Nanocalcium of *Pila ampullacea* Shell incorporated into Feed on Molting and Growth Performance of Crayfish *Cherax quadricarinatus*

F.B.A Jabbar¹*, M. Ansar¹, Ardiansyah²

¹Aquaculture Study Program, Department of Fisheries, Faculty of Animal Science and Fisheries, Universitas Sulawesi Barat, Majene, West Sulawesi, Indonesia  
²Department of Aquaculture, Pangkep State Polytechnic of Agriculture, South Sulawesi Indonesia  

*Corresponding Email: firmansyahjabbar@unsulbar.ac.id*

**Abstract.** Freshwater snail *Pila ampullacea* shell can be used as alternative for calcium source. The shell has been known as solid waste and it also has been a major problem in the agriculture field. The present study aims to determine the effect of nanocalcium made from *P. ampullacea* shell to improve the molting and growth performance of red claw crayfish *Cherax quadricarinatus*. A laboratory study was conducted for four weeks of feeding trials using 120 freshwater crayfish *C. quadricarinatus*. A total of four different levels of nanocalcium regimes including 0%, 2%, 4%, and 6% were applied for experimental treatments and each treatment regime consisted of three replicates. Data was collected on molting frequency, absolute weight growth, specific growth rate, survival rate, food conversion ratio, and water quality parameters. The results suggest that dietary nanocalcium treatments were significantly different in absolute weight growth, specific growth rate, and molting frequency, survival rate. However, the nanocalcium of *P. ampullacea* shell showed no significant difference in feed conversion ratio. The highest absolute weight growth, specific growth rate, molting frequency, survival rate was obtained from 2% of dietary *P. ampullacea* nanocalcium.

**1. Introduction**

Red claw Crayfish or *Cherax quadricarinatus* is known as one of the freshwater crayfish cultivated in Indonesia [1]. However, it is presently cultivated both in the tropic and temperate zone regions [2]. This species is vastly distributed across the continent from America, Europe, Asia to Australia [3]. It has similarities with seawater lobster [4]. Nonetheless, its meat texture is more tender than marine lobster. Red claw crayfish has high nutritional content, but it is low in fat [5]. In aquaculture, the huge cost of feed has become a major problem in aquaculture. The cost of feed in aquaculture activities ranges between 50% and 70% of the total operating costs [6]. The longer the production cycle, the greater the amount of feed required. Therefore, proper efforts are required to improve the growth performance of freshwater crayfish.

The calcium portion in commercial feeds formulation is very small, whereas crustaceans in general requires more calcium for growth. Calcium itself plays an important role in crayfish life cycle, especially in regulating the work of growth hormones [7]. Calcium is a vital nutrient requirement. It contributes to induce the molting process in shrimp, as well as helps to form a new exoskeleton in shrimp [8]. All aquatic animals need inorganic elements such as minerals for their metabolism [9].
Calcium can be obtained from the diet and the environment; however, it is predominantly influenced by feed. Molting is a critical phase in the life of all crustaceans and successful molting is necessary for optimum growth and survival [10]. Several attempts have been taken to meet the calcium requirements of freshwater crayfish. For instance, low doses of calcium are usually added to the aquatic environment in the rearing tank. However, this is inefficient [11]. A low survival rate often occurs in the aquaculture due to unable to molt properly leading to poor growth performance [12]

The freshwater snail P. amplaacea or locally known as rice field snail, has a shell which can be used as source of calcium. However, it is known as pests and waste in agricultural areas. Nonetheless, it has been widely consumed in various regions of Southeast Asia and have nutritional values including protein 51.8%, fat 13.61%, fiber 6.09%, ash content 24% [13]. In fact, mollusk shells consists of a large percentage calcium carbonate that can be used as a good source of Ca [14]. Several benefits of freshwater snail shells are collagen, antibacterial, antioxidant, stabilizer, emulsifier, thickening in the food industry [15] [16].

In order to improve the calcium absorption, it should be provided in the form of nano calcium [17]. In general, calcium is found in micro size. Particle size is closely related to the ability of absorption in the body [18]. In the present study, the calcium used is nano-sized calcium. Nano calcium is a pre-digestive mineral that is very efficient in entering body cells due to its size that is easily absorbed properly into the shrimp body. However, the calcium in the shell prior to fortification must be converted into a digestible form. The extraction process using distilled water, acid and alkaline solutions, and enzymes at high temperatures can change and soften the shell matrix structure [19][17].

Several studies related to the application of nanocalcium have been carried out in some species, including giant prawn [20] nano calcium particles obtained from Pinctada maxima as an antimicrobial [21]; nanocalcium on molting performance and viability of vaname shrimp [22]; the addition of nano CaO from mussel shell waste (Pilsbryocncha exilis) in saline media for the growth of tilapia (Oreochromis niloticus) [23]; the addition of nano CaO of mud crab (Scylla serrata) shells into commercial feed on the growth and molting frequency of giant prawns (Macrobrachium rosenbergi) [24]. However, study on freshwater crayfish shell for calcium sources is still very limited. The present study aims to investigate the addition of nanocalcium from rice field snail shells on the molting frequency and growth performance of Cherax quadricarinatus.

2. Materials and Methods

2.1. Experimental Animal

Healthy juveniles of Cherax quadricarinatus (average length 1 inch and average weight 2.8±0.66 g) were obtained from a private freshwater crayfish located at Polewali Regency district, West Sulawesi, Indonesia. Prior to study, the test animals were stocked in aquarium with sufficient aeration and maintained for 3 days. The freshwater lobsters were fed on commercial pellet diet (30% crude protein) twice daily at 08:00 am and 04:00 pm at satiation.

2.2. Experimental Diets

The shell and meat of the freshwater snail were first separated and rinsed with water. The shells were then dried under the sunlight. The nano calcium procedure was carried out using an acid solution (HCl) according to [25]. A commercial pellet feed (5% fat, 30% protein, 2% fiber, 11% water, and 13% ash) were used as basal diet. For the experiment, three experimental diets with three different concentrations each of nano calcium namely (2%), (4%), and (6%) were applied. The basal diet was maintained separately as control (0%) for each treatment. The experimental feed was added 1% of CMC (carboxy methyl cellulose) and pellet mill was using for pelleting. The formulated feed was dried using oven at 40°C for 24 h and dried pellet was stored in labelled air tight plastic bottles.
2.3. Experimental Design
A total of twelve rectangle aquariums with size 40x20x30 cm were used for experiment. Ten test animals were stocked to each aquarium with sufficient aeration and shelters. The test animals were fed twice a day with 3% of their body weight. The left-over feed and faeces were daily siphoned with a water exchange rate of 5-10%. The rearing was conducted for 4 weeks.

2.4. Data collection
The data was collected on molting frequency [26], absolute weight growth (AWG), specific growth rate (SGR), survival rate (SR), food conversion ratio (FCR) based on [27] using formula as follows:

Molting frequency: \( MFq = \frac{x_{\text{moit}}}{M_{\text{tot}}} \)

Calculation of absolute weight gain used the formula [28] as follows:

Absolute Weight Growth = \( W_t - W_o \)

The calculation of specific growth rates according to [29] as follows:

Specific growth rate: \( SGR = \frac{\ln W_t - \ln W_o}{t} \times 100\% \)

Survival rate was calculated using the formula [30] as follows:

Survival rate: \( SR = \frac{N_t}{N_o} \times 100\% \)

Feed conversion ratio: \( FCR = \frac{F}{(W_t + D) - W_o} \)

The water quality parameters were measured daily during the study period. Several water quality parameters including, temperatures, Dissolved Oxygen, pH, ammonia were observed during the study period.

2.5 Data analysis
Data was analysed using statistical software SPSS version 22. One way ANOVA was applied for the significance of differences (\( P<0.05 \)). Tukey test was carried out for post-hoc test. All means were presented with ± standard deviation (SD).

3. Results and discussion
3.1 Molting frequency (MF)
The highest molting frequency was found in 2% of nano calcium treatment (1.55 times per individual) (Table 1). This result is slightly higher than previous study where 2% of pure calcium (\( Lactas calcicus \)) reach 1.47 of molting frequency in \( Macrobrachium rosenbergii \) [31] and 1.03 of molting frequency in Cherax sp [32]. In the present study, the lowest molting frequency was observed in the 6% treatment (0.84 times per individual). Nonetheless, the 4% (0.86) and 6% (0.84) treatments were not significantly difference (\( P>0.05 \)). These molting frequency value are still higher than previous study with longer rearing period (two months) with calcium obtained from oyster, where the MF is 0.64 [20]. Conversely. A total of 2% oyster calcium is able to rise the molting frequency to 2.71 times for 60 days [26]. This result is higher compared to present study, where 2% of rice field snail shell can only reach 1.55 times per individual of molting. Calcium source and type of commercial feed play important role in promoting molting frequency. Eventually, the present results confirms that the addition of calcium is effectively induce molting.

The supplementation of nano-sized calcium through diet allows excellent absorption in the crayfish body and induce molting [31]. Reserved calcium is used for shell hardening process after molting experience. Nevertheless, the effect of calcium depends on the species. The mechanisms of calcium
absorption, transportation, and storage vary during the molting cycle. Calcium can be absorbed from the environment, discarded cuticle, feed.

In general, the crayfish stores calcium ions for calcifying parts of the new exoskeleton [33]. Many mineralized tissues fulfill structural functions [34]. Furthermore, the shell can stiffen and strengthen the tissue using available calcium [35]. Hence, calcium (Ca) is crucial inorganic element for normal growth, skeletal development and various physiological processes in aquatic organism [36]. Additionally, crayfish requires calcium every 7 to 10 days for molting cycle [37]. However, crayfish hatchlings molting began to occur at 2 - 3 weeks [38].

### Table 1. Mean ± SD values of some growth parameters of C. quadricarinatus during the experiment period.

| No. | Parameters                  | Experimental Feed (Mean ± SD) |
|-----|-----------------------------|-------------------------------|
| 1   | Molting Frequencies (MF)    | 0% (Control) 1.10 ± 0.08<sup>a</sup> 1.55 ± 0.13<sup>b</sup> 0.86 ± 0.25<sup>c</sup> 0.84 ± 0.52<sup>d</sup> |
| 2   | Average Weight Growth (g/day)| 0% (Control) 3.40 ± 0.15<sup>a</sup> 4.47 ± 0.04<sup>b</sup> 2.08 ± 0.05<sup>c</sup> 1.66 ± 0.35<sup>d</sup> |
| 3   | Specific Growth Rate (SGR) (g) | 0% (Control) 2.59 ± 0.12<sup>a</sup> 3.30 ± 0.27<sup>b</sup> 1.38 ± 0.97<sup>c</sup> 2.04 ± 1.03<sup>d</sup> |
| 4   | FCR (%)                     | 6% 1.36 ± 0.16<sup>a</sup> 1.01 ± 0.09<sup>b</sup> 2.08 ± 0.44<sup>c</sup> 2.27 ± 1.17<sup>d</sup> |
| 5   | SR (%)                      | 6% 70 80 50 60 |

Mean values with the same superscript letters in the same row are not significantly different (P>0.05)

#### 3.2 Average Growth Weight (AWG) and Specific Growth Weight (SGR)

The highest AWG was obtained from the 2% nano calcium treatment (4.47 ± 0.04) and the lowest in the 6% treatment (1.66 ± 0.35) (Table 1). This data is higher than previous study by [37], in which the highest AWG in crayfish was 2.47 ± 0.65 g in 6% of calcium treatment. In contrast, statistically, 4% (2.08 ± 0.05) and 6% (1.66 ± 0.35) of nanocalcium group in present study were slightly lower in terms of AWG than the previous study. Interestingly, the 0% nanocalcium treatment is significantly different than previous study. At the end of the experiment in present study, the best AWG was achieved in treatment group of 2% nanocalcium. This is consistent with result [31], where the best AWG was observed in 6% of calcium treatment. In fact, the AWG values is consistent with molting frequency in the present study.

Lack of calcium availability leads to more energy expenses to perform molting. As a result, the amount of energy mostly allocates to absorb more calcium from environment rather than taking from reserved calcium in the gastroliths or stone calcium. It leads to slower molting process leaving to crayfish vulnerable to be prey by other crayfish [26]. This is in line with [39], the cannibalism in crayfish eventually can be reduced by adding calcium concentration. Because the crayfish has more energy reserved to conduct molting. Furthermore, calcium in feed can accelerate the mineralisation process in shrimp [40].

The marine snail shells calcium at 2%-10% increases resistance to stress and improved growth rate [41]. However, high concentration of calcium also can be toxic on crayfish. [42]. In the other side, low calcium concentration can limit growth and production [43]. Additionally, low calcium levels hamper the shell formation. Meanwhile, high calcium levels also complicate the homeostatic process of calcium ions [44]. Hypo ionic or hyper ionic conditions of the body's calcium complicate the balance of the body's calcium ions with the environment. The energy requirement for conducting such process is greater. As a consequence, the use of energy for growth is hampered. This suggests that the calcium concentration needs to be precisely added into the feed. As said by [45], adequate calcium is required for molting.

The present study suggests that 4% and 6% of nanocalcium shows low in average weight gain as well as specific growth rate compared to control (0%) and 2% of nano calcium (Table 1). This also consistent with [46], as increasing calcium and magnesium ions, the growth and survival rates also decreased. Conversely, optimum weight and length of Cherax quadricarinatus was observed at 3-4%
of calcium [47]. However, type of calcium source might contribute to the different responses of crayfish. Calcium requirements is different with each species. Additionally, lower average weight growth also caused by higher cannibalism leading to high mortality (Table 1).

3.3 Feed Conversion Ratio (FCR)
Based on table 1, the FCR value shows no significant difference ($P>0.05$) in groups fed with control (0%), 2%, 4% and 6% nanocalcium. The FCR value of supplemented nanocalcium was 1.36, 1.01, 2.08 and 2.27, respectively.

In general, the FCR value indicates that the experimental feed is utilized optimally by the test animals. The FCR values for all treatment are within the recommended value for efficient commercial feed. Although the group fed with 2% nanocalcium supplemented feed had a better feed conversion ratio, nonetheless, it was not different from other groups of treatment ($P>0.05$) (Table 1).

3.4 Survival Rate (SR)
After 4 weeks experiment trial, the highest survival rate of freshwater crayfish was obtained from 2% treatment (80%) (Table 1). This result is lower than previous study, where the SR of crayfish reach 93.33% [31]. However, the average survival rate of crayfish is at 80% in aquaculture condition [48]. This findings also supported by previous study, 2% nanocalcium has a significant effect on the survival of giant prawns [20]. Moreover, [49] stated that adequate calcium in diet prevent both cannibalism and injury of crayfish and promoting high survival rate and molting frequency. In the present study, the SR percentage is consistent with the molting frequency. Poor survival rate was found in 4% treatment (50%). Statistically, the 4% and 6% treatments were not significantly difference. The control treatment was slightly lower than 2% nanocalcium group.

Since the animal test used in this study is red claw crayfish hatchlings, at this age, the juvenile is strongly aggressive and display a competition for resources. In fact, the major problem in crayfish aquaculture is cannibalism. Crayfish decrease their activity and vulnerable during molting [49], at the same time, crayfish stop feeding [50]. This contributes to mortality in every experimental units during the study period.

3.5 Water quality
Based on table 2 shows that the temperatures of experimental media during study period ranged from 26.68 °C to 28.14 °C. This data is in line with the temperature requirement for rearing crayfish is 24-26°C with a maximum fluctuation day and night 2-3 °C Alexander (2006). However, the thermal tolerance range from crayfish hatchlings is 22-32 °C [51][52]. In addition, the juveniles are unable to survive within 1 h at 40°C. They also cannot survive more than 4 days at 35°C. This suggests that the juvenile’s crayfish need to be reared at temperatures above 10°C and below 35°C. The temperature is within tolerable level for optimum growth. This temperature range also directly affects dissolved oxygen in the rearing media, which ranges from 8.24 mg/l to 10.59 mg/l, the dissolved oxygen is considered in the optimum level. As said by [52], the optimum dissolved oxygen range is 7-10 ppm. In aquaculture, condition, crayfish are stressed at oxygen levels below 3 mg / L [37]. Nevertheless, crayfish can tolerate broad range of water quality condition [56].

Table 2. The water quality parameters value ranges during experiment period

| No. | Parameters                  | Range               |
|-----|-----------------------------|---------------------|
| 1.  | Temperature (ºC)            | 26.68 ± 0.09 – 28.14 ± 0.13 |
| 2.  | Dissolved Oxygen (mg/l)     | 8.24 ± 0.02 – 10.59 ± 0.34  |
| 3.  | pH                          | 8.56 ± 0.03 – 8.60 ± 0.02   |
| 4.  | Ammonia (mg/l)              | 0.01 ± 0.02 – 0.04 ± 0.0038  |
In addition, dissolved oxygen value is closely related to the pH level of the rearing media, which ranges from 8.56 to 8.60. This pH value is considered to be in the optimal range for the growth of red claw crayfish [52]. Rapid change in water pH leads to stressed crayfish. Moreover, the low pH (4-5) and high pH (11) cause mortality, and optimum pH is 7-9 [53]. In addition, the ammonia value ranged from 0.01 to 0.04. No statistical differences were observed in the mean water temperature, dissolved oxygen, pH and ammonia (P>0.05). Water quality parameter values observed is acceptable for crayfish culture[54][55]. In general, crayfish is tolerance to wide variations in water quality make them an excellent for aquaculture [27].

4. Conclusions
The nano calcium of Pila ampullacea shells incorporated into diet promote better molting frequency and growth performance of red claw Cherax quadricarinatus. The amount of nanocalcium is recommended about 2 %. This finding suggests calcium content should be considered properly in formulating crayfish diet. In order to have better understanding, gastroliths of crayfish need to be measured and proximate analysis for body parts of crayfish also are required. These information need further research.

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