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The effects of feeding ration and cheliped autotomy on the growth and expression of ecdysteroid receptor in early juvenile mud crabs, *Scylla paramamosain*

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ABSTRACT

This study investigated the combined effects of feeding ration and cheliped autotomy on the intermolt duration, molting success, molt increments in size and weight, and ecdysteroid receptor gene (*SpEcR)* expression of early juvenile mud crab *Scylla paramamosain*. Newly molted second stage juvenile crabs (C2) were subjected to four feeding conditions; optimal, suboptimal (1/2 optimal), low (1/4 optimal) ration and starvation, and autotomy (intact vs. cheliped autotomy) in a 4 × 2 factorial design until all crabs successfully molted or died. A significant interaction of feeding ration and cheliped autotomy on intermolt duration was identified. With reduced feeding ration, both intact and cheliped autotomized crabs showed increased time and desynchrony of molting, but decreased carapace size and body weight. Importantly, all crabs with different feeding rations even the low ration had high rates of molting success (> 95%), while the crabs subjected to starvation died without molting. When fed optimal ration, the mean intermolt duration of the cheliped autotomized crabs was significantly prolonged, while no such effect was found between autotomized and intact crabs subjected to suboptimal or low feeding ration. The qRT-PCR revealed that the expression of *SpEcR* showed a general trend of inhibited by reduced feeding ration, which was consistent with observed significantly increased intermolt duration. Interestingly, the transcript level of *SpEcR* was only significantly affected by cheliped autotomy under the optimal and suboptimal feeding rations but not for the low feeding ration. Together, the results of this study suggest that the *S. paramamosain* early juveniles have a strong tolerance for fluctuations in food availability. In addition, the availability of food and limb autotomy could significantly affect growth, molting duration and synchrony of the crabs, which appeared to reflect in *SpEcR* expression level that involved in the regulation of molting and limb regeneration process of the juvenile crabs.

1. Introduction

Crustaceans play a crucial role in aquatic ecosystems and are also important food sources for global coastal communities through fisheries and aquaculture (Bondad-Reantaso et al., 2012; Waiho et al., 2021). In the natural environment, aquatic animals often experience periods of food scarcity due to both spatial and temporal variations in food availability, such as season changes and heterogeneous spatial distribution of their food (Buckup et al., 2008; Espinoza et al., 2016). Therefore, the availability of food is deemed one of the principal factors affecting crustacean survival and growth in the wild (Giménez and Anger, 2005). In aquaculture settings, cultured organisms may also suffer from inadequate food availability or even starvation due to insufficient feeding or conspecific competition. Past studies with decapods and other crustaceans have shown that food availability and quantity can affect their survival, growth, successful development and reproductive capacity (Camus et al., 2021; Sulkin et al., 1998; Stumpf et al., 2019; Waiho et al., 2018). In fact, crustaceans can tolerate relatively short periods of starvation, which is critical for them to survive in natural environments, especially those with wide fluctuations in food availability (Anger,

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2001).

In crustacean larvae, two critical starvation tolerance time points during the molting cycle, i.e. Point of No Return (PNR) and Point of Reserve Saturation (PRS), were proposed (Anger et al., 1981). The PNR is defined as the time point during the molting cycle when the capacity of crustacean larvae to recover from nutritional deficiency is lost as a result of irreversible damages caused by food deprivation, which means once PNR is passed, even sufficient food becomes available again, the larvae will not be able to molt to next stage and eventually die. On the other hand, when crustacean larvae have been fed for a certain period of time during the molting cycle, their development can reach PRS, a time point that even without further food intake, the development will continue with successful molt to the next stage. Past studies have not only identified PNR and PRS in larvae of various crustaceans, but also at other developmental stages, such as juveniles (Anger, 1995; Calvo et al., 2018; Genodepa et al., 2022; Souza et al., 2018). Another interesting phenomenon linked to food deprivation that is significant to aquaculture is compensatory growth (see review by Py et al., 2022): it has been found that fishes and crustaceans subjected to periodic feed deprivation can exhibit unusual accelerated growth after being fed normally again to recover to the weight similar to those counterparts never experiencing feed deprivation (Eroldoğan et al., 2006; Luo et al., 2022; Py et al., 2022). Since feeding typically represents the largest production cost (often $>$ 60%) of modern aquaculture (Hasan and New, 2010), a feeding strategy that might result in a compensatory growth response has good potential to improve food utilization efficiency and reduce production costs for the aquaculture industry (Morshedi et al., 2017; Stumpf et al., 2019; Py et al., 2022). So far, research about the effects of food deprivation on crustaceans has often focused on providing insights into developmental physiology under extreme scenarios (i.e. total deprivation of food) (Pascual et al., 2006; Ding et al., 2017; Calvo et al., 2018). In aquaculture settings, it is more likely that cultured crustaceans will experience different levels of food deprivation, hence investigating the effects of food deprivation on their growth and development at different intensities are more relevant, especially for juveniles with high metabolic and growth rate.

Crustaceans often experience appendages autotomy when displaying aggressive behavior, self-defense from predation or being subjected to acute environmental stimulation (Abello et al., 1994; Frisch and Hobbs, 2011). Such autotomy typically occurs at a specialized breakage point, i.e. between the ischiopodite and basipodite leg segments (Frisch and Hobbs, 2011). The chelipeds of crustaceans are utilized for prey capture and manipulation, territorial/aggressive behavior and self-defense, their loss or damage could reduce the foraging capability and success, leading to lesser food intake and suppressed growth (Smith and Hines, 1991; Parsons and Eggleston, 2005). Moreover, a recent study also found that limb removal could significantly affect the feeding ability of mud crabs, *Scylla olivacea* (Fazhan et al., 2022). On the other hand, in aquaculture settings, cheliped ablation or immobilization has been shown to reduce the intensity of cannibalism in cannibalistic crustaceans, such as the lobsters *Homarus americanus* (Kendal et al., 1982) and the giant freshwater prawn *Macrobrachium rosenbergii* (Karplus et al., 1989). In the Philippines, removing chelipeds is also practiced during the transportation of wild-collected juvenile mud crab *Scylla serrate* from the source to the farms to reduce damages/mortality due to fighting (Triño et al., 1999). Such an approach was also considered as a potential strategy for mitigating the high level of cannibalism experienced in *S. serrata* nursery where high stocking density and frequent molting occur at early juvenile stages (Quinitio and Estepa, 2011; Romano and Zeng, 2017), as well as for improving soft-shell crab production of another mud crab species *Scylla olivacea* (Rahman et al., 2020). However, Fujaya et al. (2020) found that although limb autotomy significantly shortened the duration of the molt cycle, it could also result in soft-shell crabs of lower aesthetic value in body size, and weight. These results indicate whether limb autotomy is efficient as traditional rearing methods need to be investigated deeply.

Crustaceans are known capable of regenerating lost limbs, for example, the missing limbs as a result of the reflexive autotomy response to predation in the fiddler crab, *Uca pugilator* reportedly could be fully regenerated within a single molt cycle (Hopkins, 2001). The regeneration process typically involves two phases: basal growth that follows the limb autotomy to form a blastema under the breakage point; proecdysial growth that usually occurs over a brief period prior to molting, during which the size of a small limb bud formed during basal growth phase increased by 3-folds as the result of protein synthesis and water uptake (Hopkins, 1993). Although the signaling pathways participating in the formation and development of limb primordium following limb loss are not well characterized, the deposition of a flexible cuticle during basal growth suggests that ecdysteroid signaling pathways are likely indispensable for the blastemal development during limb regeneration (Abdullah-Zawawi et al., 2021; Durica et al., 2002; Das and Durica, 2013). Ecdysteroids, a group of polyhydroxylated ketosteroids, are reported to regulate molt, limb regeneration, reproduction, and other biological processes, such as behavior, lifespan and stress resistance of arthropods (Subramoniam, 2000; Schwedes and Carney, 2012; Suzuki et al., 2019). For example, in the fiddler crab, *U. pugilator*, when a seriously injured appendage was cast off (autotomy), ecdysteroids were found to be directly involved in the whole limb regeneration process (Chung et al., 1998). However, to regulate the downstream genes of the ecdysteroid signaling pathway, ecdysteroids need to bind to the ecdysteroid receptor (EcR), which can form a heterodimer with the retinoid X receptor (Kim et al., 2005). Similar to the ecdysteroids, a large number of studies have shown that the EcR takes part in the regulation of many physiological processes in crustaceans, such as general development, gonadal maturation, molting, limb regeneration, and chitin synthesis, and has been served as an essential molecular indicator of molting and ovarian development (Abdullah-Zawawi et al., 2021; Gong et al., 2015b). Interestingly, although limb autotomy has been shown to hasten the onset of the next molt in crustaceans (Hopkins, 1982; Rahman et al., 2020), our study has found that autotomy one of the chelipeds in early juvenile mud crab *Scylla paramamosain* actually significantly increased the intermolt period (Gong et al., 2015a). Such a different effect of limb autotomy on intermolt duration probably can be explained by the fact that the time takes to regenerate lost limbs is dependent on the age/size (Mariappan et al., 2000), and the stage during the molting cycle at which the limbs were lost (Hopkins, 1982).

Since both feeding level and limb autotomy can affect the survival, development and growth of crustaceans, evaluating their potential interactive effects on aquaculture species, particularly cannibalistic species or live stage, should provide useful information for devising the best practice in aquaculture. The mud crab *S. paramamosain* is important commercial fisheries and aquaculture species in the southern coasts of China and many other Indo-Pacific countries because of its high market price and acceptance (Anh et al., 2011; Ye et al., 2011). Previous research has investigated the effects of limb autotomy and starvation (absent of food) as separate factors on survival and molting of *S. paramamosain* early juveniles (Gong et al., 2015a). Similarly, the effects of limb autotomy under fed vs. unfed condition on juvenile survival and development of another mud crab *S. serrata* was also studied (Quinitio and Estepa, 2011). However, the extreme situation of total absence of food in above mentioned studies is probably not common in both aquaculture settings or wild, hence this study sought to investigate the combined effects of more realistic different feeding rations and limb autotomy on the survival, molting interval and growth of early juvenile *S. paramamosain*, while the expression of EcR gene expression (*SpEcR)* was concurrently determined to help understand the underlying molecular regulating mechanisms.

2. Materials and methods

2.1. Source of juvenile crabs

20 healthy mature female *S. paramamosain* were purchased from a commercial mud crab farm in Zhangzhou, Fujian province, China. After being disinfected with potassium permanganate (50 ppm), the broodstock crabs were kept in concrete tanks until spawned. The crabs were checked daily and those spawned with eggs carried under their abdomen were disinfected and transferred into a separate incubation tank until larval hatching (Gong et al., 2015a). The newly hatched larvae from a single female were cultured at a density of 100–120 larvae L^{-1} and fed rotifers first, but switched to *Artemia* as larvae grew bigger (Zeng and Li, 1999). Upon the larvae reaching megalopal stage, about 5000 megalopae were randomly collected and transferred to five 100 L containers for rearing. After about 6 days, the megalopae began to molt and became the first stage crabs (C1), which were transferred again to a new container for culture. Hence any newly molted crabs that appeared in the new container with substantial large sizes were determined as the second stage crabs (C2). To ensure that C2 crabs used for the experiment were soon after molting and they were very similar during the molting cycle, the C1 culture vessels were checked at 00:00 am with any C2 crabs found removed. The vessels were checked again at 06:00 am and newly appeared C2 was collected for the subsequent experiments. Consequently, all C2 crabs had a similar developmental process in the experiment that molted within 6 h.

2.2. Experiment design and setup

2.2.1. Estimation of optimal feeding ration

Twenty newly molted C2 crabs were randomly selected and their weight was measured to estimate the average weight of C2 crabs. Subsequently, excessive chopped foot of Manila clam, *Ruditapes philippinarum,* were weighted and fed to these crabs at 8:00 am; after 24 h at 8:00 am the following day, the remained *R. philippinarum* foot was collected, blotted dry with an absorbent paper before being weighted to estimate the average daily food intake of C2 crab under ad libitum feeding. After two days of repeat, the average amount of feeding ration was used as the daily optimal ration in the following experiment.

2.2.2. Experiment design and procedures

A 4 (feeding rations) x 2 (intact vs. autotomy a cheliped) factorial design was applied to assess the interactive effects of different feeding rations and cheliped autotomy on the survival, intermolt duration, carapace length and width, body weight of newly molted C3 and *SpEcR* gene expression. The C2 crabs were fed chopped foot of Manila clam, *R. philippinarum,* and the four feeding conditions tested were: (1) optimal ration (see 2.2.1); (2) suboptimal ration: the crabs were fed ½ optimal ration; (3) low ration: the crabs were fed ¼ optimal ration; (4) starvation: no feeding. The cheliped autotomy was induced according to Quinitio and Estepa (2011) by crushing one of the chelipeds at the merus using a pair of tweezers. In order to keep the experiment consistent, the left cheliped of all the treatment crabs were removed. Each of 8 treatments was triplicated and each replicate consisted of 25 of C2 crabs which were individually cultured in round plastic vessels (diameter: 5 cm; height: 10 cm). The salinity and temperature were maintained at 22 ± 1‰ and 28 ± 0.5 °C, respectively. A 100% water exchange was carried out daily in the morning when survival and molting of the crabs were monitored and recorded. The carapace length, carapace width and body weight of the new molting C3 crabs were measured after their shells become hardened. The experiment terminated when all C2 crabs had either molted to C3 or died.

For each treatment, in addition to 3 replicates mentioned above for collecting survival, molt and growth data, a further replicate consisting of 50 crabs was also set up for each treatment, which was used for the RNA extraction. The crabs in these replicates were sampled at 6, 12, 24, 48, 72, 96, 120, 144, 168 h, as well as at premolt (C2) and postmolt stage (C3) and at each sampling time, 3crabs were randomly selected for RNA extraction to determine the expression level of *SpEcR* gene.

2.2.3. Expression of SpEcR gene

In this study, the samples were collected and immediately stored in liquid nitrogen. Then the crabs were ground for the RNA extraction after the sampling finished. The total RNA of each crab using the whole body was extracted using the trizol reagent according to the manufacturer's instruction (Invitrogen, USA). After the genomic contamination was eliminated by DNase I, the ND-1000 NanoDrop UV spectrophotometer (NanoDrop Technologies, inc. USA) was used to quantify the quality of the extract, which was reversely transcribed using the reversed first strand cDNA synthesis kit (Fermentas, USA) and stored at − 20 °C.

The expression level of EcR was measured by real-time quantitative PCR (qRT-PCR). Briefly, the qRT-PCR was executed with 2 μl of cDNA template, 0.8 μl of each primer, 10 μl of SYBR premix, and 6.4 μl of PCR-grade water under the following condition: 94 °C for 10 min; 40 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 40 s using ABI 7500 fast quantitative PCR system. The EcR forward primer was 5′- AAGAACAAAAGACTCCCACCATT-3′ and the EcR reverse primer was 5′-TCTCTCACTTACAGCCGACAGG-3′. As there are three isoforms of EcR found in the mud crab *S. paramamosain* (Gong et al., 2015b), the primers used in this study are designed in the common domain of the three isoforms. A β-actin fragment of *S. paramamosain* amplified by β-actin F 5′- GAGCGAGAAATCGTTCGTGAC-3′ and β-actin R 5′-GGAAGGAAGGCTGGAAGAGAG-3′, was used as the internal control. The negative control was performed with PCR-grade water to replace the cDNA template.

2.3. Data treatment and statistical analysis

Percentage molting success and the molting interval of each replicate in treatment was calculated by:

$$\text{Molting success } \% = 100\% \times \text{no·of C2 successfully molted to C3 / 25}$$

$$\text{C2 molting interval} = (N1 \times D1 + N2 \times D2 + \cdot + Nn \times Dn) / (N1 + N2 + \cdot + Nn)$$

Where D1, D2…, Dn represent days from the commencement of the experiment with the first day of the experiment defined as Day 1 while N1, N2…, Nn are the number of crabs found molted on day 1, 2…, n, respectively.

The data obtained on gene expression were calculated using $2^{-\triangle\triangle Ct}$ as described by Livak and Schmittgen (2001) and then subjected to statistical analysis.

Statistical comparisons of the data were carried out by two-way analysis of variance (ANOVA) for the growth, while one-way ANOVA and student's t-test analysis were performed for gene expression analysis, respectively. $p < 0.05$ was considered statistically significant. Prior to ANOVA analysis, Kolmogorov–Smirnov and Cochran tests were performed to test the normality and homogeneity of variances of the data, respectively. All statistics were carried out on the SPSS software, version 16.0 (SPSS, Chicago, IL. USA).

3. Results

3.1. Effects of food quantity and cheliped autotomy on intermolt duration, molting success, and molt increments in size and weight

The results in Table 1 showed that both food quantity and cheliped autotomy had a main effect on molt increments in intermolt duration, carapace length, carapace width, and body weight, but not on molting success. Moreover, significant interaction ($p < 0.05$) between feeding ration and cheliped autotomy was only detected on intermolt duration, but not other parameters measured, i.e. molting success, molt increments in carapace size (length and width) and body weight (Table 1).

Table 1
The effects of cheliped autotomy and feeding rations on intermolt duration, molting success, and molt increments in size and weight of early juvenile *S. paramamosain*.

Cheliped status	Feeding ration	Molting success (%)	Intermolt duration (days)	C3 carapace length (mm)	C3 carapace width (mm)	C3 weight (mg)
Intact	Optimal	96.67 ± 2.89[a]	5.31 ± 0.15[a]	4.79 ± 0.24[a]	6.35 ± 0.40[a]	37.93 ± 6.65[a]
Intact	Suboptimal	96.67 ± 5.77[a]	6.82 ± 0.18[c]	4.72 ± 0.18[ab]	6.14 ± 0.31[bc]	33.87 ± 4.02[b]
Intact	Low	95.00 ± 5.00[a]	10.43 ± 0.22[d]	4.48 ± 0.23[c]	5.84 ± 0.34[d]	28.00 ± 4.61[c]
Intact	Starvation	0	-	-	-	-
Autotomy	Optimal	100.00 ± 0.00[a]	5.93 ± 0.26[b]	4.66 ± 0.27[b]	6.24 ± 0.25[ab]	33.36 ± 6.91[b]
Autotomy	Suboptimal	96.67 ± 2.89[a]	7.21 ± 0.10[c]	4.63 ± 0.16[b]	5.99 ± 0.36[cd]	29.96 ± 3.74[c]
Autotomy	Low	96.67 ± 2.89[a]	9.95 ± 0.57[d]	4.30 ± 0.22[d]	5.61 ± 0.37[e]	23.68 ± 3.79[d]
Autotomy	Starvation	0	-	-	-	-
ANOVA (p-value)	Feeding Ration	0.516	0.000	0.000	0.000	
	Cheliped autotomy	0.361	0.228	0.000	0.000	
	Ration * Autotomy	0.746	0.014	0.438	0.477	0.905

Note: Values with different letters within the same column are significantly different ($P < 0.05$)

Among the cheliped intact treatments, with decreased feeding ration, the intermolt duration significantly extended while the body weight, carapace width and length of newly molted C3 crabs were significantly reduced; a similar trend was found among limb autotomy treatments (Table 1). On the other hand, under the optimal feeding ration, cheliped autotomy significantly suppressed molt increments in body weight and carapace length but not carapace width when compared to those with intact chelipeds. Under the suboptimal ration, cheliped autotomy also significantly decreased molt increment in body weight but not carapace length and width. However, when the crabs were fed low ration, limb autotomy significantly suppressed all growth parameters measured, including body weight, carapace length and width (Table 1).

Interestingly, regardless if cheliped was autotomized, the molting success of the juvenile crabs remained high (ranging from 95% to 100%) for all fed treatments with different rations (i.e. optimal, suboptimal and low). Even for the low ration treatments (only ¼ of the optimal ration), no significant difference was detected among any of these fed treatments (Table 1). However, despite that low feeding ration seems to be able to meet the nutritional requirement of molting, starvation resulted in total mortality of the crabs regardless of limb autotomized (Table 1).

As the result of a significant interactive effect between feeding ration and cheliped autotomy, for those crabs being fed the optimal ration and with cheliped autotomy, their intermolt duration significantly lengthened as compared to those fed the same ration but with intact chelipeds. Although limb autotomy also slightly lengthened the intermolt duration when the crabs were fed the suboptimal ration, the difference was not significant. Interestingly, when fed low ration, cheliped autotomy appeared to lead to a shorter intermolt duration although again the difference was not significant (Table 1).

3.2. *Molting synchrony*

The effects of feeding ration and limb autotomy on the molting synchrony of the crabs were also assessed. The results showed that the

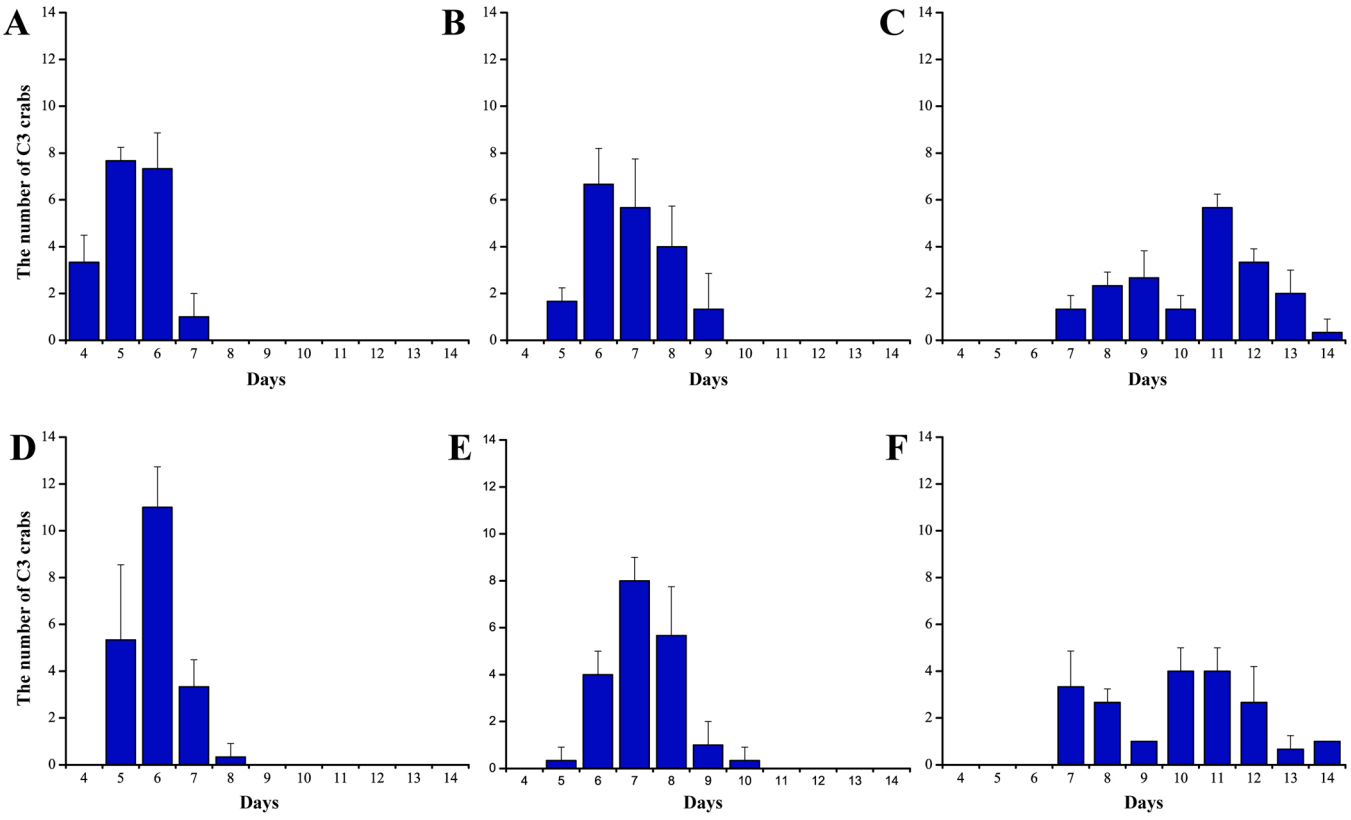

Fig. 1. The time span and frequency of molting occurrence of *S. paramamosain* early juveniles from each treatment. A: Intact + Optimal ration; B: Intact + Suboptimal ration; C: Intact + Low ration; D: Autotomy + Optimal ration; E: Autotomy + Suboptimal ration; F: Autotomy + Low ration.

feeding ration affected the molting synchrony of the crabs regardless of whether chelipeds of the crabs were autotomized. Under optimal ration, the intact crab began to molt on day 4 and molts continued to occur till day 7 (Fig. 1); for cheliped autotomized crabs, molting first occurred 1 day later (day 5) but also finished a day later, hence had the same time span of four days during which molting occurred as intact crabs. With reduced feeding ration, there was a general trend that the onset of molting was delayed while the time span of molting occurrence was prolonged regardless if limb autotomy occurred. For example, when fed a suboptimal ration, molting first occurred on day 5 for both intact and autotomy crabs, while the time spans of molting occurrence increased to 5 and 6 days for intact and autotomy crabs, respectively. As feeding further reduced to the low ration, the first molts occurred as late as day 7 while the time spans of molting occurrence increased further to 8 days for both intact and autotomy crabs (Fig. 1).

In addition, the body weight of newly molted C3 crabs molted on different days with each treatment was compared. It showed that under both optimal and suboptimal ration, crab body weights were not significantly affected by the day of molting. However, when the crabs were fed a low ration, the body weight of the crabs that molted later were significantly heavier than those molted earlier (Table 2).

3.3. Mortality pattern under starvation

When subjected to starvation, total mortality occurred for both intact and autotomy crabs. Interestingly, cheliped autotomy appears to affect the time of the crab death: mass mortality of the crabs with intact chelipeds started to occur on day 12, while for the limb autotomized crabs, it occurred on the days 11. Meanwhile, the mortality of the intact crabs started to occur on day 10 and all of them were dead by day 17, while for the autotomized crabs, both first and last mortalities occurred earlier on day 9 and 15, respectively (Fig. 2).

3.4. mRNA expression of SpEcR

The results of qRT-PCR showed that under optimal feeding ration, the expression of *SpEcR* was generally suppressed by the limb autotomy from 6 to 96 h as compared to the intact crabs with significant low expression detected at 6, 48, 72 and 96 h. However, when the crab developed to the premolt stage, the transcripts levels of *SpEcR* of the limb autotomy crabs sharply increased and became significantly higher than that of the intact crabs. After successful molting, significantly decreased *SpEcR* mRNA level was detected for the newly molted C3 crabs regardless if cheliped was autotomized and no significant difference was found between the intact and autotomy crabs (Fig. 3A). When the crabs were fed the suboptimal ration, the expression profile of *SpEcR* was similar to that of the crabs fed the optimal ration; i.e. the transcript levels of *SpEcR* of limb autotomy crabs were often significantly lower than those intact ones throughout most of the molting cycle (6–120 h); however once the crabs reached the premolt stage, *SpEcR* expression rapidly increased to a level that is similar to the intact crabs (Fig. 3B). Interestingly, the mRNA level of *SpEcR* in the crabs subjected to low feeding ration showed a difference to those fed optimal or suboptimal rations as no significant difference in *SpEcR* expression was detected between the autotomy and intact crabs except at 48 h (Fig. 3C). For those crabs subjected to starvation, a reversed trend to those crabs subjected to optimal and suboptimal rations was detected as the *SpEcR* mRNA of the limb autotomized crabs was always higher, and often significantly higher than that of the intact crabs (Fig. 3D). Finally, it is worth noting that similar to optimal ration treatments, under both suboptimal and low feeding rations, once the crabs had successfully molted, *SpEcR* mRNA level significantly decreased in the newly molted C3 crabs, and no significant difference was detected between the intact and autotomy crabs (Fig. 3).

Moreover, the transcript level of *SpEcR* was also compared across different feeding conditions for the intact and autotomy crabs. The results showed that for intact crabs, a general trend of *SpEcR* expression suppressed by decreased feeding ration was identified. Indeed, the highest expression level was always for the crabs fed the optimal ration while for crabs subjected to starvation, the mRNA level of *SpEcR* was lower than all other treatments from 48 h onward. Despite with higher degree of variations, *SpEcR* expression in limb autotomy crabs largely hold a similar trend of reduced expression level with decreased feeding ration (Fig. 4).

4. Discussion

In both aquaculture settings and natural environments, food is a critical factor that affects the survival, molting and growth of crustaceans. Juvenile crustaceans, in particular, are prone to suffer from fluctuations in food availability due to their high metabolic rates and small guts with limited capacity for storage of ingested food (Mikami and Takashima, 1993). Most marine organisms can tolerate a period of starvation and this is considered a key factor for their survival in the wild (Figueiredo et al., 2008). For example, in crustaceans, it has been reported that when the stage III juveniles of the red claw crayfish *Cherax quadricarinatus* were fed for over 1/3 of the intermolt duration, 50% of them successfully molted to the next stage, which is also known as PRS_{50} (Stumpf et al., 2010). PRS_{50} is often used as a practical way for quantifying PRS, which is defined as the duration of initial feeding within a molting cycle that leads to 50% of the experimental crustaceans becoming capable of successful molting to the next stage even food is totally withdrawn from the time point onward (Anger, 1995; Stumpf et al., 2010). PRS_{50} is species and developmental stage specific. For instant, PRS_{50} for instar 1 phyllosoma of the western rock lobster *Panulirus cygnus* reportedly was 42% of the intermolt duration, while for

Table 2
Mean body weight of newly molted C3 crabs of *S. paramamosa* molted on different days.

Treatments	Time of molting (day)									
	4	5	6	7	8	9	10	11	12	13
Optimal ration+Intact	36.11 ± 3.33^{a}	37.95 ± 3.18^{a}	38.65 ± 2.70^{a}	–	–	–	–	–	–	–
Optimal ration+Autotomy	–	31.22 ± 5.62^{a}	34.33 ± 4.04^{a}	35.52 ± 6.73^{a}	–	–	–	–	–	–
Suboptimal ration+Intact	–	30.27 ± 4.15^{a}	32.74 ± 2.98^{a}	35.23 ± 3.60^{a}	34.47 ± 6.30^{a}	36.00 ± 1.45^{a}	–	–	–	–
Suboptimal ration+Autotomy	– –		28.64 ± 1.69^{a}	30.01 ± 1.54^{a}	29.69 ± 1.47^{a}	31.1 ± 1.91^{a}	–	–	–	–
Low ration+Intact	– –		–	21.6 ± 4.45^{a}	26.84 ± 2.44^{ab}	25.64 ± 0.55^{ab}	26.80 ± 4.38^{ab}	28.18 ± 1.81^{ab}	29.30 ± 3.20^{ab}	32.89 ± 0.48^{b}
Low ration+Autotomy	– –		–	19.39 ± 2.12^{a}	24.28 ± 1.92^{ab}	25.03 ± 4.35^{ab}	23.75 ± 3.21^{ab}	23.83 ± 1.07^{ab}	24.71 ± 2.13^{ab}	25.75 ± 0.35^{b}

Data are presented as mean ± SD. Values with different letters within the same row are significantly different ($P < 0.05$)

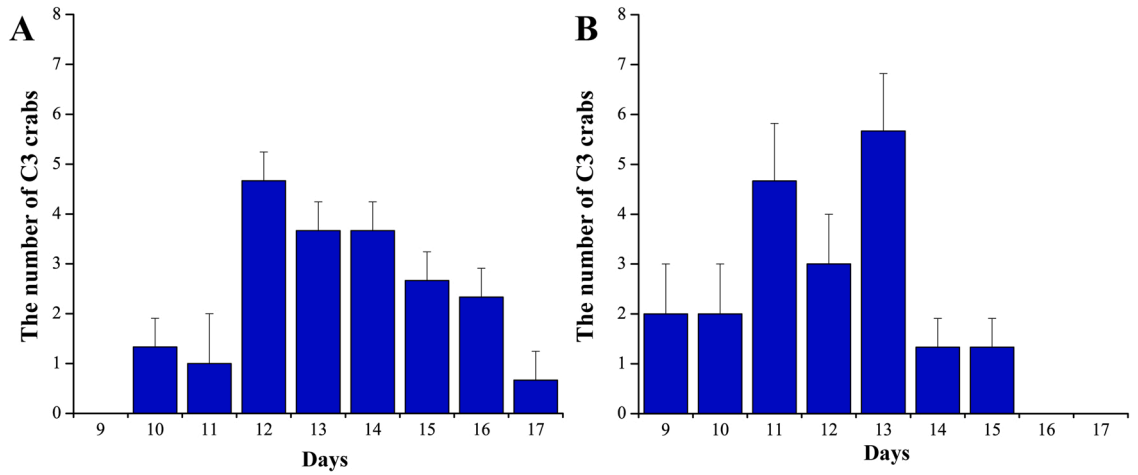

Fig. 2. The daily mortality of *S. paramamosain* juveniles subjected to starvation. A: Intact; B: Autotomy.

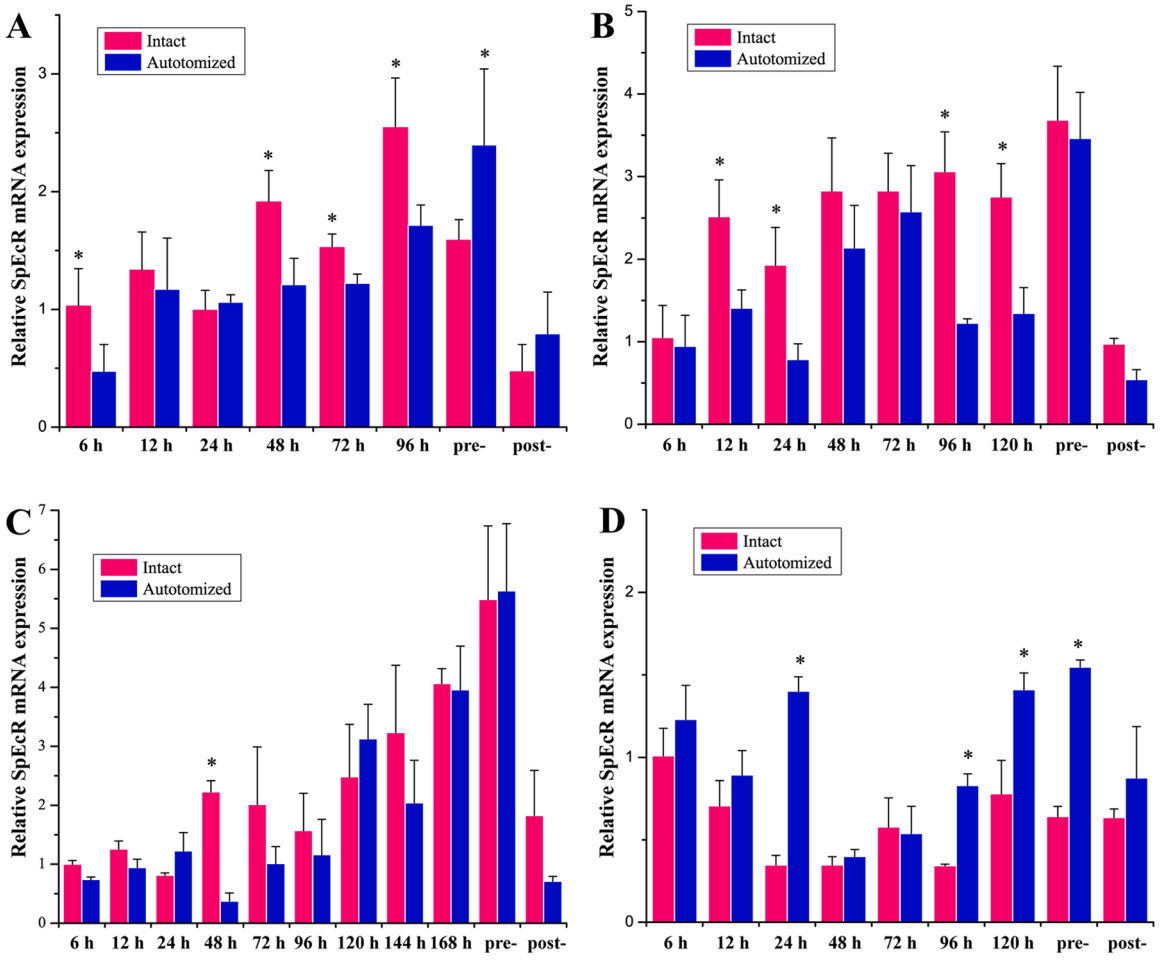

Fig. 3. The effect of cheliped autotomy on the *SpEcR* mRNA transcripts of *S. paramamosain* juveniles subjected to different feeding rations. A: Optimal ration; B: Suboptimal ration; C: Low ration; D: Starvation. Asterisks ("*") on the top of the bars indicate significant differences ($p < 0.05$). pre-: premolt; post-: postmolt (C3).

Zoea I larvae of North Sea shrimp *Crangon crangon*, it was only 23% of the intermolt duration (Liddy et al., 2003; Paschke et al., 2004). Therefore, starvation tolerance appears to be hugely variable among species and different developmental stages. In this study, it was found that both the intact and limb autotomized C2 crabs could successfully molt to the next stage with high survival (95%) even food supply was limited (e.g. under low feeding ration). This result indicates that the mud crab juveniles have a high tolerance to fluctuations in food availability and a low ration of the food supply is sufficient for them to manage to molt to the next stage successfully.

Prior to sexual maturation, crustaceans use the obtained energy from ingested food for maintenance, molting and growth, and under unfavorable conditions, individuals typically allocate energy preferentially for survival, while growth is compromised (Knowlton and Re, 1974). We

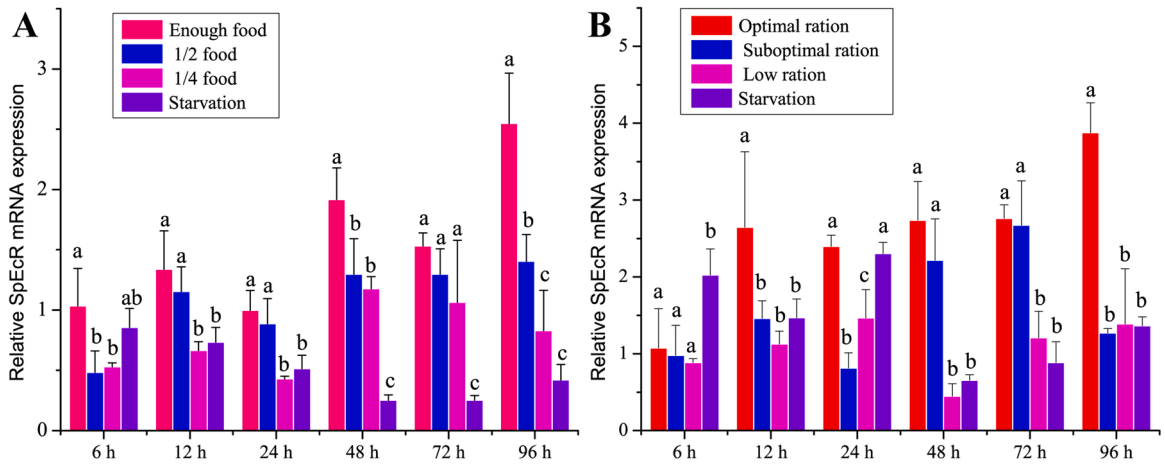

Fig. 4. The effect of feeding ration on the *SpEcR* mRNA transcripts of *S. paramamosain* juveniles. A: Intact; B: Autotomy. Values with different letters on the tops of bars are significantly different ($P < 0.05$).

found that under decreased food supply, the intermolt period of the C2 crab was remarkably increased and the body weight of newly molted C3 reduced significantly. It has also been reported that the developmental time from Zoea larvae to megalopae was negatively correlated with food ration in the porcelain crab, *Petrolisthes cabrilloi* (Howard and Hentschel, 2005). These results manifest that under unfavorable feeding conditions, crustaceans might attempt to extend their intermolt duration to accumulate more nutrition reserves to enable successful molt, even it unfortunately may not work sometimes and lead to death eventually. If this period of extended survival beyond the normal range of intermolt duration is defined as "extra feeding time", the present study showed such an "extra feeding time" increased with the food ration reducing in the mud crab juveniles. As *SpEcR* expressions of the crabs fed suboptimal and low feeding ration was significantly suppressed, it indicated that the juvenile crabs under limited feeding availability achieved a prolonged molting cycle to gain "extra feeding time" through suppressing *EcR* transcripts level.

It was unexpected that the juvenile crabs fed the low ration had very high percentages of molting success to the next stage (> 95%), which was not significantly different from those crabs fed the optimal ration of 4 times higher, although the intermolt duration was significantly prolonged. This phenomenon suggested that the juvenile mud crabs could successfully develop to the next stage under very limited feeding supply by prolonging their intermolt period to accumulate sufficient energy/nutrition to achieve a successful molt. Similarly, it has also been reported that the Zoeal larvae of *P. cabrilloi*, coped with food scarcity by increasing the intermolt duration; however, when the zoeal larvae were fed a low level of food at < 10 *Artemia* d^{-1} (optimal: 30–40 *Artemia* d^{-1}), the rate of successfully metamorphosed to megalopae stage was very low (less than 30%) despite extended intermolt duration (Howard and Hentschel, 2005). Hence it indicates that the "minimal ration" that allows crustaceans to develop to the next stage without significantly reduced molting success is likely species and developmental stage specific. In this study, the high rate of success molting of the juvenile mud crabs with the low food ration suggested that their "minimal ration" was very low, which likely was evolved to cope with the frequent lack of food in their natural habitats. Although the "minimal ration" for the juvenile was very meager, under starvation, none of the intact and autotomized crabs was able to achieve successful molt and ultimately died with significantly suppressed *SpEcR* expression. Similarly, PRS_{50} of stage III juveniles of *C. quadricarinatus* was identified as at only 1/3 of the intermolt duration, however, continuous starvation caused total mortality without a successful molt (Stumpf et al., 2010).

In crustaceans, autotomy is a fundamental adaption to cast off damaged appendages. Various aspects of limb autotomy and regeneration in crustaceans have been studied, including wound repairing after autotomy, limb blastema formation, and regenerated limb growth prior to molting (Hopkins and Das, 2015). As crustacean juveniles typically molt frequently and with their softshell, newly molted individuals are very weak, lacking self-defense capability, when cannibalism often occurs. Conspecific aggressive behavior and cannibalism are one of the primary reasons for mortality in the communal culture system for many crustaceans, such as mud crabs (Romano and Zeng, 2017). In this study, although cheliped loss could affect the growth of the crabs (i.e. increased the molting interval), the molting success and developmental synchronicity were not significantly changed with the cheliped loss and a new cheliped emerged after molting. Similarly, the autonomy of mud crab, *S. serrata*, juveniles reportedly increased the molt interval to accommodate the time needed to grow the limb blastema with a suppressed specific growth rate (Quinitio and Estepa, 2011). Moreover, in the blue crab, *Callinectes sapidus*, the autotomized cheliped can regenerate to a size similar to a normal cheliped (Smith, 1990). These results indicated that cheliped autotomy usually does not have a lethal effect and rapid growth of the regenerated cheliped occurred during the molting process. Therefore, cheliped autotomy has been used as an effective strategy to reduce the mortality caused by aggressive behavior and cannibalism during nursery culture and juvenile transportation, as well as softshell crab production of various mud crab species (Triño et al., 1999, Quinitio and Estepa, 2011, Rahman et al., 2020).

However, Fazhan et al. (2022) found that both chelipeds removing could significantly decrease the average consumption of live blood cockles in the mud crab *S. olivacea*. This result indicates that limb removal can affect the feeding ability of crabs and future optimization of the feeding practices of mud crabs during nursery culture and juvenile transportation need to be investigated deeply. Interestingly, it was found that under suboptimal feeding ration, although cheliped autotomy did increase the molting interval of juvenile crabs, the difference was not significant when compared to that of intact crabs. The regenerated chelipeds were obviously smaller (data not shown) when compared to those crabs fed the optimal ration. This result suggested that under food deficient conditions, the growth of regenerated chelipeds were compromised, and that the effects of food supply on the molting and growth might be more prominent than limb autotomy for the crab juveniles.

It has been reported that *SpEcR* gene is involved in the regulation of limb regeneration after autotomy in crustaceans (Gong et al., 2015a). In this study, under optimal ration, cheliped autotomy significantly inhibited the expression of *SpEcR* prior to the premolt stage of the crabs, which was consistent with the extended intermolt duration of the

autotomized crabs. Therefore, the low expression of *SpEcR* gene of autotomized crabs prior to the premolt stage might act as an underlying regulation mechanism for the increased intermolt duration to allow crabs more time to grow the limb blastema prior to molting. Under the low ration, the effect of limb autotomy on *SpEcR* gene expression was mostly insignificant, as the result, limb autotomy also did not significantly alter the molt interval of crabs. These results further proved that *SpEcR* was involved in regulating the molting and regeneration of autotomized limbs of the juvenile crabs.

5. Conclusion

In conclusion, this study provides new insight into the combined effects of feeding ration and cheliped autotomy on the intermolt duration, molting success, molt increments in size and weight, and ecdysteroid receptor gene (*SpEcR)* expression of early juvenile mud crab *S. paramamosain*. Significant effects of feeding ration and cheliped autotomy were observed on intermolt duration and molt increments in carapace length and width, as well as body weight of the crabs. We found that all the crabs with different feeding rations even the low ration had high rates of molting success with no significant difference was detected. Moreover, the expression of *SpEcR* showed a general trend of inhibited by reduced feeding ration, which was consistent with observed significantly increased intermolt duration. Thus, we confirmed that the *S. paramamosain* early juveniles have a strong tolerance for fluctuations of food availability, while the availability of food and limb autotomy could significantly affect growth, molting duration and synchrony of the crabs, which appeared to reflect in *SpEcR* expression level.

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CRediT authorship contribution statement

Jie Gong: Performed the main experiment and wrote the manuscripts. **Chencui Huang**: Prepared the experimental material. **Kun Yu**: Aided in part experimental work and revised the manuscript. **Shaojing Li**: Did the statistical data analysis and modified the manuscript. **Chaoshu Zeng**: Designed the experiment and modified the manuscript. **Haihui Ye**: Planed the research and designed the experiment.

Conflict of Interest

We declare that the authors do not have any conflict of interest in connection with the work submitted.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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