2. GENERAL MATERIALS AND METHODS
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2. 1. COLLECTION AND MAINTENANCE

2. 1. 1. EXPERIMENTAL ANIMALS

2. 1. 1. 1. HETEROPNEUSTES FOSSILS

The test animal *H. fossilis* (Bloch) were collected from ponds in and around Palayankottai. They were brought to the laboratory and stocked in large rectangular cement tanks (500 l capacity) for a period of 10 to 15 days in well aerated water. During the period of acclimation they were fed daily with chopped beef liver (experimental food).

These fishes were then sorted into three size groups namely A, B and C weighing 4.46 ± 0.148, 7.79 ± 0.290 and 12.11 ± 0.116 g respectively. Each size group fish were kept in separate aquaria to prevent the 'dominance' by the bigger fishes over the smaller sizes. The water was changed once daily during the period of acclimation in order to keep the dissolved oxygen content above 50% saturation level. Changing of water also helped in the removal of feces and nitrogenous wastes.

2. 1. 1. 2. CYPRINUS CARPIO

These fishes were collected from Manimuthar Dam and they were
safely transported in oxygenated polythene bags. For the sake of convenience and to differentiate from other species, the three size groups of *C. carpio* were named size group K (4.36 ± 0.412 g), L (7.79 ± 0.386) and M (12.05 ± 0.115 g). The experimental procedures were same as in the case of *H. fossilis*.

2. 1. 1. 3. **Mystus keletius**

These fishes were collected from the local ponds in and around Palayankottai. Three size groups of *M. keletius* were named X, Y & Z and the average weight of X, Y and Z size groups were 4.33 ± 0.125 g, 7.77 ± 0.306 g and 12.04 ± 0.126 g respectively. The experimental procedures followed were same as in the case of *H. fossilis* and *C. carpio*.

2. 1. 2. **Experimental food and feeding**

Experimental animals were fed with beef liver and the food was always given *ad libitum*. The food was left in the experimental troughs overnight.

2. 1. 3. **Experimental design**

Fish of the respective size groups of *H. fossilis* were grouped into different densities in logarithmic order viz., 1, 2, 4, 8 and 16 fish / aquaria (15 l capacity). Each size group thus contained five series density. However, in larger size group (B and C), the 16 - fish series was not experimented
with, since the capacity of the experimental troughs (15 l each) was found to be inadequate.

All the experiments were carried out in triplicates and conducted at room temperature (29 ± 1°C). The 'Sacrifice method' of Maynard and Loosli (1962) was followed in the present investigation to estimate fish growth. After complete evacuation of their alimentary canal by starving them for at least 24 hrs (Mohanty, 1990) healthy fishes were selected and the wet weight of the experimental fishes were determined at the beginning of the experiment. The feces released by the fish were collected, filtered and oven-dried.

Beef liver was kept frozen during the course of the experiment. Everyday the frozen liver was taken out, thawed and a known quantity was weighed and oven-dried to estimate the water content and dry weight of the food. Fish were fed with known quantity of liver.

The remnants were collected from the respective experimental troughs the next day before changing the water and were oven-dried to calculate the dry weights of the unfed. Thus the dry weight of the food consumed can be calculated, which was the difference between the dry weight of food given and that of uneaten food. Feces collected everyday (once) were dried powdered and kept in desiccator for further analyses.
All the experiments were lasted for 21 days. After the experimental period, the fish were starved for a period of 24 hrs. The final weights of the individual fish of each experimental series were taken and the fishes were oven-dried. The dried fish were powdered and subjected to estimation of energy and proximate composition.

2.1.4. ENERGY ESTIMATION

Energy estimations for fish samples were done by plain jacket oxygen bomb calorimeter (Toshniwal, India) and feces energy were estimated by wet combustion method. Necessary corrections were made for wet combustion method as suggested by Job and Gerald (1969). The energy values are represented here as Kilo Joules (KJ).

2.1.4.1. OXYGEN BOMB CALORIMETER

The oxygen bomb calorimeter used in the present investigation is a plain jacket calorimeter. A known amount of experimental material was burnt electrically using a nichrome fuse wire in a closed container (Bomb) at high pressure (25 atm.) of oxygen. The heat of combustion of the material is equivalent to the amount of heat liberated and inturn, to rise in temperature of the water in calorimeter bucket surrounding the bomb. The increase in temperature was measured accurately by a Beckmann thermometer (0.005°C sensitivity) and from this increase, the heat of combustion (the energy values of the experimental material) was calculated.
Corrections were made for the length of the unburnt wire, cotton used for ignition, acid formation and the heat loss due to radiation.

Initially, to standardise the apparatus, the water equivalent was calculated using a known calorific substance. Benzoic acid, the calorific value of which was equivalent to 6.318 Kcal / g, was used for calculating the water equivalent.

6.318 X Wt. Of benzoic acid + correction factors
Water equivalent (W. E) = \frac{\text{Temperature increase}}{\text{Temperature increase}}

The water equivalent varied from 2.419 to 2.459. Therefore the average value, 2.439 Kcal was used in the calculations. The energy values of the experimental samples were estimated using the following equation.

\[ \text{Energy value of the sample (KJ / g)} = \frac{(W. E \times \Delta \text{temperature}) - \text{correction factors} \times 0.004184}{\text{Wt. of the sample (g)}} \]

2. 1. 4. 2. **Wet Combustion Method**: (Karzinkin and Tarkovskaya, 1964)

This is an indirect method of estimating the energy value of the material. The procedures makes use of the principle that potassium iodate, when heated in the presence of concentrated sulphuric acid and organic materials, decomposes and releases nascent oxygen which inturn oxidises
the organic matter present in the sample. The decomposition of potassium iodate is proportional to the energy content of the organic material.

A sample weighing 10 - 15 mg was placed in a round bottomed flask. The 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 potassium iodate and 20 ml of concentrated sulphuric acid. The flasks were connected to the reflux 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 flasks 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 10 % potassium iodide was added after transferring them into 500 ml conical flasks. Then the flasks were kept in darkness for 10 minutes. Control and experimental flasks were then titrated against 0.1 N sodium thiosulphate using starch as indicator. The end point is the disappearance of blue colour.
1 ml of 0.1 N sodium thiosulphate corresponds to 3.567 mg of KIO₃.

1 mg of KIO₃ corresponds to 0.1869 mg of oxygen.

\[\therefore 1 \text{ ml of 0.1 N thiosulphate} = 3.567 \times 0.1869\]
\[= 0.6667 \text{ mg oxygen.}\]

Using the oxycalorific coefficient of 3.38 (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

\[(\text{Control titre value} - \text{Experimental titre value}) \times 2.25 \times 0.004184\]

\[\text{Energy value} = \frac{\text{(KJ / g)}}{\text{Sample weight (g)}}\]

2. 2. ENERGY BUDGET

The energy budget followed here is the slightly modified IBP formula (Petrusewicz and Macfadyen, 1970) represented as \(C = P + R + F\), where \(C\) is the energy consumed, \(P\) the growth (Conversion), \(R\) the energy lost as heat due to metabolism and \(F\) the feces.

2. 2. 1. ENERGY CONSUMED

It was estimated by subtracting the unfed from the energy supplied.

2. 2. 2. ENERGY ABSORBED

It was calculated by subtracting the feces energy from that of energy consumed.
2. 2. 3. ENERGY METABOLISED

Energy metabolised was estimated by subtracting the energy converted from the energy absorbed.

2. 2. 4. ENERGY CONVERTED

Energy converted was determined by subtracting the energy of fish at the commencement of experiment from the energy of fish after the termination of the experiment.

Rates of energy consumption, absorption, conversion and metabolism were calculated by dividing the respective quantities of the products of initial weight of fish (g) and duration of the experiment (21 days).

2. 2. 5. CONSUMPTION RATE \((\text{Cr})\) = \(\frac{\text{Energy consumed (KJ)}}{\text{Initial wet wt. of fish (g) X days}}\)

2. 2. 6. ABSORPTION RATE \((\text{Ar})\) = \(\frac{\text{Energy absorbed (KJ)}}{\text{Initial wet wt. of fish (g) X days}}\)

2. 2. 7. METABOLIC RATE \((\text{Mr})\) = \(\frac{\text{Energy metabolised (KJ)}}{\text{Initial wet wt. of fish (g) X days}}\)

2. 2. 8. CONVERSION RATE \((\text{Pr})\) = \(\frac{\text{Energy converted (KJ)}}{\text{Initial wet wt. of fish (g) X days}}\)
2. 2. 9. **Efficiencies of Absorption and Conversion**

\[
\text{Absorption efficiency (Ae) (\%)} = \frac{\text{Energy absorbed}}{\text{Energy consumed}} \times 100
\]

\[
\text{Gross conversion efficiency (K}_1\text{) (\%)} = \frac{\text{Energy converted}}{\text{Energy consumed}} \times 100
\]

\[
\text{Net conversion efficiency (K}_2\text{) (\%)} = \frac{\text{Energy converted}}{\text{Energy absorbed}} \times 100
\]

2. 3. **Haematological Analysis**

After 21 days of feeding, few experimental fishes from each series were used for haematological analysis, to assess the changes in fishes subjected to experimentation.

2. 3. 1. **Collection of Blood Samples**

Blood samples were obtained by severing the caudal peduncle with a sharp blade. After discarding the first drop of blood, the freely oozing blood was collected in a small watch glass containing sufficient quantity of 10\% EDTA (anticoagulant). The blood was thoroughly mixed with the anticoagulant using a thin, blunt glass rod. Haematological parameters were analysed by employing the techniques suggested by Hesser (1960) and Samuel (1978).

2. 3. 1. 1. **Total Counts of RBCs**

For RBCs count, Hayem’s solution was used. The standard RBC
diluting pipette was used for counting RBCs. Total counts of RBCs were made using an improved Neubauer counting chamber. The graticule of the counting chamber and the cover class were carefully cleaned and were absolutely free from dust. Cover glass placed in position over the graticule and the chamber was charged with the diluted blood sample by allowing the drop of flow under the coverglass. Total number of RBCs were counted in five group squares (1 group square = 16 small squares) in 80 small squares. This total number is multiplied by $10^4$. This gave the total number of cells per cubic millimeter of blood.

2.3.1.2. Estimation of haemoglobin (Hb)

Shali's haemometer was used following acid haematin method. The Shali's pipette was filled to slightly above the '20' mark; the pipette was wiped with a soft absorbent tissue to remove excess blood and the volume was adjusted to exactly '20' mark. The blood was expelled into a haemometer tube containing 0.1 N hydrochloric acid up to the '20' mark. The contents were mixed by gently drawing in and expelling the same 3 to 5 times. The contents were again mixed using a glass stirrer and allowed to stand for 30 minutes. This mixture was diluted carefully with distilled water and the colour of the solution was matched with that of the standard provided on the haemometer. The amount of haemoglobin in the blood sample was directly read in g % from graduated tube (Samuel, 1978).
2. 3. 1. 3. Estimation of haematocrit (Ht)

It was estimated adapting microhaematocrit method. After estimating ESR, the same tube was used for Ht estimation also. The sealed end of the tube was carefully inserted into a Wintrobe’s tube filled with water and having a cotton pad at the bottom. The tube was centrifuged at 6000 rpm for 20 minutes and after that the length of the packed erythrocyte column, the buffy coat and plasma column were noted. Haematocrit (%) was calculated as

\[
\frac{L_1}{L_2} \times 100,
\]

Where

\[
L_1 = \text{Height of the RBC column (mm)}
\]
\[
L_2 = \text{Total height of the column (RBC + buffy coat + plasma) (mm)}.
\]

2. 3. 1. 4. Estimation of erythrocyte sedimentation rate (ESR)

A 15 cm long corning glass capillary tube with 1 mm inner diameter and calibrated in mm was used. Blood was filled up to 100 mm by capillary action and one end was sealed with wax and secured in a vertical position and kept undisturbed. After an hour, the length of the clear plasma column at the top was measured in mm. This value was taken as the ESR in one hour.

2. 3. 1. 5. Determination of mean corpuscular volume (MCV)

This is the average volume of a single red cell in cubic microns and determined by the following method
MCV (μm³) = \frac{\text{Haematocrit}}{\text{RBC in millions / mm³}} \times 10

2. 3. 1. 6. Determination of mean corpuscular haemoglobin (MCH)

Mean corpuscular haemoglobin content of one erythrocyte is determined by

\[ MCH \text{ (pg)} = \frac{\text{Haemoglobin}}{\text{RBC in millions / mm³}} \times 10 \]

2. 3. 1. 7. Determination of mean corpuscular haemoglobin concentration (MCHC)

The mean haemoglobin concentration in g % (g / 100 ml) for 100 ml erythrocytes, as well as that of one erythrocyte is determined by the following formula:

\[ \text{MCHC} (%) = \frac{\text{Haemoglobin}}{\text{Haematocrit}} \times 100 \]

2. 3. 1. 8. Total counts of WBCs

For Leucocyte count, Shaw’s diluting fluid was used (Shaw, 1930). Solution ‘A’ (25 mg of Neutral red solution and 0.9 gm of sodium chloride in 100 ml of distilled water) and solution ‘B’ (12 mg of crystal violet and 3.8 g of sodium citrate in 100 ml of distilled water and 0.4 ml of formalin) was used for total leucocyte count. Total number of leucocytes in the four large squares were counted and this was multiplied by 500 to determine the total number of WBCs in cubic millimeter of blood (Hesser, 1960). 16
2. 3. 1. 9. **Differential leucocytes counting**

Blood smears were made using the fresh blood taken from the caudal region. Buffered Leishman’s stain (Drury and Wallington, 1967) of pH 6.8 gave excellent results of blood smears (Fixed in methanol and then air-dried).

2. 3. 1. 10. **Determination of WBC / RBC ratio**

From the total count of WBCs and RBCs this ratio was calculated:

\[
\text{WBC / RBC ratio} = \frac{\text{Total no. of WBCs}}{\text{Total no. of RBCs}}
\]

2. 4. **Proximate Analysis**

Proximate composition of the experimental fishes were determined as follows: protein by Lowry *et al.*, method (1951), lipid by Bragdon method (1950) and carbohydrate by Anthrone method (*Carrol, et al.*, 1956).

2. 5. **Respiratory Metabolism**

2. 5. 1. **Selection of size groups**

Different size groups of fishes *H. fossilis* (A, B and C), *C. carpio* (K, L and M) and *M. Keletius* (X, Y and Z) were also used in the present investigation of respiratory metabolism. Before starting the experiment, the experimental animals were starved for a period of at least 24 hrs, as the freshly fed animals show varying metabolic rates (Ponniah and Pandian, 1977).
2.5.2. **Oxygen consumption and partial pressure of oxygen (pO₂)**

The respirometers used in the present study were constructed after Job (1957) and they were of a closed type. They were wide mouthed conical flasks of 620 ml capacity closed with a one holed rubber stopper carrying a thistle funnel serving as an inlet. The outlet was a side tube fixed close to the bottom of the flask to which a rubber tube was attached and was kept closed with a pinch cock.

Experimental fishes were led into the respirometer with little stress and the respirometer was closed airtight. The interval of experimental sampling was 30 minutes and the experiment lasted for 3 hrs. At the time of sampling, the pinch cock was released, a few drops of water was allowed to flow down as it might be the stagnant water in the rubber tube and the samples were collected in the microwinkler's bottles (7 ml capacity). In view of the smaller size microwinkler bottle, the reagents were also correspondingly decreased (Wycliffe, 1976). To compensate the water loss from the respirometer while sampling, a little quantity of water was poured into it through the thistle funnel. Necessary corrections were also made for the amount of water poured into the respirometer.

Then the experimental samples were analysed for the dissolved oxygen content using Winkler's method (Ellis *et al*., 1946). The mean value
was converted into pO₂ (mmHg) at the experimental temperature following Pierce (1973).

\[
\text{mg O}_2 / \text{litre (X)} = \frac{N \times \text{ml of titrant} \times 8 \times 100}{V_s \times (V_r - V) / V_r}
\]

Where,

- \( N \) = Normality of sodium thiosulphate
- \( V_s \) = Volume of sample titrated
- \( V_r \) = Volume of reagent bottle
- \( V \) = Volume of reagents

\[
pO_2 = \frac{160 \times \text{(X)}}{7.63}
\]

Where,

- \( X \) = Oxygen volume (mgO₂ / litre)
- 7.63 = Oxygen saturation level at 30°C.

2. 5. 3. ROUTINE, STANDARD METABOLISM AND DIURNAL VARIATION

The experimental apparatus used is a simple open flow respirometer as explained by Feldmeth (1971). The respiratory chamber is thick glass tube closed at both ends with rubber stoppers. Glass tubes of smaller diameter pass through the stoppers, so that the chamber is open at both sides. At one side (outflow) the glass tube is formed into a siphon. The animal chamber is submerged in a large tank (25 l capacity) filled with water, so that the water can run out of the tanks through the animal chamber (respiratory chamber) through the siphon. Water level in the large tank
was maintained at a constant level by allowing a slow flow of water into tank. The water flow through the animal chamber is regulated by a clamp. The end of the siphon (out let) is placed at the bottom of a microwinkler bottle and the collected sample was used for further analysis. The flow rate was also calculated.

First of all, the respirometer was filled with water. Then the respective size of fishes were introduced into the respiratory chamber with its head facing the inlet. Sampling of water was carried out every half an hour continuously for three hrs and the average of 3 hrs period was taken as the routine oxygen consumption rate.

The oxygen consumption (mg O₂ / l / hr) was calculated as: Oxygen consumption (mg O₂ / l / hr) = (Concentration at inflow) - (Concentration at out flow) X flow per hour.

Thus the final differences in oxygen concentration is dependent on the metabolic rate of animals and water flow rate and not on the size of the animal chamber.

In another experiment, water sampling was done every one hour continuously for 24 hrs to examine the SMR and diurnal variation. The lowest rate of oxygen consumption during the 24 hrs period was taken as the standard metabolic rate.
2. 6. Statistical analysis

Comparisons among density groups were made by one-way analysis of variance (ANOVA) (Zar, 1984) for energy budget, haematology and proximate analysis. Where the F-value statistic was significant at 0.05 level or 5% level and differences among density means were examined by Tukey’s honestly significant difference test statistic.

For respiratory metabolism the equation $Y = aW^b$ or $\log Y = \log a + b \log W$ and two way ANOVA were used for statistical analyses of the obtained data (Zar, 1984).