliculata* exhibited different patterns when the shell was broken and calcium was added, which is affected by the interaction between calcium carbonate addition and shell breakage. The addition of calcium resulted in a significant increase in calcium content in the mantle of females with shell breakage, which resulted in the increase of the shell strength, amounts and size of eggs, but no difference for males with shell breakage among the treatments. Therefore, snails are able to repair themselves rapidly through the biomineralization of Ca²⁺ in the water environment and food intake after suffered shell damage and are able to maintain the stability of their populations and the adoption of different survival strategies. This findings may provide some references for probing invasion mechanism of *P. canaliculata* and help to implement a more targeted prevention and control on them.

Data availability

All data generated or analysed during this study are included in this published article.

Received: 14 February 2023; Accepted: 19 September 2023

Published online: 23 October 2023

References

- Zhou, X. M. *et al.* The complete mitochondrial genome of *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Mitochondrial DNA Part A* **27**(2), 884–885 (2016).
- Silva, W. D. *et al.* Potential spreading risk of an invasive snail species (*Pomacea canaliculata*) in freshwater habitats of Asia. *Curr. Sci.* **117**, 6 (2019).
- Jia-en, G. J. Z. Status, problems and countermeasures in biological control of *Pomacea canaliculata*. *Chin. J. Ecol.* **34**(10), 2943–2950 (2015).
- Xue, G. J. Z. Predation and indirect interference effects of *Trachemys scripta elegans* on *Pomacea canaliculata*. *J. South China Agric. Univ.* **37**(06), 59–64 (2016).
- Yang, S. Z. L. *et al.* A comparative study of haemocytes from different shell colours of golden apple snails (*Pomacea canaliculata*). *Sichuan J. Zool.* **32**(05), 722–728 (2013).
- Liu, Q., Yang, S., Zhao, L.-L., Yang, S.-Y. & Zhang, J.-E. Fecundity of exotic golden apple snail, *Pomacea canaliculata*, in Sichuan. *Chin. J. Ecol.* **33**(04), 1042–1046 (2014).
- Jolly, C. *et al.* Zona localization of shell matrix proteins in Mantle of *Haliotis tuberculata* (Mollusca, Gastropoda). *Mar. Biotechnol.* **6**(6), 541–551 (2004).
- Saleuddin, A. S. & Dillaman, R. M. Direct innervation of the mantle edge gland by neurosecretory axons in *Helisoma duryi* (Mollusca: Pulmonata). *Cell Tissue Res* **171**(3), 397–401 (1976).
- Huang, J. *et al.* Mantle tissue in the pearl oyster *Pinctada fucata* secretes immune components via vesicle transportation. *Fish. Shellf. Immunol.* **121**, 116–123 (2022).

10. Audino, J. A. *et al.* Mantle margin morphogenesis in *Nodipecten nodosus* (Mollusca: Bivalvia): New insights into the development and the roles of bivalve pallial folds. *BMC Dev. Biol.* **15**, 22 (2015).
11. Fang, Z. *et al.* Investigation of cell proliferation and differentiation in the mantle of *Pinctada fucata* (Bivalve, Mollusca). *Mar. Biol.* **153**(4), 745–754 (2007).
12. Fernández, M. S. *et al.* Is the snail shell repair process really influenced by eggshell membrane as a template of foreign scaffold?. *J. Struct. Biol.* **196**(2), 187–196 (2016).
13. Nkansah, M. A., Agyei, E. A. & Opoku, F. Mineral and proximate composition of the meat and shell of three snail species. *Heliyon* **7**(10), e08149 (2021).
14. Zhang, P. *et al.* Metal resistant gut microbiota facilitates snails feeding on metal hyperaccumulator plant *Sedum alfredii* in the phytoremediation field. *Ecotoxicol. Environ. Saf.* **236**, 113514 (2022).
15. Pang, C. *et al.* Effects of sediment-associated copper to the deposit-feeding snail, *Potamopyrgus antipodarum*: A comparison of Cu added in aqueous form or as nano- and micro-CuO particles. *Aquat. Toxicol.* **106–107**, 114–122 (2012).
16. Xu, Y. X., *et al.* A multiscale feature fusion method for automatic detection of eggs from two *Pomacea* spp. In *UAV Aerial Images. IEEE Geoscience and Remote Sensing Letters*, Vol. 19 (2022).
17. Li, F. *et al.* Glacial-interglacial evolution of seasonal cooling events documented by land-snail eggs from Chinese loess. *Quatern. Sci. Rev.* **284**, 107506 (2022).
18. Li, F. *et al.* Land-snail eggs as a proxy of abrupt climatic cooling events during the reproductive season. *Sci. Bull.* **66**(13), 1274–1277 (2021).
19. Deng, Y. *et al.* Biomimetic mineralization of calcium carbonate under synergistic regulation of Sr²⁺ and egg-white proteins. *Mater. Lett.* **346**, 134544 (2023).
20. Liu, H., Liu, C. & Huang, J. Characterization of the shell proteins in two freshwater snails *Pomacea canaliculata* and *Cipangopaludina chinensis*. *Int. J. Biol. Macromol.* **242**, 124524 (2023).
21. Tamburi, N. E., Seuffert, M. E. & Martin, P. R. Temperature-induced plasticity in morphology and relative shell weight in the invasive apple snail *Pomacea canaliculata*. *J. Therm. Biol.* **74**, 331–336 (2018).
22. Kaufman, A. J. *et al.* When life got hard: An environmental driver for metazoan biomineralization. *Estud. Geol. Madrid* **75**, 2 (2019).
23. Tambutte, S. *et al.* Coral biomineralization: From the gene to the environment. *J. Exp. Mar. Biol. Ecol.* **408**(1–2), 58–78 (2011).
24. Kontrec, J. *et al.* Effect of pH and type of stirring on the spontaneous precipitation of CaCO₃ at identical initial supersaturation, ionic strength and a(Ca²⁺)/a(CO₃²⁻) ratio. *Crystals* **11**, 9 (2021).
25. Li, F. *et al.* Self-mediated pH changes in culture medium affecting biosorption and biomineralization of Cd²⁺ by *Bacillus cereus* Cd01. *J. Hazard. Mater.* **358**, 178–186 (2018).
26. Schwaner, C. *et al.* Proteomic and transcriptomic responses enable clams to correct the pH of calcifying fluids and sustain biomineralization in acidified environments. *Int. J. Mol. Sci.* **23**, 24 (2022).
27. Zhu, T. *et al.* Extracellular biomineralization of uranium and its toxicity alleviation to *Bacillus thuringiensis* X-27. *J. Environ. Radioact.* **26**, 1 (2023).
28. Dammer, L. K. *et al.* Temperature impact on magnesium isotope fractionation in cultured foraminifera. *Front. Earth Sci.* **9**, 25 (2021).
29. Li, B. *et al.* Effects of temperature on biomineralization of iron reducing bacteria *Shewanella putrefaciens* CN32. *Chin. J. Geophys. Chin. Ed.* **54**(10), 2631–2638 (2011).
30. Dickinson, G. H. *et al.* Assessing the effect of salinity on barnacle adhesion and biomineralization. *J. Shellf. Res.* **34**(2), 625–625 (2015).
31. Matoo, O. *et al.* Elevated CO₂, temperature and salinity interactively affect biomineralization and shell properties of hard shell clams *Mercenaria mercenaria*. *Integrat. Comp. Biol.* **53**, E327–E327 (2013).
32. de Francisco, P. *et al.* Quantitative proteomic analyses of a Pb-adapted *Tetrahymena thermophila* strain reveal the cellular strategy to Pb(II) stress including lead biomineralization to chloropyromorphite. *Sci. Total Environ.* **8**, 91 (2023).
33. Varney, R. M. *et al.* Chitons on the cutting edge: The biomineralization of iron-clad teeth in *Acanthopleura granulata*. *Integrat. Comp. Biol.* **61**, E932–E933 (2021).
34. Shan, B. *et al.* A review on mechanism of biomineralization using microbial-induced precipitation for immobilizing lead ions. *Environ. Sci. Pollut. Res.* **28**(24), 30486–30498 (2021).
35. Zheng, Y., Xiao, C. & Chi, R. Remediation of soil cadmium pollution by biomineralization using microbial-induced precipitation: A review. *World J. Microbiol. Biotechnol.* **37**, 12 (2021).
36. Loucaides, S. *et al.* Controls on the recycling and preservation of biogenic silica from biomineralization to burial. *SILICON* **4**(1), 7–22 (2012).
37. Ripoll, J. P. Colorimetric determination of calcium in serum using methylthymol blue. *Clin. Chim. Acta Int. J. Clin. Chem.* **72**(1), 133–139 (1976).
38. Shiau, K. S. & Chen, T. L. Initial protein concentration effects on precipitation by salt. *Biotechnol. Bioeng.* **53**(2), 202–206 (1997).
39. Ismail, R. *et al.* The potential use of green mussel (*Perna viridis*) shells for synthetic calcium carbonate polymorphs in biomaterials. *J. Cryst. Growth* **572**, 126282 (2021).
40. Li, J., Xue, S. & Mao, Y. Seawater carbonate parameters function differently in affecting embryonic development and calcification in Pacific abalone (*Haliotis discus hannai*). *Aquat. Toxicol.* **257**, 106450 (2023).
41. Yang, X. *et al.* A novel matrix protein PfX regulates shell ultrastructure by binding to specific calcium carbonate crystal faces. *Int. J. Biol. Macromol.* **156**, 302–313 (2020).
42. Xu, D. *et al.* Synthesis of stable calcium carbonate nanoparticles for pH-responsive controlled drug release. *Mater. Lett.* **333**, 133635 (2023).
43. Ahammad, M. U. *et al.* Effect of oyster shell, limestone and calcium premix on egg production and egg shell quality. *Indian J. Anim. Sci.* **75**(7), 823–826 (2005).
44. de Souza, D. S. *et al.* Quail performance and egg quality at the end of production fed with varying levels of calcium. *Semina Cie. Agrarias* **37**(4), 2395–2406 (2016).
45. Heyn, A. N. The crystalline structure of calcium carbonate in the avian egg shell; and electron microscope study. *J. Ultrastruct. Res.* **8**, 176–188 (1963).
46. Jones, K. T. Ca²⁺ oscillations in the activation of the egg and development of the embryo in mammals. *Int. J. Dev. Biol.* **42**(1), 1–10 (1998).
47. Khan, S. R. *et al.* Agar and egg shell derived calcium carbonate and calcium hydroxide nanoparticles: Synthesis, characterization and applications. *Chem. Phys. Lett.* **732**, 136662 (2019).
48. de Souza, J. M. *et al.* Predation of *Diatraea saccharalis* eggs and neonates by the earwig *Euborellia annulipes*. *Biol. Control* **172**, 104953 (2022).
49. Novillo, M. *et al.* Egg predation in Antarctic fish: The ingestion by *Notothenia coriiceps* of an entire *Trematomus bernacchii* spawn identified by molecular techniques. *Estuar. Coast. Shelf Sci.* **266**, 107742 (2022).
50. Xiao, Q. *et al.* Transcriptome sequencing reveals the differentially expressed lncRNAs and mRNAs in response to cold acclimation and cold stress in *Pomacea canaliculata*. *BMC Genom.* **23**, 1 (2022).

51. Xiong, Y. M. *et al.* Immune related genes expression in juveniles of an invasive snail after challenged with Lipopolysaccharide. *Invertebrate Surv. J.* **14**, 295–302 (2017).
52. Qin, Z. *et al.* Enhanced salinity tolerance of *Pomacea canaliculata* through acclimation to lower salinities. *Hydrobiologia* **849**(13), 3015–3029 (2022).
53. Tahara, Y., Obara, K. & Kamihira, M. Calcium carbonate supplementation to chorioallantoic membranes improves hatchability in shell-less chick embryo culture. *J. Biosci. Bioeng.* **131**(3), 314–319 (2021).
54. Qiao, T. *et al.* *Clonorchis sinensis* eggs are associated with calcium carbonate gallbladder stones. *Acta Trop.* **138**, 28–37 (2014).
55. Colvin, C. N. *et al.* The effect of environmental factors on shell growth and repair in *Buccinum undatum*. *J. Exp. Mar. Biol. Ecol.* **5**, 51 (2022).
56. Chen, Y. *et al.* Repaired shells of the pearl oyster largely recapitulate normal prismatic layer growth: A proteomics study of shell matrix proteins. *ACS Biomater. Sci. Eng.* **5**(2), 519–529 (2019).
57. Xiong, X. *et al.* Transcriptome analysis reveals the transition and crosslinking of immune response and biomineralization in shell damage repair in pearl oyster. *Aquac. Rep.* **21**, 25 (2021).
58. Bosch-Belmar, M. *et al.* Short-term exposure to concurrent biotic and abiotic stressors may impair farmed molluscs performance. *Mar. Pollut. Bull.* **179**, 113724 (2022).
59. Huang, J., Xie, L. & Zhang, R. Shell repair and the potential microbial causal in a shell disease of the pearl oyster *Pinctada fucata*. *Fish Shellf. Immunol.* **86**, 934–941 (2019).
60. Guo, J. *et al.* Biological control of the exotic invasive snail *Pomacea canaliculata* with the indigenous medicinal leech *Whitmania pigra*. *Biocontrol Sci. Tech.* **27**(9), 1071–1081 (2017).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31870525, 31770484, 41871034, 31901229), Guangdong Science and Technology Project (2019B030301007). We thank the anonymous reviewers who provided helpful comments on earlier versions of this manuscript.

Author contributions

Y.C.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft. F.Y.: investigation. C.Z.: investigation. Z.Q.: data curation, review and editing. J.G.: review and editing. J.Z.: conceptualization, methodology, writing—review and editing, funding acquisition, project administration, supervision.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-023-43071-4>.

Correspondence and requests for materials should be addressed to J.Z.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2023