Chapter 2

MATERIALS AND METHODS
2.1 Systematic position

Phylum : Chordata
Sub phylum : Vertebrata
Class : Pisces
Order : Percomorphi
Sub order : Anabantoidea
Family : Belontiidae
Genus : Macropodus
Species : cupanus

2.2 Biology of experimental animal

The experimental air-breathing fish, Macropodus (Polyacanthus) cupanus (Plate 1) belongs to the suborder Anabontoidae and family Belontiidae. This fish is usually found in freshwater and estuaries in coasts of India and Sri Lanka. It is commonly called as “Spike-tailed paradise-fish” and locally it is called as “Vannathimeen”. These fish are
frequently found in paddy fields, ditches, and shallow waters and grow to the maximum of 7.5 cm in length. Body is elongated and compressed. Colouration varies widely, vary from pale reddish or dark brown, with a strong green lustre, having a small reddish circlet around the eyes and a round dark spot at the base of the caudal fin. In male fish, posterior end of dorsal and anal fins are produced to points. Posterior ends of dorsal and anal fins of female fish are rounded. *Macropodus cupanus* is an egg-laying (oviparous) species. The male builds a foam nest from air-bubbles surrounded by a hardened secretion of the mouth and usually prefers to site this among floating plants. The eggs are small and numerous. After spawning, the male drives the female away from the nest and undertakes the care of the brood alone. The young generally hatch after only 24-30 hours and are able to swim freely after 2-3 days. The youngs are very minute at first and during the earliest days must be provided with only the very finest grades of food. These fishes are very peaceful even in the community aquarium. They are active swimmers and accept any kind of food.

### 2.3 Collection and maintenance

*Macropodus cupanus* were collected from the ditches and paddy fields near the river Thamirabarani at Tirunelveli and were cultured in a pond fed on natural diet and maintained until the fishes were used for experiments.
2.4 Aquaria

The fish were collected from the pond now and then and acclimated to the laboratory condition in the aquarium tank (size: 40x25 cm, capacity: 35 litres) for a period of 4 weeks.

2.5 Feeding and water change

During the tenure of the experiment, fishes were fed with prepared dry pellet feed (35% protein diet) once in a day at 0900 hrs. Water was changed at every morning regularly.

2.6 Experimental design

Figure 2.1 illustrates the experimental design. Extensive and detailed laboratory experiments were carried out on bimodal respiration of *Macropodus cupanus* in relation to various environmental factors and pollutants. Bimodal respiration was estimated as a function of body weight (Ch.3). Ontogenic development of air-breathing organ was studied in different weight classes based on behavioural aspects (Ch.4). Oxygen consumption (aquatic and aerial) in relation to different levels of protein was estimated as a function of body weight (Ch.5). Effects of temperature on bimodal respiration was estimated in different weight classes (Ch.6). Chapter seven presents the results on the lethal toxicity of chosen pesticides in relation to body weight. Also effects of sublethal concentrations of pesticides on bimodal respiration was studied in different weight classes of *M. cupanus* (Ch.8).
**CHAPTER 3**

**Fig. 2.1 EXPERIMENTAL DESIGN**

Body weight

- Weight class
  - A: 40 mg
  - B: 200 mg
  - C: 800 mg
  - D: 2000 mg

Parameters studied: Aquatic respiration, Aerial respiration, Surfacing frequency

**CHAPTER 4**

Body weight

- Weight class
  - A: aerated water
  - B: non aerated
  - C: out of water

Parameters studied: Survival duration, Surfacing frequency
CHAPTER 5

Body weight

Weight class
- A
  - 15
  - 25
  - 35
  - 45
  - 55

- B
  - 15
  - 25
  - 35
  - 45
  - 55

Protein levels (%)
- Protein 1-35 levels I

Parameters studied: Aquatic respiration, Aerial respiration, Surfacing frequency

CHAPTER 6

Temperature

Temp. (°C)
- 22
- 27
- 32
- 37

Weight class
- A
  - B
  - C
  - D
- A
  - B
  - C
  - D
- A
  - B
  - C
  - D

Parameters studied: Aquatic respiration, Aerial respiration, Surfacing frequency
CHAPTER 7

Pesticides

<table>
<thead>
<tr>
<th>Weight class</th>
</tr>
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<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
</tbody>
</table>

Endosulfan

Methyl parathion

Carbaryl

Parameters studied: Mortality at 24, 48, 72 and 96 hrs

CHAPTER 8

Weight class

A

B

C

Endosulfan

Methyl parathion

Carbaryl

Parameters studied: Aquatic respiration
Aerial respiration
Surfacing frequency
2.7 Estimation of aquatic respiration

All fish used in the study were starved for 24 hr before experiment for feeding is known to influence oxygen consumption (Reddy and Natarajan, 1970). The fish was introduced into the respiratory chamber and allowed one hour for acclimation. The chamber was closed with a stopper air tight. After commencing the experiment, 5 ml of water was drawn from the chamber every half an hour to estimate the oxygen content of the water by Winkler's volumetric method (Welsh and Smith, 1960). The difference between the initial and the final value gave the oxygen consumed by the fish and the rate of oxygen consumption was calculated (mlO₂g⁻¹hr⁻¹). Three to five readings were taken for each fish and the mean values were calculated.

2.8 Estimation of aerial respiration

For estimating the aerial respiration, special respirometers were designed involving the principles of manometric techniques. The setup consists of a respiratory chamber connected to the graduated ‘U’ tube containing Brodie’s fluid. KOH (BDH) was used as CO₂ absorbent. The difference in the level of the fluid in the monometer for a given time was used in the following equation (adopted from Umbreit et al., 1959) and the gas utilized was calculated.

\[ V = V_i \times h/10,000 \]

where ‘V’ is the volume of the gas utilized

‘V_i’ is the volume of the gas in the respiratory chamber
Plate 2.2: Respirometer setup to estimate aquatic and aerial respiration.
‘h’ is the difference in the level of the Brodie’s fluid in the monometer.

2.9 Estimation of surfacing frequency

Air breathing behaviour was observed under laboratory conditions. Surfacing frequency of each fish was recorded along with aquatic and aerial respiration. Air-breathing fish visit the surface at regular intervals to exchange atmospheric air which is termed as surfacing (Pandian and Vivekanandan, 1976). It is considered as an indicator of aerial respiration. Number of surfacing for each 15 minutes was noted and average of 5 readings was calculated. The respirometers were filled with a constant depth of water (11 cm); the distance travelled per unit time was estimated by multiplying the mean number of visits with twice the depth of water (Vivekanandan, 1976).

2.10 Hydrobiological analysis

Water used for the experiments was clear, unchlorinated well water. During the experimental period, the water samples were analysed for dissolved oxygen by Winkler’s Iodometric method, salinity by Harvey’s titration method. pH was recorded using pH meter following the procedure given by Strickland and Parson (1972). Ammonia was estimated following the phenol-hypochlorite method (Solorzano, 1969) using de-ionised water. EDTA method was adopted to estimate the hardness of water as described by Trivedy and Goel (1986). The physico-chemical characteristics of tap water used for the experiments
were: Temperature: 28 ± 1°C; pH: 7.8 ± 0.05; Salinity: 0.13 ± 0.01 ppt; DO: 4.09 ± 0.13 ml/l; Ammonia: 0.2 ± 0.01 mg.

2.11 Statistical analyses

Results obtained were subjected to the following statistical analyses.

a. Standard deviation
b. Student's 't' test
c. Regression analysis
d. Simple correlation coefficient and
e. Analysis of variance

a. Standard deviation (SD)

\[ SD = \sqrt{\frac{\Sigma d^2}{n-1}} \]

where 'd' represents the deviation of each score from mean and 'n' the total number of samples observed.

b. Students 't' test

Student's 't' test was used to compare two means by applying the formula.

\[ t = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{SE_1^2 + SE_2^2}} \]
where $\overline{X}_1$ and $\overline{X}_2$ represents the means compared and $SE_1$ and $SE_2$ their respective standard errors. Standard error was calculated using the formula.

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

The level of significance for the 't' at corresponding degrees of freedom (df=$N-2$) was read from the probability table given in Zar (1974) where 'n' is the total number of scores in both the experiments.

c. Regression analysis

The regression equations were computed using the least square method. The basic formula followed was

$$Y = a + bX$$

where 'Y' is the dependent variable. 'X' is the independent variable. 'a' the intercept on the 'Y' axis and 'b' the slope. The formulae used to drive 'a' and 'b' are

$$b = \frac{\Sigma xy}{\Sigma x^2}$$

$$a = \overline{Y} - b\overline{X}$$

'\overline{Y}' and $\overline{X}$ represents the mean of 'Y' and 'X', $\Sigma xy$, $\Sigma x^2$ and $\Sigma y^2$ are derived from the formulae
\[ \Sigma xy = \frac{\Sigma XY - (\Sigma X)(\Sigma Y)}{N} \]

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

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

The ‘X’ and ‘Y’ denote the raw scores and ‘x’ and ‘y’

- deviation scores.

d. Simple correlation coefficient (r)

The simple correlation coefficient was determined by using the
formula.

\[ r = \frac{\Sigma xy}{\Sigma x^2 \Sigma y^2} \]

e. Two way analysis of variance (ANOVA)

Partitioning of total variance due to the different experimental
conditions was carried out following the procedure described by Zar
(1974). Values obtained for the different experimental conditions were

- tabulated in different columns and rows. For each column \( \Sigma X \) and \( \Sigma X^2 \)

- were calculated. Sum of X for all the column was squared and divided

- by the number of tabulated values and a correction factor C was

- obtained.

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

Between column SS = \( \frac{\text{Sum of } (\Sigma X)^2 \text{ of each column}}{\text{No. of values in a column}} - C \)

Between row SS = \( \frac{\text{Sum of } (\Sigma X)^2 \text{ of each row}}{\text{No. of values in a row}} - C \)

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

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

**f. Degrees of freedom (df)**

Total SS = No. of values in the table – 1

Between column SS = No. of columns – 1

Between row SS = No. of rows – 1

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

\[
F \text{ value for the variance between columns} = \frac{\text{MS between columns}}{\text{Remainder MS}}
\]

\[
F \text{ value for the variance between rows} = \frac{\text{MS between rows}}{\text{Remainder MS}}
\]

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