C H A P T E R - I

GENERAL MATERIALS AND METHODS

The technique of induced breeding and artificial fertilization was preferred over natural spawning to obtain the fertilized eggs at different stages of embryonic development of Cyprinus carpio, due to the following obvious advantages:

1. It ensures breeding as well as nearly hundred per cent fertilization of the spawn.
2. It enables the worker to know the exact time of fertilization, so that the exact incubation period (time period taken by the egg from

fertilization upto hatching) as well as the time interval between fertilization and a particular stage arrival can be noted.

3. It obsoletes the use of aquatic weeds as substratum for egg attachment, which interferes in the collection of egg samples for biochemical studies.

4. The estimation of total number of fertilized eggs is less complicated and difficult in case of stripping and artificial fertilization.

5. It prevents the eggs from mixing with the different types of particles and organisms occurring in natural tanks, which on decomposition may spoil the eggs. Moreover, the bacteria and fungi flourishing on them could also be dangerous to the developing eggs.

Induced breeding and artificial fertilization was done following the method of Woynarovich and Horvath (1980). Mature common carp fishes (Fig. 1 &2), ranging between 0.5 to 2 Kg body weight (which are easy to handle and strip), were procured from some local tanks of Raipur and Fish Seed Farm, Khutelabhata, Durg. Care was taken to provide proper space, water

level and oxygen to the fishes during transport. Breeders were brought in the breeding season, males and females segregated and kept in separate cemented stocking tanks (3x3x0.7 M) with proper space and aeration. Care was also taken to prevent any type of injury to the brood fishes during transport, handling and stocking. Breeders were acclimatized under experimental conditions for 7-8 days with proper feeding.

Unless their gonads have developed upto final resting stage, fishes would not respond to any propagation technique, hence sorting out of the right breeders was given prime importance.

4. Selection of breeders for induced breeding:

The symptoms indicative of the 'ready-to-spawn' conditions according to Woynarovich and Horvath (1980) are as follows:

Females:

(i) Well rounded and soft abdomen, the fullness of which extends posteriorly past the pelvis to the genital opening.

(ii) The genital opening is swollen, protruding and reddish or rose in colour, its edge is uneven or fringed.

(iii) The anus may also be swollen and reddish.

Males:

(i) The male releases a few drops of thick milt when its abdomen is pressed slightly.

(ii) The dorsal surface of the pectoral fin becomes somewhat rough.

All of the above mentioned features were considered while selecting the breeders.

3. Induction of ovulation and spawning:

Induced ovulation and spawning was achieved through hypophysation i.e. by injecting hypophysis, which is a 'short cut' of the natural process. In nature, ovulation in a fish is regulated and brought about by its own gonadotropic hormones, namely, follicle stimulating hormone (FSH) and luteinizing hormone (LH), produced and stored by the pituitary gland. The stored hormone is released into the blood when all the requisite conditions are favourable. The decisive natural conditions to bring about spawning in common carp according to Woynarovich and Horvath (1980) are as follows:

1. Suitable water temperature (18° to 22°C);
2. grass-bottomed spawning ground with aquatic weeds;
3. water saturated with dissolved oxygen;
4. slowly rising water level;

5. presence of the other sex and

6. absence of other fishes, specially carnivorous fishes.

In hypophysation technique, however, gonadotropic hormone, extracted from the pituitary of some other fish (donor), is injected into the breeder and this brings about the final ovulation, even if one or more decisive conditions for spawning, listed above, are less favourable.

Although hypophysation technique has its own limitations, firstly that it is effective only when the eggs in the ovary have reached the resting or dormant phase after the completion of vitellogenesis and, secondly, overmature eggs may be released leading to erroneous results, it was found suitable for present purposes.

Hypophysation technique involves the following steps:

(a) Collection and preservation of fish pituitary glands:

The pituitary gland (hypophysis) acts as an intermediary between the brain and the gonads. Its cells produce and store gonadotropins, and release them only when the gland receives the necessary command. The gonadotropin content of the pituitary gland varies during different seasons and during

different stages in the life of the fish. Immature fishes have only a small quantity of gonadotropin in their pituitary, while after natural spawning the spent fish are completely bereft of gonadotropins in their pituitary. On the other hand, the gonadotropin content is at its highest level in the pituitary of sexually ripe fishes when their gonads have reached or nearly reached the resting phase and throughout the duration of the resting phase. In view of these varying contents of gonadotropin, it is important to choose the right time for collecting the pituitary glands.

Common carps are unusual among fishes in responding only to pituitary extracts from their own species, though common carp pituitary is by far the most widely used in inducing spawning of other fish species (Hardach et al, 1972).

Considering all the above mentioned facts, fishes were selected for the collection of pituitaries and were weighed on a coarse balance. Top part of the skull was removed using a sharp knife and blood soaked by cotton. All dissecting instruments used were absolutely dry and clean. Pituitary gland was taken out carefully with the help of forceps and spatula from the ventral side of the brain below the hypothalamus and kept immediately in a dark glass phial containing

absolute alcohol. After collecting the required quantity of glands, alcohol was replaced by fresh absolute alcohol and the phials stored in refrigerator.

(b) Dosage:

Pituitary dose was fixed according to the condition of breeders. Dose was administered only when the environmental conditions for spawning were quite favourable during breeding seasons, i.e. the temperature was between 18° to 24°C (in both February and July seasons) and enough moisture was present in the air (in July season).

Total dose was worked out in accordance with the body weight of the breeder. According to Woynarovich and Horvath (1980) 0.75 mg pituitary gland is sufficient for the fishes of 0.5 to 2.0 Kg body weight. The accurately weighed pituitary gland was taken in a homogenizer tube and homogenized in known amount of normal saline (0.7 %). Total dose, prepared thus, was administered in two instalments, one preparatory (10 % of the total dose) and one decisive (90 % of the total dose) with the help of a syringe. The hormone was injected into the dorsal muscles above the lateral line and below the anterior part of the dorsal fin. Fish was kept on a foam pad, handled carefully and the needle was injected below the scale.

After injecting the preparatory dose, breeders (males and females in the ratio of 2 : 1) were transferred into a plastic pool (diameter 3 M; height 0.9 M) containing well aerated water and kept in a quiet place. The preparatory dose was usually given at 10 or 11 A.M., while the decisive dose at 11 or 12 P.M., after an interval of 12 hours or so. Males readily oozing milt were not given any dose. Only in exceptional circumstances, i.e. to increase the volume of milt or in the event of their not being perfectly mature, they were administered a single decisive dose of 1 mg per kg body weight.

'Hour grade' method was followed to know the time interval between last decisive injection and full ovulation. Hour grade is arrived at by adding the hourly temperature after the decisive injection upto full ovulation.

Vigilent watch was kept on breeders without disturbing them. Spawning response of common carp (chasing movement of males and females in a circle) was observed generally between 5.00 and 7.00 A.M. i.e. 6 to 8 hours after the last injection. However, this value was dependent upon the water temperature as well as other environmental conditions. The 'ready-to-ovulate' females were easily identified since the males follow and prod them in the region of genital opening with their snouts, trying to

induce the females to spawn. As soon as the typical jumping movement of the female carp was observed, it was taken out of the pool and stripped.

C. Artificial fertilization and stripping:

The equipments for stripping were kept ready before taking out the female breeder. The female fish particularly its hind part and tail were dried with a soft towel and held with completely dry hands. As soon as the fish was held in a vertical position, keeping a dry enamel tray below its genital opening, the eggs began to be released. The belly of the breeder was pressed very softly and slowly to strip all of the eggs. Forced stripping was strictly avoided. At the same time male fishes were also captured and dried with a soft towel. Few drops of milt, depending upon the number of eggs stripped, were allowed to fall directly on the eggs. Eggs were then mixed softly with the milt in absolutely dry condition with the help of a dry feather. Presence of water was strictly avoided, since the eggs become sticky only after coming in contact with water.

After one to two minutes appropriate amount (10 - 20 % of total volume of eggs) of fertilizing solution was added. Fertilizing solution or salt-carbamide solution was prepared by dissolving 30 g carbamide (urea) and 40 g common salt (NaCl) in

10 litres of tap water. The mixture was then stirred with a feather for about 3-5 minutes continuously. During this time the eggs absorbed the solution and began to swell. Increasing quantities of the same solution were replaced at intervals to remove the sticky material. Carbamide solution not only removes the sticky layer, but also prolongs the viability of sperms, thereby increasing the rate of fertilization. This solution also helped in dissolving the materials clogging the micropyle of eggs. Eggs were treated with carbamide solution for about one hour to ensure the complete removal of sticky layer.

Eggs were then washed repeatedly with clean water and placed immediately in incubation buckets. The hatchery and incubation buckets used (Figs. 3 & 5) were designed to fulfil the following basic requirements of developing embryos as suggested by Woynarovich and Horvath (1980):

1. The developing embryos need oxygen continuously in high concentration, specially in the later stages.

2. Adequate temperature, to which the fish is adapted in nature, is essential for normal healthy development. Lower or higher temperature adversely affects the development process.

3. Clean and plankton free water with no pollutants is essential.

4. Continuous flow of water is necessary for continuous removal of carbon dioxide and ammonia excreted by the developing embryos from time to time.

Clean tube well water was stored in an overhead tank of 5' diameter and water holding capacity of 2,000 litres. Hatchery buckets used were made up of plastic of low density polyethylene. Buckets were vertical in shape having an upper part of diameter of 44 cm and lower part of diameter of 32 cm. The height of each bucket was 43 cm and the capacity 40 litres (Fig. 5). Water from the overhead storage tank was supplied to the hatchery buckets using PVC pipes. A common pipe coming from the overhead tank was divided into several branches, each with a valve connecting from the lower surface to a bucket. Outflow from each of the buckets was connected to form a common outlet. Eggs were held in netted frames, fixed on buckets. A slow but steady flow of water was maintained, since the eggs are highly sensitive to disturbances of water.

Compressed air, from a compressor of 2 horse power, was supplied to each bucket with the help of tubing , regulators and porous aerator stones.

Adequate temperature (27° to 28°C) in the hatchery lab was maintained with the help of fans and coolers (humidifiers).

In experiments on ionic effects a modified form of hatchery was used (Fig. 4; p.153).

D. Sampling of different embryonic stages:

Different embryonic stages of Cyprinus carpio (Table 1; Fig. 5) were identified under a microscope and samples collected for the fresh and dry weight determinations as well as for certain enzymatic studies.

Sample collection was done only when more than 50 % of the embryos in a lot were observed having a particular stage. The following embryonic stages were selected for the collection of eggs/embryos.

I. Unfertilized eggs: Fully mature eggs released spontaneously by the female, while stripping, were collected.

II. Blastodisc stage: Approximately 45 minutes after fertilization 'Blastodisc' stage was identified, when the animal pole rose as a small hillock on the yolk mass and developed a dark yellow colour, and collected.

III. Early morula: About 45 minutes later (1.30 hrs following fertilization), the blastodisc

(hillock) started cleavage and successively became 2, 4, 8, 16 and 32 celled; 32 celled stage was identified as early morula and collected as such.

IV. Late morula: Further successive subdivisions of cells yielded a many celled 'blastoderm' about an hour after early morula (2.30 hrs after fertilization); this was identified as late morula and collected.

V. Blastula: As the number of cells (blastomeres) increased, their size became progressively smaller and a space, termed as segmentation cavity, appeared increasing the volume of embryo about 1.30 to 2.30 hrs following late morula stage (4.00 to 4.30 hrs after fertilization). This stage was identified as blastula and collected.

VI. Gastrula: Increasing cell multiplication led to overgrowth of yolk by micromeres 3.00 to 4.00 hrs after bastula formation (7.00 to 8.00 hrs following fertilization). This stage was identified as gastrula and collected.

VII. Closing of blastopore: Micromeres enveloped yolk mass almost completely, leaving a small area called 'yolk plug' 6.00 to 7.00 hrs after gastrula stage (13.00 to 14.00 hrs following

fertilization). This was identified as closing of blastopore stage and collected.

VIII. Comma stage: With the appearance of head and tail buds, about 11.00 to 12.00 hrs after closing of blastopore stage (about 24.00 to 25.00 hrs following fertilization), the comma stage (appearing like 'comma') was identified and collected.

IX. Eyed stage: Eyes develop as optic vesicles on the head and acquired pigmentation, while the tail bud grew about 25.00 to 26.00 hrs after comma stage (about 50.00 hrs following fertilization); this was identified as eyed stage and collected.

X. Prior to hatching stage: Head and tail regions differentiated completely and the embryo started 'twitching' movement, about 5.00 to 6.00 hrs after eyed stage (about 55.00 to 56.00 hrs following fertilization); this was identified as prior to hatching stage and collected.

Eggs/embryos thus collected, were washed thoroughly in distilled water, damp dried on whatman paper No. 1 and stored immediately in a deep frigidaire in sealed containers for fresh weight and enzymatic studies. Eggs/embryos were lyophilized to constant

TABLE - 1

Different embryonic stages of Cyprinus carpio

Stage No.	Stage Identification	Average incubation period in hours (at 24°-26°C)
I.	Unfertilized egg	-
II.	Blastodisc formation	0.45
III.	Early morula	1.30
IV.	Late morula	2.30
V.	Blastula	4.00
VI.	Gastrula	7-8
VII.	Closing of Blastopore	13-14
VIII.	Comma	24-25
IX.	Eyed	30
X	Prior to hatching	52
	Hatching	56

weight, using a lyophilizer (Teshniwal), for dry weight determinations.

Methods used for the various biochemical parameters are described in subsequent chapters. Chemicals used in analytical procedures were all of analytical grade. Glass double distilled water was used throughout in preparation of the reagents.