CHAPTER II

METERIALS AND METHODS

Broodstock preparation

The prawns used in all experiments were brought from extensive and intensive farms in Chantaburi, Chonburi, Ranong and Samutprakarn Provinces. Only those that were 5 and 8 months old were selected for these experiments. These prawns were transferred to the hatchery at the main Campus of Chulalongkorn University in Bangkok.

The handling techniques during transportation were important in ensuring the quality of live broodstocks. There were two ways by which broodstocks were transported from the farms. For those having, body weight 80 to 120 grams, they were transported in holding tank with aerating seawater at a controlled temperature (20 to 24^{o}C) to reduce physiological activities and stress. Each prawn was immobilized by inserting it into a prawn-tube in order to prevent strain. This technique could reduce injury and mortality. The prawns smaller than 80 grams body weight were transported by packing in polyethylene bags (50 X 57 cm^{2}) filled with 3 liters of seawater, pressurized with oxygen and packed in styrofoam boxes. Each bag contained 3 to 5 individuals. The rostrum of each prawn was covered with rubber tubbed to prevent puncturing . The water temperature was maintained at 20 to 24 ^{o}C. Under such conditions, the prawn could be kept alive for more than 12 hours.

Broodstocks arriving at the laboratory might be carrying some external parasites and disease. To prevent reinfection, the newly arrived prawns were quarantined and treated with 50 ppm malachite green and/or 25 ppm formalin for 10-15 minutes. The rearing tanks were 1.0 X 1.5 X 0.7 m^3 in dimensions and stocking density was 7 individuals per m^2. The prawns were allowed to acclimatize to the laboratory condition for at least 4 days or until they recovered from stress. Water quality was monitored and controlled at the optimal ranges. The salinity was adjusted to the required levels by changing rate of 5 $^{o}/oo$ per day.

Length and Weight Determination

After completely acclimatized or at minimum stress condition, the prawns were weighed and lengthened. Using electronic balance (Sartorius: Model PT-1200) for measuring body weight. Ovarian weight was measured by using electronic balance (Precisa: Model 200-A) after it was dissected from specimens.

The distance between the post-orbital margin and the medial posterior border of the carapace called carapace length (Motoh, 1981, 1985) was measured by vernier caliper. Body weight and carapace length were used to define fatness or condition index of each prawn. Fatness or condition index was calculated from the formula as follows:

$$\text{Condition index} = \frac{\text{Body weight (grams)}}{\text{Carapace length (centimeters)}} \quad \ldots\ldots\ldots(1)$$

Tagging Method

Each prawn was doubly tagged in order to identify each individual and to establish the time of molting and ovarian development. The double tagging comprised of carapace-tagged (Figure 1), color-coded plastic piece glued onto the carapace of the prawn using an epoxy resin adhesive, and eyestalk-tagged, a ring of a colored elastic silicone bearing a label inserted around the ocular peduncle of the unablated eyestalk (Figure 2). The optimum diameter and length of the tag depended on prawn size such as for subadult and adult prawn, the size of this silicone ring were 4-5 mm internal diameter and 4-5 mm length.

After molting, the carapace of the exuvia retained the carapace-tagged while the eyestalk-tagged remained intact. It was then easy to ascertain when prawn had molted. No harmful effect on the prawn was observed using these tagging methods (Primavera, 1978).

Feed and Feeding

The prawns were fed horse mussels and fresh squid meat four times daily (approximate 15-20 % of the total body weight per day) and sometimes supplemented with pelleted feed , 36-40% of protein. Uneaten food, wastes and exuvia were removed every morning before feeding.

Eyestalk Ablation Technique

Female prawns were unilaterally ablated only when their exoskeleton hardened, never ablated during postmolt (newly molted or soft shelled) or premolted (ready to molt with whitish spots on the shell) stages.

The eyestalk ablation procedures were the following:

1. Hold the prawn gently with soft cloth and firmly with one hand.

2. Eyestalk ablation was performed on either left or right eye. However, an already infected or otherwise damaged-eye should be ablated to leave one unablated healthy eye.

3. Eyestalk ablation was performed by cutting across the eye ball with scissors while holding the prawn under water to minimize stress, cut off the eyestalk about 3 mm from the base. After ablation, the ablated eyestalk immediately treated with some antibiotics, e.g. acriflavine solution 0.1% (0.1 grams Acriflavine in 100 ml of water) or thimerosal 0.1% (w/v) before release the ablated-prawn into the rearing tanks.

Rearing System

Rearing tanks were close-recirculating water system, rectangular in shape with flat bottom (Figures 3 and 4). Light intensity was controlled at the level lower than 70% of the normal light intensity by covering the tanks with black plastic sheets, continuously aerated for maintaining sufficient dissolved oxygen

Figure 1 Method of carapace tagging

Figure 2 Method of eyestalk tagging

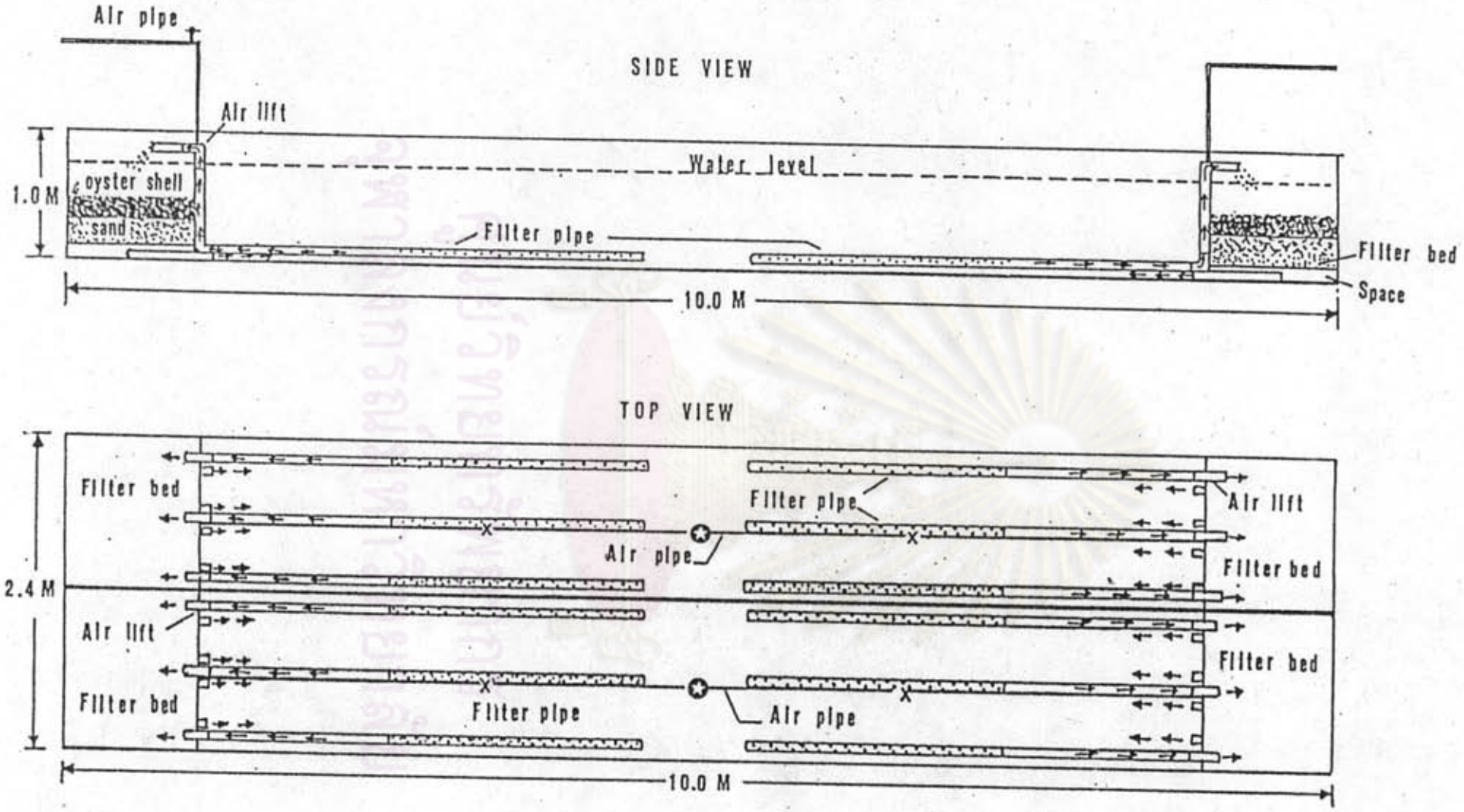

Figure 3 Closed recirculating water system, double filter bed.

A: side view, B: top view (After Menasveta *et al.*, 1989).

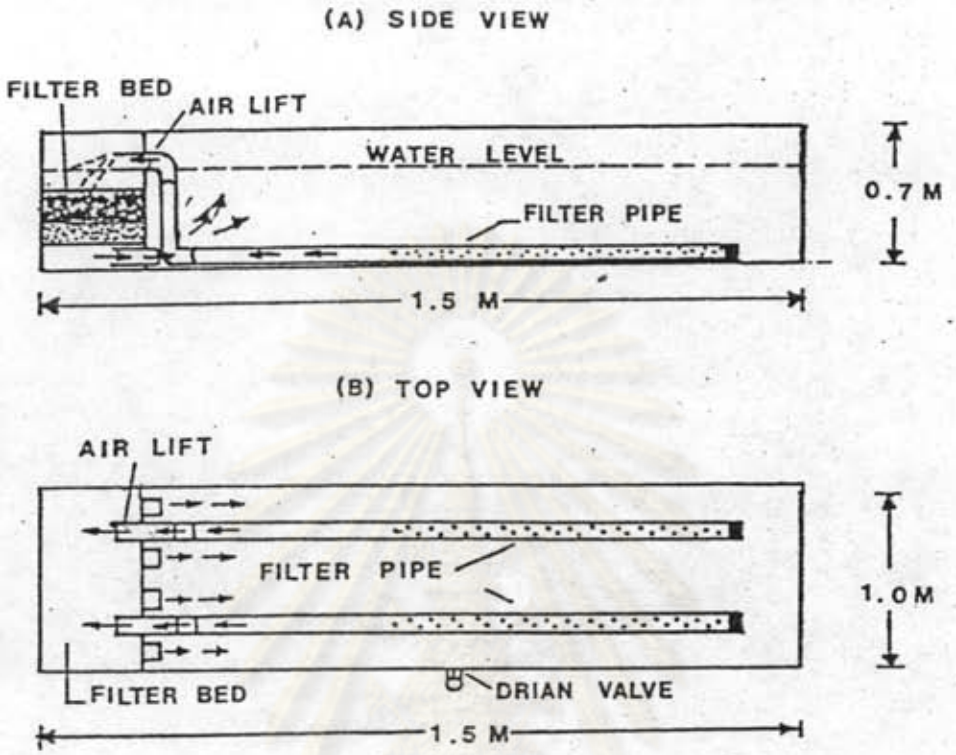

Figure 4 Schematical illustration of the closed recirculating system, single filter bed. A: side view, B: top view

concentration in water. Physico-biological filtration system of these tanks was done by recirculating seawater through sand, gravel and oyster-shell layers. The tanks with double filter bed (Figure 3) contained 8 tons of seawater and the filtering rate was 30 liters per minute while the single-filter bed tanks (Figure 4) operated at the rate of 20 liters per minute. All of the filter beds contained nitrification bacteria and some other micro-organisms. These organisms acted as biofiltration system (Spotte, 1979). Each tank contained 7 individuals per m^2 .

Water Quality

Brine water from salt farm, salinity between 55 and 85 $^{o}/_{oo}$ was used for the experiment. Aeration and addition of 50 ppm calcium hypochorite are essential to dispose organic waste and micro-organism that may cause disease. Aerated for two weeks or until excess chloride ions were changed to chlorine: $Cl_2(g)$, the chlorine determination was performed by test kit (Merck-14640). Experimental seawater was prepared from this brine water by diluting with tap water. Water salinity, temperature and pH were checked daily by using salinometer-SCT (Model YSI-33), pH-meter (Orion: Model SA-250). Nitrogen compounds such as ammonia, nitrite,nitrate were determined by test kit (Aquamerck 11120). The water quality was maintained and readjusted within the following allowable limits:

Water temperature	24-31^{o} C
pH	7.5 -8.5
NH_4^+	0-1.0 ppm

NO_2^-	0-0.50 ppm
NO_3^-	0-50 ppm

Compozyme or Biobact (trade name) was added to filter bed of the experimental tanks twice a month at the ratio of 1 tea spoon per 1 ton of seawater or whenever nitrogenous waste was overranged. Biobact should help increasing the capacity of the biofiltration system. Compozyme and Biobact are composited nitrification bacteria and some micro-organisms which usually grow under aerobic condition. Therefore, the filter bed should be always kept in an aerobic condition. Nitrification bacteria utilizes ammonia and nitrite as energy sources and oxidize them to nitrate. Ammonia and nitrite are toxic for prawns but nitrate is less toxic for prawns (Spotte, 1979).

1. Calcium Determination

Calcium ions affect molting, osmoregulation and shell formation of prawn (Deshimaru *et al.*, 1978; Wilson, 1975). It was expected that brine water which was used in the experiment had a different ratio of calcium ion to major elements from natural sea water because of precipitation. Variation in calcium concentration of the diluted brine water might result from how many times it recirculated through filter bed, depending on buffer capacity of this filtration system or on the concentration of brine sea water used. The procedure for calcium determination was based on Kremling (1976). Complexometric titration with EGTA (ethylene-glycol-bis(2-hydroxyanil) -N,N,N',N'-tetracetic acid) has been used for the direct determination of calcium in sea water. After brine water was diluted, 1 lit of diluted water with both salinity 30 $^{o}/_{oo}$ and 40 $^{o}/_{oo}$

were sampled for determining the concentration of calcium ions and calcium/salinity ratios. After recirculating for 15 and 30 days calcium concentration was determined again for comparison. .PA

Experimental Procedures

These experiments were divided into five main parts :

1. Osmotic Study of *Penaeus monodon*

In this part, osmolality of sea water and haemolymph of *P. monodon*, subadult was determined by using vapor pressure osmometer (Wescor-5500).

1.1 Osmoregulation Type

Penaeus monodon with unablated eyes (normal eyes) and unilateral eyestalk ablation were used. After acclimation of the prawns in 20, 25, 28, 30, 35, 40, and 45 $^{o}/_{oo}$ for 7 days, osmotic pressure of prawn haemolymph and culturing media were determined.

The data were then interpreted for isosmotic point and osmoregulation type of subadult *P. monodon.*

1.2 Relationship between Osmoregulation and Hypersaline Acclimation Time

P. monodon subadult were acclimated at salinities 30 $^{o}/_{oo}$ and 40 $^{o}/_{oo}$ for 24 hours, 48 hours, 7, 15, 30, and 45 days and then osmotic pressure of haemolymph and sea water were measured. The relationship between osmotic regulation and acclimation time were deduced.

2. Ovarian Maturation Experiments

2.1 Adult Prawn Maturation in Hypersalinity

Prawns used in this experiment were collected in Chantaburi province. Upon arrival in the laboratory the sizes of prawns were measured, then they were acclimated in the rearing systems for 1 month before tagging. Before starting the experiment the prawn size was determined again. The pond-reared broodstocks *Penaeus monodon* of 75 to 120 grams body weight and 4.7 to 8.0 cm carapace length were selected for the experiment. The stock density was 6-8 individual per m^2.

Rearing tanks were double filter, recirculating tanks (Figure 3) containing 8 tons of closed-recirculating water. The salinities of 30 °/oo and 40 °/oo were chosen in order to represent hypersaline condition. During the experiment lasting for 2.5 months, random samplings of 2-3 specimens were performed every 2 or 3 days in order to determine reproductive conditions. The ovarian weight, body weight (wet weight) and carapace length of each female prawn were recorded. The quantitative method for estimating reproductive activity was the ovarian index.

The ovarian index is the ratio of ovarian weight to the body weight or wet weight of prawn expressed as a percentage (De Vleming, Grossman and Chapman, 1982; Giese and Pearse, 1974; Grant and Tyler, 1983a; Vernberg and Vernberg, 1972). Thus, the ovarian index is calculated as following:

$$\text{Ovarian index (\%)} = \frac{\text{Ovarian weight (grams)}}{\text{Body weight (grams)}} \times 100 \quad \ldots(2)$$

The fatness or condition index of this broodstock is calculated by equation ..(1)

2.2 Prawn Size, Hypersaline Acclimation and Eyestalk Ablation Effects on Maturation of Subadult *Penaeus monodon*

This part was designed to examine the effects of hypersalinity (30 °/oo and 40 °/oo), acclimation period and eyestalk ablation on gonad development in two size group of subadult *P. monodon* (Table 1). Small size subadult prawns (25 to 40 grams) collected from laboratory rearing and large size group (45-70 grams) from intensive farm were used and compared. The 8-ton tanks of recirculating water system were used.

Mortality were recorded throughout the experiment. After 30 days, the prawns were dissected to determine ovarian index. Condition index of each prawn was calculated at beginning of the experiment and after being reared for 30 days. The ovarian index and condition index were then compared and discussed.

2.3 Manipulation Techniques to Induce Ovarian Maturation and Physiological Condition of Subadult *Penaeus monodon*

The prawns used in this experiment were large size subadult prawns, 45 to 70 grams body weight and 4.3 to 4.7 cm carapace length, with the age of 5 months old. The treatment methods were classified into 4 groups. First group, ablated and unablated prawns were acclimated with hypersalinity 30 °/oo or 40 °/oo for 15, 30 and 45 days (TRM-1 to TRM-4). Second group, the prawns were acclimated at

Table 1 Experimental conditions and manipulation treatments for size effects of subadult *Penaeus monodon* on maturation.

Size group	Salinity (o/oo)	Eyestalk manipulation
small size group (3.4-4.0 cm CL, 28-40 grams BW)	30	Eyestalk ablation
	30	Normal eyestalk
	40	Eyestalk ablation
	40	Normal eyestalk
large size group (4.3-4.7 cm CL, 48-60 grams BW)	30	Eyestalk ablation
	30	Normal eyestalk
	40	Eyestalk ablation
	40	Normal eyestalk

CL = carapace length (cm)
BW = body weight (grams)

30 °/oo for 30 days, then unilateral eyestalk ablated and acclimated at the same salinity for 15 days (TRM-5). Third group, (TRM-6 and 7) prawns were hypersalinity acclimated at 2 salinities, 30 °/oo and 40 °/oo. The first step acclimation time was 30 days and second step acclimation time was 15 days, and last group, prawns were also two steps acclimated with hypersalinities. The prawn were ablated before or after first acclimation in order to determine combination effects of hypersalinity and eyestalk ablation on maturation (TRM-8 to TRM-11). The TRM-12 was initial or control group. The treatment methods are summarized in Table 2.

Ovarian index and condition index were determined during experimental period as discuss above.

2.4 Studies on Ovarian Development by Paraffin Histological Method

In order to determine effects of hypersaline condition and eyestalk ablation on oocyte development, paraffin histological method was used. Mature oocyte size and oocyte morphology of subadult and adult prawns kept in salinities 30 °/oo and 40 °/oo were compared.

Dissected ovaries obtained from the experiments were preserved in 10% Buffer formalin solution for 1 to 2 weeks. After that, each gonad was cut into small pieces and gently rinsed with running tap water. The small pieces of ovaries were dehydrated by using ethyl alcohol series (50, 70 ,90, 95 and absolute alcohol, respectively). The dehydrated tissue was then clearified with toluene and infiltrated with paraplast. After this step each sample was embedded in paraplast block and cut transversely at 7-10 microns with

Table 2 Scheme of experiments of hypersaline acclimation series and eyestalk manipulation on subadult *Penaeus monodon*

Code Treatment (TRM)	Salinity (o/oo)	First hypersaline acclimation time (days)	Eyestalk Manipulation (N or A)	Second hypersaline acclimation		
				Salinity (o/oo)	Eyestalk Manipulation (N or A)	Acclimation Time (days)
1	30	15 30 45	N N N			
2	30	15 30 45	A A A			
3	40	15 30 45	N N N			
4	40	15 30 45	A A A			
5	30	30	N	30	A	15
6	30	30	N	40	N	15
7	40	30	N	30	N	15
8	30	30	N	40	A	15
9	40	30	N	30	A	15
10	30	30	A	40		15
11	40	30	A	30		15
12	25-28	0	N			

N = Normal Eyestalk
A = Eyestalk ablation

rotary microtome. The sections were mounted onto glass slides and stained with Heidenhain's ion haematoxylin and Eosin-Y (Baker, 1958; Gray, 1958; Hall, 1962; Humerson, 1967; Willey, 1971). The histological condition of the ovary was examined by light microscope and mean size of oocytes in each ovaries was determined. Sizes of 50-100 oocytes was measured using an eyepiece graticule calibrated against a stage micrometer and the mean size was obtained. Only those oocytes sectioned through the nucleus were measured (Grant and Tyler, 1983b) (see Appendix-A for the details).

Statistical Analysis

Data were analyzed by using some descriptive statistical methods. Mean values were computed as arithmetic mean $\pm$ standard deviation (SD). Regression analysis, analysis of variance, analysis of covariance and tukey-HSD multiple range test were used in testing for significant differences among treatments. The 95% confident interval was used to define a significant level. All statistical analyses were performed with SYSTAT program (System for Statistics) (Wilkinson, 1987; Zar, 1984).