STUDY OF THE EFFECT OF PROSTAGLANDINS ON REPRODUCTION OF INDIAN MAJOR CARPS AND ORNAMENTAL FISH

A THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

BY

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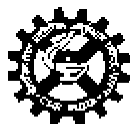
November – 2002



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ABBREVIATIONS

AA	Arachidonic acid
DHP	Dihydroxyprogesterone
Domp	Domperidone
EPA	Eicosapentaenoic acid
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GtH	Gonadotropin hormone
h	Hour
HCG	Human chorionic gonadotropin
HPLC	High performance liquid chromatography
Ip	Intraperitoneal
IU	International unit
LHRH	Luteinizing hormone releasing hormone
LHRHA	Luteinizing hormone releasing hormone analogue
MIS	Maturation inducing steroids
PG	Pituitary gland
PGs	Prostaglandins
PGB_1	Prostaglandin B_1
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
sGnRHA	Salmon gonadotropin releasing hormone analogue
µg	Microgram
gm	Gram
kg	Kilogram
ng	Nanogram

Dedicated

to

My Parents

&

Teachers

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Thesis Abstract

Thesis Title : Study of the effect of prostaglandins on reproduction of Indian major carps and ornamental fish

Fish form an important source of human diet as they provides proteins, fats and vitamins A and D. Presence of B group of vitamins, which are absent in plant food is a special feature of fish. Fish is a good source of calcium as well as polyunsaturated fatty acids (PUFA) belonging to linolenic acid (18:3). Fish oils are essential for the prevention of coronary heart diseases (Leaf and Weber, 1998).

In order to provide food to the growing population, agricultural production alone may not be sufficient. Since fish food is cheaper and rich in protein, this can possibly be exploited.

Indian major carps such as catla, rohu and mrigal are the best cultivable species among the other carps as they are the quickest growing (in terms of flesh return), non-predatory, domesticated and compatible species. Though these fishes grow and mature under pond condition, they do not normally breed in captivity. Thus, there is a need to induce Indian major carps to breed in confined waters and thereby ensure a dependable source of quality fish seed to increase fish production.

On the other hand, aquarium fish culture is being practiced all over the country to meet the domestic needs but not on commercial lines to meet the export demand. Ornamental fishes are in greater demand due to their easy maintenance in houses, offices and commercial centers and the scope for their production on large-scale is increasing day-by-day.

In view of the increasing demand both in domestic and export markets especially for freshwater ornamental fishes, if their culture is taken up scientifically and the induced breeding technique is explored to induce repeated (multiple) spawning, to reduce the gap between two spawning periods to enhance production, there is no doubt that it will be a boon to the national economy.

Till 1960s, the major part of carp seed required for culture was collected from riverine sources. Various spawning inducing agents are reported in fishes namely, fish

pituitary extract (Houssay, 1930, 1931; Cardoso, 1934; Khan, 1938; Alikunhi *et al*., 1962; Chaudhuri, 1976), human chorionic gonadotropin (Chonder, 1986), mammalian pituitary hormone (Chonder, 1994), domperidone or pimozide, ovaprim (sGnRHA) (Peter *et al*., 1986, Peter *et al*., 1988; Lakra *et al*., 1996; Pandey *et al*. 1998), ovatide (Pandey, *et al*., 2001) and pheromones (Resink *et al*., 1989; Scott *et al*., 1994; Zheng *et al*., 1995). Very few reports are available on the use of prostaglandin for spawning of carp species.

Prostaglandins are hormone-like substances derived from C_{20} fatty acids, a group of polyunsaturated, hydroxylated, long-chain fatty acids that evoke a wide spectrum of biological activity at extremely low concentrations in a variety of tissues. Prostaglandins appear to be involved in ovulation, milt production, regulation of feeding, sexual behaviour, gonadotropin secretion, endocrine regulation, pheromone production and other non-reproductive physiology of fish (Stacey and Goetz 1982; Stacey and Sorensen, 1988). All these functions are necessarily associated with breeding of fish under captivity as well as in field conditions.

Applications of natural prostaglandins in aquaculture programmes of this country, has not been studied so far. It is mainly due to their high cost and vulnerability of decomposition under variety of known and unknown conditions *viz.* their half-life in seconds, short shelf-life and storage at –70°C and above all, their non-availability in ready injectable form. These are the major stumbling blocks in the path of the use of prostaglandins in aquaculture programmes. No study has been conducted in this area to cope up with these problems neither in India nor abroad.

Therefore, there is a need to synthesize stable, long shelf-life prostaglandin analogues and study their efficacy in goldfish and Indian major carps breeding programmes and to develop alternative methods for the use of novel prostaglandins for fish breeding. This will certainly be a major breakthrough in widening prostaglandin applicability in aquaculture programmes.

This thesis is divided into six chapters.

General Introduction

Deals with the work carried out as well as the objectives and necessity of the present work.

Chapter I : Review of Literature

This part comprises literature survey with reference to induced breeding in fish with various inducing agents and prostaglandins.

Chapter II : Induced breeding of goldfish, *Carassius auratus* with prostaglandins

This chapter describes effect of various prostaglandins and their analogues as induced breeding agents for goldfish. Cloprostenol is a synthetic prostaglandin-$F_{2\alpha}$ analogue. It was administered at the dose of 1.0 μg/gm body weight of female and 0.5 μg/gm body weight of male goldfish. Induced spawning behavioural changes were observed after 4 h of injection and spawning took place after 10-12 h of injection in all the sets. Fecundity recorded as 2300 ± 104 over 183.33 ± 16.66 of the control group was highly significant ($P < 0.005$). Percentage fertilization 80 ± 2.51 over 55 ± 2.88 of the control group ($P < 0.025$) and hatching 91.66 ± 1.66 over 55 ± 2.88 of the control groups ($P < 0.01$) were also observed. Hatching occurred after 48 h of spawning. Hatchlings obtained by induced breeding appeared healthy (Table 2.4).

It was also possible to reinduce the same brooders to spawn repeatedly with the gap of 30 - 45 days by using cloprostenol.

Tiaprost is another synthetic prostaglandin-$F_{2\alpha}$ analogue which was administered at the doses ranging from 0.5 μg/gm to 2.5 μg/gm body weight. It was observed that non-injected male released with tiaprost injected (1 μg/gm body weight) female showed very prominent spawning behaviour after 1 h of injection. Behavioural changes in male were characterized by vigorous chasing of female, pairing and constant nudging of her ovipore and sides as well. During the series of spawning acts observed female approached the surface of water in head up position and male followed, both turned on their sides, they broke the water surface, male constantly chased and female gave repeated jerks (spawning acts). Eggs were not released during

those spawning acts. Males and females were again isolated after 24 h of injection and kept separately. Females injected with tiaprost ovulated after 72 h of injection. Ovulated females were again released along with male and spawning took place within half an hour after males were released. The spawning efficacy recorded at different instances as fecundity 3333.33 ± 166.67 over 183.33 ± 16.66 of the control group was highly significant ($P < 0.005$). Percentage fertilization was 81.66 ± 1.66 over 55 ± 2.88 of the control group ($P < 0.025$) and hatching percentage 91.66 ± 1.66 over 55 ± 2.88 of the control group ($P < 0.01$). Hatchlings obtained by induced breeding appeared healthy. Tiaprost with other doses induced only spawning behavioural changes (Table 2.4).

Arachidonic acid and eicosapentaenoic acid, precursors of prostaglandins, in the present study elicited only spawning behaviour in goldfish (Table 2.3).

Prostaglandin-E_2, 16-phenoxy tetranor-$PGF_{2\alpha}$, 16,16-dimethyl $PGF_{2\alpha}$, 16,16-dimethyl PGE_2 and prostodin in our preliminary study were found ineffective in inducing spawning in goldfish.

Synthetic prostaglandin analogues α-N-R, β-N-R, P-M-R,α-N-K, β-N-K and P-M-K (prepared at National Chemical Laboratory, Pune, India) in preliminary study did not induce spawning in goldfish (Table 2.5).

Chapter III : Effects of prostaglandins on breeding of Indian major carps

Cloprostenol was checked for breeding in mrigal in various ways such as Linpe method, cloprostenol in combination with Ca^{++} or pituitary priming etc. Among the selected doses, in case of cloprostenol (75 μg/kg body weight of fish) and domperidone (5 mg/kg body weight) it was observed that 50% eggs were loosed in the ovary when female was dissected after 12 h of injection. These results could give some clue to modify the doses of PGs. Egg plug effects were observed with other selected doses of cloprostenol.

Tiaprost was used in various methods (as mentioned in cloprostenol) for induced breeding. Among the selected doses it was found that when female was injected with HCG (200 IU/kg body weight) followed by second injection of tiaprost (50 μg/kg body weight) after interval of 6 h and male was injected with tiaprost (50 μg/kg body weight)

at the time of second injection of female, spawning behaviour was observed in case of male followed by milt release into the water. These activity continued for 3 h in males but female did not respond.

$PGF_{2\alpha}$, prosolvin and carboprost did not induce spawning in Indian major carps. In the preliminary study prostaglandin analogues induced spawning behaviour in male but in case of female only egg plug effect was induced. Further trials are required to standardize the doses for females of Indian major carps.

Chapter IV : Prostaglandin mediated effects on milters of Indian major carps and goldfish, *Carassius auratus*

This chapter is divided into two sections.

Section A

Effects of prostaglandins on milt volume of goldfish, *Carassius auratus*

The non-induced males of experimental groups when exposed to prostaglandin analogues namely prosolvin, tiaprost and lutylase treated females (1 μg/gm body weight), produced 52 ± 10.85, 45.6 ± 9.41 and 4.2 ± 1.43 μl milt respectively over 10.2 ± 2.46 μl of the control group after 12 h which is highly significant ($P < 0.05$, Fig. 4.1).

The non-induced males of the experimental groups when exposed to prosolvin, tiaprost and lutylase which were added directly into the water (10 μg/l) produced 40 ± 10.11, 16.5 ± 6.61 and 12 ± 2 μl of milt respectively over 10.2 ± 2.46 μl milt of the control groups after 12 h which is highly significant ($P < 0.05$, Fig. 4.2).

Section B

Quantitative assessment of milt in prostaglandin induced milters of Indian major carp, *Labeo rohita* (Hamilton-Buchanan)

Prostaglandin analogues *viz.* tiaprost and prosolvin were found to induce fourfold increase in milt volume (0.78 ± 0.20 to 3.05 ± 0.47 ml) after tiaprost administration at a dose of 50 μg/kg body weight ($P < 0.001$) in comparison with the other doses *viz.* 75 μg/kg tiaprost ($P < 0.005$) and 100 μg/kg prosolvin ($P > 0.05$) respectively. The spermatocrit values of the milt samples from the selected groups A, B, C and D were found to be 85.5 ± 6.94 ($P < 0.005$), 89.4 ± 6.14 ($P < 0.005$), 87.66 ± 3.93 ($P < 0.001$)

and 53.33 ± 7.53 respectively. The sperm count of milt of the non-induced *Labeo rohita* milters (group D) ranged from 1.8 - 2.2 × $10^7/mm^3$ with average of 2 × $10^7/mm^3$. Sperm count after tiaprost administration at the dose 50 and 75 µg/kg body weight ranged from 2.6 - 3.4 x 10^7 / mm^3 and 3.2 - 3.6 x $10^7/mm^3$ with an average of 3.0 x $10^7/mm^3$ and 3.37 x $10^7/mm^3$ respectively. Although average spermatozoa population after prosolvin administration (100 µg/kg body weight) increased up to 3.13 x $10^7/mm^3$, the volume of the milt did not increase substantially as compared to control. The sperms of rohu were immotile until the milt was diluted with water. Suddenly, the motility with excellent forward movement of sperm (70 - 90 %) increased after addition of a drop of water and was maximum (+ + + + +) for 5 - 12 seconds. Activity decreased gradually and came to an end within 60 - 80 seconds (Table 4.1).

Chapter V : Extraction of waterborne prostaglandins from female goldfish, *Carassius auratus* (Linnaeus) and their detections by High Performance Liquid Chromatography

HPLC chromatogram revealed that the test water samples in which goldfish females administered with prosolvin and tiaprost respectively were released showed presence of the peak of prosolvin and tiaprost (retention time corresponding to that of standard prosolvin and tiaprost). Peaks due to $PGF_{2\alpha}$, PGE_1, PGE_2 and arachidonic acid were absent. Control samples also showed absence of peaks due to prosolvin, tiaprost, $PGF_{2\alpha}$, PGE_1, PGE_2 and arachidonic acid. This indicated that administration of prosolvin and tiaprost to females did not result in secretion of any other prostaglandin such as $PGF_{2\alpha}$, PGE_1, PGE_2 or precursor arachidonic acid but some part of prosolvin and tiaprost respectively were released in water (Fig. 5.1, 5.2 and 5.3). Prosolvin and tiaprost when administered to female goldfish induced female sexual behaviour as well as elicited male sexual responses and increased milt volume (Jagtap *et al*., 2001). Part of injected prosolvin and tiaprost from females were released into water which was detected by High Performance Liquid Chromatography (HPLC).

Chapter VI : General Discussion

In this chapter the salient features of the thesis have been discussed with reference to the published literature on Indian major carps and goldfish induced breeding with prostaglandins. Conclusions drawn have been projected in general discussion at the end.

GENERAL INTRODUCTION

Fish form an important source of human diet as they provide proteins, fats and especially vitamins A and D. A special feature of fish is their content of vitamins -B, which are absent in plant food. Fish is a good source of calcium. Polyunsaturated fatty acids (PUFA) belonging to linolenic acid series (18:3) are normally present in fish. Fish oils are essential for the prevention of coronary heart diseases. Balanced ratios of ω_3 linolenic acid (18:3) and ω_2 linoleic acid (18:2) in fish flesh are found to be useful for maintaining a healthy heart (Crawford *et al*., 1989, Leaf and Weber, 1998). The most important fatty acid for human diet is linoleic acid (18:2n6) and linolenic acid (18:3n3), because they can not be endogenously synthesized (George and Ninawe, 2000).

In order to provide food to the growing population, agricultural production alone may not be sufficient. Since fish food is cheaper and rich in protein, this can possibly be exploited as an alternative. To do this the production of fish on a commercial basis has to be augmented, for which all out efforts are needed both from the research point of view as well as government support. The present fish eating population is about 56% with a per capita consumption of 9.5 kg/annum. To provide fish as an alternative to agricultural commodity, the production of fish in this country has to be doubled which calls for concerted efforts at all levels.

Status of Fish Production in India

India is the third largest producer of fish in the world and is second in inland fish production which contributes 45 per cent of total production in the country. The country's fish production was 28.34 and 17.89 lakhs tones respectively marine and inland fisheries sector in 2000. It has been projected that the marine resources potential is about 39 lakh tones and inland 45 lakhs tones. The production pattern of inland fisheries indicated a contribution of 54.7 per cent by major carps followed by common carp (8.1 per cent), other carps (6.1 per cent), murrels (3.6 per cent), hilsa (2.2 per cent) and other fishes (25.3 per cent). Thus carps emerge as prime species in the inland sector (George and Ninawe, 2000). During the year 1998–99, the country earned Rs. 4627 crores from this sector contributing 104 % of the total GPP.

The major species which have contributed to the production of freshwater are Indian major carps *viz*, rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus*

mrigala), Chinese carps *viz,* grass carp (*Ctenopharyngodon idella),* silver carp (*Hypophthalmicthys molitrix*), catfish (*Clarias batrachus*), climbing perch (*Anabas testudineus*), common carp *(Cyprinus carpio)*, rainbow trout, gaint freshwater prawn (*Macrobrachium rosenbergii*) and miscellaneous fish.

Indian major carps catla, rohu and mrigal are considered to be the best cultivable varieties among the other carps as they are the quickest growing in terms of flesh return, non-predatory and domesticated in nature and best compatible species.

The cultivated species of Indian major carps are riverine fishes which normally breed in rivers during the monsoon month from June to August. They do not normally breed in captivity. Fish seed, required for cultivation in ponds, consists of the eggs, hatchlings (spawns) and young fry which drift along the current of the water in rivers during floods. They are also collected from certain regions where they are found in large numbers. The extent of breeding of carps in rivers depends on the fluctuations in monsoon floods. Further breeding period of the fish is relatively short and as seed collection centers are very often situated in practically inaccessible and out of the way places, difficulty is often experienced in collecting the required quantity of fish seed. Besides, the fish seed collected from the riverine habitats is invariably a mixture of cultivable and non-cultivable species. Often the proportion of non-cultivable or uneconomic species ranges high in such collections. In the absence of any dependable method to segregate the cultivable fishes from the non-cultivable ones at the early stage, the fish farmers have no choice but rear them to an identifiable size when they could be separated. Thus the fish farmers have to take considerable chance in regard to procurement of fish seed. The rearing of appreciable number of unwanted fish involves wastage of valuable nursery space and expenditure. Thus there is need to induce Indian major carps to breed in confined waters thereby ensuring a dependable source of quality fish seed so as to increase the fish production.

On the other hand aquarium fish culture is being practiced all over the country to meet the domestic needs but not on commercial lines to meet the export demands. In spite of having the advantage of tropical climate and vast potential of aquatic resources,

it is said that India is contributing only one percent to the global trade in ornamental fish industry.

Ornamental fish are in greater demand due to their easy maintenance in houses, offices and commercial centers and the scope for their production on large-scale is increasing day-by-day. It is reported that Singapore earns more than Rs. 2000 crores from this trade, Sri Lanka is getting about Rs. 100 crores annually and India earnings are less than Rs. 25 crores (Devaraj, 2000). There is a constant demand for these beautiful ornamental fishes from European countries, America and Canada. The demand for ornamental fishes within and outside the country has been increasing rapidly during the recent years.

In India induced breeding of food fishes by using various inducing agents with well-established techniques is a routine method. However, these induced breeding techniques have not been applied to the ornamental fishes.

In view of the increasing demand both in domestic and export markets especially for freshwater ornamental fishes, if their culture is taken up scientifically and the induced breeding technique in ornamental fishes is explored to induce repeated (multiple) spawning, to reduce the gap between two spawning periods and enhance production, there is no doubt that it will be a boon to the national economy.

Till 1960s, the major part of carp seed required for culture was collected from riverine sources. Various spawning inducing agents are reported in fishes namely, fish pituitary extract (Houssay, 1930, 1931; Cardoso, 1934; Khan, 1938; Alikunhi *et al*., 1962), human chorionic gonadotropin (Chonder, 1985, 1986), mammalian pituitary hormone (Chonder, 1994), sex steroid hormones (Jalabert, 1976), thyroid hormones (Sneed and Dupree, 1961; Ramaswami and Lakshmanan, 1958), antiestrogens (non-steroidal compounds): clomiphene citrate, sexovid and tamoxifen as ovulating agents (Breton *et al*., 1975; Pandey and Hoar, 1972; Donaldson *et al*., 1978, 1981; Kapur, 1978), domperidone or pimozide, ovaprim (sGnRHA) (Peter *et al*., 1986; Peter *et al*., 1988), ovatide (Pandey *et al*., (2001) and pheromones (Resink *et al*., 1989; Scott *et al*., 1994; Zheng *et al*., 1995). Very few reports are available on the use of prostaglandins for spawning of carp species.

Prostaglandins are hormone-like substances derived from C_{20} fatty acids, a group of polyunsaturated, hydroxylated, long-chain fatty acids that evoke a wide spectrum of biological activity at extremely low concentrations in a variety of tissues. Prostaglandins are identified in gonads, semen, ovarian fluid and blood from variety of teleosts (Christ and van Dorp, 1972; Nomura *et al*., 1973; Ogata and Nomura, 1975; Nomura and Ogata 1976; Bouffard, 1979 and Coll *et al*., 1990). Unlike classical hormones, they are synthesized at the site of demand from fatty acid precursors and immediately inactivated after completing the role. Prostaglandins are produced from 5, 8, 11, 14, 17 – icosapentaenoic acid in marine animals and fish by the action of cyclooxygenase enzyme yielding the common endoperoxide intermediates prostaglandin-G and prostaglandin-H which undergo isomerisation or reduction by various enzymes in microsome (Christ and van Dorp, 1972; Mai *et al*., 1981).

Prostaglandins appear to be involved in ovulation, milt production, regulation of feeding, sexual behaviour, gonadotropin secretion, endocrine regulation, pheromone production and other non-reproductive physiology of fish (Stacey and Goetz, 1982; Stacey and Sorensen, 1988). All these functions are associated and necessary for breeding of fish in captivity as well as in field.

Majority of work on prostaglandin in relation to fish reproduction has been conducted in USA, Canada and Japan. However, natural prostaglandins in field especially in Indian aquaculture programme has not been studied so far. It is mainly due to their high cost and vulnerability of decomposition under variety of known and unknown conditions *viz.* their half-life in seconds, short shelf-life, storage at –70 °C and above all their non-availability in ready injectable form.

These are the major stumbling blocks in the path of the use of prostaglandins in aquaculture programmes to this country. No study has been conducted in this area to cope up with this problem neither in India nor abroad.

Therefore, there is a need to synthesize stable, long shelf-life prostaglandin analogues and study their efficacy in goldfish and Indian major carps breeding programmes and to develop alternative methods for the use of novel prostaglandins for fish breeding. This will certainly be a major breakthrough in widening prostaglandin applicability in aquaculture programmes.

Objectives of the present research work

1) Screening experiments conducted on goldfish for selection of promising prostaglandin analogues considering the high cost of prostaglandins.
2) Test the efficacy of prostaglandin analogues as inducing agents in the breeding programmes of Indian major carps and ornamental fish.
3) To study whether prostaglandin analogues also play a pheromonal role that can be applied in non-invasive technique of fish breeding.
4) Analysis of prostaglandins applied to female fish and their mechanism of action on reproductive behaviour and spawning by standard analytical methods like HPLC.

Carp species selected for the induced breeding experiments

Two major types of cyprinoids exhibiting two different breeding seasons in different habitats were selected,

1]. **Indian Major Carps:** (monsoon breeding riverine fishes)

Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigala*).

2]. **Goldfish (*Carassius auratus*)** (winter season)

Classification of Cyprinoids

Phylum	-	Vertebrata
Sub-phylum	-	Craniata
Super-class	-	Gnathostomata
Class	-	Teleostomi
Sub-class	-	Actinopterygi
Order	-	Cypriniforms
Sub-order	-	Cyprinoidei
Family	-	Cyprinidae

This thesis is divided into six chapters preceded by general introduction.

CHAPTER - I

REVIEW OF LITERATURE

In the freshwater sector, Indian major carps, catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) are considered to be the best cultivable species. They are riverine fishes which normally breed in rivers during the monsoon months from June to August. They do not normally breed in ponds. Fish required for cultivation in ponds consist of the eggs and hatchlings (spawn) and young fry. The fish seed collected from the riverine habitats are invariably a mixture of cultivable and non-cultivable or uneconomic species ranging high in the collection. The rearing of unwanted fishes involves wastage of valuable nursery space and expenditure. So the need to induce Indian major carps to breed in confined waters and thereby ensure a dependable source of quality fish seed has been greatly felt in India.

The technique of induced breeding which involves final gonadal maturation, ovulation, spermiation and spawning is being increasingly adapted since it was first developed to induce the Indian major carps, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* to spawn in confined waters (Chaudhuri and Alikunhi, 1957). Developments after 1970 have led to the use of "second generation technique" involving mammalian hormones, steroids and antiestrogens to spawn the cultured species for seed production. A number of reviews have recently been published which deal with induced spawning of economic species of teleosts (Chaudhuri and Tripathi, 1979; Lam, 1982; Donaldson and Hunter, 1983; Pandey *et al*., 1999).

Various chemical inducers are reported for economic fish breeding

1. Hypophysation technique
2. Human chorionic gonadotropin
3. Sex steroid hormones
4. Thyroid hormone
5. Antiestrogens (non-steroidal compounds): Clomid (Clomiphene citrate), Sexovid (Cyclofenil) and Tamoxifen as ovulating agents
6. Domperidone or Pimozide
7. Ovaprim (sGnRHA)
8. Ovatide

9. Pheromones
10. Prostaglandins
11. Indomethacin

Environmental control of reproductive activity of captive fish is feasible (or potentially feasible) but, with few exceptions, is currently impractical for most species. Therefore, chemical methods of manipulating reproductive activity continue to be widely used in fish production operations worldwide. Most research on the control of reproduction in fishes has focussed on female physiology because ovarian development and maturation are easily disturbed by environmental stresses (Patino, 1997).

Hypophysation technique

It is well known that the gonadotropin hormones (GtH-I, II) secreted by the pituitary gland play an important role in the maturation of the gonads and spawning in fishes. One of the earliest and most important success stories on the use of chemicals to manipulate fish reproduction was the development in the early 1930s of the technique known as hypophysation (Houssay, 1931).

Hypophysation technique consists of intramuscular injection of a suspension or extract of fish pituitary gland. This process elevates the level of sex hormones bringing about the maturation and shedding of the sex products in both male and female. Hypophysation as a mean of planned spawning and fry production of fishes has been widely used in Eastern Europe (Meske, 1985). Brazil was the first country to develop the technique of hypophysation.

Fish breeding by pituitary hormone injection is traced from the experiments of Houssay (1930) of Argentina who reported that the intraperitoneal injection of pituitary extract from the donor fish, *Prochilodus platensis* caused egg laying in the catfish, *Creserdon decemmaculatus*. Injection of fish pituitary gland hormones, a method of inducing breeding in fishes, is known from early thirties in Brazil (Cardoso, 1934). United States and Japan have successfully induced breeding in various species of fishes (Hasler *et al*., 1939, 1940; Kawajiri, 1946; Ball and Bacon, 1954).

The first successful attempt in inducing breeding of Indian major carps (*Labeo rohita* and *Cirrhinus mrigala*) and medium-sized carps (*Labeo bata, Cirrhinus reba and Puntius sarana*) by administering fish pituitary glands was made in India in the year 1935 by Chaudhuri and Alikhunhi, at the Central Inland Fisheries Research sub-station, Cuttack (Orissa). Khan (1938) was the first to successfully induce *Cirrhina mrigala* to spawn by injecting mammalian pituitary hormone. Chaudhuri (1955) succeeded in inducing *Esomus danricus* to spawn by intraperitonial injection of pituitary gland from catla, *Catla catla*. However, the major breakthrough achieved by Chaudhuri and Alikunhi in 1957 in induced breeding of Indian major carps using pituitary extract has greatly contributed for the rapid development of carp culture in India without having to depend mainly on the riverine collections. The hypophysation technique was later extended successfully for the breeding of silver carp and grass carp (Alikunhi *et al*., 1962).

Although, the technique of hypophysation is practiced throughout the country, there are certain inherent problems which have prevented it being taken up widely by fish farmers.

Problems of hypophysation technique

Varying potency of the pituitary gland results in unsuccessful spawning and this problem cannot be easily rectified since the farmer cannot measure the potency of the available gland. Pituitaries are to be collected at the right time and preserved properly for use. However, there are serious difficulties in large-scale collection and storage of pituitary glands owing to the limited period available for collection just before the commencement of the breeding season. There is a large gap between the supply and demand of pituitary. As a result, several fish breeders either use poor quality glands yielding unsatisfactory results or undertake only limited breeding operations due to lack of glands.

For preparations of pituitary extract basic equipments like a chemical balance and centrifuge are normally not available in several farms.

For achieving successful spawning, pituitary extract has to be administered necessarily in two split doses to female fish. This not only results in increased handling of brood fish often leading to spawning failure but also consumes considerable amount of labour and

time. Pituitary glands need to be stored in a refrigerator to avoid spoilage. However, several farms do not possess this equipment. Also pituitary extracts have poor shelf-life and are difficult to obtain. At a single large hatchery in China, as many as 80,000 carps are killed annually to provide extracts for brood stock spawning and thus a lot of fish have to be sacrificed (Gerry Porter, 1988).

Because of the above mentioned drawbacks, several fish farmers breed only common carp which does not require pituitary hormone treatment for breeding under tropical conditions. Dehadrai (1984) reported that only about 15% of the existing carp seed farms use the hypophysation technique for carps breeding. So there was need to develop alternative method to hypophysation technique of induced breeding.

Human chorionic gonadotropin (HCG)

HCG is a glycoprotein or siloprotein hormone composed of a protein core with several branched carbohydrate side chain terminating with sialic acid residues. Like fish pituitary gland HCG can be used for early ripening of ovaries and testes of Indian and Chinese major carps. The prescribed doses of HCG (activity 30 IU/mg) in the range of 4-5 mg/kg body weight for females and 2-4 mg/kg body weight for males are given every month from two or three months before the breeding season for early ripening of ovaries and testes of Indian and Chinese carps.

Chonder (1986) has described the technique of repeated breeding in Indian and Chinese major carps during the same spawning season by administering HCG injections. HCG primarily behaves as luteinising hormone. Although HCG alone has been found successful in inducing spawning in silver carp (Chondar, 1985), it has to be combined with fish pituitary for effective spawning in case of Indian major carps. However, HCG can cause immunoreactions in the recipient fish thus reducing or even eliminating the effect of the hormone in subsequent injections to the individuals of the same brood stock.

The fish breeds if the treatment is done during spawning period and right dose of HCG may vary from 100-2000 IU/kg body weight of fish. Much higher doses are generally required for catfish breeding while 100-1100 IU/kg body weight is optimum for carp breeding.

The mammalian gonadotropins have got an edge over pituitary because of their easy availability, low cost, long shelf-life and uniform gonadotropin potency. Of the various mammalian gonadotropins, HCG has given the best results. Khoo reports acceleration of vitellogenesis in goldfish by a single intramuscular implantation of HCG-cholesterol pellet (Khoo, 1980).

HCG alone was ineffective except when combined with carp pituitary homogenate in hypophysectomized goldfish (Yamazaki and Donaldson, 1968). HCG alone or in combination with fish pituitary induced spawning in silver carp and rohu (Bhowmick, 1979; Dave and Sukumaran, 1984) and grass carp and big-head (Tajudalin, 1978).

Mammalian pituitary hormones

Experiments with purified mammalian pituitary hormones in inducing spawning in hypophysectomised fish showed that the LH is very potent in ovulation and spawning in female. LH is also found to be effective in spermiation in male fish. On the other hand, FSH shows marginally positive results in case its LH contamination is taken into account. The thyroid stimulating hormone (TSH) and growth hormone (STH) are reported to be totally inert to fish spawning. Chonder (1994) reported that Syanhorin (a mixture of HCG and mammalian pituitary extract) has been used in induced breeding of Indian and Chinese carps.

Sex steroid hormones

Steroid hormones show limited success in fish breeding. Among the gonadal steroids, those used in fish breeding are: methyl testosterone, oestradial benzoate, testosterone propionate and progesterone. This may be due to conversion of progesterone *in vivo* into corticosteriods which are potent ovulating agents. It is reported that both male and female sex steroids, when administered over longer periods, bring about regressive changes in the ovaries by virtue of their feedback effect on the pituitary of the recipient (Sundararaj and Goswami, 1968). Of the various corticosteriods, the mineralocorticosteroids are far more potent than glucocorticosteroids. Deoxycorticosterone acetate (DOCA), 17-hydroxycorticosterone, corticosterone,

11-dehydrocorticosterone and 17-hydroxy-11-deoxycorticosterone are a few of the mineral adrenocorticosteroids and the prednisone, prednisolone, cortisone and hydrocortisone of the gluco-corticosteroids experimentally used in inducing ovulation and spawning in fishes. The action of gonadotropin at the ovarian level is largely mediated by the sex steroid hormones (Jalabert, 1976) which have been also used to induce maturation and ovulation in fishes.

Thyroid hormones

Thyroid hormones are known to play a role in oocyte maturation while thyroid coincides with gonadal maturation. Sneed and Dupree (1961) and Ramaswami and Lakshmanan (1958) reported that thyroid stimulating hormones (TSH) enhances the effect of HCG or fish pituitary extract in induction of ovulation in goldfish and bring about ovulation in Indian catfish.

Antiestrogens (non-steroidal compounds)

Antiestrogens are synthetic compounds that are capable of competing with estrogen for binding sites on estrogen receptors (Donaldson and Hunter, 1983). Two of these non-steroidal compounds *viz*. Clomiphene citrate and Tamoxifen have been used for inducing ovulation. Breton *et al*. (1975) showed that clomiphene citrate induced GtH release in carp. Clomiphene citrate when implanted in goldfish increased plasma gonadotropin levels which could be considered for induction of ovulation in fish (Pandey and Hoar, 1972). An optimum dose of clomiphene citrate sufficient to induce ovulation in relation to gonadal maturation of the fish is yet to be worked out and this may be the cause of failure of ovulation in several species. Kapur and Toor (1979) obtained successful spawning in common carp 40-60 hours after an injection of clomid at the rate of 10 mg/kg body weight. The feasibility of inducing final maturation and ovulation with antiestrogen tamoxifen which holds a greater promise than clomiphene citrate was examined by Donaldson *et al*. (1978, 1981) but research is needed to optimize its dosage, timing, administration route etc. While Sexovid (Cyclofenil) induced ovulation in *Labeo rohita* (Kapur, 1978), it failed in goldfish. These results indicate that excessive dosages of antiestrogens inhibit gonadotropin secretion but may work better when vitellogenesis is

complete and blood estrogens are high, however, not in fish showing the migratory nucleus stage and germinal vesicle break down when blood estrogen drop to a low level.

Domperidone or Pimozide (Dopamine antagonist)

Peter *et al*. (1986) found a major breakthrough in fish breeding research that dopamine acts as an inhibitory factor for synthesis of gonadotropins. GnRH stimulates GtH secretion while dopamine inhibits the action of GnRH. Detailed investigations on the reasons for the spawning failures when LHRH alone was used clearly indicated that dopamine interferes with the action of the LHRH on the secretion of gonadotropins. Thus blocking of dopamine action with some antagonists like domperidone or pimozide potentiates the action of LHRH resulting in successful spawning (Billard *et al*., 1983; Chang and Peter, 1983).

Ovaprim (sGnRHA)

Ovaprim is a synthetic drug (spawning hormone for fish) manufactured by M/s Syndel Laboratories Ltd., Canada, containing 20 µg sGnRH (salmon gonadotropin releasing hormone) and 10 mg domperidone in 1 ml solution.

A number of studies conducted in mammalian species have shown the neuroendocrine control of secretion of gonadal hormones. Lutinising hormone-releasing hormone (LHRH), a mammalian hypothalmic peptide, has the capacity to release gonadotropins [Lutinising hormone (LH) and Follicle stimulating hormone (FSH)] from the pituitary gland (Porter *et al*., 1980). Specific LH and FSH activity has not yet been identified in teleosts. A series of investigations conducted in China under major project funded by the International Development Research Center, Canada to Dr. R. E. Peter (Canada) and Dr. H. R. Lin (China) conclusively proved that a combination of LHRH analogue with a dopamine antagonist could be effectively used as a substitute to fish pituitary for carp breeding. These scientists named the new technique of fish breeding with above combinations as "Linpe method'' (Peter *et al*., 1988). Another significant finding is the isolation and characterization of gonadotropin releasing hormone from salmon (Sherwood *et al*., 1983). The structure of salmon releasing hormone is different from that

of mammalian releasing hormone in respect of two amino acid positions and has been shown to be more effective (Sherwood *et al*., 1983).

The first successful claim in using [D-Ala^{6}, Pro^{9}, Net] - luteinizing hormone-releasing hormone analog [LHRH-A] for induction of ovulation and spawning of cultured carp came from researchers in China (Cooperative Team for Hormonal Application in Pisciculture, 1977; Fukien-Kiangsu-Chekian-Shanghai Cooperative Group, 1977; Fish Reproductive Physiology Research Group and Peptide Hormone Group, 1978). This success immediately gained international attention and LHRH-A has been successfully used for maturation and spawning of various fish including coho salmon (van der Kraak *et al*., 1983), rainbow trout (Crim *et al*., 1983), Atlantic salmon (Crim and Glebe, 1984), seabass and rabbit fish (Harvey *et al*., 1985) and milkfish (Marte *et al*., 1987). Others have shown that secretion of gonadotropin in teleosts is regulated by a dual neurohormonal system, GnRH stimulates GtH secretion while dopamine inhibits the action of GnRH and thus using drugs which block dopamine action in combination with LHRH-A gave better results in ovulation (Peter, 1982; Lin *et al*., 1985; Sokolowska *et al*., 1985; Lin *et al*., 1986).

Peter *et al*. (1986) reported that among the various analogues of salmon releasing hormone, D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9} Net has been found to be highly effective and its particular analogue is used in Ovaprim. It has been shown to be 17 times more potent than the LHRHA.

Ovatide

Ovatide is a synthetic hormone developed for breeding of carps, catfish, mahaseer etc. It is a mixture of a sGnRH-A and domperidone. It came in market in the year 1998-99. This is an outcome of a collaborative research effort of Central Institute of Fisheries Education and M/s Hemmo Pharma, Mumbai (Pandey *et al*., 2001).

There are two basic delivery systems for spawning inducing hormones those that produce acute (bolus) target tissue exposures to the hormone and those that yield a more sustained or chronic exposure. Currently GnRH (and GtH) is most commonly delivered by bolus injections into the fish. However, in many species multiple injections of

hormones (two or more) are usually necessary to achieve satisfactory spawning results (Zohar, 1989). In this regard it seems likely that the first (priming) injection is needed to induce final differentiation of the ovarian follicle including the acquisition of maturational competence and that subsequent (resolving) injections induce maturation inducing steroid (MIS) production and the consequent maturation and ovulation of oocyte (Patino and Thomas, 1990). Even in cases where maturational competence is not of concern as the target species normally spawns several times over a prolonged breeding period then multiple injections may still be necessary to achieve full spawning potential.

A major drawback of multiple hormone injections is that some species are unable to cope up with the stress associated with repeated handling. Handling stress can cause gonadal regression and in some cases even death of the broodstock. One way to avoid excessive handling of the broodstock is to apply non-invasive techniques like using bolus-feeding strategies and also adding reproductive pheromones directly into the water. For example, dietary administration of GnRH analogues has been used successfully to induce spawning of spotted seatrout, *Cynoscion nebulosus* (Thomas and Boyd, 1989) and oral delivery of GnRH analogues also seemed to be effective in common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss)* and the African catfish (C*larias gariepinus*) (Breton *et al*., 1995). Also, feeding a diet containing GnRH analogue to spotted seatrout at 3-5 week intervals resulted in multiple spawning over their natural breeding period without any noticeable declines in fecundity, fertility or embryo survival (P. Thomas *et al*., University of Texas Marine Science Institute, unpublished data). The hormone feeding technique minimizes the handling of fish although the applicability of this technique to commercial aquaculture needs further study.

Pheromones

Another interesting possibility for the chemical control of maturation and spawning in fish concerns the use of reproductive pheromones. The interaction can be between individuals of the same species in which case they are known as pheromones (intraspecific semiochemicals). Chemical signaling systems are found in many phyla and involve many different chemicals. The reception of these signals takes place either by

olfaction or gustation (tasting). These chemical secretions, steroid or lipid in nature, may be important in species, sex or individual recognition (Liley, 1982).

There exist numerous reports on the sex pheromones among insects (Mustaparta and Almaas, 1989). Also in mammals sex odours are important. Some of these odours induce an immediate behavioural response via neural pathway (releaser effect) or have physiological effects through neural-neuroendocrine pathway (primer effect) by enhancing plasma concentrations of sex hormones followed by sexual maturity and ovulation (Wilson, 1975; Albone, 1984). However, very little is known about sex odours and their effects in fish. For the example, steroid dihydroxyprogesterone (DHP) produced by female goldfish controls not only the onset of ovarian maturation in females but also spermeation in males. Therefore, if added to water, this steroid could be used to control the timing or amount of sperm production. Indeed, preliminary tests have confirmed the feasibility of this procedure to increase male fertility in common carp and goldfish (Zheng *et al*., 1995).

There exist reports that chemicals released by one fish can alter the behaviour and physiology of the conspecifics. Majority of chemicals belonging to this category are (i) steroids (Resink *et al*., 1989; Scott *et al*., 1994) (ii) bile salts (tauroholic acid) and (iii) amino acids L-amino acid, L-serine etc. (Sorensen *et al*., 1991b).

Many studies have demonstrated that males and females of teleosts release pheromones that affect sexual behaviours and reproductive physiology of conspecifics (Liley, 1982; Stacey *et al*., 1986). Most reported sex pheromones are releasers eliciting more or less immediate behavioural changes in conspecifics. However, primer effect (physiological changes) such as the induction of ovulation in female zebra fish (*Brachydanio rerio*) by male odour have also been documented (Chen and Martinich, 1975).

With a few exceptions (*Blennius pavo*; Laumen *et al*., 1974), sex pheromones appear to originate from the gonads although whether they are released via urine or gonoducts has not been determined (Colombo *et al*, 1982). Behavioural assays of gonadal functions have led to a variety of proposals regarding the chemical nature of fish pheromones: ether-soluble substance(s) in goldfish, *Carassius auratus* (Partridge *et al*., 1976), protein pond smelt, *Hypomesus olidus* (Okada *et al*., 1978) and water-soluble

basic substance in ayu, *Plecoglossus altivelis* (Honda, 1980b). However, both cases offering good evidence for the chemical identity of fish sex pheromone suggest that it is a steroid glucuronide. In the black goby, *Gobius jozo*, Colombo *et al*. (1982) have shown that etiocholanolone glucuronide, synthesized by the mesorchial gland of the testis, attracts female and in some cases induces females to oviposit. Colombo *et al*. (1982) have also suggested that etiocholanolone glucuronide functions as an attractant for male goldfish. In *Brachydanio rerio*, males are attracted both to water-soluble extracts of ovulated ovaries and to a sub-fraction of the extract containing steroid glucuronides whereas females show no response (van den Hurk and Lambert, 1983). Results of the attraction tests using synthetic glucuronides of estradiol, testosterone and estrone suggest that the ovulated females of zebra fish attract the male by releasing a mixture of steroid glucuronides among which are glucuronides of estradiol and testosterone.

The male pheromones may be present in the urinogenital fluid caudal gland appendices of the anal fin spines or anal secretary pads. In female fishes the sexual pheromones may be present in the fluids released from the ovaries at the time of ovulation. It is possible that an endocrine involvement exists which regulates the production and release of pheromones.

Though no report on the effects of pheromones on the reproduction of Indian major carps is available, there are circumstantial evidences which suggest that pheromones secreted by Indian major carps help in effecting spawning (Ranganathan *et al*., 1967). Similarly, spawning in bundhs where few male and female brooders are injected with pituitary gland extract and released along with non-injected ones, appears to be due to the release of pheromones (Moitra and Sarkar, 1975). However, more research is necessary before the applicability of this procedure to commercial aquaculture can be properly evaluated.

Prostaglandins

Prostaglandins (PGs) are a group of hormone-like substances derived from C_{20} fatty acids, a group of polyunsaturated, hydroxylated, long-chain fatty acids that evoke a wide spectrum of biological activity at extremely low concentrations in a variety of tissues. All of the prostaglandins have in common the prostanoic acid skeleton. The credit

for the discovery of prostaglandins (PGs) goes to the Swedish scientist von Euler (1934). He established beyond doubt that the active principle, which he named prostaglandin, belongs to a completely new group of naturally-occurring substances. von Euler coined the term prostaglandin in the belief that the biologically active substance first found in human semen was a secretion of the prostrate gland. First two prostaglandins were isolated in 1960s and a vast new field of chemical, biological and clinical importance was opened up.

von Euler observed that human semen and extracts of sheep vesicular glands lower arterial blood pressure on intravenous injection and stimulate various isolated intestinal and uterine smooth muscles preparations. He showed that the active principle, prostaglandin was a lipid soluble acid and thus differed chemically from all other known substances with similar biological activity, for example, histamine, acetylcholine and adenylic compounds (von Euler, 1936).

Bergstrom and Sjovall (1960) isolated two compounds which behaved differently on partition between ether and an aqueous phosphate buffer. Those more soluble in ether was called prostaglandin-E (PGE) while the others more soluble in phosphate buffer (in Swedish spelt with an F) was called prostaglandin-F (PGF). These compounds were assigned the empirical formula $C_{20}H_{34}O_5$ and $C_{20}H_{36}O_5$, respectively. In 1962 and 1963, Bergstrom announced the chemical structure of several naturally-occurring prostaglandins. Since then research in this field has advanced rapidly.

Several natural prostaglandins and synthetic analogues are now being used as drugs. Some difficulties involved in their therapeutic application include scare natural occurrence, rapid inactivation by enzymatic degradation and non-selective widespread action on most tissues and cells.

A pharmacological activity has been found in the intestine (Suzuki and Vogt, 1965) and then identified as a mixture of prostaglandin-$F_{1\alpha}$ and prostaglandin-E_1 (Vogt *et al*., 1966). Prostaglandins have been also found in all gastrointestinal tissues of man (Bennet, 1968), rat (Coceani *et al*., 1967; Bennet *et al*., 1967), pig (Miyazaki, 1968) and other laboratory animals (Bennet and Fleshler, 1970). Bito (1972) reported that the uptake of (^{3}H) prostaglandin-$F_{2\alpha}$ was not evident in the gut of teleosts, no comparable chemical report on the digestive organs of fish is available. In addition, a prostaglandin-like

compound showing pharmacological activity on the isolated jejunum from a rat, is present in the gastrointestinal extracts from chum salmon. The finding of Christ and van Dorp (1972) also strengthens the results presented here that prostaglandin synthetase activity was found in lobster and carp intestines. From the occurrence of prostaglandins in lower vertebrates, it is thought that prostaglandins have some physiological significance in the smooth muscle motility of lower as well as higher vertebrates.

Prostaglandins are synthesized from certain C_{20} fatty acids (van Dorp *et al*., 1964; Bergstrom, 1964; van Dorp, 1969; Samuelsson, 1973). The amount of each prostaglandin may be related to the composition of the precursor fatty acids. A similar interpretation has been made by other investigators (Daniels *et al*., 1967; Lands and Samelsson, 1968; Vonkeman and van Dorp, 1968). On the other hand, Christ and van Dorp (1972) pointed out that the presence of arachidonic acid in a tissue is not always accompanied by prostaglandin biosynthesis in a tissue (Kunze and Vogt, 1971). Prostaglandins are formed from unsaturated C_{20} fatty acids providing the first double bond of the methylene chain located at ω6, (Struijk *et al*., 1966). Thus, PGE_1 and $PGF_{1\alpha}$ have been biosynthesized from C-20: 3 (homo-γ-lonolenic acid), PGE_2 and $PGF_{2\alpha}$ from C-20: 4 (arachidonic acid) and PGE_3 from C-20: 5 (Ramwell *et al*., 1968).

Prostaglandin synthetase is exclusively present in the microsomes of all mammalian cells. Most tissues release prostaglandins into perfusates or superfusates, especially after nervous or humoral stimulation (Ramwell and Shaw, 1967). The remarkable effects of prostaglandins on cyclic AMP formation and the implication of this nucleotide as an intracellular intermediate in the response of tissues to hormone suggests that the prostaglandins are involved in regulating hormone action (Bergstrom, 1967; Ramwell and Shaw, 1967).

In contrast to hormones, prostaglandins (PGs) do not circulate nor are they stored in tissues. Prostaglandins play important regulatory roles in many normal cellular functions. Rather they are synthesized locally on demand, perform a tissue-specific function and then are rapidly inactivated by metabolic enzymes.

Work on prostaglandins to fish reproduction was started in early 1970. Biosynthesis does not meet the increasing demand for prostaglandins and is also in

appropriate for production of medicinally more cultivated compounds possessing the desired tissue selectivity and higher metabolic stability. A supply of sufficient quantities of natural prostaglandins and artificial analogues relies on efficient and flexible chemical synthesis. However, no study for the isolation, identification and purification of PGs from economically important Indian food fishes has been conducted so far.

Biological significance of prostaglandins

Prostaglandins are known to be widely distributed in various species and tissues of mammals but little information is available for lower animals. Initially human seminal fluid was found to contain considerable amounts of several classical prostaglandins (e.g. PGE_1, PGE_2, PGE_3, $PGF_{1\alpha}$ and $PGF_{2\alpha}$), which are essential for normal fertility (Asplund, 1947; Hawkins and Labrum, 1961).

Among the different prostaglandin compounds isolated and identified from human tissues, it is mainly PGE_2 and $PGF_{2\alpha}$ that have been implicated in the physiological regulation of different events in the reproductive organs in human (e.g. male fertility, tubule contractility and non-pregnant and pregnant uterine contractility). The modification or stimulation of reproductive events by exogenous prostaglandins has been a major area of research. Human males who were infertile for no apparent reason possessed significantly lower concentrations of seminal prostaglandins, especially prostaglandin-E, than man of normal fertility.

The ovulation process includes the sequential events of ovum maturation, follicular rupture and development of corpus luteum. The prostaglandins appear to be deeply involved in these processes in sub-primates. Investigations from a number of laboratories have indicated that PGE_2 and $PGF_{2\alpha}$ are involved in the normal processes of ovulation in the rat and rabbit (Armstrong, 1974).

Cloprostenol and Flurostenol are phenoxyprostaglandins introduced into the United Kingdom in 1975 and 1976, respectively, by ICI as veterinary luteolytics for synchronization of estrous cycle in animals and for treatment of infertility in horses.

Prostaglandins have been examined for a variety of clinical indications, the predominant use of E-type prostaglandins has been for peptic ulcer and cardiovascular diseases and F-type prostaglandins for gynecological or fertility control applications. Prostaglandins have found limited utility in human reproductive indications and are more extensively used for farm animal estrous synchronization.

Apart from the role of prostaglandins in human reproduction, $PGF_{2\alpha}$ and its analogues are now used for the treatment of reproductive difficulties in live stocks and for the synchronization of ovulation in breeding management.

Recent information about prostaglandins in gonadal histology has now been extended from a variety of vertebrates to the maturation or spawning of marine invertebrates.

Suzuki and Vogt (1965) reported that so called “darmstoff” from frog intestine was identical to prostaglandins and remarkably high concentration of prostaglandin isomers were found in gorgorian, *Plexaura homomalla* by Weinheimer and Spraggins (1969) and other investigators (Schneider *et al*., 1972; Bundey *et al*., 1972; Light and Samuelsson, 1972). Christ and van Dorp (1972) reported the comparative aspects of prostaglandin biosynthesis in the various animals. The toxic effects of prostaglandin-$F_{2\alpha}$ against sea urchin embryos was observed by Persianino *et al*. (1973). Bito (1972) investigated the accumulation of ^{3}H-labelled prostaglandins in certain tissues of marine animals. Abramowitz and Chavin (1973) reported the relative effectiveness of the various prostaglandins in teleosts using the dermal melanophores of the black goldfish. Nomura *et al*. (1973) reported that prostaglandins exist in the testes and semen of teleosts.

Prostaglandins and fish reproduction

Prostaglandins (PGs) are identified in gonads, semen, ovarian fluid and blood from variety of teleosts. Prostaglandins (PGs) are widely distributed in animals (Christ and van Dorp, 1972; Nomura *et al*., 1973; Ogata and Nomura, 1975; Nomura and Ogata, 1976; Coll *et al*., 1990). Prostaglandins are also found in plant kingdom (Colbert, 1973; Yurin, 1990; Hayashi *et al*., 1993). Prostaglandins are involved in a variety of physiological processes. Unlike classical hormones they are synthesized at the site of demand from fatty acid precursors and immediately inactivated after completing the role.

Prostaglandins are produced from 5, 8, 11, 14, 17- icosapentaenoic acid in marine animals and fish, 8, 11, 14 -icosatrienoic acid or 5, 8, 11, 14 -icosatetraenoic acid (arachidonic acid) in mammals by the action of cylooxygenase enzyme yielding the common endoperoxide intermediates prostaglandin-G and prostaglandin-H which undergo isomerisation or reduction by various enzymes in microsome.

The biosynthesis of prostaglandins is known to depend on the prior synthesis of prostaglandin endoperoxides. Prostaglandin endoperoxides are the direct precursors of both the prostaglandins and physiologically potent thromboxanes; the endoperoxides themselves may also be physiologically active (van der Kraak and Chang, 1990). As aspirin (acetylsalicylic acid) is a well-characterized inhibitor of the fatty acid cyclooxygenase-catalyzed first step in the conversion of arachidonic acid to prostaglandin endoperoxide (van der Kraak, 1990).

Similar to mammals, arachidonic acid is precursor of prostaglandins in fish. However, reports also indicate that prostaglandins of 3rd series are the major component of prostaglandin synthesis in fish because of the presence of large quantities of icosapentaenoic acid (Christ and van Dorp, 1972). Biosynthesis of C_{22}- $PGF_{4\propto}$ from docosahexaenoic acid by trout gill is also reported (Mai *et al*., 1981). During the past 25 years there has been a marked expansion in the knowledge of the role of prostaglandins in mammalian and non-mammalian reproduction (Colbert, 1973; Guillette *et al*., 1991). Although in mid 1970s prostaglandin distribution in non-mammalian organisms (Nomura and Ogata, 1976) and especially in fish gill (Christ and van Dorp, 1972) and testes (Nomura *et al*., 1973) was proved, the majority of research work was categorically confined to the area of prostaglandins involvement only in mammalian reproduction all over the world up to late 1970s (Behrman, 1979; Thorburn, 1979; Espey, 1980).

It was beginning of 1980s when prostaglandin involvement in fish reproduction expanded dramatically all over the world (Stacey and Goetz, 1982; Stacey and Sorensen, 1988) and is still expanding taking into account various aspects of their mode of action in captivity. Role of prostaglandin-$F_{2\alpha}$ in inducing reproductive physiology of fish is well-known. Inspite of worldwide attention, majority of work on prostaglandin in relation to

fish reproduction was conducted in USA, Canada and Japan. However, very little is known about involvement of prostaglandins in the reproduction of Indian food fishes.

Prostaglandin as a fish pheromone

It was the beginning of 1986 when $PGF_{2\alpha}$ was believed to function as a pheromone in goldfish (Stacey *et al.*, 1986). It was further emphasized that $PGF_{2\alpha}$ either stimulates release of a pheromone or itself functions as a pheromone after being metabolized into an active form (Stacey *et al.*, 1986). Variety of prostaglandins were tested as olfactory stimulants with the help of Electro Olfactogram (EOG) (Sorensen *et al.*, 1988) and most potent were found to be $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$, a $PGF_{2\alpha}$ identified in goldfish (Goetz *et al.*, 1987). Stacey and Sorensen (1988) concluded that goldfish commonly use sex hormones (may be prostaglandins) and their metabolites as sex pheromones in two possible ways. Firstly, at the time of ovulation sexually receptive female goldfish releases two $PGF_{2\alpha}$ metabolites, one resembling to $PGF_{2\alpha}$ and other to 15-keto-$PGF_{2\alpha}$ (15k-$PGF_{2\alpha}$) in water and secondly this waterborne $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ stimulate male goldfish sexual behaviour. Therefore, $PGF_{2\alpha}$ first acts as hormone within the body of female to stimulate female sexual behaviour and its metabolite later acts as pheromone in water to stimulate male sexual behaviour (Stacey and Sorensen, 1988; Sorensen *et al*; 1988, 1989b; Sorensen and Stacey, 1990; Sorensen *et al.*, 1991a, b, 1992), possibly, through olfactory system (Sorensen *et al.*, 1989a; Fujita *et al.*, 1991). Olfactory responses to F-type prostaglandins in male loach, *Misgurnus anguillicaudatus* (Kitamura and Ogata, 1990, 1993), crucian carp (*Carassius carassius*) goldfish (*Carassius auratus*) (Bjerselius and Olsen, 1993; Cardwell *et al*; 1995), cyprinid, *Puntius schwanenfeldi* (Cardwell *et al.*, 1995) and other teleosts are also studied.

The cobitide loach releases $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ into water which act as male sex pheromones. This strongly suggests that cobitide loach use F-type PGs as releaser sex pheromones. Detection thresholds were 10^{-10} M for $PGF_{2\alpha}$ and 10^{-13} M for both 15-keto- $PGF_{2\alpha}$ and $PGF_{2\alpha}$ - MPM (Nikko *et al.*, 1994).

Prostaglandin-E and prostaglandin-F both known to mediate various physiological reactions which in turn regulate ovulation, fertilization, pregnancy and birth in humans

and other species (Veldhuis and Demers, 1987) are occasionally effective in inducing spawning in both male and female abalones.

It has also been hypothesized that circulating prostaglandins-F cleared to water function as a pheromone, thereby synchronizing male and female sexual behaviour (Sorensen *et al*., 1988). $PGF_{2\alpha}$ – injected female goldfish consistently released small quantities of $PGF_{2\alpha}$ as well as metabolites of this compound. One of these metabolites was 15k-$PGF_{2\alpha}$ but the identities of others were unknown. This study established that prostaglandin $PGF_{2\alpha}$ is a behavioural hormone and pheromone in the goldfish. Thus, it seems reasonable to conclude that $PGF_{2\alpha}$ functions as a hormone to directly stimulate female sexual activity. On the basis of literature it seems likely that many other species of fish, particularly the cypriniformes, use prostaglandins as both hormones and pheromones.

However, up to date maximum information regarding hormonal and pheromonal aspect of prostaglandins available is only on goldfish (*Carassius auratus*) and no generality of goldfish can be assessed or implicated until more species are investigated.

Prostaglandins and reproductive behaviour

In fish a variety of reproductive behaviours (Keenleyside, 1979; Balon, 1981) are governed by hormones (Stacey, 1981). Prostaglandins (PGs) as a chemical mediator also influence reproductive behaviour in several male and female teleosts. It is believed that the brief duration of PGs induced spawning behaviour is likely due to rapid prostaglandin synthesis and metabolism (Stacey, 1984).

Female reproductive behaviour

Indomethacin (prostaglandin synthesis inhibitor) completely blocks the sexual behaviour both in ovulated and non-ovulated females (which have been injected with ovulated oocytes). Prostaglandin injection rapidly overcomes the inhibitory effect of indomethacin (Stacey, 1976). Mechanism of PGs induced spawning behaviour in fish is not clear, however, several results on goldfish suggest that prostaglandin synthesized by the ovaries or mature oocytes, first enters the body circulation and secondly acts on brain to trigger spawning behaviour (Stacey, 1981; Goetz, 1983). Several findings indicate that

the level of spawning activity in female goldfish is a function of circulating prostaglandin levels. There is a positive correlation between the dosage of injected $PGF_{2\alpha}$ and the duration and intensity of spawning behaviour (Stacey, 1981). Frequency of spawning behaviour in goldfish is faster soon after injection and then becomes slow.

Earlier Stacey and Peter (1979) speculated that since in goldfish fertilization is external, the ovulation necessarily precedes sexual behaviour and prostaglandins released by the ovary in response to the presence of intra-ovarian ovulated eggs act on the brain by stimulating neural afferent which may function to synchronize spawning behaviour in goldfish.

Male reproductive behaviour

Patridge *et al*. (1976) showed that male goldfish placed with one ovulated and one unovulated female spends more time courting with the ovulated fish. Stacey (1981) confirmed this and demonstrated that male goldfish preferentially court PG-treated females and this preference indicates PG-mediated courtship behaviour in male. In goldfish, normally, females are receptive to male courtship and spawn only when ovulated (Stacey and Peter, 1979) as well as males can be induced by intramuscular injection of $PGF_{2\alpha}$ (Stacey, 1981). Male courtship behaviour in goldfish was stimulated with the release of a pheromone by the females (Patridge *et al*., 1976; Sorensen *et al*., 1986) which was later identified as $PGF_{2\alpha}$ (Sorensen *et al*., 1988).

Prostaglandins and gonadotropin secretion

In fish there may be one or two types of gonadotropins (GtHs). Circulatory level of GtH increases during gonadal development and maturation. Gonadotropin regulates ovarian and testicular function by inducing an exceptional steroid hormone which is 17∝, 20β-dihydroxy-4-pregnen-3-one (17∝, 20β-P). However, there appears to be a shift in GtH function, it induces synthesis and secretion of estradiol-17β during previtellogeic phase which in turn induces vitellogenesis or yolk protein synthesis while during post-vitellogenic phase GtH triggers the synthesis of 17∝, 20β-dihydroxy-4-pregnen-3-one which is responsible for final maturation leading to ovulation and spermiation (Patino, 1997).

There are a few published accounts of prostaglandin's effects on GtH secretion in fish. Peter and Billard (1976) found that injection of $PGF_{2\alpha}$ or PGE_2 but not PGE_1 into the third ventricle of the brain decreased immunoreactive serum GtH 30 min post-injection in mature female goldfish. In contrast, Singh and Singh (1976) have shown that daily intraperitoneal injection of $PGF_{2\alpha}$ or PGE_1 induces ovulation within 2-6 days in the catfish, *Heteropneustus fossilis*. As hypophysectomy blocks this ovulatory response and PGs stimulated serum and pituitary GtH activity (measured by ovarian P bioassay in hypophysectomised *Heteropneustus fossilis*), they suggested that prostaglandin exerts this ovulatory action by stimulating GtH release.

In guppy, *Poecilia reticulata* basal production of PGE and PGF was low in follicle during oocyte growth, maturation and early gestation, however, it increased in mid-and late- gestation and declined at post-parturition (Venkatesh *et al*., 1992). Gravid un-injected female silver carp had very high ovarian PGE_2 and $PGF_{2\alpha}$ levels. In contrast females that had ovulated 80-100% of their oocytes were assayed from 0-25 h, before, during and post-ovulation had significantly decreased PGE_2 levels compared to the levels in gravid controls. Ovarian $PGF_{2\alpha}$ levels did not change significantly in any group of fish. It is conceivable that the progressive decrease in PGE_2 might actually determine the time at which ovulation and spawning occur. There is considerable evidence that PGs are involved in ovulation and spawning of domesticated fish. Level of ovarian PGE_2 and $PGF_{2\alpha}$ in the ovarian tissue of silver carp have been measured by Radioimmunoassay (RIA) before, during and after ovulation. Results suggested that PGs (especially PGE_2) may affect GtH secretion in silver carp by an action on hypothalamus pituitary system (Liu *et al*., 1992).

Prostaglandins and fish ovulation

Prostaglandins (PGs) have been shown to play an important role in gonadotropin-induced ovulation in mammals, birds and fishes (Jones *et al*., 1990). They are known to be involved in ovulation in several fishes (Stacey and Goetz, 1982; Jones, 1987; Goetz *et al*., 1989). Of various prostaglandins tested, ovulatory action of $PGF_{2\alpha}$ was found to be most effective both *in vivo* and *in vitro* in a number of teleosts (Stacey and Goetz, 1982). In yellow perch, *Perca flavescens* (Goetz and Theofan, 1979), common carp, *Cyprinus*

carpio (Epler *et al*., 1985) and goldeye, *Hoidon alosoides* (Pankhurst, 1985) both PGE_2 and $PGF_{2\propto}$ have been shown to induce ovulation *in vitro*. Goetz and Theofan (1979) observed that PGE_1, PGE_2 and $PGF_{2\propto}$ all induced ovulation in the indomethacin-blocked follicles but PGE_2 was most effective.

Other *in vitro* studies indicate that $PGF_{2\propto}$ is involved in follicular rupture in rainbow trout, *Salmo gairdneri* but PGE_2 was ineffective (Jalabert and Szollosi, 1975; Kagawa and Nagahama, 1981). *In vitro* studies in goldfish, *Carassius auratus* have shown that indomethacin, an anti-inflammatory agent which blocks PGs synthesis from dihomo-Y-linolenic acid and arachidonic acid inhibits ovulation and this inhibition can be overridden by administration of exogenous prostaglandin-E_1, prostaglandin-E_2 and prostaglandin-$F_{2\propto}$ (Stacey and Panday, 1975).

Prostaglandins and milt production

In comparison to female teleosts, very little is known regarding prostaglandin involvement in milt production / spermiation in males.

Indomethacin

Most of the evidence regarding the role of prostagalndins in bringing about ovulation in animals comes from the use of inhibitors of prostaglandin biosynthesis. Indomethacin is a prostaglandin biosynthesis inhibitor. Most of the workers, however, support a direct action of indomethacin on ovary since LH failed to restore ovulation in treated rats (Tsafriri *et al*., 1972). Some workers have also presented evidence in favour of inhibitory effect of indomethacin at ovarian level (Labhetswar, 1974; Stacey and Pandey, 1975; Hamada *et al*., 1977). There may also be some possibility of indomethacin interfering with spontaneous release of gonadotropins since exogenous gonadotropins may bring about removal of indomethacin caused blockade to ovulation (Stacey and Pandey, 1975). Orczyk and Behrman (1972) reported decreased levels of prostaglandins in hypothalamus following inhibition of ovulation by aspirin in rats.

The survey of literature revealed that the use of natural prostaglandins (PGs) in aquaculture program of India has been neglected. It is mainly due to their high cost and

vulnerability to decomposition under variety of known and unknown conditions *viz*. their half-life in seconds, storage at −70 °C etc.

Thus the study of potential of prostaglandins as induced breeding agents in Indian major carps and ornamental fish is needed.

On the other hand aquarium fish culture is being practiced all over the country to meet the domestic entertainment needs but not on commercial lines to meet the export needs. In spite having the advantage of tropical climate and vast potential of aquatic resources, it is said that India is contributing only one percent to the global trade in ornamental fish industry. Ornamental fishes are in greater demand due to their easy maintenance in houses, offices and commercial centers and the scope for their expansion is increasing day by day.

Goldfish was bred by administering human chorionic gonadotropin (HCG) injections for the first time in India (Reddy *et al*., 1990) but apart from this there is no report on induced breeding of goldfish.

In India induced breeding technique of food fishes by using various inducing agents and well-established technique is a routine practice. However, these induced breeding techniques have not been applied on ornamental fishes.

In view of the increasing demand both in domestic and export markets especially for freshwater ornamental fishes, if their culture is taken up scientifically and the induced breeding technique in ornamental fishes is explored to induce multiple spawning, to reduce the gap between two spawning periods and enhance production, there is no doubt that it will be a boon to the national economy.

CHAPTER - II

INDUCED BREEDING OF GOLDFISH, C*arassius auratus* WITH PROSTAGLANDINS

INTRODUCTION

Aquarium fish culture is being practiced all over the country to meet the domestic entertainment needs but not on commercial lines to meet the export needs. In spite of the advantage of tropical climate and vast potential of aquatic resources, it is said that India is contributing only one percent to the global trade in ornamental fish industry. Ornamental fishes are in greater demand due to their easy maintenance in houses, offices and commercial centers. The scope for the expansion of ornamental fish business is increasing day-by-day.

There is a constant demand for these beautiful ornamental fishes from European countries, America and Canada. The demand for ornamental fishes within and outside the country is increasing rapidly. The world trade in ornamental fishes is estimated at Rs. 8,000 crore and the developing countries are the major sources for imports. Over 80 per cent of the trade is dominated by freshwater fish and the balance by marine ornamental fish. The leading markets are in the US, Europe and Japan.

In India, Kolkata, Mumbai and Chennai are the major production centers (Business Line, Financial Daily, August 21, 2001). The annual demand for ornamental fishes in India is always more than production.

In India induced breeding of food fishes by using various inducing agents by well-established techniques is a routine method. However, these induced breeding techniques have not been applied on aquarium fishes. In view of the increasing demand both in domestic and export markets especially for freshwater ornamental fishes, if the culture is taken up scientifically and the induced breeding technique is applied in ornamental fishes for repeated (multiple) spawning, to reduce the gap between two spawning periods and enhance production, there is no doubt that it becomes a boon to the national economy.

Goldfish, *Carassius auratus* (Linnaeus) one of the best aquarium fish world wide is native of central Europe. It was introduced into the Ooty Lake in Madras in 1874 from Central Europe for the experimental purpose. It belongs to the family Cyprinidae and is closely related to Indian major carps.

Goldfish is good model for conducting basic research work in laboratory conditions. On the basis of these results further trials were taken on other carp species those are not bred in captivity like Indian major carps, Chinese carps etc on fields.

Goldfish was bred by administering human chorionic gonadotropin (HCG) in India first time by Reddy *et al*. (1990). Many workers of Japan including Fujita *et al*. (1948), Kawamura and Montanga (1963) could successfully induce spawning of *Carassius auratus*, *Carassius carassius, Cobitis taenia, Cobitis biwae* and *Misgurnus anguillicaudatus* during the forty decades.

Prostaglandins (PGs) appear to be involved in ovulation, male and female sexual behaviour, gonadotropin (GtH) secretion and thus considerably affect the reproductive physiology of fish (Goetz, 1983; Stacey, 1981; Stacey and Goetz, 1982; Stacey *et al*., 1987). From investigations on the effects of PGs in fish it does appear that certain PGs have a stimulatory effect on ovulation. Except few references of Stacey and Sorensen on effects of natural PGs on goldfish reproduction, no report is available on synthetic PGs on goldfish breeding.

No study is made by using natural prostaglandin analogues for mass seed production of goldfish due to their high cost, unstable nature, short shelf-life and storage at –70°C. In view of this, in the present attempt was made to evaluate the role of prostaglandins and their analogues (natural as well as synthetic) as induced breeding agents in goldfish, *Carassius auratus*.

MATERIALS AND METHODS

Animals

Mature goldfish brooders were procured from local ornamental fish dealers during winter season. The male and female brood fish were identified based on the external secondary sexual characters from the available lot of brooders. Female goldfish with reddish swollen vent, bulky abdomen and active males with white tubercles on operculum and secretion of milt were chosen for experiments. They were fed daily with artificial fish pellets (fishmeal, wheat flour, rice bran, yeast, vitamin and calcium) and live tubifex worms / mosquito larvae at the rate of 10% of their body weight (Reddy *et al*., 1990).

Broodstock were maintained in separate tanks (4' diameter and 3' high) at 22 ± 1 °C on 12 L:12 D photoperiod. An aerator was provided in tank and aquarium water for regular supply of oxygen. Water was partially changed once a week and replenished with fresh dechlorinated water. Once or twice in a month, fishes were given a bath of potassium permanganate ($KMnO_4$) solution. From 1% $KMnO_4$ stock solution, 2 cc was added per gallon of water and a bath of half an hour was given to disinfect the fishes.

Chemicals

The prostaglandins their analogues and other chemicals used in the present study and their sources are mentioned in Table 2.1.

Experimental Methodology

The following aspects were studied to ascertain the role of prostaglandins (natural and synthetic) in goldfish breeding: I) Induced breeding and II) Behavioural responses.

I) Induced breeding experiments

General methods

Breeding experiments on goldfish were conducted at National Chemical Laboratory (NCL), Pune, India. Animal house (a room of size 20' x 18') was renovated and equipped with proper well water connection for plastic pools, glass aquaria, electrical connections etc (Fig. 2.1).

The male and female brooders in the age group of 8-12 months weighing 20-50 gm were properly selected for experiments from common broodstock. Brooders were selected pair-wise. Each selected pair was kept overnight in separate aquarium (36" x 12" x 15") in 50 l of dechlorinated water with temperature 20-22 ^{0}C. Fabricated glass stands woven with nylon mesh were kept at the bottom of aquarium to protect the eggs from being eaten by their parents (Fig. 2.4) and pairs were checked for ovulation after 24 h. Non-ovulated females were further subjected to hormone treatment.

The pairs were weighed separately in a suitable container with water and actual weight of each fish was determined by deducting the known weight of the container and water from the total weight. Required amounts of prostaglandins was dissolved in ethanol

and further dilution was done made in fish saline (0.6% NaCl). Brooders were netted out in a bag net and wrapped in the same bag net. They were placed on a table and carefully injected, avoiding wriggling movements. Injection was administered intramuscularly at the base of caudal fin above the lateral line (Fig. 2.2). Injected pair (1 male and 1 female) was released in 50 l aquarium and was kept under observation for 24 h. Saline injected pair of goldfish was treated as control.

Ovulation and spawning

From 6 h onwards of injection ovulation was checked when fish started chasing each other vigorously causing intermittent water splashing. The time from the last injection to ovulation was refereed to as the latency period. Upon gentle massaging of the abdomen ovulated eggs were easily extruded from the genital pore. After spawning parents were immediately removed and eggs were treated with methylene blue to prevent from fungal attack.

In case of failure of spawning within 24 h after injections males and females were separated and were again kept for observation of ovulatory changes. Various experiments were conducted using different doses for dose standardization. The results of induced breeding experiments are summarized in Table 2.3-2.6.

Ovaprim (sGnRHA), a standard spawning inducing hormone was used in the present study for standardization of induced breeding method.

II) Behavioural changes

Separate experiments were carried out for behavioural studies. Only females were injected with prostaglandins, no male from control or experimental groups was injected with PGs. Saline injected female was kept as control. Behavioural changes were observed till 24 h after injection. The temperature was maintained 24 ± 1 ^{0}C during these behavioural experiments.

a) Behavioural responses of male goldfish

Males were released with prostaglandin injected non-ovulated females and reproductive behavioural changes in males were observed. The time taken to initiate the behavioural responses in male and duration of behavioural activity were studied.

b) <u>Behavioural responses of female goldfish</u>

Various prostaglandins were injected to non-ovulated female goldfish and female spawning behavioural changes like spawning acts, jerks and duration of activity etc were observed.

Statistical Method

Standard errors for means were derived by the usual formula:

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

SE = Standard error

SD = Standard deviation

n = Number of replicates.

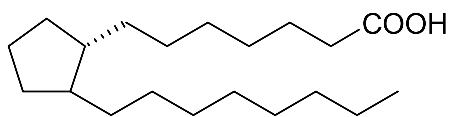
Table 2.1 List / Sources of prostaglandins and other chemicals used for goldfish breeding experiments

Name of compound	Source
Natural prostaglandins	
Prostaglandin-E_1 (PGE_1)	Cayman Chemicals, 690 KMS Place
Prostaglandin-E_2 (PGE_2)	Ann Arbor, MI 48108 USA
Prostaglandin- $F_{2\alpha}$ ($PGF_{2\alpha}$)	,,
Prostaglandin precursors	
Arachidonic acid (AA)	Cayman Chemicals, 690 KMS Place, Ann Arbor, MI 48108 USA
Eicosapentaenoic acid (EPA)	,,
Synthetic prostaglandin analogues	
16-Phenoxy tetranor $PGF_{2\alpha}$	Cayman Chemicals, 690 Place Ann Arbor, MI 48108 USA
16,16-Dimethyl PGE_2	,,
16,16-Dimethyl $PGF_{2\alpha}$	,,
Cloprostenol	,,
Iliren (Tiaprost)	Hoechst Roussel Vet. GmbHd-65203 Wiesbaden
Prosolvin (Luprostiol)	Intervet, International B.V. Boxmeer-Holland
Lutylase (${PGF}_{2\alpha}$)	Upjohn, Upjohn s.a. puurs, Belgium
Prostodin (Carboprost)	Astra IDL Ltd, Bangalore 560063, India

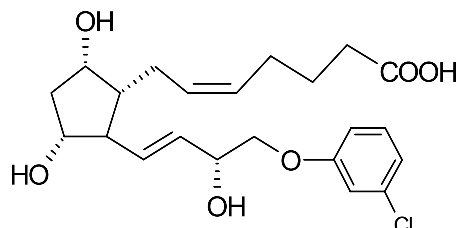
Table 2.1 Contd.

Name of compound	Source
Prostaglandin analogues synthesized at National Chemical Laboratory, Pune	
α-N-R (**11**)	β-N-R (**12**)
P-M-R (**13**)	α-N-K (**14**)
β-N-K (**16**)	P-M-K (**17**)
Misoprostol (**19**)	
Other compounds	
Ovaprim (sGnRHA)	Syndel Laboratories, Vancouver, Canada
Human Chorionic Gonadotropin	UNI – SANKYO Ltd. Gaganpahad, Bangalore Road, R.R. Dist Hyderabad-501 323
Indomethacin	E. Merck (India) Ltd. Plot No. D-116 MIDC, Trans Thane Creek Area, Thane – Belapur Road, Nerul, Navi Mumbai 400 706

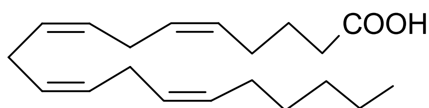
Structure of Prostaglandins



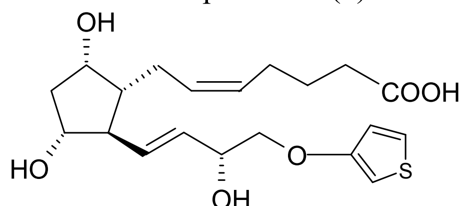
Prostanoic acid (**1**)



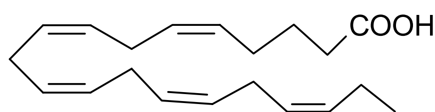
Cloprostenol (**7**)



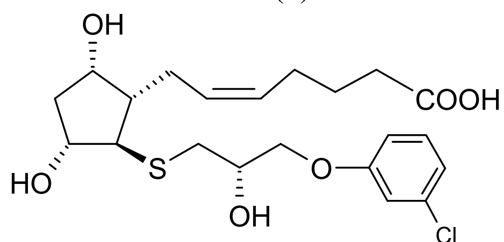
Arachidonic acid (**2**)



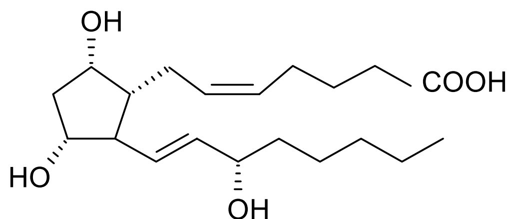
Iliren (**8**)



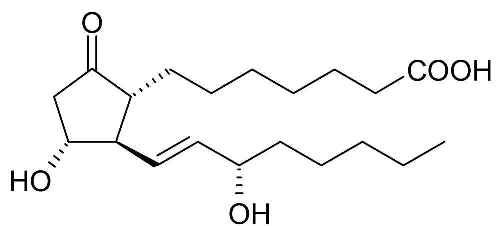
Eicosapentaenoic acid (**3**)



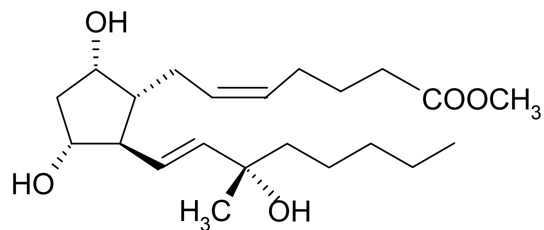
Prosolvin (**9**)



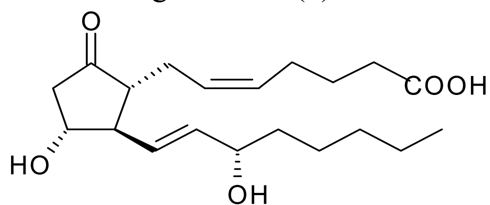
Prostaglandin-$F_{2\alpha}$ (**4**)



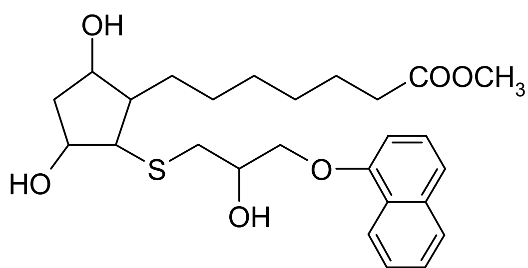
Prostaglandin-E_1 (**5**)



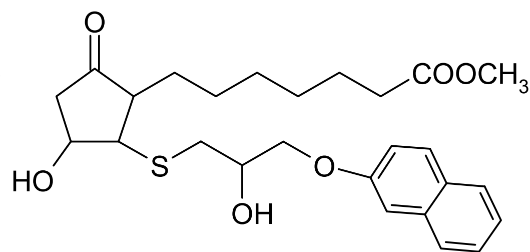
Prostodin (**10**)



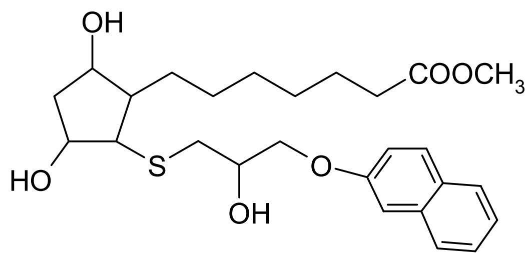
Prostaglandin-E_2 (**6**)



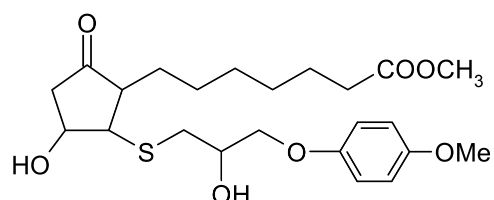
α-N-R (**11**)



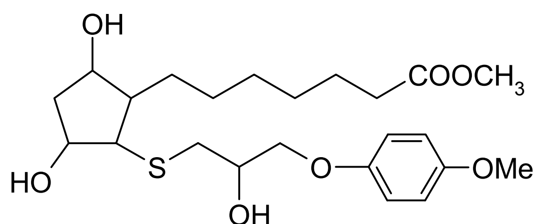
β -N-K (**16**)



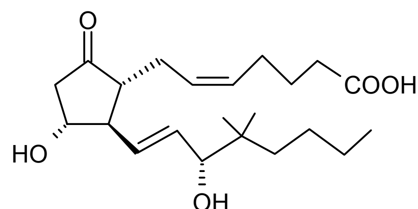
β -N-R (**12**)



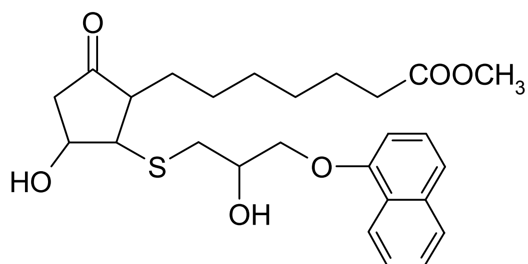
P-M-K (**17**)



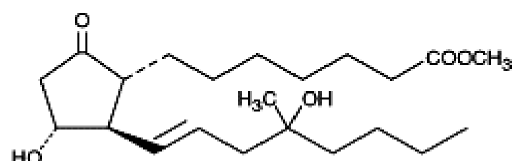
P-M-R (**13**)



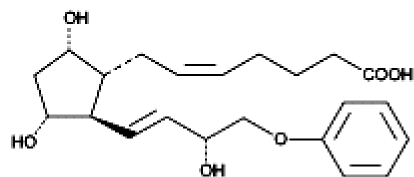
16,16-Dimethyl prostaglandin-E_2 (**18**)



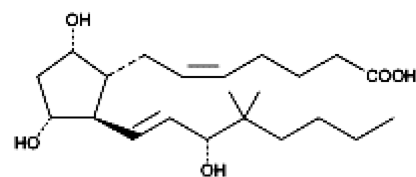
α-N-K (**14**)



Misoprostol (**19**)



16-Penoxy tetranor prostaglandin $F_{2\alpha}$ (**15**)



16,16-Dimethyl prostaglandin-$F_{2\alpha}$ (**20**)

Preparation of chemicals for injection

Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$)

$PGF_{2\alpha}$ is a natural prostaglandin. Stock solution was prepared by dissolving 500 μg of $PGF_{2\alpha}$ in 2.5 ml buffer solution (1 μg/5 μl). For further dilution fish saline (0.6% NaCl) solution was used. Dose tried for female was 1.0 μg/gm and for male 0.5 μg/gm body weight of fish.

Prostaglandin-E_1(PGE_1) and Prostaglandin-E_2 (PGE_2)

These are the natural prostaglandins. Stock solution was prepared by dissolving 1 mg of PGE_1 or PGE_2 in 1 ml ethanol (95%) (1 mg/1 ml) and diluted with 9 ml buffer saline (1μg/10 μl). Dose tried was 1.0 μg/gm and 0.5 μg/gm body weight of female and male fish respectively.

16-Phenoxy tetranor prostaglandin-$F_{2\alpha}$

It is a synthetic analogue of prostaglandin-$F_{2\alpha}$. Stock solution was prepared by dissolving 1 mg of 16-phenoxy tetranor $PGF_{2\alpha}$ in 0.1 ml ethanol. 5 μl of this stock solution was diluted with 45 μl buffer saline. Dose tried was 1.0 μg/gm and 0.5 μg/gm body weight of female and male fish respectively.

16,16-Dimethyl prostaglandin-$F_{2\alpha}$

16,16-dimethyl $PGF_{2\alpha}$ is a metabolically stable analogue of $PGF_{2\alpha}$. Stock solution was prepared by dissolving 1 mg of 16,16-dimethyl $PGF_{2\alpha}$ in 0.1 ml ethanol. 5 μl of this stock solution was diluted in 45 μl buffer saline (1 μg/1 μl). Dose tried was 1.0 μg/gm and 0.5 μg/gm body weight of female and male fish respectively.

16,16-Dimethyl PGE_2

Stock solution was prepared by dissolving 1 mg of 16,16-dimethyl PGE_2 in 0.1 ml ethanol. 5 μl of the stock solution was diluted with 45 μl buffer saline (1 μg/1 μl). Dose tried was 1.0 μg/gm and 0.5 μg/gm body weight of female and male fish respectively.

Arachidonic acid (AA)

It is the precursor of prostaglandins. Stock solution was prepared by dissolving 25 mg AA in 250 µl ethanol. 1.5 µl of this stock solution was diluted with buffer saline solution to a final volume of 50 µl (3 µg/µl). Dose range tried was 2-5 µg/gm body weight of fish.

Eicosapentaenoic acid (EPA)

It is also a precursor of prostaglandins. Stock solution was prepared by dissolving 10 mg of EPA in 0.1 ml ethanol. 1.5 µl of this stock solution was diluted with buffer saline to the final volume of 50 µl (3 µg/µl). Doses tried were 1.0 µg/gm and 0.5 µg/gm body weight of female and male fish respectively.

Cloprostenol

Cloprostenol is a synthetic analogue of prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$). Stock solution available (10 mg/ml ethanol) was used for further dilution with fish saline. It is stable for at least six months in these solvents. 25 µl of stock solution was diluted with buffer saline to 225 µl (1 µg/µl). Dose range tried was 0.5 µg/gm to 5 µg/gm body weight of fish.

Synthetic prostaglandin **11** (α-N-R)

It is a synthetic analogue of prostaglandin-$F_{2\alpha}$, synthesized at National Chemical Laboratory, Pune. Stock solution : 300 µg of **11** were dissolved in 1 ml solvent (0.5 ml ethanol + 0.5 ml saline) (1 µg/3.3 µl). Dose used for goldfish was 1.0 µg/gm and 0.5 µg/gm body weight of female and male respectively.

Synthetic prostaglandin **12** (β-N-R)

It is another analogue of prostaglandin-$F_{2\alpha}$ synthesized at National Chemical Laboratory, Pune. Stock solution : 500 µg of **12** were dissolved in 1 ml solvent (0.5 ml ethanol + 0.5 ml saline) (1 µg/2 µl). The dose used for goldfish was 1.0 µg/gm and 0.5 µg/gm body weight of female and male respectively.

Synthetic prostaglandin **13** (P-M-R)

It is also a synthetic analogue of prostaglandin-$F_{2\alpha}$ and it was synthesized at National Chemical Laboratory, Pune. Stock solution : 500 μg of **13** were dissolved in 0.5 ml ethanol and diluted with 0.5 ml buffer saline (1 μg/2 μl). Dose used for goldfish was 1.0 μg/gm and 0.5 μg/gm body weight of female and male respectively.

Synthetic prostaglandin analog **14** (α-N-K)

This synthetic analogue of prostaglandin was synthesized at National Chemical Laboratory, Pune. Stock solution: 500 μg of **14** were dissolved in 0.5 ml of ethanol and diluted with 0.5 ml buffer saline to give a stock with concentration 1 μg/2 μl. Dose used for goldfish was 1.0 μg/gm and 0.5 μg/gm body weight of female and male respectively.

Synthetic prostaglandin **16** (β-N-K)

It is a synthetic analogue of prostaglandin and was synthesized at National Chemical Laboratory, Pune. Stock solution : 500 μg of **16** were dissolved in 0.5 ml ethanol and diluted with 0.5 ml buffer saline to afford a stock solution with concentration 1 μg/2 μl. Dose used for goldfish was 1.0 μg/gm and 0.5 μg/gm body weight of female and male respectively.

Synthetic prostaglandin **17** (P-M-K)

It is a synthetic analogue of prostaglandin. It was synthesized at National Chemical Laboratory, Pune. Stock solution : 500 μg of **17** were dissolved in 0.5 ml ethanol and diluted with 0.5 ml buffer saline to give a stock with concentration 1 μg/2 μl. Dose used for goldfish was 1.0 μg/gm and 0.5 μg/gm body weight of female and male respectively.

Misoprostol

It is a synthetic analogue of prostaglandin-E_1 and was synthesized at National Chemical Laboratory, Pune. Stock solution : 500 μg misoprostol was dissolved in 0.5 ml ethanol and diluted with 0.5 ml buffer saline to give the stock solution with concentration 1 μg/2 μl. Dose used for goldfish was 1.0 μg/gm and 0.5 μg/gm body weight of female and male respectively.

Lutylase (Prostaglandin-$F_{2\alpha}$)

Commercially available ready to use form. 5 mg dinoprost contains in 1 ml (5 mg/ml). Dose used for goldfish was 1.0 μg/gm and 0.5 μg/gm body weight of female and male respectively.

Iliren

This is a commercially available ready to use synthetic analogue of prostaglandin-$F_{2\alpha}$, 1 ml iliren contains 0.150 mg tiaprost. Dose range used for goldfish was 0.5 μg/gm to 2.5 μg/gm body weight of goldfish.

Prosolvin

It is a synthetic prostaglandin-$F_{2\alpha}$ analogue. Prosolvin is a clear solution of luprostiol (7.5 mg/ml) commercially available for injection. Dose range tried was 0.5 μg to 1.5 μg/gm body weight of goldfish.

Prostodin

Prostodin is a synthetic analogue of prostaglandin- $F_{2\alpha}$ commercially available in ready to use form. 1 ml contains 250 μg carboprost (1 μg/4 μl). Dose tried was 0.5 μg/gm body weight of male and 1.0 μg/gm body weight of female goldfish.

Ovaprim

Ovaprim is a synthetic analogue of salmon gonadotropin releasing hormone. It is a known induced fish breeding agent. 1 ml of ovaprim contains 20 μg of sGnRHA and 10 mg domperidone. Dose used for goldfish was 0.5 μl/gm and 0.125 μl/gm body weight of female and male respectively.

Indomethacin

It is a prostaglandin biosynthesis inhibitor. Stock solution was prepared by adding 25 mg indomethacin in 5 ml buffer saline followed by 1 drop of Tween 80 to the turbid formed,

to give a clear solution (5 μg/μl). The dose used for goldfish was 10 μg/gm body weight (Stacey, 1976).

Domperidone

It is a dopamine synthesis inhibitor. Dopamine inhibits the release of gonadotropin releasing hormone. Stock solution was prepared by dissolving 25 mg domperidone in 1 ml dimethyl formamide (DMF) and 0.5 ml saline (17 μg/μl). Dose used was 5 μg/gm body weight of male as well as female goldfish.

Human Chorionic Gonadotropin (HCG)

1000 IU of HCG was dissolved in 1 ml saline (1000 IU/1 ml). Dose used for female goldfish was 3 IU/gm body weight and for male goldfish was 1.5 IU/gm body weight.

Methylene blue (antifungus)

Stock solution was prepared by adding 1 gm of medical grade methylene blue to 100 cc of hot water. Used 4 cc of the stock solution was used for each gallon of water to avoid fungal infection of eggs.

Fish saline

It was prepared by dissolving 6 mg of sodium chloride in 100 ml water and used for dilution of compounds (0.6% NaCl).

Buffer solution

Buffer solution was prepared by dissolving 600 mg sodium chloride (NaCl) and 20 mg sodium bicarbonate ($NaHCO_3$) in 100 ml of distilled water.

Figure 2.1. The glass aquaria for goldfish experiments.

Figure 2.2. Administration of intra-muscular injection to goldfish at base of caudal peduncle.

RESULTS

Spawning response of goldfish with Ovaprim

In the present study, the response of goldfish to ovaprim (sGnRHA) is given in Table 2.2. Various doses were tried for dose standardization. It was found that 0.5 µl/gm and 0.125 µl/gm body weight of female and male respectively, induce courtship behaviour and spawning. After 6 h of injection spawning behavioural changes were observed in male and female, characterized by vigorous chasing of female, pairing and constant nudging of her ovipore and side as well.

The series of spawning acts were observed, female approached the surface of water in head up position and male followed. Both turned on their sides, they broke the water surface, male constantly chased and female gave repeated jerks (spawning acts). Eggs and sperms were released during these acts and fertilization occurred. This activity repeated up to several hundred times during 1- 4 h. Spawning occurred generally 10-12 h after injection in all ovaprim treated groups. After spawning, parents were removed from the spawning aquarium. Fertilized eggs were counted and percentage of fertilization was determined. Fecundity was recorded as 1000 ± 115.47 per female with the fertilization rate (%) 75 ± 2.88. Hatching occurred generally after 36 – 72 h of spawning (depended upon temperature) with rate (%) 78.33 ± 4.40. Hatchlings obtained by induced breeding appeared healthy.

Fish injected with saline of control group did not show spawning behaviour or spawning at all. The latency period was observed 10-12 h in all experimental groups and the rates of fertilization and hatching were normal.

Effects of natural prostaglandins (PGs) and their precursors on goldfish breeding

$PGF_{2\alpha}$ (4)

Table 2.3 shows the effect of various natural prostaglandins on breeding of goldfish, *Carassius auratus*. It is observed from the table that among the natural PGs, $PGF_{2\alpha}$ (**4**) induced spawning.

$PGF_{2\alpha}$ (**4**) was administered at the dose level 0.5 µg/gm body weight of goldfish for both male and female. Induced spawning behaviour was observed after 6-8 h of

injection and this activity continued for 1 h but no ovulation and spawning was observed within 24 h of injection.

In another experiment it was found that $PGF_{2\alpha}$ (**4**) administered at the rate of 1.0 µg/gm body weight of female and 0.5 µg/gm body weight of male induced spawning behaviour after 6-8 h of injection and spawning took place after 10-12 h. Number of spawned eggs was 200 ± 17.32 per female with fertilization rate (%) 20 ± 1.15 (the reason of less fertilization rate was not found). No hatching occurred due to fungal infection. The latency period was 10-12 h in all sets.

In another experiment higher dose of $PGF_{2\alpha}$ (**4**) was tried in combination with indomethacin. No behavioural changes and ovulation was observed within 36 h of injection.

Prostaglandin-E_1 (5)

Prostaglandin-E_1 (**5**) was administered with priming of human chorionic gonadotropin (HCG). Dose of HCG 1 IU/gm body weight of female and 0.5 IU/gm body weight of male was given and after 6 h PGE_1 (**5**) was administered at the rate of 1.0 µg/gm and 0.5 µg/gm body weight of female and male respectively. Induced spawning behaviour in both male and female was seen after 1 h of second injection. This behavioural activity continued for 2 h but no ovulation and spawning was observed within 24 h. In other doses of PGE_1 (**5**) spawning behaviour and ovulation was not seen. PGE_1 (**5**) did not exhibit any side effects (e.g. discoloration of eyes, increased slimness, sluggishness etc.) during and after the experiments on goldfish.

Prostaglandin-E_2 (6)

In case of PGE_2 observed behavioural changes and ovulation were absent at dose 1.0 µg/gm and 0.5 µg/gm body weight of female and male goldfish respectively. No side effects were observed on goldfish during and after experiments.

16,16-Dimethyl PGE_2 (18)

16,16-Dimethyl PGE_2 was administered at the dose level of 1.0 µg/gm and 0.5 µg/gm body weight of female and male goldfish respectively. In case of male spawning

behaviour (chasing) was observed after 45-60 min of injection and this male activity continued for 1 h but female did not respond.

Arachidonic acid (2)

Arachidonic acid (**2**) induced spawning behaviour in goldfish at the dose of 3 μg/gm and 1 μg/gm body weight of female and male respectively. Spawning behavioural changes were observed after 7 h of injection but no ovulation and spawning was observed within 24 h of injection. No side effects were observed.
Arachidonic acid (**2**) in combination with indomethacin did not induce behavioural changes and ovulation.

Eicosapentaenoic acid (3)

EPA (**3**) at the dose of 1.0 μg/gm body weight of female and 0.5 μg/gm body weight of male goldfish induced spawning behaviour in males after 6 h of injection but females did not respond.

Effects of prostaglandin analogues (synthetic) on breeding of goldfish

Cloprostenol (7)

Data presented in Table 2.4 demonstrated that cloprostenol (**7**) at the dose of 1.0 μg/gm body weight of female and 0.5 μg/gm body weight of male goldfish, induced spawning behavioural changes after 4 h of injection and spawning took place after 10-12 h of injection in all cloprostenol (**7**) sets. Fertilized eggs were counted and percentage of fertilization was determined. Fecundity was recorded as 2300 ± 104.08 over 183.33 ± 16.66 of the control group was highly significant ($P<0.005$). Percentage of fertilization 80 ± 2.51 over 55 ± 2.88 of the control group ($P < 0.025$) and hatching 91.66 ± 1.66 over 55 ± 2.88 of the control groups ($P < 0.01$) was also observed. Hatching occurred after 48 h of spawning. Hatchlings obtained by induced breeding appeared healthy (Fig. 2.5). The latency period was observed 10-12 h in all cloprostenol (**7**) treated groups. No side effects of cloprostenol (**7**) were observed during and after the experiments on goldfish.

It was also possible to reinduce the same breeders to spawn repeatedly with the gap of 30-45 days by using cloprostenol (**7**). Higher doses of cloprostenol (**7**) tried in combination with indomethacin (dose: Indomethacin + cloprostenol, 10 μg/gm + 1 μg/gm and 10 μg/gm + 5 μg/gm body weight of fish) did not show spawning behaviour and ovulation.

Prosolvin (9)

Prosolvin (**9**) induced spawning behaviour at a dose of 1.5 μg/gm and 0.5 μg/gm body weight of female and male goldfish respectively and another dose of 0.5 μg/gm body weight for both female and male. Behavioural changes were observed after 1 h of injection and this activity was seen for 3 – 4 h. No ovulation was observed within 24 h.

Tiaprost (8)

Tiaprost (**8**) was administered at the doses ranging from 0.5 μg/gm to 2.5 μg/gm body weight of fish. It was observed that non-injected male released with tiaprost injected female (1 μg/gm body weight) showed very prominent spawning behaviour after 1 h of injection. Behavioural changes in male were characterized by vigorous chasing of female, paring and constant nudging of her ovipores and sides as well. During the series of spawning acts observed female approached the surface of water in head up position and male followed both turned on their sides, they broke the water surface, male constantly chased and female gave repeated jerks (spawning acts). Eggs were not released during those spawning acts. Males and female were again isolated after 24 h of injection and kept separately. Females injected with tiaprost (**8**) ovulated after 72 h of injection. Ovulated females were again released along with male and spawning took place within half an hour after males were released. The spawning efficacy recorded at different instances as fecundity 3333.33 ± 166.67 over 183.33 ± 16.66 of the control group is highly significant ($P < 0.005$). Percentage of fertilization was 81.66 ± 1.66 ove 55 ± 2.88 of the control group ($P < 0.025$) and hatching percentage was 91.66 ± 1.66 over 55 ± 2.88 of the control group ($P < 0.01$). Hatchlings obtained by induced breeding appeared healthy.

No side effects were observed in case of tiaprost (**8**) on goldfish during and after the experiments. Tiaprost with other doses induced only spawning behavioural changes (Table 2.4).

Prostodin (10)

Prostodin did not induce any behavioural changes and ovulation at the dose of 1.0 μg/gm body weight of female and 0.5 μg/gm body weight of male goldfish.

16-Phenoxy tetranor $PGF_{2\alpha}$ (15) and 16,16-Dimethyl $PGF_{2\alpha}$ (20)

These prostaglandin analogues did not induce any behavioural changes and ovulation at dose of 1.0 μg/gm and 0.5 μg/gm body weight of female and male goldfish respectively. No side effects were observed on goldfish during and after experiments.

Effects of synthetic prostaglandin analogues (prepared at NCL, Pune) on goldfish breeding

Data presented in Table 2.5 indicates that synthetic prostaglandin analogues **11**, **12**, **13**, **14**, **16**, **17** and Misoprostol (**19**) did not exhibit any behavioural changes and spawning at a dose of 1.0 μg/gm body weight of female and 0.5 μg/gm body weight of male. No side effects were observed in case of these synthetic analogues on goldfish during and after the experiments.

Effects of prostaglandin (PG) analogues on spawning behavior of goldfish, *Carassius auratus*

In this experiment only females (non-ovulated) were injected with PG analogues at dose levels 1 μg/gm body weight and none of the males of the control and experimental groups was administered with prostaglandins and observed for behavioural changes in males and females.

Results presented in Table 2.6 show that prosolvin (**9**) injected female induced spawning behaviour in male after half an hour of injection. The behavioural changes in male included vigorous chasing of female, pairing and constant nudging of her ovipores and sides. In females, induced spawning behaviour was observed after 1 h of injection.

During the series of spawning acts observed, female approached the surface of water in head up position and male followed. Both turned on their sides, they broke the water surface, male constantly chased and female gave repeated jerks (spawning acts). Spawning acts counted were 55 ± 3.53 per hour. This activity continued for 4-6 h. No ovulation and spawning were observed within 24 h of injection. No side effects were observed during and after the experiments.

In Tiaprost (**8**) treated female, male spawning behaviour was observed after 1 h of injection, behavioural changes were same as prosolvin (**9**). Spawning acts counted were 50 ± 2.23 per hour. These behavioural activities continued for 5-6 h. No ovulation and spawning was observed within 24 h of injection and no side effects were observed.

Cloprostenol (**7**) injected females induced male spawning behaviour after half an hour of injection. Spawning behavioural activity was same as tiaprost, spawning acts counted in female were 30 ± 2.28 per hour. This behavioural activity continued for 2-3 h. No ovulation and spawning were observed within 24 h of injection as well as no side effects were observed.

Lutylase (**4**) did not exhibit any behavioural changes in male and female. No ovulation and spawning were observed within 24 h of injection and no side effects were observed.

Saline injected females of the control group did not show any behavioural changes.

Figure 2.3. Prostaglandin administered goldfish showing sexual behaviour.

Figure 2.4. Close-up view of aquarium showing screen which separates eggs from parent fish.

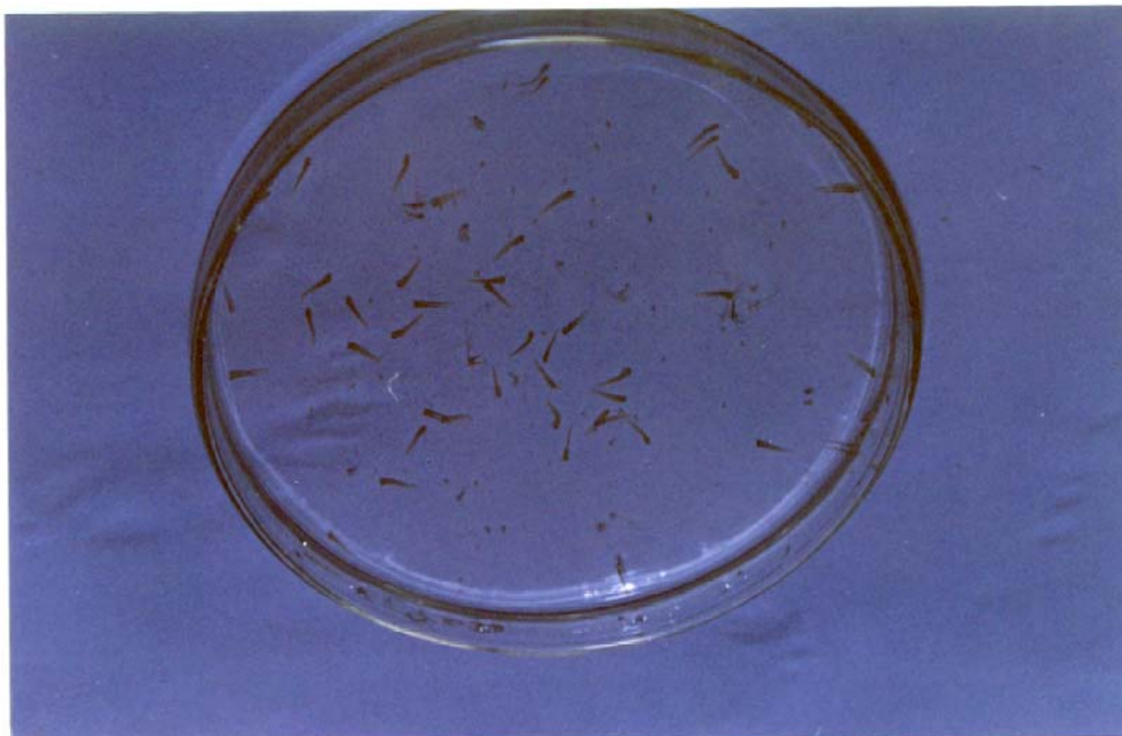


Figure 2.5. Hatchlings of goldfish, *Carassius auratus*.

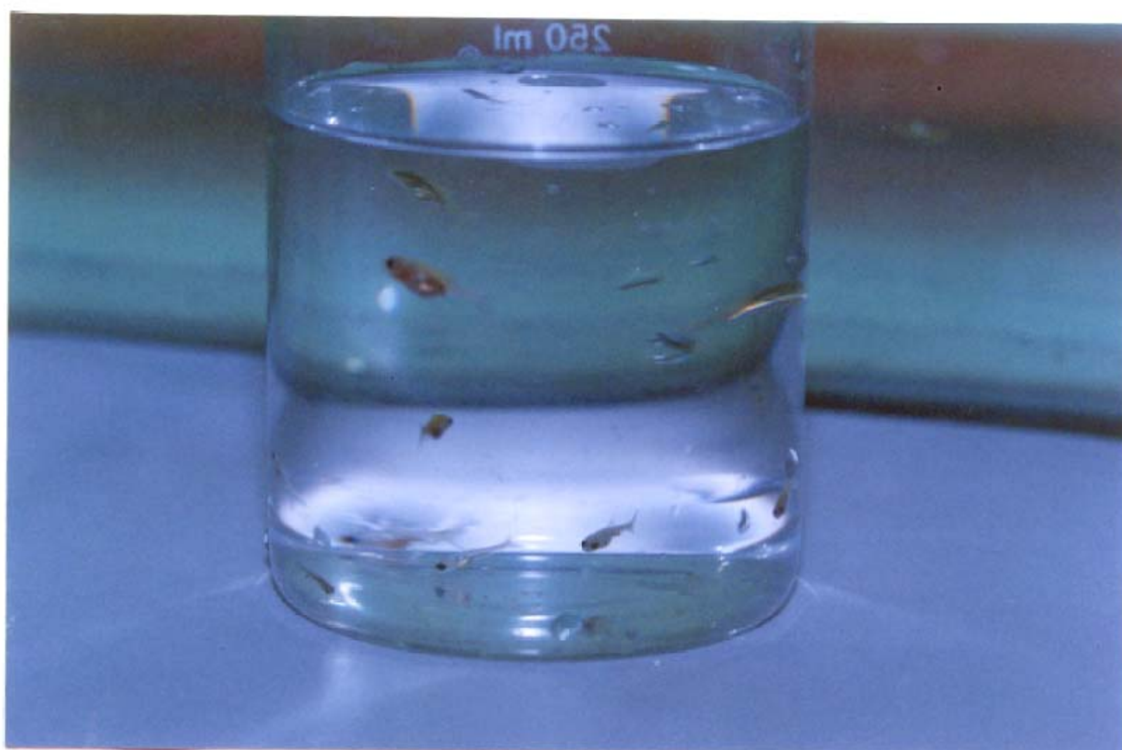


Figure 2.6. Fry of goldfish, *Carassius auratus*.

DISCUSSION

The results of present study strongly suggest a role of prostaglandins (PGs) in regulation of spawning behaviour, ovulation and spawning in goldfish, *Carassius auratus*.

As reported Ovaprim (sGnRHA) is a known spawning inducing agent in Indian major carps and other carp species (Nandeesha *et al*., 1990b; Peter *et al*., 1988). In the present study it was found that a single injection of ovaprim with dose of 0.5 µl/gm and 0.125 µl/gm body weight of female and male respectively, induced spawning in goldfish within 10-12 h of injection with fertilization rate (%) above 75 and hatchlings obtained by this method were healthy (Table 2.2).

Prostaglandins are known to be involved in ovulation in several fishes (Stacey and Goetz, 1982; Jones, 1987; Goetz *et al*., 1989). Of various prostaglandins, ovulatory action of $PGF_{2\alpha}$ was found to be most effective both *in vivo* and *in vitro* in variety of teleosts (Stacey and Goetz, 1982). Similar results were obtained in the present study in which natural $PGF_{2\alpha}$ induced ovulation and spawning in goldfish.

PGE_1 (**5**) another natural prostaglandin, individually did not induce spawning behaviour and ovulation. Spawning behavioural changes were observed when PGE_1 (**5**) was administered with human chorionic gonadotropin (HCG) priming but no ovulation and spawning was observed.

Linpe method of induced ovulation and spawning of cultured fish consists of treatment with an analogue of gonadotropin-releasing hormone (GnRH) plus dopamine antagonist (Peter *et al*., 1988). As per the Linpe method, PGE_1 (**5**) was studied in combination with domperidone (dopamine antagonist) but it was found ineffective.

Prostaglandin-E_2 (**6**), 16-phenoxy tetranor-$PGF_{2\alpha}$ (**15**), 16,16-dimethyl $PGF_{2\alpha}$ (**20**) and 16,16-dimethyl PGE_2 (**18**) in our preliminary study were found ineffective in inducing spawning in goldfish.

Arachidonic acid (**2**) and eicosapentaenoic acid (**3**), precursors of prostaglandins, in the present study induced only spawning behaviour.

Present results emphasize that $PGF_{2\alpha}$ analogue tiaprost (**8**) is effective in inducing spawning behaviour, ovulation and spawning in goldfish. It was observed that injection of tiaprost (**8**) to non-ovulated female goldfish not only elicited the spawning behaviour and ovulation in females but also stimulated male spawning behaviour. Excellent spawning

results were obtained when only female was injected with tiaprost. Here tiaprost acts as a hormone in female and same females released some parts of tiaprost or other chemicals into water which act as pheromone for male. Delayed spawning was observed after 72 h of injection. Tiaprost is more stable than natural prostaglandins.

The economic analysis carried out has indicated that natural $PGF_{2\alpha}$ costs Rs. 1.0 for 1μg while tiaprost costs Rs. 0.2 for 1 μg. Thus the tiaprost is more economical than natural prostaglandin-$F_{2\alpha}$.

Our findings in the present study on natural PGs are in agreement with earlier reports of Stacey and Goetz (1982) and Sorensen *et al*. (1988) that relate to prostaglandin induced behavioural responses in non-ovulated female goldfish.

Prostaglandin injection to non-ovulated female releases an odour that elicits male reproductive behaviour identical to that elicited by the odour of ovulated female (Sorensen *et al*., 1986). The tendency of male goldfish to contact the female genital areas (nudging) with snout may be well exposing males to high local concentration of such stimulants. Sorensen *et al*. (1986, 1995) have reported that circulating PGs are cleared to the water to function as pheromones thereby synchronizing male and female sexual behaviour in goldfish. As nothing is known about how goldfish would release injected tiaprost (**8**) into water, it is not clear whether natural release of tiaprost (**8**) itself or its metabolites during preovulatory period could result in water concentration sufficient to induce male spawning behaviour in the present study. This is not the direct action of tiaprost (**8**) but Tiaprost-mediated pheromonal action. Rapid and intense sexual behavioural responses in male goldfish when exposed to tiaprost (**8**) treated female emphasizes the pheromonal and olfactory potency of circulating tiaprost (**8**) or its metabolites.

Tiaprost (**8**) (synthetic analogue of $PGF_{2\propto}$) used in the present study has been characterized as a veterinary luteolytic prostaglandin which is 200 times more luteolytic than natural $PGF_{2\propto}$ (Dukes *et al*., 1974).

Cloprostenol (**7**), a synthetic analogue of $PGF_{2\propto}$, used in the present study has been characterized as a luteolytic agent and is more potent than $PGF_{2\propto}$ (Dukes *et al*., 1974). Present results emphasize the potential of cloprostenol (**7**) as an effective induced breeding agent in case of goldfish. In this study cloprostenol (**7**) at the dose of 1.0 μg/gm

and 0.5 μg/gm body weight of female and male respectively stimulated the ovulation in goldfish after 10-12 h of injection. Fertilization and hatching were normal in goldfish injected with cloprostenol (**7**). It is also possible to reinduce the same breeders for multiple spawning with the gap of 30-45 days by using cloprostenol (**7**).

This induced breeding technique can be employed for synchronization of spawning and mass production of goldfish seed production. To our knowledge this is the first report in which cloprostenol (**7**) has been used for induced spawning of goldfish.

The prostaglandin analogues synthesized at National Chemical Laboratory, Pune in the preliminary study failed to induce spawning behaviour, ovulation and spawning in goldfish. More research work is needed for final conclusion.

Although the role of hormones in the control of sexual behaviour in mammalian females is well established (Davidson and Levine, 1972) this is not the case with fish (Liley, 1969). We report here however, the induction of sexual behaviour in female goldfish, *Carassius auratus* by injection of PG analogues like cloprostenol (**7**), tiaprost (**8**) and prosolvin (**9**) etc.

Under normal circumstances, female sexual behaviour is seen only in fish which have recently ovulated and thus the onset of behavioural responsiveness is synchronized with the presence of mature viable eggs ready for oviposition. Most spawning occurred in the 30 min following the spawning run, there after spawning activity declined sharply.

Apart from the application of luteolytic prostaglandins such as tiaprost (**8**) and cloprostenol (**7**) as induced spawning agent for the ornamental fishes, the better understanding of the role of such luteolytic prostaglandins in vitellogenesis, ovulation and spawning of difficult to spawn fishes, can be further applied for the production of fish seed on mass scale.

Overall, the synthetic analogues used in the present studies were more effective than the natural PGs. They were more stable as well as non-toxic to experimental fish. Cost wise they are very cheaper as compared to natural prostaglandins.

Results of the present study concluded that amongst all the PGs (natural and synthetic) used, cloprostenol (**7**) and tiaprost (**8**), were found more effective as induced breeding agent in goldfish. If the presently perfected technique of induced breeding is

employed to increase the production rate it is possible not only to meet the domestic demand but also to enter the international market which will fetch foreign exchange to our country.

Table 2.2. Spawning response of goldfish, *Carassius auratus* with Ovaprim.

Name of compound	Weight of fish Mean ± SE (gm) Female	Male	Dose per gm body weight Female	Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean± SE	Remarks
Control	30.79 ± 6.0	37.84 ±10.0	--	--	No behavioural changes observed	--	--	--	No ovulation observed
Ovaprim	39.77 ±12.90	31.59 ±10.23	0.5 µl	0.125 µl	Pair was sluggish for 3-4 h after injection. Chasing activity started generally after 6-8 h, spawning took place after 10-12 h of injection.	1000 ± 115.47	75 ± 2.88	78.33 ± 4.40	Spawning took place

Values are means ± SE of 3 replicates.

Table 2.3. Effect of natural prostaglandins (PGs) and their precursors on goldfish, *Carassius auratus* breeding.

Name of compound	Weight of fish Female Mean ± SE (gm)	Weight of fish Male Mean ± SE (gm)	Dose per gm body weight Female	Dose per gm body weight Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean± SE	Remarks
Control	28.49 ± 7.39	44.45 ± 6.01	--	--	No behavioural changes observed	--	--	--	No ovulation observed
$PGF_{2\alpha}$	23.76 ± 3.87	26.32 ± 4.50	0.5 µg	0.5 µg	Spawning behavioural changes observed after 6-8 h of injection	--	--	--	,,
,,	29.29 ± 1.14	28.14 ± 3.63	1.0 µg	0.5 µg	Spawning behavioural changes (chasing, courtship) observed after 6-8 h of injection, spawning took place after 10-12 h of injection	200 ± 17.32	20 ± 1.15	(No hatching due to fungal infection)	Spawning took place
Indomethacin + $PGF_{2\alpha}$	18.91 ± 1.97	31.96 ± 1.63	10 µg + 5 µg (same dose for male and female)		No behavioural changes observed	--	--	--	No ovulation observed

Table 2.3. Contd.

Name of compound	Weight of fish Female Mean ± SE (gm)	Male	Dose per gm body weight Female	Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
16-Phenoxytetr-anor $PGF_{2\alpha}$	30.04 ± 0.55	31.90 ± 3.65	1 µg	0.5 µg	No behavioural changes observed	--	--	--	No ovulation observed
16,16-Dimethyl $PGF_{2\alpha}$	26.90 ± 1.98	23.33 ± 2.13	1 µg	0.5 µg	--	--	--	--	’’
PGE_1	27.99 ± 5.19.	25.61 ± 2.7	1 µg	0.5 µg	--	--	--	--	’’
Domperidone + PGE_1	26.30 ± 2.03	24.08 ± 2.99	5 µg + 1µg	5 µg + 0.5µg	--	--	--	--	’’
HCG + PGE_1	30.58 ± 1.68	37.43 ± 1.27	1 IU + 1µg	0.5 IU + 0.5µg	Behavioural changes observed after 1 h of injection, activity continued for 2 h	--	--	--	’’
PGE_2	25.73 ± 1.46	24.66 ± 1.45	1 µg	0.5 µg	No behavioural changes observed	--	--	--	’’

Table 2.3. Contd.

Name of compound	Weight of fish Female Mean ± SE (gm)	Weight of fish Male Mean ± SE (gm)	Dose per gm body weight Female	Dose per gm body weight Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
16,16-Dimethyl PGE_2	28.09 ± 2.06	21.00 ±1.42	1 µg	0.5 µg	Sexual behaviuoral changes observed only in male after 1 h of injection, activity continued for 1 h	--	--	--	No ovulation observed
Arachidonic acid (PG precursor)	32.61 ± 4.73	34.64 ± .82	3 µg	1 µg	Spawning behavioural changes observed after 7h of injection	--	--	--	’’
Indomethacin + arachidonic acid	25.53 ± 2.91	31.09 ±1.90	10 µg + 5 µg (same dose for male and female)		No behavioural changes observed	--	--	--	’’
Eicosapentaenoic acid (PG precursor)	32.54 ± 1.28	26.70 ± 1.43	1 µg	0.5 µg	Sexual behavioural changes observed in male after 6 h of injection	--	--	--	’’

Table 2.4. Effect of prostaglandin analogues (synthetic) on goldfish, *Carassius auratus* breeding.

Name of compound	Weight of fish Female Mean ± SE (gm)	Weight of fish Male Mean ± SE (gm)	Dose per gm body weight Female	Dose per gm body weight Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
Control	22.16 ± 3.99	28.23 ± 1.89	--	--	Spawning behavioural changes observed	183.33 ± 16.66	55 ± 2.88	55 ± 2.88	Natural spawning occurred but number of eggs is very less
Cloprostenol	27.84 ± 6.35	27.04 ± 6.15	0.5 µg	0.5 µg	Spawning behavioural changes observed	--	--	--	No ovulation observed
,,	24.42 ± 3.01	22.09 ± 1.29	1.0 µg	0.5 µg	Spawning behavioural changes observed after 3-4 h of injection, spawning took place generally after 10-12 h of injection	2300 ± 104.08***	80 ± 2.51*	91.66 ± 1.66**	Spawning took place
Indomethacin + Cloprostenol	35.66 ± 10.39	33.41 ± 2.02	10 µg + 1 µg (same dose for male & female)		No behavioural changes observed	--	--	--	No ovulation observed
	25.65 ± 3.72	29.58 ± 5.24	10 µg + 5 µg (same dose for male & female)		No behavioural changes observed	--	--	--	,,

Table 2.4. Contd.

Name of compound	Weight of fish Female Male Mean ± SE (gm)		Dose per gm body weight Female Male		Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
	Female	Male	Female	Male					
Prosolvin	40.62 ± 14.18	36.62 ± 9.08	0.5 µg	0.5 µg	Spawning behavioural changes observed after 1 h of injection, no ovulation within 24 h of injection	--	--	--	No ovulation observed
,,	42.43 ± 5.68	43.83 ± 6.53	1.5 µg	0.5 µg	,,	--	--	--	,,
Tiaprost	40.86 ± 5.06	36.33 ± 2.18	0.5 µg	0.5 µg	Spawning behavioural changes observed in male after ½ h of injection. Female started spawning activity after 1 h of injection, both came to the water surface, female gave repeated jerks,	--	--	--	,,

Table 2.4. Contd.

Name of compound	Weight of fish Female Mean ± SE (gm)	Weight of fish Male Mean ± SE (gm)	Dose per gm body weight Female	Dose per gm body weight Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
					activity continued for 6 h				
	31.65 ± 6.17	30.23 ± 2.12	2.5 µg	0.5 µg	Both male and female showed reproductive behaviour after 3 h of injection. Male actively chased the female and circling	--	--	--	No ovulation observed
	40.06 ± 1.54	31.66 ± 5.48	1.0 µg	0.0 µg	Initially spawning behavioural changes observed after 2-3 h of injection. Within 24 h of injection no ovulation observed. Same pair was kept for further ovulatory changes, ovulation took place in the same pair after 72 h of injection	3333.33 ± 166.67***	81.66 ± 1.66*	91.66 ± 1.66**	Delayed spawning took place

Table 2.4. Contd.

Name of compound	Weight of fish Female Mean ± SE (gm)	Weight of fish Male Mean ± SE (gm)	Dose per gm body weight Female	Dose per gm body weight Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
Prostodin	24.07 ± 5.49	27.88 ± 1.51	1.0 µg	0.5 µg	No behavioural changes observed	--	--	--	No ovulation observed

Data were analyzed by Students ‘t’ test.

Three replicates for each compound,

(P < 0.005)***, (P < 0.01)**, (P < 0.025)*

Table 2.5. Effect of synthetic prostaglandin analogues (prepared at NCL, Pune) on goldfish breeding.

Name of compound	Weight of fish Mean ± SE (gm) Female	Male	Dose per gm body weight Female	Male	Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
Control	28.39 ± 2.89	27.76 ± 5.82	--	--	No behavioural changes observed	--	--	--	No ovulation observed
α-N-R (11)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	,,
β-N-R (12)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	,,
P-M-R (13)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	,,
α-N-K (14)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	,,
β-N-K (16)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	,,

Table 2.5. Contd.

Name of compound	Weight of fish Female Male Mean ± SE (gm)		Dose per gm body weight Female Male		Behavioural changes	No of eggs obtained per female Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean ± SE	Remarks
P-M-K (17)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	No ovulation observed
Misoprostol (19)	39.91 ± 5.57	34.96 ± 5.37	1.0 µg	0.5 µg	,,	--	--	--	,,

Table 2.6. Effect of synthetic prostaglandin analogues on spawning behaviour of goldfish, *Carassius auratus*.

Name of compound	Weight of fish Mean ± SE (gm) Female	Male	Dose per gm body weight Female	Male	Time taken to initiate male response (h)	No of spawning acts/h by female Mean ± SE	Duration of spawning behaviour (h)	Remarks
Control	34.70 ± 2.96	34.82 ± 4.54	--	--	Nil	Nil	Nil	Nil
Prosolvin	44.05 ± 3.64	38.40 ± 5.76	1.0 µg	--	½	55 ± 3.53	4 - 6	Induced spawning behavioural responses were very prominent in both male & female
Tiaprost	31.13 ± 3.66	34.36 ± 3.98	1.0 µg	--	1	50 ± 2.23	5 - 6	Induced spawning behavioural responses in both male & female
Lutylase	30.52 ± 2.17	34.08 ± 4.55	1.0 µg	--	Nil	Nil	Nil	No behavioural changes observed
Cloprostenol	35.80 ± 1.80	42.20 ± 6.20	1.0 µg	--	½	30 ± 2.28	2 - 3	Induced spawning behavioural changes in both male & female

CHAPTER – III

EFFECTS OF PROSTAGLANDINS ON BREEDING OF INDIAN MAJOR CARPS

INTRODUCTION

Indian major carps are economically important food fish in India. Indian major carps catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) are considered to be the best cultivable species among the other carps as they are the quickest growing fishes in terms of flesh return, non-predatory and domesticated in nature and best compatible species.

Indian major carps are riverine fishes which normally breed in rivers during the monsoon months from June to August. They normally do not breed in captivity. Besides, the fish seed collected from the riverine habitats is invariably a mixture of cultivable and non-cultivable species. Often, the proportion of non-cultivable or uneconomic species ranges high in such collections. In the absence of any dependable method to segregate the cultivable fishes from the non-cultivable ones at the early stage, the fish farmers have no choice but rear them to an identifiable size when they could be separated. Thus the fish farmers have to take considerable chance in regard to procurement of fish seed. The rearing of appreciable number of unwanted fish involves wastage of valuable nursery space and expenditure.

There is need to induce Indian major carps to breed in confined waters and thereby ensure a dependable source of quality seed to increase the fish production.
Various induced breeding agents were reported for Indian major carps such as pituitary gland extract, human chorionic gonadotropin (HCG), sex steroid hormones, thyroid hormones, clomiphene citrate, sexovid, tamoxifen, ovaprim (sGnRHA), ovatide and pheromones etc. The related references are already highlighted in chapter I.

Prostaglandins (PGs) appear to be involved in ovulation, milt production, regulation of feeding and sexual behaviour, gonadotropin release, endocrine regulation and other non-reproductive physiologies of fish (Stacey and Goetz, 1982; Sorensen *et al*., 1992). All these functions are associated with the breeding of fish in captivity as well as in field.

No drug commonly used in fish breeding is as effective as prostaglandin so far as cumulative effect of it in general and reproduction in particular is concerned. Not only PGs ($PGF_{2\alpha}$, PGE_2) but also their precursors and derivatives are also effective in regulating pre- and post-reproductive physiology of fish (Amod *et al*., 1992 a, b, c).

Literature survey shows that dietary essential fatty acids containing PG precursors have been used for inducing pre- and post-reproductive physiology of fish (Fremont *et al*., 1984; Tocher and Sargent, 1984).

Food fishes like *Clarias batrachus*, *Heteropneustes fossilis, Labeo* species*, Cirrhinus mrigala, Catla catla,* etc., are abundantly available in greater part of India. Up to date literature survey shows that except few works on PGs in relation to the reproductive physiology of *Heteropneustus fossilis* (Singh and Singh, 1976, 1977) and *Clarias batrachus* (Amod, 1992), no systemic study has been conducted. From investigations on the effects of prostaglandins in fish, it does appear that certain prostaglandins have a stimulatory effect on ovulation.

Barring the work of Stacey and co-workers on the effect of indomethacin on ovulation and the spawning behaviour in female goldfish, *Carassius auratus* no other report seems to be available on any other teleost.

Reports on the effects of natural prostaglandins on the reproductive endocrinology of goldfish promoted us to undertake the research on induced breeding of Indian major carps e.g. rohu, mrigal and catla by using prostaglandin analogues.

Majority of work on prostaglandins in relation to fish reproduction was conducted in USA, Canada and Japan as mentioned in review of literature. Barring a few reports on involvement of prostaglandins in teleosts reproduction no work appears to have been done on profiles of their applicability for Indian major carps breeding.

Although use of natural prostaglandins in fish reproduction research is practiced throughout the world, there are certain inherent problems which have prevented them from being taken widely by fish farmers. The use of PGs is limited to research laboratories only because of the major problems listed below.

1. Varying potencies of the PGs result in unsuccessful spawning and their problems can not be easily rectified since the fish farmer can not measure the potencies of the available PGs.

2. For prostaglandin preparation in injectable form basic equipment like a sophisticated chemical balance (preferably electronic balance of six digit), an accurate pH meter, highly purified chemical for buffer / saline preparation and a deep freeze refrigerator

(-70 °C) etc. are the minimum requirements which are generally not available in majority of fish farms.

3. PGs once dissolved in buffer must be utilized within a short period to avoid spoilage. Generally considerable time is taken in collecting brood fish, locating site of injection etc. and during this period without proper care, the activity of PGs is difficult to retain.

4. Among other reasons PGs high cost, limited shelf-life, decomposition with environmental fluctuations and diminished action in case of heavy metals / pesticides exposed fish are well known.

In view of various known problems listed above, during the use of prostaglandins in aquaculture in a large scale, scientists began to search for stable, long shelf-life and readymade prostaglandin analogues. Systematic approach however, has never been made to develop any suitable process for the synthesis of stable analogues of prostaglandins to widen their use in fields also.

Due to the all above problems, there is need to develop a process to obtain persistently active, stable and readymade prostaglandin analogues and study their efficacy in fish reproduction.

In this chapter attempts were made to study the efficacy of natural as well as commercially available and synthetic stable, long shelf-life prostaglandin analogues in breeding of Indian major carps.

MATERIALS AND METHODS

Animals

Two Indian major carps *Labeo rohita* and *Cirrhinus mrigala* (commonly known as rohu and mrigal respectively) were selected for induced breeding experiments (Fig. 3.1. A and B).

Chemicals

The prostaglandins and other compounds used in the present study for fish induced breeding experiments and their sources are mentioned in Table 3.1.

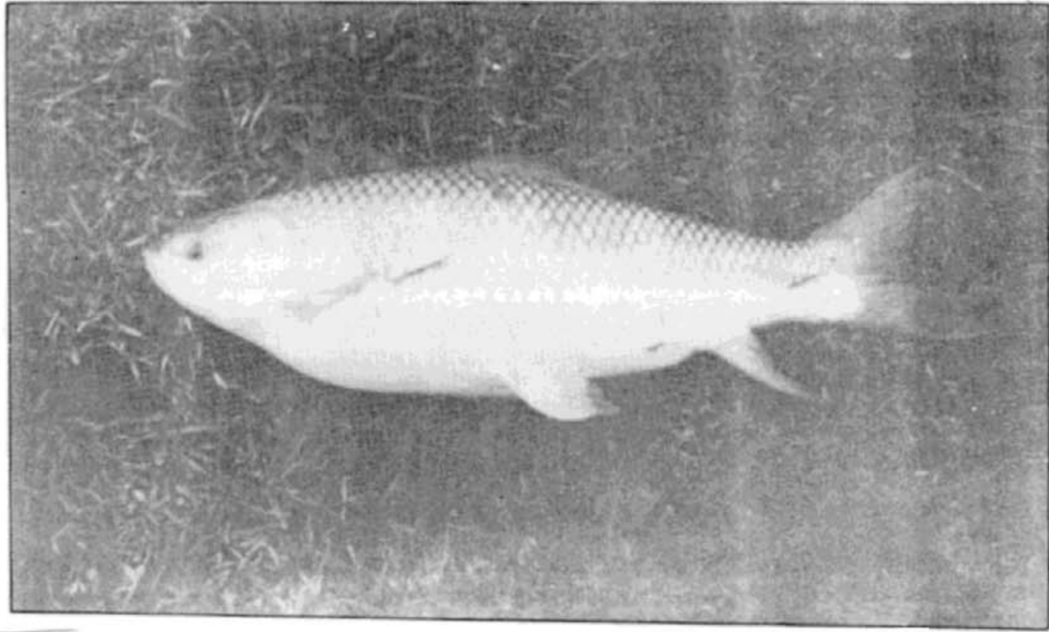
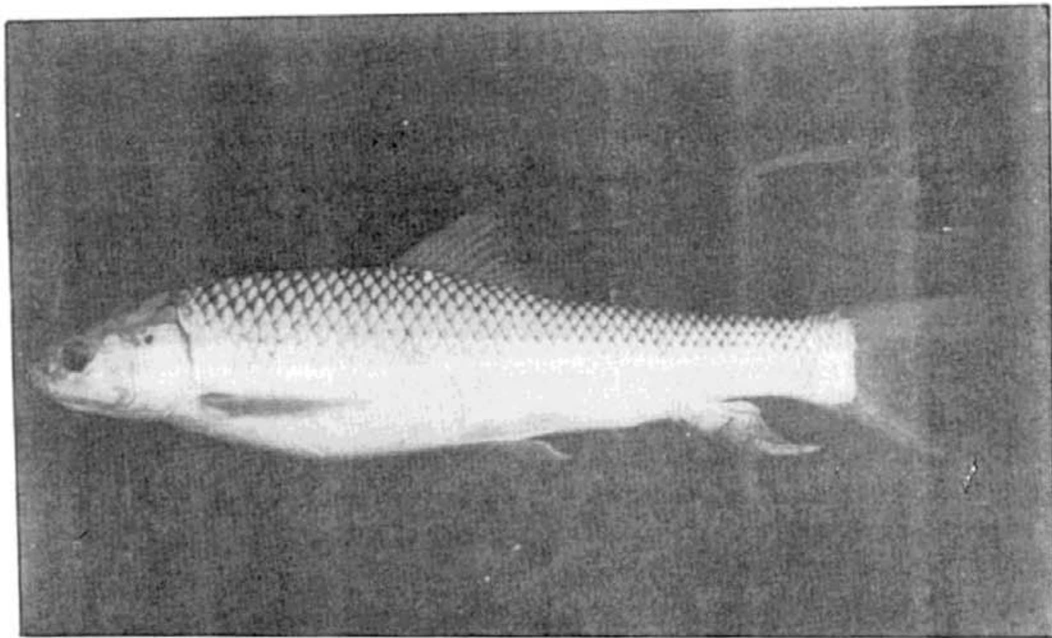


Figure 3.1. Indian Major Carps: **A)** ***Labeo rohita***



B) ***Cirrhinus mrigala***

Table 3.1. List / sources of prostaglandins and other chemicals used for Indian major carps breeding experiments.

Name of compound	Source
Natural prostaglandins	
1. PGE_1	Cayman Chemicals, 690 KMS Place Ann Arbor, MI 48108 USA
2. PGE_2	,,
3. $PGF_{2\alpha}$	,,
Prostaglandin precursor	
1. Arachidonic acid	Cayman Chemicals, 690 KMS Place Ann Arbor, MI 48108 USA
Synthetic prostaglandin analogues	
1. Cloprostenol	Cayman Chemicals, 690 Place Ann Arbor, MI 48108 USA
2. Iliren (Tiaprost)	Hoechst Roussel Vet. GmbHd-65203 Wiesbaden
3. Prosolvin (Luprostiol)	Intervet, International B.V. Boxmeer-Holland
4. Prostodin (Carboprost)	Astra IDL Ltd, Bangalore 560063, India
Prostaglandin analogues synthesized at National Chemical Laboratory, Pune	
α-N-R (**11**)	β-N-R (**12**)
P-M-R (**13**)	

Table 3.1. Contd.

Name of compound	Source
Other compounds	
1. Ovaprim (sGnRHA)	Syndel Laboratories, Vancouver, Canada
2. Human Chorionic Gonadotropin	UNI – SANKYO Ltd. Gaganpahad, Bangalore Road, R. R. Dist Hyderabad-501 323
3. Domperidone	Lupin Laboratories Ltd, 159, C.S.T., R'd, Kalina, Santa Cruz (W) Mumbai – 400 098
4. Thyroid hormone	Glaxo India Ltd.
5. Clomiphene citrate	Local, Pune
6. Pituitary gland	Hadapsar fish farm, Pune (Govt. of Maharashtra).
7. Indomethacin	E. Merck (India) Ltd. Plot No. D-116 MIDC, Trans Thane Creek Area, Thane – Belapur Road, Nerul, Navi Mumbai 400 706

Preparation of compounds for injection

Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$)

Stock solution was prepared by dissolving 1.4 mg of $PGF_{2\alpha}$ in 4.2 ml buffer saline (1 µg/1 µl). Dose range tried was 1 µg to 100 µg/kg body weight for male and 1 µg to 200 µg/kg body weight for female.

Arachidonic acid (AA)

It is the precursor of prostaglandins. Stock solution was prepared by dissolving 6.2 mg of AA in 3 ml ethanol and diluted with 15.6 ml buffer saline (1 µg/3 µl). Doses tried were 33 µg and 300 µg/kg body weight of male and female fish.

Cloprostenol

Cloprostenol is a synthetic analogue of prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$). Stock solution available (10 mg/ml ethanol) was used for further dilution with fish saline to get a solution of concentration 1 µg/2 µl. Dose used for male was 5 µg to 100 µg/kg body weight and for female 5 µg to 125 µg/kg body weight.

Human Chorionic Gonadotropin (HCG)

5000 IU of HCG was dissolved in 10 ml saline (500 IU/ml). Threshold dose range used was 100 IU to 200 IU/kg body weight of fish.

Prostodin

Prostodin is a synthetic analogue of prostaglandin-$F_{2\alpha}$ commercially available in ready to use form. 1 ml prostodin contains 250 µg carboprost (1 µg/4 µl). Doses tried were 10 µg and 20 µg/kg body weight of fish.

Iliren

This is a commercially available ready to use synthetic analogue of prostaglandin-$F_{2\alpha}$. 1 ml iliren contains 0.150 mg tiaprost. Dose range used was 5 µg to 125 µg/kg body weight of fish.

Prosolvin

It is a synthetic prostaglandin-$F_{2\alpha}$ analogue. Prosolvin is a clear solution of luprostiol (7.5 mg/ml) commercially available for injection. Dose range tried was 25 μg to 50 μg/kg body weight for male and 25 μg to 100 μg/kg body weight for female fish.

Ovaprim

Ovaprim is a known induced breeding agent for fish. Dose used for male was 0.2 ml/kg body weight and 0.3 - 0.4 ml/kg body weight of female of rohu and mrigal.

Domperidone

It is a dopamine synthesis inhibitor. Dopamine inhibits the release of gonadotropin releasing hormone. Stock solution was prepared by dissolving 25 mg of domperidone in 1 ml dimethyl formamide (DMF) and 0.5 ml saline (17 μg/μl). Doses tried were 1 mg/kg and 5 mg/kg body weight of male as well as female fish.

Clomiphene citrate

Stock solution was prepared by dissolving 12.5 mg of clomiphene citrate in 1 ml saline (1 mg/80 μl). Dose used was 300 μg/kg body weight of fish. Same dose was used for male and female fish.

Thyroxine sodium

Stock solution was prepared by dissolving 0.1 mg of thyroxine in 1 ml saline. Solution was centrifuged and supernatant obtained was collected and used for injection. Dose used was 0.1 mg/kg body weight of fish.

Synthetic prostaglandin analogue **11** (α-N-R)

It is a synthetic analogue of prostaglandin-$F_{2\alpha}$, synthesized at National Chemical Laboratory, Pune. Stock solution was prepared by dissolving 300 μg of **11** in 1 ml solvent (0.5 ml ethanol + 0.5 ml saline) (1 μg/3.3 μl). Dose range used was 5 μg to 100 μg/kg body weight of fish.

Synthetic prostaglandin analogue **12** (β-N-R)

It is another synthetic analogue of prostaglandin-$F_{2\alpha}$, synthesized at National Chemical Laboratory, Pune. Stock solution: 500 μg of **12** were dissolved in 1 ml solvent (0.5 ml ethanol + 0.5 ml saline) (1 μg/2 μl). The dose range used was 10 μg to 50 μg/kg body weight of fish.

Synthetic prostaglandin analogue **13** (P-M-R)

It is also a synthetic analogue of prostaglandin-$F_{2\alpha}$ and it was synthesized at National Chemical Laboratory, Pune. Stock solution was prepared by dissolving 1 mg of **13** in 0.5 ml ethanol and diluted with 0.5 ml saline (1 μg/1 μl). Dose range used was 10 μg to 200 μg/kg body weight of male and female fish.

Selection of brooders

Healthy disease free brooders of rohu and mrigal of the average weight 1-5 kg and 2-4 years age groups were selected.

Collection of broodstock

Broodstock was collected from Katraj Lake, Pune, Maharashtra in the month of March – April (suitable period of broodstock collection) and stored at Hadapsar Fish Farm, Pune (Govt. of Maharashtra). Drag net was used for netting (to avoid gill injury). Broodstock was carried in tarpaulin cistern tank (3 m height x 2 m diameter x 2 m height) which was fixed in tempo and filled with well water.

Brooders stocking and their maintenance

Broodstock was stored at Hadapsar Fish Farm, Pune in two earthen ponds (80 m length x 40 m diameter x 1.5 m depth). Stocking was done at the rate 1500 –2000 kg/ha.

Manuring

Initial manuring of brood stocking pond was done three weeks prior to stocking of fishes with cattle dung (20,000 kg/ha) or compost manure (10,000 kg/ha). A prophylactic quick lime treatment (200 kg/ha) was given to the pond 7 days before the

initial fertilization. Monthly dose of raw cattle dung (2000 kg/ha) was also followed (Chonder, 1994).

Supplementary feeding

Broodstock was fed on rice bran and oil cake (1:1) daily at the rate 3 - 4 % of their body weight from post-spawning months onwards and then 1-2 % of their body weight about one month before spawning and finally with a stoppage of feeding for a week before the actual breeding experiments started (Chonder, 1994).

Collection of carp pituitary glands

Pituitary glands were collected from Indian major carp females in the months of May-June. The glands were collected from the cut fish heads only. The head cut out by means of a bone cutter and removed and the foramen is clear seen. The gray matter and the fatty substances over the brain were removed by blunt end of the forceps. The anterior end of the brain was first detached by means a pair of fine forceps inserted into the foramen and then the entire brain was lifted up carefully. The pituitary gland was then picked up carefully by means of tweezers.

The pituitary glands were weighed and kept in absolute alcohol immediately after collection. Each gland was kept in a separate phial with marked. After 24 h, glands were washed with absolute alcohol and kept again in the phial with fresh absolute alcohol and stored in a cool shady place at room temperature or under refrigeration until needed (Chondar, 1994).

Pituitary gland extract preparation

For the preparation of pituitary gland extract, pituitaries obtained from mature females of Indian major carps were homogenized in 0.6% NaCl and the extract was obtained and used for injection.

Experimental Methodology

The following aspects were studied to ascertain the role of prostaglandins in Indian major carps induced breeding: 1) Behavioural responses 2) Induced breeding.

Induced breeding experiments were carried out on rohu and mrigal at two places in India: **1**) Hadapsar Fish Farm, Pune (Govt. of Maharashtra) and **2**) Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Orissa.

Induced breeding experiments were conducted in the month of June-August (which is the normal breeding period of these fishes) because in this period heavy rainfall and cool environment appeared to be conducive to spawning. In hot sunny days fishes generally did not spawn.

Selection of brooders for experiments

Selection of brooders is very important in induced breeding experiments which is effectively carried out in monsoon season. Brooders were identified and selected for the experiment on the basis of following morphological characters.

Male: Males were identified by the roughness of the dorsal surface of the pectoral fin, milt oozed through the vent on slight pressure on the abdomen which was almost flat and the vent was not swollen.

Female: Females were identified by the very smooth pectoral fin and bulging abdomen with swollen reddish vent.

Brooders (rohu and mrigal) selected for experiments were of the average weight 1-5 kg and 2-5 years age group from common broodstock. The selected brooders were kept in hapa or Chinese breeding pool for acclimatization. They were made to fast for 6-8 h before injection to release fecal matter out side the body and easy for spawning.

Spawning test

Before administration of compound to female fish, first it was checked whether the female was ready for spawning or not by following test. About 100 eggs from Indian major carps female were removed by means of a plastic catheter to determine the stage of nuclear migration. Eggs were fixed in the fixative containing 30 parts absolute alcohol and 70 parts glacial acetic acid for 4-5 minutes and then the portion of germinal vesicle was visualized under microscope and observed. If nucleus goes towards the animal pole then the female is ready for spawning. Females showing this test were selected for experiments (Gupta *et al*., 1990).

Figure 3.2. Harvest of carp from stoking pond for experiments.

Figure 3.3. Selections of brooders for breeding experiments.

Methods of breeding

Most of the breeding experiments were conducted in cloth hapas (2.5 x 1.2 x 1 m, Fig. 3.5). Cloth hapas were fixed in earthen ponds or Bangla bundh (15 – 20 m long and 5 – 8 m broad). The showers / sprinklers provided created an artificial rainy condition in the pond. The sprinklers had major effect in increasing oxygen content of water and helped in maintaining the water temperature. Some experiments were also conducted in Chinese breeding pool (6 – 8 m diameter & 1.2 - 1.5 m height) with sprinklers provided and Chinese hatchery (Fig. 3.6, 3.7, 3.8 and 3.9). Community breeding experiments were also conducted in Chinese breeding pool (Fig. 3.10).

The brooder males and females were weighed individually with hand net and the actual weight of each fish was determined by deducting the known weight of the hand net from the total weight.

Methods of injection

Required amount of prostaglandins were dissolved in ethanol and further dilutions were made in fish saline solution (0.6% NaCl). Brooders were one by one netted out in hand net. They were placed on a table and carefully injected avoiding wriggling movements.

Injection was administered intramuscularly at the base of caudal fin above the lateral line. Some times brooders were also injected intra-peritoneal at the base of pectoral fin (Fig. 3.4). Injected brooders were released pair wise (1 male and 1 female) in single hapa or Chinese circular hatchery (Fig. 3.11). Experimental brooders were observed for 36 h after injection at the interval of 6 h with respect to behavioural changes, ovulation and spawning etc. (Fig. 3.12). Ovulation was checked by manual pressure on the abdomen. Ovulation responses were catagorised in two groups. The fishes that gave a few ovulated oocytes (less than 1% of body weight) which can be considered as an abdominal response were referred to as "partially ovulated fish" while the fish which gave more than 3% of their body weight of ovulated oocytes were referred to as "normal ovulated fish". When these ovulated fish spawned they were referred to as "spent fish" (Jalabert *et al*., 1977). For hatching of eggs, incubation or hatching pool was used which was a circular cemented tank measuring 3 - 4.5 m in diameter and 1.2 – 1.5 m deep (Chondar, 1994).

Figure 3.4. Administration of injection to the brooders.

Figure 3.5. A breeding hapa fixed in water for experiments.

Figure 3.6. Chinese circular spawning pool for fish breeding experiments.

Figure 3.7. Plastic portable Chinese type carp spawnery and hatchery (West Bengal model "Amitava").

Figure 3.10. Community fish breeding experiments in Chinese breeding pool

Figure 3.11. Injected breeders in a hand net being introduced in portable circular hatchery.

Prostaglandins were tested individually or in combination with domperidone (dopamine antagonist), Ca^{++} and primed with HCG and pituitary gland extract. Control fish received the saline (0.6% NaCl).

Ovaprim (standard fish induced breeding agent) was used in the present study for comparisons with other compounds and for standardization of the set up of hapa breeding method.

The following methods were used for assessment of results.

Counting of eggs

The eggs of Indian major carps are non-floating, non-adhesive in nature and round in shape. The eggs were collected in bucket from hapa. The eggs in the bucket were then put into a rectangular piece of closed meshed mosquito net or fine meshed cloth held in water. The cloth was then slowly lifted out of the water so as to allow the water to drain off. The eggs were then measured in a beaker, mug or cup of known volume. The total volume and number of eggs laid can easily be calculated from the known volume and number of eggs of the sample beaker (mug or bati). It may be represented by the following formula (Chondar, 1994).

$$\text{Total number of eggs laid (approx)} = \text{Average number of eggs in each sample beaker (or mug)} \times \text{Number of beakers (or mugs) of eggs}$$

Fertilization rate

Fertilized eggs of Indian major carps are transparent, oval or round in shape. Unfertilized eggs are opaque. The fertilization rate was calculated through random sampling by examining 3-4 samples from each breeding hapa / pool by using following formula.

$$\%\ \text{Fertilization} = \frac{\text{Average number of fertilized eggs in a sample}}{\text{Average total number of eggs in a sample}} \times 100$$

Hatching percentage

The following formula was used for calculating the hatching percentage.

$$\text{Hatching \%} = \frac{\text{Total number of spawn}}{\text{Total number of fertilized eggs}} \times 100$$

After injection, breeding pairs were released in breeding hapa or Chinese breeding pool. Single pair was released in each hapa. Observations were taken every 3 h and checked for ovulation and spawning.

Spent fish rearing

After experiments, fishes were given dip bath of 1 ppm potassium permanganate solution for half an hour, followed by dip bath in freshwater and released back into the stocking pond. Same brooders were used for next year.

Details of water quality for experiments

The physico-chemical parameters of water checked during the period of experiments ranged as temperature 27-29 ^{o}C, pH 6-7.5 and dissolved oxygen content (DO) 6-7 ppm measured by using mercury-in-Celsius thermometer (0°C to 50°C), grip pH meter and Aquamerk kit respectively.

Statistical Method

Standard errors for means were derived by the usual formula.

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

SE = Standard error

SD = Standard deviation

n = Number of replicates

Figure 3.12. Continuous observations were taken after injection.

RESULTS

The results of various trials of prostaglandins and their analogues on breeding of Indian major carps are summarized in tables appended to this chapter.

Spawning response of *Cirrhinus mrigala* to ovaprim and carp pituitary gland extract

Ovaprim

As shown in Table 3.2 the effective dose of ovaprim was found to be 0.2 ml/kg and 0.3 ml/kg body weight of male and female respectively which induced spawning after 10-12 h of injection. Number of eggs obtained 140000 ± 8660 with fertilization (%) 95 ± 1.52 and percentage of hatching 80 ± 5.77.

Carp pituitary gland extract

Carp pituitary gland extract (CPE) was injected twice in two different doses to female carps-first dose was given 3 mg/kg body weight, followed by second injection 6 mg/kg body weight and the time interval between the two injections was 6 h. In case of male, one injection at a dose 3 mg/kg body weight was given at the time of second injection to female. Spawning occurred after 6 h of second injection. Number of eggs obtained were 80000 ± 5773 with fertilization (%) 90 ± 2.88 and percentage of hatching 75 ± 2.30 (Table 3.2).

Threshold dose of carp pituitary extract (CPE) for *Cirrhinus mrigala*

Data presented in Table 3.3 demonstrate that $1/6^{th}$ of spawning dose of pituitary extract (1.5 mg/kg and 0.5 mg/kg body weight of female and male fish respectively) and $1/8^{th}$ of spawning dose of pituitary extract (1.12 mg/kg and 0.37 mg/kg body weight of female and male respectively) did not show ovulation and spawning in mrigal female. Therefore these doses ($1/6^{th}$ and $1/8^{th}$ of spawning dose) were taken as the threshold or subminimal dose, which was sufficient to prime up the ovary (Table 3.3).

Human Chorionic Gonadotropin (HCG)

HCG is used for early ripening of gonads in Indian major carps and also as a spawning inducing agent. In the present study we used HCG for priming dose to enhance the spawning. The optimum dose for Indian major carps spawning is 100 – 1100 IU/kg body weight. We tried here the dose range 200 IU to 1000 IU/kg body weight but ovulation and spawning was not observed.

Effects of natural prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) on breeding of *Cirrhinus mrigala*

The Table 3.4 shows the effects of natural prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) on breeding of mrigal. Individual $PGF_{2\alpha}$ dose range tried was 1 μg to 30 μg/kg body weight. Among the selected doses in case of 2 μg/kg body weight of fish (same dose for male and female), spawning behaviour was observed after 6-8 h of injection but no ovulation was observed within 24 h of injection.

$PGF_{2\alpha}$ was also studied in combination with domperidone. Dose range tried was 5 μg to 200 μg/kg body weight of $PGF_{2\alpha}$ with 5 mg/kg body weight of domperidone. Among the selected doses, in case of $PGF_{2\alpha}$ (20 μg and 200 μg/kg body weight) + domperidone (5 mg/kg body weight) only egg plug effect was observed (Table 3.4).

$PGF_{2\alpha}$ (10 μg/kg body weight) was tried in combination with domperidone (5 mg/kg body weight) and Ca^{++} (300 μg/kg body weight of fish) which resulted in female showing egg plug effect after 12 h of injection (Table 3.4).

$PGF_{2\alpha}$ was studied in combination with carp pituitary extract (1/6th of spawning dose) followed by second injection of $PGF_{2\alpha}$ (20 μg/kg body weight) and the time interval between the two injections was 6 h. Egg bound effect was observed after 8 h of second injection and no ovulation was observed (Table 3.4).

$PGF_{2\alpha}$ was tried in combination with 1/6th of carp pituitary extract (CPE) spawning dose and domperidone. 1/6th of CPE spawning dose was followed by injection of $PGF_{2\alpha}$ (10 μg/kg body weight) and domperidone (5 mg/kg body weight) after the time interval of 6 h. Egg plug effect was observed after 8 h of second injection (Table 3.4).

$PGF_{2\alpha}$ was also tried with pituitary priming (1/6th of spawning dose) followed by second injection of Ca^{++} (300 μg/kg body weight) + domperidone (5 mg/kg body weight) and $PGF_{2\alpha}$ (20 μg/kg body weight) after an interval of 6 h. Egg bound effect was observed after 6 h of second injection (Table 3.4).

In another experiment, first dose was pituitary priming (1/6th of spawning dose) followed by second injection of thyroxine (0.1 mg/kg body weight) + domperidone (5 mg/kg body weight) and $PGF_{2\alpha}$ (25 μg / kg body weight) and the time interval between the two injections was 6 h. Egg bound effect was observed after 6 h of second injection.

But no ovulation and spawning were observed within 24 h of injection (Table 3.4).

Effects of prostaglandin analogues on breeding of *Cirrhinus mrigala*

The data presented in Table 3.5 shows the effects of various PG analogues on breeding of mrigal.

Cloprostenol

Individual cloprostenol dose range tried was 10 µg to100 µg/kg body weight. Almost in all experiments, egg bound effect was observed after 12 - 24 h of injection, no ovulation was observed.

Cloprostenol was also studied in combination with domperidone. Cloprostenol dose range tried was from 5 µg – 125 µg/kg body weight + domperidone (5 mg/kg body weight). Among the selected doses, egg bound effect was observed at the doses 50 µg, 100 µg and 125 µg/kg body weight after 12 h of injection but ovulation could not be induced.

Cloprostenol was tried in combination with domperidone. Among the various experiments cloprostenol (75 µg/kg body weight) and domperidone (1 mg/kg body weight) resulted in partial spawning.

Cloprostenol was tried in combination with Ca^{++} and domperidone. Cloprostenol (30 µg/kg body weight), Ca^{++} (300 µg/kg body weight) and domperidone (5 mg/kg body weight) resulted in egg plug effect after 14 h of injection.

Cloprostenol was also tried in combination with human chorionic gonadotropin (HCG) and domperidone. First dose HCG of (200 IU/kg body weight) was followed by second injection of domperidone (5 mg/kg body weight) and cloprostenol (75 µg/kg body weight) after an interval of 6 h. This combination induced an egg plug effect after 8 h of second injection (Table 3.5).

Tiaprost

Individual tiaprost dose range tried was 5 µg to 125 µg/kg body weight of fish. Among the selected doses, tiaprost at the dose 75 µg/kg body weight induced behavioural changes in female (restlessness and jumping) after 3 h of injection but could not induce ovulation within 24 h of injection.

With the dose of 100 μg/kg body weight egg bound effect was observed after 12 h of injection but no ovulation was observed. Tiaprost (25 μg to 50 μg/kg body weight) tried in combination with domperidone (5 mg/kg body weight) did not show any effect.

Tiaprost was also studied in combination with HCG priming. The first dose for female was HCG (200 IU/kg body weight) followed by second injection of tiaprost (50 μg/kg body weight). In case of male, one injection of tiaprost at a dose 50 μg/kg body weight was given at the time of second injection to female. Male exhibited spawning behaviour (chasing and courtship) after 3 h of injection and released milt into the water. The activity continued for 3 h but the female did not respond (Table 3.5).

Prosolvin

The dose ranges of prosolvin tried were from 25 μg to 100 μg/kg body weight of fish. No behavioural changes and ovulation were observed within 24 h of injection. Prosolvin was also tried in combination with domperidone but no ovulation was observed within 24 h of injection (Table 3.5).

Carboprost

Individual carboprost doses tried were 10 μg and 20 μg/kg body weight of fish. No behavioural changes were observed within 24 h of injection (Table 3.5).

Effect of synthetic prostaglandin analogues (prepared at NCL, Pune) on breeding of *Cirrhinus mrigala* (Table 3.6)

α-N-R (11)

The dose range tried was 5 μg to 100 μg/kg body weight of fish. Among the selected doses, 10 μg and 50 μg/kg body weight of fish resulted in egg plug effect after 14 h of injection but no ovulation was observed within 24 h of injection.

β-N-R (12)

Dose range tried was 10 μg to 50 μg/kg body weight of fish. The dose of 20 μg/kg body weight could induce egg plug effect after 18 h of injection. (Table 3.6).

P-M-R (13)

The dose range tried was 10 μg to 200 μg/kg body weight of fish. Among the selected doses, egg plug effect was observed at the dose of 20 μg/kg body weight. No behavioural changes and ovulation was observed within 24 h of injection. P-M-R was also tried in combination with domperidone but it could not induce ovulation within 24 h of injection

No side effects were observed in case of any prostaglandins during and after the experiments on fishes (Table 3.6).

Spawning response of *Labeo rohita* to ovaprim and carp pituitary gland extract

Ovaprim

As shown in Table 3.7 the effective dose of ovaprim was found to be 0.2 ml/kg and 0.3 ml/kg body weight of male and female respectively which induced spawning after 10-12 h of injection. Number of eggs obtained 240000 ± 4041 with fertilization (%) 90 ± 2.88 and hatching percentage 90 ± 2.30 (Table 3.7).

Carp pituitary gland extract

Carp pituitary gland extract (CPE) was injected twice in two different doses to female carps - first dose was given 3 mg/kg body weight followed by second injection 6 mg/kg body weight and the time interval between the two injections was 6 h. In the case of male, one injection at a dose 3 mg/kg body weight was given at the time of second injection to female. Spawning occurred after 6 h of second injection. Number of eggs obtained 130000 ± 7637, fertilization (%) 85 ± 1.15 and percentage of hatching 80 ± 7.63 (Table 3.7).

Effects of natural prostaglandins and their precursors on breeding of *Labeo rohita*

$PGF_{2\alpha}$

$PGF_{2\alpha}$ was studied for induced breeding in the dose range of 1 µg to 30 µg/kg body weight of fish. Among the selected doses, 20 µg/kg body weight resulted in egg plug effect in female after 18 h of injection but no ovulation was observed within 24 h of injection.

$PGF_{2\alpha}$ was also tried in combination with domperidone. No ovulation was observed within 24 h of injection (Table 3.8).

Arachidonic Acid

In case of arachidonic acid, doses tried were 33 µg and 300 µg/kg body weight of fish. No ovulation was observed within 24 h of injection (Table 3.8).

Effects of synthetic prostaglandin analogues on breeding of *Labeo rohita* (Table 3.9)

Tiaprost

Tiaprost was injected in the dose range of 5 µg to 50 µg/kg body weight of fish. Among the selected doses, 50 µg/kg body weight of fish resulted in egg plug effect after 18 h of injection. No ovulation was observed within 24 h of injection. Tiaprost was also tried in combination with domperidone at different doses but no ovulation was observed.

Tiaprost was tried with HCG priming where in first dose of HCG (200 IU/kg body weight of fish) was followed by injection of tiaprost (50 µg/kg body weight of female) after the time interval of 6 h. In the case of male, one injection of tiaprost (50 µg/kg body weight) was given at the time of second injection for female. After 18 h of injection partial ovulation was observed. Eggs were released outside the body when belly was pressed.

Various other combinations of HCG and tiaprost were studied for induced breeding but no spawning was observed.

No side effects of any prostaglandins on *Labeo rohita* were observed during and after the experiments. Follow up was continued for 48 h.

DISCUSSION

Induced breeding of Indian major carps is indeed an extremely important aspect of aquaculture as these fishes do not normally breed in captivity even if all environmental conditions are optimum. Besides, they are in great demand as food fish in India. Indian major carps breed only once a year during the monsoon which coincides with the breeding season of the majority of Indian teleosts. The precise nature of the monsoon cue is still unknown.

Various induced breeding agents such as carp pituitary extract (Chaudhuri and Alikhuni, 1937), ovaprim (Peter *et al*., 1988) etc. are known. Although prostaglandins (PGs) have been reported as one of the carp breeding inducing agents (Tripathi and Khan, 1990) so far there is no report of such breeding on large scale in India or abroad. Role of PGs in fish reproduction is well illustrated but no references are available on PGs for Indian major carps breeding. The use of natural prostaglandins is very much limited due to their high cost and short shelf-life period.

We made maiden effort to study various synthetic stable PG analogues (commercially available as well as synthesized at NCL, Pune) to induce spawning in Indian major carp females. Various methods were employed to test the efficacy of PGs in induced breeding of Indian major carps. Pituitary gland extract and ovaprim induced full spawning in *Labeo rohita* and *Cirrhinus mrigala* in the present study with normal egg count, fertilization and hatching rate (Table 3.2 and 3.7).

In the present study, threshold dose of carp pituitary extract for *C. mrigala* was found to be $1/6^{th}$ of spawning dose which was sufficient to prime up of the ovary. Higher doses (above $1/6^{th}$ of spawning dose) induced ovulation and spawning (Table 3.3).

Individual $PGF_{2\alpha}$ was checked for induced breeding of *C. mrigala* in the present work. It was found that at the lower doses of $PGF_{2\alpha}$ (2 μg/kg body weight of fish), spawning behavioural changes were observed in fish but no ovulation and spawning was observed (Table 3.4).

Secretion of gonadotropin in teleosts is regulated by dual neuro-hormonal system, GnRH stimulates GtH secretion while dopamine inhibits the action of GnRH and

thus using drugs which block dopamine action in combination with LHRHA better results were obtained in ovulation (Peter, 1982; Lin *et al*., 1986; Sokolowska *et al*., 1985). The "Linpe" method (sGnRHA and dopamine antagonist) has been used for induced ovulation in case of a number of cultured fish (Peter *et al*., 1988).

In the present study, $PGF_{2\alpha}$ was tested by Linpe method i.e. $PGF_{2\alpha}$ was used in combination with domperidone (dopamine antagonist) which could induce egg plug effect in various groups, generally after 12 h of injection (Table 3.4).

Jamaluddin *et al*. (1989) reported that addition of Ca^{++} to the Linpe method stimulates GtH release from fish pituitary cells. To get an idea with this line we have performed experiments by Linpe method plus Ca^{++} (300 μg/kg body weight) but only egg plug effect was observed in female after 12 h of injection and no ovulation was observed within 24 h of injection.

As per earlier reports (Sneed and Dupree, 1961; Ramaswami and Lakshmanan, 1958) thyroid hormones play a role in oocytes maturation. Thyroid stimulating hormones (TSH) enhance the effect of HCG or fish pituitary extract on induction of ovulation in goldfish and Indian major catfish.

In the present study experiments were done with doses of $PGF_{2\alpha}$ (25 μg/kg body weight) in combination with $1/6^{th}$ CPE spawning dose, domperidone (5 mg/kg body weight) and thyroxine (0.1 mg/kg body weight). No ovulation was observed within 24 h of injection. $PGF_{2\alpha}$ was also tried in combination with pituitary priming which resulted in egg plug effect. The exact reason of egg plugging was not found (Table 3.4).

Cloprostenol is a luteolytic synthetic analogue of $PGF_{2\alpha}$ and was checked for breeding in mrigal in various ways such as Linpe method, cloprostenol + Ca^{++}, cloprostenol + pituitary priming etc. Among the selected doses in case of cloprostenol (75 μg/kg) and domperidone (1 mg/kg body weight of fish) was observed 50% eggs were loosed in the ovary when female was dissected after 12 h of injection. These results could give some clue to modify the doses of PGs for induced breeding of Indian major carps. Egg plug was observed with other selected doses of cloprostenol (Table 3.5).

Human chorionic gonadotropin (HCG) has been used to induce final maturation of oocytes and also as a tool in aquaculture (Donaldson and Hunter, 1983; Peter *et al*., 1988).

Tiaprost is a synthetic analogue of $PGF_{2\alpha}$ used in the present study. Tiaprost was checked in various methods (as mentioned in $PGF_{2\alpha}$) for induced breeding. Among the selected doses, it was found that when female was injected with HCG (200 IU/kg body weight) followed by a second injection of tiaprost (50 μg/kg body weight) after a time interval of 6 h and the male was injected with tiaprost (50 μg/kg body weight) at the time of second injection on female, male showed spawning behaviour followed by milt release but female did not show any response (Table 3.5).

Further trials are required to standardize the dose for females. Present results are similar to our previous results obtained on goldfish with tiaprost (Chapter 2). In other doses of tiaprost, egg plug effect was observed.

Prosolvin, a luteolytic prostaglandin-$F_{2\alpha}$ analogue used in the present study, did not induce breeding in mrigal. More trials are required for conclusion.

Carboprost used in the present study is a synthetic analogue of $PGF_{2\alpha}$, preliminary trials with the drug could not give any baseline data and further trials are required.

Synthetic analogues (prepared at NCL, Pune) α-N-R (**11**), β-N-R (**12**) and P-M-R (**13**) in the preliminary trials showed egg plug effect in females, no changes were observed in males. Trials are not sufficient to final conclusions of these synthetic PG analogues (Table 3.6).

Labeo rohita

Few selected prostaglandins were tried on *L. rohita* for induced breeding. Natural $PGF_{2\alpha}$ showed egg plug effects similar to *C. mrigala*.

Arachidonic acid (AA) is a precursor of prostaglandin synthesis and in the present study it could not induce the ovulation (Table 3.8).

Tiaprost in combination with HCG priming (first dose of HCG 200 IU/kg body weight) followed by second injection of tiaprost (50 μg/kg body weight) to female after the time interval of 6 h and in case of male only tiaprost (50 μg/kg body weight) at the time of second injection to female, induced partial ovulation after 18 h of injection.

The present study indicated that among the selected prostaglandins, tiaprost and prosolvin could give encouraging results like induced spawning behavioural changes. However, trials are not sufficient to conclude the efficacy of these compounds as induced breeding agents.

Synthetic prostaglandin analogues were found to be more stable than the natural prostaglandins. They can be stored under refrigeration whereas most of the natural PGs are required to be preserved at –70 ^{o}C.

No side effects were observed of any prostaglandins on Indian major carps during and after the experiments.

Although the present results are encouraging, use of synthetic luteolytic prostaglandin analogues for induced ovulation and spawning in Indian major carps needs further verification.

Table 3.2. Spawning responses of *Cirrhinus mrigala* to carp pituitary extract and ovaprim.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
Carp pituitary gland extract (CPE)	1.4 ± 0.1	1.4 ± 0.2	3 mg 6 h gap + 6 mg	3 mg	Spawning behavioural changes was observed after 8 h of injection, spawning took place after 12 h	80,000 ± 5773	90 ± 2.88	75 ± 2.30	Spawning took place
Ovaprim	1.5 ± 0.1	1.3 ± 0.1	0.3 ml	0.2 ml	Spawning behavioural changes observed after 6 h of injection, spawning took place after 10-12 h of injection	1,40000 ± 8660	95 ± 1.52	80 ± 5.77	Spawning took place

Table 3.3. Experiments conducted to work out threshold dose of carp pituitary gland extract for *Cirrhinus mrigala*.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
Carp pituitary gland extract (full spawning dose)	1.4 ± 0.11	1.7 ± 0.1	3 mg 6 h gap + 9 mg	3 mg	Spawning took place after 9-12 h of injection	1,80000 ± 11547	90 ± 2.88	85 ± 2.88	Spawning took place
½ of spawning dose	2.0 ± 0.28	2.0 ± 0.1	4.5 mg	1.5 mg	Spawning took place after 9-12 h of injection	1,40000 ± 8660	95 ± 1.52	80 ± 5.77	Spawning took place
1/4th of spawning dose	1.2 ± 0.11	1.9 ± 0.1	2.25 mg	0.75 mg	Spawning took place after 12 h of injection	60,000 ± 5000	50 ± 5	70 ± 5.77	Spawning took place
1/6th of spawning dose	0.76 ± 0.06	0.7± 0.05	1.5 mg	0.5 mg	Female egg plugged after 24 h of injection	--	--	--	No spawning observed
1/8th of spawning dose	0.5 ± 0.0	0.7± 0.05	1.12 mg	0.37 mg	Female egg plugged within 12 h of injection	--	--	--	No spawning observed

Table 3.4. Effects of natural prostaglandins (PGs) on breeding of *Cirrhinus mrigala*.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$)	1.66 ± 0.08	1.76 ± 0.1	1.5 µg	1.0 µg	No changes observed within 24 h of injection	--	--	--	No spawning observed
2)	2.36 ± 0.1	2.2 ± 0.1	2 µg	2 µg	Spawning behavioural changes observed after 6-8 h of injection, no ovulation within 24 h of injection	--	--	--	Only spawning behavioural changes observed
3)	2.3 ± 0.1	2.1 ± 0.2	8 µg	4 µg	No changes observed	--	--	--	No spawning observed
4)	1.2 ± 0.1	0.8 ± 0.1	10 µg	10 µg	Belly of female became soft	--	--	--	,,
5)	1.0 ± 0.1	0.8 ±0.1	20 µg	20 µg	No changes observed	--	--	--	,,

Table 3.4. Contd.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
	6)	1.2 ± 0.1	1.0 ± 0.1	30 µg	30 µg	No changes observed within 24 h of injection	--	--	--	No spawning observed
$PGF_{2\alpha}$ + Domperidone (DOMP)		1.23 ± 0.1	0.83 ± 0.1	5 µg + 5 mg	5 µg + 5 mg	,,	--	--	--	,,
	2)	0.83 ± 0.1	0.7 ± 0.1	10 µg + 5 mg	10 µg + 5 mg	Belly of female became soft, no ovulation within 24 h of injection	--	--	--	No spawning observed
	3)	1.0 ± 0.1	0.7 ± 0.1	15 µg + 5 mg	15 µg + 5 mg	Belly of female became soft, no ovulation within 24 h of injection	--	--	--	,,

Table 3.4. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
4)	1.0 ± 0.2	1.0 ± 0.1	20 µg + 5 mg	20 µg + 5 mg	Female egg plugged within 24 h of injection	Plugged	--	--	No spawning observed
5)	1.2 ± 0.1	0.6 ± 0.1	20 µg + 5 mg 6 h gap + 20 µg	20 µg + 5 mg 6 h gap +20 µg	No changes observed	--	--	--	,,
6)	1.1 ± 0.1	0.8 ± 0.1	200 µg + 5 mg	100 µg + 5 mg	Female egg plugged after 12 h of injection	Plugged	--	--	,,
$PGF_{2\alpha}$ + DOMP + Ca^{++}	1.1 ± 0.1	0.8 ± 0.1	10 µg + 5 mg + 300 µg	10µg + 5 mg + 300 µg	,,	Plugged	--	--	,,
CPE priming 6 h gap + $PGF_{2\alpha}$	1.4 ± 0.1	1.2 ± 0.1	1.5 mg 6 h gap 20 µg	0.5 mg 6 h gap 20 µg	Female egg plugged after 8 h of 2nd injection	Plugged	--	--	,,

Table 3.4. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
CPE priming 6 h gap + $PGF_{2\alpha}$ + DOMP	1.1 ± 0.1	1.1 ± 0.1	1.5 mg 6 h gap + 10 µg + 5 mg	0.5 mg 6 h gap + 10µg + 5 mg	Female egg plugged after 8 h of 2nd injection	Plugged	--	--	No spawning observed
2)	1.4 ± 0.1	1.0 ± 0.1	1.5 mg 6 h gap + 20 µg + 5 mg	0.5 mg 6 h gap + 20µg + 5 mg	Female egg plugged after 14 h of injection	Plugged	--	--	,,
3)	1.0 ± 0.2	0.7 ± 0.1	1.5 mg 6 h gap + 25 µg + 1 mg	0.5 mg 6 h gap + 25µg + 1 mg	,,	Plugged	--	--	,,
CPE priming 6 h gap + Ca^{++} + $PGF_{2\alpha}$ + DOMP	1.2 ± 0.02	1.0 ± 0.1	1.5 mg 6 h gap + 300 µg + 20 µg + 5 mg	0.5 mg 6 h gap + 300 µg + 20 µg + 5 mg	Female egg plugged after 6 h of 2nd injection	,,	--	--	,,

Table 3.4. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
CPE priming 6 h gap + Thyroxine + PG $F_{2\alpha}$ + DOMP	1.3 ± 0.1	0.8 ± 0.1	1.5 mg 6 h gap +0.1 mg + 25 µg + 5 mg	0.5 mg 6 h gap + 0.1 mg +25 µg + 5 mg	Female egg plugged after 6 h of 2nd injection	Plugged	--	--	No spawning observed

Table 3.5. Effect of synthetic prostaglandin analogues on breeding of *Cirrhinus mrigala*.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
Cloprostenol		0.9 ± 0.11	1.0 ± 0.1	10 µg	10 µg	No changes observed	--	--	--	No spawning observed
	2)	0.7 ± 0.1	0.6 ± 0.1	20 µg	20 µg	Female egg plugged	Plugged	--	--	,,
	3)	0.8 ± 0.1	1.1 ± 0.1	30 µg	30 µg	,,	,,	--	--	,,
	4)	1.2 ± 0.17	1.3 ± 0.2	40 µg	40 µg	,,	,,	--	--	,,
	5)	1.1 ± 0.1	1.0 ± 0.1	50 µg	50 µg	,,	,,	--	--	,,
	6)	1.2 ± 0.1	1.3 ± 0.1	75 µg	75 µg	,,	,,	--	--	,,

Table 3.5. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
7)	1.26 ± 0.2	1.2 ±0.2	100 µg	100 µg	Female egg plugged	Plugged	--	--	No spawning observed
Cloprostenol + Domperidone	0.6 ± 0.1	0.5 ± 0.1	5 µg + 5 mg	5 µg + 5 mg	No changes observed	--	--	--	,,
2)	1.1 ± 0.1	1.0 ± 0.1	10 µg + 5 mg	10 µg + 5 mg	,,	--	--	--	,,
3)	1.3 ± 0.1	1.1 ± 0.2	30 µg + 5 mg	30 µg + 5 mg	,,	--	--	--	,,
4)	0.6 ± 0.1	0.7 ± 0.1	50 µg + 5 mg	50 µg + 5 mg	Female egg plugged after 12 h of injection	Plugged	--	--	,,
5)	1.2 ± 0.1	1.3 ± 0.1	100 µg + 5 mg	50 µg + 5 mg	,,	,,	--	--	,,

Table 3.5. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
6)	1.6 ± 0.2	1.1 ± 0.1	125 µg + 5 mg	50 µg + 5 mg	Female egg plugged after 12 h of injection	Plugged	--	--	No spawning observed
7)	1.4 ± 0.1	1.5 ± 0.1	75 µg + 1 mg	75 µg + 1 mg	After 12 h of injection female dissected observed 50 % of eggs were loosed in the ovary	--	--	--	50 % eggs were loosed in the ovary
8)	1.0 ± 0.1	1.5 ± 0.2	100 µg + 1 mg	100 µg + 1 mg	Female egg plugged after 12 h of injection	Plugged	--	--	No spawning observed
Cloprostenol + Domperidone + Ca^{++}	1.2 ± 0.1	1.3 ± 0.2	30 µg + 5 mg + 300 µg	30 µg + 5 mg + 300 µg	Female egg plugged after 14 h of injection	,,	--	--	,,

Table 3.5. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
HCG priming + Cloprostenol	1.0 ± 0.1	0.9 ± 0.1	150 IU 6 h gap + 50 µg	50 µg	No changes observed	--	--	--	No spawning observed
HCG priming + Cloprostenol + Domperidone	1.3 ± 0.1	1.0 ± 0.1	200 IU 6 h gap + 75 µg + 5 mg	200 IU 6 h gap + 75µg + 5 mg	Female egg plugged after 8 h of 2nd injection	Plugged	--	--	’’
Female Cloprostenol & Male Ovaprim	1.2 ± 0.0	1.2 ± 0.1	30 µg	0.2 ml	No changes observed	--	--	--	’’

Table 3.5. Contd.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
CPE priming + Cloprostenol + Domperidone		0.8 ± 0.1	0.9 ± 0.1	2 mg 6 h gap + 75 µg + 5 mg	0.5 mg (CPE at the time of female 2nd inj.)	Belly of female became soft, partial spawning took place after 12 h of 2nd injection	Not counted	--	--	Partial spawning took place
Tiaprost	1)	1.0 ± 0.1	0.9 ± 0.1	5 µg	5 µg	No changes observed	--	--	--	No spawning observed
	2)	1.4 ± 0.1	1.3 ± 0.1	25 µg	25 µg	,,	--	--	--	,,
	3)	0.9 ± 0.1	0.8 ± 0.1	50 µg	50 µg	,,	--	--	--	,,
	4)	0.8 ± 0.1	1.6 ± 0.1	75 µg	75 µg	After 3 h of injection female showed behavioural changes (jumping,	--	--	--	,,

Table 3.5. Contd.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
						restlessness) no ovulation was observed				
	5)	0.9 ± 0.1	1.0 ± 0.1	100 µg	100 µg	Female egg plugged after 12 h of injection	Plugged	--	--	No spawning observed
	6)	1.1 ± 0.1	1.3 ± 0.1	75 µg 6 h gap + 50 µg	50 µg (At the time of 2nd dose to female)	No changes observed	--	--	--	,,
Tiaprost + Domperidone	1)	1.0 ± 0.1	0.7 ± 0.1	25 µg + 5 mg	25 µg + 5 mg	,,	--	--	--	’’

Table 3.5. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
2)	1.1 ± 0.1	1.0 ± 0.1	35 µg + 5 mg	35 µg + 5 mg	No changes observed	--	--	--	No spawning observed
3)	0.6 ± 0.1	0.7 ± 0.2	50 µg + 5 mg	50 µg + 5 mg	,,	--	--	--	,,
Female Tiaprost & male Ovaprim	0.8 ± 0.1	0.8 ± 0.1	50 µg	0.2 ml	,,	--	--	--	,,
HCG priming + Tiaprost	1.3 ± 0.1	1.2 ± 0.1	100 IU 6 h gap + 50 µg	50 µg (At the time of 2nd dose to female)	,,	--	--	--	,,

Table 3.5. Contd.

Name of compound		Weight of brood fish (kg) Mean ± SE Female	Male	Dose per kg body weight Female	Male	Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	2)	1.5 ± 0.1	0.9 ± 0.1	200 IU 6 h gap + 50 µg	50 µg	Spawning behavioural changes observed in male after 3 h of inj. and released milt into the water. The activity continued for 3h, but female did not respond	--	--	--	No spawning observed
	3)	1.1 ± 0.1	1.0 ± 0.1	100 IU 6 h gap + 100 µg	100 µg	No changes observed	--	--	--	,,
Prosolvin	1)	1.2 ± 0.1	1.3 ± 0.1	25 µg	25 µg	,,	--	--	--	,,

Table 3.5. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
2)	0.9 ± 0.1	0.9 ± 0.1	75 µg	40 µg	No changes observed	--	--	--	No spawning observed
3)	1.5 ± 0.1	1.1 ± 0.1	100 µg	50 µg	,,	--	--	--	,,
Prosolvin + Domperidone	1.1 ± 0.1	1.2 ± 0.1	75 µg + 5 mg	40 µg + 5 mg	,,	--	--	--	,,
Carboprost	1.2 ± 0.1	1.1 ± 0.1	10 µg	10 µg	,,	--	--	--	,,
	1.0 ± 0.1	0.9 ± 0.1	20 µg	20 µg	,,	--	--	--	,,

Table 3.6. Effect of synthetic prostaglandin analogues (prepared by NCL, Pune) on breeding of *Cirrhinus mrigala*.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
α-N-R (11)	1)	1.4 ± 0.1	1.2 ± 0.2	5 µg	5 µg	No changes observed	--	--	--	No spawning observed
	2)	1.5 ± 0.1	2.0 ± 0.0	10 µg	10 µg	Female egg plugged after 14 h of injection	Plugged	--	--	’’
	3)	1.2 ± 0.1	1.1 ± 0.1	25 µg	25 µg	No changes observed	--	--	--	’’
	4)	1.2 ± 0.1	1.3 ± 0.1	50 µg	50 µg	Female egg plugged after 14 h of injection	Plugged	--	--	’’
	5)	1.2 ± 0.1	1.0 ± 0.1	100 µg	100 µg	No changes observed	--	--	--	’’

Table 3.6. Contd.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
β-N-R (12)	1)	1.4 ± 0.1	1.2 ± 0.2	10 µg	10 µg	No changes observed	--	--	--	No spawning observed
	2)	1.3 ± 0.1	1.1 ± 0.0	20 µg	20 µg	Female egg plugged after 18 h of injection	Plugged	--	--	,,
	3)	1.3 ± 0.1	1.0 ± 0.1	30 µg	30 µg	No changes observed	--	--	--	,,
	4)	1.2 ± 0.1	1.3 ± 0.1	40 µg	40 µg	,,	--	--	--	,,
	5)	1.2 ± 0.1	1.0 ± 0.1	50 µg	50 µg	,,	--	--	--	,,
P-M-R (13)	1)	0.9 ± 0.1	0.9 ± 0.1	10 µg	10 µg	,,	--	--	--	,,

Table 3.6. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
2)	1.0 ± 0.1	0.7 ± 0.2	20 µg	20 µg	Female egg plugged	Plugged	--	--	No spawning observed
3)	1.5 ± 0.1	1.0 ± 0.1	200 µg	70 µg	No changes observed	--	--	--	’’
4)	1.3 ± 0.1	1.2 ± 0.1	200 µg	200 µg	No changes observed	--	--	--	’’
P-M-R (13) + Domperidone	1.0 ± 0.2	0.9 ± 0.1	20 µg + 5 mg	20 µg + 5 mg	’’	--	--	--	’’
2)	0.7 ± 0.1	0.7 ± 0.1	30 µg + 5 mg	30 µg + 5 mg	No changes observed	--	--	--	’’
P-M-R (13) for female & ovaprim to male	2.0 ± 0.1	2.1 ± 0.1	150 µg	0.2 ml	’’	--	--	--	’’

Table 3.6. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
2)	2.6 ± 0.1	2.2 ± 0.2	200 µg	0.2 ml	No changes observed	--	--	--	No spawning observed
3)	2.2 ± 0.1	1.2 ± 0.1	250 µg	0.2 ml	,,	--	--	--	,,

Table 3.7. Spawning response of *Labeo rohita* to carp pituitary extract and ovaprim.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
Carp pituitary gland extract (CPE)	1.6 ± 0.2	1.2 ± 0.4	3 mg 6 h gap + 6 mg	3 mg	Spawning behavioural changes observed after 8 h of injection, spawning took place after 12 h of injection	1,30000 ± 7637	85 ± 1.15	80 ± 7.63	Spawning took place
Ovaprim	2.4 ± 0.1	2.0 ± 0.1	0.3 ml	0.2 ml	Spawning behavioural changes observed after 8 h of injection, spawning took place after 8-12 h of injection	2,40000 ± 4041	90 ± 2.88	90 ± 2.30	Spawning took place

Table 3.8. Effect of natural prostaglandins (PGs) and their precursors on breeding of *Labeo rohita*.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
Prostaglandin-F2α	1.83 ± 0.4	1.6 ± 0.3	1 µg	1 µg	No changes observed	--	--	--	No spawning observed
2)	1.93 ± 0.1	1.9 ± 0.1	2.5 µg	2 µg	Female showed only spawning behaviour, no changes in male	--	--	--	,,
3)	1.23 ± 0.1	0.8 ± 0.1	5 µg	5 µg	No changes observed	--	--	--	,,
4)	2.2 ± 0.1	1.3 ± 0.1	15 µg	15 µg	,,	--	--	--	,,
5)	1.23 ± 0.1	0.4 ± 0.1	20 µg	20 µg	Female egg plugged after 18 h of injection	Plugged	--	--	,,
6)	0.9 ± 0.1	1.2 ± 0.1	30 µg	30 µg	No changes observed	--	--	--	,,

Table 3.8. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
	Female	Male	Female	Male					
Prostaglandin-$F_{2\alpha}$ + Domperidone	1.3 ± 0.1	1.1 ± 0.2	2.5 µg + 5 mg	2.5 µg + 5 mg	No changes observed	--	--	--	No spawning observed
	1.23 ± 0.1	0.9 ± 0.1	5 µg + 5 mg	5 µg + 5 mg	’’	--	--	--	’’
Arachidonic acid	1.0 ± 0.1	1.0 ± 0.1	33 µg	33 µg	No changes observed	--	--	--	’’
	0.8 ± 0.1	0.8 ± 0.1	300 µg	300 µg	’’	--	--	--	’’

Table 3.9. Effect of synthetic prostaglandin analogues on breeding of *Labeo rohita*.

Name of compound		Weight of brood fish (kg) Mean ± SE		Dose per kg body weight		Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
		Female	Male	Female	Male					
Tiaprost	1)	1.2 ± 0.1	1.3 ± 0.2	5 µg	5 µg	No changes observed	--	--	--	No spawning observed
	2)	0.8 ± 0.1	0.9 ± 0.1	10 µg	10 µg	,,	--	--	--	,,
	3)	1.6 ± 0.1	1.3 ± 0.1	25 µg	25 µg	,,	--	--	--	,,
	4)	1.0 ± 0.2	0.8 ± 0.1	50 µg	50 µg	Female egg plugged after 18 h of injection	Plugged	--	--	,,
Tiaprost + Domperidone		1.0 ± 0.1	0.7 ± 0.1	25 µg + 5 mg	25 µg + 5 mg	No changes observed	--	--	--	,,
		0.9 ± 0.1	0.8 ± 0.2	35 µg + 5 mg	35 µg + 5 mg	,,	--	--	--	,,

Table 3.9. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE Female	Male	Dose per kg body weight Female	Male	Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
Female tiaprost & male ovaprim	0.8 ± 0.1	0.8 ± 0.1	50 µg	0.2 ml	No changes observed	--	--	--	No spawning observed
HCG priming + Tiaprost	1.3 ± 0.1	1.2 ± 0.1	100 IU 6 h gap + 50 µg	50 µg (At the time of 2nd inj to female)	Female egg plugged	Plugged	--	--	,,
2)	1.0 ± 0.1	0.8 ± 0.1	200 IU 6 h gap + 50 µg	50 µg (At the time of female 2nd inj)	After 18 h of injection when press the belly some eggs were released outside the body when belly was pressed	Not counted	--	--	Partial ovulation took place

Table 3.9. Contd.

Name of compound	Weight of brood fish (kg) Mean ± SE Female	Male	Dose per kg body weight Female	Male	Observations	No of eggs obtained Mean ± SE	Fertilization rate (%) Mean ± SE	Hatching rate (%) Mean±SE	Remarks
3)	0.7 ± 0.1	0.8 ± 0.1	200 IU 6 h gap + 50 μg 48 h gap + 50 μg	50 μg (At the time of female 2nd inj)	No changes observed	--	--	--	No spawning observed
4)	1.1 ± 0.1	1.0 ± 0.1	100 IU 6 h gap + 75 μg 24 h gap + 50 μg	75 μg (At the time of female 2nd inj)	,,	--	--	--	,,

CHAPTER - IV

PROSTAGLANDIN MEDIATED EFFECTS ON MILTERS OF INDIAN MAJOR CARPS AND GOLDFISH, *Carassius auratus*

SECTION – A : EFFECTS OF PROSTAGLANDINS ON MILT VOLUME OF GOLDFISH, *Carassius auratus*

INTRODUCTION

In comparison to female teleosts very little is known regarding involvement of prostaglandins in milt production / spermiation in males. Christ and van Dorp (1972) first identified PGE_1 and PGB_1 in the milt of *Cyprinus carpio*. Nomura *et al*. (1973) recorded PGE_2 in testes of a flounder, *Paralichthys olivacens*, PGE_2 and $PGF_{2\propto}$ in the testes of bluefin tuna, *Thynnus thynnus* and PGE_1 in the semen of chum salmon, *Oncorhynchus keta*. Nomura and Ogata (1976) later identified PGE_3 in the testes of carp, *Cyprinus carpio* and leopard shark, *Triakis scyllia*. Bouffard (1979a) with the help of radioimmunoassay measured PGB_1, PGE_1 and $PGF_{2\alpha}$ in the blood and testes of goldfish. Direct evidence that PGs help in milt production is not well understood. However, PGs indirectly help in the production of milt in certain teleosts which is associated with an increase in the level of blood GtH.

It has long been recognized that many animals release specialized chemicals to the environment, where there have specific behavioural and / or physiological effects on conspecifics (Darwin, 1887). Karlson and Lüscher (1959) coined the term pheromone. Pheromones were defined as substances that are excreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for example a definite behaviour or developmental process. Karlson and Lüscher (1959) reported that unlike hormones the substance is not secreted into the blood but out side the body; it does not serve humoral correlation within the organisms but communication between individuals.

Kittredge *et al*. (1971) were the first to propose that aquatic organisms might commonly use hormones as pheromones. By the early 1980s, behavioural studies confirmed that ovulation and pheromone release are closely associated in at least 10 species of oviparous fish (Emanuel and Dodson, 1979; Lee and Ingersoll, 1979; Honda, 1979, 1980a, b, 1982a, b; Sorensen and Winn, 1984).

There is evidence that teleost fish use hormones and their metabolites as reproductive pheromones (Stacey *et al*., 1987). In goldfish 17α, 20β-dihydroprogesterone (17, 20β-P), the proposed mediator of oocyte final maturation (Scott and Canario, 1987) also functions as a sex pheromone. 17, 20β-P is released to the water by females during oocyte final maturation where it functions as a potent olfactory stimulant elevating

circulating gonadotropin (GtH) in males within 15 min and increased milt production within 6 h (Stacey and Sorensen, 1986; Dulka *et al*., 1987; Sorensen *et al*., 1987). 17, 20β-P is a poor postovulatory pheromone because its synthesis and release are declining by the time of ovulation (Dulka *et al*., 1987) and because waterborne 17, 20β-P has only minor effects on male behaviour (Sorensen, unpublished results).

Another intriguing fish pheromone is prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) which circulates in the blood stream of female goldfish and is associated with follicular rupture and ovulation. When it is released into water together with a metabolite 15-keto-$PGF_{2\alpha}$, it induces increased (but short lived) aggression amongst males and courtship behaviour.

Levels of the circulating F-prostaglandins (PGF) increase in goldfish and at least several other fish at the time of ovulation (Bouffard, 1979; Cetta and Goetz, 1982), presumably reflecting a role modulating follicular rupture (Goetz, 1983). Circulating PGF appears to function as a hormonal signal triggering spawning behaviour through direct action on the brain of goldfish (Stacey and Peter, 1979; Stacey and Goetz, 1982; Stacey, 1987).

However, natural prostaglandins have not been commercially used in the field of induced breeding due to their high cost, unstable nature and short shelf-life. In view of this various synthetic, more stable analogues of $PGF_{2\propto}$ which is currently used in veterinary practice, were used in the present study. Attempts were made to evaluate the role of prostaglandins in induction of milt volume in goldfish, *Carassius auratus* and widen their use in aquaculture.

MATERIALS AND METHODS

Animals

Mature goldfish brooders were procured from laboratory broodstock, maintained on artificial fish pellets (fishmeal, wheat flour, rice bran, yeast, vitamin and calcium) and live tubifex worms / mosquito larvae at 25 ± 1 °C and 12D : 12L photoperiod as described in detail in Chapter 2.

Chemicals

Prostaglandin analogues used in the present study are commercially available in ready to use form.

1. **Iliren** (Tiaprost) **:** Purchased from Hoechst Roussel Vet GmbH d - 65203 Wiesbaden, a synthetic more stable analogue of $PGF_{2\alpha}$ (1 ml Iliren contains 0.150 mg tiaprost).
2. **Prosolvin** (Luprostiol) **:** Purchased from Intervet, International B.V. Boxmeer-Holland, is a synthetic analogue of $PGF_{2\alpha}$ (1 ml prosolvin contains 7.5 mg Luprostiol).
3. **Lutylase** (Dinoprost) **:** Dinoprost is a synthetic $PGF_{2\alpha}$ (1 ml Lutylase contains 5 mg dinoprost tromethamine) available from Upjhon S.a. Puurs, Belgium.

Experimental Methodology

From the common stock, breeding pairs weighing 20–50 gm were properly selected (females with reddish swollen vent, bulky abdomen and active males with white tubercles on operculum and secretion of milt). Selected pairs were weighed and kept overnight in separate spawning aquarium (36" x 12" x 15") in 50 l of dechlorinated water and checked for ovulation and spawning. Non-ovulated females were used for experiments. Two sets of experiments were carried out to study prostaglandin mediated effects on the milt volume of goldfish.

Experiment 1

This experiment was conducted to study effects of selected prostaglandin analogues (prosolvin, tiaprost and lutylase) on the milt volume of male goldfish when exposed to prostaglandin treated females. Treatment and control groups contained five pairs each. The females of the experimental groups were given prostaglandin at a dosage of 1 μg/gm body weight and those of control groups were given an equal volume of fish saline (0.6% NaCl). Males were exposed to prostaglandin treated females (1M:1F). The experiment lasted 12 h. None of the male of the control and experimental groups was administered with prostaglandins.

Experiment 2

This experiment was conducted to determine whether exposure to prostaglandins increases milt volume or not. Treatment and control groups each contained five males. Males were placed in 5 l of water containing prostaglandin analogues (prosolvin, tiaprost and lutylase) at a concentration 10 µg/l. The experiment was completed within 12 h. None of the males of the control and experimental groups was administered with prostaglandins.

Milt volume studies

Males were placed belly up on a moist pad and milt was removed by stripping (applying gentle pressure to the abdomen) as described by Kyle *et al*. (1985). Initial pre-treatment stripping was done and males were placed randomly in 50 l of water (separate tanks) and maintained at 25 ± 1 °C as the stock. On the day of experiment males were either exposed to prostaglandin treated female or to water into which prostaglandin was added directly. Control experiments were run simultaneously. After 12 h of exposure to the treatment, fish were removed from the test aquarium and milt was stripped. Milt was collected in sterilized, pre-weighed glass capillary tubes. Glass capillary tubes were re-weighed and weight of milt was calculated. Milt Density = 1 gm/ml was assumed for calculation of milt volume.

Comparative effect of selected leuteolytic prostaglandins on milt volume of goldfish is represented in graphs attached.

Statistical Method

Milt volume data was analyzed by Analysis of Variance (ANOVA) test. First to determine whether group shared a common starting point, pre-treatment milt volumes were compared with ANOVA. Post-treatment milt volumes were compared with ANOVA.

RESULTS

Behavioural changes observed in males exposed to non-ovulated females injected with prostaglandin analogues were very prominent. Behavioural changes in male were characterized by vigorous chasing of female, pairing and constant nudging of her ovipore and side as well. During the series of spawning acts observed, female approached the surface of water in head up position and male followed, both turned on their sides, they broke the water surface, male constantly chased and female gave repeated jerks (spawning acts).

The results obtained on the volume of milt harvested is presented in Fig. 4.1 and 4.2.

Experiment 1

There was no pre-treatment difference in milt volume between control and experimental groups. The non-induced males of experimental groups when exposed to prostaglandin analogues namely, prosolvin, tiaprost and lutylase treated females (1 μg/gm body weight), produced 52.00 ± 10.85, 45.60 ± 9.41 and 4.20 ± 1.43 μl milt respectively over 10.20 ± 2.46 μl of the control group after 12 h which is highly significant ($P < 0.05$, Fig. 4.1).

Experiment 2

There was no pre-treatment difference in milt volume between control and experimental groups. The non-induced males of the experimental groups when exposed to prosolvin, tiaprost and lutylase which were added directly into the water (10 μg/l) produced 40.00 ± 10.11, 16.50 ± 6.61 and 12 ± 20 μl of milt respectively over 10.20 ± 2.46 μl milt of the control groups after 12 h which is highly significant ($P < 0.05$, Fig. 4.2).

All males exposed to saline control failed to show a significant increase in milt volume.

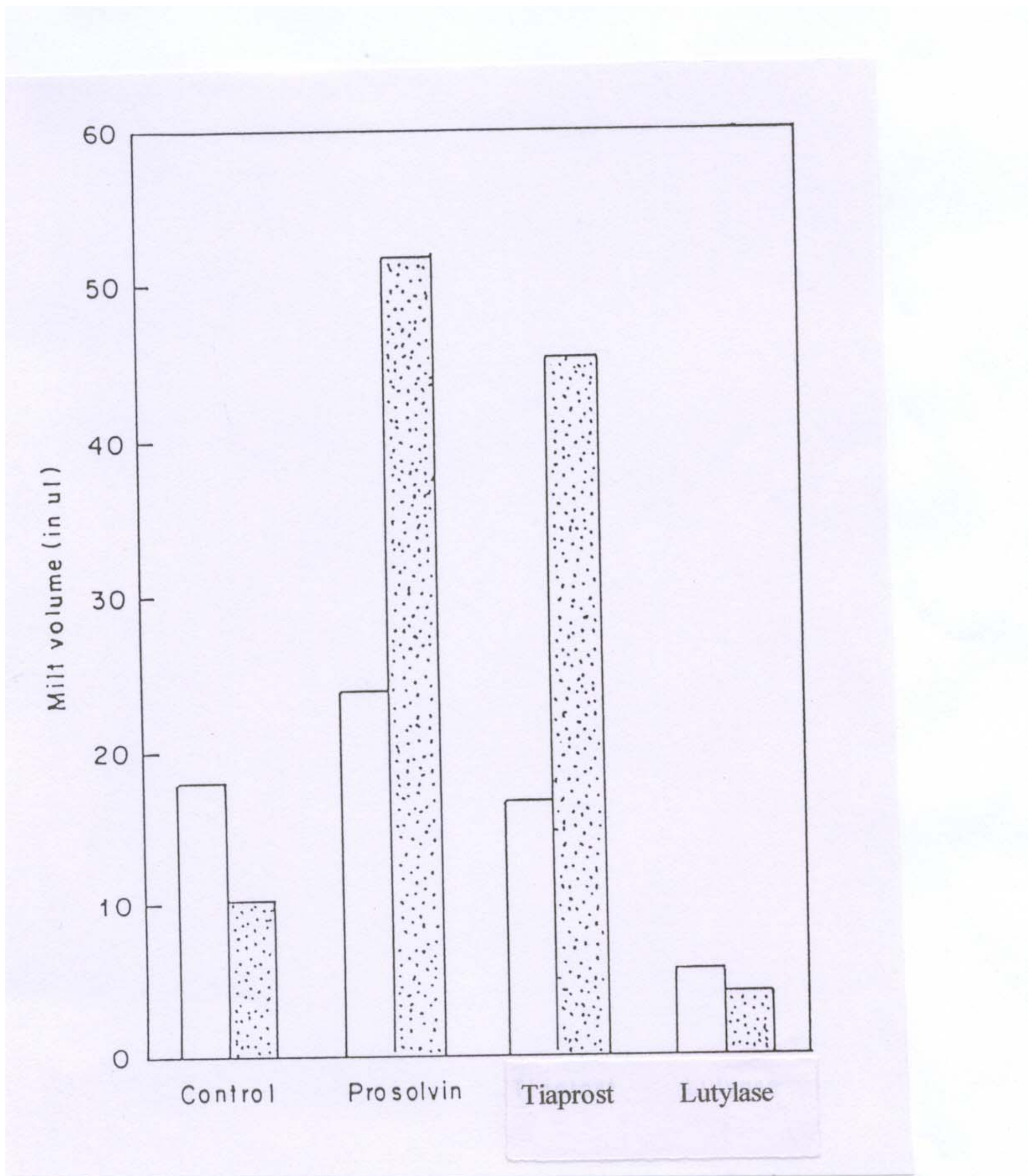


Figure 4.1. Volume of milt stripped from male goldfish before (open bar) and 12 h (stippled bar) after exposure to Prosolvin, Tiaprost and Lutylase injected (1 μg/gm body weight) female respectively. Post-exposure values were analyzed by ANOVA.

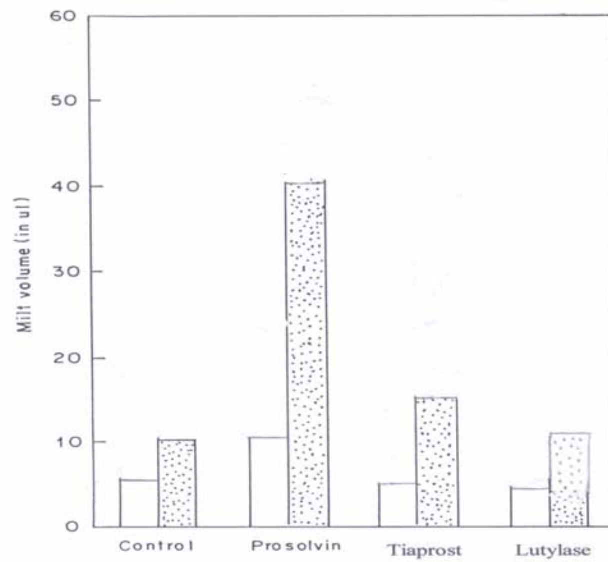


Figure 4.2. Volume of milt stripped from male goldfish before (open bars) and 12 h after exposure (stippled bars) to Prosolvin, Tiaprost and Lutylase added directly into water 10 μg/l respectively, to give final a concentration of approximately 10 ng/ml. Post-exposure values were analyzed by ANOVA.

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DISCUSSION

Although it seems apparent that male fertility during spawning should be positively correlated with milt volume, we are not aware that such a relationship has been demonstrated in externally fertilizing fish. However, sperm depletion has been suggested to cause decreased male fertility (as measured by the proportion of eggs fertilized) in spawning lemon tetra, *Hyphessobrycon pulchripinnis* (Nakatsura and Kramer, 1982). Because goldfish spawn in large groups in which many males compete for access to ovulated females, it seems likely that the volume of milt that a male has ready for release may be an important determinant of his reproductive success.

Present study demonstrated that waterborne prostaglandins are extremely effective in increasing the volume of milt that can be stripped from male goldfish. These findings strongly suggest that luteolytic prostaglandin analogues (prosolvin, tiaprost and lutylase) were reproductive primer pheromones which cause increased milt volume in male goldfish.

In the present work the volume of milt increased when males were exposed to PG injected non-ovulated females (Fig. 4.1). This is in good agreement with earlier reports of Sorensen *et al*. (1986, 1995). Prostaglandin injection to non-ovulated female goldfish releases an odour that elicits male reproductive behaviour identical to those elicited by odour of ovulated female (Sorensen *et al*., 1986). The tendency of male goldfish to contact the female genital areas (nudging) with snout might expose males to high concentration of such stimulants which resulted in an effective increase in milt volume in male goldfish. Sorensen *et al*. (1986, 1995) have reported that circulating PGs are cleared to the water to function as pheromone there by synchronizing male and female sexual behaviour in goldfish. As nothing is known about how goldfish would release injected prostaglandin analogues into water, it is not clear whether natural release of prostaglandin analogues themselves or their metabolites during preovulatory period could result in water concentration sufficient to increase the milt volume in present study. This is not the direct action of prostaglandins but prostaglandins-mediated pheromonal action. A pheromone-mediated increase in milt volume could affect reproductive success if the increase occurs prior to ovulation and spawning. We observed that the latency of the prostaglandin induced milt response was 12 h.

Rapid and intense sexual behavioural responses in male goldfish and induced milt volume when exposed to prostaglandin-treated females emphasizes the pheromonal and olfactory potency of circulating prostaglandins / metabolites.

Present results emphasize that injection of prostaglandin analogues to non-ovulated female goldfish not only elicited the spawning acts in females but also induced increase in milt volume in male goldfish. Our findings are in agreement with the findings of Stacey (1976), Stacey and Goetz (1982) and Sorensen *et al*. (1988) related to prostaglandin induced behavioural response in non-ovulated female goldfish. In male goldfish without PG treatment blood GtH and milt volume increased due to the presence of gravid female (Kyle *et al*., 1985). Female goldfish (*Carassius auratus*) releases mixture of F prostaglandins (PGFs) in aquaria water and this water- borne PGF causes an increase in the gonadotropin (GtH) and milt (sperm and seminal fluid) levels in spawning males (Stacey and Sorensen, 1988; Sorensen *et al*., 1988).

In the present study it was found that prostaglandins in water at a concentration of only 10 ng/ml are effective in increasing milt volume (Fig. 4.2). Also, although the threshold water concentration of prostaglandins required to induce the milt response has not been determined, preliminary results indicate that it is less than 10 ng/ml.

How exposure to prostaglandins causes increased milt volume is not understood. The most likely mechanisms involve either a neural stimulation of the testes and sperm ducts (Dulka and Demski, 1986) or an increase in plasma gonadotropin (Billard *et al*., 1982; Kyle *et al*., 1985).

Many other fundamental questions about sex pheromone function in fish remain to be addressed. For example, the mechanism(s) by which pheromones are synthesized (metabolized), their relationship to hormones, their complexity and the means by which they are released are unknown. The behavioural and ecological roles of pheromones and their impact on reproductive success are also not understood in any species. Finally, the basis of neurological responsiveness is poorly understood.

Rapid increase in milt volume occurs in male goldfish as a result of sexual activity (Kyle *et al*., 1985) but the sensory cues mediating this response have not been investigated.

Metabolism of sex hormones causes dramatic increases in the behaviour and circulating hormone levels of male goldfish. One (1) gm of these pheromones activates 40 billion liters of water (De Fraipoint *et al*., 1993).

The present study also indicated that picomolar concentration of waterborne prostaglandins increases the milt volume that can be stripped from male goldfish, a finding with immediate practical application as a non-invasive technique for increasing releasable sperm in culturerd carp like *Catla catla*, *Cirrhinus mrigala*, *Labeo rohita* and other species those are not bred in captivity. The clear effects of prostaglandins as a pheromone on goldfish in increasing milt volume warrants field trials under culture conditions to determine whether pheromonal milt enhancement can offer practical alternatives to existing invasive techniques.

However, under culture conditions, prostaglandin treatment would reduce labour cost and handling stress by eliminating injections and reduce risk to broodstock because the pheromone treatment activates normal endogenous mechanisms theoretically eliminating the potential for generating pharmacological high plasma hormone levels.

Tiaprost and prosolvin (synthetic analogues of $PGF_{2\alpha}$) used in the present study were stable at room temperature and had long shelf-life compared to natural prostaglandins. No side effects were observed of these synthetic prostaglandins during and after the experiments on goldfish. These prostaglandin analogues are cheaper than natural prostaglandins. Tiaprost and prosolvin used in the present study have been characterized as veterinary luteolytic prostaglandins which are 200 times more luteolytic than natural $PGF_{2\alpha}$ (Dukes *et al*., 1974).

Results of the present study indicate that tiaprost and prosolvin were very effective in inducing milt volume in male goldfish when exposed to prostaglandin-injected non-ovulated female goldfish and also when prostaglandins were directly added into the water. The maximal response was obtained with tiaprost.

Apart from the application of tiaprost and prosolvin (luteolytic prostaglandins) as induced spawning agents for ornamental fishes, better understanding of the role of such luteolytic prostaglandins in Indian major carp milters which is difficult to spawn, can be further studied.

CHAPTER - IV

SECTION – B : QUANTITATIVE ASSESSMENT OF MILT IN PROSTAGLANDIN INDUCED MILTERS OF INDIAN MAJORCARP, *Labeo rohita* (Hamilton-Buchanan)

INTRODUCTION

Indian major carps are the most commercially important culturable freshwater species: (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*). These carps do mature but not breed spontaneously in the confined water of the culture pond, thus need hormonal induction and environmental manipulation for their captive propagation. Though the induced breeding is a 4-decade-old practice in India, the deficiency of male brood is a common experience, expressed by many of our seed producers.

Both male and female brood carp are equally responsible for seed production. Male brood during breeding phase is termed as milter and the so called semen is known as milt. Mere milting by pressure on the abdomen of the mature male does not speak about the efficiency of the male. The efficiency of the milter can be worked out by evaluating the quantity as well as quality of the milt produced.

The reproduction in culture fish can be controlled both by invasive techniques e.g. the injection or feeding of drugs or hormones that directly drive reproductive events and non-invasive techniques e.g. the manipulation of environmental or social cues that regulate a species reproduction under natural conditions. Successful non-invasive techniques include the use of photoperiod manipulation to alter seasonal breeding in rainbow trout, *Oncorhynchus mykiss* (Duston and Bromage, 1988). The invasive technique most relevant to the present study in the Linpe method for induction of ovulation (Peter *et al.*, 1988). Invasive endocrine techniques also have been used to enhance sperm production in common carp (Courtois *et al.*, 1986; Saad and Billard, 1987) because males under culture conditions often produce insufficient sperm for induced breeding programmes (Rothbard, 1981).

In breeding schemes for farm animals, artificial insemination (AI) is much used to increase the selection intensity among males. In salmonids AI with fresh semen has been practiced since the dry fertilization method was pioneered by the Russian V.P. Vrasski (Soudakevicz, 1874). Recently cryopresrvation of semen from salmonids has also been developed (Scott and Baynes, 1980).

Knowledge of semen composition is a prerequisite to a better understanding of the physiology of fish spermatozoa. Chemical and physical properties are used in the

evaluation of reproductive ability of mammalian spermatozoa (Cruea, 1969). Some of these characteristics have been studied in a few fish species (Clemens and Grant, 1965; Ginsburg, 1972; Guest *et al*., 1976; Scatt and Baynes, 1980; Kazakov, 1981; Piironen, 1985).

In induced reproduction and artificial insemination, it is desirable to increase the number of available spermatozoa by stimulating spermiation. Carp pituitary extracts are often used for this purpose (Clemens and Grant, 1964, 1965; Rothbard and Rothbard, 1982) but other hormones should be tested. Among these, HCG has proved to be ineffective (F. Takashima, unpublished data) and LHRH has been widely used in Chinese carp (Chinese National Bureau of Aquatic Products, 1980).

Induced breeding of Indian major carps has been commercially adopted in India for last four decades. Efforts have been made to induce the carp milters by using various inducing agents *viz.* pituitary extract, LHRH, Ovaprim etc. (Clemens and Grant, 1964; Billard *et al.,* 1983; Rothbard and Rothbard, 1982; Nandeesha *et al*., 1990). Although prostaglandins (PGs) have been reported as one of the carp breeding inducing agents (Tripathi and Khan, 1990) the use of natural prostaglandins in fish breeding programme is very much limited due to their high cost and short shelf-life period. In view of this, synthetic and more stable $PGF_{2\alpha}$ analogues namely, tiaprost and prosolvin were used in the present study to induce *Labeo rohita* milters. Preliminary results obtained on male goldfish indicated that prostaglandins increased the milt volume. On the basis of these results it was decided to conduct the same experiments on Indian major carp milters. The attempts were made to compare the effectiveness of synthetic, more stable prostaglandin analogues on milt volume, spermatocrit values, sperm count and motility of induced and non-induced milters of an Indian major carp, *Labeo rohita*.

MATERIALS AND METHODS

Animals

Healthy rohu milters raised in pond up to the age of 2 years (weight 1.5 - 2 kg) were brought to the carp hatchery in August and stocked at room temperature. They were fed on rice bran and oil cake at the rate of 2 % of body weight.

Chemicals

Prostaglandin analogues used in the present study were commercially available ready to use.

1. **Iliren** (Tiaprost) **:** Available from Hoechst Roussel Vet GmbH d - 65203 Wiesbaden; a synthetic, more stable analogue of $PGF_{2\alpha}$ (1ml Iliren contains 0.150 mg tiaprost).
2. **Prosolvin** (Luprostiol) **:** Available from Intervet, International B.V. Boxmeer-Holland, is a synthetic analogue of $PGF_{2\alpha}$ (1 ml prosolvin contains 7.5 mg Luprostiol).

Experimental Methodology

Preliminary experiments were carried out at Hadapsar Fish Farm, Pune, (Govt. of Maharashtra) and then further experiments were carried out at Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Orissa, India, under the guidance of scientists (Carp Breeding Division).

In the present work, screening experiments were conducted on Indian major carp, *Labeo rohita* milters due to easy availability and small size for selection of promising prostaglandin analogues considering the high cost of prostaglandins.

The selected milters of rohu were weighed separately in a hand net and the actual weight of each fish was determined by deducting the known weight of the hand net from the total weight. Brooders were netted out in a hand net. They were placed on a table and carefully injected avoiding wrangling movements. Injection was administered intramuscularly at the base of caudal fin above the lateral line. Experiments were carried out in Chinese spawning pool (6-8 dia & 1.2 – 1.5 m height). Before collection of milt, they were starved for 12 h to prevent intervention of fecal matter or urine during milt collection. The selected milters (twenty) were divided equally into four groups namely A, B, C and D. Tiaprost was administered at a dose 50 µg/kg and 75 µg/kg body weight to group A and B respectively. Prosolvin was administered at a dose 100 µg/kg body weight to group C. Control group D was administered with fish saline (0.6% NaCl). Fishes from all the four groups were milted by stripping after 6 h of hormone treatment. Abdominal region was wiped with absorbent paper to remove water droplets. Samples of milt were collected in sterilized ice cooled graduated centrifuge tubes (Fig. 4.3). Spermatocrit value of milt samples was determined by centrifuging 50 µl of semen for 20 min at 10,000 rpm

(Bouck and Jacobson, 1976) in ordinary haematocrit centrifuge. Results were analyzed by Students 't' test. Motility of spermatozoa was evaluated following the method given by Guest *et al*. (1976). Number of spermatozoa was counted per unit volume by using haemocytometer.

Statistical Method

Standard deviation for means was derived by usual formula. Milt volumes and spermatocrit values of treated Vs untreated milters were subjected for Students 't' test. Following formula was used to work out Students 't' test:

$$\text{“t”} = \frac{X_1 - X_2}{\sqrt{SE_1^2 + SE_2^2}}$$

X = Mean value

SE = Standard error

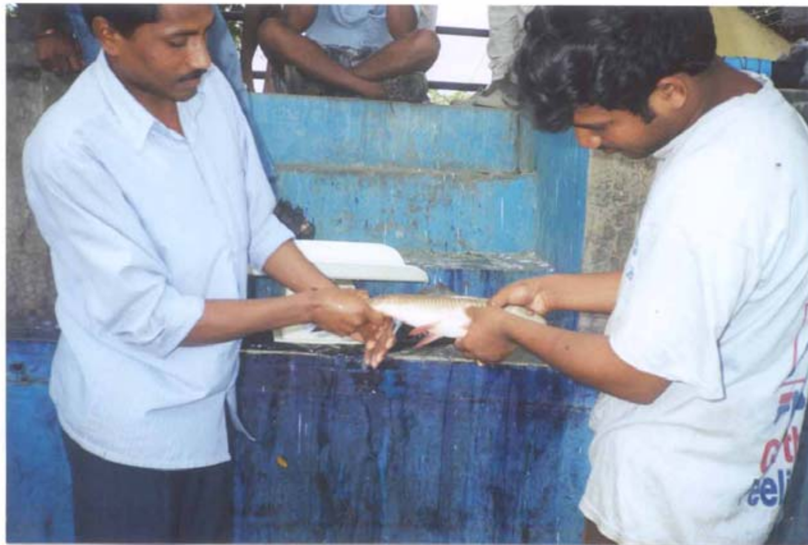

Figure 4.3. Collection of milt samples from Indian major carp, *Labeo rohita*.

RESULTS

The results of increase in volume and properties of milt of *Labeo rohita* before and after administration of prostaglandin analogues *viz.* tiaprost and prosolvin are presented in Table 4.1. Fourfold increase in milt volume (0.78 ± 0.20 to 3.05 ± 0.47 ml) was noted after tiaprost administration at a dose 50 µg/kg body weight ($P < 0.001$) in comparison with the other doses *viz.,* 75 µg/kg body weight of tiaprost ($P < 0.005$) and 100 µg/kg body weight of prosolvin ($P > 0.05$) respectively. The spermatocrit values of the milt samples from the selected groups A, B, C and D were found to be 85.50 ± 6.84 ($P < 0.005$), 89.40 ± 6.14 ($P < 0.005$), 87.66 ± 3.93 ($P < 0.001$) and 53.33 ± 7.53 respectively. The sperm count of milt of the non-induced *Labeo rohita* milters (group D) ranged from $1.8 - 2.2 \times 10^7/mm^3$ with average of $2.0 \times 10^7/mm^3$. Sperm count after tiaprost administration at a dose of 50 and 75 µg/kg body weight ranged from $2.6 - 3.4 \times 10^7/mm^3$ and $3.2 - 3.6 \times 10^7/mm^3$ with an average of $3.0 \times 10^7/mm^3$ and $3.37 \times 10^7/mm^3$ respectively. Although average spermatozoa population after prosolvin administration (100 µg/kg body weight) increased up to $3.13 \times 10^7/mm^3$, the volume of the milt did not increase substantially as compared to control. The sperms of rohu were immotile until the milt was diluted with water. The motility increased with excellent forward movement of sperm (70 - 90 %) after addition of a drop of water and was at its maximum (+ + + + +) for 5 - 12 seconds. Activity decreased gradually and came to an end within 60 - 80 seconds.

Table 4.1. Quantitative assessment of milt of *Labeo rohita* by prostaglandin induction.

Compound name	Group	Dose (µg / kg)	Milt volume/kg^{a,b} ml ± SD	Spermatocrit^{a,b} value % ± SD	Sperm count range x $10^7/mm^3$	Motility score
Tiaprost	(A)	50	3.05 ± 0.47**	85.50 ± 6.84*	2.6 - 3.4	+ + + + +
	(B)	75	2.83 ± 0.8*	89.40 ± 6.14*	3.2 - 3.6	+ + + + +
Prosolvin	**(**C)	100	1.09 ± 0.59^{NS}	87.66 ± 3.93**	2.8 - 3.4	+ + + + +
Control	**(**D)	--	0.78 ± 0.20	53.33 ± 7.53	1.8 - 2.2	+ + + + +

a = Mean ± SD of five replicates.

b = Data analyzed by Students 't' test.

*(P < 0.005) and **(P < 0.001).

NS - Not significant.

DISCUSSION

Administration of tiaprost, a prostaglandin analogue increased the milt volume in *Labeo rohita*. Significant increase in the milt volume observed in the present study by tiaprost (50 μg/kg body weight) suggests the prostaglandin induction of *Labeo rohita* milters. Prostaglandin stimulation also increased the average spermatozoa population. On induction, milt volume increased and spermatocrit value (viscosity) came down below 90% (Gupta and Rath, 1991). Tiaprost induced milt samples in the present study exhibited 85% spermatocrit value. Ginsburg (1972) reported that the viscosity of milt was influenced by sperm concentration. The optimum spermatozoa population in the milt sample of non-induced Indian major carp milters varies from 2.0×10^7 - $3.5 \times 10^7/mm^3$ (Gupta and Rath, 1991). Lesser viscosity from control sample (D) can be correlated with the less sperm count and could be a physiological variation among the selected milters. It is also evident from the present observations that milt with higher doses of tiaprost was more viscous with high sperm count. High sperm count in induced milters may be an adaptive nature to short survival time. The carp milt samples which possess spermatocrit value 70, sperm count 2.0×10^7 /mm^3 and motility + + + + are considered as quality sample.

Unlike mammals, teleostean spermatozoa are immotile in the testis as well as in seminal fluid (Morisawa and Suzuki, 1980). Present results showed that motility of carp spermatozoa became vigorous after putting a drop of water (hypotonic solution) and remained for few seconds on activation as reported in other freshwater teleost (Billard, 1978). Since only activated spermatozoa can fertilize the eggs (Terner, 1986), tiaprost induced translucent milt with proper viscosity and sperm count recorded in present study was ideal to result in successful fertilization which was observed in preliminary studies.

Tiaprost and prosolvin (prostaglandin analogues) used in present study are reported to be more luteolytic than natural prostaglandins (Dukes *et al*., 1974) and are currently used in veterinary practice. Tiaprost induced *Labeo rohita* milters effectively as

compared to prosolvin. These experimental techniques will be used to induce males for spermiation for artificial insemination.

The economic analysis carried out has indicated that the tiaprost male inducing dose costs Rs. 9.2 and ovaprim (existing inducing agent) male inducing dose costs Rs.10. Thus tiaprost is cheaper than ovaprim. Further trials were required for standardization of doses, comparative study and for cost comparison with existing inducing agents.

The results of this study clearly showed that the volume of strippable sperm and milt in *Labeo rohita* can be increased by administration of prostaglandin analogues.

Controlling milt production in cultured carps is generally less problematic than is controlling ovulation. However, the fact that hormone and drug injection are often used to increase milt volume in common carp (Courtois *et al*., 1986; Saad and Billard, 1987; Billard *et al*., 1989) and in other carp species, *Ctenopharyngodon idella* (van Eenenaam *et al*., 1990), *Labeo rohita* and *Cirrhinus mrigala* (Halder *et al*., 1991) suggest that non-invasive pheromonal techniques for enhancing milt production in the Indian major carp may have practical applications.

CHAPTER - V

EXTRACTION OF WATERBORNE PROSTAGLANDINS FROM FEMALE GOLDFISH, *Carassius auratus* (LIN.) AND THEIR DETECTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

INTRODUCTION

Pheromones of fish are secretions released into the external medium (water) conveying information usually via olfactory receptors and bringing about fairly specific reactions and behavioural changes in the recipient. Pheromones are either releasers which trigger a relatively rapid behavioural response or primers which usually involve the endocrine system and produce a more gradual and prolonged shift in the physiology of the recipient.

Ripe male goldfish are able to discriminate between ovulated and unovulated females by a pheromone contained in the ovarian fluid which is released shortly after ovulation. It is most probable that ovarian pheromones are released into the urine and hence to the exterior. Goldfish ovulate in the spring in response to a surge in gonadotropin (GtH) triggered by rising temperature, aquatic vegetation and pheromones. Male-female reproductive physiology and behaviour must be highly synchronized. This synchrony is mediated by at least two hormonally derived cues with distinctly different actions and identities. The first pheromone is released prior to spawning by ovulatory females and functions primarily as a steroidal primer. The second pheromone is released by recently ovulated (sexually active) females and stimulates male sexual activity thus functioning as a releaser. The principle function of the preovulatory pheromone appears to be to stimulate increased sperm production and motility in conspecific males and to evoke behavioural competitiveness.

Males and females of a variety of teleosts release pheromones that affect the sexual behaviour and reproductive physiology of conspecifics (Liley, 1982; Pandey, 1984; Stacey *et al*., 1986; Stacey and Cardwell, 1997).

Prostaglandins (PGs) play functional role in sexual behavioural responses and ovulation (Goetz, 1983; Stacey, 1976, 1981; Stacey and Goetz, 1982; Stacey *et al*., 1987). Prostaglandin-$F_{2\propto}$ ($PGF_{2\propto}$) is intriguing fish pheromone which circulates in the blood stream of female goldfish and is associated with follicular rupture and ovulation. When released into water together with a metabolite 15 keto-$PGF_{2\propto}$ it elicited increased (but short-lived) aggression amongst males and courtship behaviour.

The isolation of pheromones and knowledge of their activity now forms a part of the science of endocrinology. Understanding of pheromone function in teleost fish has been impeded by a lack of information on pheromone identities. No teleosts sex pheromone system is well understood but there is convincing evidence that males and females from a number of species use hormones and / or their metabolites as sex pheromones.

A prostaglandin was isolated in the gastrointestinal tract of the shark, *Triakis scyllia* and identified as prostaglandin-E_2 (PGE_2) by bioassay, thin-layer chromatography, gas-liquid chromatography, ultraviolet absorption spectrometry and mass spectrometry (Ogata and Nomura, 1975). Using gas chromatography-mass spectrometry they have definitively identified prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) in the circulation and water of ovulatory goldfish to demonstrate that it is both behavioural hormone and the precursor of post-ovulatory sex pheromones (Sorensen *et al*., 1995). Release of 13,14-dihydro-15-keto-prostaglandin-$F_{2\alpha}$ a sex pheromone to water was observed in case of cobitid loach following ovulatory stimulation. The holding water was analyzed by high pressure liquid chromatography at 4 hours intervals for 16 hours after the injections (Ogata *et al*., 1994). Methods of prostaglandin analysis with the necessary sensitivity for application to individual tissue and fluid specimens are still in the developmental stage.

The goldfish is well suited for studies of pheromone release by females and pheromonal effects on males because ovulation is synchronized with the light-dark cycle and can be manipulated under laboratory conditions.

In the previous chapter we found that when non-ovulated female goldfish was injected with synthetic prostaglandins, spawning behavioural changes were observed within half an hour of injection. Same female releases some chemicals into the holding water which acts as pheromone on male to induce spawning behaviour and increase in milt volume (Jagtap *et al*., 2001).

To ascertain the functional role of synthetic prostaglandins in fish breeding, attempts were made to study the behavioural and physiological responses and fate of injected prostaglandins in goldfish, *C. auratus.* The present chapter describes in detail the method applied for the extraction of waterborne prostaglandins and their detection by High Performance Liquid Chromatography (HPLC).

MATERIALS AND METHODS

Animals

Mature goldfish were procured from local ornamental fish dealers. From the common stock, breeding pairs weighing 20-50 gm each were selected (females with reddish swollen vent, bulky abdomen and active males with white tubercles on operculum and secretion of milt) and segregated. Segregated males and females were maintained in separate tanks (120 cm diameter and 90 cm height) at 25 ± 1 °C on 12 L : 12 D photoperiod. They were fed on a prepared diet (fish meal, wheat flour, rice bran, yeast, vitamins and calcium), live tubifex worms and mosquito larvae.

Chemicals

PGE_1, PGE_2 and arachidonic acid (prostaglandin precursor) were procured from Cayman Chemical; Ann Arbor, MI, USA. $PGF_{2\propto}$ (Lutylase) was purchased from Upjhon S.a. Puurs, Belgium and the Prosolvin used was from Intervet, International B.V. Boxmeer- Holland. Tiaprost, a synthetic $PGF_{2\propto}$ analogue was procured from Hoechst Roussel Vet GmbH d - 65203 Wiesbaden. Authentic prostaglandin samples were dissolved in a mobile phase before injecting into HPLC system.

HPLC System

The HPLC system consisted of a column Nucleosil® 100 - 5 C_{18} [250 x 4.6 mm ID]. Mobile phase (pH 3) was of triethyl ammonium phosphate (TEAP) buffer : acetonitrile (55 : 45). Flow rate of the mobile phase was 1.0 mL/minute and elution times of prostaglandins were monitored at 208 nm.

Experiment 1

Tiaprost, a more stable synthetic $PGF_{2\propto}$ analogue was administered to non-ovulated female goldfish at a dose 1 µg/gm body weight. Female was released into 5 l distilled water along with mature male in circular enamel trough. Water sample was collected after 2 h of injection for extraction of prostaglandins. Males of the experimental and control groups were not injected with prostaglandins.

Experiment 2

Prosolvin was administered at a dosage 1 μg/gm body weight to non-ovulated female goldfish. The procedure was similar to experiment 1. Males of the experimental and control groups were not injected with prostaglandins.

Detection of waterborne prostaglandins by HPLC

The device used for extraction of waterborne prostaglandins is shown in Fig. 5.1. The device consisted of following parts: A neck of round bottom flask with 29 F joint was closed with a rubber septum. A barrel of luer-lock syringe was connected at the bottom of flask. Sep-Pak (Waters) cartridge was fixed to the tip of the syringe. A small needle along with flexible rubber tube was pierced through the septum and the other end of rubber tube was connected to nitrogen cylinder or air cylinder (for stable compounds). Uniform continuous flow rate of water was adjusted by controlled pressure.

Prostaglandin-injected female goldfish along with mature male were released in 5 l distilled water (20 ± 1 °C) in circular enamel trough. Females of the control group were injected with fish saline (0.6% NaCl). To avoid possibility of superficial contamination, prostaglandin-injected females were thoroughly washed in fresh water before releasing into the experimental setup.

After half an hour of injection to female goldfish, spawning behavioural changes were observed in non-induced males, females also showed spawning behavioural changes. These activities continued for 1 ½ to 2 h. Water sample was collected after 2 h of injection and double filtered through sintered glass funnel. Round bottom flask of the device was filled with test water sample up to 3/4 of its capacity. Neck of the flask was sealed with standard septum and uniform flow of the water through activated Sep-Pak (Waters) cartridge was maintained by adjusting the pressure. 2-3 l of test water sample was passed through the cartridge. The organic compounds adsorbed on the cartridge were eluted with 10 ml HPLC grade methanol. Methanol was evaporated at room temperature by vacuum distillation and the residual sample was used for HPLC analysis. Same procedure was followed for the control water sample.

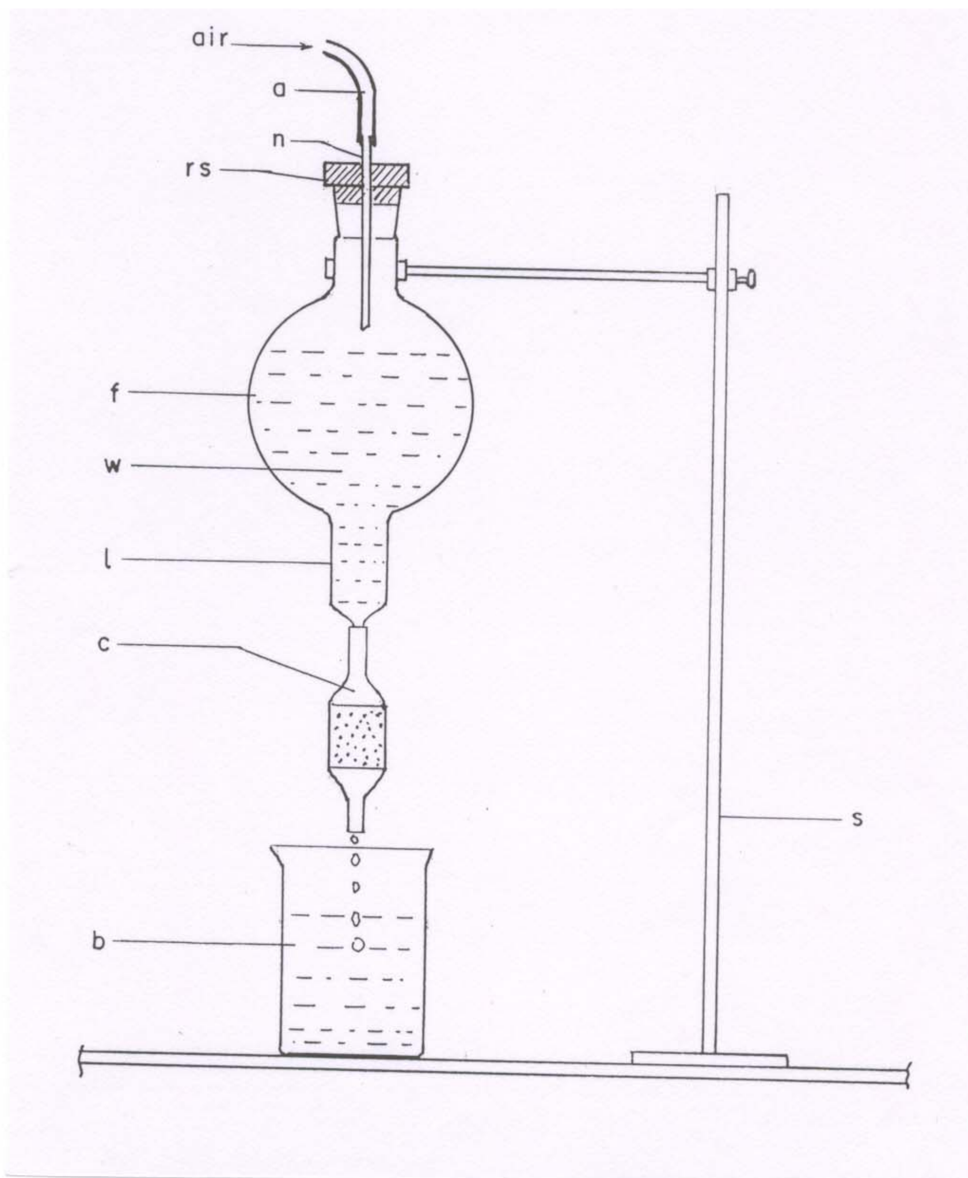

Figure 5.1. Dilute aqueous solution extraction device.

a – Air tube, b – Beaker, c – C_{18} cartridge, f – Round bottom flask, l – Luer-lock syringe, n – Needle, rs – Rubber septum, s – Stand, w – Sample water.

RESULTS

Tiaprost

HPLC chromatogram revealed that the test water sample in which goldfish females administered with tiaprost were released showed presence of the peak with retention time 11.8 min (Fig. 5.2. II) which corresponded to the authentic tiaprost retention time 11.22 min (Fig. 5.2. I). Control sample showed absence of the peak due to tiaprost (Fig. 5.2. III). This indicated that administration of tiaprost to female goldfish resulted into the release of part of injected tiaprost itself in water. It was also observed that flow rate of 1 l/h was quite effective for the extraction of prostaglandins at nanogram levels, from 2-3 l of aqueous sample for HPLC analysis. Present study indicated that a part of tiaprost injected into female is released into water which acts as pheromone for the male.

Prosolvin

HPLC chromatogram revealed that the test water samples in which goldfish females, administered with prosolvin, were released showed presence of the peak of prosolvin retention time 9.17 min (Fig. 5.3. II) corresponding to standard prosolvin retention time 9.16 min (Fig. 5.3. I). Peaks due to PGE_1, PGE_2, arachidonic acid and $PGF_{2\propto}$ were absent (Fig. 5.3. IV, V, VI and VII). Control samples also showed absence of peaks due to prosolvin, PGE_1, PGE_2, arachidonic acid and $PGF_{2\propto}$ (Fig. 5.3. III). This indicated that administration of prosolvin to female did not result in secretion of any other prostaglandin such as $PGF_{2\propto}$, PGE_1, PGE_2 or precursor arachidonic acid but some prosolvin was released in water (Fig. 5.3. II). Part of injected prosolvin from female is released into water, which was detected by HPLC in the present study.

In the experiments in which the prostaglandins were administered at lower dose (0.5 μg/gm body weight) to female goldfish, prostaglandins were not detected by HPLC. It was also found that from 5 l of water quantity prostaglandin analogues were detectable by HPLC but above 5 l water quantity it was not possible to detect the prostaglandins. Prostaglandin analogues were easily detectable by HPLC when freshly prepared samples were used for detection. Extracted samples were stored at -70^0C if immediate analysis

was not possible. Samples degraded when they were stored at 0 to 10 ^{0}C within overnight. The temperature range 20 ± 1 ^{0}C is found to be very conducive to stimulate spawning activity at dose of prostaglandin analogues 1 μg/gm body weight of female goldfish.

The waterborne prostaglandin detection was observed only in those females showing spawning behavioural changes after prostaglandin administration. Experiments conducted in winter season showed excellent results. The water samples collected after 2 h of prostaglandin analogues administration showed presence of prostaglandins easily detected by HPLC.

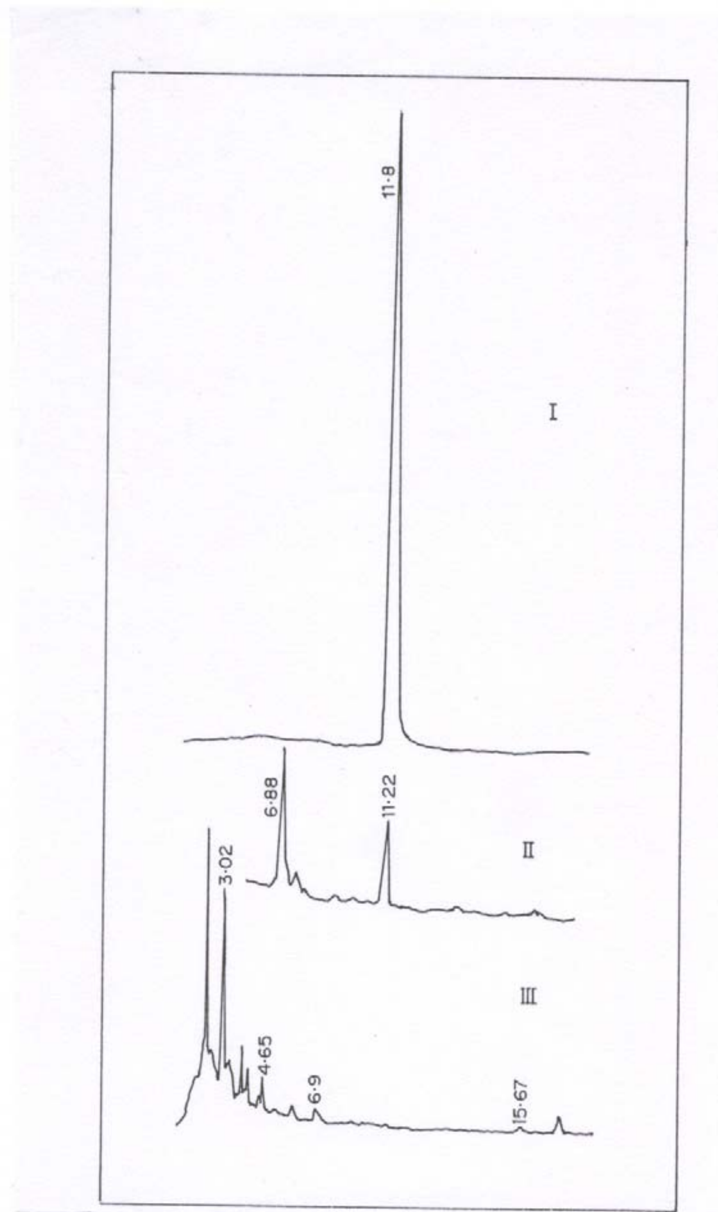

Figure 5.2. Chromatogram of Tiaprost (Prostaglandin analogue) and experimental samples. Elution on Nucleosil ®100 - 5C_{18} with TEAP buffer (pH3): acetonitrile (55: 45). Numbers indicate retention time in minutes. I - Std. Tiaprost, II - Experimental water sample, III - Control water sample.

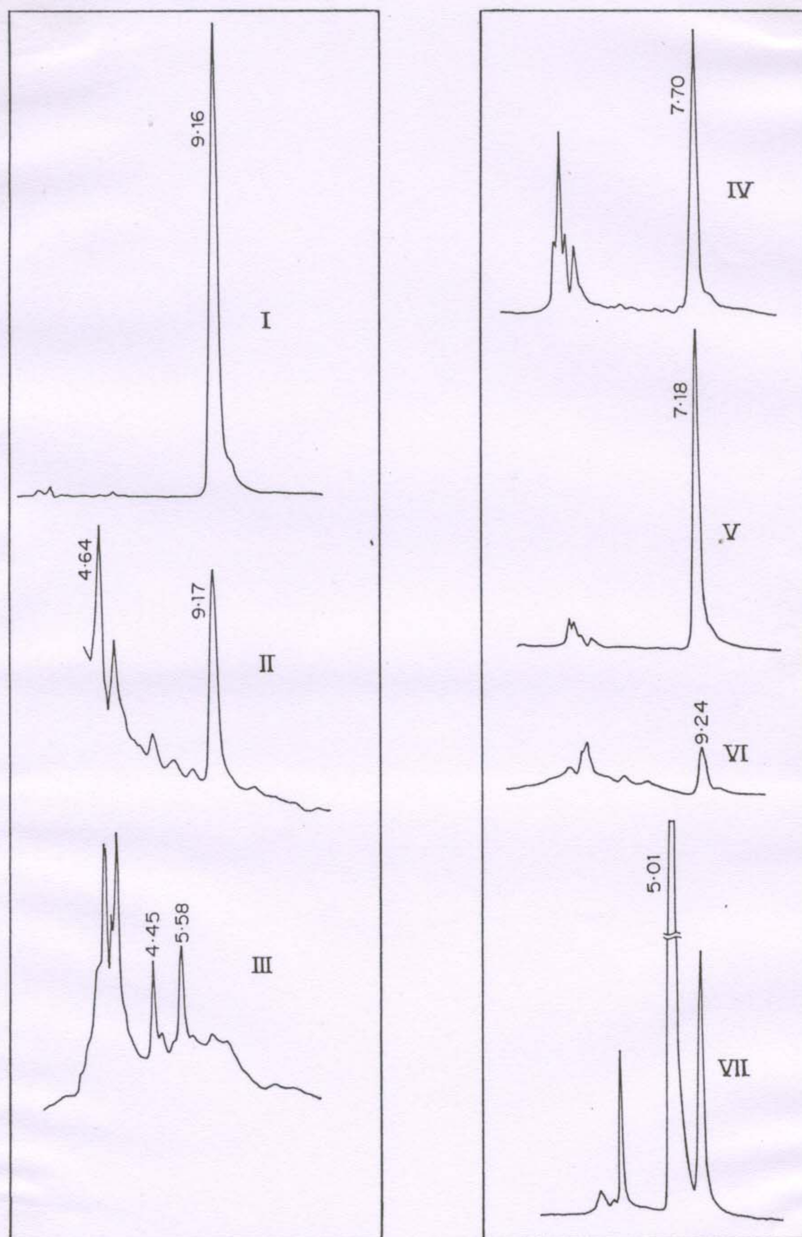

Figure 5.3. Chromatogram of various prostaglandins and experimental samples. Elution on Nucleosil® 100 - 5 C_{18} with TEAP buffer (pH 3): acetonitrile (55: 45). Number indicates retention time in minutes. I-Std. Prosolvin, II- Experimental water sample, III-Control water sample, IV-PGE_1, V-PGE_2, VI-Arachidonic acid, VII-$PGF_{2\alpha}$ (Lutylase).

DISCUSSION

Present results indicate that when prostaglandin analogues are injected (1 μg/gm body weight) to non-ovulated female goldfish, some part of injected prostaglandin analogues is released into water that acts as pheromone on male, induces sexual behavioural responses and increased milt volume in goldfish. The waterborne prostaglandins were extracted and detected by HPLC in the present study.

Circulating prostaglandins are cleared to the water to function as pheromone thereby synchronizing male and female sexual behaviour in goldfish (Sorensen *et al*., 1986). Sorensen *et al*. (1988) also reported that prostaglandins and/or its metabolites act as potent olfactory stimulants comprising postovulatory female sex pheromone in goldfish.

The prostaglandin compounds within crude lipid extracts of tissues and fluids are separated into groups (PGE, PGF, PGA with PGB) by silica acid column or thin-layer chromatography (TLC); the individuals prostaglandins are then isolated by reversed phase partition chromatography or TLC. Labeled prostaglandins added to the original tissue homogenate to indicate the efficiency of the extraction procedure will also aid in identifying the peaks of active material separated during chromatography. Following

TLC, tentative identification of the prostaglandins present can be done by analysis of eluants of those areas of chromatoplates which correspond to authentic prostaglandins concomitantly developed where sufficient quantities of prostaglandins are present (> 2 μg). Spray reagents may be employed.

Several methods for visualizing prostaglandins on chromatograms have been used. The chromatograms are sprayed with 10 % alcoholic phosphomolybdic acid and then heated to 100 °C for 5-10 min; PGE compounds are visualized as yellow-brown spots and the PGF compounds as intense blue.

The initial evidence that the pharmacological active substances within sheep vesicular glands were acidic in nature was obtained by preparative electrophoresis (von Euler, 1937). PGE and PGF compounds released into superfusates of the cat cerebral cortex were partially purified on the basis of their weak acidity by electrophoresis in a pyridine-acetic acid buffer of pH 5.4 (Ramwell and Shaw, 1966). However, neither this technique nor that of paper chromatography (Bergstrom and Sjovall, 1960; Bergstrom and Sjovall, 1960) has been exploited in the separation of prostaglandins owing to the excellent results obtained with TLC. TLC, alone and in combination with other techniques has been extensively applied to the separation of the prostaglandins either as the free acids, methyl esters, or the methylated or acetylated methyl esters.

The precise chemical identity of the postovulatory PGF pheromone has yet to be established and its true biological (behavioural) significance and mechanism of synthesis (metabolism) and release are unknown. The multi-component, metabolite-based PGF pheromone may have more sophisticated biological significance than 17α, 20βP.

The present study supported our previous results (Chapter IV) that when prostaglandins are injected to non-ovulated female goldfish, first they act as hormone in female and female releases some part of injected prostaglandins into the water (detected by HPLC) which acts as pheromone on male and induces sexual behaviour and increased milt volume.

The method described in the present work is effective for extraction of waterborne prostaglandin analogues from dilute aqueous samples of the range of 5 l. The concentrated samples thus obtained can be further analysed by HPLC for detection of

prostaglandins. It was observed that water samples collected after 2 h of prostaglandin administration showed presence of prostaglandins.

PGFs are released via both urine and gills. Release of prostaglandins via urine in pulses is likely an important factor in modifying male behaviour (Sorensen *et al*., 1988). In the present study it was not possible to determine how injected prostaglandin analogues were released by female into water.

Prosolvin and tiaprost veterinary synthetic $PGF_{2\propto}$ analogues used in the present study are reported to be more luteolytic than natural prostaglandins (Dukes *et al*., 1974). This method can be effectively used for the extraction of organic constituents from the large volumes of dilute aqueous samples and will be useful for further study of induced breeding using various synthetic prostaglandins.

CHAPTER - VI

GENERAL DISSCUSSION

Induced breeding of Indian major carps is indeed an extremely important aspect of aquaculture as these fishes do not normally breed in captivity even if all environmental conditions are optimum. Besides, they are in great demand as food fish in India. Indian major carps breed only once a year during the monsoon which coincides with the breeding season of the majority of Indian teleosts. The precise nature of the monsoon cue is still unknown.

Prostaglandins are involved in inducing ovulation (Stacey and Goetz, 1982), stimulating gonadotropin secretion (Singh and Singh, 1976; Stacey and Goetz, 1982) and in the regulation of spawning behaviour in cyprinids (Stacey and Peter, 1979).

Besides, some studies also indicate the stimulatory effect of prostaglandins on gonadotropin release mainly via their effect on hypothalamus (Sato *et al*., 1974; Harms *et al*., 1974; Eskay *et al*., 1975). Some reports are also available on the localized effect of prostaglandins at ovarian level (Osman and Dullaart, 1976; Jalabert and Szollosi, 1975; Stacey and Pandey, 1975).

So far there is no report on use of prostaglandins for induced breeding of Indian major carps in India or abroad. We made a maiden effort to observe whether prostaglandins can induce spawning in Indian major carps.

Preliminary study was conducted on goldfish (*Carassius auratus*) in laboratory conditions so that on the basis of these results, trials could be conducted on Indian major carps, initially as exploratory work and later on fields on a large scale.

Keeping the foregoing goal in mind, the results obtained in the present work may be interpreted and discussed as below.

Ovaprim (sGnRHA) is a known spawning inducing agent in Indian major carps. In the present study ovaprim was found to be a very effective spawning inducing agent in goldfish. Among the selected doses, spawning took place at the dose 0.5 μl/gm body weight of female and 0.125 μl/gm body weight of male. Generally spawning took place after 10-12 h of injection. Number of eggs, fertilization rate (%) and hatching rate (%) were normal. Hatchlings obtained by this method were healthy (Chapter II, Table 2.2).

Various prostaglandins and their analogues (natural, commercially available and synthetic) and other hormones were studied for goldfish induced breeding. Amongst the

tested PGs, cloprostenol and tiaprost (synthetic prostaglandin-$F_{2\alpha}$ analogues) induced spawning in goldfish at the dose 1 μg/gm body weight. Fertilization and hatching were normal. Another conspicuous and consistent observation was the high rate of fertilization whenever PG analogues (tiaprost and cloprostenol) were employed as compared with that of control. The high rate of fertilization appears to be due to the complete development of eggs under the endogenous gonadotropin action. Apart from the high rate of fertilization the hatching success was also generally high with cloprostenol and tiaprost.

It is also possible to reinduce the goldfish breeders after every 35-45 days by administering cloprostenol. As per our knowledge, this is the first report of cloprostenol to induce repeated (multiple) spawning in goldfish. This induced breeding technique definitely increases the production of ornamental fishes.

In case of tiaprost, induced spawning behaviour and spawning in male and female goldfish were observed even when only females were injected with tiaprost and none of the male was injected with tiaprost. These females release some chemicals into the water which act as pheromone on male to induce increase in milt volume.

On the basis of the present findings, it seems likely that many other species of fish particularly the cypriniforms use prostaglandins as both hormones and pheromones. However, the possibility of species-specific differences in production remains. It will be exciting to extend our studies to other species and to examine the specialized mechanisms which must underlie PG production and release in the goldfish. The results of the present study may have practical application in mass-scale seed production of goldfish.

In the present study it was found that when non-injected males were exposed to prostaglandin analogue (tiaprost, prosolvin and lutylase)-injected females (1 μg/gm body weight) increase in milt volume in male goldfish significant ($P < 0.05$, chapter IV, Fig. 4.1).

Present results are consistent with the other findings (Stacey, 1981; Sorensen *et al*., 1989; Sorensen *et al*., 1988) that tiaprost, prosolvin and lutylase (synthetic prostaglandin-$F_{2\alpha}$ analogues) not only induce spawning behaviour in female goldfish but also induce male to increase milt volume. Probably prostaglandin-injected females release some chemicals which act on males as pheromones.

In the present study it was also found that the non-induced males of the experimental groups, when exposed to tiaprost, prosolvin and lutylase which were directly added into the aquarium water (10 μg/l), induced males to increase milt volume ($P < 0.05$, chapter IV, Fig. 4.2). Further research work is needed to induce spawning in females by directly adding prostaglandins into the water.

Present results of prostaglandins as a pheromone in goldfish for increase in milt volume warrant field trials under culture conditions to determine whether pheromonal milt enhancement can offer practical alternatives to existing invasive techniques. Once this technique is standardized in goldfish breeding, same technique will be tried on Indian major carps and if this technique works in case of Indian major carps to induce breeding by directly adding prostaglandins into the water that will be a major breakthrough in aquaculture. Under culture conditions, addition of prostaglandins directly into water would reduce labour cost and handling stress by eliminating injections and reduce risk to broodstock because the pheromone treatment activates normal endogenous mechanisms theoretically eliminating the potential for generating pharmacological high plasma hormone levels.

Arachidonic acid and eicosapentaenoic acid (EPA) precursor of PGs induce spawning behaviour in goldfish. More trials are required to standardize the doses for induced spawning.

Prostaglandin-E_1, prostaglandin-E_2, 16-phenoxytetranor-$PGF_{2\alpha}$, 16,16-dimethyl $PGF_{2\alpha}$, 16,16-dimethyl PGE_2 and PG analogues synthesized at NCL, Pune were found ineffective in the preliminary study. Further trials are required for final conclusions.

In the present study it was found that when prostaglandin was injected to non-ovulated female goldfish, it first acts as a hormone in female inducing female sexual

behaviour and female releases some part of the injected prostaglandin into water which acts on male as a pheromone to induce male to increase milt volume. Waterborne prostaglandins were detected by HPLC. This study confirmed that prostaglandin acts as hormone as well as pheromone.

In the present study a method for extraction of prostaglandins from the large volume of dilute aqueous samples was developed. This method developed for extraction and detection of waterborne prostaglandins in the induced breeding experiments on goldfish could be employed in case of Indian major carps.

Various prostaglandins and their analogues (natural, commercially available and synthesized at NCL, Pune) were tried individually or in combination with priming (HCG and carp pituitary gland extract) for induced breeding of Indian major carps.

Cirrhinus mrigala: Among the selected compounds, cloprostenol at the dose 75 μg/gm body weight and domperidone (1 mg/kg body weight) for both male and female, resulted in release of 50% eggs in the ovary was observed by dissection after 12 h of injection (Chapter III, Table 3.5).

Tiaprost is an another analogue of $PGF_{2\alpha}$ which induced spawning behaviour in male. When female was injected with HCG priming dose (200 IU/kg body weight) followed by second injection of tiaprost (50 μg/gm body weight) after 6 h and male was administered only tiaprost at the dose 50 μg/gm body weight at the time of second dose to female, male showed spawning behaviour, chased the female actively and released milt into the water but female did not respond (Chapter III, Table 3.5).

This research work provides a base line. Further research work is needed to standardize the doses for induced spawning in Indian major carps. These analogues will be useful for inducing spawning in Indian major carps. No research work is available on effect of tiaprost on breeding of Indian major carps. It is the first report about the effect of tiaprost on breeding of Indian major carps.

With other prostaglandins, egg-plugging effect was observed in mrigal females. There are no definite known reasons for plugging. It is belived that over-dosage of

hormones and /or over handling of brood fish result in clumping of eggs which block the oviduct.

Labeo rohita: It was found in the present study that when tiaprost was (50 µg/gm body weight) administered to female with HCG priming (200 IU/kg body weight) after 6 h of gap and male was administered with only tiaprost (50 µg/gm body weight) at the time of second injection to females, partial ovulation was observed after 18 h of second injection (Chapter III, Table 3.9). This preliminary result shows that the tiaprost may induce spawning in Indian major carps but further research work is needed.

With other prostaglandin analogues, only egg plugging effect was observed in females of *Labeo rohita*.

The Indian major carp milters need induction for release of spermatozoa in captivity. Carp milters are induced for spawning by using various inducing agents *viz*, pituitary gland extract, ovaprim etc but no report is available on induction of Indian major carp milters with prostaglandins. In the present study it was found that tiaprost (50 µg/kg body weight) induced fourfold increase in milt volume (0.78 ± 0.20 to 3.05 ± 0.47, $P < 0.001$) in *Labeo rohita* after 6 h of injection. This study showed that higher doses of tiaprost (75 µg/kg body weight) result in decrease in milt volume. The sperm counts after tiaprost administration at the doses of 50 µg/kg body weight and 75 µg/kg body weights were $3.0 \times 10^7/mm^3$ and $3.37 \times 10^7/mm^3$ respectively. The volume of the milt did not increase as compared to control in experiments with prosolvin (Chapter IV, section – B, Table 4.1).

The most important fact reported here is the ability of tiaprost to induce milters of Indian major carps to increased milt volume, spermatozoa, motility etc. Further research work is needed to standardize doses in Indian major carp females for induced spawning.

It will be exciting to extend our studies to other species and to examine the specialized mechanisms which must underlie production and release of prostaglandins in the goldfish.

Till date there is no reference available on induction of milt volume in *Labeo rohita* with tiaprost (synthetic prostaglandin analogue) it is first report of induction of *Labeo rohita* milters with tiaprost. This result indicates that further work in this field will

lead to identification of prostaglandin analogues for induction of spawning in Indian major carps.

CONCLUSIONS

- The effect of various prostaglandins, natural as well as synthetic analogues, has been tested on *Carassius auratus* (goldfish), *Labeo rohita* (rohu) and *Cirrhinus mrigala* (mrigal) in view to know if they can induce behavioural changes, ovulation and spawning.
- Courtship behavioural changes induced in goldfish after administration of luteolytic $PGF_{2\alpha}$ analogues *viz*. tiaprost and prosolvin were very prominent.
- Among the tested compounds $PGF_{2\alpha}$, cloprostenol and tiaprost induced spawning in goldfish, *Carassius auratus*.
- Tiaprost showed delayed spawning effect after 72 h of injection in goldfish. Prostaglandin precursors eicosapentaenoic acid and arachidonic acid induced spawning behaviour in goldfish.
- Significant increase in the milt volume was observed in male goldfish when exposed to prostaglandin injected female and water in which prostaglandins were added directly.
- Waterborne prostaglandins could be extracted and detected by HPLC.
- Selected prostaglandins *viz*. $PGF_{2\alpha}$, α-N-R, β-N-R and P-M-R showed only oxytocic activity and was reflected by egg bound effect in Indian major carp females.
- Luteolytic prostaglandins such as tiaprost and cloprostenol could induce courtship behavioural changes in Indian major carp males.
- Tiaprost at the dose of 50 μg/kg body weight of male of *Labeo rohita* could increase the milt volume and number of spermatozoa.
- Preliminary trials indicated that prostaglandin analogues could induce Indian major carp milters.
- Overall the synthetic analogues were much more effective than the natural PGs. They were more stable as well as non-toxic to the experimental fish and cost wise they are also cheaper than natural prostaglandins.

- The present work on carp opens new perspectives in current practice of induced breeding with:
    1) The possibility to replace the classical invasive method by direct addition of prostaglandins into water which will induce carps to spawning.
    2) Reduction in the handling of brood fishes by using non-invasive pheromone breeding technique.
    3) Reduction in the cost of injection by injecting only female with inducing agents wherein the female releases pheromone which acts on male and spawning takes place without injecting the male with inducing agent.

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- Not seen in original.

LIST OF PUBLICATIONS AND PATENTS

PAPER PRESENTED

The following papers were presented in The *Fifth Indian Fisheries Forum*, held at Central Institute of Freshwater Aquaculture, Bhubaneswar, 751 002 India, on *17 – 20 Jan, 2000.*

1. H. S. Jagtap, S. S. Ghumare, H. B. Borate, R. D. Wakharkar and T. Ravindranathan. Induced breeding of goldfish, *Carassius auratus* with Cloprostenol. *Abstract No. PY- 38*, pp – 80.

2. S. S. Ghumare, H. S. Jagtap, H. B. Borate, R. D. Wakharkar, T. Ravindranathan, Bithi Mondal, S. D. Gupta and S. C. Rath. Quantitative assessment of milt in prostaglandin induced milters of *Labeo rohita* (Ham). *Abstract No. PY – 28*, pp - 77.

3. S. S. Ghumare, H. S. Jagtap, H. B. Borate, R. D. Wakharkar and T. Ravindranathan. Tiaprost mediated induced spawning of goldfish, *Carassius auratus*. *Abstract No. AQ – 65*, pp-17.

PUBLICATIONS

1. H. S. Jagtap, S. S. Ghumare, H. B. Borate, R. D. Wakharkar and T. Ravindranathan (2001). Pheromonal activity of Prosolvin in goldfish, *Carassius auratus* (Linnaeus). *Journal of Applied Aquaculture*, **11** (3): 81-88.

2. S. S. Ghumare, H. S. Jagtap, H. B. Borate, R. D. Wakharkar and T. Ravindranathan (Jul.-Sep., 2001). Extraction of waterborne prostaglandins from female goldfish, *Carassius auratus* (Linnaeus). *Indian J. of Fisheries,* **48** (3): 337-339.

3. H. S. Jagtap, S. S. Ghumare, H. B. Borate, R. D. Wakharkar and T.

Ravindranathan (Oct.-Dec., 2000). Some observations on Tiaprost mediated induced spawning of goldfish, *Carassius auratus* (Linnaeus). *Indian J. of Fisheries*, **47**(4): 371-375.

4. S. S. Ghumare, H. S. Jagtap, H. B. Borate, R. D. Wakharkar, T. Ravindranathan, Bithi Mondal, S. D. Gupta and S. C. Rath. Quantitative assessment of milt in prostaglandin induced milters of *Labeo rohita* (Ham). *Journal of Aquaculture In the Tropics* (in press).

5. H. S. Jagtap, S. S. Ghumare, H. B. Borate, R. D. Wakharkar and T. Ravindranathan. Induced breeding of goldfish, *Carassius auratus* with Cloprostenol. Accepted for publication in *Proceedings of Fifth Indian Fisheries Forum*, *No. FIFF / Proc./ 2001-17/88.*

PATENTS

1. A process for the preparation of 3 - sulfanyl cyclic ketones.
T. Ravindranathan, R. D. Wakharkar, H. B. Borate, P. D. Shinde, V. A. Mahajan, V. H. Tillu, S. S. Ghumare and H. S. Jagtap. *Indian Patent*, *Nf - 374/99*.

2. A novel 3-sulfanyl cyclic ketones. (Product patent).
T. Ravindranathan, R. D. Wakharkar, H. B. Borate, P. D. Shinde, V. A. Mahajan, V. H. Tillu, S. S. Ghumare and H. S. Jagtap. *Indian Patent*, *Nf - 80/2000*.