Chapter 2

General Materials and Methods

2. MATERIALS AND METHODS

2.1 Experimental System

All experiments were carried out in a temperature controlled recycling water system (Table 2.1 and Figure 2.1) in a tropical aquarium. The system consisted of 32 cylindrical self-cleaning centrally drained tank units (with lids) of 30-L capacity, 40-cm diameter and 25-cm depth each. Water was supplied to each tank at a rate of 1-L min^{-1} from a 250-L header tank. Water drained from the tanks through central standpipes in to a series of filtration tanks. The water was then pumped up to a 250-L header tank where it was aerated using airstones and heated using 3KW thermostatically controlled immersion heaters to maintain a temperature of 28 ± 1°C throughout the experimental period. About thirty percent of the water in the system was replaced biweekly with freshwater to adjust water quality (Table 2.4) and to avoid accumulation of faeces and uneaten feed or any excretory products. A constant photoperiod of 12 hours light and 12 hours dark was maintained and the light hours were between 8.30am to 8.30pm.

Table 2.1 Description of the Experimental System

Description	
A. Experimental Tank:	
Number	32
Volume (L)	30
Diameter (cm)	40
Depth (cm)	25
Water flow rate (L/min^{-1})	1
B. Filtration Tank:	
Number	6
Volume (L)	100
C. Volume of Sump Tank (L)	120
D. Volume of Header Tank (L)	250

2.2 Experimental Fish

A tropical fish species, African catfish, *Clarias gariepinus* (Burchell, 1822) was used as a model for this research. The brood fish (male and female) were reared in captivity to sexual maturity and bred in the tropical aquarium and hatchery complex of Institute of Aquaculture, University of Stirling. Breeding was carried out artificially using Ovaprim (Glaxo India Limited) as described by Hossain (1998). Successfully hatched larvae were kept in the hatching trays until day 3 of exogenous feeding (Haylor, 1992). First feeding was carried out with *Artemia* (Argent Chemical Laboratories, USA) hatched cysts for the next 4 days. On the following day, larvae were siphoned out from the hatching trays through a 5 mm clear plastic tubing into a bucket and then transferred to a rearing tank (by gentle pouring from the bucket) where they were reared for 2-3 months to the desired sizes for each experiment. Fish (average weight 8-13g) were obtained for the experimental work from the stock and randomly assigned into groups of 20 fish. Each fish group was placed in an individual experimental tank (30-L).

2.3 Experimental Diets

This involved diet formulation and preparation as detailed below:

2.3.1 Diet Formulation

Fishmeal (herring type) and soybean meal (dehulled, solvent extracted) were used as dietary protein sources and with wheat flour (whole wheat) were donated by BioMar Ltd. Scotland. Corn starch (Sigma 4126) and wheat flour were used as sources of carbohydrate. Lipid sources were a mix of equal amounts of fish oil and corn oil. Equal proportions of fish oil and corn oil were incorporated into the experimental diets to ensure adequate supply of fatty acids of both n-6 and n-3 series, assumed to be essential to African catfish. α-cellulose (Sigma 8002) was used as a filler and carboxymethyl cellulose (Sigma 5013) was used as a binder at a rate of 2%. 0.5% chromium (III) oxide (BDH 277572Q) was used as inert indicator for digestibility

studies. The vitamin premix (Table 2.2) and mineral premix (Table 2.3) were mixed into the diets as nutrient supplements at a rate of 1%. The premixes were those established at the Nutrition Group of the Institute of Aquaculture, University of Stirling, as the most appropriate for freshwater fish. To obtain the required protein, lipid, carbohydrate and energy levels, the amounts of α-cellulose, starch and oil were varied in the diets. The digestible energy (DE) content of the diet was estimated using published DE values for channel catfish i.e. 14.67 kJ/g protein, 33.94 kJ/g lipid and 10.48 kJ/g carbohydrate (NRC, 1993).

Table 2.2 Composition of the vitamin premix used in experimental diets

Nutrient	mg/kg*	Source
Retinol Palmitate (Vitamin A)	1000.00	Sigma R3750
Cholecalciferol (Vitamin D)	4.00	Sigma C9756
Tocopherol Acetate (Vitamin E)	7000.00	Sigma T3376
Vitamin K	1500.00	Sigma V3501
Thiamine Hydrochloride (Vitamin B_1)	4250.00	Sigma T4625
Riboflavin (Vitamin B_2)	3000.00	Sigma R4500
Pyridoxine Hydrochloride(Vitamin B_6)	1250.00	Sigma P9755
Cyanocobalamin (Vitamin B_{12})	1.25	Sigma V2876
Ascorbic Acid (Vitamin C)	37500.00	Sigma AO278
Biotin (Vitamin H)	90.00	Sigma B4501
Choline Chloride	74050.00	Sigma C1879
Folic Acid	1000.00	Sigma F7876
Calcium Pantothenate	5250.00	Sigma P9153
Niacinamide	12500.00	Sigma N3376
Myoinositol	25000.00	Sigma I5152
Ethoxyquin	200.00	Sigma E8260

* This premix was made up to 1 kg with α-cellulose

2.3.2 Diet Preparation

Dry ingredients were mixed for about 30 minutes in a Hobart mixer (Belle, Mini 150; England) to ensure that the mixture was well homogenized and then blended with oil for about 15 minutes. Water was added at 20-30% v/w to give a pelletable mixture. A steam conditioned California Pellet Mill (model CL2, San Francisco, California) was used to pellet the diets. An appropriate die was used to give pellets of desired sizes (1.0 to 3.0 mm) depending on fish size. Pellets were dried by convection at 40°C overnight in a drying cabinet. The dried pellets were then packed in polythene bags, sealed and stored at –20°C until used.

Table 2.3 Composition of mineral premix used in experimental diets

Minerals	Chemical Formula	g/kg	Source
Magnesium Sulphate	$MgSO_4 .7H_2O$	510.00	BDH291172L
Sodium Chloride	NaCl	200.00	BDH 26281
Potassium Chloride	KCl	151.11	BDH 101984L
Iron Sulphate	$FeSO_4.7H_2 O$	100.00	BDH 101124L
Zinc Sulphate	$ZnSO_4. 4H_2O$	22.00	BDH 102994R
Manganese Sulphate	$MnSO_4 .4H_2O$	10.15	BDH 101534M
Copper Sulphate	$CuSO_4 .5H_2O$	3.14	BDH 10091
Cobalt Sulphate	$CoSO_4 .7H_2O$	1.91	BDH 27801
Calcium Iodate	$CaIO_3.6H_2O$	1.18	BDH 27602
Chromic Chloride	$CrCl_3 .6H_2O$	0.51	BDH 27752

2.4 Experimental Practices

2.4.1 Acclimation and Weighing Procedure

Prior to initiation of each experiment, fish underwent a 2-week conditioning during which they readily adjusted to a commercial trout diet (Trout Fry 02 crumble 1.0- 1.5 mm, BioMar Ltd., Scotland). At the beginning of the experiment, fish were individually weighed applying anesthesia with 1% benzocaine (Ross and Geddes, 1979). Fish were gently wiped dry with soft tissue paper and weighed on a Mettler PM 6000 balance to the nearest 0.1g. Fish were weighed individually at the beginning and end of every feeding trial, and bulk-weighed fortnightly in between. Fortnightly bulk weights were used to adjust the daily feed ration for the following 2 weeks.

2.4.2 Experimental Period

All experiments were conducted for 8 weeks.

2.4.3 Fish Feeding

Fish were offered either restricted feeding (5% of body weight or 1% body weight as maintenance requirement) or appetite feeding subdivided into three equal feeds at 10:00, 14:00 and 18:00 h every day as dictated by experimental design. After a stipulated period of feeding (20 to 30 min.) unconsumed feed, if any was collected on a fine mesh sieve, dried weighed and subtracted from food offered. Fish were deprived of food on the day of weighing and tanks were thoroughly scrubbed and rinsed with water. Each treatment group had three replicates and were fed according to the experimental protocol.

2.4.4 Faeces Collection

Faecal matter was collected once a day at about 08.30am before feeding commenced. Faeces collection was performed by siphoning material from the bottom of each tank, which was then freeze-dried. Faeces collected from each tank were pooled together according to treatment. Dried faecal samples were kept in airtight containers until analyzed. Tanks were also cleaned in the evening after feeding to remove uneaten food and faeces. Faecal collection started from the third week of the study and continued until sufficient faecal matter was collected.

2.5 Water Quality Management

Dissolved oxygen, pH, temperature, total ammonia (NH_3 –N), NO_3 –N and NO_2 –N were measured as follows:

2.5.1 Dissolved Oxygen

Dissolved oxygen was monitored biweekly using an oxygen meter (YSI 57 Clandon, Ohio, USA).

2.5.2 pH

pH was monitored biweekly using a pH meter (CG 840, Schott).

2.5.3 Temperature

Temperature was measured weekly using an oxygen meter temperature probe (YSI Clandon, Ohio, USA). Water temperature was controlled using a 3 KW electric heater controlled by a Deem 10/1193 thermister linked to an on/off controller set at 28 ± 1°C.

2.5.4 Ammonia Nitrogen (NH_3 –N)

Ammonia nitrogen as NH_3 - N (mgL^{-1}) was determined using a Tecnicon Auto Analyser II according to standard methods (SCA, 1982a)

2.5.5 Nitrate Nitrogen (NO_3 –N)

This was measured using a Tecnicon Auto Analyser II according to standard method (SCA, 1982b) as NO_3- N (mgL^{-1}).

2.5.6 Nitrite Nitrogen (NO_2–N)

The procedure was the same as for NO_3-N and determined as NO_2 –N (mgL^{-1}).

Table 2.4 Water quality parameters measured during the all experiments combined together with suggested acceptable ranges.

Parameters:	**Range**	**Acceptable Ranges**
Dissolved oxygen (mg/L)	6.50 – 7.60	> 3.00[1]
pH	6.45 – 7.30	6.5 – 8.00[1]
Temperature (°C)	27.00 – 29.00	10.00[2] – 35.00[3]
Ammonia nitrogen (mg/L)	0.08 – 0.37	< 0.05[1]
Nitrate nitrogen (mg/L)	0.39 – 6.07	< 250.00[1]
Nitrite nitrogen (mg/L)	0.02 – 0.24	< 0.25[1]

1. Viveen *et al.*, (1985)
2. Hoffman *et al.*, (1991)
3. Jauncey, personal communication

2.6 Experimental Analyses

This comprised chemical analysis and biological evaluation.

2.6.1 Chemical Analysis

General chemical analyses performed consisted of proximate analysis of feedstuffs, diets and carcass, as well as energy levels in diets, faeces, and chromic oxide in faeces and diets. Energy and protein determination in faeces and diets for digestibility studies used a CHNS/O series II combustion analyzer (Perkin Elmer) according to method of Pantazis and Jauncey (1996). Energy contents of whole fish samples were calculated using gross energy values of 23.6 and 39.5 kJ/g for protein and lipid respectively (Jauncey, 1998). Proximate analyses for moisture, protein, lipid, ash and crude fibre, were performed using standard procedures (AOAC, 1990). Fish within each group were pooled for carcass analysis. All samples were analyzed in triplicate.

2.6.1.1 Moisture

This is gravimetric measurement of moisture in the feedstuffs, diets, and carcass – expressed as a percentage of the initial sample weight. A representative sample was dried to constant weight in an oven maintained at 110°C.

2.6.1.1.1 Procedures

One gram of sample was pre-weighed (W_1) on a foil tray and placed in an oven (Gallenkamp) at 110°C for 24 hours. The sample was removed from the oven, cooled in a dessicator, and reweighed (W_2). Moisture percentage was calculated according to the formula:

$$\text{Moisture (\%)} = (W_1 - W_2) / W_1 \times 100$$

2.6.1.2 Crude Protein (CP)

Crude protein was determined by the Kjeldahl method (Tecator AN 3087), the most widely used method employed for the determination of protein in organic substances. This is based on the fact that on digestion with concentrated sulphuric acid and catalysts, organic compounds are oxidized and the nitrogen is converted to ammonium sulphate. Upon making the reaction mixture alkaline, ammonia is liberated, removed by steam distillation, collected and titrated.

2.6.1.2.1 Procedure

200 mg sample was placed in a Kjeldahl digestion tube. Two mercury Kjeltabs (Fisher K/0130/80) and 5 ml concentrate sulphuric acid (BDH 45006) were added to the sample. The sample was digested at 400°C (Digestion system 40 Tecator 1006 heating unit) for one hour. 20 ml of deionised water and 5 ml of 1.33N sodium thiosulphate were added to the sample after allowing it to cool. The sample was then distilled and the ammonia liberated, after adding of 25ml of 40% NaOH collected in standard boric acid and titrated against 0.2M hydrochloric acid. Both distillation and titration were semi-automated (Kjeldahl Auto 1030 Analyser, Tecator). A blank was prepared and treated in the same manner except that the tube was free of sample. Protein percentage was calculated according to the formula:

Protein (%) = (sample titre – blank titre) $\times 0.2^{1} \times 14.007^{2} \times 6.25^{3} \times 100$ / sample weight

Where,

1. Normality of hydrochloric acid
2. Molecular weight of nitrogen
3. Nitrogen factor; since protein is assumed to be 16% nitrogen

2.6.1.3 Crude Lipid

The method employed was that of solvent extraction using a Soxhlet extraction unit (Tecator Soxtech HT 1043 Extraction unit). Crude lipid in diets was determined by extraction with chloroform : methanol (2:1) solvent, whilst in feedstuffs and fish carcasses it was determined by extraction with petroleum ether. Crude lipid in diets was not recovered well by extraction with petroleum ether. However, use of chloroform & methanol in the ratio 2:1 gave better results. The method depends upon the heating of solvent, which is allowed to pass through the sample to extract the lipid. The extract is collected in a cup and, when the process is completed, the solvent is evaporated and the remaining crude lipid is dried and weighed.

2.6.1.3.1 Procedure

One gram of sample was weighed into an extraction thimble and covered with absorbent cotton. 50 ml of solvent was added to a pre-weighed cup, which contained 5 glass balls. Both thimble and cup were attached to the extraction unit (Soxtec System 1043). Extraction unit temperature was fixed at 115°C for petroleum ether as a solvent while chloroform : methanol (2:1) was at 160°C. The sample was subjected to boiling in solvent for 30 minutes followed by rinsing for 1- 1.5 hours. Solvent was evaporated from the cup to the condensing column Extracted lipid in the cup was placed in an oven at 110° C for 1 hour and after cooling the crude lipid was calculated using following formula:

$$\text{Crude lipid (\%)} = (\text{extracted lipid/ sample weight}) \times 100$$

2.6.1.4 Crude Fibre (CF)

This method depends upon digestion of moisture free and solvent extracted sample with weak acid solution and then with weak base solution. The remaining residue is ashed and the difference in weight on ashing is considered crude fibre (hydrolysis resistant organic matter).

2.6.1.4.1 Procedure

1g of defatted sample was placed in a glass crucible and attached to the extraction unit (Fibertec system 1020 hot Extractor). 150 ml of boiling 1.25% sulphuric acid solution and 10 drops of Octanol (anti-foaming agent) were added. The sample was digested for 30 minutes and then the process was stopped, the acid was drained out and the sample washed with boiling distilled water. After this, 150 ml 1.25% sodium hydroxide solution and 10 drops of octanol were added. The sample was digested for 30 minutes then the process was stopped, the alkali was drained out and the sample washed with boiling distilled water. Finally the crucible was removed from the extraction unit and oven dried at 110°C overnight. The sample cooled in a dessicator and weighed (W_1). The sample was then ashed at 550°C in a muffle furnace (Gallenkamp Muffle Furnace) for 2 hours, cooled in a dissector and reweighed (W_2). Extracted fibre was expressed as percentage of the original undefatted sample and calculated according to the formula:

$$\text{Fibre (\%)} = [(\text{digested sample } W_1 - \text{ ashed sample } W_2) / \text{sample weight}] \times 100$$

2.6.1.5 Ash

Ash content was determined as total inorganic matter by incineration of the sample at 600° C. Remaining inorganic materials are reduced to their most stable form, oxides or sulphates and are considered 'ash'.

2.6.1.5.1 Procedure

1g of sample was weighed into a pre-weighed porcelain crucible and incinerated (Gallenkamp Muffle Furnace) overnight at 600°C. The crucible was removed from the muffle furnace, cooled in dessicator and weighed. Ash content was calculated according to the following formula:

$$\text{Ash (\%)} = (\text{ash weight} / \text{sample weight}) \times 100$$

2.6.1.6 Nitrogen Free Extract (NFE)

Nitrogen free extract (carbohydrate) was calculated by subtracting the total percentages of moisture, crude protein, crude lipid, ash and crude fibre from 100%.

$$\text{NFE (\%)} = 100 - (\text{moisture}^* + \text{protein} + \text{lipid} + \text{ash} + \text{fibre}^{**})$$

* In case of dry matter basis, moisture was excluded

** Fibre is included here so that NFE represents potentially available carbohydrate

2.6.1.7 Gross Energy

Gross energy was determined either directly calorimetrically, using an adiabatic autobomb calorimeter or was calculated from protein, lipid and carbohydrate contents.

2.6.1.7.1 Bomb Calorimetry

Gross energy was determined by automatic adiabatic bomb calorimeter (Gallenkamp & Co Ltd, England). The method is based on combustion in a 'bomb' chamber, and when the sample is burned the resulting heat is measured by increase in temperature of water surrounding the bomb.

2.6.1.7.1.1 Procedure

1g of sample was pelleted with a briquette press and weighed in a crucible. The pelleted sample was connected to the firing wire, which was fitted between the electrodes, by a cotton thread. The electrode assembly was placed into the bomb and the bomb was tightened. The circuit was tested and the bomb was filled with oxygen to a pressure of 3000 Pa (30 bar). The calorimeter vessel was filled with water (total weight 3 Kg) at 21- 23°C and the prepared bomb was placed inside the calorimeter vessel and then the calorimeter vessel was placed into the water jacket. The machine was switched on and left for a while (10-15 minutes) to warm up. Prior to firing, the initial temperature of the water was checked and recorded and 10-15 minutes after firing the final temperature was recorded. Benzoic acid was used as a standard. The energy value determined from such a standard of known energy was used to calibrate the system. Finally, the sample energy content was calculated according to the formula:

Gross Energy (kJ/g) = [(final temp. - initial temp.) × 10.82]-0.0896 / sample weight (g)

Where,

10.82 = Heat capacity of the calorimeter in kJ/K.

0.0896 = Combined energy value of nickel wire and cotton in $kJ.g^{-1}$.

2.6.1.7.2 Calculated Gross Energy

Using standard factors of 23.6, 39.5 and 17.2 kJ/g for protein, lipid and carbohydrate respectively according to Jauncey (1998), protein, lipid and carbohydrate energy contents were summed to give total or gross sample energy.

2.6.1.8 Chromic Oxide

Chromic oxide in diets and faeces was analysed according to the method of Furukawa and Tsukahara (1966). The procedure depends upon the digestion of the sample by concentrated nitric acid and subsequent oxidation of chromic oxide with 70% perchloric acid. The orange colour formed by the oxidation of chromium III to chromium VI is read on a spectrophotometer (Uvikon 810) at 350 nm against distilled water.

2.6.1.8.1 Procedure

50-100 mg sample was placed in a Kjeldahl flask. 5 ml of concentrated nitric acid was added to the flask and the mixture gently boiled for 20 minute (care taken not to boil dry). After cooling 3 ml of 70% perchloric acid was added to the flask. The mixture was then gently reheated until the solution turned from green to orange plus a further 10 minutes to ensure oxidation is complete. The oxidised solution was then transferred to a 100-ml volumetric flask and diluted to volume with distilled water. The adsorbance of the solution was determined using a spectrophotometer (Uvikon 810) at 350nm and chromic III oxide was calculated according to the formula:

Weight of chromic oxide in sample = (Absorbance – 0.0032 / 0.2089)

Chromic oxide (%) = Weight of chromic oxide/ sample weight (mg)] × 100

2.6.2 Biological Evaluation

Data collected during the experimental period and subsequent analysis of diets, carcasses and faeces were used for the determination of various nutritional parameters:

2.6.2.1 Growth

2.6.2.1.1 Weight Gain

Weight gain (g) = Mean final body weight (g) - Mean initial body weight (g)

2.6.2.1.2 Coefficient of Variation (CV) of Final Weight

This is simply the standard deviation expressed as a percentage of the mean. Its formula is:

$$CV\ (\%) = S / Y \times 100$$

Where, S is the standard deviation and Y is the mean

2.6.2.1.3 Percentage Weight Gain

$$\%\ \text{Weight gain} = (W_2 - W_1 / W_1) \times 100$$

Where, W_1 is the mean initial fish weight and W_2 is the mean final fish weight.

2.6.2.1.4 Specific Growth Rate (SGR % / Day)

SGR is the average percentage weight change per day between any two weighings provided that the growth curve is exponential in form.

$$SGR\ (\%\ /\ Day) = [(\ln W_2 - \ln W_1) / (T_2 - T_1) \times 100]$$ (Brown, 1957)

Where W_1 is the initial fish weight (g) at time T_1 (day) and W_2 is the final fish weight (g) at time T_2 (day)

2.6.2.2 Food Conversion Efficiency (FCE)

FCE is defined as the fish live weight gain per unit of food fed in dry weight. FCE was calculated as follows:

FCE = Live weight gain (g) / Food fed (g dry weight)

2.6.2.3 Protein Utilisation

2.6.2.3.1 Protein Efficiency Ratio (PER)

PER is defined as fish weight gain per gram of crude protein fed. PER gives an indication of the efficiency of protein utilization and is calculated as follows:

PER = Live weight gain (g) / Crude protein fed (g) (Osborne *et al.*, 1919)

2.6.2.3.2 Apparent Net Protein Utilisation (ANPU)

NPU expresses the percentage of ingested protein that is retained by deposition in the carcass. It is usually calculated by the carcass analysis method of Miller and Bender (1955). When no correction for endogenous nitrogen losses is made the results are expressed as apparent net protein utilisation (ANPU):

ANPU (%) = $(P_2 - P_1)$ / Total protein consumed (g) $\times$ 100

Where, P_1 is the protein in fish carcass (g) at the beginning of the study and P_2 is the protein in fish carcass (g) at the end of the study.

2.6.2.3.4 Apparent Net Lipid Utilisation (ANLU)

ANLU expresses the percentage of ingested lipid that is retained by deposition in the carcass. Since no correction for the endogenous lipid losses is made the results are expressed as apparent net lipid utilisation (A NLU):

$$ANLU\ (\%) = (L_2 - L_1) / \text{total lipid consumed} \times 100$$

Where, L_1 is the lipid in fish carcass (g) at the beginning of the study and L_2 is the lipid in fish carcass (g) at the end of the study.

2.6.2.3.5 Apparent Net Energy Utilisation (ANEU)

Apparent net energy utilisation (ANEU) expresses the percentage of ingested total energy that is retained by deposition in the carcass and was calculated as follows:

$$ANEU\ (\%) = (E_2 - E_1) / \text{Total feed consumed (g)} \times \text{Dietary energy in feed (kJ)} \times 100$$

Where, E_1 is the energy in fish carcass (g) at the beginning of the study and E_2 is the energy in fish carcass (g) at the end of the study.

2.6.2.4 Digestibility Determination

The inert indicator Cr_2O_3 was used in feeds. It passes unaffected by digestion through the alimentary tract of fish. This provides a convenient method of measuring digestibility without the need of quantitative collection of faeces. This method was applied to fish using chromium III oxide as the indicator (Furukawa and Tsukahara, 1966).

2.6.2.4.1 Dry matter Digestibility

Dry matter digestibility was calculated according to Windell *et al.*,(1978) as follows:

$$\text{Dry matter digestibility (\% DM)} = 100 - [100(\%Cr_2O_3 \text{ in feed} / \%\ Cr_2O_3 \text{ in faeces})]$$

2.6.2.4.2 Apparent Nutrient Digestibility

Apparent nutrient (protein, lipid and energy) digestibility was calculated according to the formula from Maynard *et al.*, (1979) as follows:

Apparent nutrient digestibility (%) =

100–[(%Cr_2O_3 in feed / % Cr_2O_3 in faeces) × (%nutrient in faeces / %nutrient in feed)] ×100

2.6.2.5 Organ Indices (OI)

Organ indices i.e. viscerosomatic index (VSI), eviscerosomatic index (EVSI) and hepatosomatic index (HSI) were calculated as follows:

Organ indices (OI, %) = Organ weight (g) / Body weight (g) × 100

2.7 Liver Lipid Extraction

Liver lipid was determined by a modification of the method of Folch *et al.*, (1957). This consists of homogenizing liver tissue with a 2:1 chloroform-methanol mixture.

2.7.1 Procedure

300-400 mg frozen liver sample was weighed into a glass homogeniser tube and homogenised (Ultra-Turrax-England) with approximately 20 volumes of chloroform / methanol 2:1 (v/v). One liver of fish from each tank was homogenised individually. The homoginizer with sample was surrounded by ice during homogenisation. After homogenisation the extract was filtered using pre-washed filter paper into a 15-ml test tube. The homogeniser tube was then rinsed twice with a known amount of chloroform/methanol 2:1 (v/v) and the rinsing also passed through the filter paper. Finally, the filter paper was rinsed with same amount of chloroform and methanol 2:1 (v/v) solvent. 0.88% KCl was added into the mixture as a quarter of this standing volume of chloroform/methanol solvent (ratio of chloroform : methanol : aqueous

was 8:4:3) and the vessel tightly stoppered and mixed vigorously. The stopper was removed from the tube and the tube centrifuged (Super-minor centrifuge, MSE, UK) at 1500 rpm for 3 minutes. After centrifugation the upper aqueous phase was removed and discarded by vacuum filtration. The organic phase from the bottom of the tube was then evaporated under nitrogen to dryness. The dry lipid in the tube was redissolved in a small amount of chloroform / methanol 2:1 (v/v) solvent and transferred to a pre-weighed vial (W_1). The tube was rinsed well and rinsings added to vial. The vial was then dried under nitrogen and desiccated overnight before reweighing (W_2). Total lipid in the sample was calculated:

Total lipid (%) = $(W_2 - W_1)$ / sample weight $\times$ 100

2.8 Determination of Liver Glycogen

Liver glycogen was determined by a slightly modified method as described by Seifter *et al.*, (1950) and Hassid and Abraham (1957). It is actually a modification of the method originally described by Good *et al.*, (1933) for tissue of relatively high glycogen content. The method consists of the digestion of the tissue in hot 30% KOH and precipitation of the glycogen with ethanol. The glycogen is then treated with anthrone reagent and determined colorimetrically as glucose. The final quantity of glucose is multiplied by the factor 1.11 to convert to glycogen (Morris, 1948). This method has the advantages of brevity and simplicity as compared with other procedures since it eliminates the necessity for both glycogen precipitation and hydrolysis, and requires few and simply prepared reagents.

The following reagents, blanks and standards were prepared for liver glycogen determination:

(a) 30% KOH Solution

This was prepared by dissolving 30 g of KOH in 100 ml of distilled water.

(b) 95% Ethanol

95% ethanol was used (BDH 28729 6P).

(c) 95% Sulphuric acid

This was prepared by adding 95 ml (BDH AnalaR 10378) concentrated sulphuric acid in 5 ml of distilled water and cooling the resultant solution.

(d) Anthrone-Sulphuric acid 0.2% reagent:

This reagent was prepared by dissolving 0.2 mg Anthrone (Sigma A-1631) in 100ml of 95% sulphuric acid (the reagents were surrounded by ice during preparation). The reagent is not stable in solution and was made up daily as required.

(e) Blank:

A blank was prepared by adding 0.03 ml 30% KOH solution to 0.04 ml 95% ethanol and making up to 5 ml with distilled water.

(f) Glucose Standards:

A working glucose standard (100mg/dL) solution (Sigma 635-100) was used in the determination. Several dilution's with 0.03 ml of KOH, 0.04 ml of ethanol, and distilled water were made to obtain 25, 50, 75, 100, 125, 150, 175, 200 and 225 µg concentrations of glucose in 5 ml to construct a standard curve. 5ml of each dilution was treated as sample and the colour formed was measured on a spectrophotometer (Uvikon 810) at 620 nm against a blank.

2.8.1 Procedure

2.8.1.1 Sample Preparation (Digestion of the Tissue Sample)

Two liver samples (150 - 200 g each) from each tank were pooled together prior to digestion in boiling water to minimise individual differences and to obtain sufficient solution to run the determination. 300-400 mg frozen liver sample was placed in a 12-ml centrifuge tube containing 3 ml of 30% KOH. The tubes were then placed in a boiling water bath for 20 minutes for digestion. After cooling in a cold water bath, 3.75 ml of 95% ethanol was added and mixed well. The contents of the tubes were then brought to boil in a hot water bath and again cooled in cold water bath. The samples were then centrifuged at 300 rpm for 15 minutes. The resulting supernatant liquid was decanted, filtered into a 250-ml volumetric flask, and diluted to the mark with distilled water. The contents of the flask were then thoroughly mixed.

2.8.1.2 Anthrone-sulphuric Acid Reaction

A 5-ml aliquot of the sample solution was taken from the 250-ml volumetric flask and placed in a 15-ml Pyrex tube. The sample tube was then submerged in a cold water bath and 10 ml of anthrone reagent added into it. The reactants were mixed by swirling the tubes. The tubes were immediately covered and heated for 10 minutes in a boiling water bath followed by cooling in a cold water bath. The colour thus formed was read on spectrophotometer (Uvikon 810) at 620 nm against a blank. The blank was incubated alongside the tested sample and treated in the same manner except that 5 ml distilled water was used instead of 5ml sample solution.

The resultant glucose quantity (µg / 5ml) was multiplied by dilution factor of 50 in order to extrapolate the reading to the total sample size. The final quantity of glucose was multiplied by 1.11 to convert it in to glycogen (Morris, 1948).

Percentage of glycogen in the sample was calculated according to the following formula:

% glycogen = Quantity of glucose (µg / 5 ml) $\times$ 50 $\times$ 1.11 $\times$ 100/ Sample weight

2.9 Histological Analysis

Histological analysis was performed to investigate differences or abnormalities in fish livers and intestines as a result of feeding the different experimental diets. The methods of Drury and Wallington (1980) were adopted. At the end of each experiment 4 fish from each tank were sacrificed and livers and intestines were dissected. Samples were fixed in 10% neutral buffered formalin for at least 24 hours. Samples were cassetted and processed automatically for dehydration, cleaning and wax impregnation (Appendix 1). Processed samples were embedded in paraffin wax and sectioned by microtome (5 µm thickness). Samples were then stained with haemotoxylin and eosin and examined under light microscope to evaluate any differences between samples.

2.10 Enzyme Studies

Liver and intestinal enzyme activities were assessed to investigate the effect of different experimental diets.

2.10.1 Collection of Intestine and Liver

At the termination of the growth experiment fish were dissected four hours after the first feeding and the digestive tract (intestines) and livers were removed. After as much as possible of the attached fat had been removed, intestines and livers were blotted dry with tissue paper and kept in small plastic bags, individually, before freezing at –60°C until used for enzyme determination.

2.10.2 Enzyme Extraction

Frozen intestines and livers were weighed and homogenised separately in an electrical homogeniser (Utra- Turrax-England) with ice-cold physiological saline (9 g sodium chloride /L). From each tank two samples of each tissue were pooled together prior to homogenisation in order to minimize individual differences and to obtain sufficient extract to run the enzyme assays. Samples were surrounded by ice during homogenisation, and the process carried out at 200 rpm for 3 minutes. 10 ml of physiological saline was used for each 1 g of frozen intestine or liver sample. Homogenised samples were then centrifuged (Mistral 3000i Centrifuge, MSE, UK) at 3600 rpm for 15 minutes at 4°C to remove cell debris and nuclei. The resulting supernatant was used for each enzyme assay.

2.10.3 Enzyme Assays

2.10.3.1 Protease

Total protease activity was determined according to the method of Kuntiz (1947). The principle of this method is measurement of the concentration of split products (peptides and amino acids) in a casein solution after digestion by an extracted enzyme solution. The Lowry method (Sigma protein assay kit-procedure no. p 5656) was used to calculate the concentration of split product. The following substrate was prepared and used for protease activity determination.

2.10.3.1.1 Casein Substrate

The casein substrate was prepared by dissolving 1 gram of casein (Sigma 8654) in 100 ml of 0.1M phosphate buffer (pH 7.6). The suspension was heated in a water bath at 60°C for a complete dissolution of casein.

2.10.3.1.2 Procedure

Two replicates of 0.5 ml of enzyme extract from the intestine or the liver tissue were mixed in 15-ml test tubes with 2 ml of casein solution. Then tubes were incubated in a water bath at 30° C for 15 minutes. At the end of the incubation time 1 ml of acetic acid was added to each tube to terminate the reaction. Samples were then centrifuged (Centaur 2; MSE, model MSB 020C × 1.5, UK) at 3500 rpm for 10 minutes and 1ml of supernatant was pipetted out to determine protein content based on the Lowry method. Protease activity was expressed as the amount of protein digested by 0.5 ml of enzyme solution at pH 7.6 per minute at 30°C.

2.10.3.1.3 Lowry Method

Lowry reagent solution was prepared by adding 40 ml distilled water to a bottle of Lowry reagent (Sigma 1013). The solution was then well mixed by inversion. 1ml of supernatant of digested sample was mixed with 1 ml of Lowry reagent in a 15-ml test tube. The sample was left to stand at room temperature for 20 minutes and then 0.5 ml of Folin & Ciocalteu's reagent (Sigma 9252) was added with rapid and immediate mixing. The sample was left to stand for another 30 minutes at room temperature to develop the colour. The sample solution was then transferred to a 2.5-ml semi-micro cuvette (BDH 307/380/03) and the colour formed was read on a spectophotometer (Uvikon 810) at 500nm against a blank. The blank was prepared in the same manner except 1 ml of water was used instead of 1 ml of supernatant.

2.10.3.1.4 Protein Standard

Protein standard was prepared by dissolving 1.98 mg of bovine serum albumin (Sigma P7656) in 4.96 ml of distilled water to a concentration of 400µg /ml. Several dilutions with distilled water were made to obtain 50, 100, 200, 300, and 400 µg of protein in 1 ml to construct standard curve. 1ml of each dilution was then treated as sample and blank and the colour formed measured on spectrophotometer (Uvikon 810) at 500 nm against a blank.

2.10.3.2 Lipase (EC 3.1.1.3)

Lipase activity was determined according to a Sigma diagnostic test-kit (Procedure no. 800). The procedure is based on the hydrolysis of triglycerides in olive oil into fatty acids and diglycerides.

$$\text{Olive Oil} \xrightarrow{\text{Lipase}} \text{Fatty acids + Diglycerides}$$

The amount of fatty acids formed under the specific conditions of the test, is a measure of lipase activity in the sample. The fatty acids formed are determined by titration with 0.05N sodium hydroxide.

2.10.3.2.1 Procedure

Two replicates of 1 ml of enzyme extract from the intestine or liver were mixed in 15-ml test tubes with 3 ml of Sigma lipase substrate (Sigma 800-1) and 1ml of Trizma buffer (Sigma 800-2). The tubes were shaken vigorously and were then incubated in a water bath at 30°C for 3 hours. A blank for each tank was incubated alongside the tested sample and treated in the same manner except that 1 ml of distilled water was used instead of extracted enzymes. Immediately after starting the incubation, 1 ml of enzyme extract was placed into a

Erlenmeyer flask and stored in a refrigerator (2 – 6°C) for 3 hours, this was marked as blank. At the end of the incubation period, the contents of blank tubes were poured into the blank flasks and the contents of sample tubes were poured into clean 50-ml Erlenmeyer flasks. 3 ml of 95% ethanol were used to rinse each of the two tubes (sample and blank) and were poured into their respective flasks. 3 ml 95% ethanol were added to each tube to terminate the reaction. Six drops of thymolphthalein indicator solution (Sigma 800-3) were added to each flask (sample and blank). Samples and blanks then were subjected to titration with 0.05N sodium hydroxide solution. Each flask was titrated to a slight but definite blue and the burette reading was recorded after each titration. The initial reading was subtracted from the final reading for each titration. The difference in titration volume between sample and blank determined the quantity of fatty acids liberated during incubation time by 1 ml of extracted enzyme solution.

2.10.3.3 a -Amylase (EC 3.2.1.1)

α-amylase activity was assayed according to the procedure of Tietz (1970) using soluble starch as a substrate.

Principle

The method depends upon the liberation of maltose from the substrate by extracted enzyme solution under standard conditions. Starch is hydrolysed by α-amylase to maltose and limit dextrin's which in turn become reduced to glucose by maltase. The resultant maltose reacts with 3, 5 dinitrosalicylic acid to yield nitrosaminosalicylic acid. The colour change accompanying this reaction, measured as absorbance at 546 nm, is proportional to the amount of maltose formed and hence to the catalytic action of the α-amylase on starch.

The following substrate, reagent and standards were prepared for α-amylase determination:

2.10.3.3.1 Starch Substrate

The starch substrate was prepared by dissolving 1 gram of starch (Sigma 9765) in 100 ml of 0.1M phosphate buffer (pH 7.0). Concentrated HCl and 0.5N NaOH were used to adjust the pH.

2.10.3.3.2 Dinitrosalicylic Acid Reagent

The dinitrosalicylic acid reagent was prepared by dissolving 5 grams of 3,5-dinitrosalicylic acid (Sigma 0550) and 150 grams of sodium potasium tartarate (Sigma 6170) in 150 ml of distilled water and 200 ml of 1N sodium hydroxide. The mixture was refluxed in a water bath at 60° C until all components were totally dissolved and then made up to 500 ml with distilled water.

2.10.3.3.3 Maltose Standard

The maltose standard was prepared by dissolving 20 mg of maltose (Sigma 9171) in 20 ml of distilled water. Various dilutions with distilled water were made to obtain a 1000, 500, 400, 300, 200, 100, and 50 µg concentrations of maltose in 1ml to construct curve. 1ml of each dilution was treated as sample and the color formed was read on spectrophotometer (Uvikon 810) at 546 nm against a blank.

2.10.3.3.4 Procedure

Two replicates of 200 µl of enzyme extract from the intestine and liver were mixed in 15-ml test tubes with 1 ml of starch substrate solution (in 0.1M phosphate buffer, pH 7.0). The tubes were incubated in a water bath at 30°C for 15 minutes. At the end of the incubation time 2 ml of dinitrosalicylic acid reagent was added to terminate the reaction. Samples were then incubated in a boiling water bath for 5 minutes and left to cool at room temperature to develop

the colour. The sample solution was then transferred to 2.5-ml semi-micro cuvette (BDH 307/380/03) and the colour formed was read on spectrophotometer (Uvikon 810) at 546 nm against a blank. A blank was incubated alongside the tested sample and treated in the same manner except that 200µl of distilled water was used instead of extracted enzyme solution.

α-amylase activity was expressed as the amount of maltose liberated by 200µl of enzyme solution at pH 7.0 per minute at 30°C.

2.11 Blood Plasma Assays

Blood plasma assays were designed to investigate the effect of different experimental diets.

2.11.1 Collection of Blood

At the end of the experiment, four fish from each tank were randomly selected 17 hours after the last meal to determine plasma composition. The fishes were anaesthetised in a weak solution of benzocaine (1% benzocaine) until the fish just lost orientation. Blood was then withdrawn from the vein under the lateral line by inserting a needle (25 gauge) of a sterile disposable syringe (1ml capacity). Approximately 0.5 - 0.8 ml of blood was quickly withdrawn from each fish and transferred into a 0.75-ml stoppered plastic Micovette CB 1000 S (SARSTEDT 17.1521) tube containing heparin.

2.11.2 Plasma Preparation

The plasma was separated from blood cells by centrifugation at 8,000 rpm for 5 minutes (using a Microcentaur Centrifuge, MSE Ltd., Sussex, England) within 30 minutes of collection to minimize glycolysis. Plasma samples of four fish from each tank were pooled together prior to preservation in order to minimise individual differences and to obtain sufficient plasma samples to run the all determinations. The pooled plasma aliquots were quickly transferred to two stoppered plastic Eppendorf tubes and preserved by freezing at –60°C until assay.

2.11.3 Plasma Assays

2.11.3.1 Glucose

Concentrations of plasma glucose were assayed with the aid of a Sigma diagnostic test kit (Procedure no. 315) and an ELISA reader (Dynatech MR 5000). The plasma glucose concentration was quantified spectrophotometrically by the glucose (Trinder) enzymatic reaction. Initially glucose is oxidised to gluconic acid and hydrogen peroxide catalised by glucose oxidase. The hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulphonate to form a quinoneimine dye, which can be read at its maximum absorbance of 505 nm. The intensity of the colour produced is directly proportional to the concentration of glucose in the sample. The enzymatic reactions involved in the assay are as follows:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{p-Hydroxybenzene Sulphonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$

2.11.3.1.1 Procedure

To quantify multiple samples more rapidly, a modification of the test-kit method was made. An ELISA reader (MR5000, Dynatech, UK) was used to read the samples, standard and blank at 550 nm in a multiscan spectrophotometer by using 12 × 8 well micotitre plates (Nunc, UK). The glucose standard (Sigma 16-300) was used as a standard in this assay. Three replicates of 5 µl of each plasma sample were mixed with 150 × 2 µl of Trinder reagent into a microtitre plate. Three replicates of blank and standard samples were then also mixed separately with 150 × 2 µl of Trinder reagent into the same microtitre plate in the same manner except that 5 µl of distilled water for blank and 5 µl of standard solution was used instead of plasma sample. After 18 minutes at room temperature, the OD of the samples, standards and blanks were read at 550 nm in a multiscan spectrophotometer (MR5000, Dynatech, UK). The concentration of the glucose in each sample (mg/ dL) was calculated as described in the test-kit instructions.

Glucose Concentration (mg/dL) of Sample =
(Sample absorbance - Blank absorbance) / (Standard absorbance - Blank absorbance)×300*
* Concentration of standard

2.11.3.2 Triglycerides

Plasma triglycerides concentration was assayed by using the procedure of a Sigma diagnostic test-kit (Procedure no. 339). Triglycerides (GPO-Trinder) reagent was used for the quantitative enzymatic determination of triglycerides in plasma at 540 nm. Methods for triglycerides determination generally involve enzymatic or alkaline hydrolysis of triglycerides to glycerol and free fatty acids followed by either chemical or enzymatic measurement of the glycerol released. Triglycerides are hydrolised by lipase to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions catalysed by glycerol kinase, glycerol phosphate oxidase and peroxidase.

The enzymatic reaction involved in the assay is as follows:

(A) Triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol.

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol + Fatty Acids}$$

(B) The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-1-phosphate and adenosine diphosphate (ADP).

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-1-phosphate + ADP}$$

(C) Glycerol-1-phosphate is oxidised to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing hydrogen peroxide.

$$\text{Glycerol-1-phosphate} + O_2 \xrightarrow{\text{G PO}} \text{DAP} + H_2O_2$$

(D) A quinoneimine dye is produced by the peroxidase (POD) catalized coupling of 4-aminoantipyrine (4-AAP) and sodium n-ethyl-N (3 sulphopropyl) m-anisidine (ESPA) with H_2O_2 , which shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly propotional to glyceride concentration of the sample.

$$H_2O_2 + \text{4-AAP + ESPA} \xrightarrow{\text{POD}} \text{Quinonemine Dye} + H_2O$$

2.11.3.2.1 Procedure

The triglycerides (GPO-Trinder) reagent was prepared by dissolving 10 ml of deionized water in the vial. The reagent was then warmed in a water bath to 30°C. Three replicates of 5 µl of plasma sample were mixed with 1 ml Triglycerides (GPO Trinder) and 10 µl of deionized

water in a 10-ml test tube. The tubes were mixed by gentle inversion and then incubated in a water bath at 30° C for 10 minutes. Glycerol standard (Sigma G1394) was used in this assay. Three replicates of blank and standard were incubated alongside the tested sample and treated in the same manner except that 5 µl of distilled water for blank and 5 µl of standard solution for standard was used instead of plasma sample. At the end of the incubation time the samples, the blanks and the standards were then read and absorbance recorded on spectrophotometer (Uvikon 810) at 540 nm against distilled water as a reference. The concentration of triglycrides in the plasma sample was calculated as described in the test-kit instructions according to the following formula:

Triglycerides (mg/dL) =

(Sample absorbance - Blank absorbance) / (Standard absorbance - Blank absorbance)× 250*

***** Concentration of standard

2.11.3.3 Cholesterol

Concentrations of plasma cholesterol were assayed with the aid of a Sigma diagnostic test kit (Procedure no. 352) and were quantified spectrophotometrically by the cholesterol reagent enzymatic reaction at 500 nm.

Enzymatic reactions involved in the assay system are as follows:

(A) Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase (EC 3.1.1.13) to cholesterol and free fatty acids.

$$\text{Cholesterol Esters} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol + Fatty Acids}$$

(B) The free cholesterol, including that originally present, is then oxidised by cholesterol oxidase (EC 1.1.3.6) to cholesterol-4-en-3-one and hydrogen peroxide.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

(C) The hydrogen peroxide produced is then coupled with the chromogen, 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase (EC 1.11.1.7) to yield a quinoneimine dye which has an absorbance maximum at 500 nm.

The intensity of the colour produced is directly proportional to the total cholesterol concentration in the sample.

2.11.3.3.1 Procedure

The cholesterol reagent was prepared by dissolving 20 ml deionised water in the vial as described in the kit instructions. The reagent was then warmed in a water bath at 30°C. Three replicates of 5 µl of plasma sample were mixed with 1 ml cholesterol reagent and 10 µl of deionised water in a 10-ml test tube. The tubes were mixed by gentle inversion and then incubated in a water bath at 30°C for 10 minutes. Cholesterol calibrator (Sigma C 0284) was used in this assay. Three replicates of blank and standard were incubated alongside the tested samples and treated in the same manner except that 5 µl of distilled water for blank and 5 µl of Cholesterol calibrator (200mg/dL) for standard was used instead of plasma sample. At the end of the incubation time the samples, the blanks and the standards were then read and absorbance recorded on a spectrophotometer (Uvikon 810) at 500 nm against distilled water as reference. Concentration of cholesterol in the plasma sample was calculated as described in the test-kit instructions according to the following formula:

Cholesterol (mg/dL) =

(Sample absorbance - Blank absorbance)/(Standard absorbance - Blank absorbance)× 200*

***** Concentration (200 mg/dl) of cholesterol in calibrator

2.12 Amino Acid Analysis

All the analyses were carried out using an LKB 4151 Alpha-Plus Amino acid analyser (LKB Bichrom Ltd, Cambridge). The amino acid contents of the blood plasma and the experimental diet samples were analysed according to the procedures described below:

2.12.1 Sample Preparation for Blood Plasma Samples

Two replicates of 0.05 ml of plasma sample were mixed in 1.5-ml Eppendorf tube with 0.05 ml of 10% sulphosalicylic acid to deproteinised the samples. The samples were then well mixed and placed in a fridge for 30 minutes. Samples were removed from the fridge and centrifuged (IEC, Model Micromax, USA) at 15000 rpm for 20 minutes to remove the protein precipitates. The supernatants then removed and filtered through a centrifugal microfilter in a centrifuge (Centaur 2; MSE, Model no. MSB020C × 1.5, UK) at 2000 rpm for 5 minutes. The resulting supernatant filtrate was used for amino acid analysis.

2.12.2 Sample Preparation for Experimental Diets Samples

Diet samples for amino acid analysis were hydrolysed with 5.7N HCL for 24 hours at 110°C *in vacuo*, following the procedures given in the LKB 4151 Alpha-Plus Instruction manual (1983).

2.12.3 Principles of Analysis

These analyses were based on the following principles. The prepared sample was loaded on to a column of cation-exchange resin and the amino acids were sequentially eluted by buffers of varying pH and ionic strength. In a high temperature reaction coil ninhydrin was reacted with the column eluent to form coloured compounds, the colour intensity being directly proportional to the quantity of an amino acid present in the sample. The amount of each

coloured compound is determined by a photometer measuring the amount light absorbed at 570nm. Amino acids were identified by comparison of peak retention times to a known standard and were quantified by comparison of peak areas to the same standard mixture.

2.13 Statistical Analysis

All data were subjected to analysis of variance (ANOVA) using Minitab statistical software for Windows (release 12, 1998). Comparisons among treatment means were carried out by one way analysis of variance followed by Tukey's test (0.05). Standard deviation (±SD) was calculated to identify the range of means. A dose-response analysis was also employed to determine the nutritional requirements for lipid to carbohydrate ratio (Zeitoun *et al.,* 1976). Percentage data were transformed by arc-sine transformation (Zar, 1948) prior to ANOVA and reversed afterwards.