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Abstract. In a new commercial commodity aquaculture, tropical freshwater crayfish *Cherax quadricarinatus*, it is known that female has a slower growth rate than male. One way to increase production rate is through monosex cultures comprising all-male population by using genital reversion technique. This biotechnology method can be applied by using hormones and natural products. The natural hormones extracted from gamma sea cucumber, *Stichopus variegatus* has bioactive substances including steroid compounds, while honey bee contains chrysin compounds that act as aromatase inhibitors. Therefore, the combination of both substances expected can increase in testosterone level and accelerate production in the male monosex population of juvenile crayfish. The purpose of the study is to determine the most effective dose of honey bee in masculinization of juvenile freshwater crayfish combined with 2 mg. L^{-1} steroid extract of gamma sea cucumber. Furthermore, a Completely Randomized Design (CRD) was designed in the experiment with five different dose of honey bee treatment and dipping method in 2 mg. L^{-1} steroid extract of gamma sea cucumber for 18 h. Each treatment with four replications. The highest male formation was found in the dose of 20 ml. L^{-1} honey bee at 83.75% and the lowest was in control. The present of honey bee at different doses in 2 mg. L^{-1} of gamma steroid extract did not significantly give an effect on percentage of female, intersex formation, survival, and growth rate of juvenile redclaw crayfish.

Keyword: masculinization, steroid, chrysin, aromatase inhibitors, testosterone

1. Introduction

One type of tropical freshwater lobster which has been cultivated in Indonesia is redclaw crayfish (*Cherax quadricarinatus*) from Queensland and the Northern of Australia [1]. This species has several advantages, such as easy to cultivate, not susceptible to disease, fast growth, large size, omnivorous, relatively high fecundity and can tolerate wide ranges of water quality conditions [2]. The females crayfish has a slower growth rate than males at the same age [1, 2, 3, 4]. One way to produce this cultivated aquaculture commodity used monosex-male cultivation technique by using the method of

genital reversion (*sex reversal*). This biotechnology method can be applied by giving hormones and other natural ingredients to increase the aquaculture production. There are some problems using synthetic hormones, the price is relatively expensive [5] and has a negative impact to the environment [6, 7]. One of natural sea source hormones that is safe for aquaculture and environment is hormone extracted from visceral organs of gamma sea cucumber, *Stichopus variegatus*. Sea cucumber extract was known contains steroids type testosterone which is an androgen hormone [8, 9, 10, 11]**,**which have been shown can increase the ratio of male in some species of fish and crustacean. The use of sea cucumber as a natural steroid is very important in aquaculture. Rasyid [12] showed that the yield of gamma sea cucumber, *S. variegatus* extract was 2.78 % and he found secondary metabolite contents were steroids and saponins. Steroid extract induced in the crustacean group can stimulate increased testosterone [4], that leads to male genital formation [13]. Honey is a substance that more natural, secure, economically inexpensive and environmentally friendly as an alternative ingredient. This material has the potential to drive sex the fish is predominantly male [5]. Honey contains potassium and chrysin which acts as an aromatase inhibitor [14, 15]. Aromatase is a type of enzyme that catalyzes the conversion of testosterone to estradiol and then because of the presence of chrysin, the estrodiogenesis process will be inhibited [15]. If the aromatase activity is low, the individual will differentiate towards the testes, so that the testosterone production will be increasing and stimulating the growth of male genital organs and male nature [7, 16]. Types of natural steroid ingredients that utilized in sex reversal including, such as honey bee in guppy [15,17], in freshwater crayfish [18], in tilapia [19] and bee resin through artificial diet in tilapia [20]. The combination of these two natural ingredients, steroid extract of gamma sea cucumber and honey bee will increase the percentage of male and will accelerate the profitability of large scale production of freshwater crayfish. Therefore, the main purpose of the study is to determine the most effective dose of honey bee mixed with 2 mg. L^{-1}steroid extract of gamma sea cucumber, *S. variegatus* in the formation of monosex-male of juvenile crayfish *C. quadricarinatus*.

2. Materials and Methods

2.1. Extraction of gamma sea cucumber

The extraction of gamma sea cucumber was categorized based on body weight and length in terms of species and age to determine the existence of testosterone. The raw materials was approximately 8 kg of mature gamma sea cucumber, each weight around 300 g to 800 g with a length of 25 cm to 45 cm, obtained from Sari Ringgung fisherman, Lampung. Extracts of powdered sea cucumber were obtained by using solvents including n-hexane, diethyl eter, and methanol. After 24 h exposure period in n-hexane, the extract was concentrated under low pressure at 30°C by rotary evaporation. The diethyl ether extract was ready after 48 h and then the solved was removed by rotary evaporation at 35°C. The methanol extract was ready after 72 h and then the solvent was removed by rotary evaporation at 40°C. Ether-methanol was formed by adding ether in order to separate the methanol-aqueous extract, and then the upper phase was separated by separating funnel. The upper phase was combined with n-butanol and aqueous extract was separated by separating funnel. Each extract was shaken by mechanical shaking at room temperatur (25°C). All processes were accomplished under dark condition and crude extract were kept in the freezer [21]. The process of gamma sea cucumber extraction was performed at the Integrated Laboratory and Technology Innovation Center, University of Lampung.

2.2. Animal maintenance test and treatment

The study was conducted in the research laboratory of Aquatic Biology, the University of Lampung. The juvenile freshwater crayfish approximately 2-3 weeks-old with the size body length of 2-2,5 cm were obtained from Gemma Farm cultivators, Central Java. The juvenile crayfish was acclimatized by feeding and supplying oxygen and good sanitation. The selectian was based on morphological characteristics, such as body length, healthiness and completeness of body organs. The juvenile crayfish stocked in separate fiber tanks containing recirculating water (27±1°C) and exposed to a 12 (light): 12 (dark) photoperiod, where oxygen concentration was kept above 5 mg. L^{-1} by aeration. The

experimental stocking density was adjusted to twenty juveniles per aquaria. Each experimental aquarium was filled up to a culture volume of 5 L with dechlorinated water. Daily, faeces and other particles were extracted out from the bottom of each aquarium by siphoning and 40-50% of water volume was changed every two days. In this treatments control only given a 2 $mg.L^{-1}$ gamma sea cucumber extract solution and another treatment given 2 $mg.L^{-1}$ gamma sea cucumber steroid extract and different honey bee doses of 5 $ml.L^{-1}$, 10 $ml.L^{-1}$, 15 $ml.L^{-1}$, and 20 $ml.L^{-1}$ and all the treatments were soaked for 18 h. The maintenance of juvenile crayfish was done for 40 days, until the genital organs can be identified. Feeding is given twice a day in the morning and evening. Water quality parameters were measured, such as pH, dissolved oxygen, temperature and hardness.

2.3. Research design

The research design in this study using the experimental method with Completely Randomized Design (CRD) using 5 treatments and 4 replication. The treatment in this study used combinations of gamma sea cucumber steroid extract at dose of 2 $mg.L^{-1}$ and different dose of honey bee: control (0 $ml.L^{-1}$), 5 $ml.L^{-1}$, 10 $ml.L^{-1}$, 15 $ml.L^{-1}$ and 20 $ml.L^{-1}$ water. The juvenile crayfish were kept for 40 days to monitor its growth performance and at the end of the experiment the sex ratio was determined by secondary sex characteristics between male and female. Growth performance was monitored by recording the morphometric characteristics, such as body weight gain, daily growth rate and total length of juvenile crayfish.

2.4. Research parameters

The parameters in this study are:

1. Percentage of sex ratio (male, female, and intersex) of jevenil crayfish [22]

$$\text{Percentage of sex ratio} = \frac{number\ of\ male,\ \ female,\ \ or\ intersex}{number\ of\ crayfish\ samples} \text{ x } 100\ \%$$

2. Percentage of survival rate of juvenile crayfish [22]

$$\mathbf{SR = Nt / No \times 100\ \%}$$

SR : crayfish survival rate (%)
Nt : number of juvenile crayfish at the end of the study
No : number of juvenil crayfish at the beginning of the study

3. Growth rate includes total weight (TW), daily growth rate (DGR) and total length (TL) [23]

$$\mathbf{TW = Wt - Wo}$$

TW : total weight gain (g)
Wt : average weight at the end of study (g)
Wo : average weight at the beginning of study (g)

$$\mathbf{DGR = \frac{Wt - Wo}{t}}$$

DGR : daily growth rate ($g\ d^{-1}$)
Wt : average weight at the end of study (g)
Wo : average weight at the beginning of study (g)
t : time between observations/maintenance time (d)

$$\mathbf{TL = Lt - Lo}$$

TL : total length (cm)
Lt : average length at the end of study (cm)
Lo : average length at the beginning of study (cm)

4. Water quality during maintenance
   Water quality measurements include: pH measured using pH meter, water temperature using thermometer kit, and dissolved oxygen using DO meter.

2.5. Data analysis

The data including the percentage of sex ratio, survival rate, growth rate then processed for the analysis of variance, if there is a real difference the continued with LSD test (the smallest real difference with $\alpha = 5\%$) using SPSS 16 software.

3. Results and Discussions

The complete results of the combination of gamma sea cucumber extract at 2 mg.L^{-1} and different doses of honey bee on sex formation, survival and growth rate of juvenile freshwater crayfish are shown in Table 1. From the results of the LSD test indicated that the highest percentage of juvenil males was found in the treatment of giving honey dose of 20 ml. L^{-1} by 83.75% and the lowest was found in the control. The treatment does not give significantly effect on percentage of female, individual intersex, and survival rate as seen in Table 1. The percentage of male sex formed in juvenile freshwater crayfish follows a positive linear pattern. It means the higher the dose of honey bee given, the higher male sex formation produced.

Table 1. The formation of sex (male, female, intersex), survival rate and growth rate of juvenile freshwater crayfish for 40 days maintenance

Treatments (honey bee doses) (ml L^{-1})	Male formation (%)	Female formation (%)	Intersex formation (%)	Survival (%)	Growth Rate		
					Total weight gain (g)	DGR (g d^{-1})	Total length (cm)
0	55.35±2.19[a]	30.08±4.82	14.57±1.24	76.25±4.36	1.152	0.0288	3.145
5	56.25±3.21[a]	21.87±5.72	21.88±1.96	86.25±3.58	1.188	0.0297	3.418
10	62.50±2.20[a]	31.25±2.65	6.25±1.18	76.25±2.75	1.216	0.0304	3.456
15	67.41±3.02[a]	28.12±3.73	4.47±1.62	81.25±4.29	1.206	0.0302	3.457
20	83.75±4.33[b]	14.94±2.69	1.31±0.52	72.50±3.66	1.181	0.0295	3.691

Notes: different letters in the table indicate a difference between treatments (LSD test)

According to Ferreira [24] honey contains polyphenolic compounds such as flavonoids, flavonols, phenolic acids, catechins, and cinnamic acid derivatives. One of the flavonoid derivatives is chrysin. Honey also contains potassium and chrysin which acts as an aromatase inhibitor [14]. The increase in the percentage of males is thought to be due to honey containing chrysin compounds. Chrysin acts as an aromatase inhibitor, chemicals that can be used to manipulate sex differentiation by inhibiting aromatase enzyme activity. Aromatase inhibitors inhibit the expression of the aromatase gene (cytochrome P450) which plays a role in aromatization of androstenedione to estrogen and testosterone to estradiol-17β [25, 26]. Server [25] added that fish larvae exposed to low aromatase activity lead to the formation of testes, whereas at high aromatase activity lead to the formation of ovaries. The effect of inhibiting the formation of estrogen can lead to masculinization. Chrysin acts as an inhibitor, meaning that it can inhibit the action of the aromatase enzymes involved in the production of estrogen resulting in an increase in the formation of the testosterone hormone. The testosterone can stimulate the formation of male genital organs and male secondary sex characteristics [15, 16, 27].

The natural hormone testosterone contained in the steroid extract of gamma sea cucumber (*S. variegatus*) also plays a role in the successful formation of male juvenile crayfish. Based on research by Meydia et al [11], the extract of sea cucumber is proven to contain steroids. The highest steroid concentration was 62.80 mg. mL^{-1} in ethyl acetate solvent. According to Kustiariyah [9], the steroid content in body tissues and blood vessels can be in the form of steroid hormones, free fatty acids, triglycerides and cholesterol. Extract of steroid hormones induced in crustaceans can stimulate an increase in testosterone which leads to the formation of male genitalia [13].

Giving steroid extract of sea cucumber at a dose of 2 mg. L^{-1} is the most effective dose in the formation of male genitalia of freshwater crayfish. This is in accordance with study by Susanto [4] that giving sea cucumber extract at a dose of 2 mg. L^{-1} at a temperature of 27°C gives the highest male sex formation by 75.16%. The mechanism of hormone action through the immersion method will enter the body by diffusion pass through the skin, gills and digestive organs. Crustacean gills generally consist of lamina branches, while the gill filaments are lined with thin cuticles and cytoplasm which divides the gill cells [28]. Water-soluble steroid extract will enter the body through the mucosal tissue of the gill cells and distributed to the target tissue [29]. According to Connell and Miller [30] the absorption of dissolved components in water through the gills is quite large when compared to absorption through the skin and digestive organs.

The difference among treatments in terms of sex ratio of juvenile crayfish were determined on the basis of secondary sex characteristics. The secondary sex characteristics used included the appearance of genital papillae in male and genital openings in female, as seen in Figure 1.

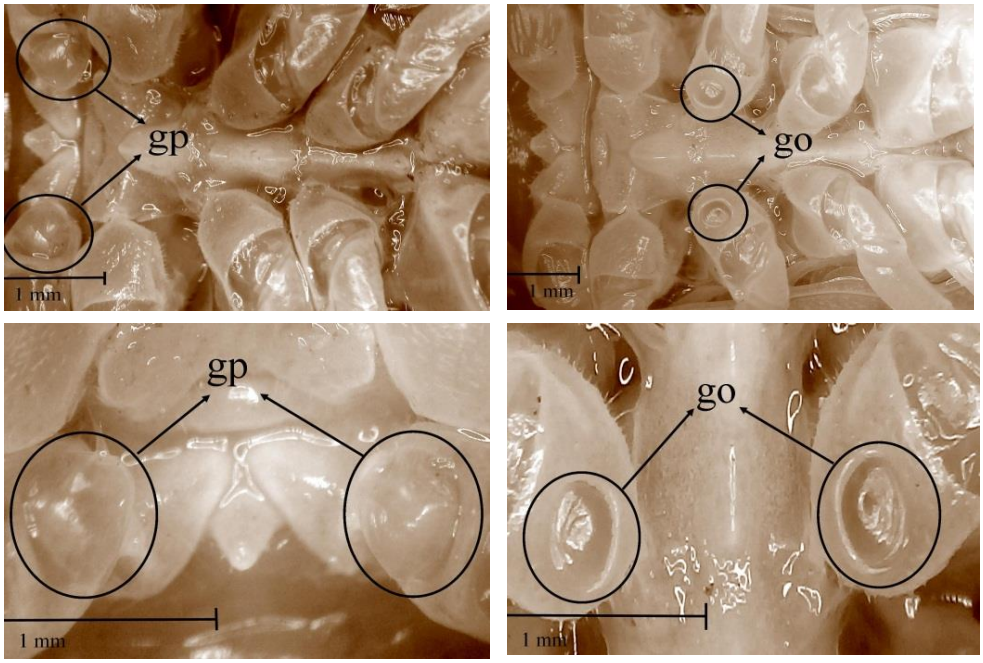

Figure 1. The difference between genital organ of male (left) and female (right) of juvenile freshwater crayfish. Male crayfish has *genital papillae* (gp) at the base of the fifth pair of walking legs (periopod) and female *has genital openings* (go) at the third pair of walking legs (periopod).

Base on statitical analysis, the treatments giving 2 mg. L^{-1} steroid extract of gamma sea cucumber and honey bee with different doses did not give significantly effect on female and intersex formation.

In dipping method if the dose of hormone is too high the larvae will be stressed and die, but if the dose is too low then the ability of hormones in sex reversal is not effective and the juveniles become steril (intersex individual) [27, 31]. It is known that the addition of honey with different doses in the steroid extract of 2 mg. L^{-1} sea cucumber does not statistically significant effect on the formation of female genitalia and intersex bodies in juvenile freshwater crayfish. This is presumably due to the small amount of chrysin in honey, so that a dose of less than 20 ml. L^{-1} of water is considered less effective in the formation of male genitalia. In addition, in the immersion method not all chrysin compounds and testosterone hormones can be properly absorbed by crayfish body.

Based on the analysis of variance, the addition of different doses of honey in the steroid extract of sea cucumber at 2 mg. L^{-1} has no significant effect on the survival rate of juvenile freshwater crayfish. The highest percentage of survival rate was found in the dose of honey 5 ml. L^{-1} water (86.25%). The lowest was in the dose of 20 ml. L^{-1} (72.50%). In the high doses with high honey

density, it can cause a decrease in the function of body tissue. It caused the organisms stress and die. Besides that the mortality also dominated by cannibalism, the cannibalism occurs during moulting cycle due to weak of body condition, stressful, and susceptible to environment changes. Therefore, it has an effect on the function of enzymes that play a role in metabolism [32]. The factors that influence the level of survival are biotic factors (population density, age, and ability to adaptation) and abiotic factors (environment maintenance) [33, 34, 35, 36]. The mineral content in honey is very important in supporting during juvenile life of the freshwater crayfish. Sudrajat [37] stated that effective immersion in genital direction needs to get pay attention on the concentration of honey bee given, the higher the honey dose given does not determine the high survival rate of fish larvae. According to Bogdanov [38] every 100 g of honey contains 205-1676 mg. L^{-1} of potassium (K), 49-51 mg. L^{-1} of calcium (Ca), 19-35 mg. L^{-1} of magnesium (Mg), and 18 mg. L^{-1} of sodium (Na). The minerals in honey are absorbed by juvenile crayfish are necessary to increase the body's resistance or body's immune system. Honey acts as a bactericidal, bacteriostatic, antifungal, antiviral, scolicidal, antioxidant, antitumor, and anti-inflammatory [39]. However, when giving honey at a dose of 20 ml L^{-1} of water, the survival rate was not high, this is presumably because the higher the dose of honey given, the water concentration will also increase so that it disrupts the metabolic system in the body. The high doses of honey concentration can cause stress and reduce the function of the body tissue in fish larvae [37].

The growth is characterized by the increase of total weight gain, daily growth rate (DGR) and total length in the body. The highest of total weight was found in the treatment of giving honey dose of 10 mg. L^{-1} (1.216 g) and the lowest was in control (1.152 g). The highest DGR was found in the treatment giving honey dose of 10 mg. L^{-1} (0.0304 g d^{-1}) and the lowest in control (0.0288 g d^{-1}). The highest of total length was found in the treatment giving honey dose of 20 mg. L^{-1} (3.691 cm) and the lowest was in control (3.145), as seen in Table 1. The total body length of juvenile freshwater crayfish is caused by the mineral content that is absorbed during immersion. Some of the minerals content in the honey that have a function in growth are potassium, magnesium, natrium and vitamin C [18]. Minerals in honey has a function in the formation of body structure, such as exoskeletone in crustacean and give impact on the growth [40]. Testosterone in the steroid extract of gamma sea cucumber give an effect on growth and amino acids in gamma sea cucumber also useful in protein synthesis for muscle formation and androgen hormones in the body of the organism [41].

Water quality during 40 days of maintenance (as seen in Table 2): pH ranged between 7.61-7.91, is still relatively stable. The optimum pH for the growth of freshwater lobster is 6-8 [42]. The water temperature during rearing has a range of 28.5-30.8^{o}C.

Table 2. Water quality measurements during 40 days maintenance

Parameters	Treatments (honey bee doses) (ml. L^{-1})				
	0	5	10	15	20
pH	7,61-7.91	7.62-7.88	7.73-7.85	7.72-7.90	7.71-7.89
Temperature (^{0}C)	28.5-30.5	28.8-30.6	29.0-30.6	29.4-30.6	29.5-30.8
DO (mg. L^{-1})	5.68-6.34	5.91-6.78	6.42-6.85	6.06-6.75	6.68-7.18

The temperature value is relatively stable because the freshwater crayfish kept indoors so they are not directly influenced by changes in water temperature. The optimum temperature for growth has a range of 24-31^{o}C and the temperature below or above this range will be dangerous [22]. The range value of dissolved oxygen during maintenance is 5.68-6.85 mg. L^{-1}. The optimum ranges of dissolved oxygen for crustacean growth is > 5 mg. L^{-1} [22, 42]. Oxygen is important for oxidation of nutrients that enter the body [43].

4. Conclusions

Combination of steroid extract of gamma sea cucumber and different doses of honey bee have a significantly effect on increasing masculinization of juvenile crayfish with the dose of 20 ml/L give the highest male formation of 83.75%. Combination of steroid extract of gamma sea cucumber and different doses of honey bee does not give significantly effect on survival and growth rate.

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