The Journal of Animal & Plant Sciences, 24(1): 2014, Page: 159-166
ISSN: 1018-7081

INDUCED BREEDING, EMBRYONIC AND LARVAL DEVELOPMENT OF CRITICALLY ENDANGERED FISH *PUNTIUS SARANA* (HAMILTON, 1822) UNDER CAPTIVE CONDITION

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ABSTRACT

In the present study, *Puntius sarana* collected from Khandepar River were transported in live condition and acclimatized to captivity. The captive brood stock was successfully raised in FRP tank with a specially balanced fish diet and intermittently fed with *Tubifex* warms for a period of one month. Sex of brooders was identified based on morphological features – the abdomen was swollen in females, while it was firm and round in males. Induced breeding of this species, with ovatide at 0.2 ml per male (180 g) and 0.3 ml per female (232-240 g) was achieved. Sex ratio of 2:1 and 1:1 (male: female) were maintained in two trials. The interval between injection and spawning was 8-9 hours. The average fertilization and hatching rates observed were 90.5% and 75.39 % respectively. The hatching period was observed to be 15-16 hours at water temperature 26.5-28.5°C. The diameter of fertilized eggs was 1.3mm. The result presented in this study acquaint with a step ahead for the inventory and conservation of commercial freshwater food fish, *Puntius sarana* in Indian Rivers. The originality of this work and its application is for the conservation purpose reside in the scope of studied species. The present work contributes to cover deficient information for *Puntius sarana.*

Keywords: *Puntius sarana,* brooders, breeding, conservation, hatchery

INTRODUCTION

Among the fresh water species, carps are the back bone of Indian freshwater aquaculture, comprising around 90% of the total fresh water fish production (FAO, 2008). Carp culture account for about 95% of the country's total aquaculture production (FAO, 2008). Among the minor carps, *Puntius sarana* is a medium sized carp species and reported to have moderate growth rate compared to the major carps. Higher consumer preference, even at smaller size of 100–200 g, makes the species a suitable candidate for diversifying carp culture (Gopakumar *et al.,* 1999; Chakraborty *et al.* 2003). It has also been proven that these species could be cultured along with carps (polyculture) which would increase the total production (Jena *et al*. 2008). It is a tasty, the most popular and favourite table fish among barb species having high nutritional and market value in south Asian countries (Chakraborty *et al*. 2006). *Puntius sarana* is distributed in the Gangetic river system and its eastern region of the country (Mohanta *et al*., 2008). It was once common in ponds, rivers, streams, reservoir and lakes of India. Of late, the natural stocks of this species have dwindled to a great extent (David *et al.* 1974). Natural breeding of *P. sarana* was reported by Bhatnagar (1963, 1979) who spotted spent female and spawn in nature. However, spawning did not seem to have contributed to recruitment as evidenced by the progressive decline in population of these fishes. Chaudhuri and Alikunhi (1957) and Chaudhuri (1962), had successfully bred *P. sarana* in India through carp pituitary injection. However, no efforts were made on its brood stock development, breeding, seed rearing and grow-out culture in the country.

Due to less fry survival and indiscriminate over-exploitation, the minor carp resources have been drastically reduced from abundance to vulnerable in South–East Asian regions (Chondar, 1999; Mahanta *et al*., 1994). *P. sarana* conservational status has been referred to as critically endangered (IUCN Bangladesh, 1998; Ameen *et al*., 2000; Hussain and Mazid, 2004). (Mijkherjee *et al.* 2002) categorized it as a vulnerable species. These minor carps can be conserved by ranching and introduction of these species into aquaculture systems as a diversified species. In order to conserve this species, a comprehensive approach needs to be followed starting from, brood stock development with breeding technology standardization. The proposed study was therefore undertaken to develop captive brood stock, artificial breeding, fry and fingerling rearing for *P. sarana*

MATERIALS AND METHODS

Collection and Experimental site for animals: Khandepar River emerges in the Western Ghats, moving westward and meets the Arabian Sea at Goa (India). The Khandepar River has a rich biodiversity and the genus *Puntus* is one of them. A total of eight specimen of *P. sarana* were collected, out of which three were males and

five were females. The fishes were packed in polythene bags filled with 1/3 water and 2/3 pure oxygen and to prevent any damage to the bags, they were kept in cartons. The experiments were conducted in the wet lab (breeding units) of Central Institute of Fisheries Education (CIFE), Old Campus, Versova, Mumbai. The fishes were brought to the CIFE wet laboratory and given bath treatment in 5 ppt saline water for about an hour. Thereafter the fishes were transferred to 1000 litre capacity FRP tank filled with 600 litres of filtered fresh water. The fishes were fed with formulated pelleted feed manufactured by M/s. Godrej with brand name Jalapari having approximately 32% protein. The feed was given at the rate of 5% body weight. The fish were also fed with tubifex worms intermittently once in two to three days. A 30 days trial was conducted for maturation of the species by intensive feeding. The fishes were regularly observed for their maturation.

Induced Breeding: A 500 liters capacity circular FRP tank was used for breeding of *P. sarana*. The tank was filled with 450 liters of filtered fresh water and arranged aeration provided. As the eggs of *P. sarana* are adhesive in nature, it needs suitable egg collectors. For this purpose 120 gauge polythene strips were used. The polythene strips were 1.0 to 1.5 cm in width and 45 to 50 cm in length. The strips were made into bunches and tied to sinkers to keep the strips in vertical position in the breeding tank. The quantity of strips used was 3 to 4 times the body weight of female. 'Ovatide' an inducing agent manufactured by M/s. Hemmo Pharma, Mumbai was used for spawning. In the first set, the female weighing 240 g was administered with 0.3 ml and two males weighing 180 g and 185 g were administered with 0.2 ml for each and subsequently in second set the female weighing 232 g was administered with 0.3 ml and one male weighing 180 g administered with 0.2 ml. In the both set inducing agent was administered at 10.00 P.M.

The diameter of eggs at various developmental stages was measured with ocular micrometer and stage micrometer fixed to Motic-image plus microscope connected with computer. For hatching of eggs, 500 l capacity circular FRP tank was used. The tank was filled with 500 l of filtered fresh water with aeration. The egg collectors with fertilized eggs were transferred to the hatching tank. Micro-photography of embryonic developmental stages of *P. sarana* was studied by using Phase contrast microscope (Zeiss Company) attached with C-mount camera connected with computer. Water quality of brood stock tank, breeding tank and hatching tank were scrupulously maintained by exchanging water at regular intervals. In order to maintain water quality at optimum level, the water quality parameters viz., temperature, pH, dissolved oxygen, total alkalinity, and ammonia were recorded and analyzed at fortnightly intervals as per the procedure of APHA (1998).

Estimation of spawning fecundity and fertilization rate: The spawning of fish was confirmed the next day by examining the eggs on the plastic strip. After spawning, the spent brooders were removed from tank. Then the spawning fecundity and fertilization rate were estimated using random sampling method. Polythene strips attached with eggs were randomly collected from five different places from the hatching tank after 8 hours of spawning. The fertilized and unfertilized eggs from five samples were counted separately. The spawning fecundity and percentage of fertilization were estimated by using the following formulae:

$$\text{Fecundity} = \frac{\text{No.of eggs per g of polythene strips} \times \text{Total wt.of polythene strips in g}}{\text{Wt of fish in g}} \times 1000$$

Note: Fecundity per Kg. body weight.

$$\text{Fertilization rate (\%)} = \frac{\text{Number of fertilized eggs}}{\text{Total no of eggs in sample}} \times 100$$

Harvesting of spawn: Yolk sac was absorbed after 60 to 65 hours of hatching. After yolk absorption, the spawn was harvested and randomly counted from different locations of the tank in the presence of thorough aeration for uniform distribution. The quantity of spawn was calculated by using the following formula:

No. of spawn harvested= No. of spawn per litre X volume of water in spawning tank.

Hatching rate was calculated after harvesting of spawn on third day after hatching. The hatching rate was calculated by using the following formulae.

$$\text{Hatching percentage} = \frac{\text{No. of spawn}}{\text{No. of fertilized eggs}} \times 100$$

A 1500 liters capacity FRP tank was filled with fresh water and fertilized with organic slurry normally used for culture of zooplankton.

Preparation of egg custard: Various authors have formulated egg custard with different ingredients keeping chicken eggs as a major component. The ingredients were mixed thoroughly in blender and cooked in a boiling water bath for about 15 to 20 minutes to make it semi-solid form. The egg custard was stored in a refrigerator until further use. The protein content of the egg custard was found to be 45.2%. The egg custard was passed through 0.2 to 1.0 mm size sieves to feed various larval stages.

Statistical analysis: All data presented are expressed as means± standard error subjected to two way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test with the help of SPSS-16.0 version software

RESULTS

Brood stock development: The fishes were observed regularly for morphological indicators of maturation. During maturation it was observed that males were smaller in size than females. Male and female brooders were identified by following morphological indicators. The females, with slightly smaller and brighter pectoral fins, bulging abdomen and discharged ova on applying slight pressure on abdomen whereas males, with slightly larger and dull colour pectoral fins, linear body and oozing milt on applying slight pressure on abdomen.

Induced breeding: Two sets of fish were bred successfully in 500 liters capacity FRP tanks through induced breeding method by using 'Ovatide' as an inducing agent. The details of breeding trials are given in Table- 1**.** The fish were spawned after 8 to 9 hours of administration of inducing agent. The spawning was observed by the presence of eggs on the polythene strips (egg collectors) and whitish foam on the water surface in the breeding tank. Fishes were spawned after 8 - 9 hours of hormone administration at 26-28°C. Spawning details are presented in Table -1.

Fecundity: The fecundity was estimated by random sampling method. The spawning fecundity of two bred females was 78,500 and 72,800 for 240 g and 232 g of fish respectively. On the basis of present experimental results, average fecundity of *P. sarana* is 3, 20,438 Nos. / Kg body weight. The detail of fecundity is given in Table- 1. The fertilization rate was found to be 92 % for the first set and 89.5% for the second set. The average fertilization rate was found to be 90.5 %.

Embryonic and larval development: The following embryonic developmental stages were observed and their characters discussed:

Morula stage; cleavage started in the fertilized egg after 30 minutes. Gradually the cleavage furrow is restricted to animal pole of cytoplasm. After repeated successive cleavages, a large number of cells formed as group at animal pole. This stage of embryonic development is called 'morula' (Figure -1 A). At this stage, the mean diameter of eggs was 1.29±0.015 mm. **Blastula stage;** after morula, the developing embryo further divided into numerous cells and arranged in the form of a layer called blastoderm. Gradually, the blastoderm formed into several layers due to further cell division which is called blastodisc. At this stage, a space was formed between yolk and blastoderm, which is called blastocoel. This stage of embryo is called 'blastula' (Figure -1 B). The mean diameter of eggs was 1.3±0.010 mm. **Early gastrula stage;** at this stage, the blastoderm started invading and slowly spread over the yolk in the form of a thin layer ((Figure -1 C). This is beginning of gastrulation. The eggs measured 1.3±0.012 mm. **Mid gastrula stage;** in this stage, the lower rim of the blastodisc thickened and formed a ring. The ring is called 'germinal ring' (Figure -1 D). This was clearly visible around the yolk. The mean diameter of eggs was 1.3±0.012 mm. **Late gastrula stage;** in the late gastrula stage, blastoderm covered more than 80 percent of the yolk. The embryonic shield was clearly visible. Optic rudiment was clearly visible (Figure -1 E). The eggs measured 1.3±0.012 mm in diameter.

Yolk plug stage; at this stage, yolk invasion was completed by gradual spreading over the germ layer. Rudimentary head and tail were formed, and differentiated from the yolk (Figure-1 F). It measured 1.3±0.012 mm in diameter.

Organogenesis; the embryo was elongated. Head and tail end were clearly differentiated from the yolk, and heart beat was noticed. Yolk sac was clearly visible. Rudiments of various body organs were formed at this stage (Figure-1 G).

C- Shaped embryo; the embryo elongated and gradually differentiated head and tail. The body formed into C-shape. The yolk was attached between tail and head. Myotomes development was observed. The embryo started occasional movement (Figure-1 H).

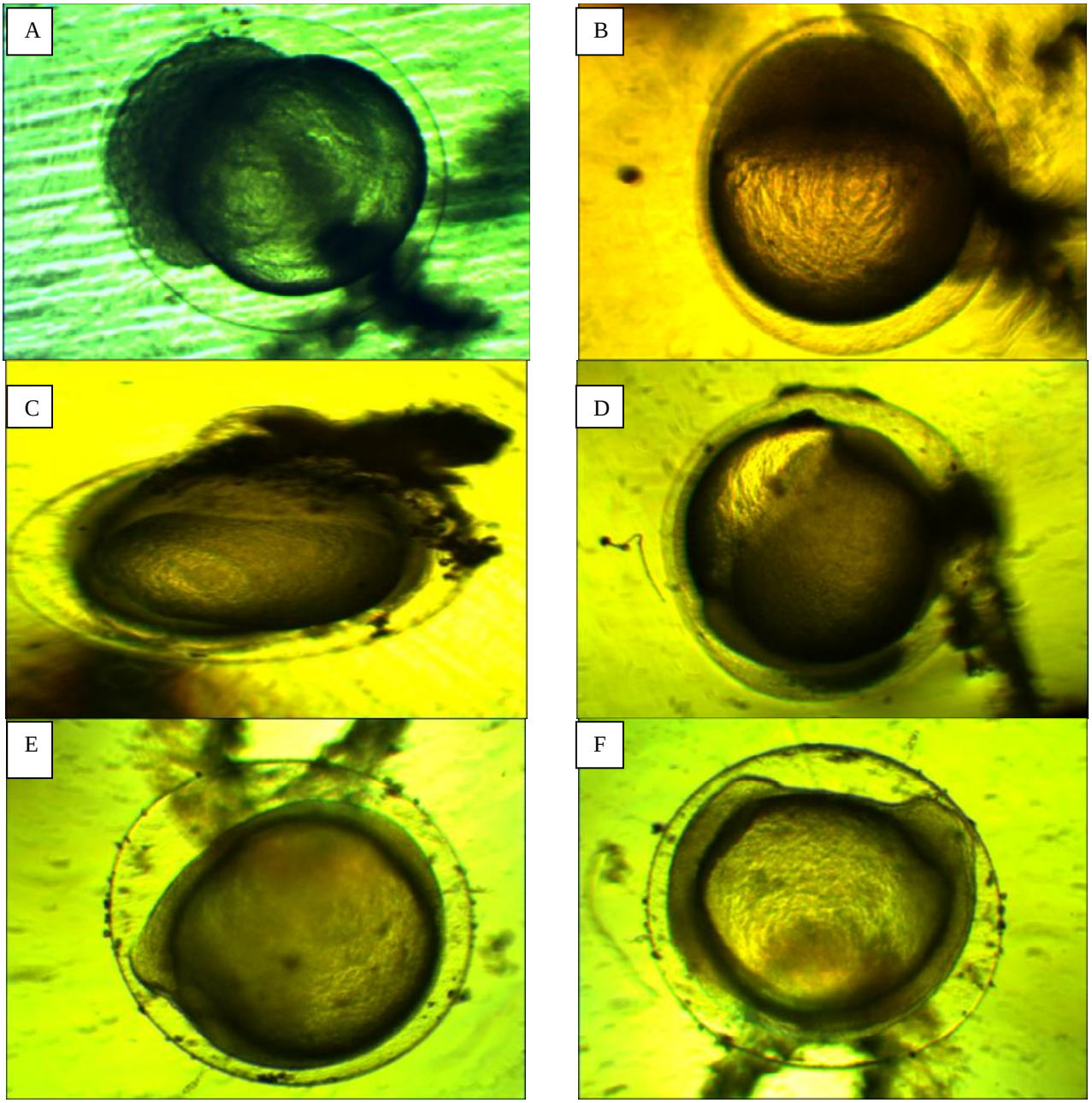
Twitching stage; the tail was completely detached from the yolk. The yolk sac was restricted to head region. The number of myotomes increased. The embryo became active and exhibited continuous twitching movement (Figure-1 I).

Hatching of embryo; as the embryonic development completed, the twitching movement started vigorously and the embryo hatched out within 15-17 hrs at water temperature 26.0-28.0^0 (Figure-1 J). **Newly hatched larva;** the vigorous movement of fully developed embryo ruptured the egg shell and larva emerged. The length of freshly emerged larvae ranged between 3.0 mm and 3.5 mm (Figure-1 K). **Hatching;** the development and hatching of P. sarana eggs were same as that of carps. The eggs which showed asymmetrical development died during the course of hatching. Hatching of the fertilized eggs was observed after 15 hours of spawning and continued up to 17 hours at 26 to 28°C. The details are presented in Table-1.

Rearing of spawn; the yolk sac absorbed fry were harvested and stocked in well prepared FRP tank for further rearing. The fry were grown to 12 to 15 mm in 18 days.

Table 1: Result of induced breeding trials of *Puntius sarana* administered with ovatide

Breeding trials	Wt of Brooder	Sex ratio	Dosage of inducing agent 'Ovatide'(ml / individual)	Spawning period (hrs)	Fecundity	Fertilization (%)	Incubation period (hrs)	Hatching (%)	Spawn obtained (nos.)	Water temperature (°C)
1st trial	F = 240 g M1 = 180 g M2 =182g	2:1	F = 0.3 M =0.2	8 – 9	78,500	92.00	15-16	72.28	52,200	26.5 – 28.0
2nd trial	F = 232 g M1 = 180 g	1:1	F = 0.3 M = 0.2	8 – 9	72,800	89.50	15-16	78.50	51,150	27.0 - 28.5
Average		...	...	...		90.50	...	75.39	51,675	27.5



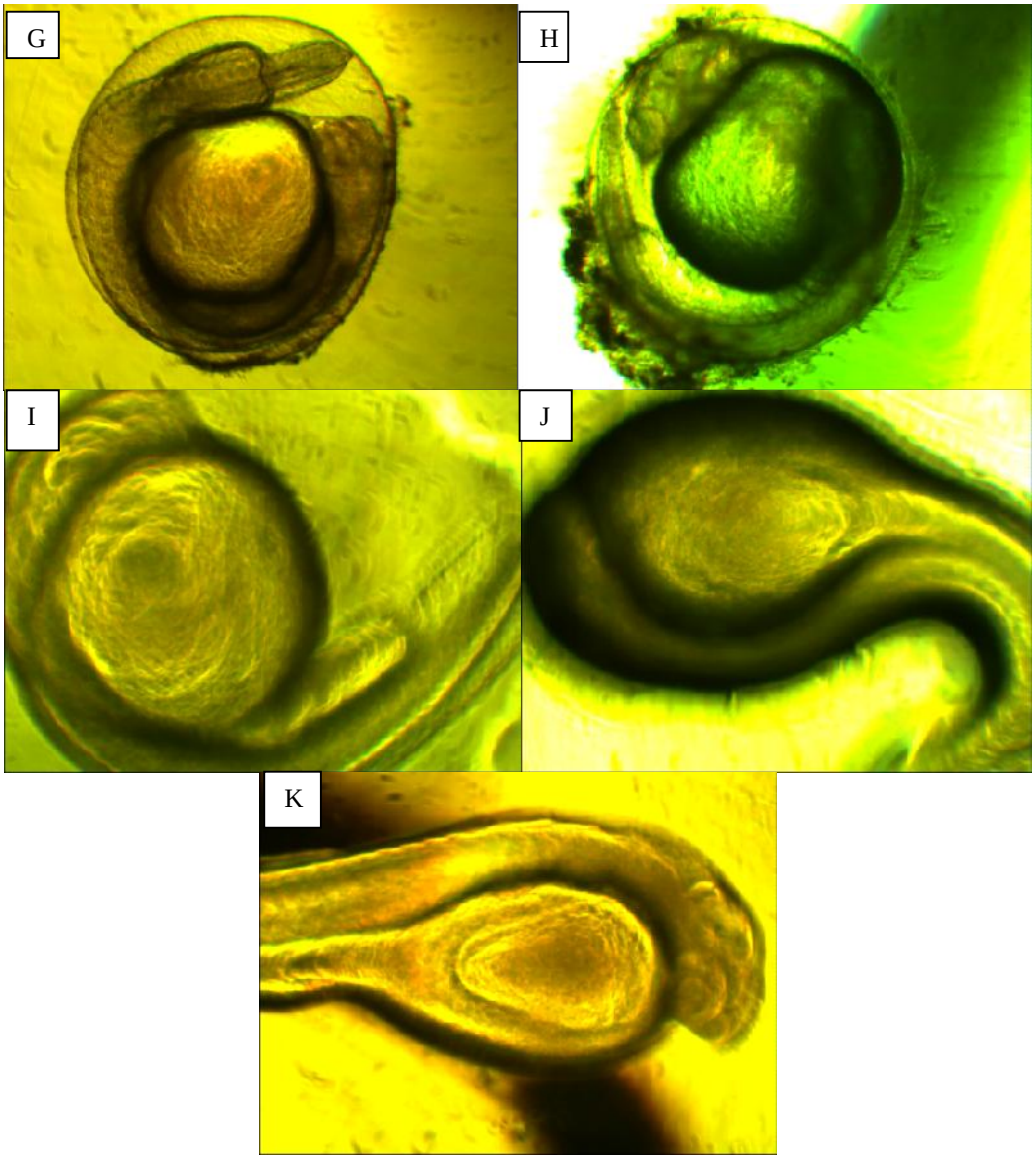


Figure 1: Embryonic developmental stages of *P. sarana.* (A) Morula stage, (B) Blastula stage, (C) Early gastrula stage, (D) Mid gastrula stage, (E) Late gastrula stage, (F) Yolk plug stage, (G) Organogenesis, (H) C-Shaped embryo, (I)Twitching stage, (J)Hatching of embryo, (K) Newly hatched larva.

DISCUSSION

The olive barb *Puntius sarana* is one of the commercially important barb species which has great potential for aquaculture in the Eastern and North Eastern parts of India. In the recent years there has been a growing concern about the non-availability of quality fish seed for sustainable fish production. Management of brood stock has, therefore, to be taken into consideration. By improving and controlling the quality of brood stock and breeding activities in hatcheries, aquaculture productivity could be improved and impacts on capture fisheries and biodiversity reduced (Mourente and Odriozola, 2006). The reproductive cycle of *P. sarana* was found to follow the normal pattern, timing of growth phase and maturation stage of germ cells in both male and female and was comparable to that of the other medium carps (Chakraborty *et al*., 2007). The testicular development of *P. sarana* occurs much earlier than that of the ovary (Chakraborty *et al*., 2007). The maturation of oocytes in *P. sarana* was found to be asynchronous to partially synchronous because all the oocytes matured at different intervals (Chakarborty *et al*., 2007). Some of these eggs became fully matured while some remained in developing condition (Chakraborty *et al*., 2007). The proportion of developing oocytes were found to be higher during May to mid-September and smaller oocytes passed through vitellogenesis and reach final maturation

and got released during second spawning in the months of August and September (Chakraborty *et al*., 2007). Based on this, it was presumed that this species is having a prolonged breeding period. The observations in the present study that males matured in April and females in the last week of May are in concurrence with the finding of (Chakraborty, *et al*., 2007). Similar observations of varying maturity stages of oocytes were reported in *P. carnaticus* by Manoj kumar and Kurup 2009.

The results of the present study have shown that 'Ovatide' at 0.3 ml/240g female and 0.02 ml/180g male is sufficient to induced spawning in *P. sarana.* For induced breeding of *P. sarana*, a single dose of 5-8 mg pituitary gland/kg body weight is recommended for the female, while a single dose of 4.0 mg pituitary gland/kg body weight was recommended for the male (Mazid and Kohinoor, 2003). Chaudhari and Alikunhi (1957) spawned Indian major carps and *Puntius sarana* through hypophysation technique. (Bhuiyan *et al*., 2006) used different doses of pituitary gland extract for breeding of *Puntius gonionotus.* The result of present study shows that *P. sarana* could be successfully induced bred with ovatide and it is probably the first report on fish caught from Khandepar river (Goa), India.

Spawning response of *P. sarana* in the present study was comparable to that of the major carps where 95-100% breeding response is easily achieved (Basavaraja *et al*., 1999). In *P. sarana*, female with a length of 23.8-38.0 cm and weight of 180-792 g, the fecundity ranged between 16,000 and 2, 90,000 (Chandrasoma and De Silva, 2008). In the present study the spawning fecundity of *P. sarana* was 78,500 and 72,800 for 240 g and 232 g of fish respectively. On the basis of present experimental results the average fecundity of *P. sarana* was 3, 20,438 Nos. / Kg body weight. The fecundity recorded in the present study is almost similar to the results reported by Chandrasoma and De Silva (2008). The fecundity of *P. gonionotus* ranged between 3, 00,000 to 5, 00,000 eggs/kg body weight and size of eggs was small (Mohanta *et al*., 2008). The fecundity of *P. sarana* is almost similar to that of *Labeo gonius* where fecundity was reported to be 2, 45, 000 and 5, 40, 000 for fish weighing 800 to 900 g and 1500 to 1600 g respectively (Chondar, 1999).

The percentage of fertilization depends on the quality of brood stock. In the present study, the average fertilization rate was 90.5% and hatching rate was 75.39 %. The low hatching rate may be attributed to hatching of eggs in confined water. The fertilization and hatching percentages were almost similar to that of the major carps, where up to 80% survival could be obtained from eggs to early fry i.e., spawn (Chaudhuri and Singh, 1984) .In the present study, egg incubation period ranged between 15 and 16 hours at 26.5-28.5^{0}C. Hossain *et al*. (2007) observed hatching period between 16 to 18 hours at 27-28^{0}C for the medium carp, *L. bata*. The size and colour of fertilized eggs of *P. sarana* found to be similar to that of common carp, *Cyprinus carpio.* The present study revealed that the colour of fertilized eggs was transparent initially and change to creamy as the embryonic development proceeded. The fertilized eggs were small and after hardening, the size ranged between 1.27 and 1.39 mm. The fertilized eggs were transparent and unfertilized ones were opaque and white. The size of fertilized swollen eggs of common carp ranges 1.5 to 2.5 mm (Woynarovich and Horvath, 1984). The size of freshly hatched larvae of *P. sarana* was 3.0 to 3.5 mm whereas in common carp it was 4.8 to 5.0 mm (Woynarovich and Horvath, 1984).

In the present study, the hatchlings of *P. sarana* were absorbed yolk sac within 60 t0 65 hours at water temperature at 27.0 to 28.5^o C. However, the yolk absorption period was 3-4 days for Indian major carps at water temperature 24-31^o C (Woynarovich and Horvath, 1984). The results indicate that the yolk absorption period depends on water temperature and quantity/size of yolk sac. As the yolk sac of *P. sarana* was smaller than that of Indian major carps, it was absorbed at a faster rate.The growth and survival rate of early fry (spawn) of carp greatly depend on the availability of quality live food organisms, preferably zooplankton (Mumtazuddin *et al*., 1982). In the present study, the early fry (spawn) were harvested from the hatching tank and stocked in well prepared 1500 litres capacity FRP tank for further rearing. The fry attained 12-15 mm in 18 days. No supplementary feeding was done during early growth period. The fry were fed with unicellular microscopic zooplanktonic organisms i.e., infusoria. As the mouth of early fry of *P. saeana* is small as compared to major carps, the infusoria was continued for a period of 4-5 days. At certain occasions the fry were fed with boiled chicken egg yolk during shortage of Infusoria. As early nutrition play a major role in growth and survival of fish, the fry were fed with freshly hatched *Artemia* nauplii once in a day from day 5th to day 10.

Conclusion: *Puntius sarana* can be easily matured and bred successfully under captive conditions similar to that of carps. Brood stocks management and hatcheries should be established for conservation and ranching initiated for sustained natural recruitment of the species. Establishment of proper sanctuaries in selected areas of rivers, floodplain and reservoirs is recommended for conservation of this species. Further studies like genetic characterization will help in conservation of *P. sarana* in natural environment. Therefore, for diversification of carp culture *P. sarana* need to be developed as a candidate species with the help of captive breeding and well established hatchery technology.

Acknowledgements: The authors are grateful to the Director, Central Institute of Fisheries Education,

Mumbai, for providing necessary facilities for carrying out this experiment

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