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INDUCTION OF OVARIAN DEVELOPMENT AND SEX DIFFERENTIATION IN THE GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii*, BY SEROTONIN, METHYL FARNESOATE, AND PHYTOECDYSONE.

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ABSTRACT

Serotonin (5-HT) and methyl farnesoate (MF) are neurohormones that play important roles in stimulating somatic and gonadal development in decapod crustacea. These agents also accelerate molting that is associated with the reproductive cycle. In addition, ecdysteroids has been reported to regulate molting and concurrent female reproduction, but the stage-specific effects of ecdysteroids on both molting and reproduction during a single molting cycle have not yet been determined for any crustacean. Our study was performed in the giant fresh water prawn, *Macrobrachium rosenbergii*, after randomly dividing sexually mature female prawns into six groups immediately following ovarian stage I. The groups were: (1) a non-injected normal control group (NC); (2) and (3) control groups injected intramuscularly with crustacean physiological saline (VC1), or CPS plus 5 % ethanol (VC2), respectively; (4) and (5) groups injected with 5-HT or MF at a dose of 0.1 μg/g BW, respectively; and (6) a group injected with phytoecdysone (PE) at a dose of 0.05 μg/g BW. The injections were repeated every five days until spawning. It was found that, the ovarian maturation period (OMP) of the three hormone-treated groups was significantly shortened comparing with the controls. They were 23 ± 3.4, 19 ± 6.6, and 21 ± 10.3 days, respectively, while the OMPs of NC, VC1, VC2 groups were 32 ± 3.4, 31 ± 3.7, and 31 ± 3.7 days, respectively. Average duration of embryonic development period (EDP) from spawn to hatching of each group was not significantly different to the controls. Furthermore, the offspring of the 5-HT- treated group exhibited increased male to female sex ratio at 1.1: 1, while MF and PE treated group promote sex ratio at 1:1. These findings demonstrated that, 5-HT, MF and PE could induce ovarian development in giant freshwater prawn, *M. rosenbergii.* The vitellogenin (Vg) and vitellin (Vt) content during ovarian development in female brood stock during reproductive activity is also discussed.

Keywords: *Macrobrachium rosenbergii,* serotonin, methyl farnesoate, phytoecdysone, ovarian development, sex differentiation, molting

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INTRODUCTION

At present, the giant freshwater prawn, *Macrobrachium rosenbergii*, is an important crustacean cultured in Thailand and other Asian countries such as India, Vietnam, and China (Office of Agriculture Economics, 2013). Nowadays, there are two sources of freshwater prawn production, namely natural collection from rivers, lakes, and reservoirs, and from aquaculture. Consumers demand large sized prawns which are mostly collected from natural sources. However, prawns collected from nature are inadequate in consumer credentials, thus there is a growing demand for animals produced by aquaculture. Thus, aquaculture methods should be developed to fulfill the demand.

In Thailand, the popular giant prawn is *M. rosenbergii.* Generally, male prawn of this species is larger than female at the same age due to physiological factors during culture, i.e., females use many nutrients during gonadal growth and ovarian development (Holthuis, 1980). The larger size of male causes culturists to concentrate on growing male prawns rather than females. Currently, there are several techniques to produce all-male offspring, such as removing the androgenic gland (AG) in the crayfish, *Cherex quadricarinatus* (Sànchez and Lòpez, 2010), and *M. rosenbergii* (Alfalo et al., 2006). An all-male culture yielded 473g/m pond within 150 days, whereas all female and mixed populations produced 248 and 260 g/m pond, respectively (Sagi and Alfaro, 2005). However, this technique is accomplished by manual segregation of juveniles resulting in the need for extremely skilled labor.

The complex interactions between several neuroendocrine and endocrine organs play a key role in controlling gonadal and secondary sexual characteristics development in both male and female crustaceans. Several neurotransmitters affect the release of reproductive hormones in crustaceans, such as serotonin (5-hydroxytryptamine; 5-HT) (Hirai et al., 1988; Meerattana et al., 2006; Tinikul et al., 2008), methyl farnesoate (MF) (Olmstead and Le Blanc, 2001; 2003; Laufer et al., 1998; Laufer et al., 2005). These agents also accelerate the molting following the gonadal maturation. In addition, Lachaise et al. (1981), Lachaise and Hoffmann (1982); Okumura et al. (1992) have reported that ecdysteroid is an important hormone controlling molting and concurrent female reproduction but its function is influenced by during a single molting cycle.

Hence, this research was focused on hormonal manipulation efficiency for inducing the brood stock female of *M. rosenbergii* to give all male offspring prawn. In addition, ovarian development of this female brood stock was also studied.

MATERIALS AND METHODS

Experimental animals and determination of ovarian stage

Adult males and females *M. rosenbergii* were obtained from the Bangpakong River. They were stocked in the same tank with the ratio of blue-claw males and females at 1:5. The prawns were acclimated for two weeks, during which female prawns that developed to ovarian stage I of the ovarian cycle, and some at stage IV, were collected for experiments. Stages of ovarian development were based on the criteria classified by Meeratana and Sobhon (2007).

Solutions of the test chemicals were prepared, as follows. First, 5-HT was dissolved in crustacean physiology saline (CPS; NaCl 29 g, KCl 0.71 g, $CaCl_2 2H_2O$ 2.38 g, $MgSO_4 2H_2O$ 3.16 g, $NaHCO_3$ 0.5 g, $MgCl_2.6H_2O$ 0.17g, HEPES 4.76 g in 1 L of distilled water). MF and PE were dissolved in ethanol plus CPS (5% V/V).

The female prawns at ovarian stage I were randomly divided into six groups. They were identified by different colors of plastic beads tying on the eyestalk. The groups included: (1) a non-injected normal control group (NC); (2) and (3) control groups injected intramuscularly with crustacean physiological saline (VC1), or CPS plus 5 % ethanol (VC2), respectively; (4) and (5) groups injected with 5-HT or MF at a dose of 0.1 μg/g BW, respectively; and (6) a group injected with phytoecdysone (PE) at a dose of 0.05 μg/g BW. The injections were repeated every five days until spawning.

Effects of 5-HT, MF, and PE on Vg levels

The hemolymph of treated and control prawns

were collected from the substernal hemolymph sinus every four days. A 100 µl sample of hemolymph was mixed with 100 µl of anticoagulant (containing 7.94 g sodium citrate, 19.64 g NaCl, 3.35 g EDTA in distilled water). The sample was then centrifuged at 10,000 x g at 4 ˚C for 10 min. The supernatants were collected and then stored at -20 ˚C until assayed.

Purification and characterization of vitellin (Vn) from the giant freshwater prawn *M. rosenbergii*

Stage IV ovaries were extracted and homogenized (35% w/v) in 50 mM Tris-HCl, pH 7.4; containing 0.1 mM PMSF, 0.5 mM DTT, and 150 mM NaCl, in a Teflon homogenizer. The homogenate was centrifuged at 15,000 xg for 90 min at 4 ˚C. The supernatant yielding was prepared for determined Vn by Bradford method (1987) as follows. Next, 300 ml of the supernatant was loaded onto a column of hydroxylapatite (10 x 1.6 cm.) and separated using a stepwise gradient of potassium phosphate buffer 0.1, 0.4, and 1.2 M, pH 6.8. Fractions of 1.5 ml. were collected at a flow rate of 1 ml/min, and then individually measured for protein concentration at 595 nm in a spectrophotometer.

The fraction containing vitellin from hydroxylapatite column was purified by using sephacryl S-300 column (30 x 1.5 cm). The sephacryl column was equilibrated with Tris-HCl buffer, pH 8 at room temperature. Aliquots of each fraction obtained from the elution step were separated by 10% SDS-PAGE. Then determine an apparent molecular mass by passing the purified vitellin through a sephacryl S-300. The molecular mass of the vitellin was calculated by comparing the distribution coefficient to standard proteins.

Protein concentrations were determined using the Bradford reagent method (Bradford, 1987) against a bovine serum albumin curve. A 10 ml sample protein was added in each well and then filled with 0.2 ml of dye reagent. Next, the solution was mixed and left for 5-30 min at room temperature. The protein concentration of each solution was determined using absorbance of wavelength at 595 nm.

ELISA assay

Vitellogenin levels determination were performed by using an indirect ELISA. In brief, approximately 10 µg/ml hemolymph was prepared in 10 mM sodium phosphate (pH 7.0). Then, 50 µl of protein solution was added to each well and incubated overnight at 4 ˚C. The plate was washed with PBS three times. A 150 µl aliquot of the blocking buffer was added and the plate incubated for 1 h, after which it was washed three times with PBS. Then, 50 µl of polyclonal antibody against Vn (anti-Vn) was added and the plate incubated overnight at 4 ˚C. The plate was washed three times with PBS. A 50 µl aliquot of GAM-HRP (diluted 1:1,000 in PBS) was added to each well and the plate incubated overnight at 4 ˚C. The plate was washed three times with PBS. A 100 µl aliquot of OPD and 0.006 % H_2O_2 was added in 0.1 M citrate buffer to each well and the plate incubated for 5 min. Then, 50 µl 1N H_2SO_4 was added to every well to stop the reactions. For binding quantization, the results were read at 490 nm absorbance.

Histological observations

Ovaries, i.e., stages I, II, III, and IV of the control group, 5-HT, MF, and PE prawn group were cut into small pieces and fixed in Davidson's fixative for histological observation. Tissues were dehydrated in a graded series of ethanol, i.e., 50%, 70%, 80%, 90%, and absolute ethanol, respectively. The dehydrated tissues were subsequently cleared with xylene, infiltrated, and embedded in paraffin. The histological sections were cut into a 5-7 µm thickness using a rotary microtome. The sections were stained with Harris's Hematoxylin and counter-stained with Eosin.

Comparison of growth rate and sex differentiation in *M. rosenbergii* offspring from treated parents and untreated parents.

Five thousand post larvae from each hormonal injected female (10 animals/L) were randomly sampled and reared in 0.75 m diameter concrete pond for 60 days. The survival rate, growth rate, and sex ratio were determined at the end of the reared date. The sex was examined by observing the presence of male sex structures, namely, the

appendix musculini on the medial site of endopodite at distal end of the second pair pleopods. The examination was identified by cutting off the second pair pleopods and observing the structures under stereomicroscope.

Statistical analyses

Data were presented in the forms of mean ± SD. The experimental data were analyzed using a SPSS program using a one-way analysis of variance (ANOVA) and Duncan's New Multiple Range Test. A probability value less than 0.05 ($p < 0.05$) indicated a statistical significance. Data were presented in the form of mean ± SD.

RESULTS

Effects of 5-HT, MF, and PE on ovarian development

The results showed that, at 49 days post-injection, 5-HT-injected prawns exhibited various ovarian developmental stages from stage I of ovarian maturation to spawning. The 5-HT, particularly at 0.1 μg/g body weight resulted in a decreased to a variable degree and a significant difference was recorded at 23 ± 3.4 days compared with the control (NC 32 ± 3.4, VC1 31 ± 3.7 days). The MF-injected group exhibited spawning at 19 ± 6.6 days, and the PE injected group was 21 ± 10.3 days. The OMP of the NC and VC2 prawns exhibited spawning at 32 ± 3.4 and 31 ± 3.7 days, respectively.

The ovarian development period (ODP) after at five days injected with 5-HT, MF and PE (10 ± 2 %, 18 ± 4 % and 10 ± 2 %) is shown in Fig. 1A. In the first development stage, it was found significant difference ($p < 0.05$) compared to NC, VC1, and VC2 (30 ± 2 %, 30 ± 4 %, and 30 ± 4 %), respectively.

At 10 days, after injection female prawns with 5-HT, MF, and PE was induced from stage I into stage II (8 ± 2 %, 6 ± 3%, and 4 ± 1 %); however, there was no significant difference observed ($p > 0.05$) as compared to NC group (12 ± 3 %). But there was significant difference ($p < 0.05$) when compared to VC1 and VC2 group (2 ± 2 % and 2 ± 2 %) as shown in Fig. 1B. At 15 days, after injection with MF and PE, these hormones induced ODP into stage III (6 ± 2 % and 5 ± 2 %), while there was no significant difference ($p > 0.05$) found as compared to NC group (6 ± 3 %), VC1 group (9 ± 2 %), and VC2 group (8 ± 2 %). The 5-HT treated group showed significant difference of ODP (2 ± 2 %) (Fig. 1C). At 20 days of injection with 5-HT, ODP change was 7 ± 2 % which was significant difference ($p < 0.05$) as compared to NC, VC1, VC2, MF, and PE group; 5 ± 2 %, 6 ± 2 %, 6 ± 2 %, 3 ± 2 %, and 5 ± 2 %, respectively (Fig. 1D).

Mean duration of embryonic developmental period (EDP; from spawning to hatching) of each group was 18 days, and was not significant different compared with the controls. Furthermore, an analysis of data for percentage of sex differentiation of *M. rosenbergii* among the groups also showed not significant differences in sex ratio. 5-HT 0.1 μg/g BW was shown male 51.25 ± 7.80% and female 48.75 ± 7.80%, MF 0.1 μg/g BW was shown male 47.50 ± 5.19% and female 52.50 ± 5.19%, and PE at 0.05 μg/g BW was shown male 45.25 ± 8.73% and female 54.75 ± 8.75%. While of NC was shown male 52 ± 5.71% and female 52 ± 5.71% not different with those of treatments ($p>0.05$) (Table 1).

The Vg concentrations after injected with 5-HT, MF, and PE at 0.1 μg/g BW exhibited a gradual increase from stage I to stage IV (Fig. 2). At stage I, the Vg concentrations were 1.88 ± 0.20 to 4.35 ± 0.38 μg/ml. For the stage II, Vg concentration gradually increased (between 9.51 ± 0.73 to 11.28 ± 0.83 μg/ml and ovarian stage III (16.98 ± 0.65 to 18.56 ± 0.58 μg/ml), and finally reached the highest levels (18.89 ± 0.29 to 21.28 ± 0.79 μg/ml) in stage IV. The changes in the Vg content of hemolymph were differed significantly during the ovarian stage ($P < 0.05$).

Table 1. The percentage of survival and sex differentiation of the larvae *M. rosenbergii*.

	Survival rate (%)	Sex ratio	
		Male (%)	Female (%)
NC	55[a]	48 ± 5.71	52 ± 5.71
5-HT	51[a]	51.25 ± 7.80	48.75 ± 7.80
MF5	44.66[a]	47.50 ± 5.19	52.50 ± 5.19
PE**	37**[b]	45.25 ± 8.73	54.75 ± 8.75

Survival rate n = 5,000 animals, ** n = 1,000 animals, Sex ratio n = 100 animals.

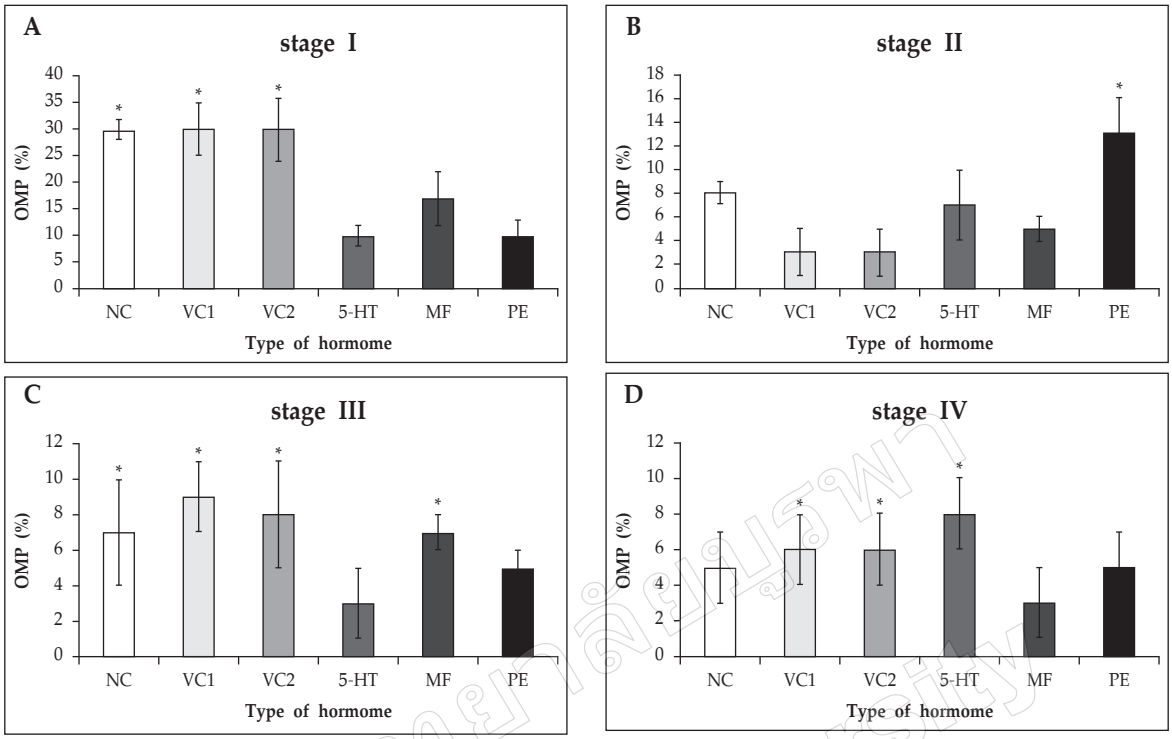

Figure 1. Percentage of ovarian development of prawn stimulating with 5-HT, MF, and PE and control groups (NC, VC1, and VC2)
* = Statistically significant difference $p < 0.05$

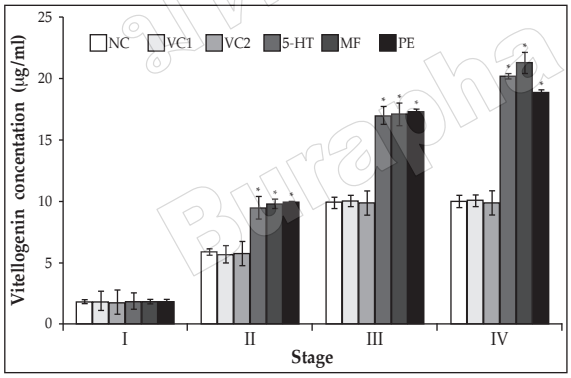

Figure 2. The changes of Vg concentration in hemolymph of *M. rosenbergii* after injections with serotonin and methyl farnesoate for 0, 5, 10, 15, 20, 25, and 30 day ($p < 0.05$)
* = Statistically significant difference $p < 0.05$

Stages of the ovarian cycle

Histologically, the ovarian cycle could be classified into five stages: stage 0 (spawn), I (spent), II (proliferative), III (premature) and IV (mature), respectively. In stage 0, the ovary immediately following spawning appears loose and empty. It contains collapsed ovarian pouches with only strands of follicular cells and connective tissue remaining. Most of the follicular cells return to their original ovoid shape. The main hemolymph vessel in the central ovarian area is still present and appears intact. However, small hemolymph sinuses are dispersed throughout ovarian tissue, but their boundaries are not clearly defined. Groups of oogonia at the central ovarian core are frequently seen. Thickening and folding of the ovarian capsule is apparent around the ovary. During stage I (spent), the ovary is filled with primary oocytes occupying the oogenic zone, and previtellogenic oocytes (Oc1 to Oc2) occupying the previtellogenic zone. Follicular cells are rarely seen at this stage of the oocytes. The hemolymph vessels and sinuses reconvert to the more intact form (Fig. 3 F). In stage II (proliferative), the ovary exhibits faint orange color. The oocytes are mainly the late previtellogenic (Oc2) and the early vitellogenesis (Oc3) steps. The division between oogenic and previtellogenic zone becomes clearly visible. Follicular

cells increase in number and become more apparent (Figs. 3A, B). In stage III (premature), the ovary is bright orange in color and increases in size. Most of the oocytes are in late vitellogenesis (Oc4). The follicular cells elongate to spindle shape and are less frequently seen (Figs. 3C, D, E). In stage IV (mature), the ovary appears in deep orange color and its size increases remarkably when compared to earlier stages. The ovary contains mostly mature oocytes (mOc), while islets of oogonia and primary oocytes are frequently seen in the central ovarian core, and other oocytes (Oc1–Oc4) are absent. While those remaining appear as thin spindle shapes closely surrounding each oocyte (Fig. 3F).

The ovarian capsule and trabeculae are very thin and the ovarian pouch boundaries are hardly discernible. The oviducts are seen on the ventro-lateral side of each ovarian lobe. Their walls continue with the extremely thin ovarian capsule (Fig. 3B).

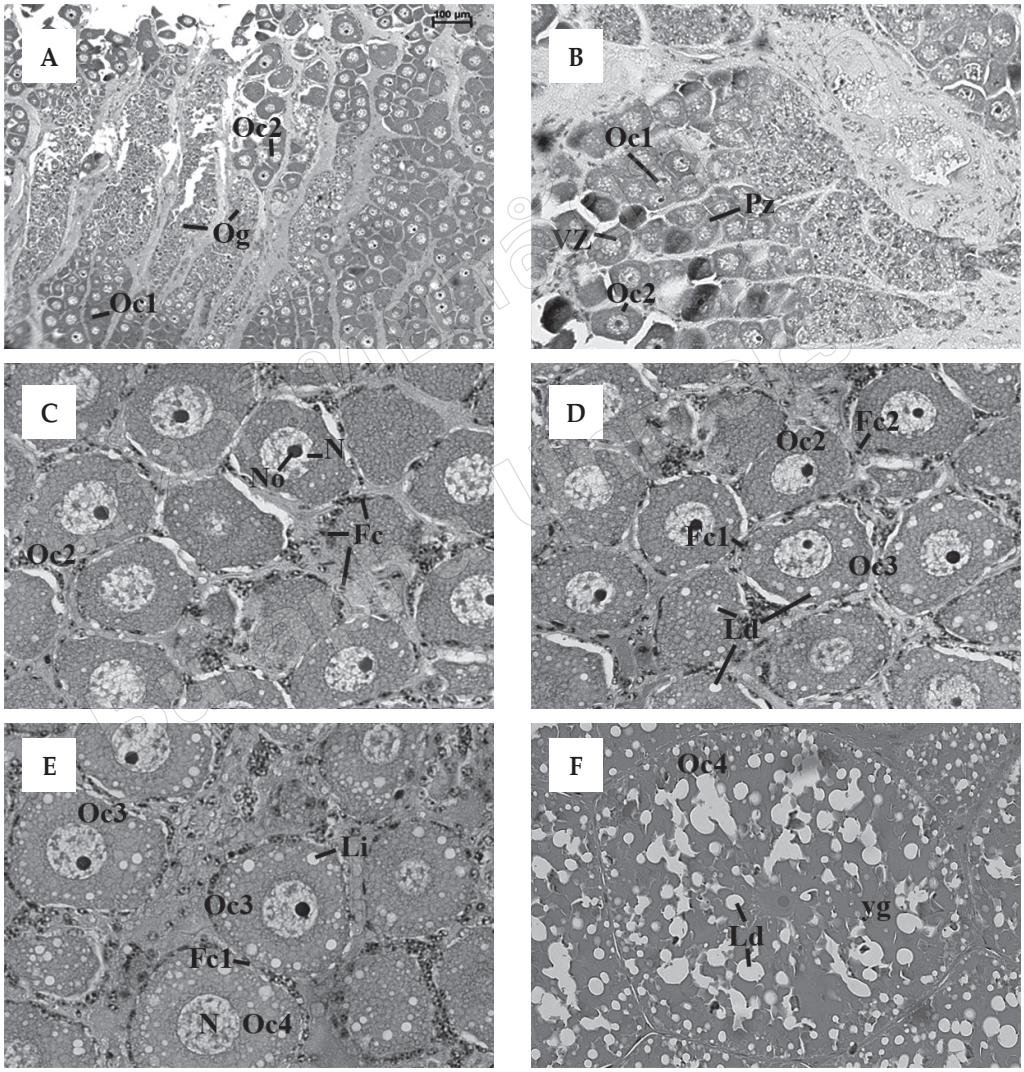

Figure 3. Light micrographs of H&E-stained ovarian sections showing histology of various stages of ovarian cycle and steps of oocyte development. A: oogonia (Oog) ; B: early previtellogenic oocyte (Oc1), oogenic zone (Oz) , previtellogenic zone (Pz) C: late previtellogenic oocytes (Oc2), nucleus (No) follicular cells (Fc); (type I and type II) ; D: early vitellogenic oocyte (Oc3); E: late vitelloginic oocytes (Oc4); F: mature oocytes (mOc), yolk granules (yg), lipid droplets (Ld).

DISCUSSION

Based on our results, it was found that the ovarian stage influenced on the effect of chemical and hormone injection, and was observed in ovarian histology using H&E stained. The ovarian stages in *M. rosenbergii* were classified to five stages (Domrongphol et al., 1991; Meerattana and Sobhon, 2007). The spawn ovary (stage 0) has enlarged in size more than spent (stage I), II, and III showed oogonia, primary oocytes and previtellogenic oocyte. The accumulation in numbers of previtellogenic oocytes (Oc1, Oc2) is the distinguishing characteristic of proliferative ovary (stage II). An increasing in size of premature (stage III) and mature (stage IV) ovaries is due to the increased numbers of vitellogenic oocyes (Oc3, Oc4, mOc), which sizes are enlarged significantly due to the uptake of yolk protein from exogenous sources. The islets of oogonia and previtellogenic oocyte among fully mature oocytes in stage IV reflect the reserve capacity of the ovary in multiple spawning species and start new cycle.

In the ovaries of prawns injected with 5-HT and MF, it was found the fastest ovarian advance was 23 days for 5-HT and 19 days for MF. Both 5-HT and MF injections at the dose of 0.1 μg/g BW daily, ovary was faster developed compared with the control group. This result is consistent with Rodriguesz et al. (2002) in which they injected the red swamp crayfish (*Procambarus clarkii*) with 10^{-8} mol of MF twice a week for three weeks. They found that ovarian development increased. Laufer et al. (1998) fed *Procambarus clarkii* with 1 and 2 μg/individual/meal with methyl farnesoate in food for 30 days. Marsden et al. (2008) fed tiger black prawn, *Penaeus monodon*, with 5.5 μg/g BW MF in food and found that it could accelerate the development of the reproductive system. In the same way, Abdu et al. (2001) fed *Cherax quadricarinatus* with 4.5 and 9 μg/individual/week with methyl farnesoate mixed in food during their winter reproductive arrest period and found that it had no effect on reproductive. In 1998, Abdu et al. fed *M. rosenbergii* during stage nine with artemia dipped 0.21, 0.35, and 0.59 μg/ml methyl farnesoate and found that the growth rate of larvae fed with low dose of methyl farnesoate was higher than those fed with high dose methyl farnesoate. While this study induced female brood stocks by injection with 0.1 m g / g BW of MF ovarian development shown significantly shorter than those of control ($p < 0.05$). In addition, this concentration affected the growth rate of post larvae at 90 days.

In the case of the PE treatment, the results showed that it could induce ovarian development in 21 days which was shorter than those of control ($p < 0.05$). Phytoecdysteroid extracted from *Vitex glabrata* was studied for its effect on molting and reproduction and found that it has a similar chemical structure to that of the crustacean molting 20-hydroxyecdysone (Werawatanamatin et al., 1986; Suksamran et al., 1998).

When prawns were injected with 0.1 μg/g BW PE every three days, they sloughed faster compared with the control group. Their shell softened and ovarian development did not progress. Thus, it was studied on the effect of lower concentration by injection with the concentration of 0.05 μg/g BW every five days, their sloughing was not different from those of control group.

The high dose of PE exhibited toxicity and may have caused the prawns to die. The presence of sloughed and persistent soft shell brood stock was observed at the dosage of 0.1 μg/g BW PE, which is consistent with the findings of Vuthiphandchai et al. (1993). They found that prawns injected with 0.125 μg/g BW ecdysone died but did not slough. However, eye stalk ablated prawns injected with 0.01 and 0.05 μg/g BW sloughed faster. Armstrong (1972) injected ecdysone in *Palaemonetes pugio* at concentrations of 0.25, 0.3, 1.0, 3.0, and 5.0 μg/g BW and found all of shrimps died within five days. Whereas the shrimp during ovarian development injected with 0.1 μg/g BW survived but did not slough, and the shrimp carrying developed ovary could lay eggs within 12 days. Pholpunthin et al. (1990) injected star-shaped kite black shrimp with 0.1 μg/g BW of β-ecdysterone and found they could slough safely. In *Penaeus merguiensis*, 0.3 μg/gBW of β-ecdysterone injection caused the shrimp died within three days. They explained that hormone

stimulated sloughing too fast and the animals did not have enough energy to throw off the old shell.

In *M. rosenbergii* and most species of crustacean, reproduction is thought to be under the control of various hormones, including vitellogenesis inhibiting hormone (VIH) and vitellogenesis stimulating hormone (VSH). So, the correlation of vitellogenin (Vg) variation and ovarian maturation was studied. Vg content in the hemolymph of *M. rosenbergii* in each group was compared to those stimulated by 5-HT, MF, and PE, it could be shortening the ovarian development period with nine days, 12 days, and 10 days, respectively compared with control. The result indicated that 5-HT is the best ovarian development stimulant for *M. rosenbergii* considered with shortest ovarian development period, survival rate, and fecundity. Vg level accumulated in the ovary at stage IV (Tinikul et al., 2008; Vaca and Alfaro, 2000; Laufer et al., 1998). Furthermore, Songsangjinda (1989) studied the relationship between Vg, 20-hydroxyecdysone (20-HE) and egg development in *Penaeus merguiensis* and found that 20-HE at the dose of 50 ng/g BW could increase blood Vg level, but it was decreased at higher concentrations of 100 and 150 ng/g BW. In crustaceans, vitellogenesis is an important process in the female reproductive cycle (Okumara, 2004), which is characterized by the appearance in the hemolymph of Vg, the precursor of major yolk protein, vitellin (Vn) (Tsukimura, 2001). Thus, hemolymph Vg level is important indicator of ovarian maturation.

In nature, male and female ratio is about 1:1 (Malecha et al., 1992). However, sex differentiation in crustacean was interesting because male *M. rosenbergii* is bigger than female and get better price. In this study, we would like to study the effect of hormone on sex differentiation by inducing female brood stock by injection with different hormones. The effect of 5-HT at 0.1 μg/g BW on sex differentiation was evaluated. The studies showed that induced female brood stock produced male and female offspring in the ratio of 1.1:1. In contrast, the study of Olmstead and Leblanc (2000) showed 100 % induction in male of *Daphnia magna* with methyl farneasoate (400nM). Phytoecdysone at 0.05 μg/g body weight and MF at 0.1 μg/g BW also induced male and female 1:1 and was not different when compared to the control group.

Finally, the effect of serotonin, methylfarnesoate, and phytoecdysone in promoting the ovarian development was clearly observed but it was not clear if there was any sex reversal induction.

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