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# The effect of molting hormone (20-hydroxyecdyson) on molting of mud crab (*Scylla Olivacea* Herbst, 1976)

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**ABSTRACT**

Molting in crustaceans is influenced by molting hormone. This study aims to examine the effect of adding the molting hormone 20- hydroxyecdyson (20-HE) against the concentration of ecdysteroid haemolymph and molting of mud crab. The study is designed using Complete Randomized Design (CRD), consisting of four dose treatments of 20-HE, namely: (A) 0 µg/mL (without hormone), (B) 1.0 µg/mL, (C) 1.3 µg/mL and (D) 1.6 µg/mL; each treatment is repeated three times. The addition of molting hormone 20-HE is done by injection. Parameters measured are ecdysteroid haemolymph concentration post-injection, the percentage of molting, molting duration, weight and width of carapace, survival rate, as well as water quality as the growth medium. Analysis of the sample of molting hormone and haemolymph is performed using Ultra-Fast Liquid Chromatography (UFLC). Concentration of ecdysteroid haemolymph pre and post injection of vitomolt is performed by t-test. To determine the effect of molting treatment on mangrove crab, analysis of variance (ANOVA) is done. The results show that the addition of 20-HE molting hormone increases the concentration of molting hormone (ecdysteroid haemolymph), increases the percentage of molting, accelerates molting duration, increases the weight and width of the carapace, and increases survival of mangrove crabs. The higher the dose of molting hormone injected, the higher the concentration of ecdysteroid haemolymph post-injection. The 20-HE hormone dose of 1.0 µg/mL results in the highest percentage of molting, fastest molting duration, the highest increase of weight and width of carapace, and the highest survival rate.

***Key words :*** *Mangrove crab, Ecdysteroid, Haemolymph, Molting hormone 20-HE*

## Introduction

Phases of the moon affect the concentration of ecdysteroid haemolymph of mud crabs (*S. olivacea* Herbst, 1796). The highest concentration of ecdysteroid is found during the weak gravitational force of the moon that is in phase ½ I and ½ II, and the lowest concentration is found during the strong gravitational force of the moon, i.e. the dark phase of the moon and the full moon (Hasnidar, 2014). The dynamics of ecdysteroid haemolymph are suspected to be the cause of fluctuations of molting crabs. The cycle of the moon and tides contribute to molting of mud crabs in the ponds (Fujaya and Alam, 2012). Molting is the process of skin sloughing or shedding of many invertebrates, including the crabs; the new skin replaced the old one. Growth in the crab is a function of molting; the more often the molting happens means the faster the growth is. Molting affects the growth process of red king crab (*Paralithodes*

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*camtschaticus*) (Stevens, 2012). At first molting process, the growth and increase in size of the crabs significantly improves, as well as on subsequent molting, which also promotes growth and increases the size of crabs though not as good as the first molting. At high tide (spring tide) on the phase of the full moon and new moon, crabs actively eat yet the number of molting crabs is very small. In contrast to the conditions of low tide (neap tide), crabsreluctantly eat, yet the number of molting crabs is increasing. The crab molting fluctuations cause discontinuities in the soft-shell crab production. Mud crabs are soft-shell crab; they are cultivated and harvested shortly after molting before a new skin or shell undergoes hardening. Therefore, the sooner the molting, the shorter the cultivation period will be-so, the cultivation is more efficient.

Factors affecting molting are molting hormone, which can control the cycle of skin turnover of crustaceans, such as ecdysteroid hormone that can induce crabs from intermolt phase towards the premolt phase (Skinner, 1985). Therefore, the addition of molting hormone can induce molting acceleration time. Additionally, cholesterol, which serves as the primary precursor for biosynthesis of molting hormone (ecdysteroid) on organ Y also has an effect on the process of molting on crabs (Imayavaramban *et al.*, 2007). Crabs cannot synthesize cholesterol, so it must be supplied from food or directly with the addition of molting hormones from outside the body. The effectiveness of molting hormone to stimulate molting is strongly associated with the dose given. Hormones are chemical signals, usually produced in very small quantities, but can cause major changes in the target cell. The effect can be stimulating or inhibiting (Meyer, 2007). Therefore, improper dosage can cause inhibiting effect or may cause abnormal condition. This study aims to examine the effect of addition of molting hormone (20-HE) to concentrations of ecdysteroid haemolymph and percentage of molting, the duration of molting, the growth on weight and width of carapace, and the survival rate of mud crabs. For the more efficient cultivation of soft-shell crab, information about the application of molting hormone that can accelerate the growth and increase molting is required. The use of molting hormone 20-HE is not economical for large-scale and is very limited in continuity. Therefore, the results of this study are expected to become the basis for the application of molting hormone, which is more economical, continuous, and harmless to consumers.

## Materials and Methods

Mangrove crab (*Scylla olivacea*), as many as 100, carapace width of 61-62 mm and weighing 50-51 grams were obtained from the local crab catcher. Crabs were put in a crab box, one box for each crab; then, they were put in ponds by floating them on the water surface as soft-shell crab cultivation procedure. During cultivation, crabs were fed with trash fish at a dose of 10% of their body weight, on a frequency of once a day, i.e. in the afternoon before the evening (Fujaya *et al.*, 2012).

The crabs that have been acclimated were subsequently given molting hormone 20-hydroxyecdyson (20-HE) from SIGMA®. Before 20-HE hormone was given, it was dissolved in 70% ethanol for the stock solution (5mg/mL). Concentrations of 20-HE injected were calculated based on the highest ecdysteroid concentration subtracted with the lowest ecdysteroid concentration (Hasnidar, 2014) multiplied by the estimated volume of haemolymph (Gleeson and Zubkoff, 1977), that was 15% perbody weight (1.0 µg/mL), 20% per body weight (1.3 µg/mL), and 25% per body weight (1.6 µg/mL). The volume of haemolymph became consideration to determine the exact volume of injection, because there had not been any information on the haemolymph of mangrove crabs (*S. olivacea*). Weight of crab ± 50 g per crab, 20-HE solution was injected at the base of the swimming legs (pereiopods number 5) (Fujaya and Alam, 2012), using 1 mL volume syringe with 27-gauge size.

Haemolymph was taken once at the beginning (before injection of 20-HE hormone), and three times post-injection. Observations post-injection were (1) observationaI I on moon phase ¾ I, (2) observation II, on moon phase I, and (3) observation III, on moon phase ¾ II. Haemolymph of crab was taken from the basal area of the fifth pair of legs using a 1-mL syringe with a syringe of 27-gauge. The 1 mL haemolymph was stored in eppendoft and mixed with anticoagulant on the ratio of 1:1 (Fujaya and Trijuno, 2007). Further, samples were stored in a freezer with a temperature of -200°C, and then the samples were ready to be extracted. The extraction procedure of haemolymph is as follows. First, 1 mL of haemolymph was addedwith 3 mL of diethyl ether. Second, the mixture was shuffled using vortex for 30 seconds and then allowed to stand for 2 minutes. The layer top is an ether phase containing steroids. Then, residue that remainedwas extracted

back 3 times and then collected and dried at a temperature of up to 400°C (Fujaya and Trijuno, 2007).

Procedure on the measurement of ecdysteroid is as follows. First, dried residue of samples as extraction product was diluted with methanol pro UFLC, then put in an auto sampler vial of UFLC. Second, samples were analyzed using Ultra-Fast Liquid Chromatography (UFLC) Shimadzu LC-20 AD with the condition of the tool as follows: (a) column: Shim Pack ODS C18 250x4,6 mm; (b) the system: reversed phase; (c) mobile phase: methanol-water (80:20 v/v); (d) flow rate: 1 mL/min; (e) temperature column: 40 °C ; (f) detector: photodiode array (UV), 246 nm; and d(g) injection volume: 10 mL. Quantification of ecdysteroid was done using standard series of 20-hydroxyecdyson (Sigma®) (Fujaya and Trijuno, 2007).

The study was designed using Complete Randomized Design (CRD), consisting of of four dose treatments of 20-HE, namely: (1) 0 µg/mL (without hormone), (2) 1.0 µg/mL, (3) 1.3 µg/mL and (4) 1.6 µg/mL; each treatment was repeated three times. Parameters measured were(1) ecdysteroid haemolymph concentration post-injection, (2) the percentage of molting, (3) molting duration, (4) weight and width of carapace, (5) survival rate, as well as (6) water quality as the growth medium.

Comparison between the initial and post-injection haemolymph ecdysteroid concentration was performed by t-test (Sokal and Rohlf, 1969). Meanwhile, to determine the effect of treatment on the haemolymph ecdysteroid concentration, percentage of molting, molting duration, growth in weight and width of carapace, as well as survival, analysis of variance (ANOVA) was employed (Steel and Torrie, 1993). Water quality parameters were analyzed using quantitative description used to describe the feasibility of water as the growth medium of mangrove crabs.

## Results and Discussion

### The Effectiveness of the Addition of the Molting Hormone 20-HE is influenced by the Phases of the Moon

Response of mangrove crabs post-injection of molting hormone 20-HE can be seen in the increase in the concentration of ecdysteroid haemolymph. Result on t-test showed that the average initial concentration of ecdysteroid haemolymph (1.15 ± 0.17) was not significantly different (p> 0.05) from the average post-injection concentration of molting hormone 20-HE doses of 0 µg/mL (without hormone) on observations of the moon phase¾, full moon, and moon phase ¾ II. However, the average initial haemolymph ecdysteroid concentration (1.15 ± 0.17) was significantly different (p <0.05) with the average concentration of ecdysteroid haemolymph post-injection dose of 1.0 µg/mL, 1.3 µg/mL and 1.6 µg/mL in all moon phases of the observation (Fig. 1).

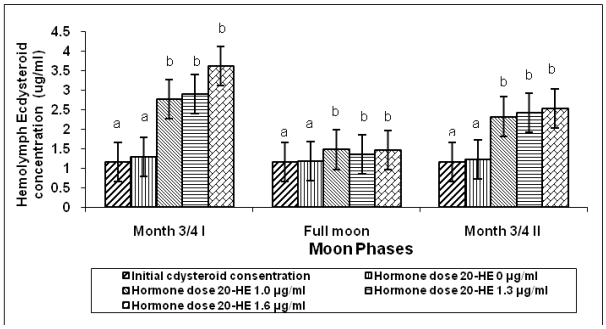


**Fig. 1.** Comparison on concentration of ecdysteroid haemolymph (*S. olivacea*) before and after treatment with molting hormone 20-HE, letters above the graphs showed significant difference (p<0.05) based on t-test.

The higher the dose of 20-HE molting hormone injected, induced the higher the concentration of ecdysteroid haemolymph. However, in treatment A (dose 0 µg/mL) or without the addition of molting hormone, concentration of ecdysteroid haemolymph showed no increase. Post-injection, concentration of ecdysteroid haemolymph differed by phases of the moon. The higher the dose of exogenous molting hormone 20-HE injected induiced the higher the concentration of ecdysteroid haemolymph post-injection. Previous result suggest that ecdysteroid was a major steroid hormone in the arthropods that have a primary function as a molting hormone, yet it also regulates physiological functions, such as growth, metamorphosis, and reproduction (Gunamalai *et al.*, 2004). This hormone secretes by the Y organ in the form of ecdysteroid. In the haemolymph, this hormone is converted into an active hormone 20-hydroxyecdysone by 20-hydroxylase enzyme found in the epidermis of organs and other tissues.

Concentration of ecdysteroid haemolymph on observation I (moon phase ¾I) decreased on observation II of the full moon and increased again in observation III of moon phase ¾ II (Figure 2). The

dynamics in the increase of ecdysteroid were allegedly linked to the phases of the moon and the half-life of ecdysteroid hormones. Increasing concentration of ecdysteroid haemolymphpost-injection was highest in observation Iof the moon phase ¾ I, followed by observation III of the moon phase ¾ II, and the lowest was observation II at full moon phase. The dynamics of the increase in ecdysteroid has been allegedly linked to the phases of the moon and the half-life of ecdysteroid hormone. Based on the phases of the moon, the addition of molting hormone 20-HE was only able to increase the concentration of ecdysteroid haemolymph when the gravitational force of the moonwas weak in moon phase ¾I and ¾ II. However, it was not able to increase the concentration of ecdysteroid haemolymph when gravitational force of the moon is very strong during the full moon. Thus, the effectiveness of the addition of exogenous molting hormone was influenced by the phases of the moon.

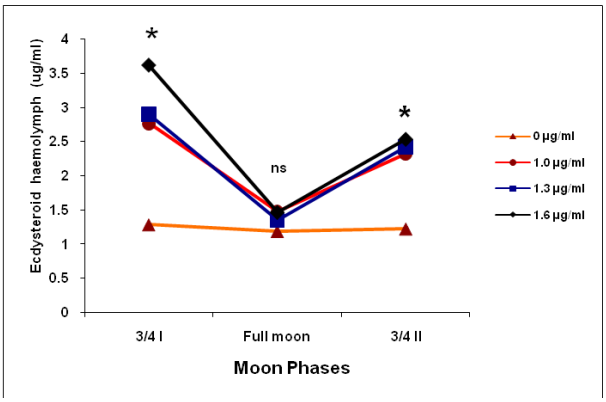


**Fig. 2.** Concentration of ecdysteroid haemolymph (µg/mL) of mangrove crabs (*S. olivacea*) based on post-injection. The asterisk (*) shows significant difference ($\alpha<0.05$), ns shows non-significant difference ($\alpha>0.05$) based on Duncan test.

If compared to the increase in ecdysteroid haemolymph post-injection during moon phase ¾ I and ¾ II, ecdysteroid concentration in the moon phase ¾ I was higher than in the moon phase ¾ II. Changes in ecdysteroid concentrations were higher in early post-injection observation and this has been allegedly linked to the working mechanism of hormone injection method in which the hormone gets directly into haemolymph to be further transported to a target organ. While in the full moon phase, addition of exogenous molting hormone does not increase ecdysteroid haemolymph. This is may be because the synthesis of hormones in the body (endogenous) is very low in that phase—it is strong gravitational force, and even exogenous ecdysteroid rapidly undergoes a process of diffusion at the digestion system and is excreted through feces or urine. Ecdysteroid is a steroid easily metabolized in the gastrointestinal tract. This causes the hormone that goes through the digestive tract mostly accumulated in the hepato pancreas to subsequently undergo metabolism, and is partly absorbed by the intestine before being taken to the target organ (Fulierton, 1980).

While on the moon phase ¾ II, ecdysteroid concentration increases back, and this is allegedly linked to ecdysteroid synthesis in organ Y that has become active again due to the gravitational force of the moon that has already started to disappear. In addition, in the previous phase of the moon, i.e. the full moon, high tide occurs where crab feeding is very high. High feeding activity will lead to energy assimilation causing growth increase. The condition is thought to be responded by the crabs to produce ecdysteroid to prepare for molting back.

The addition of molting hormone 20-HE brought significant ($p<0.05$) effect on time and percentage of molting. Molting percentage and time for the dose of 1.0 µg/mL was significantly different with the dose of 1.6 and 1.3 µg/mL and the dose of 0 µg/ml (Figure 3). The addition of 20-HE molting hormone showed an increase on ecdysteroid haemolymph. However, the high increase in ecdysteroid haemolymph concentration was not in line with the increase in molting percentage and duration. The highest molting percentage and the fastest duration were indicated by the lower dose of 1.0 µg/mL compared to a dose of 1.6 and 1.3 µg/mL. It shows that the 20-HE molting hormone dose required by mangrove crabs is determined by the proper dosage. The higher the dose given is thought to give inhibiting feedback toward the work of ecdysteroid to stimulate molting. High concentration of exogenous hormones from the optimal needs can lead to abnormal molting. Similar result has been reported in yellow crabs (*Cancer anthonyi*) on a 20-HE hormone concentration higher than 400 mg (McConaugha, 1979); in the larvae crabs (*Rhithropanopeus harrisii*) at a concentration of the 20-HE hormone higher than 5 µg/mL (McConaugha and Costlow, 1980).

The highest molting percentage and the fastest molting duration shown by the mangrove crabs on the treatment of molting hormone 20- HE injection

with a concentration of 1.0 µg/mL is believed to be the most appropriate concentration to increase the concentration of molting hormone produced by organ Y. Meanwhile, the addition of 20-HE molting hormone > 1.0 µg/mL resulted in a lower molting percentage and slower molting duration; it may be due to the r concentration is higher than optimal concentration to induce molting. Response of lobsters (*Homarus americanus*) on the treatment of ecdysteroid is influenced by the dose and season.

The best results are in early spring treatment only with a dose of 0.5 µg/g of body weight, and at the end of autumn with a dose of 1.5 µg/g of body weight (Aiken, 1980). Crustacean has a unique mechanism in regulating the balance of hormonal levels, including the level of ecdysteroid that controls molting. Excessive hormone concentration in their haemolymph can be excreted through urine or feces. Some organisms give negative feedback through various means, i.e. the reduction of receptors or inactivation of the existing hormone (Fujaya *et al.*, 2013).

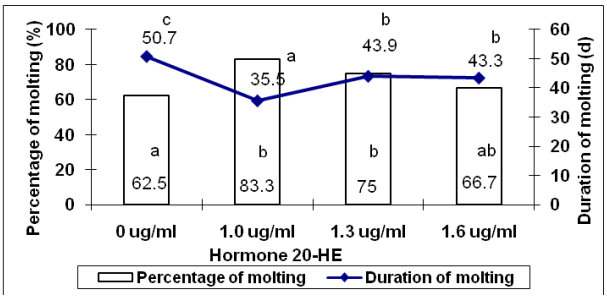


**Fig. 3.** The average of molting percentage and duration of mangrove crab (*S. olivacea*) post-injection of molting hormone 20-HE. Different letters above the value show significant difference ($\alpha$<0.05) based on Duncan test.

Application of Molting Hormone 20-HE at the Right Dose Affects Molting Percentage, Molting Duration, and Weight Gain and Carapace Width

The addition of molting hormone 20-HE with different doses showed differences in the growth of absolute weight and absolute width of carapace. Results of analysis of variance showed that the dose of the molting hormone 20-HE significantly (p <0.01) affected weight and absolute carapace width. Weight gain and absolute carapace width after molting treatment at the dose of 1.0 µg/ml 20-HE was higher and different from the dose of 1.6 and 1.3 µg/mL and 0µg/mL.

Results of analysis of variance showed that molting hormone 20-HE significantly (P <0.05) affected the growth of the biomass, but did not significantly (p> 0.05) affect the survival rate of biomass. The growth of biomass at the dose of 1.0 µg/mL was higher and different from the dose of 1.6 and 1.3 µg/mL. Treatment without molting hormone 20-HE showed the lowest biomass growth. In line with biomass growth, the survival rate of mangrove crabs showed that molting hormone at the dose of 1.0 µg/mL was higher as 83.3% but no different to other treatments.

Growth is one of the important components in cultivation. Growth in crabs is the change in carapace width and weight that occurs periodically at every molting process. The growth of mangrove crab weight after molting ranges between 16.67-26.93 gram or 33.169% to 52.879%, and the increase in carapace width ranges 9.8-12.38 mm or 13.780% to 19.728%. The highest growth of the weight and width of carapace happens at treatment of 20-HE molting hormone injection at the dose of 1.0 µg/mL and the lowest is at the treatment without hormones. 20-HE molting hormone injection besides accelerates the molting duration also increases the weight and width of the post-injection carapace. Similar result occured on mangrove crabs feed with artificial feed enriched with extracts of spinach (phytoecdysteroid) (Aslamiyah and Fujaya, 2010). Ecdysteroid is a type of steroid hormone that is thought to have an anabolic effect by increasing protein synthesis. Previous research suggested that the most prominent metabolic steroids are the intensified protein metabolism (Donaldson *et al.*, 1981). Some research indicates an increase in the growth of farmed fish fed with synthetic 17-metyltestosteron steroid hormone. The application of 17 i-metyl testosteron effectively decreases the use of feed protein to 20% and has been proven to improve growth and feed efficiency of Betutu fish (Aslamiyah and Fujaya, 2010). The Ecdysteroid also serves to increase protein production by increasing mRNA synthesis (Preston and Dinan, 2002). Ecdysteroidalso stimulates the metabolism of carbohydrate, lipid biosynthesis, and acts as an immune stimulant and antioxidant (Lafont and Dinan, 2003).

Reproduction and molting of decapoda is aseasonal event that is controlled by hormone (Nagaraju, 2011; Subramoniam, 2011). Both physiological mechanisms are thought to be stimulated by ecdysteroid. Synchronization occurs between the

**Table 1.** The average value, the daily average value, and the optimal value of water quality parameters of mangrove crab (*S. olivacea*) cultivation

| Parameter | Average Value | Daily Average Value | Optimal Value |
|---|---|---|---|
| Temperature (°C) | 20 –35.7 | 31.04 ± 2.535 | 25-35°C[1] |
| pH | 7 – 8.5 | 7.35 ± 0.431 | 6.8-8.2[1] |
| Salinity (ppt) | 15 – 35 | 29.25 ± 3.390 | 25 ppt[2] |
| Dissolved oxygen (ppm) | 2.1 – 6.43 | 4.82 ± 1.338 | >5 ppm[3] |
| Ammonia (ppm) | 0.00 – 1.38 | 0.98 ± 0.15. | <0.1[4] <0.25[4] |
| Nitrite (ppm) | 0.1 – 1.227 | 0.400 ± 0.103 | 0.0-0.5[3], 0.5-1.0[5] |

**Note :** [1]: Fujaya *et al.* (2012), [2]: Karim (2008), [3]: Gaudé and Anderson (2011), [4]: Wedemeyer dan Mcleay (1981), [5]: Turano *et al.* (2008), [6]: Shelley and Lovatelli (2011).

growth and development of vitellogenesis of palaemonid shrimp as freshwater prawns (*Macrobrachium idella* and *M. rosenbergii*) (Tsukimura and Kamemoto, 1991; Okumura and Aida, 2000). This indicates that ecdysteroid besides playing a role in molting also playing a role in stimulating vitellogenesis. However, it is not in crabs (*Carcinus maenas*) (Styrishave *et al*., 2008), the American lobster (*Homarus americanus*) (Tiu *et al*., 2009),. and crabs (*Uca triangularis*) (Sudha *et al.*, 2012), so that the involvement of ecdysteroid in reproduction crustacean is not settled (Subramoniam, 2000; Hopkins, 2009). In addition to acting as molting hormone, ecdysteroid also acts as a regulator of physiological functions such as growth, metamorphosis, and reproduction (McConaugha, 1979). As a molting hormone, ecdysteroid serves to increase protein production by increasing mRNA synthesis (Gunamalai *et al.*, 2004; Klein, 2004).

The average value, the daily average value, and the optimal value of water quality parameters during the study are presented in Table 1. Observed water quality parameters are parameters that allegedly affect molting, growth, and survival rate of mangrove crab. Biomass growth of mangrove crabs increases sharply from 220,000 g (without the addition of molting hormone) to 589,000 g with the addition of molting hormone at the dose of 1.0 µg/mL. This shows that the addition of 20-HE molting hormone with the right dose becomes a trigger for faster molting and higher growth. The same also happens on survival rate, in which at a dose of 1.0 µg/mL, growth was 83% higher than other treatments. The addition of 20-HE molting hormone with the right dose increases the survival rate of crabs, as observed in larvae of the yellow crab (*Cancer anthonyi*) (McConaugha, 1979), crabs (*Rhithropanopeus harrisii*) (McConaugha and Costlow, 1980), and crab larvae (*P. pelagicus*) (Azra *et al.*, 2012). Further, a dose of 20-HE hormone higher than optimal requirement, while helping to lower survival rate, also causes abnormal molting. The need for 20-HE hormone can vary between species and stages of development of crabs. The higher the stage of development towards adult crabs, the higher the needs of 20-HE hormone will be (Dvoretsky and Dvoretsky, 2010; Azra *et al.*, 2012).

## Conclusion

In this study, the addition of 20-HE molting hormone through injections has led to increasing concentrations of ecdysteroid haemolymph post-injection when the gravitational force is weak duringthe moon phase ¾ I and ¾ II. However,ecdysteroid haemolymph concentration does not increase when the gravitational force is so strong during the full moon phase. Molting hormone 20-HE dose of 1.0 µg/mL is the best to induce higher molting than the dose of 1.3 and 1.6 µg/mL.

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