2. GENERAL MATERIALS AND METHODS

2.1. BIOLOGY OF EXPERIMENTAL ANIMAL

Phylum : Chordata
Subphylum : Vertebrata
Class : Pisces
Order : Cyprinodontiformes
Family : Poeciliidae
Genus : Xiphophorus
Species : helleri

One of the most successful live-bearers (Ovo-viviparous), the swordtail, was brought to Europe from Mexico and Guatemala. Scientifically, the swordtail is known as *Xiphophorus helleri*. *Xiphophorus* means “sword-bearer”, and the second part of the name is in honour of the scientist, Carl Heller. With its striking appearance and lively posture, the swordtail is a delight to watch in the aquarium. It is also biologically interesting. It is omnivorous in nature and eats mosquito larvae as well as leaves and fruits that happen to fall into the water. With its ability to adapt itself to a wide range of habitats, it can thrive in brackish lagoons as well as in the freshwater ponds, rivers, streams and lakes. However, in response to different habitats it takes on different colours and shapes. The male individual of the species grows to a length of 8-10 cm (excluding the sword) (Plate 2.1) and the female to a length of 12 cm (Plate 2.2). The swordtail has a very short maturation period, taking only a few months to become sexually
mature. The male develops a long spike at the bottom of the caudal fin. The anal fin also begins to change and it develops into the gonopodium, which is the intromittent organ by which the male fertilizes the egg within the female. During courtship, the male performs an interesting pre-mating act. Placing itself in full view of the female it performs a sort of mating dance. The purpose of this dance is to attract the female’s attention and position her for a gonopodium strike, by keeping her relatively immobile during the time. The mating lasts for only a few seconds. When a productive female does not have a fertile male during maturation, the ripe ovary degenerates, and the female grows a sword to become a fully functional male within 1-2 months. No reverse transformation has been known to occur.

For rearing the swordtail in aquaria, the water should be clean, with a neutral to slightly alkaline pH (7.3-7.9) and a temperature between 26-30°C. Before parturition, the mature female can be recognized by the increased size of its belly and a conspicuous, dark pregnancy spot on its ventral side. During parturition, the female may be seen hiding or may not feed well. It is better if two or three females are released per male for breeding in a heavily planted cement tank. A fully developed female may produce as many as 150-200 young ones per breed. Although swordtail parents are not highly predatory upon their young ones, they have no built-in inhibitions about eating them. Floating plants are the best type of protection for the fry, as parents spend most of their time near the surface, sometimes feeding on their young ones. The fry are fed on planktonic organisms for first seven days and later on with chopped beef liver twice a day. After 20 days young fish are transferred from smaller tanks to larger ones where they reach maturity in four months. The interval between each breeding cycle is 4-5 weeks.
**Brief details of sword tail (Xiphophorus helleri)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origins</td>
<td>Mexico and Guatemala</td>
</tr>
<tr>
<td>Size</td>
<td>8 to 12 cm</td>
</tr>
<tr>
<td>Temperature</td>
<td>26 – 30°C</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 – 7.9</td>
</tr>
<tr>
<td>Water hardness</td>
<td>20° to 28° dH</td>
</tr>
<tr>
<td>Temperament</td>
<td>Active and Peaceful</td>
</tr>
<tr>
<td>Diet</td>
<td>Live food, flakes and freeze dried</td>
</tr>
<tr>
<td>Reproduction</td>
<td>Live bearers, floating plants and production for the fries – sometimes feeding on their young ones.</td>
</tr>
<tr>
<td>Breeding tank size</td>
<td>Prefer large tanks</td>
</tr>
<tr>
<td>Life span</td>
<td>4 – 6 years</td>
</tr>
<tr>
<td>Tank region</td>
<td>All over the tank</td>
</tr>
<tr>
<td>Gender</td>
<td>Males are slender, develop a long spike at the bottom of the caudal fin, female larger than the male, abdomen enlarged</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>1 male : 3 female</td>
</tr>
</tbody>
</table>

### 2.2. COLLECTION OF EXPERIMENTAL ANIMAL

Thirty females and ten males of *Xiphophorus helleri* were procured from local aquarium, Tuticorin, Tamil Nadu, India. They were brought to
the laboratory in plastic polythene bags containing aerated water. The broods of *X. helleri* were stocked in 1:3 ratio of male and female separately in ten cement tanks (width: 58 cm; height: 40 cm; 120 l capacity) with sufficient quantity of the macrophyte *Hydrilla* species. They were well acclimated and from these broods, juveniles were obtained for the experimental studies. Female *X. helleri* released their young ones at dawn once in 28 or 35 days (James, 1998) and they were collected from breeding tank and maintained in large cement tanks (width: 175 cm; height: 40 cm; 400 l capacity). Juveniles of uniform size (45 days old) were used for all the experimental studies.

**2.3. FEEDING AND WATER CHANGE**

The fry of *X. helleri* fed with *Artemia nauplii, Daphnia* sp., *Moina* sp., *Cyclops*, depending on their availability from day 0 to 13 thrice a day. After 13 days, the juveniles were fed on minced pieces of fresh beef liver twice in a day at 0600 and 1700 hr until the commencement of experiment (45 days). The medium was changed before feeding once in 2 days. Unconsumed feed was removed by pipette after 1 hr of feeding and dried in hot air oven at 80°C. The water used was clean, unchlorinated well water and its quality was monitored biweekly. The tanks were drained twice a week and replenished with freshwater, to remove the accumulated faeces at the bottom. The fish were kept under a natural photoperiod of about 12 hrs every day throughout the experiment.

**2.4. EXPERIMENTAL DESIGN**

Five series of experiments were conducted to investigate the effect of dietary supplementation of *Spirulina* on feed intake, growth, gonad development, fertility, leucocytes count, metabolic rates in relation to ration levels
and time and duration of feeding *Spirulina* for maximum coloration in *X. helleri*. In each series of experiments, selected feeding and growth parameters, gonad weight and fertility, coloration were estimated. In addition, gut enzymes, leucocytes count, digestibility, proximate composition, respiratory metabolism and ammonia excretion were also studied in the doctoral work.

### 2.5. ANALYSIS OF HYDROBIOLOGICAL PARAMETERS

Water used for the experiments was clean, unchlorinated well water. During the experimental period, biweekly samples of water were analysed for dissolved oxygen by Winkler’s Iodometric method, for salinity by Harvey’s titration method, pH was recorded using pH meter following the procedure given by Strickland and Parson (1972). Ammonia was estimated following the phenol-hypochlorite method (Solarzano, 1969) using de-ionised water. EDTA method was adopted to estimate the hardness of water as described by Trivedy and Goel (1986).

### 2.6. EXPERIMENTAL DIETS

Feed formulation was done by square method (Hardy, 1980) and 45% basal protein diet was prepared by using ingredients like fish meal, ground nut oil cake, tapioca flour, Maida, cod liver oil (lipid source) and vitamins and mineral mixtures (Table 2.1). The diets contained 45% basal protein for maximum growth and reproductive potential in *X. helleri* (James and Sampath, 2004c). At first dried and powdered ingredients were blended to make a homogenous mixture. Subsequently, the feed ingredients were mixed with an aliquot of boiled water and then cooked in steam for 20 minutes. The dried *Spirulina* powder was used as a source of *Spirulina* and it procured from Antenna ornamental fish farm,
Madurai, TN, India. Five diets were prepared with different levels of *Spirulina* – 0, 5, 10, 15 and 20%. All the diets were mixed with 1% chromic acid as a marker for digestibility determination. The pellets (2 mm size) were prepared with a hand operated pelletizer and dried in sunlight. The dried diets were stored in a refrigerator until use. The stability of the experiment diets was averaged to 92% during the 1 hr of feeding (Table 2.2).

Protein and lipid contents of experimental diets were determined in spectrophotometer following Lowry *et al.* (1951) and Bragdon (1951) respectively. Moisture content was analysed by drying in an electric hot air oven at 100\(^\circ\)C. Mineral content of the feed / fish samples was estimated following the method of Paine (1964). Nitrogen free extract (NFE) was calculated by subtracting the total of the protein, lipid and mineral contents from the dry weight of the feed / fish samples.

### 2.7. WATER STABILITY TEST

Water stability of the experimental diets was measured at 10 minute intervals for 60 minutes as described by Pascual *et al.* (1978). Triplicates of 3 to 4 gm samples of each test diets were weighed, placed in separate watchglass and then immersed in a trough of water for 10, 20, 30, 40, 50 and 60 min respectively (Table 2.2). The percentage water stability was determined as follows:

\[
\text{Water stability (\%)} = \frac{\text{Final dry weight of feed}}{\text{Initial dry weight of feed}} \times 100
\]
2.8. METHODOLOGY

Juveniles of *X. helleri* (mean body length: 22.58±1.26 mm; mean body weight: 0.132±0.02 g) were collected from the laboratory bred brooders and divided into 5 groups each consisting of 25 individuals. They were stocked in a circular cement cistern tanks (width: 58 cm; height: 40 cm; 120 l capacity) containing 100 l throughout the experiment. Three replicate tanks were randomly assigned to dietary *Spirulina* levels or other treatments. In each aquarium, fishes of similar body weight were reared.

2.8.1. Chemical analysis

Determination of ash

Ash content of the test diets was estimated following the method of Paine (1964).

Principle

The sample is ignited at 550°C, burning off all organic materials. The inorganic materials which does not volatilize at that temperature is called ash.

Apparatus

1. Muffle furnace
2. Silica crucibles
3. Desiccator with magnesium perchlorate dessicant.

Procedure

- Place clean crucibles in a muffle furnace at 550°C for 1 hr. Transfer crucibles from furnace to a desiccator and cool to room temperature. Weigh as quickly as possible to prevent moisture absorption. Use metal tongs to move the crucibles after they are ashed or dried.
• Weigh by difference 1 g of sample into tarred silica crucibles. Place in a muffle furnace and hold the temperature at 550°C for 6 hr. Transfer the crucibles to a desiccator and cool to room temperature.

• Then weigh the crucibles as quickly as possible to prevent moisture absorption.

\[
Ash(\%) = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100
\]

2.8.2. Biological evaluation

During the experimental period, fish were fed with known quantum of feed twice in a day (07:00 and 16:00 hrs) in a feeding tray for 2 hrs after which unconsumed feed was removed and dried in a hot air oven at 80°C. Feed consumption (mg) was estimated by subtracting the amount of unconsumed dry feed from the total dry weight of the feed offered. Feeding rate was calculated from the following formula.

\[
\text{Feeding rate (mg}^{-1} \text{ g live fish}^{-1} \text{ day)} = \frac{\text{Total feed consumed (mg)}}{\text{Initial wet weight of the fish (g)} \times \text{No. of days}}
\]

Before beginning the experiment, total body length (mm) and weight of the fish in each aquarium were measured. Total length of the 3 individuals was determined using a graph sheet and the average was taken as mean body length (mm). Mean body weight (g) was calculated by wet weight of test animals divided by total number of animals in the aquarium at that time.

\[
\text{Mean body weight (g)} = \frac{\text{Wet weight of fish (g)}}{\text{No. of fish in the aquarium}}
\]

Weight gain (growth) was calculated as the difference between the wet weight of the fish at the beginning of the experiment and that on the day of
calculation. Specific growth rate (SGR) was calculated according to the following expression.

\[
\text{Specific growth rate (SGR\%)} = \frac{\ln W_{t_1} - \ln W_{t_0}}{t_1} \times 100
\]

Where \( \ln W_{t_0} \) and \( \ln W_{t_1} \) were the logarithms of weight of the fish at the commencement and end of each sampling period and \( t_1 \) was duration (days).

Feed conversion ratio (FCR) was calculated by relating the feed consumption to gain in wet weight of fish.

\[
\text{Feed conversion ratio (FCR)} = \frac{\text{Feed consumed (g)}}{\text{Wet weight gained (g)}}
\]

From the time of gonad development and till the commencement of breeding, three females from each treatments were sacrificed at 25 days interval. Their ovaries were removed and weighed and the gonadosomatic index (GSI) was computed according to the formula of Dahlgren (1979).

\[
\text{Gonadosomatic index (\%)} = \frac{\text{Wet weight of gonad (mg)}}{\text{Wet weight of fish (mg)}} \times 100
\]

After that, ovaries (brood pouch) were dissected; eggs / embryos were separated, counted and staged according to Meffe (1985). Using a dissection microscope, eggs / embryos were sorted out into one of six categories, primarily for detection of superfetation: (i) immature ova (ova in the process of yolkig but sub-mature in size), (ii) mature ova / fertilized egg (full sized ova, unfertilized or recently fertilized), (iii) primitive streak (primitive streak present, but not yet distinguishable, (iv) early eyed (eyes present, but not full sized; little dorsal pigmentation), (v) middle eyed (eyes full sized; moderate to extensive dorsal pigmentation) and (vi) late eyed (little yolk remaining, near parturition).
Prior to sacrificing the fish for gonad estimation and the determination of embryo’s stages, caudal peduncle of sample test individuals was cut with sharp and sterilized knife to collect the blood for counting the different types of leucocytes. Muscle, skin and fins were separately collected and subjected to the color estimation following Bjerkeng (1992). Simultaneously, muscle and gonad were also isolated from the test individuals and subjected to the estimation of energy and proximate composition. Foregut, midgut and hindgut were also separately dissected out from the same test individuals to determine the chosen gut enzymes activities (amylase, protease and lipase).

Fish, feed samples, unconsumed feed and chosen tissues were weighed in an electric monopan balance to an accuracy of 1 mg.

Three females were randomly chosen from each replicate and reared with single male in a separate tank containing sufficient quantity of macrophyte, *Hydrilla* species, until the completion of the experiment. The remaining test animals were removed from the experimental tanks. Female broods of the swordtail released young ones at dawn once in 28 – 35 days intervals and they were isolated from their parents and counted. The number of intrafollicular embryos produced by female fish is known as fertility (Dahlgren, 1979).

Relative fertility = \[ \frac{\text{Total fry production at experimental period (days)}}{\text{Mean wet weight of female (g)}} \]

2.8.3. Estimation of carotenoid content

Total carotenoids in the fins, skin and muscle of *X. helleri* were analysed following the method Bjerkeng (1992). All the fins from the three fishes were cut with sterilized scissors. Fishes were then dissected and the muscle adhering to the skin was removed carefully with a sharp knife. The fins, skin and
muscle were separately kept in small packets and stored in a deep freezer. Tissues of more than one fish were pooled separately to obtain the required quantity.

Tissues (30-50 mg) were cut into small pieces and homogenized in a tissue homogenizer for 3 times in one hour duration with 20 ml of acetone. The extracts thus obtained were filtered. Carotenoid content was measured in UV spectrophotometer. Calculations were made using the method of Bjerke (1992). Absorbance was measured at 475 nm.

2.8.4. Estimation of digestive enzymes

Preparation of enzyme source

Prior to commencement of breeding, 3 test fish from each tank were removed and starved for 24 hrs and sacrificed. The whole alimentary tract was dissected out in ice cold fish ringer solution and thoroughly washed externally. The tissue was rinsed with cold distilled water and a portion of alimentary canal, foregut midgut and hindgut was ligatured, split open and washed thoroughly. The tissues were homogenised separately with distilled water using mechanical dispencer. The homogenate was centrifuged at 40,000 rpm for 15 min at 0°C using high speed refrigerated centrifuge (Remi Model K-II) to prepare 1 to 10% of aqueous extracts. The clear supernatant was used as the crude enzyme extract for subsequent assay.

Quantitative assay

Amylase activity (Bernfield, 1955)

The reaction mixture containing 1 ml enzyme extract, 9.5 ml phosphate buffer (pH 7.2) and 0.5 ml of 2% soluble starch solution was incubated
at $37^0$C for 3 min. The enzyme reaction was interrupted by adding 2 ml of 3, 5 dinitrosalicylic acid. After adding 20 ml of distilled water, the brown product was read at 580 nm. Maltose solution (0.2 to 2 mg) was used as standard. The specific activity was expressed as $\mu$g maltose mg$^{-1}$ of protein hr$^{-1}$. Protein was determined by standard method (Lowry et al., 1951).

**Protease activity (Jany, 1976)**

The reaction mixture contained 1 ml of 1% casein (as substrate), 0.5 ml of 0.1 M phosphate buffer (pH 7.6) and 1 ml of crude enzyme extract. The reaction was terminated after 30 min by adding 5 ml of 5% TCA solution. The mixture was centrifuged and tyrosine in an aliquot of sample mixture was determined by the color given by Folin-Ciocalteu reagent in alkaline solution and the optical density was measured at 650 nm. The color was compared against a standard tyrosine solution in 1 M HCl. Protein was estimated by standard method using BSA as standard and specific activity was measured as $\mu$g of tyrosine mg$^{-1}$ of protein hr$^{-1}$ at $37^0$C.

**Lipase activity**

The lipase activity was determined by titrimetric method (Teitz and Friedrick, 1966). The assay system consisted of 1.5 ml of pure olive oil (99.9%) with 1.5 ml of 1 M Tris-HCl buffer (pH 8) to which 1 ml of crude enzyme extract was added and incubated for 6 hr at $37^0$C. The reaction was arrested by addition of 3 ml of 95% ethyl alcohol. This mixture was titrated against 0.01 N NaOH using 0.9% (w/v) thymolphthalein in ethyl alcohol as indicator. The specific activity was expressed as lipase mg$^{-1}$ protein hr$^{-1}$ (1 ml of 0.01 N NaOH is taken as corresponding 100 lipase units).
2.8.5. Estimation of energy

Wet Combustion Method – Karzinkin and Tarkovskaya (1964)

Principle

This is an indirect method of estimating the energy value of the material. The procedures make use of the principle that potassium iodate, when heated in the presence of concentrated H$_2$SO$_4$ and organic materials decomposes and release nascent oxygen which in turn oxidises the organic matter present in the sample. The decomposition of potassium iodate is proportional to the energy content of the organic material.

Reagent

1. 5% KI\textsubscript{O$_3$}
2. 10% KI
3. Con. H$_2$SO$_4$
4. 0.1 N Na$_2$S$_2$O$_3$
5. Starch indicator 1%

Procedure

A sample weighing 10-15 mg was placed in a round bottomed flask. A flask also contained exactly 3 ml of 5% potassium iodate solution and 20 ml concentrated sulphuric acid. The analyses were done in duplicates. The control flask contained 3 ml of KI\textsubscript{O$_3$} and 20 ml of concentrated sulphuric acid. The flask were connected to the reflex condensers without shaking and heated on heating mantles. Boiling continued for an hour (Organic substances oxidised can be judged by the vigorous release of free iodine).

After combustion, the experimental and control flask were cooled and 50 ml of distilled water was added separately. The liquid was mixed well and
the flasks were heated (not boiled) until complete disappearance of the pink colour and the smell of iodine. The contents were cooled and each flask was diluted with 250 ml of distilled water and 10 ml of potassium iodide was added after transferring them into 500 ml of conical flask.

Then the flask were then titrated against 0.1 N sodium thiosulphate using starch as indicator. The end point is the disappearance of blue color.

**Calculation**

1 ml of 0.1 sodium thiosulphate corresponds to 3.567 mg of KIO₃.

1 mg of KIO₃ corresponds to 0.1869 mg of O₂.

1 ml of 0.1 n sodium thiosulphate = 3.567 x 0.1869 = 0.6667 mg O₂.

Using the oxy-calorific coefficient of 3.33 (Winberg, 1956) the amount of oxygen utilized can be converted into calories. Multiplying the caloric value with 0.004184 gives the values in kilo joules. Energy values were estimated using the following formula.

\[
\text{Energy value} = \frac{(\text{Control titre value} - \text{Experimental value}) \times 2.25 \times 0.004184}{\text{Sample weight}}
\]

**2.8.6. Estimation of ammonia**

Ammonia excretion of test animals was estimated following the phenol hypochlorite method (Solarzano, 1969).

**Principle**

Water containing ammonia reacts with phenol and hydrochlorite under alkaline condition to produce indophenol blue colour. The intensity of the colour depends on the concentration of the ammonia present in the sample. Ammonia nitrogen can be determined by this method over a range of 0.001 – 0.1
mg/l using a 10 cm cuvette or 0.1-1 mg/ l using 1 cm cuvette. All glasswares must be washed thoroughly with deionized water. The amino acids or urea present in the sea water don’t interfere to any practical degree.

Total ammonia includes ionized form and unionized form of ammonia. The proportion of these two forms depends upon the pH and temperature of the water. The unionized form of ammonia is non toxic to fishes. To ionized form of ammonia could easily pass through the biological membrane and causes toxicity.

**Reagents required**

1. **Phenol – alcohol 10%**

   Add 10 gm of phenol to 100 ml of absolute alcohol or to a 100 ml mixture containing 5% propanol and 95% alcohol.

2. **Sodium nitroprusside**

   Dissolve 1 gm of sodium nitroprusside in 200 ml of distilled water. The reagent remains stable for about one month and it should be stored in amber bottle.

3. **Trisodium citrate solution**

   Dissolve 100 gm of trisodium citrate and 5 gm of NaOH in 500 ml of ammonia free water.

4. **Hypochlorite solution**

   Fresh commercial grade bleaching powder containing atleast 5.25% of sodium hypochlorite should be used. It slowly decomposer in due course. It should not be used if the free chloride falls below 1.5 M.
5. Oxidizing solution

Add 100 ml of trisodium citrate solution to 2.5 ml of hypochlorite solution. It should be prepared fresh daily.

6. Standard ammonia stock solution

Dissolve 3.81 gm of anhydrous NH₄Cl in ammonia free water and dilute it to 1 litre. 1 ml of the solution contains 1.22 mg.

Procedure

Take 50 ml of the water sample and add 2 ml of phenol solution. 2 ml of sodium nitroprusside and 5 ml of oxidizing reagent successively. Mix it well and keep it without disturbances for colour development. Measure the intensity of the colour developed in a colorimeter using a red filter (640 nm).

2.8.7. Statistical treatment

The data collected were subjected to the following statistical analyses.

i. Standard deviation

ii. Student’s ‘t’ test

iii. Simple correlation coefficient

iv. Simple regression

v. Analysis of variance

vi. Duncan’s multiple range test

i. Standard Deviation

\[ SD = \sqrt{\frac{\sum d^2}{n-1}} \]
Where ‘d’ refers to the deviation of each score from mean and ‘n’ the total number of observations.

**ii. Student’s ‘t’ Test**

Student’s ‘t’ test was used to compare two means by applying the formula:

$$ t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}} $$

where $\bar{X}_1$ and $\bar{X}_2$ represent the means compared and $SE_1$ and $SE_2$ are their respective standard errors.

Standard error was calculated using the formula,

$$ SE = \frac{SD}{\sqrt{n-1}} $$

The level of significance for the ‘t’ at corresponding degree of freedom ($Df = n-1$) was read from the probability table given in Zar (1974). ‘n’ is the number of scores in both the experiments.

**iii. Simple Correlation Coefficient (r)**

The simple correlation coefficient ‘r’ was calculated from the formula.

$$ r = \frac{\sum xy}{\sqrt{\sum x^2 \sum y^2}} $$

**iv. Simple Regression**

The regression equation was computed using the least square method. The basic formula followed was
\[ Y = a + bX \]

Where \( Y \) is the dependent variable, \( X \) is the independent variable, \( a \) is the intercept on \( Y \) and \( b \) is the slope. The formulae used to derive the values of \( a \) and \( b \) are

\[
b = \frac{\sum xy}{\sum x^2}
\]

\[
a = \bar{Y} - b\bar{X}
\]

Where \( \bar{Y} \) and \( \bar{X} \) denote the means of \( Y \) and \( X \). \( \sum xy \) and \( \sum x^2 \) are derived as follows:

\[
\sum xy = \sum XY - \frac{(\sum X)(\sum Y)}{N}
\]

\[
\sum x^2 = \sum X^2 - \frac{(\sum X)^2}{N}
\]

\[
\sum y^2 = \sum Y^2 - \frac{(\sum Y)^2}{N}
\]

Capital \( X \) and \( Y \) denote the raw scores and small \( x \) and \( y \), the deviation scores.

v. Analysis of variance (ANOVA)

Two-way analysis of variance

Partitioning of total variance into variance due to the different experimental conditions (e.g. rearing period and feeding rate or specific growth rate) was carried out following the procedure described by Zar (1974). Various values obtained for different experimental conditions, were tabulated in columns and rows. For each column, \( \sum x \) and \( \sum x^2 \) were calculated. Sum of \( X \) for all columns was squared and divided by the number of tabulated values and a correction factor was obtained.

\[
\text{Correction factor (C)} = \frac{(\sum x)^2}{N}
\]
Total SS = Sum of $X^2$ for all columns – C

Between column SS = \( \frac{\text{Sum of } (\sum X)^2 \text{ of each column}}{\text{Number of values in a column}} - C \)

Between row SS = \( \frac{\text{Sum of } (\sum X)^2 \text{ of each row}}{\text{Number of values in a row}} - C \)

Remainder SS = Total SS – (Between column SS + Between row SS)

Considering the degrees of freedom for each source of variance, mean square (MS) was calculated.

**Degrees of freedom (DF)**

Total SS = Number of values in the Table – 1.

Between column SS = Number of columns – 1

Between row SS = Number of rows – 1

Remainder SS = Total SS Df – (Between Column Df + Between rows Df)

F value for the variance between columns = \( \frac{\text{MS between columns}}{\text{Remainder MS}} \)

F value for the variance between rows = \( \frac{\text{MS between rows}}{\text{Remainder MS}} \)

Significant level at the corresponding Df was read from table D.11 given by Zar (1974).

**vi. Duncan’s multiple range test**

The Duncan’s multiple range test using SPSS (version 13.5) software was performed to find significant level of 0.05.
Table 2.1. Formulation and percentage composition experimental diets. Each value is the mean ($\bar{X} \pm SD$) of three observations.

<table>
<thead>
<tr>
<th>Ingredients (g / 100g diet)</th>
<th>Spirulina levels (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Fish meal</td>
<td>50</td>
<td>35</td>
<td>30</td>
<td>25</td>
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<tr>
<td>Groundnut oil cake</td>
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<td>Maida</td>
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<td>10</td>
<td>15</td>
<td>20</td>
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<td>Vitamin and mineral mixtures</td>
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<td>1</td>
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<tr>
<td>Cod liver oil</td>
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<td>1</td>
<td>1</td>
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</tr>
</tbody>
</table>

**Proximate composition (%)**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
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<tbody>
<tr>
<td>Protein</td>
<td>45.02 ± 0.14</td>
<td>46.99 ± 0.11</td>
<td>48.35 ± 0.16</td>
<td>50.08 ± 0.27</td>
<td>51.38 ± 0.12</td>
</tr>
<tr>
<td>Lipid</td>
<td>6.96 ± 0.07</td>
<td>5.74 ± 0.02</td>
<td>5.35 ± 0.03</td>
<td>4.76 ± 0.03</td>
<td>4.60 ± 0.02</td>
</tr>
<tr>
<td>Ash</td>
<td>15.83 ± 0.76</td>
<td>17.67 ± 0.29</td>
<td>18.50 ± 0.50</td>
<td>19.95 ± 0.84</td>
<td>22.17 ± 0.29</td>
</tr>
<tr>
<td>Nitrogen free extract</td>
<td>32.19 ± 0.82</td>
<td>29.60 ± 0.63</td>
<td>27.80 ± 0.74</td>
<td>25.21 ± 0.43</td>
<td>21.85 ± 0.34</td>
</tr>
</tbody>
</table>
Table 2.2. Water stability (%) of experimental diets.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>98.42 ± 0.63</td>
<td>98.46 ± 0.74</td>
<td>98.42 ± 0.51</td>
<td>98.43 ± 0.44</td>
<td>97.72 ± 0.69</td>
</tr>
<tr>
<td>20</td>
<td>95.79 ± 0.38</td>
<td>95.82 ± 0.87</td>
<td>95.74 ± 0.46</td>
<td>95.20 ± 0.77</td>
<td>95.02 ± 0.64</td>
</tr>
<tr>
<td>30</td>
<td>92.88 ± 0.16</td>
<td>92.94 ± 0.70</td>
<td>92.53 ± 0.41</td>
<td>92.07 ± 0.80</td>
<td>92.00 ± 0.38</td>
</tr>
<tr>
<td>40</td>
<td>89.00 ± 0.45</td>
<td>89.79 ± 0.22</td>
<td>89.62 ± 0.14</td>
<td>89.59 ± 0.62</td>
<td>88.99 ± 0.55</td>
</tr>
<tr>
<td>50</td>
<td>87.60 ± 0.16</td>
<td>89.35 ± 0.12</td>
<td>89.40 ± 0.58</td>
<td>88.99 ± 0.50</td>
<td>87.65 ± 0.72</td>
</tr>
<tr>
<td>60</td>
<td>86.42 ± 0.33</td>
<td>87.22 ± 0.54</td>
<td>87.29 ± 0.22</td>
<td>88.70 ± 0.61</td>
<td>87.20 ± 0.45</td>
</tr>
</tbody>
</table>