The Seybold Report
ISSN 1533-9211
DOI: 10.5281/zenodo.10200690

STUDY OF 17β-ESTRADIOL HORMONE CONCENTRATION AND HISTOLOGY OF GONAD TISSUE IN RELATION TO FEMINIZATION AND GROWTH RATE OF PASIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*, BOONE 1931) POSTLARVAE

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Abstract

This study was conducted to determine the effect of different concentration of estrogen hormone, 17β-estradiol (E2) on feminization for the production of all female and growth rate of Pasific white shrimp (*Litopenaeus vannamei*) postlarvae. *L. vannamei* PL1 were randomly stocked at a density of 10 ind.L^{-1} into a 10L of seawater for each experimental flask with three replicates each. PL1 was immersed in seawater containing 0.2, 0.4 and 0.6 mg/L estrogen hormone (E2) for 4 hours. The experiment was continued for 60 days. At termination of experiment, the specimens in each treatment group were individually weighed and measured for their wet body weight (BW) and total length (TL) to estimate the weight gain, length gain and the specific growth rate (SGR). The mean sex values of female gained from control till the highest E2 hormone concentration of 0, 0.2, 0.4 and 0.6 mg.L^{-1} were 38.89%, 52.22%, 63.33% and 78.89% respectively. The immersion dose treatment in the E2 hormone also had a significant effect ($P<0.05$) on the weight and length growth of white shrimp post-larvae compared to the control. The weight gain and length gain were 126824% and 900% for control, 135748% and 954% for 0.2 mg.L^{-1} concentration, 149662% and 961% for 0.4 mg.L^{-1} concentration and 151029% and 964% for 0.6 mg.L^{-1}. The mean SGR BW and SGR TL for control till the highest E2 hormone concentration of 0, 0.2, 0.4 and 0.6 mg.L^{-1} were 11.91 and 3.84, 12.02 and 3.93, 12.18 and 3.94 and 12.20 and 3.94 respectively. This difference is thought to be caused by differences in weight and length between male and female individuals. The increase in the percentage of female individuals and the growth rate of *Litopenaeus vannamei* post-larvae in various treatments compared to controls in this study was influenced by differences in the concentration or dose of the E2 hormone given. This is related to the ratio of the amount of the E2 hormone that is absorbed into the body of the post-larvae osmotically. The higher concentration of the E2 hormone used in post-larva immersion caused higher amount of E2 hormone absorbed into the post-larva's body. The E2 hormone concentration of 0.6 mg.L^{-1} turned out to be the highest level of absorption into the post-larval body, namely an average of 105 pg.mL^{-1} and it is what causes the percentage of female sex and the growth rate of vaname shrimp post-larval to be the highest compared to other treatments. Histological tests on vaname shrimp gonad tissue samples resulting from the study did not reveal any hermaphrodite individuals.

Keywords: 17β-Estradiol, Feminization, *Litopenaeus Vannamei*, Postlarvae, Growth Performance, Gonad Tissue.

INTRODUCTION

Aquaculture activities in Indonesia continue to be developed, especially shrimp cultivation, where consumer demand continues to increase from time to time, namely to meet the needs of the export market. The high world market demand for this commodity is a potential opportunity

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for Indonesia's natural resources to increase the country's foreign exchange value from the cultivation sector. Globally, the amount of vaname shrimp (*Litopenaeus vannamei*) production continues to show an increasing trend, from around 2.64 million tonnes in 2010 to 4.96 million tonnes in 2018 (FAO, 2020). At the regional level, the volume of Indonesian shrimp exports also shows a fairly significant pattern of increase from around 162,256 tons in 2015 to 239,227 tons in 2020, where this increase in export volume is also followed by an increase in the value of economic income from around USD 1.45 billion in 2020. 2015 to more than USD 2 billion in 2020 with the main markets for export purposes being the USA, Japan, the European Union and China (Comtrade, 2020).

Female Penaeid shrimp generally have faster growth and larger sizes than male shrimp at the same age (Gopal et.al., 2010; FAO, 2006; Perez-Rostro and Ibarra, 2003). Vannamei shrimp (*Litopenaeus vannamei*) grown out in ponds have average body weight of 46.8 ± 7.3 grams for females and 41.2 ± 5.9 grams for males, while white vaname shrimp grown out in tanks have average body weights of 40.2 ± 7.9 and 35.4 ± 6.3 grams each for female and male (Andriantahina, et.al., 2012; Chow and Sandifer, 1991). This is because female vaname shrimp have higher feed conversion efficiency and energy digestion than males and size is a determining factor in competition for food (Moss and Moss, 2006). Therefore, it is very important to know sexual dimorphism in vaname shrimp due to significant growth differences in harvest size (Perez-Rostro et.al., 1999), so that it is hoped that cultivating monosex vaname shrimp (all females) will be able to increase profits cultivators based on the size and weight of shrimp on the market.

Efforts to reverse the sex from male to female (feminization) using commercial 17β-estradiol (E2) orally or mixing feed with hormones have been carried out on several Penaeid shrimp, for example in the post-larval stage of tiger shrimp (*Penaeus monodon*) and banana shrimp (*Fenneropenaeus merguiensis*) and have been proven to be able to increasing the percentage of the number of female individuals by 55.56 - 82.23% and 77.6 - 100% respectively and increasing their growth rate (Hafiz et.al., 2012a, b). Meanwhile, treatment using the soaking method was carried out by Aktas and Genc (2011) on white shrimp (*Penaeus semisulcatus*) with a percentage of female individuals of 50.88%; Sutaman (2002) on tiger prawns (*Penaeus monodon*) produced the highest percentage of female individuals of 78.7% and Sugestya et.al., (2018) on vaname shrimp (*Litopenaeus vannamei*) produced a percentage of female individuals of 70.67 - 100% and increase its growth rate.

The engineering of feminization technology must continue to be developed because of demands for the development of science and technology which can help increase the sex change of shrimp to female to increase production. The use of the E2 hormone has been widely used in the fisheries sector. In this study, we will examine the use of E2 hormone in feminization and the growth rate of vaname shrimp in relation to E2 hormone concentration and histology of gonad tissue so that it can be used as a reference to speed up the feminization process and it can support the continuous acceleration of increasing white shrimp (*Litopenaus vannamei*) production.

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MATERIALS AND METHODS

Preparation of test animals

The test animals used in this research were 1-day old vaname shrimp (Litopenaus vaname) post-larvae (PL1) obtained from spawning at the Nucleus Center Unit of National Broodstock Center for Shrimp and Mollusc Karangasem, Bali where this research was carried out, so that in the process there were no requires a long acclimatization time for white shrimp post-larvae to adapt to their environment. A total of 30 post-larval samples were taken randomly from the aquarium to measure and record initial total length (mm) and wet body weight (g).

Preparation of hormone solution

Preparation of the hormone solution was carried out by following the method used by Rosmaidar et.al., (2014) and Aktas and Genc (2011) by weighing the E2 hormone (Argent Laboratories Inc. Philippines) using a digital scale with a weight of 0.2; 0.4 and 0.6 mg, then each dissolved in 1 mL of 95% ethanol and then homogenized. The mixture of the E2 hormone solution with 95% ethanol was then poured into 9 aquariums containing 1 liter of sea water and given aeration for 20 – 30 minutes to remove the alcohol content. This process is said to be perfect if there is no longer the smell of ethanol in the water.

Soaking test animals with hormone solution

White shrimp post-larvae were randomly selected and stocked in immersion aquariums at a density of 200 individuals/liter for all treatments and replications. Each soaking was carried out for 4 hours for each treatment and repetition. Next, the post-larvae are filtered and rinsed with clean sea water and placed back in an aquarium containing 10 liters of new sea water. The control treatment was prepared without the addition of E2 hormone.

A total of 100 post-larvae and 50 ml of soaking water were taken and stored in a sample bottle wrapped in aluminum foil and stored in a refrigerator for testing 17β-estradiol levels in post-larval meat and soaking water using the ELISA method in the laboratory.

Rearing of test animals

The rearing of vaname shrimp post-larvae was carried out in 12 aquarium units, each containing 10 liters of sea water with a density of 10 individuals/liter, so that the number of post-larvae stocked in each aquarium was 100 individuals. All aquariums are placed in a 10 m^3 concrete tank filled with fresh water to create the same environmental conditions for all maintenance aquariums (water bath). To maintain the temperature in the range of 28 – 32^oC and the surface of the concrete tank is covered with dark colored plastic to keep the temperature stable and preventing 90% of light entering media during the test. The aeration bubble setting is done in such a way as to produce fine air bubbles by adjusting the aeration tap in each aquarium with an aeration stone so that the post-larvae are not disturbed.

Feeding is carried out five times a day with a dose of 10% body weight/day at 08.00, 12.00, 16.00, 20.00 o`clock given a mixture of feed made from the Frippak brand (INVE (Thailand) Ltd.) with a protein content of 42% and Seastar (Hiprox Enterprises Co. Ltd..) and at 24.00

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o`clock, 20 individuals/liter of Great Salt Lake brand (USA) artemia naupli were given as recommended by Kian et.al., (2004). Feeding in the form of artemia nauplii is only given from PL1 to PL10.

The maintenance of rearing media was changed every two days at 40-50% by siphoning it using a small hose and replacing it with new, clean, sterilized water. During the study, daily water quality parameters were maintained in the salinity range of 28 – 32 ppt, dissolved oxygen >4 mg/L, temperature 28 – 32ºC, and pH 7.5 – 8.5. To remove leftover food and shrimp waste, siphon the bottom of the aquarium periodically. Dirt from the bottom of each siphoned aquarium is collected in a fine filter and placed in a bucket to separate the dirt from the larvae that are sucked in. The larvae that have been separated from the feces are returned to the aquarium.

At the end of the rearing period (PL60), the post-larvae in each aquarium are collected by first siphoning 1/3 of the water level of the water level in the rearing medium and attaching a fine scoopnet to the end of the siphon hose til the rest of post-larvae can be collected. The length of the post-larvae collected from each aquarium was measured and their body weight was weighed to analyze the growth rate. Next, the post-larvae were soaked in a 10% formalin solution which was stored in a sample bottle and given a label according to each treatment before observing the secondary and primary gender histologically.

Determination of estradiol-17β levels

Post-larvae of vaname shrimp after soaking for 4 hours with hormones, the estradiol-17β content was measured by grinding the flesh and hepatopancreas using a mortar. Next, the estradiol content analysis was measured using the COAT-ACOUNT estradiol kit made by Diagnostic Product Corporation Los Angles, USA. The ground meat samples were centrifuged at 6000 rpm for 5-10 minutes. Measuring the levels of estradiol-17β absorbed in the meat and remaining soaking water used the ELISA method at the Physiology Laboratory, Faculty of Medicine, Brawijaya University.

Sex determination and sex ratio

Observation of the sex of white shrimp post-larvae treated with the E2 hormone by immersion was carried out at the end of the study. External sex identification is carried out by observing differences in the external sex structure of the post-larvae morphologically under a microscope based on the method used by Garza-Torres et.al., (2009) and Campos-Ramos et.al., (2006). The ratio of the number of males and females is determined in percent.

Internal gender identification which includes the ovaries and testicles is carried out following the method used by Garza-Torres et.al., (2009), King (1948) and Dall et al. (1990) which was carried out when the post-larvae were 60 days old. Histological observations of the gonads tissue organs of the of test animals were observed using a microscope after being stained with hematoxylin and eosin were carried out in the Pathology Laboratory Anatomy, Faculty of Medicine, Brawijaya University.

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Determination of the growth rate

At the end of the study, individual post-larvae from each treatment group were weighed and measured to determine body weight and total length to calculate the final average body weight (BW) and total length (TL) and specific growth rate (SGR). Body weight was measured using a digital scale with an accuracy of 0.0001 grams and total length was measured using a caliper with an accuracy of 0.1 mm. The results of measurements of body weight, total length and specific growth rate were calculated using the equation used by Bautista-Teruel et.al., (2003) as follows:

$$GR\ (\%) = \frac{Wt - Wo}{Wo} \times 100$$

$$SGR = \frac{100\ [\ \ln\ Wt\ - \ln Wo\ ]}{d\ (days)}$$

where:

GR = growth rate (%)

SGR = specific growth rate

Wo = initial weight / length (grams or cm)

Wt = weight / final length (grams or cm)

d = rearing periode (days)

Statistical analysis

To determine the differences among treatments, one-way Analysis of variance (ANOVA) was used. Post host test and Tukey's test were used to determine the significantly different among treatment. The significance level of the results was set at ($P < 0.05$). All statistics were performed using SPSS (version 16).

RESULT AND DISCUSSION

Sex ratio

In this study, observation and gender identification of vaname shrimp (*Litopenaeus vannamei*) were carried out after rearing for 60 days, where the weight and secondary sexual characteristics as well as the gonad tissue had developed perfectly so that identification errors could be avoided. This refers to the opinion of Garza-Torres et.al., (2009) that post-larvae of vaname shrimp are 50 days old with a weight of 0.5 - 0.6 g and a body length of 45 - 50 mm and Campos-Ramos et.al., (2006) which states that the sex determination of juvenile vaname shrimp starts from day 50 to 60 or when the juveniles develop to a body weight of 150 - 200 mg and a body length of 20 - 25 mm while males and female can be differentiated... Where female individuals can be distinguished by the formation of a pair of curved sharp ridges in thelycum and gonad histology shows the presence of ovaries (Fig. 1) while in male individuals it can be characterized by the presence of a gonopore located at the base of the fifth walking

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legs (pereiopod) and endopodites on a first pair of swimming legs (pleopod), the structure is rounded and gonad histology shows the presence of testes (Fig. 2).

Results of study on soaking post-larvae (PL1) of vaname shrimp in a solution of the hormone estradiol-17β at a dose of 0.2; 0.4 and 0.6 mg/L respectively during these 4 hours, indicating that the percentage of female vaname shrimp from all treatments was higher compared to the control. The results of observing the percentage of sex ratio of vaname shrimp can be seen in Fig. 3.

Based on Fig. 3, it can be seen that the post-larvae (PL1) of vaname shrimp were soaked in the E2 hormone at a dose of 0.2; 0.4 and 0.6 mg/L each for 4 hours had a very significant effect ($P < 0.05$) on all treatments and controls. The increasing concentration of the E2 hormone given during immersion resulted in an increase in the percentage of female individuals. And it was found that the best dose to get the highest percentage of female vaname shrimp in this study was treatment at a dose of 0.6 mg/L with a number of female individuals of 78.89%. Apart from that, based on the results of observations of gonad tissue samples, it turned out that no sterile or hermaphrodite gonad tissue was found. All male testicular tissue (Fig. 2b) and female ovaries (Fig. 1b) showed complete gonad tissue.

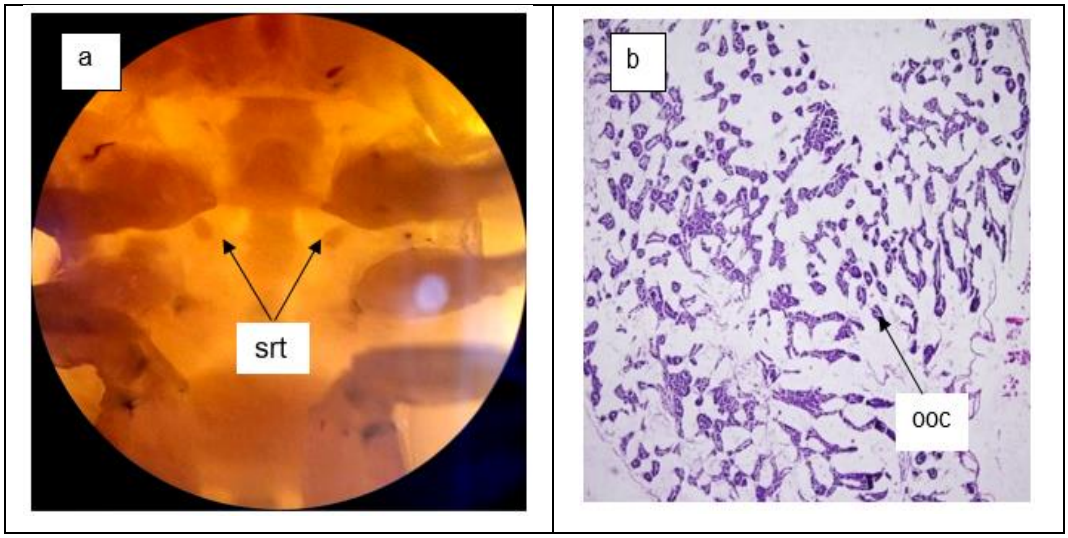

Figure 1: Post-larval secondary sex characteristics of female vaname shrimp (a) shows the presence of *sharp ridges in thelycum* (srt) and (b) shows the presence of an *oocyte* (ooc)

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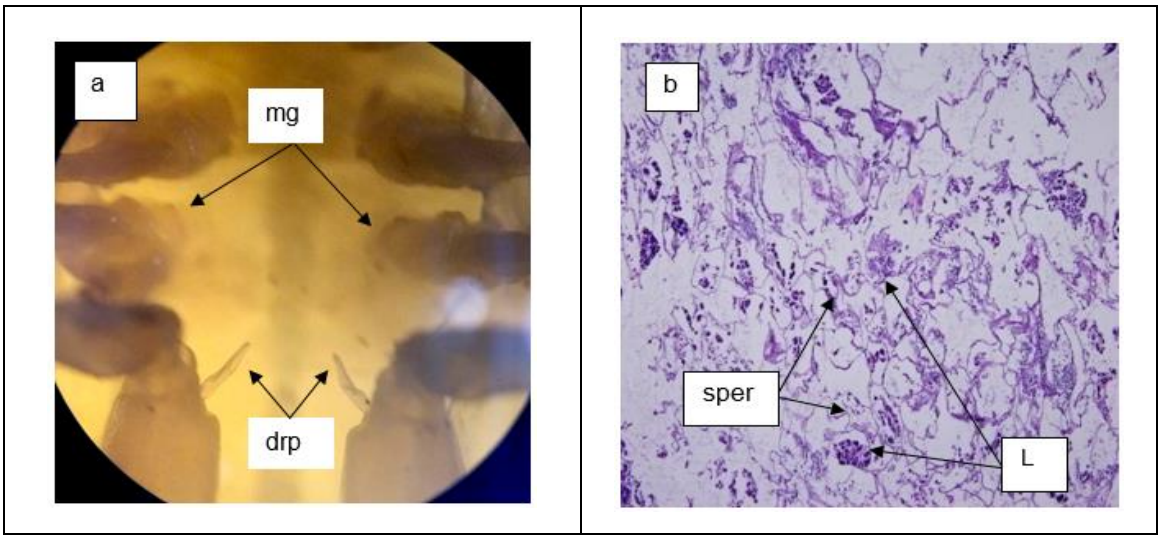


Figure 2: Post-larval secondary sex characteristics of male vaname shrimp (a) shows the presence of an *apical dense region of petasma* (drp) and the presence of *male gonophores* (mg) on the male sternite and (b) shows the presence of spermatogonia (sper) around the lumen (L)

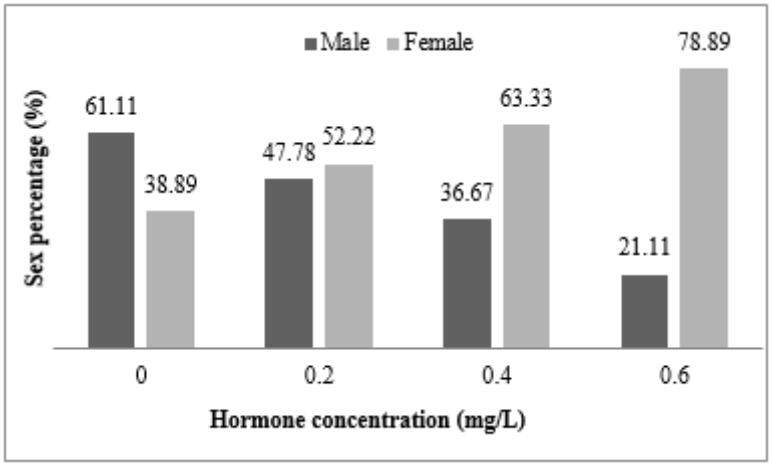


Figure 3: Percentage of sex ratio of vaname shrimp in various treatments and controls

This shows that the administration of the hormone estradiol-17β using the immersion method at the PL1 stage has been proven to be able to influence the hormonal system in the body of vaname shrimp. This is in accordance with the opinion of Garza-Torres et.al., (2009) that sex reversal can be carried out in vaname shrimp during the labile period or when the sex chromosomes are not clearly differentiated, namely before PL12. In addition, the results of this study show that soaking PL1 in the E2 hormone has been able to direct white shrimp post-larvae to become female individuals due to the androgen glands not developing from the start and resulting in the automatic formation of ovaries (Sagi, 1988; Sagi et.al ., 1997), and is also reinforced by the opinion of Baeza, (2006) and Sagi and Aflalo (2005) who state that a decrease

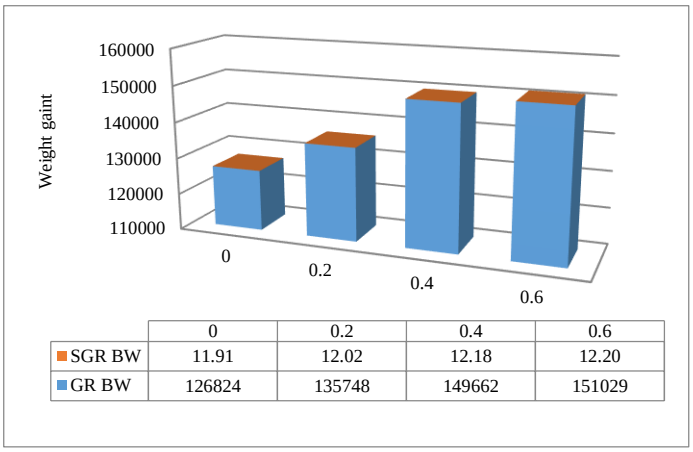
ISSN 1533-9211

DOI: 10.5281/zenodo.10200690

in the concentration of androgen hormones in the hemolymph over a certain period can inhibit the formation of male genitalia in a crustacean organism, so that it can result in changes or automatic sex reversal to female.

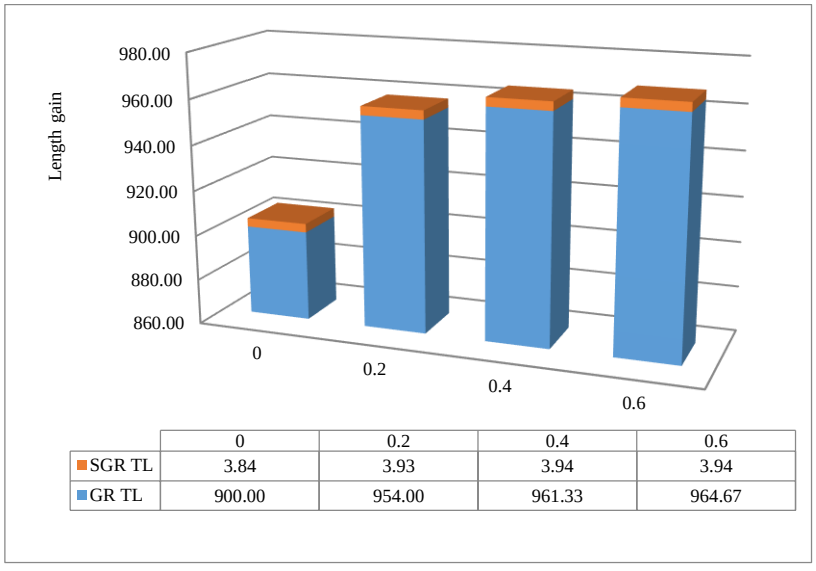
Growth rate

Data analysis carried out on the specific growth rate (Specific Growth Rate) of growth in weight and final length of the average vannamei shrimp individual showed that there were differences in weight and length between treatments and were higher than controls, respectively as presented in Fig. 4 and 5.



	0	0.2	0.4	0.6
SGR BW	11.91	12.02	12.18	12.20
GR BW	126824	135748	149662	151029

Figure 4: Average weight gain and SGR BW of individual vaname shrimp



	0	0.2	0.4	0.6
SGR TL	3.84	3.93	3.94	3.94
GR TL	900.00	954.00	961.33	964.67

Figure 5: Average length gain and SGR TL of individual vaname shrimp

From the results of this study, it can be seen that in all treatments, immersion of post-larvae in the hormone estradiol-17β had a significant effect ($P<0.05$) on the average final weight of

ISSN 1533-9211

DOI: 10.5281/zenodo.10200690

vaname shrimp individuals for all treatments and controls. However, this was not the case for the average growth in length, which only showed a significant effect ($P<0.05$) only on the control. These results show that the higher concentration of the E2 hormone given in the treatment will result in a higher average growth in weight and length compared to the control. This shows that the difference in growth is more caused by differences in growth between male and female individuals as well as differences in the percentage of male and female individuals.

The increase in the average growth of vaname shrimp larvae when compared with controls in this study shows that administration of the E2 hormone by soaking has been able to have an effective influence on the weight and length growth of vaname shrimp. This is in accordance with the opinion of Piferrer (2001), that steroid hormone treatment apart from influencing sex changes can also have an impact on growth and Wang et.al., (2008) who state that the E2 hormone not only influences the occurrence of sex reversal but also becomes growth promoter in *L. macrochirus* shrimp.

Effect of Dosage on Sex Reversal and Growth

The increase in the percentage of female individuals and the growth of *Litopenaeus vannamei* post-larvae in various treatments compared to controls in this study was influenced by differences in the concentration or dose of the E2 hormone given. This is related to the comparison of the amount of the E2 hormone that is absorbed into the post-larval body osmotically and the amount of the E2 hormone remaining in the soaking media water, where the test results are using the ELISA method in the laboratory.

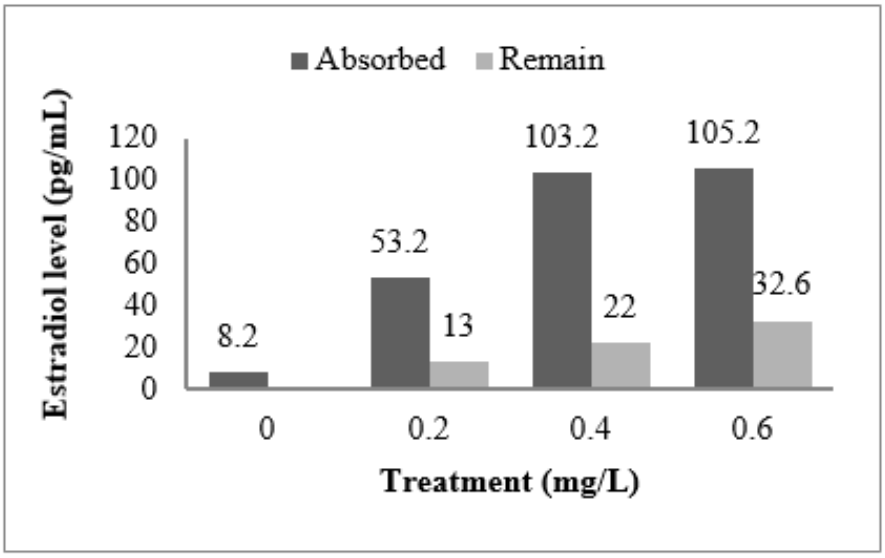


Figure 6: Comparison of the concentration of the E2 hormone between treatments with the amount of hormone absorbed in the post-larval body and the hormone remaining in the soaking media water (pg/mL)

ISSN 1533-9211

DOI: 10.5281/zenodo.10200690

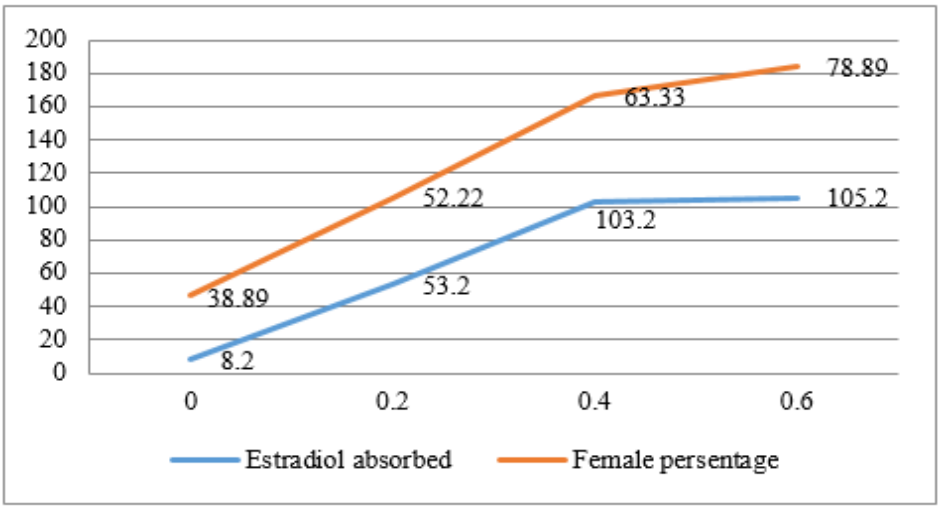


Figure 7: Comparison of the percentage of female vaname shrimp with the amount of hormone absorbed in the post-larval body (pg/mL)

From Fig. 6 it can be seen that the higher the concentration of the E2 hormone used in soaking post-larvae, the higher amount of hormone absorbed into the body of the post-larvae. The hormone concentration of 0.6 mg/L turned out to be the highest level of absorption into the post-larval body. So it is suspected that this is what causes the percentage of female sex and the degree of post-larval growth of vaname shrimp at a concentration of 0.6 mg/L to be the highest compared to other treatments. Because the use of steroid hormones to change sex is influenced by several factors including the type and dose of the hormone used, the method of giving the hormone, the length of time it is given, the type of fish, the age of the fish, environmental conditions, especially water temperature and the type of feed used (Vanyakina, 1969; Hunter and Donaldson 1983). A comparison of the sex percentage of female vaname shrimp with the levels of estradiol-17β absorbed in the post-larval body can be seen in Figure 7.

The hormone estradiol-17β is a natural hormone derived from cholesterol, while cholesterol and sterols are essential components of cell membranes and steroid hormones, however cholesterol ($C_{27}H_{45}OH$) is not synthesized *de novo* in the shrimp body so it must be obtained from food to stimulate the synthesis of steroid hormones (Belles et al., 2005; Seybold and Tittiger, 2003; Tobe and Bendena, 1999; Grieneisen et.al., 1994) namely ecdysteroids produced by the Y-organ. It is a pair of glands that secrete ecdysteroids (ecdysone) or molting hormone (MH). Located in the cerebral and thoracic ganglion in the cephalothorax of all types of crustaceans including white shrimp whose production is influenced by MIH from the X-organ sinus gland (XOSG) (Snyder and Chang, 1997a, b; Blais et.al., 1994; Mattson and Spaziani, 1985). So, the exogenous presence of cholesterol derived from the hormone estradiol-17β in this study will increase cholesterol levels in the post-larval body and stimulate the synthesis of ecdysteroids by the Y-organ as well as inhibit the production of MIH from the sinus gland of the X-organ, resulting in molting and growth. in post-larvae. A comparison of the growth in

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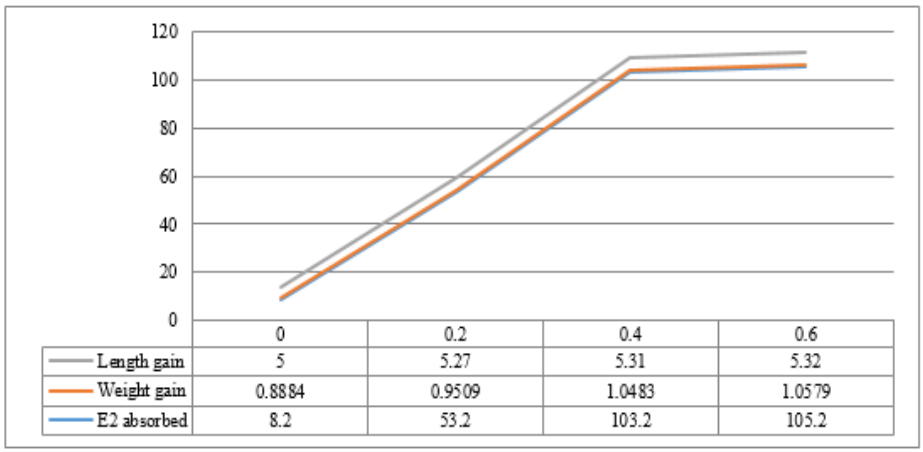
DOI: 10.5281/zenodo.10200690

weight and length of vaname shrimp with the levels of estradiol-17β absorbed in the post-larval body can be seen in Figure 8.



	0	0.2	0.4	0.6
Length gain	5	5.27	5.31	5.32
Weight gain	0.8884	0.9509	1.0483	1.0579
E2 absorbed	8.2	53.2	103.2	105.2

Figure 8: Comparison of growth in weight and length of vaname shrimp with the amount of hormone absorbed in the post-larval body (pg/mL)

CONCLUSION

The present study demonstrates that soaking the post-larval stage (PL1) of white shrimp (*Litopenaeus vannamei*) in a solution of the E2 hormone for 4 hours can increase the percentage of female individuals. The highest percentage was achieved in treatment with a dose of 0.6 mg/L, namely 78.89% and gave the highest degree of growth compared to other treatments with a weight gain of 151029 % and SGR for body weight was 12.20, while length gain reached 964.67% and total length SGR reached 3.94.and also histological tests on gonad tissue samples did not reveal any hermaphrodite individuals.

Acknowledgement

The authors wish to thank all the staff from National Broodstock Center for Shrimp and Mollusc Karangasem, Bali and Faculty of Fisheries and Marine Science for all the help which made this study possible.

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