CHAPTER 6

Experimental Breeding
INTRODUCTION

In recent years induced breeding has been profitably used for aquaculture programmes. But its standardization is an important prerequisite for any species. The role of pituitary in the reproduction of vertebrates was first understood from the experiments of Aschheim and Zondek (1927), when they investigated that pituitary implants accelerate sexual development in mice (for details see Allen, 1939 and Pickford and Atz, 1957). In the year 1929, Wolf on *Rana pipiens* and Houssey *et al.* (1929) on *Bufo marinus*, performed pioneering experiments demonstrating the induced ovulation in anurans by injecting or implanting homoplastic pituitary glands. Subsequently, many biologists such as, Adams (1931); Wills *et al.* (1933); Shapiro (1936); Bellerby (1933); Rugh (1934, 1935a, b, 1939, 1962); Creaser and Gorbman (1935); Gallian (1937); Ramaswamy and Lakshman (1958, 1959); Wright (1945, 1950); Wright and Hisaw (1946); Wright and Flathers (1961); Nieuwkoop and Faber (1967); Hock and Wen (1970); confirmed ovulation in anurans by pituitary hormones and steriod injections. Based on this principle certain biological companies in U.S.A. (such as Carolina, Burlington NC and Turtox Chicago) supply pituitary kits for induced breeding in frogs.
While working on *Rana tigrina*, *Rana hexadactyla* and *Rana cyanophlyctis* Ramaswamy and Lakshman (1959) expressed concern that in India techniques of induced breeding are not adequately developed and prescribed a more favourable technique using the homoplastic as well as fish pituitaries, in combination with threshold dosage of mammalian hormones for induced ovulation in these species. But even now induced breeding is not conveniently practiced in many Indian laboratories.

Rugh (1948) proposed that the pituitaries can be stored in absolute ethyl alcohol without losing their potency. Use of preserve pituitaries is now a routine for induced breeding in fishes (see Jhingran, 1975). Khare, Roy and Kumar (1981) demonstrated that frog pituitaries preserved in absolute ethyl alcohol retain their potency for several years and can be used profitably for induced breeding in frogs. The procedure followed in the present investigation for *Rana cyanophlyctis* is based on the combination of techniques prescribed by Rugh, (1934) Osche, (1968) and Jhingran (1975). According to this technique, a stock of anterior pituitaries of male and female frogs preferably of same species preserved in absolute ethyl alcohol is built up and a measured quantity of these pituitaries is used at the time of induced breeding experiment. *Rana cyanophlyctis* has a prolonged breeding
period - March to October at Shillong and still longer at Gauhati. This chapter describes how these preserved pituitaries are successfully used for induced breeding of this species under laboratory conditions.

Induced breeding technique can be used for experimental breeding of the animal on large scale. As gonado somatic index and fecundity may be helpful in experimental breeding programmes they have also been included in the present chapter.

REVIEW OF LITERATURE

The concept of the use of pituitary injection for successful spawning in frogs came up in 1929 through the work of Wolf on *Rana pipiens* and Houssay *et al.* on *Bufo marinus*. The following year 1930, Houssay demonstrated spawning by pituitaries in fishes also. Wolf (1929) observed that continuous transplantation of a pituitary for 3 – 4 successive days in female *Rana pipiens* either lead to ovulation or gorging of egg in uterus. He also experimented and found that the transplantation of brain or neural tissue had no effect on gonads or spawning. Wills *et al.* (1933) observed that toad can be induced ovulated by fish pituitaries. Rugh (1934, 1935)
demonstrated that injection of homoplastic pituitary glands directly in the abdominal cavity brought about amplexus and ovulation. The egg thus obtained were artificially inseminated. With the help of Homoplastic and Heteroplastic pituitaries, Rugh (1935) successfully induced ovulation in *Rana clamitans*, *Rana catesbeiana*, *Rana palustris* and *Bufo floweri* during different months and noted that the dosage of pituitaries varied during different months. He also noted that the pituitary of *Bufo floweri* was smallest in size and least potent for other species and that of *Rana catesbeiana* was largest and strongly effective in other species. He further demonstrated that extract of mammalian anterior pituitary (sheep gland and anturian - 5 from urine of pregnant individuals are effective in all species of frogs. Rugh (1934) used meshed pituitary extract in distilled water or 10% ethyl alcohol for induction of ovulation in anurans, and observed that homogenate enhanced pituitary action by 300-400%. He observed that hypophysectomization in frog showed of gonads but on injecting its own pituitary later enhanced some gonadal maturation and ovulation. While concluding he also mentioned that the degree to which ovaries are emptied depends upon the temperature. Subsequently he investigated the breeding behaviour of different species of frogs found in North America, and stated that anuran eggs and larvae can be obtained in all months of a year. Rugh (1935)
further observed that inter and intra-specific amplexes can be achieved in frogs, but not among frogs and toads. He believed that the warts and poisonous skin of toad had been the plausible reason for the failure of amplexes. Rugh in the year 1939 described in detail the technique of obtaining anuran eggs by anterior pituitary during breeding period of frogs (for details of technique see Rugh, 1962). Landgrebi and Pusser (1941) described the technique of breeding *Xenopus* in laboratory. In 1942, Robinson and Hill made some modification in the injection technique proposed by Rugh (1934), for successful induced ovulation of *Rana pipiens* in the laboratory conditions. Adams and Granger (1938) showed that *Triturus viridescens* pituitary induces ovulation in *Rana pipiens* and felt that there was no zoological specificity of gonads stimulating hormones.

Creaser and Gorbman (1939) recorded that fish pituitaries are capable of spawning prawn. Further, Wills, Riley and Stubb (1933); Stroganor and Alpatov (1951), and Picford and Atz (1957) and Chaudhuri (1960, 1963) have also reported that spawning in fishes can be induced by frog pituitaries. In the year 1942, Creaser achieved ovulation in *Rana pipiens* by bird pituitary preparation. On placing the ovaries "in vitro" in the cultural medium having pituitary hormones. Wrights (1945) demonstrated growth and maturation of ova. In the year 1950, he demonstrated that the length of the exposure of ovaries in
cultural medium having pituitary hormones leads to "in vitro" ovulation. Barr and Hobson (1967) regulated dosage of the gonadotrophin injection in *Xenopus laevis* so as to obtain desirable number of egg. They also formulated a method for the estimation of numbers of egg laid by any species. Alonso-Bedata and Serrano (1970) described a technique for experimental ovulation and fertilization in *Rana ridibunda* and noted that keeping female of the species at room temperature for larger period deteriorates the ovaries and ovulation capabilities. Hook and Wen (1970) demonstrated artificial breeding in *Rana limnochasis* following Rugh's technique and described its early development up to tadpole stages. Though there is no zoological specificity, earlier worker, Barth (1933); Creaser and Grobman (1935); Rugh (1933, 1935) mentioned that sheep or other cattle pituitaries extract and/or pregnant mare serum and/or human pregnancy urine and chorionic gonadotropin do not induce ovulation in mature *Rana pipiens*. But subsequently many workers have successfully induced ovulation using heteroplastic pituitaries including mammalian pituitaries and various gonadotropic steroids, such as Rugh (1935) in *Bufo floweri* and *Rana catesbeiana*; Creaser and Grobman (1935) in *Hyla aurea*; Ballerby (1933) in Cunnigham and Smart (1934) and Gallian (1937) in *Rana temporaria* and Shapino (1933), Shapiro and Zwarenstein (1939) in *Xenopus laevis*. Wright and Hisaw (1946) in
ovulating *Rana pipiens* with a mixture made up of mammalian pituitary and gonadotropinsteriods. In the year 1961 Wright and Fleather observed that injection of homoplasic anterior pituitary gland in combination with progesterone compels complete ovulation in *Rana pipiens*.

Contribution on induced breeding on frog species available in India are relatively few. Ramaswamy and Lakshman (1958, 1959) presented injection of homoplasic pituitaries in combination with threshold mammalian hormones for successful induced breeding in *Rana tigrina*, *Rana hexadactyla* and *Rana cyanophlyctis*. They also noted that pituitary collected from females having regressed ovaries showed indifferent result and the frog freshly collected give better result than those stored for 8 to 10 days. Further they mentioned that by altering the dosage the ovulation could also be achieved during winter month in *Rana cyanophlyctis* as gravid females could be procured throughout the year.

Gangadharma and Ramiah (1968) observed that Hypophysectomy and ovariectomy in *Rana cyanophlyctis* resulted in the atrophy of the oviduct and decrease in alkaline phosphate activities. Methallibure a non steroid pituitary inhibitor reduced the oviducal alkaline phosphate activity. Further, starvation destroyed the mature follicle in the ovary and caused depletion of ovarian
cholesterol and oviducal alkaline phosphate activity. Hence they concluded that reproduction in female skipper frog is under hormonal and nutritional control as in mammals. Kasinath and Basu (1977) investigated that different dosage of steroid had significant role on spermatogenesis of the frog *Rana hexadactyla*.

Gopalkrishnan and Rajasekharsetty (1977) observed that *Rana cyanophlyctis* is a unique frog which maintains the gravity of ovary round the year. However, its active breeding season is recorded to be a prolonged one extending from July till the end of September in South India. Further they recorded that the ovary of the skipper frog shows two distinct phases. No. (i) ovarian phase during which ovary releases gravid eggs (ovulation). No. (ii) oviductal phase during which the released egg passes through the oviduct causing spawning. They also noted that pituitary homogenate induces spawning better than steroids. While the steroids acts as an effective agent for oocyte maturation (Roy, 1979) induced ovulated *Rana limnochares* at Shillong under laboratory conditions using homoplastic and heteroplastic pituitaries homogenates and observed that large number of viable eggs can be obtained from the female frogs during the month of April and May, soon after the termination of it hybernating phase. During later months the frogs act indifferently due to spent
ovaries. Hence she has recommended April and May for successful induce ovulation in *Rana limnochanis* of Shillong.

The dosage calculation and preservation of the pituitaries have been attempted by the biologists world over. Strogonov and Alpatov (1951) proposed frog unit as the unit quantity of gonadotropin that would bring about appearance of sperm in cloaca of 50% of male frogs and toads and/or that exhibit mature spermatozoa in their spermduct. However, this technique was not found suitable due to indifferent behaviour in anuran population during different months and from different ecological conditions. Jhingran (1975) mentioned that this has been the reason for not deriving any chemical standardization of pituitary gonadotropin which insures successful spawning in animals.

Alikunhi *et al.* (1960); Choudhuri (1960, 1963) described preservations of fish pituitary in alcohol and acetone and observed that such pituitary retain its potency hence can be conveniently used later when and where required. Ibrahim and Choudhuri (1966) devised a technique for preservation of pituitary extract in glycerine and thereby providing readymade suspension for induce ovulation in animals. The technique, however, was found to be not so effective as its potency diminishes after the storage of 9 to 61 days (see Jhingran, 1975).
Body length and its relationship with gonadal condition/weight have little been worked out amongst anuran. Liu (1950) estimated the number of egg masses and clutches size in *Amolops chunganesis* per ovulation per female and noted that an average female of 54.0 mm lays an approximately 2180 ova. Among temperate Ranids, Terentjev (1960) developed relationship between clutch size and the size of female and noted a linear relationship that can be expressed by a linear expression:

\[
\log F = -1.7428 + 2.1670 \log L
\]

where \( F = \) Fecundity, \( L = \) SV length of female in cm

Inger and Greenberg (1966) and Inger and Bacon (1968) derived relationship between body length and clutches sizes among temperate anuran and reptiles and noted linear expressions, identical to Terentjev's (1960) empirical formula. Inger and Bacon (1968) observed that in sararid forest, rain frogs breed throughout year and show similar annual seasonal and behavioural pattern throughout the year. Moreover the changes in their seasonal pattern can be known by Kruskal Wallis analysis (Seigel, 1956). Schroder (1974) derived correlations of weight and size of tistis with the body size of male *Rana catesbiana*, throughout its reproductive cycle, to find out the period
for its active breeding. Koskala and Pasanen (1975) derived correlation coefficient and regression equations in *Rana temporaria* among following structures:

**Body length and weight of ovary**

\[ Y = 0.01766 \times X + 1.28563 \]

\((P = 0.001; N = 18)\)

**Body length and weight of oviduct**

\[ Y = 0.02954 \times X + 0.64478 \]

\((r = 0.912; P = 0.001; N = 29)\)

**Body length and numbers of egg**

\[ Y = 33.394 \times X - 1480.498 \]

He noted that volume of spawn size and numbers of eggs are dependable on the size of female and observed that female of SVL 78.0 mm would produce 13.5 - 14.0 gm of spawn comprising about 1360 eggs of size 2.01 mm containing about 1.0 mg of magnesium; 0.2 mg of zinc and 0.04 gm of copper.
MATERIAL AND METHODS

Collection of frog:

The specimens of *Rana cyanophlyctis* collected both at Gauhati and Shillong were used for induced breeding experiments. It was easier to get mature frog more abundantly at Gauhati than at Shillong (see Chapter III and Fig. 3.3). The frogs from Gauhati were transported to the laboratory in 5 and 10 litre plastic jar half-filled with pond water and covered with perforated lid or cheese cloth. It was observed that over crowing, more than 15 frogs in small and more than 30 in large container, resulted in some mortality. The pond water was replenished thrice during 101 km journey from Gauhati to Shillong, by draining out about 2/3 of water at a time.

At the laboratory they were maintained in large glass aquaria (13″ x 18″ x 18″) covered with win mesh lid (Fig. 4). The aquaria were filled up to 6″ pond water and were set with steep sand base at one side, and stones to maintain amphibious environment. Algae and aquatic plants were placed in the aquarium. Insects and earthworms were given to the frogs as food. Diseased frog, specially those with red leg disease (Mohanty-Hymedi, 1974), were removed periodically, as soon as such symptom was first
observed. The water of the aquarium was replenished at weekly intervals and temperature was maintained at approximately 20°C by electric bulb.

Removal of pituitaries:

The frogs were pithed, and sometimes just dead or dying frogs were also used as pituitary donors as they also yielded good result. The pituitaries were dissected out as follows:

Head with upper jaws were cut transversely behind the angle of jaw and placed upside-down, in amphibian ringer solution, if experiments are to be performed with fresh pituitaries, otherwise in absolute ethyl alcohol. The parasphenoid bones were cleared and with a tip of fine scissors inserted in foramen magnum, the parasphenoid were cut longitudinally along its lateral edges and deflected gently, taking care every time not to injure underlying organs. The pituitary is located behind optic chiasma, as a transferred kidney shaped pinkish structure surrounded by some endolymphatic tissue (Fig. 65). The pituitaries were carefully removed and placed in amphibian ringer solution or absolute ethyl alcohol, as the case may be.
Fig. 6.4: Maintenance of frog in the laboratory condition (Aquarium) before experimental breeding.

6.5: Position of pituitary.
Preparation of pituitaries homogenate and injection technique:

Mature healthy female (SVL 5.8 cm weight more than 25.0 gm) with enlarged abdomen were selected for experiment and placed separately in 2 litre clean and sterilized corning beakers, with some pond water. Required amount of pituitaries were homogenized in 0.5 ml distilled water in a sterilized crucible or glass homogenizer and the homogenate was injected with sterilized 2.0 ml hypodermic syringe fitted with number 21 needle in the dorsal femoral lymph sac of the frog (Fig. 6.4). The needle was withdrawn slowly using cotton swab dipped in 70% alcohol, with a finger tip and at the point of injection to prevent loss of injected homogenate from the lymph sac. The injected females were replaced in the beaker. (Fig. 6.7)

Stripping of ova and fertilization:

The stripping was attempted about 20 hours of the injection. The frog was held in left hand and gentle pressure and backward movement was applied with the palm of right hand. As soon as some eggs were seen coming out of the cloaca, the frog was again placed back in the beaker and sperm suspension were quickly prepared by macerating 4 testis of mature males in 200 ml of pond water in finger bowls. The sperm suspension was checked under microscope
and as soon as the sperms regain the mobility, eggs from the tested female were stripped into the sperm suspension. First cleavage was taken as the test for the successful fertilization.

**Fecundity and gonado somatic index:**

For fecundity estimations, both ovaries, of the frogs, collected and preserved during different months were taken out, washed and blotted on the filter papers to remove the excessive moisture. Extraneous, tissues were removed from each ovary under binocular microscope. The volume of the ovaries was measured with the help of measuring cylinder, through water displacement technique. Thereafter the ovaries were divided into four aliquots and numbers of ova in each were counted, to find out the total numbers of ova in the ovaries of each frog. Numbers of eggs in the ovaries of each frog and its total length/weight were plotted to prepare scatter graph and regression was worked out to find out the fecundity. Similarly, with the help of scatter diagram and regression equation the relationship between the SV length and weight of the ova has also been calculated. Terentjev's (1960) empirical formula for the calculation of fecundity has also been applied on its log 'n' and log 'e' bases.

The gonado-somatic index for each frog has been calculated as:

\[
\text{Gonado-somatic index} = \frac{\text{Weight of ova}}{\text{Weight of frog}} \times 100
\]
EXPERIMENTS AND RESULTS

In 1977, some pilot experiments were performed to find out the approximate dosage (see Table 6.1), in which, 3 - 8 pituitaries were homogenated in 0.5 ml distilled water and were administered in the frogs weighing from 29.50 gm to 32.50 g (SV length 5.85 cm - 6.10 cm). It was observed that a dosage of about 0.08 mg/g weight of the female caused successful induction of ovulation and spawning.

Subsequently it was confirmed that females having SV length 5.8 cm or above and weight more than 25.0 g responds to pituitary injection and others do not. A general observation was that less agile females with enlarged abdomen and while at rest keeping hind limbs extended widely, show better response to pituitary injection than others.

Based on the preliminary findings, following experiments were performed:

1) Experiments with fresh homoplastic pituitaries.
2) Experiments with homoplastic pituitaries preserved in absolute ethyl alcohol.
3) Experiments with homoplastic pituitaries preserved for one to two years.
Table 9.1

Pilot experiments to calculate the dosage of homoplastic pituitaries for induced ovulation in *Rana cyanophlyctis*

| Exp. No. | Injection Date | Injection Time | Atm. Temp. °C | Sex | Number | Weight g | Pituitary weight/weight of injected female mg/g | Volume of homogenate SVL Weight cm | Condition of female | Stripping Date | Stripping Time | Number of ova obtained |
|----------|----------------|----------------|----------------|-----|--------|----------|-----------------------------------|---------------------------------|-------------------|-----------------|----------------|----------------|--------------------------|
| 1_a      | 8.6.77         | 11.30          | 21.5           | M   | 4      | 0.0126   | 0.05                                      | 0.5                         | 5.45             | 23.40          |                |                |                          |
| 1_b      | 8.6.77         | 12.00          | 21.5           | F   | 3      | 0.0148   | 0.05                                      | "                            | 5.85             | 29.50          |                |                |                          |
| 1_c      | 8.6.77         | 12.20          | 21.5           | F   | 4      | 0.0204   | 0.06                                      | "                            | 6.10             | 32.10          |                |                |                          |
| 1_d      | 8.6.77         | 12.45          | 21.5           | F   | 5      | 0.0264   | 0.08                                      | "                            | 6.00             | 30.70          | 9.6.77         | 8.30           | 105                       |
| 1_e      | 8.6.77         | 12.40          | 21.5           | F   | 6      | 0.0313   | 0.10                                      | "                            | 5.90             | 31.20          | 9.6.77         | 6.00           | 193                       |
| 1_f      | 8.6.77         | 16.40          | 21.5           | F   | 8      | 0.0431   | 0.15                                      | "                            | 6.05             | 32.20          | 9.6.77         | 6.40           | 79                        |
4) Experiments with heteroplastic pituitaries.

5) Experiments with homoplastic pituitaries collected from immediately dead frogs.

6) Experiments to see induction of ovulation second time.

**Experiments with fresh homoplastic pituitaries:**

The female frogs of SV length varying from 5.80 cm to 6.20 cm and weight 26.00 gm - 32.90 gm, were injected with the homogenate made up of 5 to 8 freshly collected homoplastic males and female pituitaries, of the weight varying from 0.0296 gm to 0.15 gm during June and July months, at laboratory temperature varying between 20.8°C to 21.5°C. The data has been compiled in Table 6.2.

On the next day, after a lapse of 14-24 hours stripping were achieved in all the experiments frogs. The number of ova released varied from 29 to 196 in different experiments. It was noted that male and female freshly collected homoplastic pituitaries are capable of inducing ovulation in frogs and a dosage of 0.1 mg/gm body weight leads to successful spawning during rainy season.

**Experiments with homoplastic pituitaries freshly preserved in absolute ethyl alcohol.**

The female frogs having SV length 4.9 cm to 6.2 cm and weight between 11.70 gm and 32.00 were injected with
Table 6.2

Experimental breeding with fresh pituitaries in *Rana cyanophlyctis*

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Injection Date</th>
<th>Injection Time</th>
<th>Atm. Temp. °C</th>
<th>Pituitary weight/weight of female frog mg/gm</th>
<th>Volume of homogenate injected ml</th>
<th>Condition of female SVL cm</th>
<th>Weight gm</th>
<th>Date Time obtained</th>
<th>Stripping Date Time</th>
<th>Number of ova obtained</th>
</tr>
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<tbody>
<tr>
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<td>21.6.77</td>
<td>19.10</td>
<td>21.5</td>
<td>0.0384</td>
<td>0.1</td>
<td>0.5</td>
<td>6.10</td>
<td>31.00</td>
<td>22.6.77 12.10</td>
<td>78</td>
</tr>
<tr>
<td>_2_b</td>
<td>21.6.77</td>
<td>20.00</td>
<td>21.5</td>
<td>0.0391</td>
<td>0.1</td>
<td>0.5</td>
<td>6.20</td>
<td>32.70</td>
<td>22.6.77 9.00</td>
<td>29</td>
</tr>
<tr>
<td>_2_c</td>
<td>6.7.78</td>
<td>11.00</td>
<td>20.8</td>
<td>0.0343</td>
<td>0.1</td>
<td>0.5</td>
<td>6.10</td>
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<td>7.7.78 8.10</td>
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<td>0.15</td>
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<td>0.5</td>
<td>5.85</td>
<td>30.00</td>
<td>16.7.78 13.00</td>
<td>67</td>
</tr>
</tbody>
</table>

* Frogs subjected to homoplastic injection for second time.
the homogenate made up of 3 to 8 male and female preserved homoplastic pituitaries of the weight varying from 0.0138 to 0.0397 gm, during July at an approximately 21.0°C to 22.0°C laboratory temperature. The dosage of pituitary homogenate of approximately 0.1 mg/gm body weight were successful to induce ovulate the mature frog. The data has been tabulated in Table 6.6. Out of the six experiments performed Exp. No. 6a, 6b and 6f were successful, whereas experiments No. 6c, 6d and 6e were unsuccessful. These results indicate that *Rana cyanophlyctis* of size group 5.9 cm and above and weight 30.0 gm and above, could be induced breed upon freshly preserved homoplastic pituitaries.

**Experiments with homoplastic pituitaries preserved for one year in absolute ethyl alcohol:**

These series of experiments were performed during June and July (Laboratory temperature 21.5 - 22.5°C) with homoplastic pituitaries preserved for one year in absolute ethyl alcohol. It was observed that the pituitaries of *Rana cyanophlyctis* remain potent in absolute ethyl alcohol for one year. The pituitary dosage of 0.1 mg/gm weight of the female were found to be effective. The data has been compiled in Table 6.7. It is further noted that the skipper frog having SV length 5.8 cm in length and
Experiments performed with the freshly preserved homoplasic pituitaries in Rana cyanophlyctis.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Injection Date</th>
<th>Injection Time</th>
<th>Atm. Temp. °C</th>
<th>Pituitary Sex</th>
<th>Number</th>
<th>Weight gm</th>
<th>Pituitary weight/weight of female frog mg/gm</th>
<th>Volume of homogenate injected ml</th>
<th>Condition of female SVL cm</th>
<th>Stripping Date</th>
<th>Stripping Time</th>
<th>Number of ova obtained</th>
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</thead>
<tbody>
<tr>
<td>6a</td>
<td>21.7.77</td>
<td>16.00</td>
<td>22.0</td>
<td>F</td>
<td>5</td>
<td>0.0296</td>
<td>0.09</td>
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<td>5.90</td>
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<tr>
<td>6b</td>
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<td>F</td>
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<td>22.0</td>
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<td>0.5</td>
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<td>11.70</td>
<td>22.7.77</td>
<td>-</td>
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<td>15.50</td>
<td>22.7.77</td>
<td>-</td>
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<tr>
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<td>6.20</td>
<td>31.90</td>
<td>22.7.77</td>
<td>12.10</td>
</tr>
</tbody>
</table>
### Table 6.7

Experiments of induced breeding with homoplastic pituitaries preserved for one year in *Rana cyanophlyctis*  
(Pituitaries preserved on 19.6.77)

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Injection Date</th>
<th>Atm. Temp. °C</th>
<th>Sex</th>
<th>Number</th>
<th>Pituitary Weight gm</th>
<th>Pituitary weight/weight of female frog mg/gm</th>
<th>Volume of homogenate injected ml</th>
<th>Condition of female SVL cm Weight gm</th>
<th>Stripping Date</th>
<th>Stripping Time</th>
<th>Number of ova obtained</th>
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29.20 gm in weight have only responded to one year preserved pituitaries, the experiments 7d and 7e (see Table 6.7) were found unsuccessful, due to immature size and under weight of the experimental frogs.

Experiments with homoplastic pituitaries preserved for two years in absolute ethyl alcohol:

The present series of experiments were performed in the months of May and June 1979 (laboratory temperature 20.6-22.0°C) with the pituitaries of *Rana cyanophlyctis* preserved in absolute ethyl alcohol since July 1977 (two years). During preservation the portion of absolute ethyl alcohol evaporated was replaced by fresh one. The required dosage of such pituitaries were homogenised in 0.5 ml glass distilled water and injected in the dorsal lymph sac of the female frogs. The data has been compiled in Table 6.8. It was observed that the pituitaries preserved in absolute ethyl alcohol retained its potency for a minimum of 2 years and act as efficiently as fresh or one year old preserved pituitaries. In this series, all the six experiments were found successful. The dosages during the experiments was observed varying between 0.08 mg/gm to 0.09 mg/gm weight of the female.
Table 8.8

Experimental breeding with homoplastic pituitaries preserved for two years in *Rana cyanophlyctis*

(Pituitaries preserved on 8.5.77)

<table>
<thead>
<tr>
<th>Exp. No.</th>
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<th>Atm. Temp. °C</th>
<th>Sex</th>
<th>Pituitary weight/gm</th>
<th>Weight of frog mg/gm</th>
<th>Volume of homogenate injected ml</th>
<th>Condition of female SVL cm</th>
<th>Weight gm</th>
<th>Stripping Date Time</th>
<th>Number of ova obtained</th>
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Experiments with the help of preserved homoplastic pituitaries in different months:

Induced breeding was attempted in *Rana cyanophlyctis* for 14 continuous months, from June 1977 to July 1978. In all the 43 experiments performed, the preserved homoplastic pituitaries were used. Data has been compiled in Table 6.9. The observation made are as follows:

1. Induction of ovulation was achieved in the frogs having a measurement SL length 5.8 cm or over and weight 25.0 gm and over. Female frogs smaller in size and weight did not respond to pituitary injection.

2. A dosage of 0.08 - 0.2 mg/gm weight of the female was found effective to achieve ovulation in *Rana cyanophlyctis*. A slightly increased dosage of 0.15 mg/gm - 0.2 mg/gm was found to be effective during March/April and September/October months (Pre and Post breeding phase) and approximately 0.08 mg/gm was found effective during peak rainy season May/June (breeding phase) this also coincides with its peak active phase (see Chapter 3).

3. Although the frogs could be induced ovulated in each month from March till October, but the amounts of egg laid ovulated were found varying in different months. The maximum numbers of 667 ova were achieved in the frog from May month and minimum of 27 ova from the frog in March month. The frogs collected and
<table>
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<th>Exp. No.</th>
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<th>Sex</th>
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induced breed during May and June months were the best in obtaining number of ova and the percentage of success in experimental breeding. Further, the maximum success in the ovulation were observed in the month, when it rained heavily and when the abundance and the activities of the frog on the land were maximum.

4. No ovulation could be achieved during winter months from late October till late February, perhaps because of physiological inertness in adult frogs.

5. The frogs (serial number 34, 35, 45, 47, 48 of Table 6.9) were collected and stored in plastic jar for a week, and then were subjected to induced breeding. Out of 5 frogs only one (serial No. 47) could ovulate with a poor spawn of 31. Hence, it can be concluded that the frogs stored for over one week without proper feeding in the laboratory cannot be induced ovulated.

Experiments with heteroplastic pituitaries of Rana limnocharis:

This series of experiments, were performed with freshly collected and absolute ethyl alcohol preserved
<table>
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<th>Injection Date</th>
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<th>Pituitary Number</th>
<th>Pituitary Weight/g</th>
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pituitaries of *Rana limnocharis*. Data has been compiled in Table 6.4. It was observed that a dosage of 0.1 mg/gm to 0.15 mg/gm weight of the females of *Rana cyanophlyctis* were found effective to achieve ovulation in the frogs. In the experimental frogs $6_{41}$ and $6_{4j}$ did not respond to ovulation and died. The failure is attributed to higher dosage of pituitary injection. However, in the above series of experiments a minimum of 36 ova and a maximum of 109 ova (see Exp. No.$6_{4g}$ and $6_{4e}$ of Table 6.4) were procured.

Experiments of induce ovulations with the homoplastic pituitaries collected from dead *Rana cyanophlyctis*:

In the present series of experiments the pituitaries collected from the frogs (male and female) died for 12 hours to 18 hours were homogenated and injected with 0.5 ml of distilled water. The data has been compiled and presented in Table 6.3. 4 out of such 6 experiments performed were found successful. It was observed that a pituitary dosage of 0.09 mg/gm female body weight leads to successful ovulation. A minimum of 67 ova and maximum of 170 ova can thus be obtained. Further, it can be concluded that the frog although dies, its pituitary retains the gonadotrophic activities for some time over 18-20 hours.
Experiments of induced breeding in *Rana cyanophlyctis* with the help of homoplastic pituitaries collected from the frog died 4 to 6 hr before of experimentation

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Injection Date</th>
<th>Atm. Temp. °C</th>
<th>Injection Temp. °C</th>
<th>Pituitary Sex</th>
<th>Pituitary Number</th>
<th>Pituitary Weight g</th>
<th>Volume of homogenate injected female mg/g</th>
<th>Condition of female SVL cm</th>
<th>Weight g</th>
<th>Stripping Date</th>
<th>Stripping Time</th>
<th>Number of ova obtained</th>
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<td>M</td>
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<td>30.50</td>
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<td>-</td>
</tr>
<tr>
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<td>&quot;</td>
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<td>22.80</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>19.8</td>
<td>F</td>
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<td>0.09</td>
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<td>0.0199</td>
<td>0.07</td>
<td>&quot;</td>
<td>5.70</td>
<td>26.00</td>
<td>11.6.67</td>
<td>11.30</td>
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</table>
Experiments of induce ovulations in the frogs which has already ovulated once:

The frogs which has already ovulated with the help of homoplastic fresh pituitaries, once (Table 6.2) have been subjected to the experimental breeding for the second time, with the help of preserved homoplastic pituitaries. The data of the experiments have been compiled and presented in Table 6.5. Only the asterisk marked experimental frogs (Table 6.2) which have once ovulated with the help of fresh homoplastic pituitaries extract were found to be reovulating with preserved homoplastic pituitary extract, and it is noted that the frog No.6.2b, which ovulated 29 ova on 22.6.1977 re-ovulated 100 ova on 25.6.1977 (see Exp. frog No. 6.5b). The experimental frog No. 2 (Table 6.2) which ovulated 176 ova on 7.7.1978, on induction re-ovulated 68 ova on 9.7.1978 (see Exp. frog No. 5e); the experimental frog No. 2d (Table 6.2) which ovulated 71 ova on 7.7.1978 induction re-ovulated 49 ova on 9.7.1978 (see Exp. frog No. 7.5d) and finally experimental frog No. 2 (Table 6.2) which ovulated 196 ova on 16.7.1978 on induction re-ovulated 72 ova on 18.7.1978 (see Exp. frog No. 6.5e). Hence, it can be concluded that the frogs, that has already induced bred once can be induced to breed for the second time, with a short gap for second injection.
Experiments of induced breeding in *Rana cyanophlyctis* for the second time, that has already induced once

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Injection Date</th>
<th>Atm. Temp. °C</th>
<th>Pituitary weight/weight of injected female mg/g</th>
<th>Volume of homogenate injected</th>
<th>Condition of female SVL</th>
<th>Weight cm</th>
<th>Stripping Date Time</th>
<th>Number of ova obtained</th>
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<td>6.20 32.70</td>
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<td>5b</td>
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<td>F 6 0.0337 0.1</td>
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<td>6.20 31.00</td>
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<td></td>
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<tr>
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<td>F 5 0.0313 0.1</td>
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<td>14.30</td>
<td>M 9 0.0298 0.09</td>
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<td>18.7.78</td>
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<td>5.85 29.85</td>
<td>18.7.78</td>
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</tr>
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</table>
Gonado sometic index:

The gonado sometic index, the ratio between the weight of the gonad and the weight of the animal has been calculated in 20 female frogs of SV length and body weight ranging from 5.45 cm to 7.15 cm and 18.17 gm to 49.02 gm respectively (Table 6.10).

The graph plotted between SV length versus weight of ova (Table 6.10; Fig. 6.2) showed a linear relationship and is expressed as:

\[ Y = mX + c \]

where

\[ Y & X \] represent variables,
\[ X & Y \] represent weight of ova and SV length respectively.

\[ m \& c \] - constants of the equation.

The relationship between weight of ova and SV length of frog has been calculated, applying the sum of least square method and was noted as follows:

\[ \log W_1 = -4.506 + 6.4369 \log L \]

(See Fig.4.2)

where

\[ W_1 = \text{weight of ova} \]
\[ L_1 = \text{SV length of frog} \]
Fig. 6.1 (A) : Relationship between Body weight of female *Rana cyanophlyctis* and gonadosomatic index.

6.2 (B) : Relationship between ova weight of female *Rana cyanophlyctis* and gonado somatic index

6.1 (C) : Relationship between SV length of female and gonadosomatic index.
Body weight
\[ r = 0.7050 \]

Ova weight
\[ r = 0.9092 \]

SV Length
\[ r = 0.8431 \]

Fig. 6.1
### Table 6.10

Relationship of SV length and weight of frog with number, weight of ova and gonado somatic index in *Rana cyanophlyctis*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Body's Length</th>
<th>Body's Weight</th>
<th>Weight Measured</th>
<th>Weight Calculated</th>
<th>O V A</th>
<th>Number By counts</th>
<th>Number By regression equation derived</th>
<th>Taren'tjev's equation at loge</th>
<th>Taren'tjev's equation at logn</th>
<th>Gonado somatic index</th>
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<tr>
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<td>1.6291</td>
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<td>6.2982</td>
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<td>12427</td>
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</tbody>
</table>
The weight of each frog has been calculated in relation to its SV length with the help of above derived relationship. The frog of SV length measuring 7.15 cm showed maximum weight of ovary 9.8265 gm and the frog, with SV length 5.45 cm showed minimum weight of ovary 1.7119 gm (Table 6.10). However, the weight of ova measured through aliquots showed, the minimum ovary weight (1.6291 gm) female frog of SV length 5.45 cm and maximum ovary weight (11.5879 gm) in female frog of SV length 7.15 cm. The correlation coefficient driven between SV length and weight of ovary was found 0.9581 (Fig. 6.2), highly significant at 1% and 5% levels.

Table 6.10 also show the comparison between the measured and calculated weight of ova (see Fig. 6.3) and measured and calculated members of ova (see Fig. 6.3). The comparison reveals little variation in observed and calculated values. The scatter diagram plotted between gonado somatic index, and the body weight of the frogs; gonado somatic index and ovary weight of the frog and gonado somatic index and snout-vent length of the frog have been best expressed by linear regression (Fig. 6.1 A, B, C). The correlation coefficient between gonado somatic index and body weight; gonado somatic index and ovary weight and gonado somatic index and snout-vent length of female frogs have also been calculated and were found to be 0.7050; 0.9092 and 0.8431, respectively.
Fecundity:

The graph plotted between log of SV length and log, numbers of ova, female frogs (Table 7.10) showed a linear relationship and can be expressed by the formula:

\[ Y = mx + c \]

where

\[ Y \text{ & } X = \text{represent variables namely ova number and SVL respectively.} \]

\[ m \text{ & } c = \text{equation constants} \]

The relationship between fecundity and SV length of the frog has been found out applying the sum of least square method, from the above linear expression:

\[ \log F = -1.4169 + 6.0851 \log L \]

where

\[ F = \text{number of eggs in thousand} \]

\[ L = \text{SV length in cm} \]

The fecundity in each frog has been calculated with the help of the above equation. A maximum number of
Fig. 6.3: Correlation coefficient and relationship between weight of frog and number of ova.
Fig. 6.3

\[ r = 0.82 \]

- Number of Ova
- Weight of Frog (gm)
6048 eggs were calculated from the frog of SV length 7.15 cm and minimum of 1159 eggs from the frog of SV length 5.45 cm (Table 6.10). However, the fecundity noted through gravimetric method showed the minimum count of 1262 eggs from the frog of SV length and weight of 5.45 cm and 18.17 gm respectively and maximum 6695 count at 7.05 cm and 47.27 gm respectively (Table 6.10). The correlation coefficient between the two variates length and fecundity was found to be 0.9432 (Fig. 6.2A) and was significant at 1% and 5% level of confidence.

The graph plotted between weight of ova and numbers of egg as 'Y' and 'X' axis showed linear expression ) with a significantly high correlation coefficient of 0.8628. The correlation coefficient derived between the weight of ova calculated and number of ova (r = 0.9180) and was also found to be significant at 1% and 5% probability level.

Terentjev's (1960) empirical equation for the calculation of fecundity and clutch size for temperate frogs, has also been applied at log_{e} and log_{n} bases in *Rana cyanophlyctis*. The fecundity observed by gravimetric measurements, calculation by derived linear regression formula and by Terentjev's equations at log_{e} and log_{n} bases have been tabulated and compared in the Table 6.10,
Fig. 6.2 (A): Linear relationship and regression equations between SV length and number of ova of Rana cyanophlyctis.

6.2 (B): Regression equation, and linear relationship between SV length and weight of ova of Rana cyanophlyctis.

6.2 (C): Regression equation drawn at \( \log_2 \) in accordance to Terentjev's formula at given SVL of Rana cyanophlyctis.

6.2 (D): Regression equation drawn at \( \log_n \) in accordance to Terentjev's formula at given SVL of Rana cyanophlyctis.
A
\[ \log F = 1.42 + 6.09 \log L \]
\[ r = 0.94 \]

B
\[ \log W = 4.51 + 6.44 \log L \]
\[ r = 0.96 \]

C
\[ \log N = -1.74 + 2.17 \log L \]

D
\[ \log N = -1.42 + 6.09 \log L \]

Fig. 6.2
and Fig. 7.2 C and D. It is noted that fecundity calculated through Terentjiev's equation does not hold good for *Rana cyanophlyctis* and varies greatly with the fecundity calculated by linear regression and gravimetric method.

**DISCUSSION**

Induced breeding by pituitary injection is now a routine procedure in many laboratories all over the world. Eversince, Ascheium and Zordek demonstration that pituitary implant enhances sexual development in mice (Allen, 1939) and Wolfe (1929) experiment that pituitary glands cause induction of ovulation in *Rana pipiens*, different techniques have been evolved for induced breeding (see Rugh, 1962; Nieuwkoop and Faber, 1967; Jhingran, 1975). Nieuwkoop and Faber (1967) and Roy (1979) respectively described detailed technique of induce ovulation in *Xenopus laevis* and *Rana limnocharis*, following Rugh (1962) technique. The experiments in the present investigation on the induced breeding of *Rana cyanophlyctis* has been followed in accordance to the technique prescribed by Nieuwkoop and Faber (1967) and Roy (1979). Though Rugh
(1962) prescribed use of fresh pituitary, he did mention that pituitaries preserved in ethanol can be used for this purpose. However, Rugh's (1962) technique was found to be somewhat cumbersome as in every set of experiment one has to collect a number of frogs, decapitate some of them to collect required numbers of pituitaries and then inject it along with some distilled water in the abdomen of the experimental female.

In fishes acetone preserved pituitaries are used (Alikunhi et al., 1960; Choudhuri, 1960, 1963 and Ibrahim and Choudhuri, 1966), but in frogs pituitaries preserved in absolute ethyl alcohol yield successful results. It is difficult to explain such differential response, in fishes and frogs. In the present investigation response to fresh, preserved as well as pituitaries taken out from just dead or dying frogs was tested. As reported earlier (Roy 1979; Khare, Roy and Kumar, 1981) the use of frog pituitaries preserved in absolute ethyl alcohol was found very convenient as they can be effectively used even after 2-3 years of preservation. This investigation reveal that:

(1) The frog can be induced breed for eight continuous months, namely March, April, May, June, July, August, September and October.

(2) The frog of minimum measurements of SV length 5.8 cm and weight 25.0 gm is required for successful induced spawning.
(3) The pituitary dosage of approximately 0.08 mg/g body weight for fully mature and slightly for somewhat immature female frogs can be effectively used for induced ovulation.

(4) The number of ova obtained by induced breeding during rainy season are more, maximum being 667, than at the beginning or end of rainy season, minimum being 24.

(5) At cold weather of Shillong induction of ovulation occurs in 20 to 24 hours.

(6) The pituitaries preserved in absolute ethyl alcohol remain equally potent for over two years as fresh ones.

(7) The frogs kept in captivity for a larger duration show more failure and indifferent results than those freshly collected.

(8) The frogs do not show zoological specificity and can be induced bred by heteroplastic pituitaries.

(9) The pituitaries of dying or just dead frogs also maintain their potency.

A large number of frog in the tropical regions are reported to breed throughout the year (see Chruch, 1960 a,b; Zeller, 1960; Inger and Greenberg, 1963; Berry,
1964; Inger and Bacon, 1968; Brown and Alcala, 1970; and Duellman, 1970). Berk (1930); Bragg (1950 and Ballinsky (1969) have reported that many tropical anurans breed in the nature at any time of the year and at places were temperature and rainfall are sufficiently high. McCann (1933) and Ramaswami and Lakshman (1959) mentioned that the aquatic frog, *Rana cyanophlyctis* is capable of breeding throughout the year if suitable conditions are provided to them. Thus in the present investigation induced breeding of *Rana cyanophlyctis* was attempted every month from January to December, and successful response was recorded from March to October. The failure of breeding during November, December, January and February may be attributed to very low atmospheric temperature (Fig. 1.2.3).

Heusser (1961); Gunther (1969); Smith (1969); Wahl (1969) and Van Gelder and Hoedemackers (1971) reported that in warmer regions, a population of frogs, abiding in permanent water bodies, may have several short period of peak breeding activity. *Rana cyanophlyctis* seems to belong to the same category, and has prolonged breeding season at Shillong, which coincide with the rainy months.

The failure to achieve induced spawning in *Rana cyanophlyctis* weighing less than 5.8 cm and 25.0 gm, is attributed to its immature age having small immature oocyte which are not competent to respond to the gonadotropin
stimulus as also reported earlier by Schuetz (1969),
Evennett and Thornton (1971). Gangadhara and Ramiah
(1968) observed that starvation in the Rana cyanophlyctis
destroyed the mature follicle in the ovary. Jorgensen
(1976) noted degeneration of non-ovulated egg rapidly due
to starvation. Failure in the induced ovulation of the
stored frog, during present investigation may be due to
the poor feeding in captivity.

**Fecundity:**

Terentjev (1960) observed linear relationship
between fecundity and SV length in certain Russian frogs
and subsequently derived a regression equation to calculate
fecundity of the available frogs from its known SV length.

\[
\log F = -1.7428 + 2.1670 \log L
\]

where

- \( F \) = Fecundity
- \( L \) = SV length of frog

Inger and Greenberg (1966); Inger and Baccon (1968)
noticed that fecundity observed in the natural conditions
and fecundity calculated in accordance to Terentjev's
regression equation, showed little variation in rain forest
frogs. In the present investigation a linear relationship between fecundity and SV length of female frogs have been observed (Fig. 4.2). Further, the bivariate correlation coefficient derived between the two variables described above have also been found to high (r = 0.94 at 19 degree of freedom, 'df'). The relationship between fecundity and SV length (Fig. 4.2) in Rana cyanophlyctis is found to be expressed by \( \log F = -1.4169 + 6.0851 \log L \).

Applying SV length of the Rana cyanophlyctis measured during present investigation to the Terentjev's equation at \( \log \) base 'e' and \( \log \) base 'n' the fecundity in the various size group of the frog has been calculated (Table 6.10). The fecundity calculated accordingly showed great variations from the observed and calculated fecundity in the frog derived in accordance to the regression equation calculated earlier for the Rana cyanophlyctis. However, in both the cases of frogs from different populations, the linear relationship and linear regression in bivariates have been observed. The relationship between SV length and weight of the ova has also been derived (Fig. 6.3) and was observed to be expressed by the linear regression equation, with fairly high correlation coefficient (\( r = 0.96 \) at 19 df.)

\[
\log W = -4.506 + 6.4369 \log L
\]
Further, significant correlation coefficient and linear relationship between, weight of the frog and weight of ova observed \( (r = 0.86; \text{19 df.}) \) weight of the frog and the weight of ova calculated \( (r = 0.92; \text{20}) \) has been observed. Koskela and Pasanen (1975) working on *Rana temporaria* observed a linear regression and high correlation coefficient between (1) SV length of the frog and weight of ova (2) SV length and weight of oviduct; (3) SV length and number of egg; (4) SV length and size of egg \( (r = 0.8 \text{ to } 0.9; P < 0.01) \). They also concluded that the value of spawn size and number of eggs depend on the size of female frog and calculated that *Rana temporaria* of SVL 78 mm would produce 13.5 to 14.0 gm of spawn, consisting of about 1360 eggs of a size of 2.01 mm.

Similarly, with the help of equation derived for *Rana cyanophlyctis* it is noted that a female measuring 7.15 cm would produce 9.8 gm of spawn comprising of 6048 of size 1.5 mm each. However, aliquots analysis and gravimetric measurement at the same SV length it was observed that it had 8.12 to 11.58 gm of ova, consisting of 4465 to 5808 eggs of an approximate size of 1.5 mm.

The linear relationship in the bivariates (1) gonado somatic index and weight of ova (2) gonado somatic index and weight of frog (3) gonado somatic index and SV length of frog, have been observed. The correlation
The coefficient of the three relationships described above have been 0.91, 0.71, and 0.84 respectively which is recorded to be significantly high at 1% and 5% probability level. Hence, with the help of these derived equations, ova size, ova weight, ova number can be known at a given SV length or body weight of frog, without sacrificing the animals.
SUMMARY

This chapter deals with induced breeding and gonadosomatic index. Homoplastic pituitaries preserved in absolute ethyl alcohol were used for induced breedings. They were found to retain their potency for a period of 3 years and the frogs responded to them as effectively as to fresh ones. Induced breeding could be performed for 8 months, from March to October. The females having SVL 5.8 cm and above and weight 25.0 gm and above, responded successfully to the pituitary infection. 0.03 mg/gm wt of the female was effective in breeding season. During pre and post breeding period a larger dose 0.15 mg/gm - 0.2 mg/gm wt of the females was found to be effective. The maximum number 667 ova were obtained during May and minimum, 27 ova during March. In some experiments, induced breeding for the second time, was also successfully performed on the same frogs.

Significant correlation coefficient ($P < 0.01$) and linear relationship has been obtained between SVL and number of ova, SVL and weight of ova, body weight and number of ova, body weight and size of ova, gonadosomatic index and body weight, gonadosomatic index and SVL; and gonadosomatic index and weight of the ovaries.
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