CHAPTER II

MATERIALS AND METHODS

The study comprised of two experiments. The first experiment (Experiment I) dealth with the effect of different astaxanthin and canthaxanthin concentrations on growth, coloration, moult frequency and food conversion in juvenile giant tiger prawns, and the second experiment (Experiment II) dealth with the effect of astaxanthin on gonad maturation in giant tiger prawns.

Experiment I : Effect of astaxanthin and canthaxanthin on coloration of juvenile giant tiger prawns.

Preparation of Juvenile Prawns

Giant tiger prawns used in this experiment were collected from an intensive culture farm in Bangpakong, Cholburi Province, and subsequently they were transferred to and reared in a large concrete tank in a laboratory at the Marine Science Department, Chulalongkorn University, Bangkok. The prawns had the weight ranging from 9-12 g. The prawns were quarantined with 100 ppm formalin and 3 ppm malachite green for 5 to 10 minutes. The prawns were fed with a commercial feed containing 42.5% protein. Feeding

was continued until all prawns turned to a light blue colour.

The blue colour giant tiger prawns were divided into 18 groups of 15 prawns each. These groups were stocked in the 18 two hundred litre round fiberglass tanks. The water system of these tanks was a closed recirculating water system (Figure 1).

Experimental Diets

The experimental diets used in this feeding trial were processed by the Marine Biotechnology Research Unit, Chulalongkorn University. The experimental diets consisted of 9 formulations with the same basal ingredients but containing different concentrations of astaxanthin and canthaxanthin. Of these nine diets, four of them contained 25 ppm, 50 ppm, 75 ppm and 100 ppm astaxanthin, another four of them contained 50 ppm, 100 ppm, 150 ppm and 200 ppm canthaxanthin, and the rest (one) of them contained none of the two chemicals (control). The nine formulations of these diets were shown in Table 2.

The diets were prepared by mixing dry ingredients in a column mixer, then cod liver oil was added with mixing and blending for another 15 min. The blended ingredients was added with approximately 35% (by weight) of cold water and mixed to form a dough like mass. This dough was later extruded throngh a meat chopper with the sieves of 2.4 mm diameter. This process increased the temperature to 40-50 ^{o}C. The spagetti like processed feeds were dried in an oven at the temperature of 60 ^{o}C for 17-18 hrs. Then the feeds were taken out and softly ground to make short pellets. The pellets were put in sealed contained and kept in a refrigerator at 10 ^{o}C until use.

Experimental Design

With nine experimental diets and 18 test units, the feeding trial formed a complete randomized design of 9 treatments and two replications. The trial period was two months. The prawns were fed at satiation three times daily at about 08.00, 12.00 and 16.00 h. Uneaten food and fecal waste were removed before the first daily feeding. The following parameters were observed and recorded:

1. Individual growth of prawns by length and weight was measured at every 15 days interval.

2. The moulting rates were determined daily, by using the equation

$$MR = \sum_{i=1}^{t=7} [m/n]$$ (modified from Guary *et al.*, 1976).

where MR = individual moulting rate (week)

m = total number of moults in a day

n = number of living prawn

i,t = day

3. Feed conversion ratios (FCR) were calculated at every 15 days. FCR can be determined from prawns weight and food intake by using the equation:

$$FCR = F/(Wt-Wo)$$ (Ofojekwn and Ejike, 1984).

F = The amount of total feed concumed by the prawns.

Wo = initial weight of each period.

Wt = final weight of each period.

4. Daily mortalities were recorded.

5. Visual observation of the live prawns and boiled prawns was done periodically. Photograph was taken for recording the coloration of these prawns.

6. A pooled sample of six prawns was sacrificed at the beginning. Thereafter three prawns from each test unit were sacrificed at the end of each month. The prawn samples were analysed for astaxanthin and total carotenoids by the HPLC method and by the spectro photometric method, respectively. The analyses were carried out by F. Hoffmann - La Roche, Switzerland.

Water Quality Measurements

Water temperature, dissolved oxygen and salinity in the experiment tanks were monitored daily. Water temperature and dissolved oxygen were determined using a YSI oxygen meter. Salinity was determined by a reflectometer. Ammonium, nitrite, nitrate were checked by colorimetric methods (Merck's Test Kits No. 11 102).

Proximate Analysis of Diets

Proximate analysis of nutritional values such as moisture content, crude protein, lipid and crude fibre of the experimental diets were done by the AOAC method (AOAC, 1980).

Statistical Analysis

The statistical analysis used in the experiment was descriptive statistics, analysis of variance, analysis of covariance and regression analysis. The Statistic Analysis System (SAS) program (SAS, 1985) was used for all statistical analysis.

Experiment II : Effect of astaxanthin on gonad maturation and reproduction.

The objective of this experiment was to determine the effect of astaxanthin on ovarian maturity, spawning and egg quality of adult female giant tiger prawns. The experiment was conducted in two seperated trials. The two trials had the same experimental protocal. The difference was only the timing.

Trial I was conducted first, then followed by Trial II. In Trial I. all biological data concerning gonad maturation and spawning were collected. Nevertheless, because of the malfunction of a freezer at Sichang Marine Research and Training Station (SMaRT), the hepatopancreas and gonad samples of Trial I thawed and could not be used for the analysis of astaxanthin. With this regard, Trial II was conducted as a repetition of Trial I but the emphasis was merely on the collection of these two organs for studying the role and mobilization of astaxanthin between the organs.

Preparation of Experimental Prawns

Adult female prawns used in this trail were collected from a semi-intensive farm at Klung, Chantaburi Province,

while adult male prawns were collected from the Andaman sea, Stoon Province. The prawns were acclimated in 4 m^3 concrete tanks at Sichang Marine Research and Training Station, Sichang Island, Cholburi for 15 days. The prawns were quarantined by the treatments with 100 ppm formalin and 3 ppm malachite green for 10 to 20 min. The initial weight of females ranged from 88 to 115 g while males ranged from 88 to 95 g. Subsequently The prawns were divided into two groups and were placed in two closed recirculating seawater system. Each tank received 40 non-ablated males, 40 non-ablated females, and 40 ablated females. Each female was eye-tagged by labeled rubber tube around the eyestalk, and was tagged at the carapace. The latter tag was done by attaching the tag with epoxy glue on the carapace. The prawns were raised in the maturation tanks for 80 days with stocking density at 3.16 individuals/m^2 and sex ratio at 1 male : 2 female. An untagged eyestalk of each female was ablated by cutting with a scissors.

Recirculating Seawater System

This experiment was conducted in two 35 m^3 recirculating seawater tanks. The closed recirculating seawater system was designed by Piamsak menasveta *et al.*(in press). The system is shown in Figure 2.

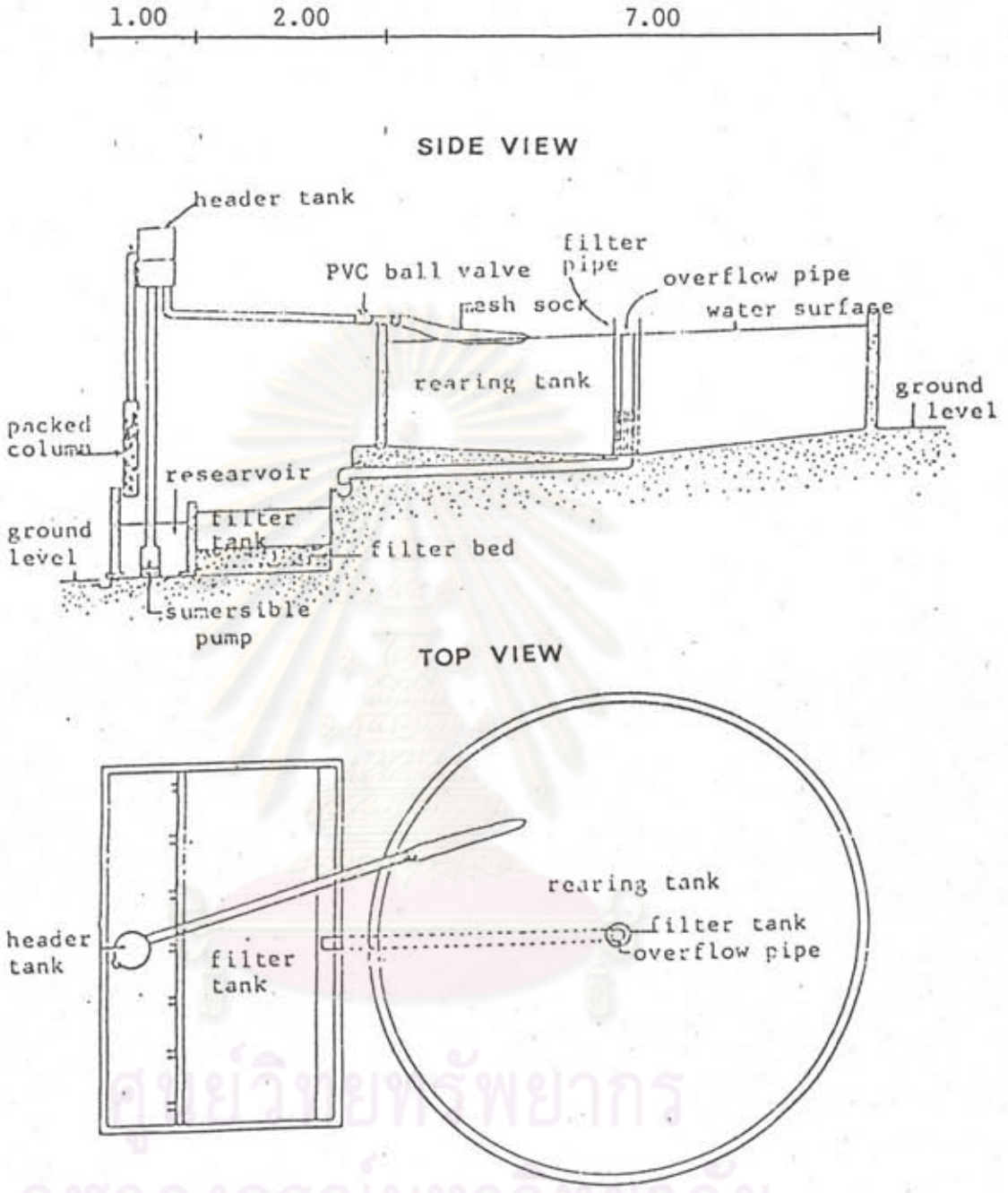

Figure 2 Schematic illustration of maturation tank for Experiment II. The scale in metres. (source: Piamsak Menasveta *et al.*, in press).

Feed preparation

Two formulated feeds were used. The two feeds had the same basic composition excepted that one formulation contained 100 ppm of astaxanthin and another did not. The feed composition is shown in Table 3. The two formulated feeds were processed in the same manner as described in Experiment I, excepted that the EPA oil (W3 HUFA) was sprayed onto the pellets right after the pellets were oven dried.

Experimental design

The experiment was a 2x2 factorial design. The first factor was two feed formulations i.e. non-astaxanthin added feed and astaxanthin added feed. The second factor was the two conditions of eyestalks, i.e. one was the unilateral ablated and another was non-ablated. The experimental period was 80 days. The prawns were fed with two kinds of diets in combination per day. The feeding rate of the formulated diets was at 5-6% per day, and the feeding rate of the fresh natural diet was at 4-5% per day. The experimental design was summarized in Table 4.

Table 3 Composition of the prepared diets used for in the *Penaeus monodon* maturation experiment.

Ingredients	Non-Astaxanthin diet (%)	Astaxanthin diet (%)
Fish meal	20.0	20.0
Fish meal : SP 55	20.0	20.0
Squid meal	20.0	20.0
Squid meal : SLP	10.0	10.0
Shrimp head meal	10.0	10.0
Soyabean meal (USA)	10.0	10.0
Wheat gluten	6.0	6.0
Lecithin	1.0	1.0
EPA oil (Marine Omega)	5.0	5.0
Cholesterol	0.5	0.5
Potassium phosphate	1.0	1.0
Dicalcium phosphate	2.0	2.0
Sodium alginate	1.5	1.5
Sodium hexametaphosphate	1.0	1.0
Vitamin Mix	1.6	1.6
Vitamin C	0.4	0.4
Astaxanthin (ppm)		100.0

Table 4 Summary of experimental design for *Penaeus monodon* maturation experiment of Trial I.

Treatment	Tank condition			Prawn Number		Prawn Density (no./m^2)	Feeding type and Feeding time
Feed types	Shape	Tank bottom Area (m^2)	Water Vol. (m^3)	Males	Females		
100 pmm Astaxanthin added diet	round	38	40	40	40 eyestalk ablated	3.16	Shrimp, squid and clam at 9.00-9.30 am. Pelleted feed at 3.30-4.00 am. and 11.00-11.30 pm.
					40 non ablated		
No Astaxanthin added diet	round	38	40	40	40 eyestalk ablated	3.16	
					40 non ablated		

Data Collection

Ovarian Maturity and Spawning

Two days after being eye-stalk ablated, female prawns were netted out and checked for ovarian maturity by exposing the flash light beam through the abdominal part of the females. This procedure was done underwater at 6:00-7:00 pm once every three days.

Stage III and IV matured (gravid) females were recorded and tranferred individually to a circular fiber-glass spawning tanks with 150 litre of new filtered seawater of 28-30 ppt with moderate aeration. The females with other stages of maturity were left in the maturation tanks.

The following morning, females in spawning tanks were checked for spawning by observing the presence of floating proteinaceous scum released during spawning and the water was sampled in a glass container for egg observation.

Visual examination of the ovaries of spent females was done again to determine the completion nature of spawning. Completely spawning females were returned to their respective tank. Partial spawning females and stage III or Iv matured females that did not spawn were still kept in the spawning tanks for another 1-2 nights and thereafter they

were returned to the tank after spawning or when ovaries were reabsorped.

Eggs Quantity and Quality

Spawned eggs of individual spawner were gently siphoned from the spawning tank into an egg washer with a series of 2 nets, 1. coarse net (0.4 mm mesh), retaining large dirt particles but allowed eggs to pass through. 2. fine net (0.15 mm mesh): retaining the eggs but allowed finer particles to pass through. The eggs were always kept immersed in seawater and processed gently to avoid mechanical damage.

Cleaned eggs of an individual spawner were collected separately in 500-ml seawater in beaker. Three 1-ml aliquot eggs were sampled in a counting chamber after stirring the beaker to ensure uniform distribution. The number of fertilized and unfertilized eggs were determined microscopically by the method described by Primavera and Posadas (1981). The average egg count of the three samples (=no. eggs/ml) multiplied by 500 (=no. eggs/beaker) gave the estimated total number of eggs spawned. Percent fertility was therefore computed as :

$$\% \text{ Fertility} = \frac{\text{Total no. of fertilized eggs}}{\text{Total no. eggs spawned}} \times 100$$

Remaining eggs were tranfered to hatch in the spawning tank with continuous aeration. Hatching rate was determined 36 hrs after spawning by counting the number of nauplii in three 50-ml aliquot samples.

$$\text{Hatching Rate} = \frac{\text{Total no. nauplii}}{\text{Total no. eggs spawned}} \times 100$$

The nauplii in the spawning tanks were incubated to develop for 48 hrs, at which time the number of protozoea metamorphosed was determined as the same manner as nauplii. Percent metamorphoris from egg to the first protozoea stage was therefore computed as followed :

$$\% \text{ Metamorphosis from egg to protozoea} = \frac{\text{Total no. protozoea}}{\text{Total no. eggs spawned}} \times 100$$

Moulting and mortality

Dead prawns and their splited old exoskeleton in the culture tanks were taken off every morning. Number of moulting and dead prawns were recorded. Dead prawns were determined microscopically. Retagging of moulting prawn was done every 3-4 days.

Total Carotenoid and Astaxanthin Contents in Hepatopancreas and Ovaries

During the experiment a number of female prawn were sacrificed for the analyses of total carotenoid and astaxanthin contents. The same number of female prawns were taken out from the two treatment tanks, and This was done covering the whole period of the experiment. The prawns that were taken out during the course of the experiment bore stage I to IV ovarian development. The prawns were dissected and hepatopancreas and ovary were taken out and were frozen for later analyses.

It should be noted that the sacrificed prawns were excluded from the observed data on spawning. The HPLC was used for analysing astaxanthin contents. The total carotenoid contents were determined by a spectrophotometric method. The analyses was conducted by F. Hoffman-La Roche Laboratory in Switzerland.

Water Quality Measurements

Certain physical and chemical properties of seawater in the culturing system were measured. Water temperature, salinity, dissolved oxygen and pH were measured once every two days while ammonium, nitrite and nitrate, were measured

weekly by the methods described by Strickland and Parsons (1972).

Statistical Analysis

The statistical methods for this experiment was the same as described in Experiment I.