GENERAL METHODOLOGY

I. Source of fish stock and method of sampling

Live and young *Heteropneustes fossilis* belonging to 0+ year-class (average total length 5-14 cm) were captured from some local ponds at Aligarh (Lat. 27°34'30" N Long. 78°4'26"E) and were brought to the laboratory in earthen pots. The fish were given a bath in KMnO₄ (5 mg/lit) and randomly stocked in outdoor cemented tanks (2.7 x 1.5 x 1.2 m). Prior to the commencement of the feeding trials, the fish were acclimatized to laboratory conditions in large glass aquaria. During acclimatization period they were fed minced meat ad libitum once a day. From the above acclimatized stock, fish of the desired size range were procured as and when required for feeding trial. Care was taken to select healthy fish only. At the time of measurements the fish were anaesthetized with MS 222.

II. Procurement of feed ingredients and processing of meals

Brans, and oil cakes of various types like groundnut, peanut, til, soybean and mustard were obtained from the market and dried in an electrical oven at 90°C for 24-30 h. The
dried material was carefully grounded to a fine powder and preserved in polythene bags.

Fresh plants of berseem (*Trifolium alexandrium*) were collected from the University Agriculture Farm, whereas water hyacinth (*Eichhornia crassipes*) and duckweed (*Lemna sp.*) were obtained from a neighbouring sewage-fed pond. For the preparation of the leaf meal the plants were washed, the leaves air-dried and powdered.

III. Chemical analyses

(1) Estimation of crude protein

The estimation of crude protein was made by a slight modification of Wong's microkjeldahl method as adopted by Jafri, *et al.* (1964). 0.1 g sample was digested in 5 ml of 1:1 sulphuric acid and heated till fumes appeared. 0.5 ml of saturated potassium persulphate was then added to oxidize the digesting mixture. The digestion was continued for about 14-20 h till the solution in the kjeldahl flask became water clear, indicating that all the nitrogenous material present in the sample has been converted into ammonium sulphate. The clear solution was transferred to a volumetric flask and diluted to 50 ml. An aliquot (0.5 ml) of this solution was then directly nesslerized by adding Bock and Bendict Nessler's reagent (Oser, 1965). The solution was kept at room temperature for 10 minutes for complete colour development and the
colour intensity read on a Bausch and Lomb Spectronic 20 Spectrophotometer at 480 μm. The intensity of colour developed was proportional to the amount of ammonium sulphate contained in the solution. The values of optical density obtained for various samples were read off against a standard calibration curve (Fig. 1) prepared by taking readings of a series of different dilutions containing known amount of nitrogen. This method gave a direct measure of the total nitrogen present in the sample. The crude protein was calculated by multiplying the nitrogen value with the conventional protein factor (6.25). The values were recorded as percentage on dry or wet weight basis.

The above method was, however, found unsatisfactory for the estimation of crude protein in materials with high chlorophyll pigment. The method of Lindner (1944) was adopted for estimating the crude protein in such materials. 100 mg sample was digested in 2 ml of conc. sulphuric acid in kjeldahl assembly for 3-4 h till it turned light yellowish in colour. This was cooled in a dessicator, and 0.5 ml hydrogen peroxide (3%) added to oxidise the digesting mixture. The heating was resumed for another 15 minutes and 2-3 drops of hydrogen peroxide was again added to enhance the reaction for complete conversion of nitrogenous material to ammonium sulphate. The water clear digested sample thus obtained was diluted to 100 ml
to serve as stock solution. Aliquot (10 ml) from the above stock solution was mixed with 1 ml of 10% sodium silicate, 2 ml of 2.5N NaOH, and the volume finally made to 100 ml with distilled water. From the above solution, 5 ml aliquot was nesslerized by adding 1 ml of Bock and Bendict Nessler's reagent and the volume made to 10 ml with distilled water. The solution was kept at room temperature for 15 minutes for complete colour development and the intensity of colour measured spectrophotometrically at 525 μm. The values of nitrogen thus obtained were read off against the calibration curve and the crude protein calculated in the manner as described above.

Protein in fish tissue was estimated using the method of Lowry et al. (1951). A weighed quantity (3-4 g) of tissue sample was homogenized in 5-6 ml distilled water. This was treated with two volumes of trichloroacetic acid (TCA) to precipitate the protein. The content was centrifuged at 3000-4000 rpm for 15 minutes and the supernatant discarded. The process was repeated two to three times for complete removal of acid soluble compounds. In order to remove lipoidal compounds, the pellet was washed 3-4 times with 95% ethanol and the residue repeatedly treated with solvent ether. Finally, the solvent was discarded and the tissue sample dried in an oven at 100°C for a minimum of 12 h. The above process yielded a white fat-free dry powder which was used for estimation of
protein as well as for RNA and DNA.

0.01 g dry fat-free sample was treated with 1 ml of 1N sodium hydroxide and the content heated for 15-20 minutes in boiling water bath to digest the tissue residue. After digestion, the tubes were cooled to room temperature and the volume raised to 10 ml with distilled water with constant mixing. Aliquot (0.1 ml) from this mixture was taken and made to 2.0 ml with distilled water. 1 ml of carbonate-copper solution was then added, mixed and the content allowed to stand for 10 minutes at room temperature. This was followed by the addition of 0.1 ml Folin's reagent with mixing. A blank was likewise prepared by taking 0.1 ml of mixture containing 1 part of 1N sodium hydroxide and 9 parts of distilled water. After an incubation period of 30 minutes at room temperature the colour was read on the spectrophotometer at 500 μ. A standard curve (Fig.2) was prepared relating the optical density to micrograms of protein with a highly processed bovine serum albumin as the standard.

(ii) Estimation of RNA

Method for the extraction of RNA from the tissue and its determination by the Orcinol reaction was the same as given by Schneider (1957). 0.2 g of dry fat-free pellet obtained after the removal of acid soluble and lipoidal compounds was suspended in 2.0 ml of 1N potassium hydroxide
and incubated at 37°C for 20 h. 0.4 ml of 6N hydrochloric acid and 2.0 ml of 5% TCA were added to it after incubation. The contents were then mixed and centrifuged at 4000 rpm for 15 minutes. The supernatant was taken for the determination of RNA by the Orcinol reaction. A known volume (1.0 ml) of the aliquot of RNA extract was raised to 2 ml with distilled water and an equal volume of the Orcinol reagent added to the diluted aliquot. The tubes containing the reacting mixture were placed on a boiling water bath for 30 minutes. A blank was also prepared by taking 2 ml of distilled water and 2 ml of the Orcinol reagent. The intensity of the greenish colour of the test solution was read at 660 μm on a spectrophotometer. A calibration curve (Fig. 3) was prepared relating the optical density to micrograms of RNA using processed yeast RNA as the standard. The quantity of RNA was calculated as μg/100 mg of dried sample.

(iii) Estimation of DNA

DNA was extracted from the tissue by the method of Webb and Levy (1955). Following the dehydration of tissue residue by ether and drying in electric oven, 0.05 g of the sample was suspended in 5 ml TCA (5%). The contents were heated in boiling water bath for 30 minutes and allowed to cool to room temperature. The evaporation loss in the reacting mixture
was compensated by adding 5% TCA. The contents were filtered and the filtrate taken for DNA estimation using the method of Ashwell (1957) as adopted by Mustafa and Mittal (1982). A measured (0.5 ml) volume of the filtrate was mixed with 0.05 cysteine reagent (5%). To this was added, with constant mixing, 5 ml of 70% sulphuric acid, and the content incubated again for 30 minutes at room temperature. During incubation, the mixture was shaken 3-4 times. The colour developed was read spectrophotometrically at 490 μm, after adjusting the instrument with a blank solution wherein the sample aliquot was replaced with 0.5 ml distilled water. A calibration curve (Fig. 4) was prepared for comparison relating the optical density to micrograms of DNA, using highly polymerized calf thymus DNA as the standard.

(iv) Estimation of moisture

For the determination of moisture content, a known quantity of sample taken in pre-weighed vitreosil crucible was placed in a hot air oven at 100°C for 20-22 h. The crucible containing the dried sample was cooled to room temperature in a dessicator and reweighed. To ensure that the sample had become completely dried, the entire process was repeated till a constant weight was obtained. The loss of weight gave an index of water from which the percentage of water was calculated.
(v) **Estimation of ash**

Dried powdered sample (2-5 g) was ignited in a muffle furnace at 600°C till the sample became completely white and free from carbon. The crucible was cooled in a dessicator and reweighed to estimate the amount of ash. The quantity of ash was expressed as percentage on dry weight basis.

(vi) **Estimation of crude fat**

Crude fat in various sample was extracted with petroleum ether (BP 40-60°C) using the continuous soxhlet extraction technique. Prior to extraction, the sample was finely powdered and dried in an oven at 100°C. A weighed (5 g) quantity of the dried sample was taken in Whatman fat extraction thimble and plugged with cotton. The extraction was carried out for about 10-12 h. The extraction of certain plant materials, like berseem was, however, continued till the extract became pigment free. At the end of extraction, the solvent was recollected from the flask and placed in an oven for complete removal of solvent traces. The flask was cooled in a dessicator and reweighed. The increase in the weight of flask gave the quantity of fat extracted from the known weight of the dried sample.

(vii) **Estimation of total carbohydrate**

No direct estimation of carbohydrate was performed. The
percentage of total carbohydrate was, however, calculated by subtracting the sum of percentage value of water, fat, protein and ash from hundred (Jafri et al., 1964).

IV. Assessment of energy value

The energy value was calculated by using the factor 4 for protein and carbohydrate and 9 for fat, and expressed as kcal or Joules (Hastings, 1979).

V. Determination of protein digestibility

Chromic oxide indicator method (Furukawa and Tsukahara, 1966) was adopted to estimate the digestibility of protein in the formulated feed. The percentage of nutrient, and the corresponding percentage of the indicator substance, in the feed and faeces were analysed, and the digestibility calculated using the following formula:

\[
\text{Digestibility} = \frac{\% \ Cr_2O_3 \ in \ feed}{\% \ Cr_2O_3 \ in \ faeces} \times \frac{\% \ nutrient \ in \ faeces}{\% \ nutrient \ in \ feed} \times 100
\]

Pooled and well-mixed faecal matter (100 mg), containing chromic oxide, was digested in 5 ml of conc. nitric acid to remove the organic matter. To prevent the content from complete drying more nitric acid was added, if required. After cooling, 3 ml of perchloric acid (60%) was added to the mixture and the heating resumed till all traces of nitric acid disappeared.
Oxidation of chromium oxide to dichromate was indicated by the development of a brilliant golden yellow colour. The content was washed thoroughly into a 100 ml volumetric flask and the volume made to mark with distilled water. After keeping the solution for 10 minutes at room temperature for complete colour development, the optical density was measured at 350 nm on a spectrophotometer. The concentration percent of chromic oxide was read from a standard curve (Fig. 5) prepared separately by using different dilutions of chromic oxide.

VI. Formulation of feed from locally available ingredients and preparation of pellets

Square method (Hardy, 1950) was adopted for calculating the ingredient percentages to obtain the desired dietary protein level in the feeds formulated from locally available ingredients. This method was also applied separately for achieving the different levels of energy in the feeds.

All the ingredients, in finely grounded form, were sieved and the required quantities weighed. Water stability of the pellets with pellet binders like boiled rice, potato starch and rice polish was not found adequate. During the present experiment carboxymethyl cellulose was, therefore, used as a binder. The binder was boiled in a measured quantity
of water and homogeneous mixture of ingredients added to it with constant stirring. The feed mixture was then cooked on moderate heat over a steam bath for nearly 10-15 minutes. The slow heating of the mixture, in addition to ensuring better formation of colloidal bond, preserved the nutritive value of the diet. The cooked feed mixture in dough form was hand pressed, and strands of food obtained on to a stainless steel tray were dried at 90°C. The moisture level of the feed was adjusted to an acceptable level. Pellets of 2-3 mm diameter were found most acceptable to the fish.

VII. General experimental design

All experiments were conducted in well-aerated glass aquaria (60 x 28 x 30 cm), containing 30-1 fresh water, under natural photoperiod. The water was exchanged on alternate days or twice a week as required. The aquarium water was siphoned off every day to remove the faeces. Thorough scrubbing and cleaning of each aquarium was carried out weekly. Dissolved oxygen and water temperature were monitored.

In most cases the density of fish per aquarium was calculated on the basis of formula given by Ford (1981) for the purpose, the water surface area of aquarium was measured in cm square (X) and the value divided by the factor 140, the latter being worked out on the basis of the standard length
of the fish stocked in the aquarium.

\[
\text{Stock Density} = \frac{X}{140}
\]

All experiments were replicated. Other details including diet formulation have been dealt with in specific chapters.

VIII. Assessment of nutritional parameters

One of the criteria chosen to ascertain the performance of feed was the assessment of growth obtained by subtracting the initial weight of biomass from its final weight over a particular duration of feeding trial.

The specific growth rate was calculated using the formula:

\[
SG = \frac{G}{D}
\]

where,

- \( G \) was the gain in live weight of fish, and
- \( D \) was the duration of the feeding trial.

Using the length and weight records, the 'condition coefficient' in fish was estimated by the formula:

\[
K = \frac{\text{Weight(g)}}{\text{Length(cm)}^3} \times 100
\]

The 'food intake' was calculated from the actual quantity of
food consumed by the fish over the experimental period.

Food conversion factor was calculated on the basis of the ratio of food consumed to live weight gained by the fish. The gross growth efficiency represented the percentage of the ratio of live weight gain to food consumed, while the protein efficiency ratio was the ratio of wet weight gain to weight of protein fed.

The protein digestibility was estimated using chromic oxide indicator technique (see also page 25).

Protein deposition, protein conversion efficiency, energy retention and energy conversion efficiency were assessed using the following formulae:

Protein deposition (\%) \[ \text{Protein deposition} = \frac{\text{Final body protein} - \text{Initial body protein}}{\text{Protein consumed}} \times 100 \]

Protein conversion efficiency (\%) \[ \text{Protein conversion efficiency} = \frac{\text{Final protein of fish}}{\text{Protein consumed}} \times 100 \]

Energy retention (\%) \[ \text{Energy retention} = \frac{\text{Final body energy} - \text{Initial body energy}}{\text{Total dietary energy fed}} \times 100 \]

Energy conversion efficiency (\%) \[ \text{Energy conversion efficiency} = \frac{\text{Final energy in fish}}{\text{Total dietary energy fed}} \times 100 \]

The calculation of calories required to produce a kilogram of fish was based on the method suggested by Phillips and Brockway (1959).
IX. **Evaluation of body composition**

The estimation of proximate body composition have been made using the standard techniques described earlier.

X. **Statistical analysis**

Standard deviation, standard error, regression equation, correlation coefficient, analysis of covariance, t-Test for estimating the significance of 'r', factorial analysis of data and calculation of confidence limit were made using standard statistical techniques (Fraser, 1958; Graybill, 1961; Snedecor and Cochran, 1968; and Zeitoun et al., 1976). All analyses were made on Casio FX-39.
Fig. 1. Calibration curve of Nitrogen.
Fig. 2. Calibration curve of Protein.
Fig. 3. Calibration curve of RNA.
Fig. 4. Calibration curve of DNA.
Fig. 5. Calibration curve of chromic oxide.