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Tidal Study of Hormonal Conditions Related to Puberty Molting in Mating Behavior of Mud Crab *Scylla serrata*

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ABSTRACT

This study aimed to analyze the effect of tidal time and/or depth of mating media on hormonal conditions related to puberty molting on mating behavior and molting latency of mud crabs *S. serrata*. A two-factorial completely randomized design was used to assess the effects of tidal conditions, with treatments including no tide (P1), 12-hour high tide followed by 1-hour low tide (P2), and 24-hour low tide followed by 1-hour high tide (P3). The water depth consisted of 3 treatment levels, namely a depth of 30cm (K1), a depth of 60cm (K2), and a depth of 90cm (K3). The treatment was a combination of factors from all levels with three replications. Observation parameters included ecdysteroid titer, molting latency period, and absolute carapace width. A two-way ANOVA analysis was used to analyze the data and continued with Duncan's test. The results showed that the highest ecdysteroid concentration value, 1,573.12± 20.85ngmL^{-1}, was observed at P3K3. The shortest molting latency period was 14.17± 1.04 days in P3K3. Moreover, the total carapace width was not significantly different. It was concluded that the mating medium with a high tide of 24 hours, a low tide of 1.0 hour with a rearing water depth of 90cm affected the hormonal conditions and molting latency of the mud crabs *S. serrata*.

INTRODUCTION

The development of mud crab cultivation is one alternative to increase production intensively and sustainably. The success of a sustainable and economically profitable mud crab culture requires a continuous supply of quality seeds from hatchery units **(Millamena & Quinitio, 2000; Djunaidah *et al*., 2004; Herlinah *et al*., 2016)**. Low broodstock quality is a problem in seed production which results in low quality and

survival rate of mud crab larvae **(Herlinah *et al*., 2015; Waiho *et al*., 2015)**. The broodstock used in the hatchery at this time is adult female mud crabs that have matured gonads or have spawned in nature, so that low-quality broodstock are often obtained, even though a good selection process has been carried out **(Yi *et al*., 2009; Usman *et al*., 2015)**. Efforts to provide superior broodstock through a controlled mating process in hatcheries have begun, but have not yet given optimum results **(Waiho *et al*., 2015)**. Management and provision of superior broodstock for mud crabs is highly dependent on understanding their reproductive strategies. Reproductive strategy is a complex process related to biological characteristics, especially behavior and reproductive physiology **(Fahmi, 2001)**. Mating behavior in mud crabs is part of reproductive behavior and physiology which includes several stages, namely the pre-copulation stage, the molting stage, the copulation stage, and the post-copulation stage **(Waiho *et al*., 2015)**. Puberty molting is a stage of mating behavior that determines the success of the copulation process and the beginning of the reproductive process of female mud crabs. The molting stage of crustaceans is a complex process that involves external and internal factors. External factors are related to environmental cues that are physiologically responded to in a balanced way to stimulate hormonal processes. Crustaceans are arthropods that can adapt to various types of aquatic environments **(Takemura *et al*., 2010; Techa & Chung, 2015; Hosamani *et al*., 2017)**. Mud crabs primarily inhabit mangrove areas, where environmental conditions fluctuate due to tidal cycles, affecting water depth and physical characteristics **(Shelley & Lovatelli, 2011; Hubatsch *et al*., 2015)**.These environmental conditions directly affect the physiological conditions and behavior of mud crabs to grow and reproduce, hence it is necessary to study tide analysis under laboratory conditions with a controlled environment on mating media on hormonal conditions related to molting for the development of sustainable hatchery technology engineering.

At this time, adult female mud crabs with matured gonads or those that have recently spawned in nature are often obtained as broodstock. Despite thorough selection processes, these broodstock may still be of lower quality **(Yi *et al*., 2009; Usman *et al*., 2015)**. Efforts to provide superior broodstock through a controlled mating process in hatcheries have begun, but have not yet given optimum results **(Waiho *et al*., 2015)**. Management and provision of superior broodstock for mud crabs is highly dependent on understanding their reproductive strategies. Reproductive strategy is a complex process related to biological characteristics, especially behavior and reproductive physiology **(Fahmi, 2001)**. Mating behavior in mud crabs is part of reproductive behavior and physiology which includes several stages, namely the pre-copulation stage, the molting stage, the copulation stage, and the post-copulation stage **(Waiho *et al*., 2015)**. Puberty molting is a stage of mating behavior that determines the success of the copulation process and the beginning of the reproductive process of female mud crabs. Moreover, the molting stage of crustaceans is a complex process that involves external and internal

factors. External factors are related to environmental cues that are physiologically responded to in a balanced way to stimulate hormonal processes. Crustaceans are arthropods that can adapt to various types of aquatic environments **(Takemura *et al*., 2010; Techa & Chung, 2015; Hosamani *et al*., 2017)**. The mud crab habitat is mostly in mangrove areas, where the environment physically fluctuates at various time scales and water depths due to tidal cycles **(Shelley & Lovatelli, 2011; Hubatsch *et al*., 2015)**. These environmental conditions directly affect the physiological conditions and behavior of mud crabs to grow and reproduce, hence it was necessary to study tide analysis under laboratory conditions with a controlled environment on mating media on hormonal conditions related to molting for the development of sustainable hatchery technology engineering.

MATERIALS AND METHODS

The research was conducted from February to November 2021 at the Balai Besar Perikanan Budi Daya Air Payau, Jepara, Cik Lanang Street, Bulu Village, Jepara District, Jepara Regency, Central Java Province of Indonesian. A completely randomized design (CRD) factorial with two factors was used in this study. The first factor is the length of the tide and low tide which consists of 3 levels of treatment. The second factor is the water depth which consists of 3 treatment levels. The treatment in this study was the result of a combination of factors from all levels of treatment, hence in this study there were 9 combinations. The variables studied consisted of independent variables including the length of tidal time which consisted of P1 = no treatment of tidal time, water changes were carried out every three days, P2 = 12-hour high tide and 1-hour low tide, P3 = 24 tide time hour and at low tide for 1.0 hour, and the water depth at high tide consists of K1 = 30cm water depth, K2 = 60cm water depth, K3 = 90cm water depth. The dependent variable included physiological responses consisting of ecdysteroid titers, molting latency period, growth and absolute carapace width, and water quality as supporting parameters. The test animal that was used in this study is the mud crab *S. serrata* which has reached sexual maturity or has a carapace width > 80mm with a male and female ratio of 1: 2 **(Waiho *et al*., 2015)**. Mud crabs were adapted for 3 days before treatment. The container used in this study was a white round fiberglass tub with a volume of 1,000L, a height of 100cm, and a diameter of 100cm. The rearing tank was equipped with salinity of 25gL^{-1}, equipped with an aeration equipment, and a 5cm thick sand substrate. The feed used was fresh feed in the form of fish *Sardinella* sp. Feed was given with a frequency of once a day, with a restricted feeding method, namely the amount of feed given was 10% of the biomass **(Nghia *et al*., 2007; Pattiasina *et al*., 2012)**. Hemolymph sampling was carried out for ecdysteroid titer testing. Hemolymph samples were taken once every five days until the mud crabs have tested molting. Hemolymph was collected using a 5mL syringe equipped with a 27-gauge syringe at the base of the fifth leg. A total of 1mL of hemolymph mixed with anticoagulant with a ratio of 1:1 was stored in a 2mL Eppendorf

tube. Additionally, each tube was labeled, then the samples were stored in a refrigerator at –20º C until they were ready for extraction **(Herlinah *et al*., 2015; Fujaya *et al*., 2019; Hasnidar & Tamsil, 2019)**.

Ecdysteroid titer

The ecdysteroid titer test method uses the enzyme-linked immunoassay (ELISA) method with the 20-hydroxyecdysone ELISA kit with the procedure including dilution by adding concentrate assay buffer in deionized water or distilled water in a ratio of 1:5. Sample preparation was carried out by adding methanol to the hemolymph sample in a ratio of 1:3, then vortexed for 30 seconds and centrifuged the solution at 11,200 xg for 10 minutes at 4°C. The supernatant was removed, then the pellet was dried thoroughly using a centrifugal concentrator at 30°C for 2-3 hours. Standard dilutions were prepared by adding 10µL of 20-hydroxyecdysone to 990µL of 1X assay buffer, resulting in a concentration of 25,000pg/ mL. To create additional standards, 300µL of 1X assay buffer was added to tubes labeled 10,000, 4,000, 1,600, 640, 256, 102.4, and 0pg/ mL. For antigen binding, 50µL of standard or sample was added to the appropriate wells, followed by 75µL of 1X assay buffer to detect non-specific binding (NSB) and 50µL to detect maximum binding (standard B0 or zero). Next, 25µL of 20-hydroxyecdysone conjugate was added to each well, and 25µL of 20-hydroxyecdysone antibody was added to each well except the NSB well. The plate was sealed and shaken for 2 hours at room temperature. After thoroughly aspirating the solution, the wells were washed 4 times with 300µL of 1X wash buffer. For chromogen binding, 100µL of TMB substrate was added to each well, resulting in a blue solution. After a 30-minute incubation at room temperature, 50µL of stop solution was added, changing the color from blue to yellow. Absorbance was read at 450nm after 10 minutes.

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Molting latency period

The molting latency period is the time required from treatment until molting occurs in the tested animals **(Suryati *et al*., 2013)**.

Absolute carapace width

The absolute carapace width was calculated based on the difference between the carapace width after molting or at the end of the study and the crab carapace width at the beginning of the study **(Herlinah *et al*., 2015)**.

Water quality

Water quality parameters were measured from the beginning to the end of the experiment. Observations of water quality include temperature, salinity, pH, and

dissolved oxygen every day, namely in the morning, and in the afternoon. Measurement of water alkalinity parameters was carried out every 3 days.

Data analysis

All study data were presented as mean ± standard deviation and analyzed by the statistical analysis software. Before analysis, Shapiro-Wilk test and Levene test were used to test the normality of distribution and homogeneity of variance of raw data. Analysis of diversity two-way ANOVA was used to determine the effect of treatment. If the treatment had a significant effect, the analysis was continued with Duncan's post-hoc test. All treatment effects were considered to be significantly different at the error rate ($P< 0.05$) and were recorded in letters.

RESULTS

1. Ecdysteroid titer

The mud crab ecdysteroid titer value by ELISA test during the study is presented in Table (1). The results of the Shapiro-Wilk normality test showed that the standard residual value is normally distributed ($P> 0.05$). Furthermore, Levene's variance homogeneity test showed that the ecdysteroid titer value had the same or homogeneous variance ($P> 0.05$). Analysis of variance shows that the tidal time factor (P) and the water depth factor (K), as well as the interaction factor (P*K) significantly affected the mud crab ecdysteroid titer value. The error rate for the P factor is equal to 0.000 ($P< 0.05$), which means that based on the tidal time treatment, there is a very significant difference in the titer value of mud crab ecdysteroid. The ecdysteroid titer values in mud crabs, based on Duncan's post hoc test at the 5% significance level, revealed significant differences among the tidal time treatments. The highest titer was observed at P3 with 1,388.06± 162.49ng/ mL, while the lowest was at P1 with 1,163.01± 81.16ng/ mL. The value at P2 was 1,305.18± 87.25ng/ mL.

Table 1. Mud crab ecdysteroid titer value

Treatment	Repeat ($ngmL^{-1}$)			Mean±Standard deviation ($ngmL^{-1}$)
	1	2	3	
P1K1	1,133.74	1,175.78	1,204.79	$1,171.44 \pm 35.72^{ab}$
P1K2	1,249.72	1,303.38	1,115.17	$1,222.75 \pm 96.96^{abc}$
P1K3	1,138.42	1,115.66	1,030.42	$1,094.83 \pm 56.93^{a}$
P2K1	1,209.33	1,205.13	1,445.46	$1,286.64 \pm 137.56^{bc}$
P2K2	1,245.67	1,346.26	1,252.23	$1,281.39 \pm 60.37^{bc}$
P2K3	1,272.66	1,362.83	1,399.94	$1,345.14 \pm 65.46^{c}$
P3K1	1,269.59	1,316.20	1,138.29	$1,241.36 \pm 92.25^{abc}$
P3K2	1,261.46	1,464.24	1,323.39	$1,349.70 \pm 103.92^{c}$
P3K3	1,549.58	1,580.54	1,589.26	$1,573.12 \pm 20.85^{d}$

*Different letters show significant differences according to Duncan's test at the 5% level.

The results of the analysis of variance on the K factor had an error rate equal to 0.046 ($P < 0.05$), which means that based on the water depth treatment, there is a significant difference in the ecdysteroid titer value of mud crabs. K3 treatment based on Duncan's post hoc test at the 5% level had an effect on the average value of ecdysteroids of 1,337.70± 211.93ngmL^{-1}, not significantly different from K2 which had an average value of ecdysteroids of 1,285.40± 94.78ngmL^{-1} and significantly different from treatment K1 with an average value of ecdysteroids of 233.15± 98.51ngmL^{-1}. Furthermore, in the K2 and K1 treatments, the average value of ecdysteroids was not significantly different. The results of the analysis of variance on the *P**K factor had an error rate equal to 0.004 ($P < 0.05$), which means at a 95% confidence level based on the interaction of treatment with tidal length and water depth, there is a significant difference in the ecdysteroid titer value of mud crabs.

2. Molting latency period

The molting latency of mud crabs during the study in each treatment is presented in Table (2). The results of the Shapiro-Wilk normality test showed the standard residual value was normally distributed ($P > 0.05$). Furthermore, Levene's variance homogeneity test showed that the molting latency had the same or homogeneous variance. The *P* factor, K factor, and *P**K factor based on the results of the variance analysis had a significant effect on the molting latency of mud crabs. The error rate for the *P* factor was equal to 0.000 ($P < 0.05$), which means that based on the treatment of tidal time there is a difference in the latency of the mud crab molting. The average value of mud crab molting latency based on the results of Duncan's post hoc test showed a significant different ($P > 0.05$) between treatments with the shortest mud crab molting latency at P3 which was 15.89 ± 2.50 days. Additionally, the longest molting latency of mud crabs at P1 was 26.06± 2.63 days, while the molting latency of mud crabs at P2 was 19.06± 1.83 days.

Table 2. Mud crab molting latency

Treatment	Repeat (days)			Mean±Standard deviation (days)
	1	2	3	
P1K1	29.50	28.50	30.00	29.33 ± 0.76^{e}
P1K2	24.50	23.50	25.00	24.33 ± 0.76^{d}
P1K3	24.50	26.00	23.00	24.50 ± 1.50^{d}
P2K1	22.00	19.00	19.50	20.17 ± 1.61^{c}
P2K2	19.50	21.00	19.00	19.83 ± 1.04^{c}
P2K3	17.00	18.50	16.00	17.17 ± 1.26^{b}
P3K1	18.50	19.00	19.50	19.00 ± 0.50^{bc}
P3K2	13.50	16.00	14.00	14.50 ± 1.32^{a}
P3K3	13.00	14.50	15.00	14.17 ± 1.04^{a}

*Different letters show significant differences according to Duncan's test at the 5% level.

The results of the analysis of K factor variance showed differences in the molting mass of mud crabs based on water depth ($P< 0.05$). The molting period of mud crabs based on Duncan's post hoc test showed that it was the shortest in K3 and was not significantly different from K2, each having a molting period of 18.61± 4.74 days and 19.56± 4.36, respectively. The longest molting latency period at K1 was 22.83± 4.99 days. The interpretation of the analysis of variance on the *P**K factor showed that there was an interaction between tidal time and water depth which caused the mud crab molting latency to be significantly different ($P< 0.05$). P3K3 treatment had the shortest mud crab molting latency, with 14.17± 1.04 days, which was not significantly different from P3K2 treatment with a molting latency period of 14.50± 1.32 days. However, it was significantly different from other treatments. While the longest molting latency period was obtained in the P1K1 treatment, with 29.33± 0.76 days, that were significantly different from the other treatments.

3. Absolute carapace width

The absolute carapace width of mud crabs during the study is presented in Fig. (1). The results of the Shapiro-Wilk normality test showed that the standard residual value is normally distributed ($P> 0.05$). Furthermore, Levene's variance homogeneity test showed that the molting latency had the same variance or was homogeneous ($P> 0.05$). The *P* factor, K factor, and *P**K factor based on the results of the variance analysis showed that the absolute carapace width of mud crabs had no significant effect ($P> 0.05$).

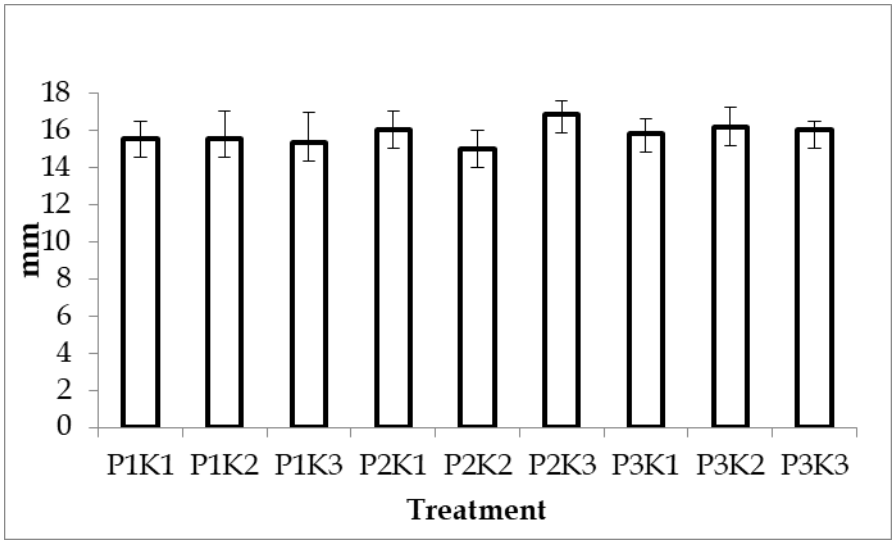

Fig. 1. Diagram of mud crab absolute carapace width (mean ± standard deviation)

4. Water quality

Table (3) below presents the results of water quality measurements during the study.

Table 3. Results of water quality measurements during research

Treatment	pH		Dissolved oxygen (mgL^{-1})		Temperature (^0C)		Alkalinity (mgL^{-1} $CaCO_3$)
	Measurement time						
	04.00	14.00	04.00	14.00	04.00	14.00	
P1K1	8.12±0.03	8.22±0.02	4.24±0.01	4.36±0.03	26.11±0.24	29.79 ±0.33	104.12 ±2.88
P1K2	8.11±0.02	8.20±0.02	4.27±0.03	4.49±0.03	27.45±0.12	29.24±0.18	108.36±0.84
P1K3	8.09±0.01	8.17±0.01	4.29±0.02	4.49±0.02	27.71±0.09	29.51±0.17	110.67±0.52
P2K1	8.10±0.03	8.16±0.02	4.30±0.07	4.39±0.05	26.59±0.32	28.50±0.30	105.27±1.63
P2K2	8.17±0.01	8.22±0.01	4.28±0.09	4.50±0.04	27.44±0.22	29.71±0.22	113.03±0.15
P2K3	8.16±0.02	8.22±0.02	4.21±0.02	4.48±0.02	27.78±0.56	29.55±0.09	119.46±3.23
P3K1	8.09±0.01	8.15±0.00	4.33±0.04	4.60±0.02	26.85±0.06	28.48±0.05	119.15±1.60
P3K2	8.15±0.01	8.21±0.01	4.16±0.01	4.43±0.05	27.52±0.05	29.24±0.16	128.14±2.37
P3K3	8.19±0.03	8.23±0.02	4.22±0.07	4.51±0.04	28.72±0.15	30.58±0.30	142.30±1.97

Mean ± Standard deviation

Temperature values ranged from 27.44 to 28.72ºC in the morning and from 29.24 to 30.58ºC in the afternoon. pH values ranged from 8.09 to 8.19 in the morning and from 8.16 to 8.23 in the afternoon. Dissolved oxygen ranged from 4.16 to 4.30mg/ L in the morning and from 4.36 to 4.52mg/ L in the afternoon. Alkalinity ranged from 104.12 to 142.30mg/ L CaCO3.

DISCUSSION

The length of the tidal period and the depth of the waters based on the research results show an effect on the ecdysteroid titer value of the mud crabs ($P< 0.05$). The results of the analysis showed that the highest ecdysteroid titer value in the P3K3 treatment was 24 hours high tide and 1-hour low tide with a water depth of 90cm. The length of the tide and the water depth is some of the environmental cues from external stimuli that influence Y-organ (YO) to synthesize and secrete ecdysteroid hormones into the hemolymph. Ecdysteroid concentration in mud crab hemolymph is related to mud crab molting activity for growth and reproduction. A high mean ecdysteroid value is an indication of ongoing molting activity. Furthermore, **Chang and Mykles (2011)** explained that ecdysteroids are hormones that play a role in the molting process and a high ecdysteroid titer value indicates the start of the molting process. Additionally, **Swetha *et al*. (2011)** explained that ecdysteroids in crustaceans can function to accelerate premature molting.

An increase in the concentration of ecdysteroid hormone in hemolymph is a sign of the start of molting and results in a shorter molting interval and an accelerated frequency of the molting period **(Lemos & Weissman, 2020; Maulianawati *et al*., 2020)**. The pre-molting process is triggered and coordinated by an increase in the titer of ecdysteroids in the hemolymph, including synthesis of new exoskeleton, degradation and resorption of old exoskeleton, atrophy of claw muscles, and growth of regenerating limbs. It is further explained that the integration of external cues mediated by the brain and eyestalk ganglia in controlling the release of MIH from the X-Organ/sinus gland (XO/SG) and internal cues determines the decision to initiate molting in crustaceans **(Mykles, 2021)**. The temporal and physiological relationship between molting and reproduction in crustaceans and especially crabs is not clearly understood. However, female crabs must molt to mate and store sperm that can be used for subsequent spawning without molting and subsequent mating until the second and third spawning **(Thomton, 2005)**.

The results of statistical analysis through Duncan's test at a significance level of 0.05 showed the shortest mud crab molting latency in the P3K3 treatment, namely 24 hours of high tide and 1-hour low tide with a water depth of 90cm. The different molting latency period was caused by the difference in the average value of the ecdysteroid titer due to the effect of treatment in this study. The variation in the mean value of ecdysteroid titers during the molting cycle is determined by the combined effects of biosynthesis, metabolism, and excretion. The balance between synthesis and excretion affects the concentration of ecdysteroids in the hemolymph. Furthermore, in the peripheral tissue, ecdysteroid synthesis is converted to the active form (20 hydroxyecdysones and Ponasterone A) and excreted through the antennal gland. Increased biosynthesis and conversion to active ecdysteroids affect the increase in concentrations in hemolymph **(Chang & Mykles, 2011)**. The molting cycle in crustaceans begins in the pre-molt when MIH secretion is reduced or stopped by environmental cues **(Mykles, 2021)**. In this study, environmental cues that play a role in the molting process are most likely the treatment of the length of the tide and the depth of the water. External signals from environmental cues will be responded to and integrated with internal signals by SG Neuropeptides to initiate molting **(Shrivastava & Princy, 2013)**. Increased ecdysteroid synthesis in YO and secretion of ecdysteroid titers to hemolymph shortens the molting latency period and accelerates the frequency of the molting period **(Techa & Chung, 2015)**. This is consistent with the results of this study, where the average value of the highest ecdysteroid titer in the P3K3 treatment had the shortest molting latency, which was 14.17± 1.04 days.

The results showed that the absolute carapace width had varying values in each treatment but did not show a significant difference. The average absolute carapace width ranged from 15.33± 1.61 to 16.83± 0.76mm. Water quality criteria that must be met in mud crab cultivation are pH 7.0 to 8.0; dissolved oxygen > 2.5mgL^{-1}, temperature 23 to 32ºC, and alkalinity > 50mgL^{-1} CaCO3 **(Ario *et al*., 2019)**. Based on the results of the

measurement of water quality parameters, the results obtained in general still support the mud crab maintenance media in the optimal range. Water quality in crustacean aquaculture is an important factor influencing survival, reproduction, and optimum growth. Water temperature plays a role in regulating the metabolic processes of aquatic biota. The increase in temperature causes an increase in the metabolic rate, oxygen consumption, and a decrease in the solubility of oxygen in the water. High temperatures with tolerable levels can shorten the molting period of mud crabs. But if the water temperature is lower than 20ºC then growth, activity, and appetite will stop **(Gong *et al*., 2015)**. Mud crabs in the waters of mangrove forests are found in waters with a temperatures of 28 to 36ºC and lagoon waters in the range at temperatures of 13 to 40ºC. Mud crabs grow in a temperature range of 25 to 35ºC and optimally at 30ºC **(Shelley & Lovatelli, 2011)**. Carbon dioxide and alkalinity are related to the pH of the water. High pH values can inhibit or delay the molting process in crustaceans **(Lemos & Weissman, 2020)**. The pH value that can be tolerated by mud crabs ranges from 5 to 9, while optimal growth are in the range of 7.5 to 8.5 **(Heasman & Fielder, 1983; Shelley & Lovatelli, 2011)**. Oxygen in water is absolutely needed by aquatic organisms for respiration, metabolic processes or the exchange of substances that produce energy for growth and reproduction **(Mubarak *et al*., 2010)**. The decrease in dissolved oxygen value under optimum conditions will result in a decrease in consumption levels and affect behavior and physiological processes such as respiration, growth, reproduction, molting and survival. The optimum dissolved oxygen for mud crab culture $> 3 mgL^{-1}$ **(Pedapoli & Ramudu, 2014)**. A high alkalinity value indicates a greater capacity of the water to buffer changes in pH, thereby reducing pH fluctuations (Chen *et al.,* 2006). Optimal alkalinity for aquatic life generally ranges between 50 and 150mg/ L **(Boyd, 2020)**.

CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that the length of tidal time and water depth affect hormonal conditions and the molting latency of mud crabs *S. serrata*.

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