Chapter 3

3.0 MATERIALS AND METHODS

3.1 Experimental Facilities

All feeding experiments were conducted in the Freshwater Aquarium Laboratory of the Institute of Biological Sciences, Faculty of Science, University of Malaya. The proximate analysis, diets and carcass were conducted at the Fish Nutrition Laboratory of Freshwater Fisheries Research Center, Glami Lemi, Jelebu district, state of Negeri Sembilan, Malaysia.

3.1.1 Experimental System for Growth Trial

Plastic aquaria of 30 L capacity were used (45 x 30 x 25 cm) in the experiment. Aquaria were each fitted with oxygen inlets for aeration (Appendix 1 (a); Figure 12). Water from all experimental aquaria was changed periodically. In addition, the aquaria water was siphoned out regularly to remove any feaces materials. Each aquarium contained a bottom filter (Code No.139, Guppy Plastic) system with aeration by air pump (EK-8000, Eiko President) to maintain a dissolved oxygen concentration in the water at a constant state of approximately 5.5 – 7.0 mg/L. Temperature was maintained in the 25 – 27 °C range. Water quality parameters including dissolved oxygen, pH, nitrate and ammonia were monitored biweekly.

3.1.2 Digestibility system

In this study, a settling column system similar to the Guelph system (Cho *et al*., 1985) was employed for faeces collection. It was modified and adapted to the 30 L glass aquarium used (Figure 3.1). This collection system employed valves fitted to the bottom of the rearing aquarium where the faeces were deposited after settling. Deposited faeces were collected by opening the valve at the tip end and carefully draining the faeces into beakers. The collectors were assigned to the rearing aquaria the night before and faeces collected early the next morning. Wet settled pooled solids of faeces were frozen at -20

°C to prevent bacterial decomposition and to avoid any deterioration in its nutrient content. Faecal samples were later defrosted and oven dried at 60 °C, ground and analysed for crude protein (CP), crude lipid (CL) and dry matter.

Figure 3.1: Modified 30L aquarium used for digestibility study

3.2 Experimental Animal & Feeding Trials

Oreochromis sp. juveniles at uniform sizes and weights were used in this research. Tilapia fry were obtained from the Hatchery Research Center, state of Pahang, Malaysia. Prior to the start of the experiment, all juvenile fish were acclimatized to laboratory conditions for one week in a tank. Before the start of the experiment, fish were treated with 0.5% sodium chloride to prevent any fungal infection and attack form parasites (Rowland and Ingram, 1991). During the acclimatization period, fish were fed commercial feed (Takara Sakana-II, Fish Food, Kian Weng Trading Co.). The feeding rate consisted of 5% of the fish biomass and the ration was adjusted biweekly each time after the fry were weighed on an electronic top pan balance (AND EW-I Series). Feeding was carried out twice daily throughout the experimental period. Fish were hand-fed twice a day at 10.00 am and 5.00 pm. The amount of feed used was recorded throughout the experimental period of 56 days each.

3.2.1 Weighing Procedure for Sampling

Fish were weighed individually at the start and final of the experiment and bulk weighed for each 14 days or two weeks with the ration adjusted accordingly. An initial sample was taken at the start of the feeding trial, and after the final week of experimental period the final sampling was performed. Fish were netted, gently blotted on the soft paper tissue before individual weighing on the electronic top pan balance (AND EW-I Series) (Appendix 1 (a); Figure 8).

For all intermediate weight measurement, fish were bulk-weighed once in two weeks. All fish in each aquarium were netted using fine mesh hand net. Excess water was then removed from the fish by blotting the net on the soft paper tissue. Fish were transferred to a tarred, water-filled container and weighed collectively to the nearest 0.01 g. The biweekly mean weight of fish was used to calculate the daily food ration for

the following week. 10 fish with uniform sizes and weight gain were stocked in 15 aquaria of 30 L (45 x 30 x 25 cm) according to the diet treatment before the start of experiment. After weighing, fish were transferred again to each aquarium treatment in triplicate.

3.3 Diet Formulation and Preparation

Super worm meal (*Zophobas morio*) used as protein sources in this study were obtained from an Aquarium Shop in Petaling Jaya district, state of Selangor, Malaysia. They are locally available and commonly used as reptile and bird food supplements in Malaysia. The common super worm available in abundance is *Zophobas morio*, commonly found widely in Malaysia nowadays as the climate was suitable for their growth. The super worm were then sacrificed by lowering the body temperature in a freezer, stored in plastic bags for each 10 kg and frozen before drying at 70 °C in the oven for 24 hours. The dried super worm then was mashed through the hammer mill to get the super worm meal in a fine and homogeneous form. All the raw ingredients and the super worm meal were used within one year and proximate composition was analysed before any diet formulation to check the nutritional quality. The ingredients were kept in ventilated room under ambient temperature but super worm meal was stored at 20 °C (temperate condition) to avoid bacterial contamination.

Generally, all experimental diets were isonitrogenous (crude protein 32% as fed basis). All diets contained the minimum requirement of all essential nutrients to satisfy the needs of Nile tilapia fry or fingerlings. The diets were formulated using the WinFeed version 2.8 Software (Least Cost Feed Formulation). Fish meal (aquaculture grade) was supplied by Freshwater Fisheries Research Centre, Glami Lemi. Soybean meal and rice bran were bought from a local supplier, Fajama Trading and used as protein sparing source and carbohydrate source respectively. Di-Calcium Phosphate, vitamin premix (Table 3.1) and mineral premix (Table 3.2) as well as corn starch acted

as a binder to avoid nutrient leaching after being given to fish in the feeding treatment. For digestibility study, chromic oxide was added as an indigestible marker (Cho *et al*., 1974). For certain experiment that aimed to improve the use of super worm as a protein sparing source to fish meal, DL-Methionine (Appendix 1 (a); Figure 13) and mushroom stalks were utilized as amino acid and prebiotic supplements to enhance the growth performance of target species. Diets were prepared by mixing the dry ingredients, followed by the addition of water slowly until dough was formed. Prior to that, the dry ingredients were then weighed out according to a formulation and measurement calculated, placed in the appropriate bowl accurately. The steam conditioned Pellet Mill (KCM, Y132M-4, See Appendix 1 (a); Figure 9) was used to pellet the diets using a die size 2 mm. The resulting pellets were then dried on the shelves of the oven at 70 °C for the whole day. The diets were stored in plastic bags under ambient conditions over the experimental period. Prepared diets samples were analyzed for proximate analysis.

Table 3.1 Composition of vitamin premix used in this experiment*

Vitamin	mg / kg of premix
Vitamin A (Retinol palmitate)	500 IU
Vitamin D_3(Cholecalciferol)	100 IU
Vitamin E (Tocopherol acetate)	75000
Vitamin K_3	20000
Vitamin B_1 (Thiamine)	10000
Vitamin B_2 (Riboflavin)	30000
Vitamin B_6 (Pyridoxine)	20000
Vitamin B_{12} (Cyanocobalamin)	100
Vitamin D (Pantathenate)	60000
Nicotinic Acid (Niacin)	200000
Folic Acid	5000
Biotin	0.235

*Obtained from Fajama Trading Sdn. Bhd.

Table 3.2 Composition of mineral premix used in this experiment*

Minerals	g / kg of premix
Selenium	0.2
Iron	80
Manganese	100
Zinc	80
Copper	15
Potassium Chloride	4
Magnesium Oxide	0.6
Sodium Bicarbonate	1.5
Iodine	1.0
Cobalt	0.25

*Obtained from Fajama Trading Sdn. Bhd.

Table 3.3: Proximate analysis and prices (RM / kg) of feed ingredients used

Ingredients	**DM**	**CP**	**CL**	**Moisture**	**Ash**	**Price (RM / kg)**
Fish meal	89.25	55.64	5.32	10.75	12.75	3.50
Soybean meal	88.70	53.04	1.34	11.30	7.6	2.20
Super worm	92.49	42.83	40.01	7.51	3.54	20.00
Mushroom stalk meal	88.55	12.01	0.39	11.45	10.75	-
Rice bran	89.61	11.19	9.15	10.36	9.17	1.20
Corn starch	90.40	0.60	0.14	9.61	0.05	3.00

DM = Dry matter; CP = Crude protein; CL = Crude lipid; Price = (exchange rate: RM3.04 = 1 USD in 2011)

3.4 Methods of Proximate Analysis

Proximate analysis of diets, ingredients and whole body were carried out using the procedures of AOAC protocols (1990):

3.4.1 Moisture

Moisture content was determined by air-drying the samples in an oven at 105 °C for 24 hours. It is a gravimetric measurement of water in the feedstuffs, diets and carcass expressed as a percentage of the initial sample weight. Moisture was handled under equipment (Carbolite Oven) showed in Appendix 1 (a); Figure 7. Moisture and dry matter were quantified as follows:

% Dry matter = $(W_3 - W_2 / W_1 - W_2)$ X 100

% Moisture = 100 - % Dry Matter

W_1 = Sample weight (g); W_2 = Extraction cup; W_3 = Extraction cup weight after 105 °C heated.

3.4.2 Ash

This measured the total inorganic matter by high temperature of incineration (AOAC, 1990). Approximately 1.0 g of sample was weighed into a pre-weighed crucible and incinerated overnight at 600 °C using a Naberthem muffle furnace (Appendix 1 (a); Figure 6). The increase in the final weight of crucible after incineration represented the ash and was expressed as percentage of the original sample. Ash was measured as follows:

% Ash = $(W_3 - W_2 / W_1)$ X 100

W_1 = Sample weight (g); W_2 = Crucible weight (g); W_3 = Crucible weight + ash residue after 600 °C

3.4.3 Crude Protein

The Kjedahl method according to AOAC (1990) was used for the determination in duplicate as follows; 150 mg sample was digested in concentrated sulphuric acid and two Kjeltabs performed as catalyst. Then ammonia from the digestion process (FOSS Tecator Digester Auto, Appendix 1 (a); Figure 3) was released after reaction with 40% sodium hydroxide and distilled using FOSS Kjeltec 2200 (Appendix 1 (a); Figure 3), trapped in 4% boric acid and quantified by titration against Digitrate 0.1M hydrochloric acid – titration (Appendix 1 (a); Figure 4) was operated manually and run according to the operation manual from the manufacturer. Crude protein was established by mutliplying the total nitrogen with the conversion factor of 6.25. Crude protein was calculated as follows:

% Nitrogen = [(S-B) (N) X 1.4007] / g of sample

% Protein = % Nitrogen x 6.25

S = HCL titration for sample; B = HCL titration for blank; N = Neutrality for HCL

3.4.4 Crude Lipid

The method relatively was solvent extraction using Soxhlet extraction (FOSS Soxtec 2055, Appendix 1 (a); Figure 5). Approximately about 1.5 g sample was weighed into an extraction thimble and corked with cotton. 70 ml petroleum ether (40 °C – 60 °C) was added to a pre weighed extraction cup. The thimbles were placed into the unit by fixing the metal adapters to the magnetic rings at the bottom of each condenser and the corresponding cups then fitted into the unit underneath the thimbles. Extraction which involved boiling, rinsing and evaporation was conducted following the instructions in the manufacturer's manual. Extracted lipid in the cup was dried in an

oven (100 °C) for at least an hour before weighing and expressed as a percentage of the original sample. Crude lipid was measured as follows:

$$\% \text{ Lipid} = (W_3 - W_2 / W_1) \text{ X } 100$$

W_1 = Sample weight (g); W_2 = Extraction cup; W_3 = Extraction cup weight after 100 °C heated.

3.4.5 Crude Fiber

About 0.6 g of defatted sample in a pre-weighed Scintaglass crucible was used for crude fiber determination using acid-base hydrolysis. The crucible was fitted to the Fibertec 2022 Fibercap and run according to the manufacturer's operating instructions. Hydrolysed and oven-dried sample was later ashed in the muffle furnace at 600 °C for 4 hours and crude fiber in the defatted sample expressed as a percentage of the original undefatted sample. Crude fiber was determined as follows:

$$\% \text{ Fiber} = \frac{W_3 - (W_1 \text{ x } C) - (W_5 - W_4 - D)}{W_2} \text{ x } 100$$

W_1 = Capsule weight (g); W_2 = Sample weight (g); W_3 = Capsule weight after 130 °C; W_4 = Empty crucible weight; W_5 = Crucible weight after 600 °C.

3.4.6 Nitrogen Free Extractives

This was estimated by subtracting the total of moisture, crude protein, crude lipid, ash, and crude fiber from 100.

3.5 Amino Acid Analysis

The amino acid contents of the ingredients used for food formulation were analyzed according to the procedures described below:

The PICO TAG method with slight modification was adapted for determining the amino acid profile of the samples (Khan *et al*., 1994). 0.25 g of ingredients were hydrolysed in evacuated sealed ampoules with 15 ml of 6N hydrochloric acid (HCL) in a closed test tube shaken and then flushed with nitrogen for 1 minute prior to being put in an oven at 110 °C for 24 hours. After cooling, 10 ml of internal standards (α-amino butyl acid) were added to the mixture. The mixture was then poured into the volumetric flasks and deionized water was added until 50 ml. The mixture was then filtered using Whatman No. 1 filter paper. The hydrolysed sample can be kept for 4 weeks at -20 °C.

Then, 10 µl sample was put into the vial before the sample was dried under a vacuum for 30 minutes. 20 µl of the redrying solution (methanol: water : triethylamine, 2: 2: 1, v/ v/ v), was added to the dried sample and the mixture was shaken vigorously for 1 minute. The sample was dried again for 30 minutes followed by the addition of derivatization reagent (methanol: triethylamine: water: phenylisocyanate, 7: 1: 1: 1, v/ v/ v/ v). After derivatisation, the samples and standard were added with 100 µl sample diluents; injected into the HPLC (Jasco, CO-2065 Plus, Intelligent Column Oven, Appendix) using column (Purospher STAR RP-18 encapped, 5µm) in the volume of 20 µl and 8 µl respectively. The amino acids were determined by comparison of peak retention times to a known standard. Peak data was detected by the MD-2010 Plus, Multi Wavelength Detector and processed using appropriate software (BORWIN-PDA). The operating temperature was 43 °C. The absorbance at 254 nm was used for calculations. Tryptophan could not be analyzed because of its destruction during acid hydrolysis. The eluent A (0.1M ammonium acetate, pH 6.5) and eluent B (0.1M

ammonium acetate containing acetonitrile and methanol, pH 6.5) were used as mobile phases. Gradient conditions were used as shown in Table 3.3 below:

Table 3.3: The gradient table of Buffer A and Buffer B

Time (Min)	Buffer / Eluent A (%)	Buffer / Eluent B (%)
0	100	0
15	90	10
30	60	40
40	50	50
50	0	100
55	0	100
57	100	0
60	100	0

3.6 Fatty acid analysis

Methyl esters were prepared by sodium methoxide method. In the sodium methoxide method, sodium methoxide was used as a catalyst to interesterify fatty acid. This method is applicable to saturated and unsaturated fatty acid containing from four to twenty four carbons atoms. Fatty acid methyl esters (FAME) were analyzed using a HP 6890 Series (Hewlet Packard) gas chromatography equipped with flame ionization detector and fused silica capillary column (30 mm x 0.32 mm) with 0.25 μm (Model BPX70). The injection port and detector were maintained at 220 °C and 250 °C. The oven was programmed at initial temperature 115 °C hold 0 min, ramp 1 : 8c/min 180c hold 10 min, ramp 2 : 8c/min 240c hold 10 min The carrier gas was helium (1.6ml/min). Identification of fatty acids was made by comparing the relative retention times of FAME peaks from samples with standard from SUPELCO. The peak areas were determined by the Agilent ChemStation. Data were measured using the normalized peak area percentages of fatty acids.

3.7 Chromic Oxide Analysis

Chromic oxide was determined according to the method of Furukawa and Tsukahara (1966). The procedure depends upon the digestion of the sample by concentrated nitric acid and oxidizing chromic oxide with 70% perchloric acid. The orange colour formed by the oxidation of chromium III to chromium IV is read on a spectrophotometer at 425 nm against distilled water.

100 mg of sample was weighed into a Kjedahl flask. 5 ml of concentrated nitric acid were added to the flask and the mixture was boiled on the heater for about 60 minutes until yellowish vapour stop rising. After cooling the sample, 3 ml of 70% perchloric acid was added to the flask. The mixture was then gently heated on 220 °C again until the solution turned from a green to an orange colour after which it was left to boil for a further 10 minutes to ensure oxidation was completed. The solution was

transferred to a 50 ml volumetric flask and added with distilled water until it was filled up to 50 ml. The absorbance of the solution was determined by spectrophotometer (UV-1700 PharmaSpec, Shimadzu Corporation) at 425.4 nm against distilled water. Chromic oxide was measured according to the formula:

Chromic oxide (%) = [(absorbance-0.0032) / 0.2089 / sample weight (g)] x 100

3.8 Analysis of Experimental Data

Experimental data gathered during the growth trial and results from analysis of diets, faeces and carcasses were used to determine various biological parameters namely: growth performance; food conversion ratio and protein efficiency ratio.

3.8.1 Growth performance

Parameters used to evaluate growth performance in this study were weight gain by fish and specific growth rate (SGR). SGR is the most commonly used expression of fish growth.

Weight gain (WG): The difference between the final body weight and the initial body weight of fish. Live weight gain also reported as percentage gain, which is usually expressed as a percentage of final weight gain divided by the initial weight gain.

WG = (FBW- IBW/IBW) x 100

FBW is final body weight (g); IBW is initial body weight (g). These weights are average body weight.

Specific growth rate (SGR): The instantaneous change in weight of fish expressed as the percentage increase in body weight per day over any given time interval. It is calculated by taking natural logarithms of body weight and expresses growth as % / day (Ricker, 1979).

SGR = (ln FBW – ln IBW / *t*) x 100

t is the number of experimental period.

Survival rate: The difference between the final number of juveniles surviving after the end of the experiment and the number of juveniles at the start of experiment. Number of fish mortality was deducted from the initial. Unless the percentage is divided by the time of the experiment, survival is not reported as a rate (Glencross *et al.,* 2007).

3.8.2 Feed Conversion Ratio (FCR)

FCR is defined as the amount of dry feed fed per unit live weight gain. It often serves as a measure of efficiency of the diets. The more suitable the diet for growth, the less food is required to produce a unit weight gain, lower value indicates an improved outcome and less discharge of phosphorus wastes (De Silva and Anderson, 1995). It was calculated as:

FCR = food fed (g) / live weight gain (g)

The main problem was that FCR usually given in wet weight of both food and weight gain. Some foods contain much more moisture than others. This may cause bias that is not necessarily related to the nutrient content.

3.8.3 Protein Efficiency Ratio (PER)

Protein efficiency ratio is an expression which relates the gram of weight gained to the gram of crude protein fed according to the formula given (De Silva and Anderson, 1995):

PER = weight gain (g) / crude protein fed (g)

Although this method makes no allowance for protein used for maintenance, it is still widely used as a method of determining appropriate protein sources for fish diets. Thus, this parameter gives a measure of how well the protein source in the diet provides for the essential amino acid requirement of the fish.

3.8.4 Body composition of fish

Whole body proximate analysis was used to determine body composition of fish. The proximate analysis followed by methods described in Section 2.4 and components such as moisture, crude protein, crude lipid and ash were analysed as percentage of fresh weight. At the end of each experiment, 10 fish were randomly selected from each treatment, including the control.

3.9 Statistical Analysis

The experimental design used in this study was mainly completely randomized design (CRD) where different dietary treatments were randomly assigned to the experimental units. The null hypothesis tested in this study was: there is no significant different between dietary treatment means. All growth data were subjected to one – way analysis of variance (ANOVA). The significance of difference between means was determined by Duncan's multiple range test ($P<0.05$) using SPSS for Windows (version 12). Values are expressed as means ± SE.