# CHAPTER II

# MATERIALS AND METHODS

## Broodstock Preparation

Prawns used in ovarian development experiment were brought from extensive farms in Khlong Cone Samut Songkhram province and in Samut Sakorn Province, and prawns captured by commercial trawlers in the Upper Gulf of Thailand, Chon Buri province. Only those 6 to 7 months old or average carapace length of 5 cm and body weight range 70 to 120 g were used in this experiment.

For moulting experiment, prawns from an intensive farm in Samut Prakarn Province were used. The size of these prawns was between 20 and 40 g with an average age of 3 or 4 months. The prawns were transferred to the laboratory at the Department of Marine Science, the main campus of Chulalongkorn University in Bangkok.

The handling techniques during transportation were important in ensuring the quality live broodstock. Two methods of prawns transportation were used: The first technique, prawns were transported by packing in polyethylene bags (45 x 50 $cm^2$) filled with 3 liters of seawater, pressurized with oxygen and packed in

styrofoam boxes. Each bag contained 10 individuals. The rostrum of each prawn was covered with rubber tube to prevent puncturing the bag. The water temperature was controlled at 20 $^{o}$C. The second technique, prawns were transported in holding styrofoam boxes with continuously aerated seawater at temperature 20 $^{o}$C. Under such conditions, the prawns could be kept alive for more than 6 hrs.

Broodstock arrived at the laboratory supposedly carried with them some micro-organisms and could infected the other. Thus, these prawns were always quarantined with 50 ppm malachite green and 25 ppm formalin for 10 to 15 minutes. Then bathed with clean seawater and stored in stocking tanks with the diameter of 1.0 x 1.5 x 0.7 $m^3$ stocking density was 8 individuals per tank. Acclimating the prawns in the experimental condition for at least 4 days or until they recovered from stress. The salinity was adjusted to 30 ppt by acclimation rate of 5 ppt per day.

Weight and Length Determination

After completely acclimatization, the prawns weight and length were determined by individual. Using electronic balance (Satorious: Model PT-1200) for weighing body weight while ovarian weight was determined by using electronic balance (Model 200-A).

The distance between the post-orbital margin and the medial posterior border of the carapace called carapace length was measured by vernier caliper.

Tagging Method

Each prawn was double tagged in order to identify individuals, and to determine timing of moulting and ovarian development. The double tagged comprised of carapace-tagged, colour-coded plastic piece glued onto the carapace of prawn, and eyestalk-tagged, an insertion of a elastic silicone ring with coloured labeled code of number, around the eyestalk.

After a day of moulting, the carapace-tagged would lost be with old exoskeleton, new tag with same number and colour was replaced periodically. The eyestalk-tagged provided a permanented secondary means of identification as they rarely come off during moulting. This was an advantage to ascertain which prawn had moulted. No harmful effect on the prawn was observed when using these tagging techniques. Every morning fresh moults were removed from the tank and identified, so that the new tag could be done in the next day.

Feed and Feeding

The prawns were fed fresh (or fresh-frozen) horse mussels and squid meat, three times daily at 08:00, 12:00 and 16:00 h. The feeding ration was about 20 % of total body weight of prawns per day. Uneaten food and fecal matter were removed daily by siphoning technique in the morning before feeding.

Rearing System

Rearing or experimental tank, used in this experiment were closed-recirculating system. The rearing tanks were rectangular shape, and flat bottom with a size of 1.0 x 1.0 x 10.0 m, and filter tank at both end of the main tank. The stocking density was 5 individuals per square meter. The details of this system was described by Menasveta (1980).

Light intensity was controlled at the level lower than 80 % of normal by covering the tanks with black plastic sheets. High saline water from salt pond salinity 65 to 80 ppt was diluted to prepare experimental water at salinity 30 ppt. Before the experiment, a rearing compartment of the system was treated with 50 ppm calcium hypochloride. The system then aerated and sodium thiosulphate was added to neutralize hypochloride. Water quality, such as salinity, temperature and pH were checked daily. Nitrogenous as wastes, especially ammonium ($NH_4^+$), nitrite ($NO_2^-$) and nitrate($NO_3^-$) were determined weekly by colorimetic method (Aquamerck-11120). The water quality was maintained at the following allowable ranges salinity 30±1 ppt, pH 7.5±0.5, $NH_4^+ < 0.5$ ppm, $NO_2^- < 0.1$ ppm, $NO_3^- < 10$ ppm.

Compozyme or Biobact (trade name) were added to filter bed of the experimental tanks twice a month at the ratio of 1 tea spoon per filter bed. Compozyme was composed nitrification bacteria, *Nitrosomonas* and *Nitrobacter* species, which usually grow under

aerobic condition. Nitrification bacteria utilizes ammonia and nitrite, which are toxic to prawns, as an energy source and oxidized them to nitrate, a less toxic matter to the prawns.

Hormone Preparation

progesterone (4-Pregnene-3, 20-dione)(SIGMA), β-estradiol17-(β-D-Glucuronide)(SIGMA), 2-deoxyecdysone (SIGMA) and β-ecdysone extracted from bark of *Vitex glabrata* stock solution were dissolved in pure ethanol at a concentration of 1 μg/ul or 1mg/ml. Dilution of hormones was made by using isosaline water 0.65 % (consist of NaCl 0.65 g, KCl 0.42 g, $CaCl_2$ 0.025 g and distilled water 100 ml). In order to have a final concentration of 0.01 μg, 0.1ug, 0.2 ug, 0.4 μg, respectively. In each experiment, prawns were divided into; control group (no treated prawn), sham (prawns treated with only solvent) and treated group. Amount of hormone needed for each individual was calculated from the body weight of each prawn. These prepared hormones were injected intramuscularly between the connection of the exoskeleton of the $3^{rd}$ and $4^{th}$ lateral abdominal segment with a microsyringe (Figure 11). Hormone was prepared just prior to injection. The stock solution and aliquoted stocks of hormones were kept at $0^{o}$ C.

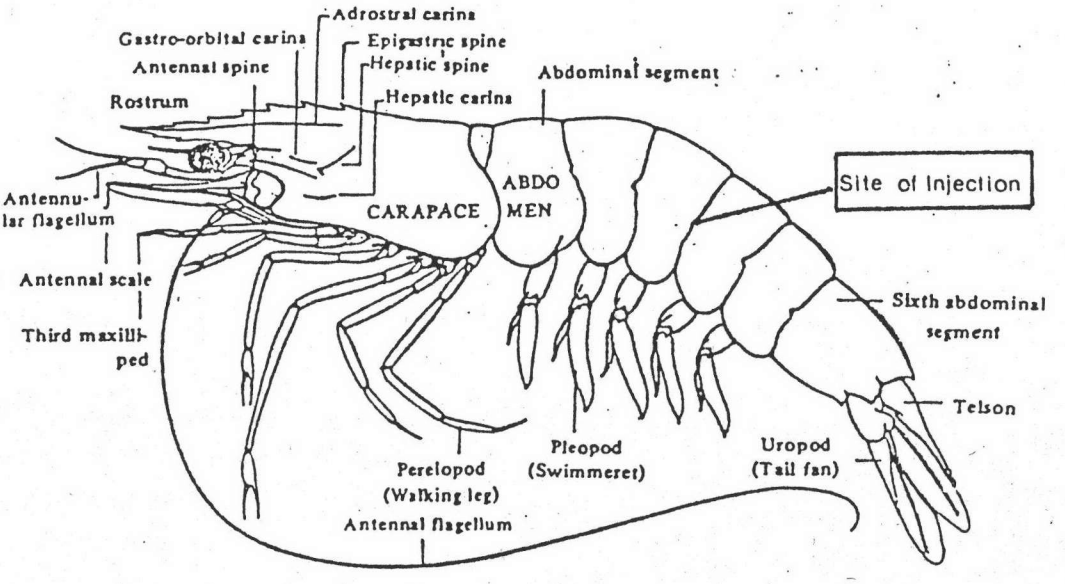


Figure 11. Lateral view of adult giant tiger prawn, *Penaeus monodon* showing site of injection.

Experimental Procedures

Part I. Study of progesterone and β-estradiol17 on ovarian development in *Penaeus monodon*.

This experiment was designed to study effects of progesterone and β-estradiol17 on ovarian development of premature prawns in different sources. Female prawns used in this experiment had been collected from extensive pond in Khlong Cone-Samut Songkharm, Samut Sakorn and commercial boats in Chon Buri province. The females were randomly divided into 14 equal group. The experimental design described in Table 1. Females collected from Khlong Cone were test in group 1 to 14, female from Samut Sakorn were tested in group 1, 2, 6, 7, 8, 9 and 14. And females from Chon Buri were test in group 1, 2, 4, 5, 6, 7 and 14. Each group has 8-10 individuals.

On days 7, 14 and 21, after injecting hormones, two prawns from each treatment were randomly sampled. Ovaries from the prawns then were dissected and the degree of ovarian development was observed visually to identify ovarian stage and weighed to determine gonad index.

Gonad index calculation can be done using an expression described by Omori and Chida (1988);

$$\text{Gonad index} = \frac{\text{fine wet ovary weight (g)}}{\text{fine wet body weight (g)}} \times 100\ \% \quad \text{----(1)}$$

The index of ovarian development could be used to identify ovarian stage as; 1 % = stage I, 2 % = stage II, 3 % = stage III and > 4 % = stage IV.

Table 1. Showing the experimental protocol for studying the effect of progesterone (P) and β-estradiol17 (Es) on ovarian development.

| Group | Treatments | Description |
|---|---|---|
| 1 | Control | No injected. |
| 2 | Sham I | Each prawn was injected once with solvent 0.1 µl/g body weight. |
| 3 | Sham II | Each prawn was injected twice with solvent 0.1 µl/g body weight farther one week. |
| 4 | P 0.01 | Each prawn was injected once with progesterone 0.01 µg/g body weight. |
| 5 | Es 0.01 | Each prawn was injected once with β-estradiol17 0.01 µg/g body weight. |
| 6 | P 0.1 | Each prawn was injected once with progesterone 0.1 µg/g body weight. |
| 7 | Es 0.1 | Each prawn was injected once with β-estradiol17 0.1 µg/g body weight. |

| Group | Treatments | Description |
|---|---|---|
| 8 | PII 0.1 | Each prawn was injected twice with progesterone 0.1 μg/g body weight farther one week. |
| 9 | EsII 0.1 | Each prawn was injected twice with β-estradiol17 0.1 μg/g body weight farther one week. |
| 10 | P 0.2 | Each prawn was injected once with progesterone 0.2 μg/g body weight. |
| 11 | Es 0.2 | Each prawn was injected once with β-estradiol17 0.2 μg/g body weight. |
| 12 | P 0.4 | Each prawn was injected once with progesterone 0.4 μg/g body weight. |
| 13 | Es 0.4 | Each prawn was injected once with β-estradiol17 0.4 μg/g body weight. |
| 14 | Initial | Prawn sampling at the beginning of the experiments. |

Part II. Study of 2-deoxyecdysone and β-ecdysone on moulting in *Penaeus monodon*.

This experiment was designed to examine the effect of commercial ecdysone and plant extracted ecdysone on moulting of *Penaeus monodon*. The study was also designed to look at effect of ecdysone on inter-moult period of the prawn, so moulting stage checking is important.

Moulting stage checking

Moulting stage check was done in each experimental prawn by using photomicroscopic observation of the uropods. A technique (Figure 12) was used as described by Smith and Dall (1985) and identified as in Figure 7. For detail, after moulting all prawns were checked moulting stage everyday to ensure that the stage of injection was correct. This observation could be done directly to the uropods of the prawn by compound stereomicroscope without cutting a piece of uropod.

Prawns moulting were divided into 3 groups of moulting stage as; postmoult (B), intermoult (C), and premoult ($D_1$"). The experimental design in this study was shown in Table 2. After injection, each treatment was closely observed in the morning and evening for moulting records.

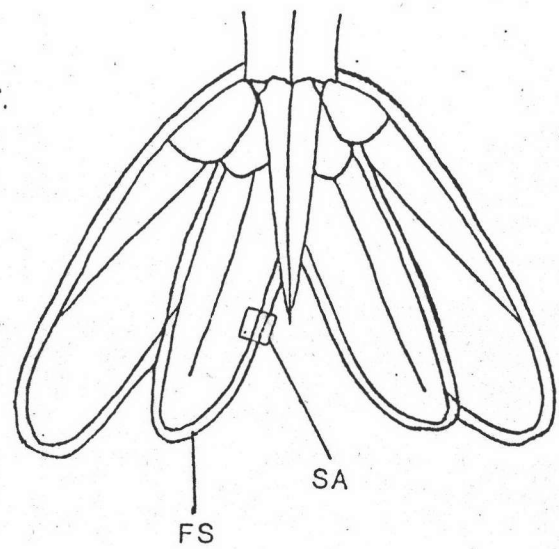


Figure 12. Uropods showing a sample area (SA) used for moulting stage observation and fringing setae (FS) (Source: Smith and Dall, 1985).

Table 2. Showing the experimental protocol for studying the effect of 2-deoxyecdysone (Ec) and β-ecdysone (βEc) on moulting.

| Group | Treatments | Description |
|---|---|---|
| 1 | Control | The prawn were not injected . |
| 2 | Sham | Each prawn was injected with solvent 0.1 µl/g body weight. |
| 3 | Ec 0.01 | Each prawn was injected with 2-deoxyecdysone 0.01 µg/g body weight. |
| 4 | βEc 0.01 | Each prawn was injected with β-ecdysone 0.01 µg/g body weight. |
| 5 | Ec 0.1 | Each prawn was injected with 2-deoxyecdysone 0.1 µg/g body weight. |
| 6 | βEc 0.1 | Each prawn was injected with β-ecdysone 0.1 µg/g body weight. |
| 7 | Ec 0.2 | Each prawn was injected with 2-deoxyecdysone 0.2 µg/g body weight. |

| Group | Treatments | Description |
|---|---|---|
| 8 | βEc 0.2 | Each prawn was injected with β-ecdysone 0.2 µg/g body weight. |
| 9 | Ec 0.4 | Each prawn was injected with 2-deoxyecdysone 0.4 µg/g body weight. |
| 10 | βEc 0.4 | Each prawn was injected with β-ecdysone 0.4 µg/g body weight. |

Statistical analysis

Data were analyzed by using some descriptive statistics. Mean values were computed as arithmetic mean ± standard deviation (SD). Analysis of variance was used in testing for significant differences between treatments of the experiments. All statistical analyses were performed with SAS (Statistical Analysis System).