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Spawning biology, embryonic development and captive breeding of vulnerable loach *Botia dario* (Hamilton)

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Abstract
Botia dario is an vulnerable species (CAMP, 1998) [4] having both ornamental as well as edible value. Breeding experiments in captivity were conducted successfully for the ornamental fish *B. dario* in May 2013-2015 using synthetic hormone. This fish spawned only in running water system. The fecundity of the females ranged from 13,880 to 27,510. The average fertilization rate was found to be 82.09 %. The average Gonado-Somatic Index of B. *dario* for female was 13.21 and for male 3.4. About 14.30-14.40 hours after fertilization the embryo hatched out from the chorion of the egg. The present work contributed to the deficient information on embryonic development, induced breeding and breeding behaviour of *B. dario*.

Keywords: *Botia dario*, Captive breeding, Embryonic development and Conservation.

1. Introduction
Botia (Indian loach) is a genus of freshwater fish of the loach family Botiidae. It is a large genus with about 20 species. *Botia dario* is one of the most active loach being distributed in Bangladesh, Bhutan and North-East India. *B. dario* known as "Queen loach", with yellow golden stripes on a black background look very attractive. *B. dario* is an vulnerable species (CAMP, 1998) [4] having both ornamental and edible value. Nine species of genus *Botia* were recorded from India (Kottelat, 2004) [13]. One special characteristic of this loach group is the ability to produce a loud "cracking" sound which is commonly heard during feeding time. This sound stems from a special type of pharyngeal teeth that are used to extract snails from their shells. The fish of this genus possess a pair of razor-like spines under their eye sockets. These spines normally lie flat, but may be extended when the loach feels threatened. The snail eating loach is one of the many natural controls of the freshwater snail population and plays a great role in controlling the disease. The proposed study was therefore undertaken in embryonic development, standardizing artificial breeding and fry and fingerling rearing of *B. dario*. There is scanty information available on loaches. Literature available show results on spawning biology and fecundity of *Cobitis taenia* by Juchno and Boron (2006) [11], fecundity of Botia *dario* by Hossain *et al*. (2007) [7], spawning behaviour of *Sabanejewia vallachica* by Bohlen (2008) [3], spawning biology of Botia *almorhae* by Joshi and Pathani (2009) [10] and diversity of loaches in Darjeeling, West Bengal by Acharjee and Barat (2014) [1]. Literature is on behaviour, breeding or conservation aspects of loaches is lacking. These lacunae inspired us investigate the behaviour, embryonic development and conservation of the *Botia dario* loach species which may contribute to some extent to the information database and conservation approach of the natural resource.

2. Materials and methods
Collection and Experimental site
B. dario weighing on an average from 3 gm to 5 gm were collected(May 2013-2015) from sampling sites located at Bhelakopa, Dwitia Khanda of Cooch Behar district, West Bengal, India lying at $26^{0}18$'North latitude and $89^{0}34$'East longitude. After collection the fishes were oxygen packed in sterile polythene bags and kept in cartons for transport to the Fishery Wet Laboratory of Uttar Banga Krishi Viswavidyalaya, Cooch Behar. In the laboratory the fishes were transferred to suitable aquariums for regular rearing and maturation.

Water quality parameters for broodstock management

The water quality parameters are very important for the rearing and breeding of *B. dario*. Fresh, dechlorinated and well aerated water was used for domestication of the fish in all the tanks. In all the experimental tanks for rearing and breeding pH, Specific conductivity and Total Dissolved Solids (TDS) were determined by using portable meter (Eutech). Total hardness (EDTA method) and Dissolved Oxygen (Winkler's method) were determined by Standard Methods (APHA, 2012) [2]. In the rearing tanks temperature of 27-32^0 C was maintained with the help of regulated water heaters (Thermostat). In the present study, the broodstock were fed with plankton, blood worm, tubifex and commercially available fish feed at 5 – 10% of the total body weight per day. The fishes were monitored regularly for morphological indicators of maturation.

Gonado-Somatic Index (GSI) and Correlation analysis amongst weight, fecundity, gonad weight and gonad length

To determine the relation between gonad weight and body weight Gonado-Somatic Index (GSI) was calculated. Correlation was determined by MS-Excel. GSI was expressed according to de Vlaming (1982) [17] method for assessing the development of gonads and calculated as:

Gonado-Somatic Index = Weight of the ovary/ Total weight x 100

Induced Breeding

20 pairs of matured fish were injected with synthetic hormone WOVA-FH (Biostadt India Limited, Mumbai) at the base of the pelvic fin. Different Set-up protocol details were followed as shownn in Table 1.

Fig 1: Matured female fish

Fig 2: Matured male fish

Fig 3: Injected hormone in pelvic fin

Table 1: Details of experimental set-up and design for induced breeding of *B. dario*.

Experimental Set-up	Sex ratio	Experimental Design	Number of Fish	Dose of hormone (WOVA-FH)
A	1:1	500 litre aquarium with aeration.	5 pairs	0.025ml /fish
B	1:1	500 litre aquarium with aeration and shower.	5 pairs	0.025ml /fish
C	1:1	Chinese hatchery of diameter 2 m and height 0.5 m having running water facility with increasing and decreasing speed of water flow of 1000 L^{-1} hr maintained throughout.	5 pairs	0.025ml /fish
D	1:1	Chinese hatchery of diameter 2 m and height 0.5 m having running water facility with increasing and decreasing speed of water flow of 5000 L^{-1} hr maintained throughout.	5 pairs	0.025ml /fish

Fecundity and Fertilization rate

Absolute fecundity was calculated according to the method of Hartman and Conkle (1960) [6] using F=nG/g where, 'F' is fecundity; 'n' is mean numbers of eggs in all samples, 'G' is weight of ovaries and 'g' is weight of samples. Eggs were collected from three regions of the gonad like anterior, middle and posterior. After 1 h of spawning, 2 litre of water and eggs were collected from the hatchery and continued for 4 hours. Counting of the fertilized egg and unfertilized eggs were done. Fertilization rate was estimated by using the following formula (Udit *et al.*, 2014) [16].

Fertilization rate (%) = Fertilized eggs/ Total no of eggs in sample x 100.

Embryonic development

Egg samples on hatching were examined hourly and the developing stages were documented through micro-photograph. Eggs were collected as the fish were spawning. Initially photographs were continuously taken for the first two hours to capture the eggs getting fertilized and the zygote stage undergoing cell division in the different stages and time frame. The standardization of rearing and culture of larvae and spawn to get healthy fingerlings was ranched in natural habitat. The yolk sac absorbed fry were harvested and stocked in well prepared cemented tank for further rearing. Feed was given twice daily. The monthly total length, body depth and body weight were taken. Before breeding season the adult fishes were ranched in different regions of Terai rivers of West Bengal, India.

3. Results

Water quality for broodstock management

The study of the water quality parameters is very important for the rearing and breeding of *Botia dario*. In all the experimental tanks for rearing and breeding experiments, pH was maintained in the range of 7.5 to 8.5, Total hardness in the range of 20-35 mg l^{-1}, Specific conductivity in the range of 110-180 µS, Total Dissolved Solid (TDS) in the range of 80-165 mg $l^{-1}$and Dissolved oxygen in the range of 7.6 - 8 mg l^{-1}. In the rearing tanks a temperature range of 27-32^0 C was maintained with the help of thermostat. Broodstock was managed in aquarium to promote gonad development. Morphological indicators of maturation of females were known through enlarged abdomen. During maturation it was observed that males were smaller in size than females. Males

matured earlier than females. The female had enlarged abdomen, which was absent in male. A common secondary sexual character was the brighter body colour of the male than that of the female fish. The yellow bands on the skin of male were deeper in colour during the breeding season, while such colour was absent in female. During the breeding period, the ripe male oozed out milt when slight pressure was applied on the vent. Eggs oozed out with slight pressure on the abdomen of ripe female.

Gonado-Somatic Index and Correlation between length, weight, fecundity, gonad weight and gonad length

Spawning period was confirmed by the Gonado-somatic index (GSI). GSI increased from April to July. The average GSI of *Botia dario* for female was 13.21 and for male it was 3.4.Gonado-somatic index was higher in female than male.

To establish the mathematical relationship between body weight, gonad weight, gonad length, fecundity and Gonado-somatic index, the values of correlation coefficient (r) were established by using the statistical formula using Excel. The scatter diagram of female and male gonado-somatic index (Figure. 4), fecundity and gonad weight (Figure. 5), gonad length and gonad weight (Figure. 6) and body weight and fecundity (Figure. 7) showed a straight line relationship and is expressed as Y = a+ bx, where, 'a' and 'b' are constants and X and Y are the variables. The coefficient of correlation(r) showed significance at $p \leq 0.01$.

Table 2: Correlation between the variables total length, body weight, gonad weight, gonad length, fecundity and Gonado-Somatic Index of the fishes.

Correlation between Characters	X	Y	r
Female and male Gonado-Somatic Index	Male GSI of the fish	Female GSI of the fish	0.990*
Fecundity and Gonad weight	Gonad weight of the fish	Fecundity of the fish	0.606*
Gonad length and Gonad weight	Gonad weight of the fish	Gonad length of the fish	0.972*
Body weight and Fecundity	Fecundity of the fish	Body weight of the fish	0.672*

* Show significance at $p \leq 0.01$

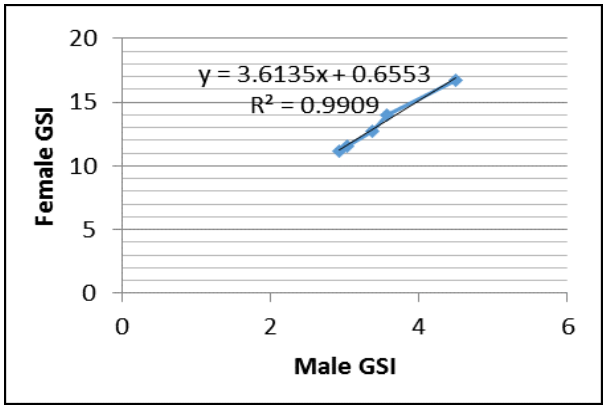

Fig 4: Relationship between Female and Male Gonado-somatic index

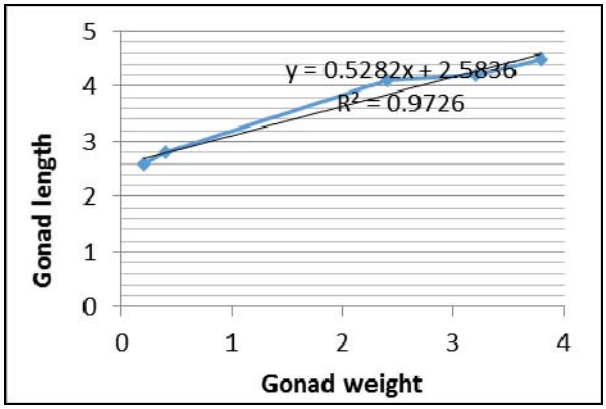

Fig 6: Relationship between Gonad length and Gonad weight

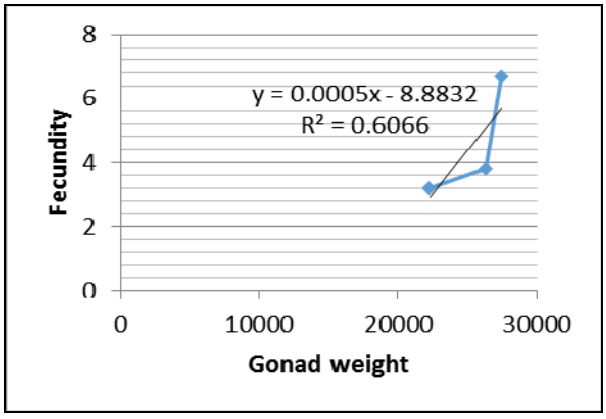

Fig 5: Relationship between Fecundity and Gonad weight

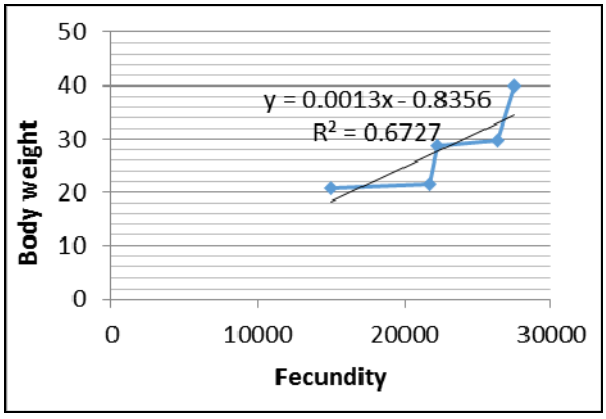

Fig 7: Relationship between Body weight and Fecundity

Breeding in different experimental set-ups

Breeding experiments in captivity were conducted successfully for the ornamental fish *Botia dario* in May 2013 and May 2015 using synthetic hormone WOVA-FH. Fertilisation was external and spawning occurred once a year during the monsoon months May to August with a peak in July. Observations of spawning were done at hourly intervals. The spawning pattern was observed in both male and female fish

during the night. The male was found constantly hitting the female on the abdomen with its head while chasing her all around the aquarium. Cracking sound was heard every now and then. Females were being chased by more than one male at the same time and there was in fighting amongst the males**.** Spawning started after half an hour of chasing.

Table 3: Summary of the stages of breeding of *Botia dario* at different time schedules in the experimental Set-up A, B, C and D.

Time Schedule	Set-up-A	Set-up -B	Set-up -C	Set-up -D
9.00p.m	Fishes cuddled up in a corner	Fishes cuddled up in a corner	Fishes were swimming against flow of water	Fishes were swimming against flow of water
10.00p.m	Same	Same	Same	Same
11.00p.m	Females swelled up but males were not active.	Females swelled up; slight activity could be seen.	Females were chase by the males at and at the same time the males in fighting	Females were chased by the males at the same time the males were fighting infighting
12.00a.m.	Same	Same	Cracking sound was heard every now and then.	Cracking sound was heard every now and then Spawning had started; the paired fishes were swimming with the current and appeared at the surface clinging to each other hooked with the spine below the eyes which was making the cracking sound.
01.00a.m.	Same	Males were chasing the females but no spawning.	Spawning had started	All the fishes were spawning
02.00a.m.	Same	Same	Same	Same
03.00 a.m.	Same	Same	Same	Stopped spawning
04.00a.m.	No spawning	No spawning	Stopped spawning	Stopped spawning

Fecundity and fertilization rate

The fecundity was estimated by random sampling method. The fecundity of females ranged from 13880 to 27510. The fertilization rate was found to be 93.75%, 80.55%, 78.20% and 75.86% for each hour respectively. The average fertilization rate was found to be 82.09 %.

Embryonic development

The embryonic development of *Botia dario* was divided into eight stages namely Zygote, Cleavage Blastula, Gastrula, Segmentation, Pharyngula, Hatching and Early larval period. The recorded embryonic development is described as follows:

Zygote period: The fertilized eggs were not adhesive, whitish in colour and optically transparent. Fertilization activated the cytoplasmic movements. The diameter of the zygote was 1m. (Fig. D).

Cleavage period: The first cleavage occurred 25 min after fertilization. The two blastomeres rounded just after first cleavage. After the first cleavage, the blastomeres divided synchronously at intervals of 4-10 min. Cleavage period was observed to be 25min. -1.15 hours and completed 64 cell stage (Fig. E- I).

Blastula period: Blastula period began with the 128-cell stage and ended with the beginning of the gastrulation. Blastula period was observed to be 1.15-3.10 hours and completed 30% of the epiboly stage (Fig. J- L).

Gastrula period: In the gastrula period, extensive cell movements were observed, including involution, convergence and extension, producing the three primary germ layers and the embryonic axis. Gastrulation began with cell involution at around 50% epiboly and completed bud stage. Gastrula period was observed to be 3.10-6.40 hours (Fig. M- N).

Segmentation period: The segmentation period was characterized by the sequential formation of the somites, and this period lasted to just prior to hatching. Segmentation period was observed to be 6.50-14.35 hours (Fig. O-S).

Pharyngula period: In this period the embryo was bilaterally organized, with a well-developed notochord and a newly completed set of somites that extended to the end of a long post-anal tail (Fig T-W).

Hatching period: Just after hatching from the chorion the larva at 14.40 h measured 2.6 mm.

After 21 h of hatching larvae started swimming and feeding (Fig X-Y).

Firstly larvae fed *Paramecium* and then *Artemia* (Fig.Z) and after 3days the larvae preferred small size zooplankton (Fig.A1). There was complete resorption of the yolk sac and minute pectoral fins were observed. The eyes were well set in the optic sockets. The dark lateral band was more prominent by seen between the operculum and the caudal fin base. 30 day old fish (A2) measured 2-3cm and 60 day old fish (A3) completely resembled the adult fish in all the features and could be easily identified. The horizontal stripes matched the

adult fish and attained the colourful pigmentation, which characterized it as an ornamental fish. In 7months the male fish had matured for breeding whereas, the (A4) female had matured fish in 8 to 9 months. The testicular development of B. *dario* occurred much earlier than that of the ovary. The present study reported that size of the two ovaries were not the same. The results of the present study had shown that WOVA –FH at 0.025ml per female and male was sufficient to induce spawning in *B dario*. Similar type of embryonic development was reported by Kimmel *et al*. (1995) [12] on *Danio rerio*, Udit *et al*. (2014) [16] on *Puntius sarana,* Dey *et al.* (2014) [5] on *Devario aequipinnatus,* reproductive biology of *Ompok bimaculatus* by Malla and Banik (2015) [14].

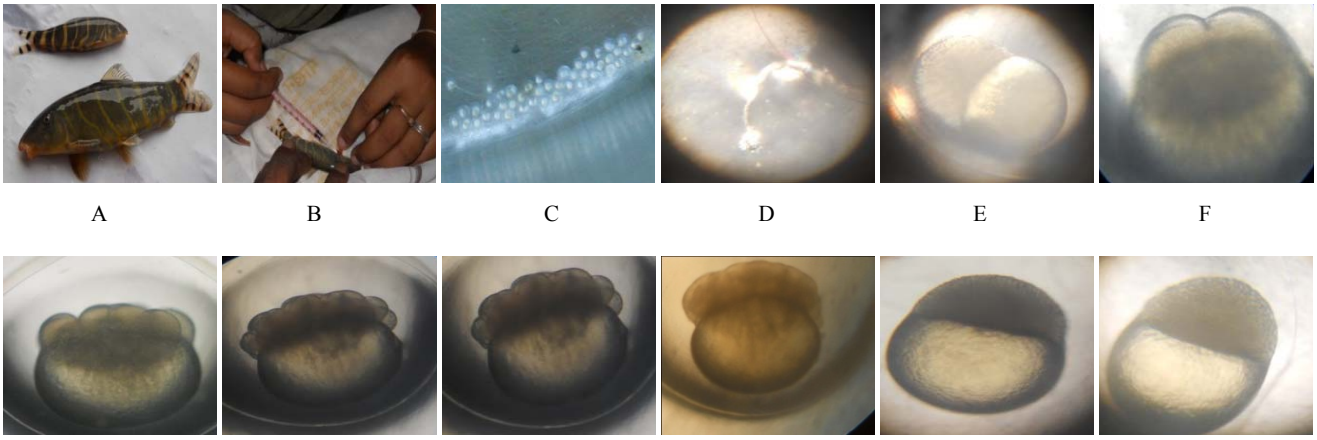

Fig 1: A= brood pair, B= injection of hormone, C= fertilized eggs, D= sperm entry of egg cell, E= 2 cell stage, F=4 cell stage, G=16 cell stage, H= 32 cell stage, I= 64 cell stage, J=128 cell stage, K= oblong stage, L= sphere stage.

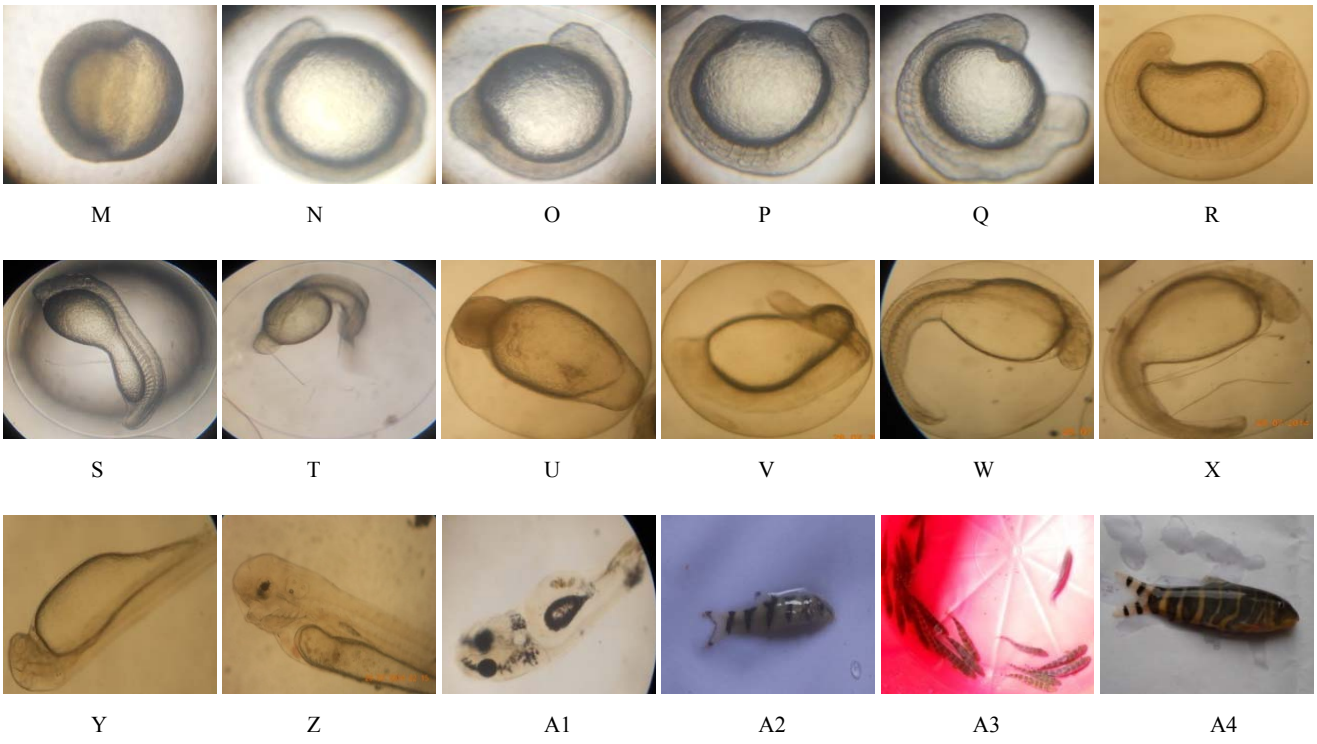

Fig 2: M=epiboly stage, N= bud stage, O= 1 somites stage, P=8 somites stage, Q =12 somites stage, R=14 somites, S=24 somites, T=Pharyngula stage, U= Pharyngula stage, V= twitching stage, W= C, shape embryo, X= Before hatching stage, Y=newly hatch larva, Z= take artemia, A1= take zooplankton, A2=one month old larva, A3= two month old fish and A4= Seven month old fish.

4. Discussion

Experimental Set-up A and B did not release any eggs but Set-up C and D released eggs. Best result was observed in experimental set-up D where the flowing water was present. Present study revealed that flowing water was essential for induced spawning of *B. dario*. The spawning behaviour of *Botia dario* was similar to Indian Major Carps like flowing water systems. The latency period was 5 to 6 hours in fish injected with 0.025ml WOVA-FH per fish. The latency period of *Puntius sarana* (Udit *et al*., 2014) [16] was 8 to 9 hours of

administration of inducing agent. The latency period of Ompok *pabda* (Purkayastha *et al*., 2012) [15] was 6 to 8 hours of administration of Ovatide.

The fertilized eggs were transparent and unfertilized ones were opaque and white. Similar type of captive breeding, embryonic development, fecundity, fertilization rate and hatching rate were reported by Kimmel *et al.* (1995) [12] in *Danio rerio*, Udit *et al*. (2014) [16] in Puntius *sarana*, Dey *et al*. (2014) [5] in *Devario aequipinnatus* and reproductive biology of *Ompok bimaculatus* (Malla and Banik, 2015) [14].

The first cleavage occurred at 25 min. after the eggs were fertilized of *Botia dario*. Udit *et a*l. (2014) [16] reported first cleavage occurred after 30 min. in *Puntius sarana*, Dey *et al.* (2014) [5] reported first cleavage to occur after 45 min. in *Devario aequipinnatus* and Kimmel *et al.* (1995) [12] reported first cleavage to occur after 40 min. in Danio *rerio.* Present study reported that first cleavage occurred after a few minutes of fertilization. The incubation period of *Botia dario* lasted for 14.30 to 14.40 hours. The incubation period was also low from the others species. The incubation period reported for *Danio rerio* was 48 hours (Kimmel *et al.,* 1995) [12], *Puntius sarana* was 15-17 hours (Udit *et a*l., 2014) [16] and *Devario aequipinnatus it* was 36 hours (Dey *et al*., 2014) [5]. The incubation period of *Botia dario had* taken less time than other ornamental and food fish.

5. Conclusion

B. dario can be easily matured and bred successfully under captive conditions similar to that the Indian Major Carp. Brood stock management and hatcheries should be established for conservation, and ranching be 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. This study documents the breeding strategy of ornamental fish *B. dario* in captivity using synthetic hormones and embryonic and post embryonic development up to 60 days till it completely resembles the adult fish. The subject matter in this paper is useful for fish breeders, aquarium keepers and those involved in or interested in the study of fish larval and fry development.

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