CHAPTER 2
MATERIAL AND METHODS
2.1 Experimental fish:

The cyprinodontid fish *Poecilia reticulata* (Peters), commonly called the "Guppy" was chosen for the present studies. *Poecilia reticulata* belongs to the family poeciliidae and it is originally a native of the West Indies and the Northern South America (Gibson, 1954). However, in recent years it is known to have successfully colonized the aquatic habitats of several tropical and subtropical countries. Introduced into the Indian waters as early as in 1906 (Prashad and Hora, 1936; Gerberich and Laird, 1965), it is known to have established itself very well to the local conditions and is abundant in varied habitats of the sub-continent (Jhingran, 1977). It is widely used as an aquarium fish, the varieties of which are innumerable. In addition to this, since *P. reticulata* is also known to serve as an efficient tool for the biological control of mosquito larvae and the intestinal parasitic infection of *Schistomiasis* or *Bilharziosis*, in the recent past its intentional propagation is also widely promoted. (Frazer and Brunner, 1953, Hora and Mukerji, 1953; Brunner, 1953).
P. reticulata was particularly chosen during the present studies for the following reasons:

1. It is widely distributed in different habitats of India in general and South India and Bangalore in particular (Reddy, 1973; Nijaguna et al., 1986).

2. Its abundance, ready availability, hardy nature, capacity to withstand a wide range of fluctuations in the physical and chemical conditions (Lewis, 1970; Joshi et al., 1976) render it highly suitable for experimental studies and it is also easy to rear the species in captivity.

3. Its small size, easily recognisable sexual dimorphism, short lifecycle, possibility of controlled breeding and already standardised feasible laboratory rearing techniques (Winge, 1927; Eloff, 1932; Krishnamurthy, 1978) are highly suitable for laboratory handling.

4. Studies on aspects of its biology, particularly that pertaining to population structure, reproduction and developmental biology in tropical situations are not worked out in detail.
2.2 **Places of fish collection**:

Field studies and collections of the experimental fish were carried out in two habitats (Fig. 1).

2.2.1 **The Vrishabhavathi stream**:

A slow flowing lentic habitat which streams through the Jnana Bharathi Campus of the Bangalore University, formed the first site of collection. The water of this stream flows over rocky bottom in several regions and here and there, in shallow areas, the habitat is covered with thick macrovegetation of floating plants (mainly *Eichhornia* spp) which also gives the fishes and other aquatic macrofauna a natural cover. The water of this stream is considerably polluted and harbours a rich fauna of mosquito larvae as also other aquatic organisms.

2.2.2 **The Garden tank**:

A man-made artificial system in front of the Department of Geology, located on the Jnana Bharathi Campus of the Bangalore University, South taluk (latitude 12° 56'30" North and longitude 77°31' East) was the second site of collection. This tank is a cement system, measuring 43 ft in length, 14 ft in width
Fig 1: Topography to show the locations of the two habitats selected for study.

A. Garden Tank.
B. Vrishabhavathi Stream.
2.5 ft in depth. As the water from this tank is regularly used for purposes of gardening, the water level keeps fluctuating. Except for some planktonic unicellular and filamentous algae, the tank is devoid of any other kind of macrovegetation. A few species of fishes (mainly carps and cichlids) are being incidentally cultured in this tank.

For purposes of the present investigations in relation to embryonic nutrition of *P. reticulata*, collection of the experimental fish were made for a period of eight months in both the above habitats. Long term studies on the reproduction and population structure of *P. reticulata* were carried out only in one habitat (garden tank) as habitat variations are known to exist (Peterson and Peterson, 1990). Bimonthly collections of fish inhabiting the garden tank, served as the material for analysing population structure through time, reproductive cycling and reproductive performance of both males and females of *P. reticulata*.

2.3 **Field collections, transportation and laboratory maintenance**:

A hand net made of nylon netting (mesh size 1 x 1 mm) was used for the collection of the specimens. After
collection the fish were transferred (in convenient densities) to plastic containers filled with freshwater. During transportation from the field to the laboratory, sufficient aeration and aquatic vegetation cover was provided. The specimens collected were stocked in the laboratory in glass aquaria, each filled with 10 to 15 litres of freshwater. The fish were maintained at a water temperature of $23^\circ \pm 1^\circ$C and fed on an *ad libitum* diet of the oligochaete tubificid worm *Tubifex tubifex*. The aquarium water was changed once every two days and aerated every alternate day.

2.4 Analyses of population parameters:

Investigations on the population structure of *Poecilia reticulata* were carried out for a period of 12 months (from September 1988 to August 1989). Numerical abundance of each category of fish (juveniles, males, non-pregnant and pregnant females) was determined by enumeration method. During each collection, the population was segregated into juveniles, males, pregnant and non-pregnant females based on the following features:

1. Morphological features such as size (less than 14mm TL: Juveniles; above 14mm TL either males or females).
2. Body colouration (males are brilliantly coloured compared to the females) (Fig.2, 3, 4 and 5) sexually dimorphic.

3. Presence or absence of "gonopodium" (in the males the anal fin is modified into an elongated structure, the gonopodium, which helps in the transfer of sperms during copulation) and

4. Presence or absence of "pregnancy mark" in the pregnant females (a dark patch on the ventrolateral abdomen near the vent).

Further percentage occurrences of juveniles, males and females were also tabulated.

A random sub sample of each category of fish classified as above was used for estimating the length-weight relationship. The total lengths (TL) were measured to the nearest mm using an mm graphic scale. After removing the moisture adhering to the body surface, the total body weight (W) was recorded to the nearest mg using a triple beam single pan balance. Scattergrams were plotted for the data on length and weight, and the straight lines were fitted following the method of least squares (Lewis and Taylor, 1967). The length-weight relationships for juveniles, males and
Fig 2: Photograph of a Juvenile *Poecilia reticulata*

Fig 3: Photograph of an adult male *Poecilia reticulata* (Scale in centimeters).
Fig 4: Photograph of a non-pregnant female *Poecilia reticulata*.

Fig 5: Photograph of a pregnant female *Poecilia reticulata*. Note the 'Pregnancy mark' on the ventrolateral region of the abdomen.
(Scale in centimeters).
females were calculated for monthly collections as well as for the pooled data collected over the entire study period.

Based on the availability and sample size through different months, the length-frequency distributions of the species were computed following the modified method of Peterson, as described by Bagenal (1978). Using this data, monthly mean-modal length-frequency distributions and growth rate were calculated.

The 'condition factor' (an index of the plumpness of the fish (Le cren, 1951) is based on the hypothesis that heavier fish of a given length are in a better condition. During the present studies the Fulton's condition factor $K_{ii}$ of each category of $P. reticulata$ was calculated as follows:

$$K_{ii} = \frac{W}{L^b} \times 100$$

Condition factor was also calculated following the equation of Linfield (1979):

$$CF = \frac{W}{L^b} \times 10^3$$

Where $CF = K_{ii}$ is the condition factor

$W =$ Mean weight of the fish
2.5. **Analyses of reproductive parameters:**

From the bimonthly stock of the fish a sub sample was used to collect information on various aspects of reproduction. Male to female ratio in each collection was calculated and represented as the sex-ratio. In order to study the reproductive performance in different sized fishes, the males were classified into three size classes based on their total length as follows:

- **Size class 1:** < 20 mm
- **Size class 2:** 20 - 25 mm
- **Size class 3:** > 25 mm

Similarly, the females were also classified into five size classes as follows:

- **Size class 1:** < 20 mm TL
- **Size class 2:** 20 - 25 mm TL
- **Size class 3:** 25 - 30 mm TL
- **Size class 4:** 30 - 35 mm TL
- **Size class 5:** > 35 mm TL

For purposes of studying the reproductive cycling during each collection, a minimum of three to six fishes...
of the above size classes were sacrificed. After dissecting the fish, the testes and the ovaries were carefully removed and the gonadosomatic index (GSI: which is an index to the relative reproductive potential of an individual) was calculated as follows:

\[
\text{GSI} \% = \frac{\text{Live weight of the Gonad (mg)}}{\text{Live weight of the fish (mg)}} \times 100
\]

The dissected ovaries and testes were then preserved in aqueous Bouin's solution for further analyses.

2.5.1 Analyses of Intra-ovarian eggs and staging of eggs for studying the reproductive cycling:

During each fortnightly collection a random sample of females in each size class was used for the analyses of ovarian sac and staging of intraovarian eggs/oocytes. Ovarian sacs, dissected from females were analysed in vitro. After dissection, the entire ovisac preserved in aqueous Bouin's solution was weighed to determine the ovisac weight. Thereafter, the ovisac was carefully analysed under a Nikon microscope to expose the different stages of eggs. For purposes of identification, the intraovarian eggs were classified into 8 convenient developmental stages (i, ii, iii stages...
of unfertilized recruiting eggs and 1,2,3,4,5 stages of incubating fertilized eggs). All the morphometric measurements of the eggs including the lengths and widths of the eggs, eyes and yolk sac in case of fertilized eggs were measured using a stereo microscope (Nikon Model No.2 LA 380). The measurements are expressed in mm after conversion using a stage and occular micrometer.

Stage i. Opaque, spherical, light cream coloured and unfertilized.

Stage ii. Still opaque, spherical, yellow in colour. slightly bigger than that in the stage i, unfertilized.

Stage iii. Opaque, spherical, yellow in colour, still unfertilized, comparatively bigger than that in the stage ii.

Stage 1. Spherical, transparent, fertilized, yellowish eggs, yolky, with prominent oil globules.

Stage 2. A small portion at one margin of the spherical egg differentiated into the clear blastodermal region, where a pair of non-pigmented eyes (light cream coloured) could be seen. Embryo
in the form of a pale white, opaque streak could be distinguished. Faint vascularization was also noticed.

Stage 3. The egg still spherical in shape, the development of vascularization was clearly observed which extended all over the yolk sac and even to the cephalic end of the embryo. Actual streaming of the blood was evident in live eggs. The growing embryo had developed darkly pigmented eyes, occasional beating of the heart was also observed.

Stage 4. Embryonic differentiation clearly noticed. The embryo overlying the yolk sac was pigmented, melanophores distinctly distributed over the head region. Eyes large and spherical, fins were also noticed, yolk sac was considerably diminished in size.

Stage 5. Eggs more or less elliptical, embryo characteristically pigmented, melanophores irregularly distributed all over the embryo, concentrated on the head region, caudal fin lying close to the mouth due to the flexing of the embryo over the yolk sac, dorsal and
ventral fins evident. Occasional jerky movements of the embryo noticed, yolk sac almost completely resorbed.

The data on the numerical abundance of the different stages of oocytes in every individual ovarian sac was tabulated in a specially prepared data sheet as follows:

DATA SHEET FOR POECILIA RETICULATA

<table>
<thead>
<tr>
<th>Date of Collection</th>
<th>Pregnancy Mark</th>
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<tbody>
<tr>
<td>Habitat</td>
<td>Groups/size-class</td>
</tr>
<tr>
<td>Fish number</td>
<td>Calculated GSI (%)</td>
</tr>
<tr>
<td>Total length of the fish</td>
<td></td>
</tr>
<tr>
<td>Total weight of the ovary</td>
<td></td>
</tr>
<tr>
<td>Ovary length</td>
<td></td>
</tr>
<tr>
<td>Ovary width</td>
<td></td>
</tr>
<tr>
<td>Notes</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oocyte stage</th>
<th>Number of oocytes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 20 30 40 50 60 70 80</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td></td>
<td></td>
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<tr>
<td>ii</td>
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</table>
Percentage of different stages of eggs in ovaries of the females of each size class in relation to seasons was tabulated along with percentages of unfertilized and fertilized eggs, the latter being represented as a line drawing.

2.5.2 Staging of fertilized, incubating eggs for studies on the yolk utilization during embryogenesis:

The investigations on the biochemical changes and the pattern of yolk utilization during embryogenesis were carried out by selectively choosing six suitable stages of fertilized (incubating) eggs/fry. Five stages of fertilized developing embryos as classified in section 2.5.1. (Stage 1, 2, 3, 4, 5) and stage 6 was the young fry, a few hours after birth (fig.6). The young at birth measured 8.79 mm in total length and did not indicate the presence of any yolk sac. With the yolk sac already absorbed and the fry having developed fully formed oral aperture, the young one could readily exhibit oral feeding right from the day one of its birth (see also Krishnamurthy, 1978). The fry of P. reticulata measuring 6.5 mm in length and with comparable morphometry as above have also been reported by Goodrich et al., 1934.
Fig 6: Stages of fertilized incubating eggs/fry chosen for studying embryonic nutrition.

a, b, c, d, e: Developing eggs in stage 1, 2, 3, 4, 5.

f: Fry (stage 6).
2.6. Histological analyses:

Testes collected from the individual males of chosen 3 size classes were processed for histological studies during April through July 1990. The dissected tissues were fixed in aqueous Bouin's fluid to prevent post-mortem changes. Further, the tissues were gradually dehydrated by upgrading in concentrations of alcohol. After one or two changes in xylene, the tissue was incubated with paraffin wax, for one hour and convenient blocks were prepared by embedding the tissue in molten paraffin wax. Sectioning of the embedded tissue was done at 8 microns using a rotary microtome and the sections were mounted on to a slide smeared with egg albumin. Sufficient water was poured on the sections to get uniform spreading of sections and the slides were warmed over a slide warmer for 2 to 5 minutes. The sections were stained in Delafield's Haematoxylin and counterstained with Eosin thereafter.

2.7. Determination of live and dry weights of developing eggs/fry:

Different stages of eggs/fry were weighed in an electric balance to determine their live weights. Individual eggs in similar stages of development,
collected from several mothers were transferred onto a pre-weighed coverslip and reweighed after blotting the adhering water. The actual live weights of the eggs were determined after subtracting the weights of the coverslip. The average live weights of eggs in six stages of development were determined separately and standard deviations were calculated.

The live eggs were dried in a hot air oven at $60^\circ C$ for 24 hours and the constant dry weight/egg or fry was determined. The dried and powdered material of each stage was stored in glass containers in a dessicator and used for further biochemical analyses.

2.7.1 **Biochemical analyses**:

Biochemical analyses were carried out to determine the content of various constituents in different stages of development.

The difference between the wet weights and their corresponding dry weights/egg or fry yielded the amount of water content. By incinerating the dry powdered tissue (20-40 mg) in a Muffel furnace at $56^\circ C$ for 5 hours, (Paine, 1964) the ash (= salt) content was determined. The difference between the dry matter and the ash content was considered as the total organic
matter. Estimation of total carbohydrates in the dry tissue was done using the phenol method (Dubois et al., 1956). Total proteins were estimated according to the method of Lowry et al., (1951). Since the carbohydrates and protein contents were estimated, that of the fat was derived by subtracting the pooled weights of the carbohydrates and proteins from the total organic matter. The calorific values (= energy content in calories) per egg or fry was derived by taking the energy equivalents of each of the 3 organic constituents (Prosser and Brown, 1962).

2.8. **Statistical analyses**:

Data obtained during the study period were subjected to the following statistical analyses (Gupta and Kapoor, 1977) wherever necessary.

a. Standard deviation (SD) from the mean:

\[
SD = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n-1}}
\]

where \( \bar{X} \) is the mean of the sample and \( n \) is the sample size.
b. Correlation co-efficient: A measure of relationship between two variables 'correlation' is given by:

\[ r = \frac{\text{Cov}(X, Y)}{\sigma_x \sigma_y} \]

Where \( \text{Cov}(X, Y) = \frac{1}{n} \sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i \)

\[ \sigma_x^{-2} = \frac{\sum x_i^2 - n\bar{x}^2}{n} \quad \text{and} \]

\[ \sigma_y^{-2} = \frac{\sum y_i^2 - n\bar{y}^2}{n} \]

c. Test for equality of means: The significance of the difference between two means is given by the
paired 't' represented by the equation:

\[ t = \frac{\bar{X} - \bar{Y}}{S^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)} \]

Where \( S^2 = \frac{1}{n_1 + n_2 - 2} \left[ (n_1 - 1) S_1^2 + (n_2 - 1) S_2^2 \right] \)

Where \( S_1^2 = \sum_{i=1}^{n_1} \frac{X_i^2 - n_1 \bar{X}^2}{n_1 - 1} \) and

\[ S_2^2 = \sum \frac{Y_i^2 - n_2 \bar{Y}^2}{n_2 - 1} \]

\( S_1^2 \) and \( S_2^2 \) are sample variances

Decision rule \( \phi = \begin{cases} \text{reject } H_0 & \text{if } t \geq t_{\alpha} \\ \text{accept } H_0 & \text{if } t < t_{\alpha} \end{cases} \)
d. Tests for significance of an observed correlation co-efficient: The significance of the relationship between the two variables is given by the student 't' test represented by the equation:

\[ t = \frac{r}{\sqrt{n - 2}} \]

Where \( r \) = correlation co-efficient of two variables.
\( n \) = degrees of freedom

e. Test for equality of correlation co-efficients: The equation used to test for equality of correlation co-efficients is

\[ Z = \frac{Z_1 - Z_2}{\sqrt{\frac{n_1 - 3}{n_2 - 3}}} \]

Where \( Z_1 = \frac{1}{2} \log_e \left( \frac{1 + r_1}{1 - r_1} \right) \) and

Where \( Z_2 = \frac{1}{2} \log_e \left( \frac{1 + r_2}{1 - r_2} \right) \)

where \( r_1 \) and \( r_2 \) are correlation co-efficients of I and II samples respectively.
f. Construction of linear model (Draper and Smith 1966): The curvilinear scattergram was plotted for the collected experimental data. The curvilinear model was transformed into a linear model by converting the actual values into logarithmic values and the regression equations were computed using the least square method:

\[ Y = b_0 + b_1 \]

The expression for \( b_0 \) and \( b_1 \) are

\[
\begin{align*}
    b_1 &= \frac{\sum_{i=1}^{n} X_i Y_i - \left(\sum_{i=1}^{n} X_i\right) \left(\sum_{i=1}^{n} Y_i\right)}{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2} \\
    b_0 &= \bar{Y} - b_1 \bar{X}
\end{align*}
\]

where \( Y \) is the dependent variable
\( X \) is the independent variable
\( b_0 \) the intercept on \( Y \)
\( b_1 \) the slope

2.9. **Photographic illustrations**:

Representative fishes (Juveniles, males, non pregnant and pregnant females of \( P. \) reticulata, the dissected ovisacs and the different stages of incubating
eggs were photographed with an Olympus OM 1 N camera fitted with a closeup lens. The stained tissue sections were photographed using Leitz microscope fitted with a photographic equipment.