3

MATERIALS & METHODS

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3.1. SELECTION OF SITE :

The present study (field trial) was conducted at the Darjeeling Pulbazar Block, Darjeeling, West Bengal, situated in the foot of Eastern Himalayas range and at the basin of the Little Rangeet River (Figure 1). Latitude and longitude of this area are 27^0 N and 88^0 E respectively. The altitude of this area is about 2500 to 3000 feet MSL. The area of the ponds are ranging from 0.0032 ha-0.0040 ha, perrineal, outlet in one side, 'Jhora fed', depth 1.0-1.5 meters and in some cases ponds are in a series. Laboratory experiment was carried out at the Post Graduate Department of Zoology, Darjeeling Government College and Vidyasagar University for digestibility study of the diets and physiological and biochemical estimation.

3.2. SELECTION OF PONDS :

Total Six(6) ponds were selected for present investigation viz. control pond and ponds ED01, ED02, ED03, ED04 & ED05. The same series of ponds were named as control pond and ED06 (instead of ED01), ED07 (instead of ED02), ED08 (instead of ED03), ED09 (instead of ED04) and ED10 (instead of ED05) in the next year experimental trial (Figures 2, 3 & 4).

3.3. SELECTION OF FISH SPECIES :

The Indian major carps, *Catla catla, Labeo rohita* and *Cirrhinus mrigala* were selected for the present investigation. Fingerlings and adults of these species are available throughout the year in the viscinity through fish seed traders of Siliguri, Darjeeling district, West Bengal (Figures 5, 6 & 7).

Systematic Position of *Catla catla* (Ham.)

Phylum - Chordara

Subphylum – Vertebrata

Grade - Telestomi

Class – Actinopterygii

Subclass – Neopterygii

Division – Teleostei

Order – Cypriniformes

Suborder – Cyprinoidae

Family – Cyprimidae

Genus – *Catla*

Species – *catla.*

Type Characters

1. Upper jaw border with premaxilla only, protrusible.
2. Lateral line represented by pitted scale.
3. Scales are comparatively large, dorsal fin with 3-4/14-16 rays.
4. Lateral transverse scale 7 ½ / 9.
5. Dorsal profile more convex than the ventral.
6. Anal fin shorter than dorsal fin with 8 rays.

7. Lateral line with 40-43 scales in the longitudinal series.
8. 5 ½ – 6½ rows of scales between the lateral line and pelvic base.

Systematic position of *Labeo rohita* (Ham.)

Phylum – Chordata

Subphylum – Vertebrata

Superclass – Gnathostomata

Grade – Teleostomi

Class – Actinopterygii

Subclass – Neopterygii

Division – Teleostei

Order – Cypriniformes

Suborder – Cyprinoidae

Family – Cyprinidae

Genus – *Labeo*

Species – *rohita*

Type Characters

1. Dorsal tubular nerve cord, pharyngeal gill slits present.
2. Presence of vertebral column,

* Presence of jaw

3. Basbels may or may not be present.
4. Snout obtuse and depressed.
5. Lip thick and fringed.
6. Dorsal fin with 15 to 16 rays.
7. Lateral line with 40-42 scales in the longitudinal and 6 ½ / 9 in the transverse series.
8. Dorsal profile more arch than ventral profile.

** Weberian apparatus connects the ear and air bladder.

9. Pelvic fin origin below the 3rd or 4th rays of dorsal fin.

Systematic position of *Cirrhinus mrigala* (Ham.)

Phylum - Chordata,

Subphylum – Vertebrata

Superclass – Gnathostomata

Grade – Teleostomi

Class – Actinopterygii

Subclass – Neopterygii

Division – Teleostei

Order – Cypriniformes.

Suborder – Cyprinoidae

Family – Cyprinide

Genus – *Cirrhinus*

Species – *mrigala*

Type characters

1. Upper jaw border premaxilla only, protrusill.
2. Lateral line represented by pitted scale.
3. Dorsal fin short with 8 – 15 rays.
4. Dorsal profile slightly more convex than the ventral profile.
5. Presence of 2 barbels.
6. Origin of dorsal fin much in front of pelvic origin, nearer to snout that to caudal base.
7. 4 ½ / 6 scales between lateral line and pelvic base, and 40 – 45 scales in the longitudinal series.

3.4. EXPERIMENTAL TRIAL DURATION :

The experimental period was normally July-December of every year. And the experiment was conducted for growth, conversion efficiency and bio-chemical

changes in Indian major carps with different formulated diets (ED01 – ED10). The time schedule for rearing of IMC were as follows :

01.07.95 – 31.12.95 (control and ED01-Ed05),

03.07.96 – 02.01.97 (control and ED01-ED05),

02.07.97 – 01.01.98 (control and ED06-ED10) and

01.07.98 – 31.12.98 (control and ED06-ED10)

3.5. FEED

3.5.1. Selection of the feed ingredients:

Twenty-one (21) feed items of both plant (14) and animal (07) origin were screened for the present investigation. Among the feed items of plant origin, oil cakes (mustard oil cake, linseed oil cake and ground nut oil cake), wheat bran, rice bran, cheap quality soybean meal, cotton seed, *Hydrilla, Spirodela, Azolla, Nymphoides, Nechamandra, Eichhornia & Cynodon* and among the items of animal origin, goat blood & slaughter house waste (from the slaughter houses, pulbazar, Darjeeling, West Bengal), silk worm pupae (from the silk production centres, Kurseong, Darjeeling, West Bengal), trash fish (from the Pulbazar market, West Bengal) and carcass wastes (cattle and pig; procured from Kalimpong, Darjeeling, West Bengal), Feather meal & meat meal (Pulbazar market, Darjeeling, W.B.) were used. All these feed ingredients are available locally throughout the year at a comparatively cheaper rate. After drying, the feed ingredients were made into powder by a grinder and stored in plastic jars separately for subsequent analysis.

3.5.2. Proximate analysis of the feed ingredients :

Each feed item was analysed for moisture, dry matter (DM), crude protein (CP) (Kjeldahl N x 6.25), crude lipid (CL) (ether extract), crude fibre (CF), nitrogen

free extract (NFE) and ash as per Weende proximate analyses scheme following the methods outlined in the "Official Methods of Analysis" published by Association of Official Agricultural Chemists (AOAC, 1980). Calcium and phosphorus contents were estimated following the Clark – Collip modification of the Kramar – Tisdall and Fiske and Subba Row method respectively as described by Oser (1960) (Figure – Winde proximate analysis scheme).

The methods are outlined as follows :-

3.5.2.1. *Moisture* :

For the determination of moisture content, weighed amount of feed ingredient was dried in a hot air-oven at a temperature of 100 ± 5^{0}C for 24 to 34 hours till a constant weight was obtained. The loss in weight was reported as moisture content of the feed ingredient.

$$\text{Moisture content (\%)} = \frac{\text{Weight fresh sample - weight dry sample}}{\text{Weight fresh sample}} \times 100$$

3.5.2.2. Dry matter (DM) :

Dry matter (DM) content of the individual feed ingredients was determined as the difference between the fresh feed sample and moisture content (DM = fresh sample – moisture). The crude protein, crude lipid, crude fibre nitrogen free extract, ash, calcium and phosphorus contents of the feed ingredients were calculated as percent of dry matter.

3.5.2.3. **Crude protein** :

The amount of crude protein content present in the feed ingredients was calculated from it's nitrogen content which was analysed by modification of the

Kjeldahl technique. 100–200 mg. of feed sample was accurately weighed and placed in a long neck 100ml digestion flask. To this, 0.1g of catalyst (triple catalyst, Se : $HgO:CuSO_4$: : 10:3:5) and 1.5 g of potassium bi-sulphate ($KHSO_4$) were added followed by 5.0 ml of concentrated sulphuric acid (H_2SO_4, AR). The mixture was then gently heated until frothing subsides. The mixture was then boiled vigorously until the solution becomes clear. The digestion mixture was cooled to room temperature and diluted with 10.0 ml of distilled water. The flask was immediately connected with the distillation bulb and an excess (20.0 – 25.0 ml) of 50% sodium hydroxide (NaOH) was added slowly through the funnel provided for this purpose. The tip of the condenser was immersed in 25.0ml of standard N/20 H_2SO_4 in the receiving flask. The solution was steam distilled for 40-45 minutes. After completion of distillation, the receiving flask was lowered and the condenser tube was washed with distilled water. The excess acid was then titrated with standard N/20 NaOH solution using phenolphthalin as an indicator. For each analysis, blank tritations were performed are respective volumes of titrants were corrected. The percentage of nitrogen was calculated as follows:

$$\%N = \frac{100 \times (V_1 - V_2) \times N \times 14.01}{W \times 20 \times 1000}$$

Where, V_1 = Volume of titrant for 25.0 ml of blank H_2SO_4

V_2 = Volume of titrant for the distillate collected in the receiving flask.

W= Weight of the sample taken in g.

N= Strength of alkali.

The crude protein was estimated by multiplying Kjeldahl Nitrogen with 6.25.

3.5.2.4. **Crude lipid** (ether extract)

The crude lipid content of the different feed ingredients was estimated using soxhlet extraction apparatus. 2.0 g dried feed ingredient was taken in a thimble and placed in the soxhlet extraction apparatus. The initial weight of the soxhlet flask was recorded and filled with 100-150 ml of petroleum ether (Boiling point 60-80^{0}C). The soxhlet apparatus was then placed in a heating mantle and the petroleum ether allowed to boil for circulation through the thimble by Siphon process. The siphoning process was repeated at least 20 times. The flask was then taken out and the petroleum ether was allowed to evaporate. The final weight of the flask was then recorded. The difference in the final weight of the flask and blank gave the weight of the crude lipid present in the feed sample.

$$\text{Crude lipid (\%)} = \frac{\text{weight of crude lipid}}{\text{weight of sample}} \times 100$$

3.5.2.5. *Crude fibre:*

After extraction of lipid by petroleum ether extraction in soxhlet apparatus, the defattened feed ingredient was used for the estimation of crude fibre. Weighed amount (2.0 g) of defattened feed ingredient was treated with 200ml of 25% boiling H_2SO_4 for 30 minutes and washed repeatedly with boiling distilled water and cold distilled water until washings are no longer acidic. The material was then treated with 200ml of boiling 25% NaOH solution for 30 minutes and washed repeatedly with hot and cold distilled water as described earlier until the washings are no longer alkaline. After thorough washing, the material was filtered through ash less filter paper (Whatman No. 42), dried in a hot air-oven and weighed.

3.5.2.6 Nitrogen free Extract (NFE) :

Nitrogen free extract (NFE) was calculated "by difference" following the quantitative analysis of crude protein, crude lipid, moisture, ash and crude fibre fractions. NFE=100 – (% crude protein +% crude lipid + % moisture + % Ash + % crude fibre).

3.5.2.7. Ash :

The ash content was determined by igniting the weighted amount (2.0g) of feed sample at 500 ± 50^0C in a muffle furnace for atleast 06-10 hours till a constant weight was obtained.

3.5.2.8. Calcium :

The amount of calcium present in the feed ingredients was estimated following the Clark – Collip modification of the Kramer-Tisdall method as described by Oser (1960). 2.0 g of the sample was first made into ash in a Muffle furnace at 550^0 ± 50^0C. The ash was dissolved in 20.0 ml of 6 (N)HCl, boiled and filtered through whatman filter paper No. 42. 2 ml of this filtrate was diluted with 98.0 ml of distilled water to which 2 drops of methyl red indicator was added. To this mixture, diluted ammonium hydroxide (NH_4OH) was added drop wise till the colour changes to brownish orange. Subsequently, 25% hydrochloric acid (HCl) was added drop wise to have a Pink colour. The entire mixture was then diluted with distilled water to make the volume upto 150 ml and after boiling, 10.0 ml of hot saturated ammonium oxalate solution was added. This was kept overnight to allow the precipitate to settle. Next day, after filtering through ash less filter paper, the beaker was washed with 50.0 ml of diluted NH_4OH and again filtered. After filtering, the precipitate was washed with 125 ml of distilled water and subsequently 5.0 ml of concentrated H_2SO_4 was

added. The entire mixture was then heated to 70^0C. It was then titrated with N/10 $KMnO_4$ to the first appearance of pink colour. The calcium content was calculated as:

Ca (%) = amount of titrant x 0.02 x 10 x 50

3.5.2.9. Phosphorus :

The phosphorus content was estimated following the method of Fiske and Subba Row as described by Oser (1960). 2.0g of feed sample was made into ash in a muffle furnace at $550^0 \pm 50^0$C and taken into a 100 ml volumetric flask. To this, 70.0 ml of distilled water and 10.0 ml of ammonium molybdate solution were added. After mixing the mixture by gentle shaking, 4.0 ml of 1,2,4 – aminonaphthosulphonic acid was added and made upto 100 ml with distilled water. Simultaneously, a separate flask was filled with 5.0 ml of standard phosphate solution, containing 0.4 mg of phosphorus, 65.0 ml of distilled water, and the same reagents that were added to the food sample. The mixture was diluted with distilled water to make the volume upto 100 ml, mixed well by inversion, and allowed to stand for 5 minutes. A blank was prepared by treating 70.00 ml of distilled water in a 100 ml volumetric flask with the same reagents, diluted to the mark with distilled water, and mixed by inversion. The optical density of the standard and of the unknown was determined in a spectrophotometer (Systronics 106) at 660-720 nm. The amount of phosphorus present in the feed sample was calculated as :

$$\frac{\text{O.D. of Unknown}}{\text{O.D. of standard}} \times 0.4 = \text{mg of inorganic phosphorus (as P)}$$

$$\text{Phosphorus (\%)} = \frac{\text{mg of inorganic phosphorus} \times 50}{1000}$$

3.5.2.10. Organic matter (OM) : The organic matter content of the feed ingredients was estimated as follows :

OM= % Dry matter (DM) – % Ash.

3.5.2.11. Caloric values :

The caloric values of the individual feed ingredients in terms of the Kcal/g were estimated using the average caloric conversion factors of 4.10, 9.45 and 5.65 Kcal/g for carbohydrate, lipid and protein respectively (Jobling, 1983).

3.5.3. Preparation of the diets after compounding :

On the basis of proximate composition and caloric value of the individual feed ingredients, availability in the large quantity and digestibility of the ingredients. I have selected ten (10) feed items for final preparation of the experimental diets & the different diets were compounded on the following basis :

The diets were formulated following the square method (Hardy, 1980). The feed ingredients were weighed in a chemical balance as per requirements and mixed thoroughly with luke warm water until a dough like mass was obtained. Some vitamin and mineral mixture (vitaminate forte, Roche India Limited) were added to the dough. The compounded feeds were made water stable using wheat flour (5%) as binder.

3.5.4 Pelletization and nutrient leaching :

The feed mixtures thus obtained were passed through a pellet-making machine. The size of the pellets was determined according to the size of the fish. The pellets were then dried in a hot air-oven at mild temperature and crumbled. After screening, the finished diets were kept in air-tight plastic containers for future use (Figures 8, 9 & 10).

To evaluate the leaching of nutrients (mainly protein), if any, weighed amount of pelleted feeds were kept in water for four to five hours and the water was analysed for protein following the methods of Lowry *et al.* (1957).

3.5.5. Sinking rate of the pelleted diets :

The sinking rates of the different pelleted feeds, in dried condition, was determined in an aquarium soon after their preparation. The pellets of uniform size were dropped into the aquarium and the time taken by them to pass through the depth of the water column was recorded using a stop watch. The average time taken by each type of pellet were calculated separately and the average sinking rate of the pellets was expressed in cm/sec.

3.5.6. Storage effect of the pelleted diets :

In order to find out the self life of the feeds, samples were drawn from each type of feed and analysed for various parameters at the initial and at the end of the long-time storage period i.e., 3 months. The proximate composition of both fresh and stored feeds were determined following the methods as stated earlier.

3.6. EXPERIMENTAL AQUARIA (For digestibility study)

The system consists of six rectangular aquaria. The base and frame of each aquarium are made up of fibre glass of neutral character and the side walls of good quality thick glass. Each aquarium measures 76 x 41 x 37 cms. The base of the each aquarium is gently sloped towards the centre where an outlet is present. Each bottom outlet is connected with a drain pipe of 0.2 cm inner diameter which can be controlled by stoppers. An overflow outlet is provided with each aquarium on the front wall and

each connected with GI pipe through which the excess water can be drained out. The outlet pipe from each aquarium ends in a funnel for filtering the uneaten feed materials and faecal matters. Each of these funnel is connected with an outlet GI pipe through which the filtered water is drained out. The aquaria were fed with tap water through the shower provided at the top (Figure - 11).

3.7. DETERMINATION OF THE FEEDING RATE AND TIME

The fishes were stocked in separate aquarium in a batch of 20 fishes in each. They were fed with the pelleted feeds at the rate of 5% of their total body weight at different time intervals, viz., '00' hours, '06' hours, '12' hours and '18' hours. Daily feeding allowance was adjusted each week on the basis of average body weight of the fish. Any left over feed were collected after 3 hours and the water was drained out through the outlet situated at the bottom of the aquarium through a filter of 22 Bolt silk cloth to collect the uneaten feed materials. The collected feed materials were taken in a petridish, dried in an air-oven at 100 ± 5^0C and weighed to determine the actual amount of feed taken by the fish (Figure -12).

3.8 PERFORMANCE OF THE PREPARED DIETS

3.8.1. Digestibility study :

The digestibility experiments were conducted in specially designed fibre glass aquaria as described earlier. Each experiment consisted of two replications. The fishes were fed with the pelleted feeds once daily at a rate of 5% of their total body weight throughout the experimental period and the actual feeding rate was determined. Daily feeding allow once was adjusted each week on the basis of average weight of the fish in each treatment. Any left over feed were collected after 3-4 hours and weighed after

drying in an air-oven at 100 ± 5^{0}C to determine the amount of feed consumed by the fish. The water from all the aquaria was drained out every 24 hours through the outlet situated at the bottom. During this process, the water was allowed to letout through a filter of 22 Bolt silk cloth to collect the faecal matter. Any nutrient leached out into the water was determined by analysing the water sample during the time of faeces collection. The faecal matter thus collected was taken in a petridish, dried in an air-oven at 100 ± 5^{0}C and weighed. For each feeding trial, a 15 day composite faecal matter was pooled for subsequent analysis. Digestibility study was terminated after 15 days. The faecal samples were analysed for proximate composition following the methods as described earlier. The digestibility of the nutrients in the feed was estimated by using the following equation :

$$\text{Digestibility} = \frac{\text{Nutrient intake - nutrient in the faecal matter}}{\text{Nutrient intake}} \times 100$$

3.8.2 Feed conversion ratio (FCR) :

To determine the feed conversion ratio (FCR) of the formulated feeds, the feeding experiment was continued for a period of 60 day. After acclimatization, the fish were kept in separate aquaria in a group of 15 fishes (*Catla* – 5, *Labeo* – 5 & *Cirrhina* – 5) in each. Each experiment was tried in duplicate. Initial weight and length of the each fish were recorded prior to commencement of the experiment. The fishes were fed daily @ 5% of their total body weight. The water of each aquarium was changed daily. The fish from each aquarium were sampled at regular intervals for monitoring the length and weight increment. The amount of feed given was adjusted on the basis of the weight gain of the sampled fish. The amount of feed consumed by the fish was determined by the method as stated earlier. At the termination of the

experimental period, the final gain in length and weight of the individual fish was recorded. The absolute conversion rate was then calculated as :

$$\text{Absolute conversion rate} = \frac{\text{Dry weight of the feed given}}{\text{Increase in wet weight of fish}}$$

3.8.3. Protein efficiency ratio (PER):

PER of the formulated diets was estimated by the gain in wet weight of the fish per gram of crude protein consumed on dry weight basis:

$$\text{PER} = \frac{\text{Wet weight gain of the fish}}{\text{Gram of crude protein consumed}}$$

3.8.4. Nitrogen balance :

The Nitrogen (N) balance was calculated as :

N balance = Total N intake – N in the faecal matter.

3.8.5. Energy balance :

The Energy balance was calculated as :

Energy balance = [Gross energy intake through food – Energy released in the faecal matter]

3.8.6. Measurement of growth parameters:

The growth of the experimental fish fed on different diets was determined on the basis of weight increments. The fishes from each of the pond were sampled each 30th day (Control and ED 01 to ED10) to record the increment in weight (g) and to calculate the specific growth rate %, Live weight gain (%) and daily weight gain (g).

$$\text{Specific growth rate (\%)} = \frac{\text{Ln final weight - Ln initial weight}}{\text{Days on trial}} \times 100$$

$$\text{Live weight gain (\%)} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Daily weight gain} = \frac{\text{Wet weight gain}}{\text{Days on trial}}$$

3.9. BIOCHEMICAL COMPOSITION OF THE FLESH :

The specimens from each of the replicate were collected at regular intervals for estimation of different biochemical parameters of the flesh of the fish fed on the prepared diets ED01 – ED10.

Moisture, dry matter (DM), crude protein (N x 6.25), crude lipid (ether extracts), and ash were estimated following the methods stated earlier AOAC, 1989).

3.10. WATER QUALITY:

The temperature, pH, dissolved oxygen (DO_2) and combined carbondioxide (CCO_2, hardness) of the water were recorded periodically (i.e. at every 7^{th} day) throughout the experimental period. The standard methods of APHA (1984) were followed to study the water quality.

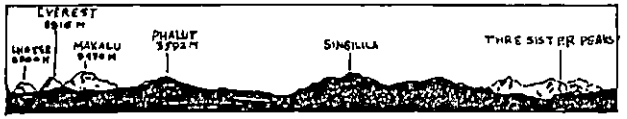
3.11. COST EFFECTIVENESS OF THE DIETS :

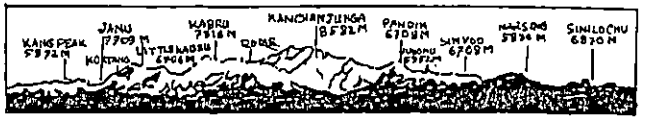
The cost of feed and fish as per the local market value, were taken into consideration to determine the cost effectiveness of the different experimental diets (ED01 – ED10). After the experimental period (180 days) the cost of unit production (Rs./Kg.), gross output (Rs.) and net output (Rs.) were estimated.

3.12. STATISTICAL ANALYSIS :

To test the goodness of fit and other statistical analysis of the experimental data, were done between the initial (during stocking) and final (after rearing period) values of different parameters.

The linear regression equation, correlation between control and ED01 – ED10 were analysed for the best fit curve and to study the rate of increment by using the software package like Microsoft Excel, Statistica, SPSS and ASP etc.





DARJEELING HILL AREAS

N

Distance in KM

INDEX

- MOTORABLE Road
- JEEPABLE Road
- TREK
- RIVER

NOT TO SCALE

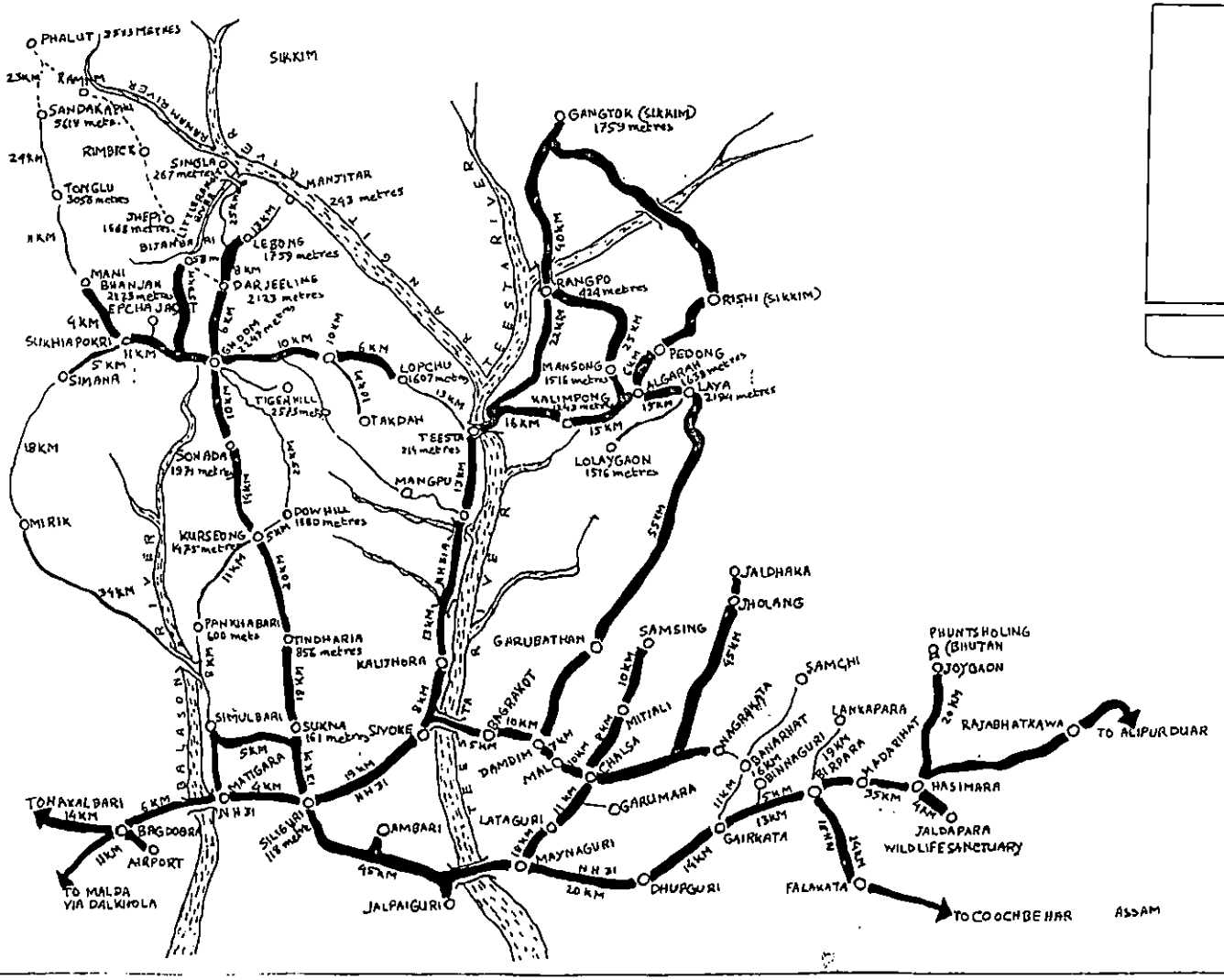


Fig.2. Experimental pond at the basin of Little Rangeet river.

Fig. 3. Experimental pond at the agricultural fields of Darjeeling hills.

Fig. 6. The Indian major carp *Labeo rohita* (Ham.).

Fig.7. The Indian major carp *Cirrhinus mrigala* (Ham.).

Fig. 8. Grinding of the different feed ingredients through hand grinder.

Fig. 9. Pelletization of the formulated diets through hand pelletizer.

Fig. 10. Dried pellet (before storage).

Fig. 11. Experimental continous flow aquaria with air, temperature, photoperiod and water flow meter.

Fig. 12A. Feeding of the fish in aquaria.

Fig.12B. Collection of faecal matter for digestibility study.