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Stimulation of Ovarian Maturation Using Serotonin (5-Hydroxytryptamine) Hormone on Banana Shrimp, *Fenneropenaeus merguiensis* (De Man, 1888)

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Abstract: This study was conducted in order to investigate the potential of using serotonin, 5-hydroxytryptamine (5-HT) hormone to induce ovarian maturation on *F. merguiensis*. Control group consisted of two subgroups: a normal group and an eyestalk ablation group. 5-HT group consisted of two subgroups; one group treated with 20 µg hormones per gram body weight (5-HT20) and another group treated with 40 µg hormones per gram body weight (5-HT40). 100 µl of 5-HT solution was injected at first abdominal somite of shrimp at Day 1 and Day 11. After 20 days, the ovarian stage of shrimp was determined by physical observation using transmission light method and by histological analysis, conducted for further confirmation of ovarian stages. Oocytes diameter was measured under advance research microscope (Nikon Eclipse 80i) using NIS-Elements D 2.30 computer software. The mean size of oocytes was analyzed using One-Way ANOVA and Tukey Test at $\alpha = 0.05$. Results show that there is a significant difference between oocyte diameter of 5-HT treatment group and control group and no significant difference between both concentrations of 5-HT used. However, signal of maturation wasobserved from the 5-HT40 group (histological analysis) in 20 days experiment. This experiment suggests that 5-HT can induce faster maturation in *F. merguiensis;* however a further study mus be conducted to find the effect of 5-HT to the seed quantity and quality of *F. merguiensis*.

Key words: *Fenneropenaeus merguiensis* · Oocyte · Ovarian maturation · Reproductive biology · Serotonin hormone

INTRODUCTION

The rapid growth in the production of shrimp has been driven by globalizing trade and favorable economics of larger scale intensive farming. Besides, limited and inconsistent availability of wild shrimp postlarvae together with an urgent need of establishing selective breeding programs has increased the interest in captive reproduction of penaeids shrimp [1].

In this study, a neurotransmitter hormone (Serotonin) was used to stimulate the ovarian maturation of banana shrimp, *Fenneropenaeus merguiensis,* which was assumed important mainly due to its large size and export value [2]. According to [3], *F. merguiensis* is a good candidate for prawn farming and domestication or selective breeding program. Therefore, further research on the reproductive biology of this species is needed in order to assist hatchery production.

Until now, the production of shrimp and prawn livestock cannot still fulfill the global demand that is increasing. Therefore there is an urgent need to find a reliable technique to apply in order to boost up the production of shrimp. Various techniques have been developed in order to speed up and maximize the larval production of shrimp, such as eyestalk ablation and artificial insemination. However eyestalk ablation technique has caused deteriorations of spawners, eggs and larval quality and quantity over time [4]. Moreover, eyestalk ablation is not repeatable and sometimes causes high mortality [5] maybe due to stress during handling.

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The same problem is reported with the use of the artificial insemination method. Thus we need to develop new, safer, faster and less stressful protocols. Hormonal manipulation could be a more reliable method to apply for enhancing gonad development. Unfortunately the information of using hormone injection to manipulate penaeid broodstock maturation is not well established. Studies on the use of serotonin hormone on the banana shrimp itself is still limited [2].

In an effort to increase the production of the shrimp, it is important for us to know the proper and safe way for shrimp seed production. This could be achieved by understanding the gonadal development of shrimp itself. One best way to induce shrimp ovarian maturation is through hormone injection. According to [6], with the aim to synchronize the production of high quality and quantity of seed at a particular time, hormone stimulation through injection can be a practical alternative to eyestalk ablation. Beside, reducing shrimp stress during handling, hormone stimulation to the ovarian maturation can result in production of high quality larvae compared to eyestalk ablation. Since there is a lack of information about hormone manipulation through injection method to the banana shrimp, *F. merguiensis*, this study could improve the current knowledge that ovarian maturation can be enhanced using serotonin hormone, 5-hydroxytryptamine (5-HT) injection. Thus, the main objective of this study was to stimulate ovarian maturation of *F. merguiensis* using the serotonin hormone (5-HT). The study also investigates the effect of different dosages of 5-HT on the period of ovarian maturation in the *F. merguiensis*.

MATERIALS AND METHODS

Gravid Females: Gravid females of *F. merguiensis* were collected from estuaries of Kota Kuala Muda, Kedah, Malaysia. After that they were brought to the marine hatchery located at Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Terengganu, Malaysia. Shrimp were held into fiberglass tanks of 5 ton capacity until their first spawning in hatchery. After spawning, they were transferred into other holding tanks of 5 ton capacity and acclimatized for 2-5 days to make sure all the eggs were released and they were in healthy condition. During acclimatization, they were fed daily at 10% biomass with squid (*Loligo* sp.) and water was changed about 70% every day.

Experimental Design: 40 specimens of healthy *F. merguiensis* females, in the size group of 15-19 cm in total length (TL), with uniform ovarian condition (spent stage) were selected for the experiment. This experiment was divided into two groups which were control group represented as Group A and hormone treatment group represented as Group B (Figure 1). There were two sub groups for control group. One group is normal control group without any treatment. Another one is positive control group treated with unilateral eyestalk ablation with one of the shrimp's eyestalk ablated by cutting the eyestalk with a sterile scissor. Iodine was applied immediately after the ablation was done. After 15 to 20 minutes, shrimp were put back into their holding tank. There were also two sub groups in the hormone treatment group. Both received the same neurotransmitter hormone (5-HT), but at 2 concentrations. One group received 20 µg of 5-HT per gram body weight and the other group of 10 shrimps received 40 µg of 5-HT per gram body weight. The hormone was diluted in a crustacean physiological saline prior to experiment. Prior to experiment, every animal was sampled and weighed. They were then put into a holding aquarium receiving aeration. Each shrimp received 100 µl of hormone solution and injection was done by using a sterile 1 ml syringe. The hormone was injected at first abdominal somite to ensure the hormone is properly injected into the shrimp body. Iodine was swapped onto the injection area immediately after injection to avoid contamination or entering of any pathogenic agent. After about 15 to 20 minutes, shrimp were put back into a rearing tank. Compared to eyestalk ablation, injection of hormone was applied twice during 20 days experiment, on the first day (Day 1) and on Day 11. This is to optimize the stimulation effect of hormone on ovarian development in the shrimp. Animals were reared in a rectangle, fiberglass rearing tank (5,000 L capacity) placed at the dark area to avoid direct illumination of sunlight during the daylight.

Shrimps were reared in separate fiberglass rearing tanks (5,000 L capacity) according to different treatment. Each tank contains 10 animals, receiving an adequate supply of oxygen. They were fed with a high protein diet, squid at about 10% of body weight (BW) everyday. About 70% of water was changed everyday. Water quality was monitored by taking data on temperature, salinity, pH and dissolve oxygen (DO) using YSI meter.

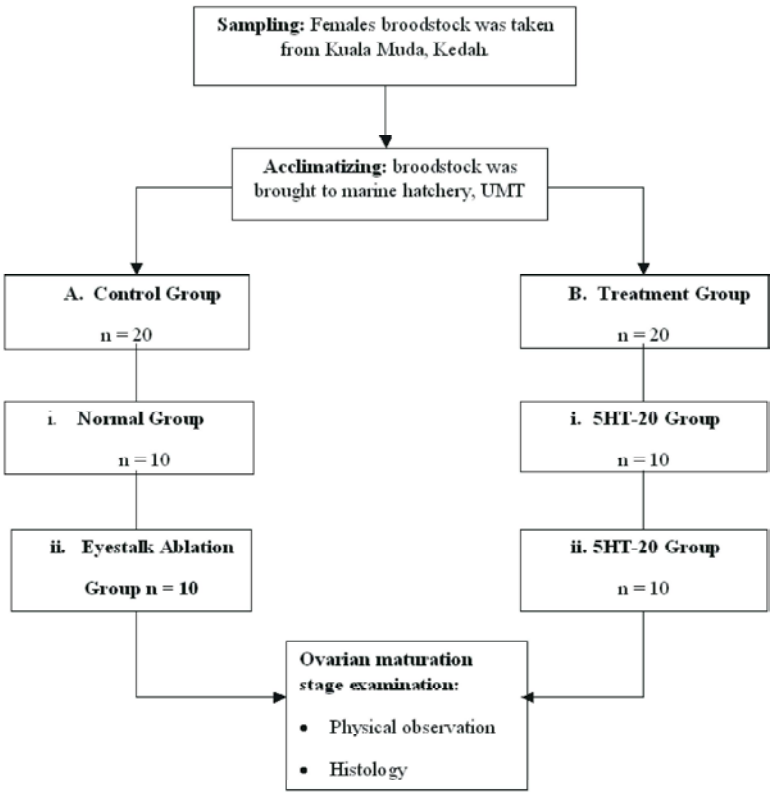

Fig. 1: Flow chart of experimental design used in the study

At the first day of experiment, all the broodstock were treated with different treatment for each group. After that the development of ovary was observed physically every five days till twenty days study period by exposing the shrimp on the beam light either by sunlight or torchlight to collect the information on the performance of shrimp ovarian development. To avoid sacrificing the sample until the last day of treatment, within development of ovary was observed only physically based on characteristic described by [7]. At the end of the 20 days study period also, the shrimp ovarian development stages was further confirmed using histology technique.

Histology: Histology method was referred to [8] with slight modification. Immediately after weight measurement, the ovaries were fixed into Bouin's solution prepared into different sampling bottle for each shrimp. The proportion of ovary sample to Bouin's solution is 1:10. After 21 hours, the samples were taken out and cut for about 2.5 centimeter (cm) each. The sub samples of ovary was then put into a histology cassette and immediately transferred into 70 % alcohol to eliminated excessive colour of Bouin's on the ovary. After that the sample were brought to laboratory for tissue processing.

Tissue processing was take place in an automated tissue processor. The aim of tissue processing is to infiltrate the fixed tissue specimen with a medium that can give enough support to allow thin sections to be cut from that tissue. The ovary sample was going through the dehydration, clearing and impregnation step with series of alcohol, xylene and paraffin wax during this tissue processing. Tissue processing period time is about 19 to 24 hours.

Ovary tissue that was processed was put into a warming tank of the embedding machine. Using a forceps, the tissue was taken out from the cassette and put into a steel mould filled with molten paraffin wax. The cassette was put on the mould and paraffin wax was added until it reached three quarter of the cassette. After that, the mould was put onto a cold plate until the paraffin wax become solid. The wax block was trimmed to remove surplus wax above the tissue and exposed the complete surface area of the specimen.

The ovary tissue block from previous step then was sectioning (cut) with the thickness of 4-5 µm using rotary microtome. The ribbons formed during sectioning were taken carefully from the microtome and put onto a clean slide. Several drops of alcohol were pipette slowly just

under the ribbons to avoid attachment of the ribbons to the slide. After that a few drops of hot water (40°C) was pipette under the ribbons so the ribbons slowly goes into the water bath containing hot water. This process is called floating. After the tissue spread well, a new frosted glass slide was used to collect (fishing) the ribbons from hot water. The slide was labeled and put upright on a wooden stand to allow slide to drain completely following by further drying on the hot plate (60°C) overnight.

In the next day, the dried slide was stained using haematoxylin and eosin stain. The slides were arranged properly in the slide rack and put into a first staining jar containing xylene. The slides were immersed for three minutes and taken out and put back into second staining jar contain xylene solution again. The purpose of immersed in xylene is to detach the wax from the tissue and slides. Xylene cannot mix with water therefore the slides were taken out again and put into series of alcohol absolute alcohol (2 times), 95% alcohol, 90% alcohol and 70% alcohol. Each step takes time for about 3 and half minutes.

The slides then were washed through the slow running water for 1 minute and half before immersed into hematoxylin stain for 14 minutes. Next the slides were taken out again and put into a container pass through the running tap water. The slides then were dipped fast 3 times into another staining jar containing 1% of acid alcohol. This acid alcohol was used to reduce the dark colour of hematoxylin. The slides were put into 2 % of potassium acetate after washing under the running tap water. After 3 minutes, the slides were washed again and immersed into a second stain, eosin. Eosin colour had cover the part in which did not stained by hematoxylin earlier. The differential colour allows us to see the shape of cell in the ovary. After 3 minutes, the slides were washed for the last time and left to air dried completely. The last step performed was mounting where the slides were put in a xylene for 1 minute before mounting using DPX. The slides then were left overnight before observed under an advanced microscope.

Data Analysis: The data taken by physical observation of the external appearance of ovary was collected every 5 days at Day 5, Day 10, Day 15 and Day 20 based on the characteristic described by [7]. Slides from histology at Day 20 were observed under advanced microscope (Nikon ECLIPSE 80i) and images were taken using NIS-Elements D 2.30 Software. Types of oocyte present in the ovary determined the stage. 30 oocyte diameters were measured and presented as mean before analyzed using one-way ANOVA and significance different between treatments used was determined.

RESULTS AND DISCUSSION

Ovarian Development: In this study, the highest ovarian development stage observed is stage III (nearly mature ovary). Some oocytes stage III shows the sign to further developed to stage IV. To confirm the stage observed done by illumination light of physical observation, histological analysis were done. Oocytes diameter calculated was used as a parameter for analysis of any significance difference by statistical analysis.

Physical Observation: Data of physical observation of the ovarian development stage using a beam light (light illumination method) was showed in Table 1. Figure 2 represent example of the characteristic of ovary from stage I to stage III that have been observed in study using this light illumination method.

Stage I ovary is white translucent in colour therefore it is difficult to distinguish it through the carapace. The abdominal region of this gonad is reduced and usually does not extend further than third abdominal somite. The ovary in stage II (developing) filling some of the abdominal cavity, the ovary is clearly better developed when compared to stage I. Two longitudinal and parallel lobes are observed along the abdominal portion of the gonad. Now the ovary can be observed through carapace by a thin band.

Stage III ovary represent by thick band, dark green in colour which filling most part of the abdominal cavity. In the cephalothorax region, the ovary covers part of stomach. The ovary is easily to seen by naked eyes.

Histological Observation: Further observation based on histological technique was done to confirmed the stage of ovarian development obtained in every individual shrimps (Table 2). Three different stages were observed, named stage I (immature), stage II (developing), stage III (ripe) and stage IV (spent). Figure 3 to 8 show the cross section (5 μm) of the *F. merguiensis* ovary for different stage obtained from this study. 30 oocytes diameter from every shrimps was measured and data of mean oocytes diameter (Table 3) was analyzed using one way ANOVA (analysis of variance).

Table 1: Numbers of individual shrimp in different treatment groups at different stage of ovarian development based on physical observation (external appearance) using illumination light after 20 days of the experimental period

	Stage of Ovarian Development			
Stage of Ovarian Development	Stage I	Stage II	Stage III	Stage IV
Control (non treated)	10	0	0	0
Eye-stalk ablation	8	2	0	0
5-HT20	0	7	3	0
5-HT40	0	6	4	0

Table 2: Numbers of individual shrimp in different treatment groups at different stage of ovarian development based on histological observation after 20 days of the experiment

	Stage of Ovarian Development			
Stage of Ovarian Development	Stage I	Stage II	Stage III	Stage IV
Control (non treated)	10	0	0	0
Eye-stalk ablation	8	2	0	0
5-HT20	0	7	3	0
5-HT40	0	6	4	0

Table 3: Mean oocytes diameter of ovary in different treatment and control group of *F. merguiensis*

Treatment	Oocyte diameter ± sd (µM)	No. of Samples
Control (non-treated)	34.742±6.730	10
Eye-stalk ablation	40.981±23.66	10
20-5-HT	103.921±30.50	10
40-5-HT	115.173±42.63	10

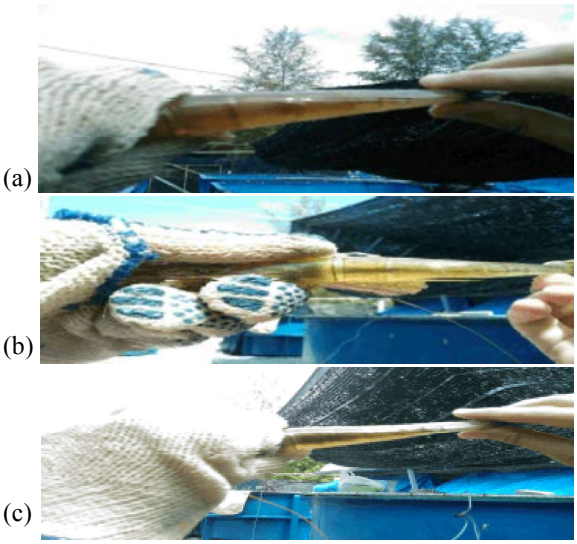

Fig. 2: A) The external appearance of stage I (immature) ovary showing clear dorsal surface. The ovary is not easily to see using naked eyes; B) Stage II (developed) ovary. The ovary appeared as a thin dense midline from the cephalothorax region to the third body segment; C) Stage III (nearly ripe ovary). Shadows of ovary appeared as a light green colour with a thick band due to early accumulation of yolk in the ovary.

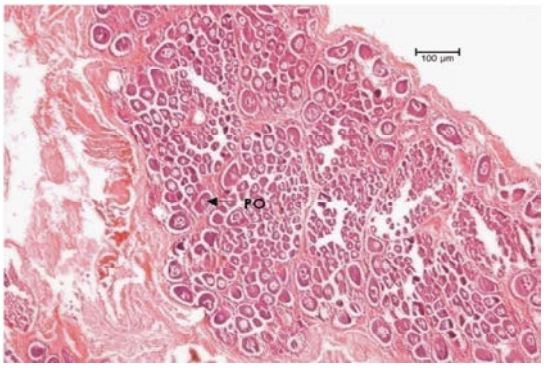

Fig. 3: Predominance of perinucleolar oocytes (PO) in the stage I ovary

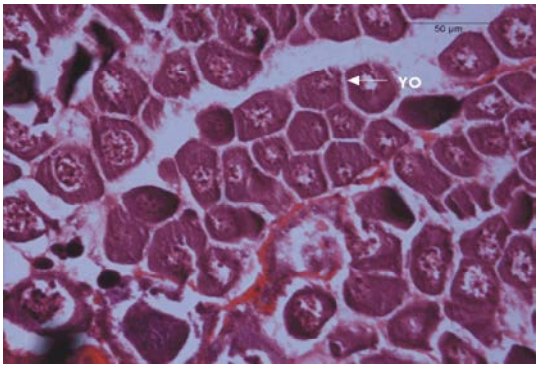

Fig. 4: Stage II ovaries showing abundant yolkless oocyte (YO)

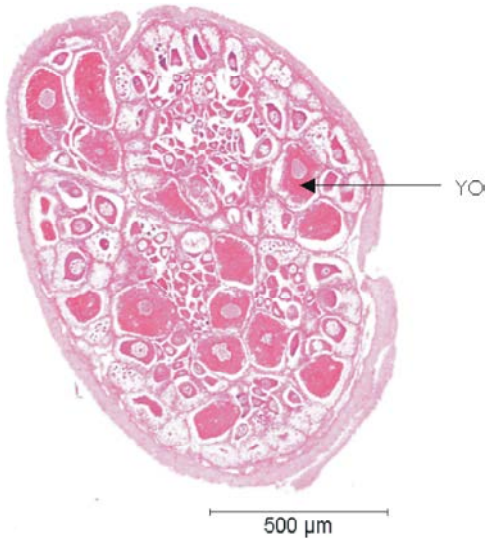

Fig. 5: Stage III ovary with presence of yolky oocytes (YO). Size range from about 100 µm to 200 µm in diameter

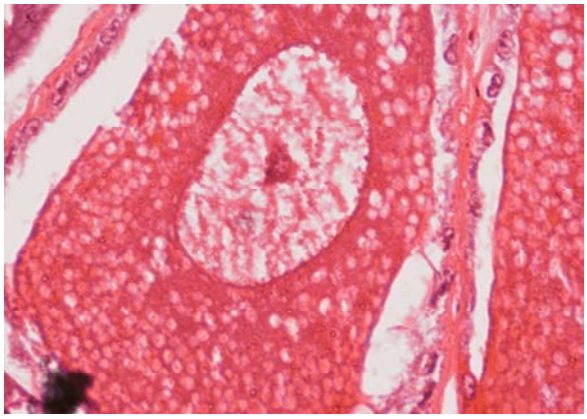

Fig. 6: Yolky oocytes in ovary stage III. (100x magnification)

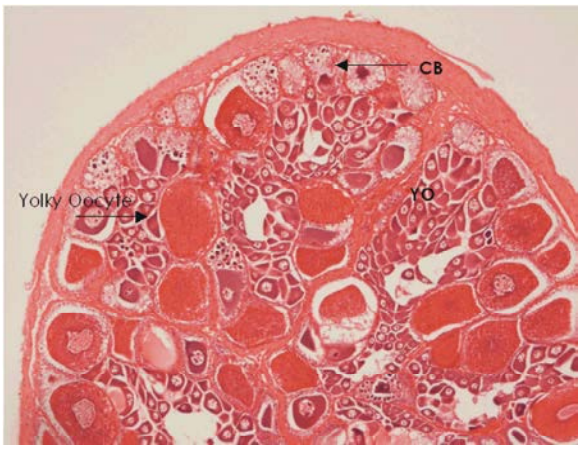

Fig. 7: Later stage III with presence of oocytes with corticol bodies (CB) (10x magnification)

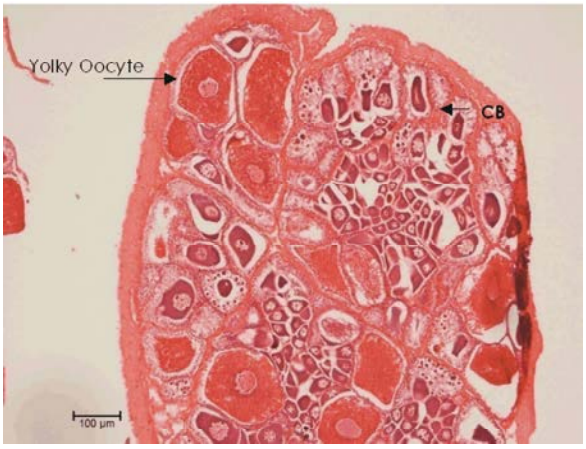

Fig. 8: Late stage III ovary (10x magnification)

In Table 2, mean of control group ovaries show that most of individual ovary in this group is in stage I, same with the mean reading of eyestalk ablation group. For hormone treatment group, size of oocytes show that most of ovaries of shrimp in this group fall into stage II to stage III. A Table 4 show result obtains from one-way ANOVA analysis is there is significance different between all experimental group.

Further analysis using Tukey test (Table 5) showed that there is significance difference between oocytes size in hormone group and eye-stalk group and between hormone groups with control group. In the other hand, there is no significance different between hormones in different concentration.

This study suggests that 5-HT promote ovarian maturation in *F. merguiensis*. Eventhough there is no stage IV ovary achieved by individual shrimp; observation on the histological work determined the presence of corticol rod in on some oocytes from stage III ovary. This indicates that 5-HT triggered the maturation of ovary in this experiment. This was supported by statistical analysis that oocytes sizes in the hormone treatment group have a significance difference with control group. In this study, stage of ovarian development was verified by two methods. First is illumination light method based on ovarian shadows appearing at the shrimp exoskeleton reflight to the light transmission. The ovarian stage also was study by using histological method as mentioned earlier. This method was established and has been widely used for a long time in hatchery work. Study by [7] supported this histological method is a reliable technique to determine ovarian stage without sacrifice the animal.

Table 4: Results shows statistical analysis on mean of oocytes diameter in different treatment. P value is greater than α value such as in red box showed significance difference between treatments

Source of variance	SS	df	MS	F	P	Ho
Treatment	52215.77223	3	17405.26	20.76452	5.55126E-08	Reject
Residual	30175.95215	36	838.2209			
Total	82391.72438	39				

Confidence level = 95%, α = 0.05

There is significant difference if P value > α value

Table 5: Tukey test analysis. Red box showed when value for calculated q is greater than value of q in table, there is significance different in treatment and therefore H0 is rejected

Treatment	D	Q-cal	Q-table	Ho
5-HT40 vs Control	80.431	8.78504979 >	3.809	Reject
5-HT40 vs Eyestalk Ablation	74.192	8.103597051 >	3.457	Reject
5-HT40 vs 5-HT20	11.252	1.2289966038 >	2.868	Accept
5-HT20 vs Control	69.179	7.556053758 >	3.457	Reject
5-HT20 vs Eyestalk Ablation	62.940	6.874601014 >	2.868	Reject
Eyestalk Ablation vs Control	6.239	0.681452744 >	2.868	Accept

Ho = is rejected when q-cal > q-table, means there are significance difference between the two treatment

In the present study, only portion from middle lobe of ovary were removed for histology since oocytes are reported to be distributed homogenously among all lobes in the ovaries of penaeid shrimp [9]. Based on the present study, we can see in the stage I ovary, there are two types of oocytes present which are perinucleolus oocyte and chromatin nucleolar oocytes. With a dominance presence of perinucleolar oocytes, we can consider the ovary as stage I. Stage I oocytes is abundant in control group and none in the hormone treatment group. In stage II oocytes, abundant of yolkless oocytes size range from about 65 µm to 125 µm appears [10]. This oocytes can be known with colouration of hematoxylin attach to it cytoplasm means this oocytes is basophilic [11]. Perinucleolar oocytes significantly increase in size (100 µm to 200 µm) and becomes as yolky oocytes which is abundant in stage III ovary. These yolky oocytes are sign of early vitellogenesis and it indicates by eosin stained oocytes in histological sections. Perinucleolar oocytes also presents but in the small number. Late stage III (early mature) oocytes can be denoted by presence of some oocytes with cortical body such as shown in the results.

Previous study by [2] also confirm that 5-HT stimulate the ovarian maturation in *F. merguiensis*. In their 15 days experiment, concentration 15 µg/g body weight 5-HT was used to induce ovarian maturation and at the end of the experiment, their shrimp can develop until late stage II. This may indicates that used of higher concentration (20 µm and 40µm) can resulting in better performance. In the control group, the ovary can reach early stage II in 20 days. Early or faster development can be seen in eye-stalk ablation group. Either their ovaries have been reabsorbed or they have matured and starting a new cycle, they show faster development compared to the other group. Eventhough there is no significance difference between different hormone concentration group, the study suggest that 40 µg/g per shrimp body weight maybe have a better performance however further study need to be conducted to investigated this matter.

In other study, [12] dsigned a method to develop a reliable technique for inducing ovarian maturation and spawning in *L. stylirostris* and *L. vannamei* as an alternative to the traditional and destructive eyestalk ablation. After 51 days experiment they suggested that the combined injection of 5-HT and spiperone not only induces maturation and spawning, but also stimulates the release of maturation promoting pheromones into the water, resulting in maturation of non injected females in the same tank at the same rate of injected females compared to non injected females in another tank (external control). This finding that 5-HT gives a better performance compared to eyestalk ablation was supported by many authors. In a study conducted by [13] an advantage of 5-HT injection over eye-stalk ablation is the higher hatching rate, resulting in higher production of *P. monodon* nauplii. Recently, similar results were reported for *Fenneropenaeus indicus* [14]. The reason behind this may be due to the fact that eyestalk ablation is a procedure that destroys the optic lobe and could disrupt several physiological processes besides reproduction, whereas 5-HT injection is a much less invasive and more specific procedure.

Since the complexity of most biological problems to obtain oocytes and oocytes for the required artificial amplification and is defined manufacturing processes more oocytes consider the knowledge of biology and ovarian development is especially important [15]. There a several factors affecting ovarian maturation of a broodstock such as size of broodstock, broodstock nutrition, environmental changes and stress factor. The broodstock used in this study is a gravid females (15 cm to 19 cm) total length that allowed spawning first in the hatchery so that they can start a new ovarian development cycle. From the personal observation, bigger broodstock had a little faster development compared to smaller broodstock. However, oocyte size was not affected by body weight [16]. In other study, investigations were made on the ovarian maturation of eyestalk ablated shrimp maintained under different environmental conditions. The results obtained indicated no signs of maturation when females of size smaller were ablated. Larger ablated females showed a delay in maturation during the colder months in contrast to those ablated during the warmer months [8].

In addition to size, broodstock nutrition is also an important element to be considered. In period of ovarian development, sufficient nutrients need to be accumulated into the egg yolk to sustain the normal development of the embryos and pre-feeding larvae. Unbalanced or incomplete diet can causes poor reproductive performance or may even stop animals from reproducing. Physiological stresses from the malnutrition could trigger oocyte resorption or reduce reproductive fitness of the broodstock. Additionally, mineral malnutrition could also cause altered composition and quality of the eggs [1]. Fresh or fresh-frozen marine organisms are often used for acceptable maturation and reproduction outputs. These marine organisms are found to give the best results when they are in a reproductive stage. Squid and bivalves, mussel and oyster are generally the main food items, fed at high daily ratios. Crustaceans like shrimp, crab and krill are also fed to shrimp spawners, but due to the risk of disease transmission, they are used less frequently nowadays [17]. In this study squid have been given to feed experimental animal due to the fact that this mollusk have better nutritional value. Furthermore, squid also have been commonly used as part of the maturation diet and a steroid-like compound found in this mollusk and it has been proposed to be responsible for its enhancing effect on maturation in shrimp [17]. According to [1] squid have 50 percent protein and 11 percent of lipid concentration which fulfill the nutrient requirement of *F. merguiensis* which is about 34 percent to 50 percent. Protein requirement of shrimp is about 34 percent to 50 percent [18]. However, during rainy day, fresh, healthy squid cannot be obtained from any market so that incomplete nutrient content in the squid maybe loss by the time it was feed to the shrimp.

Studies indicate that a reproductive response is also produced through a relative interaction of environmental factors such as temperature, light, salinity and endogenous factors of an organism. [8] mentioned in their report that a system incorporating treatment, high salinity, good water quality, reduced light intensity and optimum temperature are sufficient to induce maturation in females above the length of first maturity of *F. merguiensis* in captivity [8]. [3] reported that temperature and light intensity give strong effect to the maturation of shrimp followed by photoperiod. In this study, water quality parameter falls into a suitable range for rearing *F. merguiensis*. The temperature measured was around 27°C and 28°C, salinity was maintained around 27 to 29 ppt, water was not change during rainy day to avoid fall of salinity level and pH is about 8.2 to 9.0. On the other hand, [19] stated that tank size, substrate type and shrimp population density have also been shown to affect maturation and spawning of penaeids.

Stress factor may become a possible reason to the resorbtion occur such as in eyestalk ablation group. Stress may be due to the sampling, during water exchange and also either frequent human disturbance or any other predator that enter their rearing tank. This can be seen when about first 10 days they brought to the hatchery, they become very active to the food given which they catch it as soon as the food enter the water. Eventhough water exchange is in process they still hold to the food and feed peacefully. However in later day, they become slightly inactive to the food given when they act a little slow before start response to the food given.

Overall, since there is significance different by injection of serotonin hormone compare to control, this technique may become a good attempt for inducing ovarian maturation and spawning in penaeid shrimp since it first attempt by [6] supported by any other research discussed earlier.

In this study, application of 5-HT have significant effect on the ovarian development of *F. merguiensis* by inducing the ovarian development to faster develops compared to control group. The study shows the role of 5-HT has a promising future in the shrimp culture

industry. However, a further study is required especially at a molecular level to investigate other matters like the effect of serotonin on brooders health and effect to the offspring so on before we can established that this technique fulfill the sustainable aquaculture requirement. Further study need to be conducted to find the optimum dosage of 5-HT should be applied in order to get full ovarian maturation of *F. merguiensis* within shorter period of time. We would like to recommend that higher dosage or frequence application should be tried. Combination treatment between hormone attempted and manipulation of environmental factors in a free pathogen condition also should be put in consideration. From this study, it may be concluded that the application of 5-HT have significant effect on the ovarian development of the banana shrimp, *F. merguiensis* by inducing the ovarian development of *F. merguiensis* to faster develops compared to control group. The role of 5-HT as discussed in another study also give promising future in the shrimp culture industry. However, further study is required especially at a molecular level to investigate other matter like the effect of serotonin on consumer health and so on before we can established that this technique fulfill the sustainable aquaculture requirement.

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