CHAPTER III

MATERIALS AND METHODS

Diet and its preparation

Diet ingredients shown in Table 6 (except fish oil), were ground to powder and mixed by a twin bead rolling mixer for 15 min. Then, the different types of fish oil were added to certain levels according to a formula of Table 7. The seven formulations contained various sources of oil in the HUFAs concentration ranging from 0.35 to 1.5% (w/w). Subsequently, they were pelletized by a pelleting machine at 80°C, streamed at 90°C for 5 min, and dried in an oven at 90°C for 2 hr. Dry peliets were separated through a sieve with the pore sizes of 710, 530 and 300 μm, respectively. Each size of pellets was kept in -20°C under dark until being used.

Experimental animals

Nauplius of *P. monodon* were obtained from Daowthong farm, Chanburi province. They were reared at the hatchery of the Department of Marine Science, Chulalongkorn University and were fed on *Chaetocerous sp.* and newly-hatched *Artemia*. After developed into postlarvae stage, the laevae were fed on only newly-hatched *Artemia*. When the larvae reached day 20th (PL-20), they were randomly counted and used in the experiment.

Table 6. Ingredients of the experimental diets.

Ingredients[1]	(% w/w) dry weight
Fish meal	32
Soya bean meal	25
Shrimp head meal	10
Wheat flour	20
Soy lecithin	2
Wheat gluten	4
Vitamin premix[2]	2
Fish oil[3]	5

[1] provided by Unicord feed Co. Ltd., Thailand.

[2] provided by Rovithai., Thailand. It contained selenium, $MgCO_3$, KI, ZnO, SiO_2, antioxidant, pigment, $CoSO_4$,$MnSO_4$, $CuSO_4$, $FeSO_4$, choline, biotin, Ca, niacin, folic acid vitamin A, D, E, K, B_2,B_6, B_{12}.

[3] provided by T.C. union agrotech Co., Ltd., Thailand.

Table 7. Quantity of fish oils and EPA/DHA ratio in experimental diets.

Diet	Amount of oil (%, w/w)			EPA/DHA	%(w/w) n-3 HUFAs in diet
	Corn oil	Semi-refined oil	Refined tuna oil		
1*	5.0	-	-	1:3	0.35
2	2.5	2.5	-	1:3	1.0
3	2.5	1.25	1.25	1:2	1.0
4	2.5	-	2.5	1:1	1.0
5	-	5.0	-	1:3	1.5
6	-	2.5	2.5	1:2	1.5
7	-	-	5.0	1:1	1.5

* Control diet

Feeding trials, experimental design and controlled conditions

Twenty circular rearing tanks filled with 150 litres of filtered and disinflection seawater (30 ppt) were used in the present study. Each tank contained 73 postlarvae (100 PL-20/m^2). The completely randomize design (CRD) experiment was designed with 7 diet treatments (Table 7). Three replicates was done in each treatment, except for diet 1 (control diet) only duplicate was done. Duration of the experiment was 30 days. Shrimp was fed 5 times a day (8.00, 11.00, 14.00, 17.00 and 20.00). Uneaten food and fecal matters were removed by siphon every morning before the first feeding. The rearing system in this experiment was a closed recirculating water system.

Seawater was filtered through a biological filter unit before re-entering culture tanks. During the experiment, shrimp mortality was recorded daily, and at the end of the experiment (30 days), shrimps were individually weighed.

Proximate analysis of the experimental diets

The experimental diets were analysed for protein, fat, ash, fiber and moisture contents with methods described by AOAC (1990) (Appendix A).

Analysis of total fatty acids composition in oils, diet and shrimp tissues.

Table 8. Lists of chemicals used in fatty acid analysis.

Chemicals	Purchased from
Reference standard GLC 68B methylester[1]	NU CHEK PREP, INC., USA
Eicosapentaenoic acid (C 20:5)	NU CHEK PREP, INC., USA
Internal standard nonadecanoic acid(C19:0)	NU CHEK PREP, INC., USA
Acetyl chloride[2]	Sigma, USA
Sep-Pak silica	Waters Associates, Milford, MA, USA
Chloroform (AR grade)	Mallinkrodt., USA
Methanol (AR grade)	Mallinkrodt., USA
Na_2SO_4 anhydrous (AR grade)	Mallinkrodt., USA
KCl (AR grade)	Mallinkrodt., USA

[1] See Table 9.

[2] It was dissolved in methanol (5%, V/V) and prepared in cold container.

Table 9. The fatty acid compositions of reference standard GLC methylester.

Chain	Fatty acid	% by weight
C14:0	Methyl myristate	3.0
C14:1	Methyl myristoleate	1.0
C16:0	Methyl palmitate	10.0
C16:1	Methyl plamitoleate	2.0
C18:0	Methyl stearate	15.0
C18:1	Methyl oleate	25.0
C18:2	Methyl linoleate	10.0
C18:3	Methyl linolenate	4.0
C20:0	Methyl arachidate	2.0
C20:1	Methyl 11-eicosenoate	2.0
C20:2	Methyl 11-14 eicosadienoate	2.0
C20:3	Methyl homogammalinolenate	4.0
C20:4	Methyl arachidonate	4.0
C22:0	Methyl behenate	4.0
C22:1	Methyl erucate	2.0
C24:0	Methyl lignocerate	2.0
C22:6	Methyl docosahexaenoate	4.0
C24:1	Methyl nervonate	4.0

Fatty acid analysis of fish oil

Fish oil in this experiment were from 2 sources; semi-refined oil produced from fishes in Europe and refined tuna oil produced in Thailand. These two types of fish oil were analysed for fatty acid profiles by Gas Chromatography (G.C).

Lipid extraction

Samples containing 30 mg of total lipid were dried in an oven at 70 ^{o}C for 48 hr. Dry samples were extracted with 90 ml of chloroform/methanol (2:1 V/V). Internal standard nonadecanoic acid (C19:0) (0.2 ml, 2000 ppm) was added in solutions and stirred for 15 min, then filterd through filter paper (Whatman no.1). Filtrate was transferred into a separatory funnel containing 40 ml of KCl (0.1 M). After shaking the funnel for 1-2 min and leaving the funnel at room temperature, two phases of liquid in the funnel were observed. The lower phase ($CHCl_3$-lipid) containing lipid was collected. Two extractions were carried out in the same manner as previously described, in order to ensure a good recovery of lipid.

The $CHCl_3$-lipid fractions were pooled, and filtered through Na_2SO_4 anhydrous previously dried at 60 ^{o}C for 12 hr. These solutions were evaporated to dry at 40 ^{o}C by a vaccum evaporator (modified from Artemia Reference Center, 1993).

Esterification

The dry lipid was dissolved in 1 ml of hexane, and 5 ml of 5% acetyl chloride (V/V) in methanol was added to the lipid solution. Nitrogen gas (O_2 free) was flown into a vial of lipid solution, and a vial was sealed under nitrogen. Then, the mixture was heated at 100 °C for 1 hr. After the reaction, a vial was cooled in water, 5ml of hexane and 5ml of 6% (w/V) K_2CO_3 solution were slowly added to stop the reaction and to neutralize the mixture. These solutions were centrifuged at 4000 rpm for 5 min, and an aliquot of the hexane was filtered through Na_2SO_4 anhydrous. Each aliquot was concentrated by flowing nitrogen to a vial, and analysed for fatty acids with G.C (modified from Artemia Reference Center, 1993).

Analysis of polar lipid and non-polar lipid

Extraction of lipid was carried out by the mixture of chlorofrom/ methanol described above. Part of crude lipid was diluted in hexane to obtain a solution containing about 30 mg of lipid in 500 µl of solvent. A solvent was loaded onto the top of Sep-Pak silica cartidge. After complete adsorption of the sample, a syringe containing 20 ml of chloroform was connected to the top of cartidge. Chloroform was pushed through the cartidge and the fraction containing the non-polar lipid was collected. Methanol (30 ml) was used for eluting polar lipid. Solutions containing polar lipid and non-polar lipid were evaporated at 40 °C until they were nearly dry. Dry polar lipid was dissolved in methanol then esterifed. Both polar and non-polar lipid were esterified as

described above. Then they were analysed for fatty acids with G.C as described below (Juaneda and Roequelin, 1985).

Gas Chromatography conditions

Instrument : G.C HRGC MEGA 2 series (Fison instrument, Italy)

Detector : Flame ionization detector (Temperature at 300 °C)

Injector : Split 20:1 (Temperature at 250 °C)

Column : DB-WAX 30 mϕ. ID 0.25 mmϕ. film thickness 25 μm
(J&W scientific, USA)

Operation conditions: Temperature 180 °C for 4 min
180 °C --(5 °C/min)--> 200 °C for 65 min
200 °C --(20 °C/min)--> 220 °C for 15 min

Carrier gas : N_2 2 ml/min

Make up gas : N_2 30 ml/min

Hydrogen : 30 ml/min

Air : 300 ml/min

Peak integration was interpretted and calculated with the Chrom Card software version 2.1 (Fison Instrument, Italy) (Appendix B).

Resistance to osmotic stress of *P. monodon*

After terminating of the feeding experiments, *P. monodon* postlarvae were challenged to osmotic shock using a technique described by Ree et al. (1994). Thirty postlarvae were randomly sampled from each tank and transferred into a 2-liter container filled with freshwater. Survival was monitored

at every 10 minutes after exposing the shrimps to freshwater. Gill of shrimps were used as the indicator of mortality. The dead shrimp, gill filaments did not move, while that of alive ones were still moving. Cumulative mortality index (CMI) was calculated to determine effect of the treatmented diets.

Quality of rearing water

Ammonia, nitrate, nitrite, in water were determined weekly by using test kits (Merck, USA). Temperature, salinity and pH of seawater were recorded during the experiment period.

Statistical analysis

Weight and length gains, fatty acids and survival rates of shrimps from each group were statistically analysed by the Statistical Analysis System (SAS), using analysis of variance and Duncan multiple range test.