8. MATERIALS AND METHODS

8.1 ORIGIN OF FISH

*Mystus gulio*, the long-whisker's cat fish (Day, 1877) is locally called uppang kelethe (Jayaram et al., 1982). This fish is found in freshwaters, estuaries and even in seas throughout India, Pakistan, Bangladesh, Burma, Thailand, Sri Lanka and the Malay Archipelago. This is the only species of *Mystus* in India which is often caught in the sea, but not very far or deep (Pandian, 1970). Mostly estuarine (Fig.2), inhabiting mouths and tidal parts of rivers and adjacent coastline, it can inflict dangerous wounds. Lurid bluish brown above, dull white below (Fig.3) the dorsal and pectoral fins have spines. It is found to be a bottom feeder, eating worms, mud and small fish (Pasha, 1964).

8.2 COLLECTION

The fish is abundant, inhabiting mouths and tidal parts of rivers and adjacent coastline in and around Adirampattinam (Fig.2). Live specimens were caught from their natural habitats of local backwaters of Agniar estuary (Fig.1) which is situated in the Palk strait on the east coast of India (Lat. 10° 20'N; Long. 79° 23'E) of Thanjavur District, Tamil Nadu, India and reared in large aquarium tanks with continuous circulation of the estuarine water.

8.3 MAINTENANCE

*M. gulio* of both sexes (3-30 cm; 1-30 g; N=1200) were transferred to the laboratory from the large aquarium tanks and acclimated in the experimental tanks (PVC tanks, 500 l) in three groups. Fish weighing ≤ 1g are fingerlings; > 1 g and ≤ 8.5 g are immature and > 8.5 g are mature (Michael, 1984).
Fig. 1 Map showing the Agniar estuary located near Adirampattinam, Tanjavour District, Tamil Nadu, India.
Fig. 2 A natural estuarine habitat of *Mystus gulio* located near Adirampattinam, Thanjavur District, Tamil Nadu, India.
Fig. 3 *Mystus gulio* Lateral view.
Fish were held in circular PVC tanks and supplied with continuously aerated and running brackish water (temperature 27-28.5°C; water flow 5-8 l/min; dissolved O₂: 5.7 – 7.5 mg/l; pH 7.4 – 7.6) under a natural photoperiod for 3 months prior to experimentation. The fish were fed with boiled hen’s eggs and earthworms daily at noon. Fish maintained this way survived well without any disease problem.

8.4 GROSS ANATOMY OF GILLS AND AIR-BLADDER

A few catfish were given longitudinal incisions at the abdominal regions and preserved in 10% formalin. For study, the fish were dissected from the lateral side, so as to expose the air-bladder. Gas bladders of fresh and preserved *Mystus* were inspected for the presence of a specialized respiratory surface. The organ shape and size, the extent of vascularization, and the position and size of the pneumatic duct were also observed.

Twelve fish were also sacrificed by over-anaesthesia (MS 222; 1:10,000 buffered to pH 7.5 with sodium bicarbonate). They were thick sectioned while frozen either longitudinally or in serial transverse sections, along their body lengths. All sections were thawed, fixed and stored in 10% formalin. A few specimens were examined fresh in order to avoid the effects of fixations or preservatives on the gills.

8.5 BLOOD-WATER DIFFUSION BARRIER

Fishes of different size (4.5-16.3 g) were sacrificed and pieces of 1st; 2nd; 3rd and 4th gill arches were taken and fixed in Zenker’s fixatives. The fixed materials were processed by the conventional procedure and paraffin sections were cut at 6 µ thickness in horizontal plane (with reference to the axis of the gill filaments) and stained with haematoxylin followed by counter staining with eosin. Many photomicrographs were taken at various levels of gill filaments of each gill
arch. Magnification of the photomicrographs of secondary lamellae was determined in relation to the photomicrograph of the stage micrometer scale. The total thickness of the blood water-barrier was measured at various levels of these photomicrographs and an average value was obtained. Data were analysed by logarithmic transformations on the basis of least squares using the exponential equation,

\[ T = a w^b \]

Where \( T \) = thickness of blood / water pathway ( \( \mu \text{m} \))
- \( a \) = intercept
- \( w \) = weight of the fish (g)
- \( b \) = slope of the regression.

8.6 Gill parameters

The fish were filled with an overdose of anaesthetic (0.1 % benzocaine). The gill arches were quickly removed and fixed in bouin’s solution. The number of gill filaments, total filaments length, lamellar frequency / mm of filament and area of individual lamellae were calculated following the procedures established by Gray (1954) and Hughes (1984 a). For each gill arch from the left side, the filament number of each hemi branch was counted and every 10\(^{th}\) filament including the first and last were measured with a micrometer eyepiece.

The lamellar frequency per mm of filament and the bilateral lamellar surface area from the base, middle and tip of measured filaments were estimated after the shape of lamellae had been delineated using serial histochemical slides (5 \( \mu \)) from sagittal, longitudinal and transverse sections of filaments.
The gill area was calculated by employing the equation of Muir and Hughes (1969);

\[ \text{Gill area} = 2 \left( \frac{L}{d'} \right) b_l \]

Where \( L \) = total length of filaments

\( 2/d' \) = secondary lamellae on both sides of gill filaments and

\( b_l \) = average bilateral surface of the secondary lamellae.

The relationships between every gill filament (\( Y \)) and body weight (\( W \)) were analysed by fitting the equation:

\[ Y = a \ w^b \]

applying a linear regression analysis to logarithmically transformed data where \( Y \) is the parameter analysed, \( w \) is the body weight and \( \log a \) and \( b \) are the intercept and slope of the regression line, respectively.

8.7 Survival out of water and Evaporative Water Loss (EWL)

Samples of \textit{M. guliio} were taken from the aquaria and lightly blotted on paper towels to remove excess water. They were placed individually on a Whatman filter paper and cut to fit a litre volume plastic box. The individuals were immediately transferred to glass desiccator containing 250 ml of the following solutions for desired humidity levels using graded solutions of KOH as described by Solomon (1951). Water giving 95 to 97 \% relative humidity (R.H.); mean 95 \%. Sodium chloride giving 72 to 76 \% relative humidity (R.H.) mean 75\%. Calcium chloride giving 28 to 31 \% relative humidity (R.H.) mean 35\%.

Faster equilibration of the humidity levels was achieved by the use of the magnetic stirring bar for 5 minutes to agitate the solution at the bottom of the humidified chamber, was then placed in an incubator at a constant temperature of 28±1°C. With determinations of survival and body weight made at regular
intervals. Serial weighings were made to determine rates of evaporative water loss. The containers were weighed at intervals to the nearest 0.1 mg and weight loss was assumed to equal water loss. Criteria for life used in survival studies were either visible small spontaneous movements or recovery of life when placed in water within a few minutes.

8.8 Asphyxiation

Laboratory maintained, healthy *M. gulio* ranging in weight from 8-35g formed the material for the present study. They were fed at regular intervals but not for at least 12 h before, or during the experiments. Asphyxiation time for the fish was recorded at 26, 30, 35 and 40° C. The asphyxiation time for the fish at each temperature was recorded by placing horizontal diaphragm near water surface in the experimental jar to prevent the fish from coming to the surface and gulping the air. Fishes were kept individually in continuous flow of water (DO 7 mg / l; pH 7.3). The temperatures of the experimental tank (Fig.4) were maintained by an electrically driven thermostat. To ensure that water runs at a constant speed (250-300 ml/min), a constant level cylinder was introduced in between the tapwater and the experimental jar. Each jar was provided with a glass tube from the tap and an overflow tube connected to the experimental jar. Precautions were taken so that there might be no leakage of air-bubbles in the jars from any place. O₂ concentration was measured with a Philips O₂ meter (No 1530); calibration was checked by the Winkler method and found accurate within 0.1 mg / l.

8.9 AIR-BREATHING INTERVAL (ABI)

Air-breathing intervals were recorded at 28° C for over a month. Care was taken to control the temperature at 28±1° C. The experiments were terminated within 10 days so as to minimize the influence of seasonal variations.
Fig. 4: Experimental arrangement used for asphyxiation—aquarium with wire netting (diaphragm) and continuous flow of water.
The observations were carried out under the same conditions of illumination during the day time to avoid the changes possibly associated with the diel of light intensity. Fishes were kept individually and were observed from behind a blind. The O$_2$ content was maintained at 7.2 mlO$_2$/l (Normoxic)

8.10 RESPIRATION

8.10.1 Aquatic respiration under forcibly submerged condition

Jobs (1955) continuous flow apparatus was slightly modified to serve the present needs. The apparatus consisted of a large isolated rectangular tank with a capacity of 100 ml in which the respiratory chambers were immersed. Water was continuously introduced into the tank and passed into respiratory chambers, coming out through their outlets. Since the rate of flow through the respiratory chambers had to be constant, the depth of water in the tank was maintained constant by a constant level bottle. The desired temperature in the insulated tank was maintained to within plus or minus 0.5° C by adjusting the temperature of the water supply. The water in the tank was aerated vigorously by air diffusers. The tank and the respiratory chambers were covered during experiments by a lid.

8.10.1.1 Respiratory chambers

The respiratory chambers consisted of glass jars with three hole stoppers for the inlet, outlet and thermometer chamber (Fig.5). The capacities of these chambers varied from 1 to 6 l. The rate of flow of water into the chamber was controlled by screw clamp on a short length of rubber tubing at the free end of the glass inlet tube. The outlet of the respiratory chamber was protected by a wire gauze screen so that any excreta dropped by the fish did not block the flow. The flow of water through the respirometer [150 ml min$^{-1}$ (l h$^{-1}$)] was adjusted
Fig. 5: Respiratory chamber with experimental set up for respiratory studies.
according to the size of the fish to avoid possible suffocation and stress. The fishes were acclimated to the respirometer at least 24 h before the readings were taken. Water samples were collected from the bottles connected at the end of the respirometers. A preliminary check on the CO₂ and the pH of the water entering and leaving the chambers showed no significant difference. Each experiment lasted for 24 h or a little more. All fish used in the study were starved for 24 h before the experiments. Except for the few samples taken in order to adjust the ratio of flow about 3 h after enclosing the fish, the regular readings were begun 12 h later towards late evening and were continued until well past noon the next day. The continuous flow method used here cut down to a minimum the chances of the fish being disturbed in the course of the experiments. The pressure changes in the respiratory chamber due to extraneous factors were corrected by a thermo barometer.

The concentrations of the DO in the samples were analysed by an O₂ analyser. O₂ uptake per unit time was calculated with the help of the equation

\[ VO_2 = V_w [C_{IO_2} - C_{EO_2}] \]

Where \( VO_2 = O_2 \) uptake (ml O₂ h \(^{-1}\) )
\( V_w = \) water flow (ml / min)
\( C_{IO_2} = O_2 \) concentration of inlet water and
\( C_{EO_2} = O_2 \) concentration of outlet water

### 8.10.2 Aerial respiration

For studying the respiration of animals in air, respirometers (Figs.6-8) were designed involving the principles of manometric techniques. The construction of the apparatus is featured in the figures 9-10. The set-up consists of a respiratory chamber connected to a graduated "U" tube containing Brodie's
Fig. 6 Aerial respiratory chambers.

Fig. 7 Aerial respiratory chambers fixed to stand.
Fig. 8 Manometer arrangement used for study of aerial respiration.
Fig. 9 Set up used for study of aerial respiration.

Diagrammatic representation
Fig. 10 Manometer set up used for study of aerial respiration during outdoor work.
fluid. Carbon dioxide was absorbed in a 20 % KOH (BDH) solution. As the O₂ was utilized the pressure in the respiratory chamber was reduced displacing the manometer level. The difference in the levels of the liquid in the manometer for a given time is used in the following equation (adopted from Umbreit et al., 1959) and the gas utilized is calculated.

\[ V = \frac{V_i \times h}{10,000} \]

where 'V' is the volume of the gas utilized

- \( V_i \) is the volume of the gas in the respiratory chamber and
- 'h' is the difference in the levels of the Brodie's fluid in the manometer

Pressure changes in the respiratory chamber due to extraneous factors were corrected by a thermo-barometer. The water baths and the respiratory chambers were placed in a darkened temperature controlled cabinet during all measurements. The fishs were acclimated to the respiratory chamber for 24 h prior to each experiment. This will also reduce the possible build-up of excretory products during the experiments.

8.10.3 Bimodal respiration

Aerial and aquatic respirations were also measured simultaneously when the fish was in water with access to air using an experimental set up designed for enabling the fish to respire from both air and water. The construction of the apparatus is featured in Fig. 11 and glass desiccators (6 l) were used as respirometers (Fig.5). Water was filled to about 4500 ml and a liquid paraffin layer added leaving an air phase of 1500 ml. Preliminary tests showed no transfer of O₂ from the air, water or both media and the paraffin caused no adverse effect. A similar arrangement was used by Reddy and Natarajan (1970)
for bimodal respiration studies of air-breathing fishes. The O$_2$ consumption of
the fish in air was measured by connecting the air-phase through the lid with
a simple respirometer involving the principles of manometric techniques as
outlined earlier under aerial respiration heading. Brodie's fluid was used in the
manometer. The amount of gas utilized by the fish was calculated by multiplying
the change in pressure by the volume of the gas phase (adopted from
Umbiat et al., 1959).

The VO$_2$ from water was measured by determining the difference
between the inflowing and outflowing O$_2$ concentrations with an O$_2$ analyser
(Model PE-130) and the values used in the following equation:

\[
VO_2 = F \left[ Y_1 - Y_2 \right] + V \left[ Y_{2,0} - Y_{2,t} \right] / \text{fish wt.}
\]

Where
\( F \) = volume of water flow through respirometer
\( V \) = volume of respirometer (water phase only).
\( Y_1 \) = Inlet O$_2$ concentration
\( Y_2 \) = outlet O$_2$ concentration

The subscripts O, t indicate readings at the start and of 30 min interval
over which the readings were made. \( V \left[ Y_{2,0} - Y_{2,t} \right] \) is a correction factor for lag
in the system (Fry, 1971). This flowing water system was poorly buffered and
large change in pH occurred in a sealed system. Throughout the period of
testing, temperature, salinity, O$_2$ tension and pH were maintained at regular
intervals to make sure that these factors were relatively constant. Thus, except
for the feeding of the fishes, all conditions were maintained uniformly. The cyclic
changes in the level of illumination being the only (overt) rhythmic
factor involved. Throughout the experiments the light periods themselves
(12:12 light-dark) did not change.
8.10.4 Air-exposure

The effects of air-exposure (2 and 3½ h) on the bimodal respiration were studied by siphoning the water from the tank without handling the fish, thus minimising the handling disturbance, prior to determination of O₂ consumption. The R.H. inside the tank was 100% (at 28°C). The air movement over the tank was nil. After air-exposure, they were returned to individual respirometers. The bimodal O₂ uptake of the fishes along with the controls were determined separately using the apparatus and adopting in the procedures described above.

8.10.5 Hypoxic exposure

The fishes were divided into 3 batches. The first batch served as controls and exposed to normoxic conditions (7.2 ± 0.1 mg/l) at 28 ± 1°C. The second batch (10 fishes) was subjected to 98 h hypoxic conditions. The third batch (10 fishes) was acclimated to 30 days hypoxia. All hypoxia fish were exposed to an O₂ concentration of 2.0 mg/l. This is 20% saturation.

Hypoxic conditions in the acclimation tanks were created by bubbling compressed gases (N₂) directly into the aquaria (Fig.12) before delivering into the respiratory chambers. The level of DO was controlled by varying the water flow and flow rate control. An O₂ electrode positioned in the flow tube that circulated water into the respiratory chamber was used to monitor water O₂ content during all tests. Water flow from this system was also periodically withdrawn through a sampling port, for determination of water O₂ and CO₂ content and pH using a gas analyser that has been standardised with known gas mixtures and buffer solutions at the experimental temperatures. The respiratory rates of control and hypoxia acclimated fishes were measured for a period of atleast 1h. The air-phase of the respiratory chamber was flushed with fresh air before each period of the measurement. The O₂ electrodes were checked at the
Fig. 12: Experimental set up used to produce hypoxic gas for bimodal respiration studies. The diagram is not to scale.
beginning and at the end of each run. No fish was used more than once. After each run, the fish were blotted with a paper towel and weighed to the nearest 0.1 g. All VO₂ figures have been converted to standard temperature and pressure. During hypoxia acclimation both control and experimental fishes were fed on alternate days. The tanks had to be cleared to prevent toxic conditions. No mortality was recorded. Bimodal respiratory rates were determined as described earlier.

8.10.6 SALINITY EXPOSURE

Laboratory maintained fish were isolated and starved 24h prior to the measurement of bimodal O₂ consumption so as to eliminate the influence of differential feeding on their O₂ consumption. Bimodal O₂ consumption was measured (as outlined earlier) in sea water (S.W.) of 100% (= 32.43%), 50% and in tapwater. Change from one salinity to the next lower one (starting from 100% S.W.) was abrupt. Each fish was allowed to remain for 30 min in each medium before respiratory rates were recorded. Only one fish was taken into each respiratory chamber. Immediately after measuring in the three media, each fish was removed and blotted and weighed. 50% salinity was made by dilution of 100% S.W. with tap water. All measurements were made at 28 ± 1°C.

8.10.7 TEMPERATURE

The effect of acclimation to warm (35°C) and cold (20°C) temperature on the bimodal O₂ consumption of the fish was studied. For warm acclimation, fish were kept in a thermostatically controlled water bath adjusted to 35°C(±0.5°C) and for cold acclimation in a B.O.D. type incubator adjusted to 20°C(±0.5°C). The room temperature was 28°C. Fish were given an equilibration time of about 30 min in each temperature. The respiratory rates were determined by the methods outlined earlier.
8.10.8 CIRCADIAN RHYTHM

Samples of *M. gulio* were isolated from the stock into individual jars and fed with earthworms at regular intervals at a daily rate of 2 - 3% of body weight. Fish were last fed at 0900 h to day prior to sampling. Artificial illumination was achieved using fluorescent strip-lights located 130 cm above the water surface of the jars controlled by time clocks and set for a 12 L : 12 D photoperiod.

Small (1watt) night lights were kept on continuously to prevent total darkness. Throughout the test, O₂ tension (7.1 ± 1 mg l⁻¹), pH (7.3 ±0.1) and free CO₂ level (0.2 ±0.1 mg l⁻¹) were kept constant and the fishes were in post absorptive state.

The diurnal rhythm of bimodal O₂ consumption was studied, in 3 weight groups ranging in weight from 3.4g to 24.6 g. Each test was run at 28 ±1°C and there was a 48h acclimation period in the respiratory chamber prior to the measurement of respiration rates. The cyclic change in illumination was the only (extrinsic) agent involved. The experimental fish were weighed and placed in the respiratory chambers. The experimental set-up as described earlier enabling the fishes to respire from both the air and the water, as in natural conditions was used in the investigations. The amounts of O₂ consumed under water and in air were separately determined for a day at regular intervals of three hours each. The total O₂ consumption at each time was obtained by summing up the values for aquatic and serival respirations obtained at the corresponding time. Throughout the present study, the initial O₂ content of the water was kept constant (7.1 mg/l). The three weight groups were continuously subjected to rhythmic studies separately for 3 consecutive days.
8.11 HAEMATOLOGY

Hematological studies were carried out only on apparently healthy fishes. Inactive and emaciated specimens and those with apparent signs of infection such as ulcerous skin lesions, haemorrhages, mutilations etc. were discarded.

8.11.1 Collection of blood Sample

The method of caudal peduncle severing was adopted to collect blood samples. Each fish was stunned by a sharp blow on the head and was blotted dry, with a clean towel. After determining the sex, the posterior aspect of the body was laid on a dissection board and the caudal peduncle was severed with a sharp heavy knife. Blood freely oozing from the cut end was collected into a small cavity block containing sufficient quantity of anti-coagulant. A mixture of ammonium oxalate and potassium oxalate in the ratio 6:4 and at the rate of 0.5 mg / ml of blood was used as anti-coagulant. Cavity blocks were prepared by air-drying in each, 0.1 µl of 10mg heparin sodium salt in 8 ml of distilled water.

Only the freely oozing blood was collected. Collection was stopped as soon as the blood oozing out from the cut end began to show indications of clotting. So also, samples accidentally contaminated in any way were discarded. Care was taken to thoroughly mix the blood sample with the anticoagulant during the collection of the sample and immediately thereafter. A thin, blunt glass rod was used for mixing the blood sample with the anticoagulant. After thoroughly mixing the blood sample thus, the cavity block containing the sample was properly covered with its lid and kept in a dry place, away from sunlight and other sources of heat.

Samples for haematological analyses were prepared within 15 minutes after collection of blood. Basic peripheral haematological variables namely total
RBC count, total leucocyte count, DLC, haemoglobin content (Hb), packed cell volume (PCV), erythrocyte sedimentation rate (ESR) were determined employing the methods suggested by Hesser (1960) and Blaxhall and Daisley (1973). From the values of RBC, Hb and PCV, corpuscular constants (mean corpuscular volume – MCV, mean corpuscular haemoglobin - MCH and mean corpuscular haemoglobin concentration -MCHC) were computed.

8.11.2 STUDIES ON PERIPHERAL BLOOD

8.11.2 Total Count Of Corpuscles (TEC and TLC)

The haemocytometer method was employed for determining cell contents. A haemocytometer with improved Neubauer ruling (Superior, Germany) was used (Fig.13). Hendrick's fluid (Hendricks, 1952) lightly coloured with methylene blue, and 1:200 dilution were used for diluting blood samples in standard Thoma pipettes (Mitsubishi, Japan). Erythrocyte and leucocyte counts were made simultaneously with the same diluted sample and at 10 x 45 magnification. Duplicate count was made for each sample and sex was taken into consideration.

8.11.3 Haemoglobin content

Sahli’s acid haematin method was employed since sophisticated equipment were not available at the beginning of the work. Later, Hb content of each sample was determined spectrophotometrically with a haemoglobinometer (Type 175, Systronics India LTD) and Drabkin’s reagent (BDH). The earlier estimated Sahli’s values were corrected employing correction factors (regression coefficients) calculated by estimating the Hb content of the same sample by both methods.
8.11.4 Erythrocyte Sedimentation Rate (ESR)

ESR was determined by the micro-method because the quantity of blood available from individual fish was insufficient to adopt any macromethod. A non-heparinized micro-haematocrit tube was filled to 50 mm with anticoagulated blood. One end of the tube was sealed with sealing wax and the tube was then kept in a vertical position (Fig. 14), in a glass beaker stuffed with cotton. After one hour, length of the plasma column was measured with a ruler graduated in 0.5 mm.

8.11.5 Packed Cell Volume (PCV) or Haematocrit (Hct)

PCV or Hct was estimated by the micromethod, using non-heparinized micro-haematocrit tubes. Tubes were sealed with sealing wax. The tubes were spun in a microhaematocrit centrifuge (Toshiniwal India LTD). Hct was directly read with the microhaematocrit reader.

8.11.6 Erythrocyte constants

These were computed as shown below:

(i) Mean Corpuscular Volume (MCV) = Mean volume of erythrocyte

\[
\text{MCV} = \frac{\text{PCV}}{\text{RBC}} \times 10 \text{ (fl)}
\]

(ii) Mean Corpuscular Haemoglobin Concentration (MCHC) = The % of haemoglobin in unit volume of erythrocyte

\[
\text{MCHC} = \frac{\text{Hb}}{\text{PCV}} \times 100 \text{ (g/dl)}
\]
Fig. 13 Haemocytometer and Haemoglobinometer used for blood studies.

Fig. 14 E.S.R. tube with stand.
(iii) Mean Corpuscular Haemoglobin (MCH) = Average weight of Hb in an erythrocyte

\[
MCH = \frac{Hb}{RBC} \times 10 \ (\mu g)
\]

8.11.7 Erythrocyte dimensions

Erythrocyte dimensions were measured using air dried blood smears stained with the buffered Wright's stain following procedures recommended by Seiverd (1961). Ten cells were selected at random from each slide and the length and width of the cells were measured under 970 X magnification with a calibrated ocular micrometer (Figs 15 and 16).

8.11.8 Osmotic fragility

The method was based upon a procedure in which the percentage of haemolysis is plotted against the percentage of NaCl. The volumes used were 25 ml blood 2.5 ml of saline (% v/v NaCl of 0, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.85). After mixing, the samples were kept on ice for 1 h and were then centrifuged (2000 g for 15 min at 1° C) and the absorbance read at 540 nm. Standard curves for the Hb were prepared for each concentration of the saline solutions as the molar extinction coefficients varied slightly with different saline solutions. Thus, the cell fragility was expressed by the concentration of Hb dispersed in the aqueous samples, and the Hb value was expressed by the concentration in water. For each sample the percentage of NaCl solution causing the initial and the final degree of haemolysis was recorded. The final fragility values refer to a calculated value of NaCl whereby 50% of the sample was haemolysed. All individual fish blood samples were analysed in duplicate and average values determined.
Fig. 15 Microscope arrangement for measuring erythrocyte dimensions.
Fig. 16  Ocular micrometer – enlarged set up used to measure erythrocyte sizes.
8.11.9 Surface tension

The surface tension of fish blood was measured in duplicate by the capillary rise method at room temperature by drawing into 25 μl micropipettes placed vertically in small 1 ml test tubes. Two pipettes were used together and in the other the blood was drawn up and allowed to fall. After about 5 min with very gently tapping, the two levels were nearly at the same height, and an average value for height was determined. The radius of the tubes was determined from a microscopic measurement of a cross section of the tubing (r = 0.036 cm). The surface tension (σ) was then calculated:

\[
\sigma = \frac{1}{2} (hdg r)
\]

Where \( h \) = height of column (cm),
\( d \) = density of the blood (g cm\(^{-3}\)),
\( g \) = acceleration due to gravity at 980.67, and
\( r \) = tube radius (cm)

The final value is expressed in dynes per centimetre.

8.11.10 Refractive index

The plasma was analysed with an Abbe refractometer at 28° C, and multiple readings were taken for each sample.

8.11.11 Density

Specific gravity is measured by the cupric sulfate drop method (Phillips et al., 1950). In the present study blood was measured with 100 ml pipettes at 28° C. The sample weight was obtained, and the densities were calculated.
8.11.12 Standard bicarbonates

The blood bicarbonate was determined by the manometric method of Vanslyke and Cullen (1969).

8.11.13 Differential leucocyte count (DLC)

The blood films of normal and air-exposed fishes were stained by Giemsa's in phosphate buffer of pH 6.8 for DLC. The slides were first fixed in methanol for 5 min and then stained. The specimens were treated for 2 min with commercial Leishman's stain, water washed and treated for 5 min with Giemsa's stain. The Giemsa's stain solution consisted of Giemsa's powder (1g), glycerin (66ml) and methanol (66ml) which was then mixed (3.5 ml) with phosphate buffer (50 ml, pH 6.8, 0.1 m). The slides were finally rinsed with phosphate buffer and then with water and were then dried in air. Blood cells were identified as per the classifications of Ellis (1976).

8.11.14 Blood glucose

Blood glucose was estimated by Folin-wu method. Glucose on boiling with alkaline copper solution reduces copper from the cupric to cuprous state (cuprous oxide). The cuprous oxide so formed reduces phosphomolybdic acid to blue coloured molybdenum blue, which is measured colorimetrically. The intensity of the blue colour is proportional to glucose concentrations. Protein free filtrate of blood was prepared and after following the procedure, the intensity of the colour developed was read in a spectrophotometer (Bosch and Lomb) at 620 nm (Klontz and Smith, 1968).
8.11.15 **Blood lactic acid**

Blood samples were collected by severing the caudal peduncle and analysed for lactic acid using an enzymatic technique (Sigma Co., 1974). Measurements were made in quartz cuvettes at 340 nm with a Beckman DU spectrophotometer (Fig.17). Standard curves were made on the day the blood lactic acid determinations were made.

8.11.16 **Gill metabolites**

Fishes were killed by pithing. The gills were quickly excised and the tissues scraped from the gill filaments on a cold surface maintained at 0-2° C. Samples were immediately put into 10 parts (W/V) of ice-cold perchloric acid (2%). The homogenized samples were centrifuged at 10,000 rpm for 15 min. at 4° C. An aliquot of the resulting supernatant fluid was neutralized with 5 mol 1⁻¹ Na₂CO₃. The glucose content was determined by the method of Bergmeyer et al., (1965).

For extraction and determination of glycogen from the gill samples the procedures adopted by Lim and Ip (1989) was followed. For the release of glycosyl units from glycogen, 0.1 ml of the sample was incubated in 10 m mol⁻¹ acetate buffer (pH 4.5) with 10 ml α amyloglucosidase at 50° C for 15 min. The glycogen content was expressed as μ mol glycosyl units g⁻¹. To calculate exact lactate content of the gill tissues, the amount of lactate in the blood of the sample must be subtracted from its total lactate content.

8.11.17 **Whole body lactate content**

Fish (n=6 in each different treatments) were individually assayed for whole body lactate concentration (μ mol lactate g⁻¹) by being frozen in liquid N₂ and homogenized 1:10 in 1M ice-cold perchloric acid in a blender. Supernatant fluid samples were assayed by the UV-spectrophotometric method with a kit from Sigma chemical Co.
Fig. 17  Beckman DU – spectrophotometer.

Fig 18  Cooling centrifuge for blood metabolite studies.
8.12 Biochemical Analysis

8.12.1 Plasma protein content

Plasma samples were obtained promptly, immediately sealed in glass vacutainers and kept at \(-20^\circ C\) before analysis. All analyses were carried out in less than 2 weeks. The method adopted by Soivio and Oikari (1976) was used for the determination of the plasma protein content.

8.12.2 Tissue protein content

The tissues (gills, heart, liver, muscle, brain and air-bladder) from control and air-exposed (2 and 3 ½ h) fish were dissected out and a known amount (50 mg) of tissues was homogenized separately with ice-cold 0.32 M sucrose solution by using a Potter-Elvehjem type teflon homogenizer. The homogenate was sonicated (25,000 times/sec) for 1 min in an ultra sonicater (Philips model S2) and centrifuged at 15,000 rpm to 30 min at 4\(^{\circ}\) C. The soluble protein content of the tissue homogenates were determined by the method of Lowry et al. (1951) with crystallline bovine serum albumin (BSA) as the standard as modified by Zak and Cohen (1961).

8.12.3 Acid phosphatase

Acid (acid orthophosphoric phosphohydrolase) (EC 3.1.3.2) phosphatase was assayed following the procedure of Morton (1955).

8.12.4 Alkaline phosphatase

The alkaline phosphatase (orthophosphoric monoester phosphohydrolase) (EC 3.1.3.1) activity was determined by the method of Morton (1955) using p-nitrophenyl phosphate so colourless in solution but upon hydrolysis the phosphate group liberates p-nitrophenyl which is highly coloured in alkaline
solution. The reaction production, p-nitrophenol in acid phosphate was measured spectrophotometrically at 415 nm against reagent blank. The enzyme activity was calculated from the standard curve and expressed as micromoles of p-nitrophenol formed per hour per milligram protein. The rate of hydrolysis of p-nitrophenyl phosphate is proportional to the enzyme present in the tissue.

\[
p\text{-nitrophenyl phosphate + NaOH} \xrightarrow{\text{Phosphatase}} p\text{-nitrophenol + phosphate}
\]

The colour developed in alkaline phosphatase activity was read at 410 nm against reagent blank spectrophotometrically.

8.12.5 Na\(^+\) K\(^+\) - ATPase, Mg\(^{2+}\) - ATPase and Ca\(^{2+}\) - ATPase

The specific activities of sodium, potassium, magnesium and calcium dependent ATPase were assayed according to the methods described by Watson and Beamish (1980) and Boese \textit{et al.} (1982). Adenosine triphosphatase catalyses the conversion of ATP into ADP. During this conversion, one molecule of phosphorus is liberated.

\[
\text{Adenosine triphosphate} \xrightarrow{\text{ATPase}} \text{Adenosine diphosphate} + \text{Pi}
\]

The inorganic phosphorus liberated was assayed according to the method of Fiske and Subbarow (1925). In this method the protein is precipitated with trichloro acetic acid. The protein free filtrate is treated with acetic acid molybdate solution and the phosphoric acid formed is reduced by the addition of 1-amino 2 napthol 4- sulphonic acid (ANS) reagent to produce the blue colour. The activity of the blue colour was read at 680 nm against reagent blank using a spectrophotometer. Suitable standards were run through each batch of assays. The enzyme activity was expressed in terms of µg of inorganic phosphorus formed per hour per mg protein.
8.13 NITROGEN EXCRETION

8.13.1 Ammonia and urea estimation

For measurement of ammonia and urea excretion in normal fish, individual fish were placed in glass jars containing 100 ml of water without food for 24 h. The water samples were collected after 24 h and analysed for ammonia and urea. Aliquots (1 ml) was then analysed for ammonia at room temperature using Conway micro diffusion units (Conway, 1962). Urea was first hydrolysed to ammonia by urease, two BDH urease tablets were dissolved in water, the solutions filtered and 0.2 ml of the filtrate used in each assay. Ammonia was liberated with 1 ml of 40% KOH, absorbed in 1.5 ml of 0.001 N HCl indicator solution (1 ml of Tashiro's reagent in 100 ml HCl) and titrated with standard Ba(OH)$_2$. Tashiros reagent—4 vol of 0.1% alcoholic methyl red plus 1 vol. of 0.1% alcoholic methylene blue.

In the experiment for determining of NH$_3$ and urea excretion during aerial exposure, the fish were transferred to 100 ml freshwater (sterilized) after being kept on land for 2 and 3½ h. The concentration of each nitrogen compound out of water throughout the whole period was determined.

8.13.2 Blood ammonia and urea

Blood was obtained from anaesthetized fish with MS 222 (Sandoz) by severing the caudal peduncle. Blood from control and air-exposed fish was used for each analysis separately and 5% heparin was added to the blood. The blood from six fish was mixed together and centrifuged for 10 min at 5000 rpm and 1.5% TCA solution of about 20 vol. was added to the centrifuged blood. The solution was centrifuged for 10 min at 15,000 rpm and filtered. The filtered solution was messed up to defined volumes by adding 1.5% TCA solution. Urea was determined using 1-phenyl 1,2-propanedione–2–oxime (Archibald, 1945).
NH₃ was determined using the micro diffusion technique of Nathan and Rodkey (1957) in combination with the phenyl-hypochlorate reagents described by Chaney and Marbach (1962).

8.13.3 Liver Ammonia and Urea

Liver (100 mg) from control and air-exposed fish was removed immediately after experimentation and stored at 20°C until required for extraction. They were homogenized in 5 vol of buffer using Potter–Elvehjem homogenizer. They were deproteinized with 5% TCA. Homogenates were clarified by centrifugation at 27,000 rpm and confined to 4°C for 3 min. followed by filtration of the supernatants. All assays were performed in duplicate. Chemicals, all of analytical grade, were supplied by BDH, Madras. Ammonia and urea levels were determined by the methods of Bergmeyer (1965).

8.13.4 Free amino acid (FAA) content of blood

The blood (0.1 ml) for FAA estimation was collected from control and air-exposed fish and deproteinized in 80% alcohol and centrifuged. The supernatant was mixed with thrice its volume of chloroform and again centrifuged. The supernatant aqueous layer containing the amino acids were measured and used for spotting. The residue was hydrolysed with six times its weight of 6NHCICl for 16 h in boiling water bath. The hydrolysate was then diluted with distilled water and evaporated repeatedly over a water bath. Finally the residual activity was renewed under vacuum and the contents were dissolved in a measured quantity of 10% isopropyl alcohol and used for analysis. For the quantitative estimation of amino acids, the two dimensional paper chromatographic technique was adopted. Whatman No.1 filter paper (20X20 cm) was used for spotting and separating the amino acids. A known quantity of the solution containing the amino acids was spotted. The solvent systems used were n-butanol - acetic acid –water mixture (4:1:1) (V/V/V) for the
first phase and buffered phenol for the second phase. After irrigation was completed, the papers were dried to remove all traces of phenol, sprayed with 0.5% ninhydrin in 95% aqueous acetone and colour developed at 90°C for 15 min. Each amino acid spot was cut out and eluted separately with a mixture consisting of 7.2 ml of 80% alcohol and 0.8 ml of 0.1% copper sulphate. The optical density of each of the sample was read in a spectronic – 20 (Baush and Lomb) colorimeter at 550 filter. The quantity of amino acid was calculated from the optical density by making use of a constant and expressed in micrograms per gram of wet tissue.

8.13.5 Ornithine – urea cycle enzymes

The method adopted is a modification of that of Janssens and Cohen (1966). Fish killed by decapitation, body cavity opened, liver rapidly removed, weighed and placed in 1 vol 0.1% cetylmlethyl ammonium bromide (CTB). It was homogenized ten times its weight of ice-cold water and centrifuged for 30 min at 12,000 rpm (2-3°C). To prevent bacterial growth, 1 mg of potassium penicillin G and 1 mg of streptomycin were added, to each millilitre of supernatant solution. The supernatant of fresh tissue was kept on ice prior to use. Frozen tissue was used only occasionally in arginase and ornithine transcarbamylase assays. Rattus indicus liver was treated in the same way and used as control.

8.13.5.1 Carbamoyl phosphate synthetase

ATP: Carbamate phosphotransferase (CPS; 2.7.2.5). The method is based on Brown and Cohen (1959). The assay system consisted of 25 μ moles of ATP, 25 μ moles of magnesium sulphate, 25 μ moles of L-citrulline (pH 7.3), 25 m moles of L-aspartic acid; 20 cents of urease, 20 units of bovine arginase (BDH) and 250 μ moles of potassium phosphate buffer (pH 7.3) in a final volume of 5 ml. The incubation period was 1 h at 37°C.
8.13.5.2 Arginino Succinate Lyase – L-Arginino succinate arginine-Ligase (ASL; 4.3.2.1)

The method is based on Brown and Cohen (1959). The assay system contained 4 \( \mu \) moles of argininosuccinate, 20 units of urease, 20 units of arginase and 100 \( \mu \) moles of potassium phosphate buffer (pH 7.3). An incubation period of 60 min at 37°C was employed. The amount of free ammonia was measured as in the argininosuccinate synthetase.

8.13.5.3 Arginase = Arginine Amidino Hydrolase (Arg : 3.5.3.1)

This is estimated by the method based on Balinsky and Baldwin (1962). The assay system contained 150 \( \mu \) moles of L-arginine, 0.05 \( \mu \) moles of manganous chloride contained 200 \( \mu \) moles of ammonium bicarbonate, 20 \( \mu \) moles of ATP, 20 \( \mu \) moles of N-acetyl-L-glutamate, 20 \( \mu \) moles of L-ornithine, 40 \( \mu \) moles of magnesium sulphate, 20 units of urease (Enzyme unit: a unit of enzyme is the concentration of enzyme that produces 1 micromole of product/h), 90 units of beef liver ornithine transcarbamylase and 200 \( \mu \) moles of potassium phosphate buffer (pH 7.4) in a final volume of 4 ml. Because the activity was low when detected at all, incubation time was 2h at 37°C.

8.13.5.4 Ornithine transcarbamoylase

Carbamoyl phosphate - L-ornithine carbamoyl transferase (OTC ; 2.1.3.3) method is based on Brown and Cohen (1959). The assay system contained 20 \( \mu \) moles of L-ornithine, 20 \( \mu \) moles of dilithium carbamoyl phosphate and 100 \( \mu \) moles of Tris buffer (pH 7.6) in the total volume of 2 ml. The incubation period was for 15 or 30 min at 37°C.
8.13.5.5 Argininosuccinate synthetase

L-citrulline ; L-aspartate ligase (AMP), { ASS; 6.3 4.5.} The assay and 100 μ moles of Tris buffer (pH 9.3) in a total volume of 2 ml. After 10-15 min incubation at 37° C, the amount of urea or ammonia produced was assayed.

All figures given are in the following units- μ moles product formed / h per g wet wt of liver at 37° C and are the mean of the number of observations given in brackets. Where appropriate, the SE of the mean has also been calculated.

8.14 STATISTICAL CALCULATIONS

Throughout the present study, unless otherwise mentioned, the control and experimental values are the replicates of at least six independent observations. The $\overline{X} \pm$ S.D. or $\overline{X} \pm$ S.E. were calculated wherever necessary. Whenever needed, estimates of degrees of statistical significance of differences between groups were made by analysis of variance. The significance of deviations from the control was determined using paired (or unpaired as appropriate) Student's 't' test (Zar, 1984). All regression calculations used standard programmes for linear least square regression and comparisons of the slopes of the regression lines were performed. Correlation analysis was used to note any interaction between the parameters under scrutiny and body weight of the fish. Standard statistical techniques were adopted using an Electronic Programmable Fx – 100 v (Super Fx – Casio) calculator. Wherever necessary, the data was also subjected to computer analysis using an Armada M 700 computer developed by Compaq, Bangalore, India.