EFFECTS OF SPINACH (*Amaranthus* sp.) EXTRACT SUPPLEMENTATION ON MOLTING DURATION AND PHYSIOLOGICAL RESPONSES OF MUD CRABS (*Scylla* sp.) REARED IN CRAB APARTMENT SYSTEMS

Bagas Gagat Rahina Asihing Sutrisno[1], Supriatna[1], Hafiz Muhammad Uzair[2], Azfar Ismail[3], and Asus Maizar Suryanto Hertika[1*]

[1]Department of Fisheries and Marine Resources Management, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Malang 65145, Indonesia
[2]Depertment of Zoology, University of Sargodha, Sargodha 40100, Pakistan
[3]Depertment of Aquaculture, Universiti Putra Malaysia, Selangor 43400, Malaysia,

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ABSTRACT

This study investigates the effects of dietary supplementation of spinach (*Amaranthus* sp.) extract, rich in phytoecdysteroids, on the molting process and physiological parameters of mud crabs (*Scylla* sp.) cultivated in crab apartment boxes integrated with a recirculating aquaculture system. This experiment was designed using a completely randomized design (CRD) with four different doses of spinach extract as treatments (0, 300, 700, and 1000 ng g^{-1} of crab) and three replications. Some parameters measured were molting duration, hormone levels, including ecdysteroid and molt inhibiting hormone (MIH), reactive oxygen species (ROS), and water quality. The results showed that higher doses of spinach extract significantly shortened the molting duration ($p < 0.05$), with the fastest molting observed at 1000 ng g^{-1} (11.83 ± 1.26 days). Increased spinach extract doses also significantly affected MIH and ROS levels. Specifically, MIH levels increased from 12.05 ng mL^{-1} in the control to 20.88 ng mL^{-1} in treatment D, while ROS levels rose from 116.80 to 147.33 μmol mL^{-1}. Overall, the administration of phytoecdysteroids from spinach presents a promising, eco-friendly approach to enhance soft-shell crab production efficiency, although careful dose optimization is necessary to balance production acceleration with animal welfare.

KEYWORDS: molting; phytoecdysteroids; *Scylla* sp.; soft-shell crab; spinach extract

*ABSTRAK: **Pengaruh Suplementasi Ekstrak Bayam (**Amaranthus* **sp.) terhadap Durasi Molting dan Respons Fisiologis Kepiting Bakau (**Scylla* **sp.) yang Dipelihara dalam Sistem Apartemen Kepiting***

*Penelitian ini mengkaji pengaruh suplementasi pakan dengan ekstrak bayam (**Amaranthus** sp.) yang kaya akan fitoekdisteroid terhadap proses molting dan parameter fisiologis kepiting bakau (**Scylla** sp.) yang dibudidayakan dalam kotak apartemen kepiting terintegrasi dengan sistem akuakultur resirkulasi. Percobaan ini dirancang menggunakan rancangan acak lengkap (RAL) dengan empat dosis ekstrak bayam sebagai perlakuan (0, 300, 700, dan 1000 ng g^{-1} kepiting) dan tiga ulangan. Beberapa parameter yang diukur meliputi durasi molting, kadar*

*Korespondensi: Department of Fisheries and Marine Resources Management, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Malang 65145, Indonesia
Email: asusmaizar@ub.ac.id

hormon termasuk ekdisteroid dan molt inhibiting hormone (MIH), reactive oxygen species (ROS), serta kualitas air. Hasil penelitian menunjukkan bahwa peningkatan dosis ekstrak bayam secara signifikan memperpendek durasi molting ($p < 0,05$), dengan waktu molting tercepat pada dosis 1,000 ng g^{-1} (11,83 ± 1,26 hari). Peningkatan dosis ekstrak bayam juga berpengaruh nyata terhadap kadar MIH dan ROS. Secara khusus, kadar MIH meningkat dari 12,05 ng mL^{-1} pada kontrol menjadi 20,88 ng mL^{-1} pada perlakuan D, sedangkan kadar ROS naik dari 116,80 menjadi 147,33 µmol mL^{-1}. Secara keseluruhan, pemberian fitoekdisteroid dari bayam menunjukkan potensi sebagai pendekatan ramah lingkungan untuk meningkatkan efisiensi produksi kepiting cangkang lunak, meskipun demikian optimasi dosis tetap diperlukan agar tercapai keseimbangan antara percepatan produksi dan kesejahteraan hewan.

KATA KUNCI: ekstrak bayam; fitoekdisteroid; kepiting soka; molting; *Scylla* ***sp.***

INTRODUCTION

Mud crab (*Scylla* sp.) represents a high-value fishery commodity with strong demand in both domestic and international markets. Notably, Indonesia's export value of crab increased significantly from USD 447,651,200 in 2023 to USD 513,352,780 in 2024 (Satu Data KKP, 2025). Among its processed products, soft-shell crab holds exceptional market potential due to its unique characteristic: an entirely edible soft exoskeleton (Fujaya, 2025). The production of soft-shell crabs relies on the molting process—a critical phase in the crab's life cycle—during which they shed their old shell. However, molting is often slow and unpredictable, leading to prolonged culture periods, higher operational costs, and an increased risk of mortality from cannibalism or inadequate nutrition (Rachmawati *et al*., 2021). This makes research on molting acceleration highly relevant to enhance production efficiency.

Molting in *Scylla* sp. is hormonally regulated, primarily involving the molt inhibiting hormone (MIH) secreted by the sinus gland and ecdysteroid hormones like 20-hydroxyecdysone produced by the Y-organ (Fujaya et al., 2018). These ecdysteroids directly trigger ecdysis—the shedding process (Purnama & Haslianti, 2016). Interestingly, similar bioactive compounds called phytoecdysteroids are also found in certain plants, such as spinach (*Amaranthus* sp.) (Hidayat *et al*., 2024). Previous studies have demonstrated that phytoecdysteroids exhibit structural and functional similarities to endogenous ecdysteroids in crustaceans and have potential in promoting molting (Kim *et al*., 2025).

Incorporating phytoecdysteroid-rich plants like spinach into crab diets offers an innovative and environmentally friendly alternative to synthetic hormones for stimulating molting. A dose of 1 µg of 20-HE molting hormone is equivalent to 42 µg mL^{-1} of spinach extract (Hasnidar, 2021). Increased levels of ecdysteroids in the hemolymph provide a signal for the body to start the molting process (Habibi *et al*., 2013). This approach is expected to be safer, more sustainable, and cost-effective. Despite its promising potential, detailed insights into how spinach-derived phytoecdysteroids influence the molting process in mud crabs remain limited, especially at the hormonal and physiological levels. Thus, it becomes crucial to conduct research and analyze relevant data to formulate effective strategies for optimizing phytoecdysteroid use in soft-shell crab culture.

Most previous studies have focused solely on observing molting duration and crab survival rates without exploring the underlying hormonal mechanisms. Therefore,

this study offers a novel approach by analyzing MIH and ecdysteroid hormone levels in crabs, which play a role in molting regulation. This approach allows the study to not only examine the effects of spinach on molting duration but also understand the biological mechanisms involved at the hormonal level. These findings can provide new insights into optimizing soft-shell crab cultivation more scientifically and effectively.

MATERIALS AND METHODS

Time and Place

The research was conducted in June-July 2025. The research was taken place at the Sumberpasir Fisheries Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University.

Research Materials

The research materials used in this study included mud crabs with an average weight of 102.92 ± 2.57 g as the primary experimental animals. Spinach was used as a natural source of phytoecdysteroids to stimulate the molting process. The feed used in this study was boneless and skinless tilapia filleted to facilitate crab consumption. Before being fed to the animals, the feed was weighed at 5% of their body mass once daily, enriched with spinach extract at different doses (0, 300, 700, and 1,000 ng g^{-1}). Additional materials included equipment for preparing and extracting spinach, such as blenders, rotary evaporators, and analytical balances.

To analyze physiological responses, reagents and kits were used for measuring hormone concentrations (ecdysteroids and MIH) and oxidative stress indicators (ROS). Instruments included ELISA readers, spectrophotometers, and other laboratory apparatus to support biochemical analyses (Mahdaliana *et al*., 2022). Crab apartment boxes made of PVC were used for individual maintenance of crabs, ensuring controlled experimental conditions throughout the research period (Agustiyana *et al*., 2024).

Research Design

This study used a method where the design was a non-factorial completely randomized design (CRD) with four treatments and three replications, which included independent and dependent variables that show how they affect each other (Candra et al., 2023). Each treatment consisted of three replications, with one crab placed individually in each box, resulting in a total of 12 crabs used in the experiment. Treatment A (spinach extract 0 ng g^{-1} crab), Treatment B (spinach extract 300 ng g^{-1} crab), Treatment C (spinach extract 700 ng g^{-1} crab), and Treatment D (spinach extract 1,000 ng g^{-1} crab). The independent variable in this study was the addition of spinach extract, while the dependent variables included molting duration, ecdysteroid hormone levels, molt inhibiting hormone, and reactive oxygen species (ROS).

Work Procedure

The research process was carried out in two main stages. Stage I includes preparation of infrastructure and research materials. The process began with a literature study on the characteristics of mud crab maintenance, followed by the construction of a crab apartment box measuring 36 × 20 × 17 cm and the installation of a recirculating aquaculture system (RAS) that includes physical, chemical, and biological filters. The mud crabs used were mud crabs weighing 100.0 ± 5.0 g per individual (Rahmadiah *et al*., 2023). The average initial weight of the crabs used in this study was 102.92 ± 2.57 g per individual. The mud crabs were obtained from fishermen at the marine fisheries landing in Probolinggo, Indonesia. Each crab sample used was in the intermolt phase and had complete body parts. The mud crabs used in this study were selected based on clear visual indicators of the intermolt phase, including a hard and fully calcified carapace,

absence of soft or pliable exoskeleton areas, and lack of visible signs of imminent molting such as loosened sutures or swelling at the limb joints. Selection was further confirmed by observing normal, active behavior without lethargy, which is typical during the premolt phase. Crabs with complete body parts were defined as individuals with no missing or regenerating limbs, no damage or deformities to the chelipeds or walking legs, and an intact, undamaged carapace free from cracks or lesions. This combination of physical and behavioral criteria ensured that only healthy intermolt crabs in optimal condition were included in the experiment, thereby enhancing the reliability and reproducibility of the study. Seawater was taken from the Probolinggo mangrove ecosystem, then analyzed and prepared as a rearing media. The filtration process includes a mechanical filtration stage to remove coarse particles such as sand and mud, followed by fine filtration using micron-sized filters to remove plankton, small particles, and bacteria. Based on the measurement results, the seawater temperature was monitored at 29°C with a pH of 7.5, dissolved oxygen (DO) levels of 6.5 mg L^{-1}, salinity of 20 ppt, and ammonia of 0.3 mg L^{-1}.

Initial analysis includes the phytochemical and phytoecdysteroid content of spinach, which will be used as a feed additive. Based on the research results of Kusmiati *et al*. (2014), it can be seen that green spinach (*Amaranthus hybridus*), red spinach (*Amaranthus tricolor*), and prickly spinach (*Amaranthus spinosus*) leaves powder contain various secondary metabolites that have the potential to provide biological benefits. These three types of spinach contain alkaloids, flavonoids, saponins, tannins, carotenoids, 15 coumarins, and phenols, which are known to have antioxidant and antimicrobial activities and a role in stimulating metabolism (Santoni *et al.*, 2023). Additionaly, a dose of 1 μg of 20-HE molting hormone is equivalent to 42 μg mL^{-1} of spinach extract (Hasnidar, 2021).

Stage II begins with feed preparation. Fresh spinach leaves are dried in an oven at 60°C for 24 hours, then crushing them into a fine powder with an approximate particle size of 0.5 mm (Tamsil, 2021). The powder was macerated in 80% ethanol at a ratio of 100 g of spinach simplicia in 400 mL of ethanol for 48 hours at room temperature with occasional stirring (Saptarini *et al*., 2017). The extract was then filtered, and the solvent was removed using a rotary evaporator at 50-70°C under reduced pressure (Ridwan & Lisnawati, 2021). The concentrated extract was stored in an amber glass container at 4°C until use, with a maximum storage duration of 2 weeks to maintain stability. Spinach extract was dissolved with 80% ethanol in a 1:1 ratio and then homogenized. The solution was added with 80% ethanol up to 20 mL kg^{-1} of feed and evenly marinated into the test feed, then the feed was air-dried (Aslamyah & Fujaya, 2011). The feeding process involves hanging the food using wire and keeping it out of the water. The crabs were then allowed to feed themselves using their claws. The RAS was turned off during feeding to make it easier for the crabs to consume and to prevent contamination (Ningsih & Affandi, 2023).

The mud crabs used to weigh ± 100 g and were in the intermolt phase. The crabs were acclimatized, their initial length and weight were measured, and then they were kept in a crab apartment and fed twice a day. During the maintenance process, water quality monitoring (temperature, pH, DO, salinity, and ammonia) was carried out every 12 hours, as well as observations of behaviour and the molting process every 3-4 hours. Temperature, DO, and pH measurements were performed using a multiparameter probe (LUTRON YK-2001PHA, Taiwan), salinity measurements were performed using a refractometer (Atago NO2383 MASTER-20M Handheld Refractometer, Japan), and ammonia measurements were performed using a spectrophotometer (Thermo Scientific GENESYS 10S Series UV-Visible Spectrophotometers, USA). All excess feed and feces were carefully removed using a nylon sieve. After the crabs had molted, they were sampled. The parameters observed

hemolymph analysis to measure the content of ecdysteroid hormones, MIH, and ROS using the ELISA method. All treatments were carried out in three replications to ensure data validity.

Ecdysteroid, MIH, and ROS levels were measured using commercial ELISA kits (Diatek DR-200BC) following the manufacturer's instructions. The assays employed specific monoclonal antibodies for each target, with assay sensitivities of (insert detection limits, e.g., 10 pg mL^{-1} for MIH, 250 ng mL^{-1} for ecdysteroid, and 10 μmol mL^{-1} for ROS). Hemolymph samples were collected individually from each crab and analyzed separately without pooling to ensure the accuracy of individual measurements. All samples and standards were run in duplicate to minimize analytical variability.

Data Analysis

Data analysis was performed using ANOVA to determine the significance of the differences between treatment groups with a confidence level (α) = 0.05. If the results are significantly different, the Tukey HSD test is continued to see the differences between treatments with a confidence level (α) = 0.05.

RESULTS AND DISCUSSION

Molting Symptoms

Observation of molting symptoms was carried out every 1-2 hours during the treatment. Crabs that were about to molt did not want to eat from day-1 (D-1) until the process was complete. Symptoms of molting can also be seen in the physical color of the lower part of the crab's body, which is increasingly white, not transparent. In addition, symptoms of molting can also be seen in the back carapace of the crab, which is cracked. All of these symptoms can be seen from D-1 of the molting process until the molting process occurs.

The molting process begins when epidermal cells respond to hormonal changes by increasing the rate of protein synthesis. The increase in the rate of protein synthesis due to the stimulation of molting hormones causes apolysis which causes the separation of the epidermis layer from the old endocuticle and initiates protein synthesis to form a new procuticle (Belles, 2020). When the new exoskeleton is ready, ecdysis occurs when the crab releases the exuvia through muscle contractions and air or water intake, which causes the body to swell and split along the ecdysial sutures and finally the body with the new exoskeleton comes out of the old exoskeleton (Kubo & Klocke, 1986). After molting, the new cuticle hardens and undergoes sclerolysis (Belles, 2020). A crab undergoing molting or ecdysis is shown in Figure 1.

Molting Duration

Molting duration of mud crabs treated different doses of spinach extract are presented in Figure 2. The results show that a phytoecdysteroid at a dose of 0 ng g^{-1} (A) had the highest molting duration of 39.17 ± 4.37 days and was significantly different ($p < 0.05$) compared to all other treatments. Meanwhile, treatment D (1,000 ng g^{-1}) had the lowest molting duration of 11.83 ± 1.26 days. Treatment B (300 ng g^{-1}) with an average molting duration of 22.67 ± 2.08 days results in intermediate value, which is not different from treatment C (700 ng g^{-1}) with a molting duration of 15.00 ± 3.00 days. This pattern indicates that increasing the phytoecdysteroid dose tends to reduce the molting duration significantly.

Several studies have explored different methods of administering spinach extract, including through feed and injection. An artificial feed was developed using food waste enriched with spinach extract (700 ng g^{-1} of crab), and found that a diet containing 30.62% protein and 49.13% carbohydrate was most effective in inducing molting (Aslamyah & Fujaya, 2010). This result is related to the previous study, which found a molting percentage of 54% in crabs given spinach

Figure 1. Old exoskeleton shedding and new exoskeleton formation (molting) in crabs (*Scylla* sp.) reared in crab apartment boxes and fed with different dietary supplementations of spinach (*Amaranthus* sp.) extract

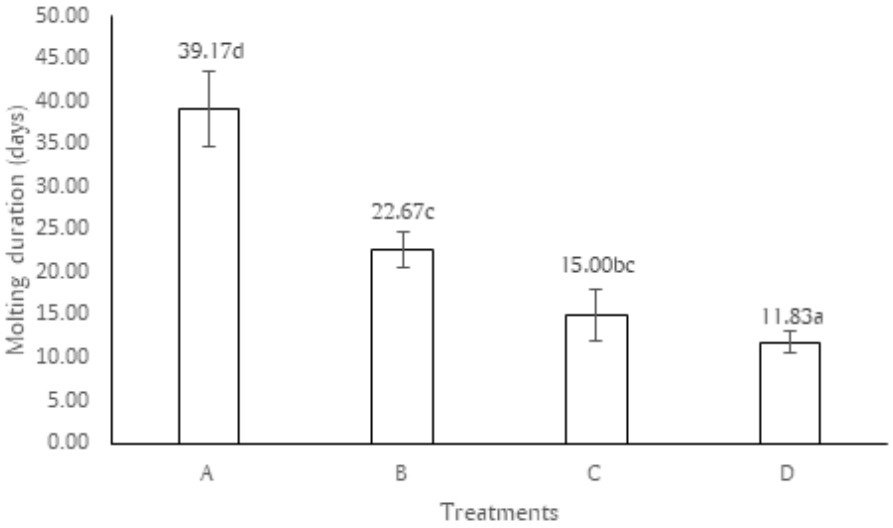

Figure 2. Molting duration of mud crabs (*Scylla* sp.) reared in crab apartment boxes and fed with different dietary supplementations of spinach (*Amaranthus* sp.) extract. Different letters on each bar indicate significant differences ($p < 0.05$). A (spinach extract 0 ng g^{-1} crab), B (spinach extract 300 ng g^{-1} crab), C (spinach extract 700 ng g^{-1} crab), and D (spinach extract 1,000 ng g^{-1} crab)

extract of 250 ng g^{-1} crab, compared to the control only with a molting percentage of 15% (Wahyuningsih, 2008). This study supports the hypothesis that spinach-derived phytoecdysteroids can accelerate molting in mud crabs. The results of the study showed that the dose of spinach extract significantly influenced the crab's physiological response, especially on its molting duration.

Ecdysteroids Levels

The bar chart in Figure 3 presents the results of the analysis of ecdysteroid hormone

levels contained in mud crab's hemolymph using the ELISA method from each treatment in this study. Treatment D (1,000 ng g^{-1}) had much lower ecdysteroid level (2,214.67 ± 105.46 ng mL^{-1}) compared to treatment A (control) with an ecdysteroid level of 2,502 ± 45.21 ng mL^{-1} ($p < 0.05$). These findings indicate that a high dose of spinach extract (1,000 ng g^{-1}) significantly reduced ecdysteroid levels. This drop might be due to a process that speeds up molting, since lower ecdysteroid levels before molting is a normal part of how hormones work in crustaceans. Hormones not only stimulate molting but also inhibit it. A negative feedback mechanism is involved in hormone function (Herold, 2023). High circulating hormone concentrations signal cells inhibit their activity in order to maintain balance (homeostasis) (Keller-Wood, 2015). High hormone concentrations reduce the production of hormone receptors and the hormone's ability to bind to target cell receptors. Consequently, adenylate cyclase activation is inhibited, which impairs the hormone's effects (Catt *et al*., 1979).

In the Dungeness crab (*Cancer magister*), peak ecdysteroid concentrations reach approximately 2,000 ng mL^{-1}, and levels as low as 1,886.5 ng mL^{-1} are sufficient to stimulate molting (Thomton *et al.*, 2006). In mud crabs, the highest concentrations occur during the premolt phase, ranging from 2,005 to 2,821 ng mL^{-1} (Fujaya & Trijuno, 2007). Tanner crabs, by contrast, show the lowest values during the intermolt stage, with hemolymph concentrations of approximately 500 ng mL^{-1} (Chang & Mykles, 2011). Dietary supplementation with mulberry leaf extract also influences ecdysteroid titers: a dose of 3.5 mg g-^{1} of feed elevates hemolymph levels to approximately 1,700 ng mL^{-1}, while 1.1 mg g^{-1} produces only a modest increase of 100 ng mL^{-1} (Fujaya *et al.*, 2018).

In mud crabs, basal molting hormone concentrations are low, around 500 ng kg^{-1} body weight (Aslamyah & Fujaya, 2011). Without additional ecdysteroids, the molting cycle is prolonged. Ecdysteroids enhance protein synthesis, thereby accelerating molting and promoting growth. For example, 1 μg of 20-HE is equivalent in effect to 42 μg mL^{-1} of spinach extract (Hasnidar, 2021). An increase in ecdysteroid levels in the hemolymph provide

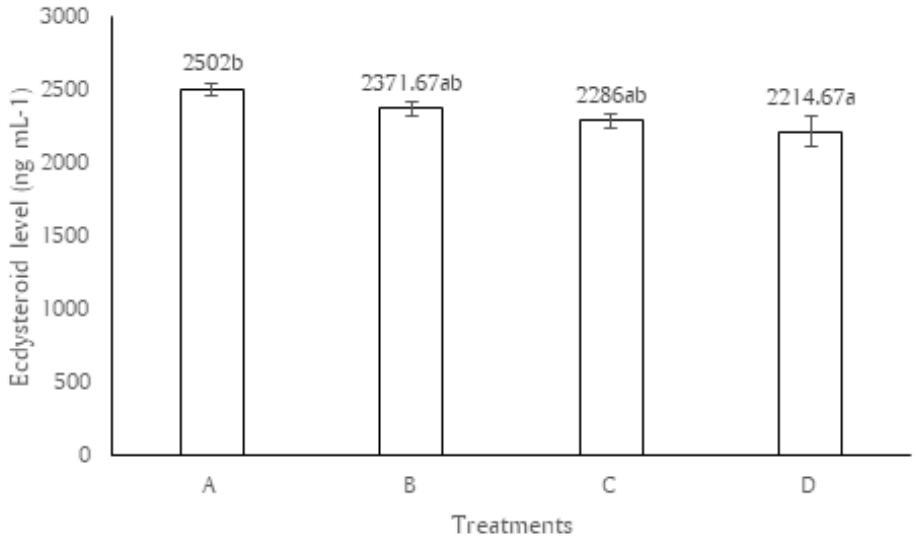

Figure 3. Ecdysteroid levels of mud crabs (*Scylla* sp.) reared in crab apartment boxes and fed with different dietary supplementations of spinach (*Amaranthus* sp.) extract. Different letters on each bar indicate significant differences ($p < 0.05$). A (spinach extract 0 ng g^{-1} crab), B (spinach extract 300 ng g^{-1} crab), C (spinach extract 700 ng g^{-1} crab), and D (spinach extract 1,000 ng g^{-1} crab)

the signal to initiate the molting process (Habibi *et al.*, 2013).

Ecdysteroids also exert inhibitory effects through negative feedback regulation (Herold, 2023). Elevated circulating hormone levels trigger homeostatic mechanisms that reduce receptor abundance and binding capacity, thereby diminishing responsiveness (Keller-Wood, 2015). This desensitization involves suppression of adenylate cyclase activation, which in turn weakens downstream hormonal effects (Catt *et al.*, 1979).

The pattern of ecdysteroid concentration in mud crab hemolymph mirrors the effects of spinach extract supplementation. Levels rise sharply during premolt and then decline rapidly during ecdysis (Fujaya & Trijuno, 2007; Fujaya *et al.*, 2018). Beyond their role as molting hormones, ecdysteroids also stimulate mRNA transcription and protein synthesis (Chang *et al.*, 1993), supporting their role in growth regulation. The primary metabolic action of steroids is the enhancement of protein turnover (Mauras, 2009), and large-scale protein synthesis is indispensable for somatic growth (Hapsari *et al.*, 2021).

Spinach extract acts as a molting stimulant due to its phytoecdysteroid content. These compounds mimic the endogenous steroid hormones of arthropods, which regulate molting as well as growth, metamorphosis, and reproduction (Song *et al.*, 2017). Ecdysone, secreted by the Y-organ, is converted in the hemolymph into the biologically active form 20-hydroxyecdysone by 20-hydroxylase enzymes located in the epidermis and other tissues. The concentration of circulating 20-hydroxyecdysone fluctuates with the molting cycle, remaining low after ecdysis and throughout the intermolt stage (Chang & O'Connor, 1978).

Molt Inhibiting Hormone Levels

The bar chart in Figure 4 presents the results of the analysis of MIH levels in mud crab's hemolymph from each treatment in this study using the ELISA method. Treatment A (control) demonstrated MIH level (12.05 $\pm$ 0.53 pg mL^{-1}), which was not significantly different ($p > 0.05$) from treatment B (300 ng g^{-1}) with an MIH level of 13.68 $\pm$ 0.93 pg mL^{-1}, but it was significantly different ($p < 0.05$) from treatments C (700 ng g^{-1}) and D (1,000 ng g^{-1}) with MIH levels of 18.27 $\pm$ 1.08 pg mL^{-1} and 20.88 $\pm$ 1.29 pg mL^{-1}, respectively. Additionally, all treatments that included the extract (B, C, and D) had noticeable differences, with higher doses leading to higher levels of MIH. This evidence indicates that the spinach extract administration can significantly increase MIH concentrations, especially at medium and high doses.

Spinach extract, which contains phytoecdysteroids, can stimulate molting when incorporated into artificial feed. A study reported that feed with lower protein content (30.62%) and higher carbohydrate content (49.13%) enhanced molting activity when supplemented with spinach extract at a dose of 700 ng g^{-1} crab (Aslamyah & Fujaya, 2011). However, higher doses of spinach extract (700–1000 ng g^{-1}) may not significantly reduce MIH levels, possibly due to the short molting duration. This indicates a complex interaction between spinach extract dose, molting mechanisms, and physiological responses (Aslamyah & Fujaya, 2010).

The molting cycle in crustaceans is regulated by the interaction between ecdysteroid hormones and neuropeptide hormones, particularly MIH and crustacean hyperglycemic hormone (CHH) (Techa & Chung, 2015). MIH, produced in the eyestalk ganglia, suppresses ecdysteroid biosynthesis in the Y-organ through extracellular signal-regulated kinase (ERK)phosphorylation and the involvement of protein kinase C (Imayavaramban *et al.*, 2007). During the molting cycle, MIH expression increases from the postmolt stage to the intermolt stage and then decreases sharply in the premolt stage, showing an inverse relationship with hemolymph ecdysteroid levels (ChunJian *et al.*, 2013). Interestingly, ecdysteroids can also regulate MIH expression through a feedback

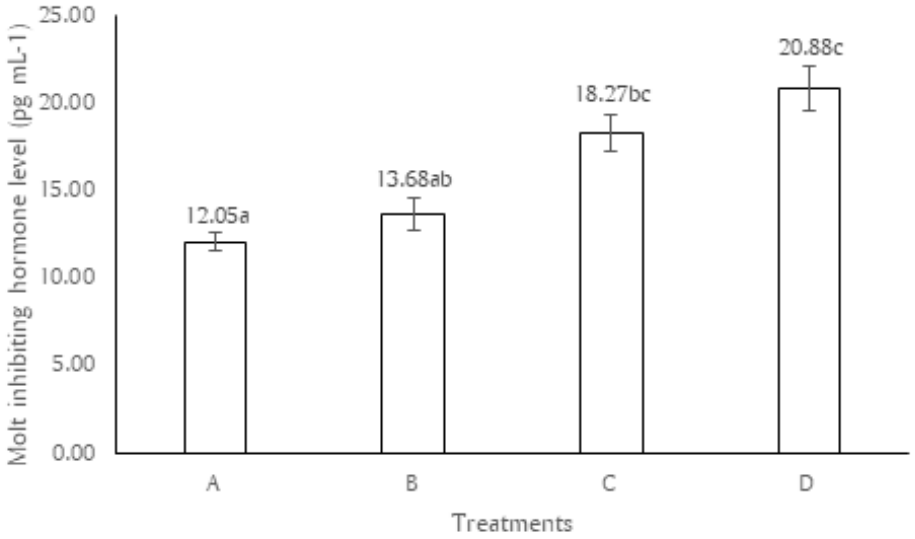

Figure 4. Molt inhibiting hormone levels of mud crabs (*Scylla* sp.) reared in crab apartment boxes and fed with different dietary supplementations of spinach (*Amaranthus* sp.) extract. Different letters on each bar indicate significant differences ($p < 0.05$). A (spinach extract 0 ng g^{-1} crab), B (spinach extract 300 ng g^{-1} crab), C (spinach extract 700 ng g^{-1} crab), and D (spinach extract 1,000 ng g^{-1} crab)

mechanism, as demonstrated by *in vitro* incubation and RNAi experiments (Techa & Chung, 2015). These hormonal interactions ensure the cyclical nature of molting in crustaceans. In addition, MIH may also play a role in ovarian maturation, as its neural expression varies across the ovarian cycle (Huang *et al.*, 2015).

In green crabs (*Carcinus maenas*), MIH levels during intermolt and early premolt stages range from 27 to 90 pg mL^{-1} of hemolymph (Chung & Webster, 2005). The sinus glands, which store MIH secreted by the X-organ in the eyestalk, contain 30–50 pg mL^{-1}. From these stores, approximately 1.2% of the total MIH is released per hour, corresponding to 7–12 pg mL^{-1} into the hemolymph (Habibi *et al.*, 2013). In blue crabs (*Callinectes sapidus*), MIH concentrations in the hemolymph also vary significantly throughout the molting cycle. At the intermolt and early premolt stages, concentrations remain relatively high (126.9–136.8 pg mL^{-1}) and are not significantly different. However, during early ecdysis, MIH levels drop significantly around 52.2 pg mL^{-1}, before increasing again in the postmolt stage to 330.3 pg mL^{-1}, indicating a rebound after molting (Techa & Chung, 2015).

Another study reported that MIH concentrations in the hemolymph of blue crabs differ markedly between ovarian stages. During the mid-vitellogenesis stage, MIH levels increased nearly fourfold compared to the pre-vitellogenesis stage, reaching 176.4 pg mL^{-1} versus only 51.3 pg mL^{-1} in pre-vitellogenesis (Zmora *et al.*, 2009). This phenomenon reflects the reproductive phase accompanied by terminal anecdysis (cessation of the molting cycle), which occurs in only a few decapod crustaceans, including *Maja squinado*, *Libinia emarginata*, and *Chionoecetes bairdi* (Zmora *et al.*, 2009).

Reactive Oxygen Species Levels

The bar chart in Figure 5 presents the results of the analysis of ROS in mud crab's hemolymph using ELISA from each treatment in this study. ROS levels in treatment A (control) (116.8 ± 8.27 μmol mL^{-1}) were not significantly different from treatment B (300 ng g^{-1}) at a level of (121.8 ± 4.49 μmol mL^{-1}), but those

were significantly different from treatments C (700 ng g^{-1}) and D (1000 ng g^{-1}) with values of (146.4 ± 1.76 μmol mL^{-1}) and (147.33 ± 1.69 μmol mL^{-1}), respectively. Conversely, there was no significant difference between treatments C and D, indicating that increasing the dose from 700 ng/g to 1000 ng/g no longer resulted in significant changes in ROS levels. The increase of ROS levels supports the idea that high doses of spinach extract can greatly boost ROS production, which might be linked to more activity in mitochondria, cell growth or reactions to physical stress after molting. In the molting phase which physiologically causes the opening of body structures and increases the risk of infection, ROS functions as an antimicrobial molecule to protect organisms from pathogens (Yusof *et al.*, 2020). However, it can also cause cell damage, if it exceeds the body's antioxidant capacity (Sinaga, 2017).

Research on spinach extract has demonstrated its potential to stimulate molting and growth in mud crabs. Artificial feed enriched with spinach extract (700 ng g^{-1}) significantly increased molting rates compared to the control (Aslamyah & Fujaya, 2010). However, higher doses of spinach extract (700–1000 ng g^{-1}) can elevate ROS levels, indicating oxidative stress. Red spinach has been identified as a source of exogenous antioxidants. Phytochemical screening of red spinach leaves revealed bioactive compounds such as flavonoids and phenolics with antioxidant potential. Toxicity testing yielded an LC_{50} value of 275,810 μg mL^{-1}, suggesting possible inhibitory effects on cell division (Marcella *et al.*, 2023).

In *Scylla paramamosain*, hydrogen peroxide (H_2O_2) levels in muscle tissue were reported at 44.3 ± 2.9 μmol mL^{-1} (Yusof *et al.*, 2020), reflecting an imbalance between antioxidant defenses and ROS production. ROS include superoxide anions, hydrogen peroxide, and hydroxyl radicals. Among these, hydrogen peroxide is a strong oxidizing agent capable of damaging proteins, lipids, and DNA (Davies, 1995). H_2O_2 is frequently used as a model compound to study oxidative stress both *in vivo* and *in vitro* (Kalpana *et al.*, 2009). In the same study, total glutathione levels in muscle

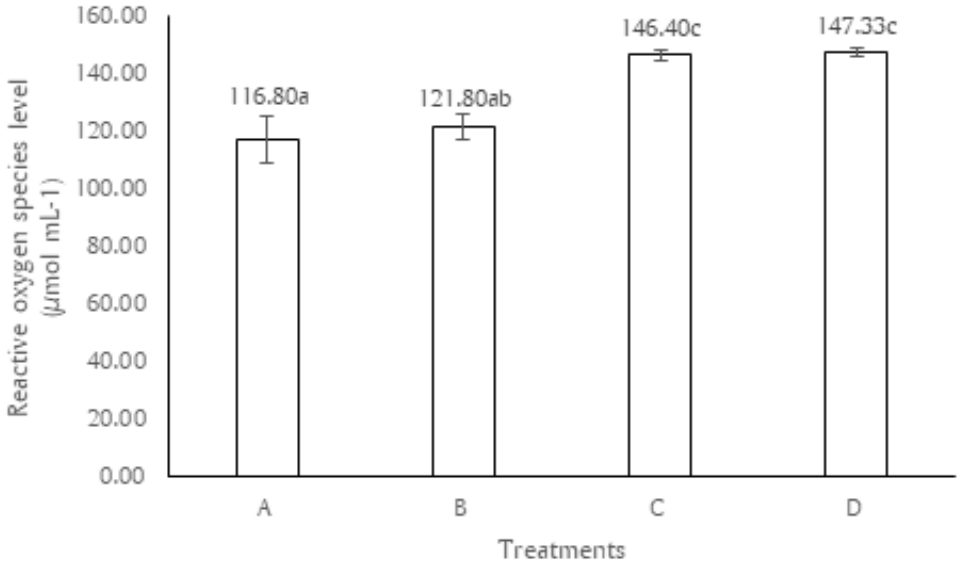

Figure 5. Reactive oxygen species levels of mud crabs (*Scylla* sp.) reared in crab apartment boxes and fed with different dietary supplementations of spinach (*Amaranthus* sp.) extract. Different letters on each bar indicate significant differences ($p < 0.05$). A (spinach extract 0 ng g^{-1} crab), B (spinach extract 300 ng g^{-1} crab), C (spinach extract 700 ng g^{-1} crab), and D (spinach extract 1,000 ng g^{-1} crab)

extract were reported at 0.5 μg mL^{-1}, confirming its role as a key antioxidant that maintains cellular redox balance during oxidative stress, including under hypoxic conditions. Previous studies have shown that antioxidant enzyme activity in crabs is influenced by salinity, season, tissue type, and sex (Geihs *et al.*, 2016).

The molting process in crabs involves major physiological adjustments, including elevated ROS production. In *Scylla serrata*, lipid peroxide (LPO), an oxidative stress marker, increased significantly during the premolt stage (Salaenoi *et al.*, 2015). Reported LPO activities (mean ± SD) ranged from 0.32 ± 0.02 to 1.50 ± 0.10, 0.08 ± 0.09 to 6.35 ± 0.25, and 1.31 ± 0.05 to 14.61 ± 0.45 U mg^{-1} protein in hemolymph, gills, and hepatopancreas, respectively. Similar patterns were observed across tissues: gradual increases from intermolt to peak activity at premolt, followed by decreases during ecdysis, and a subsequent rebound in postmolt. Among these tissues, the hepatopancreas consistently showed the highest LPO activity.

During premolt, crabs accumulate organic and inorganic reserves as energy sources for molting, a process that enhances free radical formation (Chang, 1995). This may contribute to the weakened condition of crabs during this stage, requiring increased antioxidant defenses. Crabs generally remain inactive until ecdysis. In early postmolt, antioxidant activity increases steadily, peaking at 24 hours postmolt, when crabs absorb large volumes of water to expand their body size. This expansion increases susceptibility to microbial invasion and the risk of ROS accumulation. Endogenous antioxidant production at this stage is therefore critical to counteract oxidative damage (Salaenoi *et al.*, 2015).

ROS, including O_2^-, H_2O_2, and OH^-, are byproducts of normal cellular metabolism. Their production rises sharply during premolt and ecdysis due to intensified metabolic activity (Paital & Chainy, 2012). This is accompanied by a transient decline in antioxidant activity from intermolt to early postmolt, followed by recovery in late postmolt (Head *et al.*, 2019). Proteomic studies of the Y-organ, the site of ecdysteroid synthesis, revealed dynamic shifts in protein expression across the molting cycle, with greater abundance of proteins involved in energy metabolism and ROS responses after molting (Hamer, 2015). Furthermore, because molting temporarily weakens body structures and increases infection risk, ROS also act as antimicrobial molecules to protect against pathogens (Yusof *et al.*, 2020).

Thus, ROS play a dual role in crustacean physiology. On one hand, they function as damaging oxidants; on the other, they act as signaling molecules (second messengers) that regulate apoptosis of old cells, regeneration of new epidermal tissue, immune activation, and stress responses under environmental challenges such as osmotic stress (Rivera-Ingraham *et al.*, 2024). This underscores the delicate balance between ROS generation and antioxidant defenses in crustacean molting and stress adaptation.

Water Quality

Table 1 presents the results of the study's analysis of the water quality in the media from each treatment. Water quality during the maintenance of mud crabs in the crab apartment box system integrated with RAS was consistently maintained within favorable ranges to support crab health and growth. The observed temperature remained stable between 28 and 29°C, which fits within the optimal temperature range of 25–35°C recommended by FAO (2011) for supporting metabolism and molting processes in crabs. Water pH across all treatments varied slightly from 7.8 to 8.7, reflecting a mildly alkaline environment suitable for mud crabs and well within the acceptable tolerance range of 7.5–9. Additionally, dissolved oxygen (DO) levels were observed between 6.3 and 7.5 mg L^{-1}, comfortably exceeding the minimum requirement of 5 mg L^{-1} for sustaining effective respiration and overall physiological activity in the crabs (Shelley & Lovatelli, 2011).

Salinity levels during the study were maintained between 20 and 23 ppt, falling

Table 1. Water quality of rearing media of mud crabs (*Scylla* sp.) reared in crab apartment boxes and fed with different dietary supplementations of spinach (*Amaranthus* sp.) extract

Parameters	Filter recirculating aquaculture system	Treatments				FAO (2011)
		A	B	C	D	
Tempreature (°C)	28	29	29	28.5	28.5	25—35
pH	7.8	8.2	8.7	8.4	8.5	7.5—9
Dissolved oxygen (mg L^{-1})	6.3	7.5	6.9	7.3	7.2	> 5
Salinity (ppt)	20	22	21	23	22	10—25
Ammonia (mg L^{-1})	0.2	2.8	2.7	3.1	2.5	< 3

Note: A (spinach extract 0 ng g^{-1} crab), B (spinach extract 300 ng g^{-1} crab), C (spinach extract 700 ng g^{-1} crab), and D (spinach extract 1,000 ng g^{-1} crab)

comfortably within the optimal tolerance range of 10–25 ppt for mud crabs. This suggests that RAS effectively controlled salinity stability throughout the trial period. On the other hand, the measured ammonia (NH_3) concentrations were relatively higher, ranging from 2.5 to 3.1 mg L^{-1}, with the peak observed in Treatment C. Although these values remain close to the maximum limit recommended by the FAO (< 3 mg L^{-1}), elevated ammonia levels warrant close monitoring due to their potential to induce physiological stress, disrupt the molting process, and damage gill tissues in mud crabs (FAO, 2011)

CONCLUSIONS

This study demonstrated that supplementation of mud crab's diet with spinach extract significantly influenced the molting process and several physiological parameters. Higher doses of phytoecdysteroids derived from spinach extract, reduced the duration of molting and also increased MIH and ROS levels, indicating a complex balance between accelerating molting and potential physiological stress. Overall, supplementation with natural phytoecdysteroids presents a promising, eco-friendly strategy for enhancing soft-shell crab production efficiency, although careful dose optimization is essential to maximize benefits while minimizing potential stress effects.

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AUTHOR CONTRIBUTION

BGRAS: conceptualization, methodology, investigation, formal analysis, and writing – original draft. S: resources, data curation, and visualization. HMU: resources, data curation, and visualization. AI: resources, data curation, and visualization. AMSH: supervision, validation, writing – review & editing, project

administration, and funding acquisition. All authors have read and approved the final version of the manuscript.

DECLARATION OF COMPETING INTEREST AND USE GENERATIVE AI

The authors declare no competing interests. During the preparation of this work, the authors used Quillbot Premium Grammar Checker to assist in improving language clarity, ensuring coherence in scientific writing, and structuring technical explanations. The authors reviewed and edited the content generated by the AI as needed and take full responsibility for the integrity and accuracy of the final manuscript.

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