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Induced breeding of threatened Indian medium carp *Puntius pulchellus*

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ABSTRACT

The reproductive biology of the Indian Medium Carp (*Puntius pulchellus*) was studied and induced breeding of the fish accomplished. Males were dark and exhibited pinkish red prominent tubercles on the snout in contrast to the plain snouted females which however, had a deep pink lateral band. The gonado somatic index observed in females indicated a prolonged spawning period. The fish was successfully induced bred for the first time using a preparation consisting of Salmon Gonadotropin Releasing *Hormone* Analogue. and Domperidone (0.5ml/kg body weight) followed by pituitary extract @ of 8mg/kg and 2mg/kg for females and males respectively. The larvae appeared (hatching) after 48 h with a heavily laden yolk sac which was absorbed completely after 8days. The embryonic and larval developments are discussed.

Key words: Breeding, Carp, Eggs, Larvae, *Puntius pulchellus*, Sexual Dimorphism

The principal riverine systems of Deccan and Peninsular India formed by the rivers Godavari, Krishna, Cauvery and their tributaries supported a very rich fish fauna comprising mainly of large and medium sized carps. The construction of numerous dams and barrages, indiscriminate exploitation of rivers and lack of conservation and stocking measures have put many of these fishes into the red list of threatened, vulnerable and rare species (Kowtal 1994). Among these *Puntius pulchellus* (Day),(family: Cyprinidae) is one of the prominent fish fauna which once formed an important fishery of the river Tungabhadra and fed exclusively on submerged grass (David *et al.* 1970). The local name, i.e., 'Haragi' or 'Hullu gende' (also) is referred to *H. pulchellus* as reported earlier (Anonymous 2002). Though the fish was classified as data deficient(DD) earlier (Devi and Boguskaya 2007), it has been recently reclassified as critically endangered (Devi and Ali 2013). The species is known only from its type locality in south canara, Karnataka.

The reported consumption of aquatic weeds, grass and water hyacinth roots (*Eichhornia*), *Spirogyra* and *Vallisneria* and filamentous algae by both the juveniles and adults of *P.pulchellus* (David and Rahman 1975, 1982) placed the fish next to the Chinese grass carp *Ctenopharyngodon idella* (Val) as a weed eater (Hora 1955, Hickling 1962). The fish which is capable of attaining 8 kg could thus become a welcome addition to pond culture practices of India, especially for composite fish culture (David and Rahman 1975). The induced breeding of pulchellus assumes significant importance specifically in the context of not only adding another species for diversification of the limited numbers of cultured species in Indian aquaculture but also for alleviating the threatened status of this fish. Since the past fifty years, the efforts for induced breeding of this fish were unsuccessful (Sukumaran *et al.* 1987, Ayyar *et al.* 1988). In the present work we describe the reproductive biology and the successful induced breeding of this fast disappearing freshwater species under culture conditions which could replenish their dwindling stock in the rivers of Karnataka, India .

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MATERIALS AND METHODS

Fingerlings of mean length 97.4±10.5 mm and mean weight of 13.14±4.2 g were caught by cast netting from the Tunga river near Gajanur, Shimoga, Karnataka,India. They were acclimatized for 24 h in a cement tank with flowing water facilities. The fish were given prophylactic treatment with series of antifungal and antibacterial preparations consisting of 1 % solution of malachite green, methylene blue, acriflavin, 0.5 % copper sulphate solution and released into the tanks with a final dip treatment of $KMNO_4$ (2%). After 24 h the fish were given a repeat prophylactic treatment, packed under O_2 and transported to the regional center of CIFA, Hesserghatta, Bangalore and stocked at 20/ m^3 in cement cistern of $1M^3$ capacity, after repeating the

prophylactic treatment once again. The fish fingerlings were periodically monitored for growth and were transferred to 0.1 ha earthen ponds after 80 days, attaining a mean size of 128.5±12.9 mm in length and 33.78±10.51 g in weight and reared to maturity. The water depth of the earthen pond was maintained at a minimum of 90 cm through the study period of three years.

Reproductive biology of Puntius pulchellus

The reproductive biology of the fish with respect to gonadosomatic index, fecundity. ovary morphology and identification of mature males and females (sexual dimorphism) were carried out as given below.

Gonadosomatic index and fecundity: The total body length and weight of the fishes were recorded after which the ovaries were dissected out and weighed. The gonadosomatic index (GSI) was estimated using the formula GSI = {Weight of the ovary/ Total Weight of the fish} ×100.

The fecundity of the fish was estimated by gravimetric method. The sub samples obtained from the ovaries were weighed and immersed separately in Gilson's fluid (Simpson 1951) for the release of oocytes from the clusters. The number of eggs was counted and fecundity calculated using the formula F= ni × gw/swi, where ni =number of eggs in the sub sample, gw = total weight of ovary and swi =weight of sub sample. (Lagler 1956, Reddy and Rao 1991).

Sexual dimorphism: The presence or absence of tubercles between the eyes spreading to the snout and colour variations of the pectoral, pelvic and anal fins were used to determine the sexual dimorphism exhibited by the two sexes at the time of maturity and breeding.

In addition a new approach of using truss analysis for identification of males from females was also attempted by the systemic measurement of distances (truss lines) between pairs of land marks across the body thus forming a sequential series of connected polygons termed a truss box as per the method of Strauss and Bookstein, (1982). The distances between the land marks provide more comprehensive coverage of form for greater discriminating power between individuals.

Induced breeding: The fish as and when showing signs of maturity were used in the breeding experiments. The injection schedule consisted of a combination of Salmon Gonadotropin Releasing *Hormone* Analogue and Domperidone (hormone preparation) followed by pituitary gland extract. The selected breeders were weighed to calculate the dosage of the hormones. One female (1.9 kg) and two males (1.9 kg) were selected. The first injection of the hormone preparation was given to both sexes at a dosage of 0.5 ml/kg body weight (b.w). After 6–12 h when the belly of the females became soft and swollen pituitary injections were administered. Females were injected @ 8 mg and males @ 2 mg/kg body weight. All injections were given intramuscularly between the base of the dorsal fin and lateral line by lifting the fish scale (since scales of *P.pulchellus* are larger than that of Indian Major carps) to insert the needle and after injection and withdrawal of the needle the area was gently massaged to aid distribution of the extract/hormone into the musculature and prevent any backflow.

After 6 h of the pituitary injections the breeders were anaesthetized for stripping by keeping in 25 ppm solution of clove oil. Clove oil was dispersed by dissolving 5 ml of clove oil: ethanol solution (1:4) in cold water. The brooders were immersed in this water till the opercular movement became slow and the fish non responsive to touch. Dry method of stripping was followed. The female fish was taken in a soft towel, gently wiped to remove excess water and with the ventral side held upwards at an angle. A slight pressure was applied at the genital opening to strip the eggs into a clean dry enamel basin. Immediately after stripping the eggs, the procedure was repeated with the males and the milt directly stripped on to the eggs. The milt was allowed to fertilize the eggs by slow orbital rotation of the basin for a period of 15 min. Alternatively the gametes were mixed with the help of a feather. The eggs were then washed 2 to 3 times with freshwater, till the washings become clear of the milt.

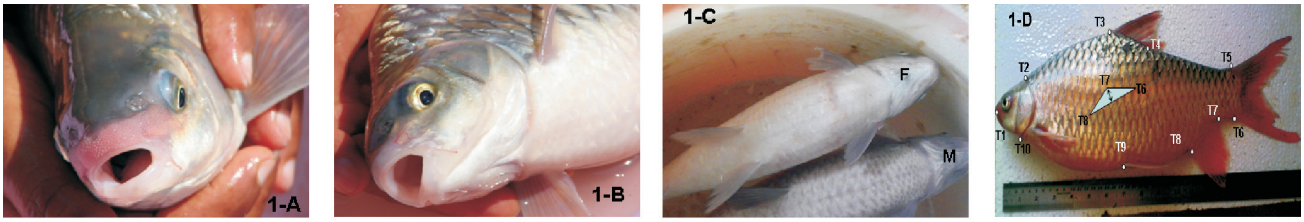
After fertilization, the bright orange eggs were transferred to a hatching unit designed for heavy eggs on the model of a bell jar unit similar to the one used in a carp hatchery (Jhingran and Pullin 1985). The eggs are given an upward lifting movement by a bottom water inlet.

RESULTS AND DISCUSSION

Pre-monsoon and monsoon surveys had indicated the availability of breeders of *P.pulchellus* in the river Tunga and Bhadra rivers in Shimoga and at Bandebele, Koochakkal and Hebbbe in Chikkamagalore District (Sukumaran *et al.* 1987, Ayyar *et al.* 1988) and has very restricted distribution today(Devi and Boguskaya 2007). Our own observations corroborated the dwindling stocks of this species in catches especially from areas adjoining Shimoga and Bhadra regions of Karnataka.

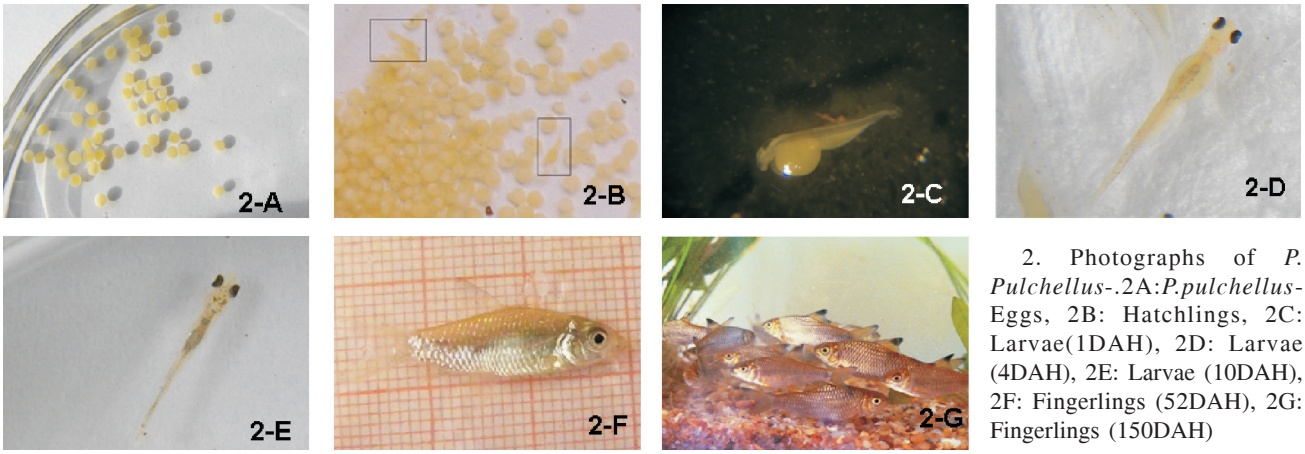
Reproductive biology of Puntius pulchellus

Sexual dimorphism: Sexual dimorphism was exhibited very distinctly by mature males and females in *P. pulchellus*. Males during the breeding season could be distinguished by the presence of pink snout with prominent tubercles between the eyes spreading to the snout (Plate 1-A) while the females exhibited a smooth snout (Plate.1-B) from the month of June onwards as observed in our study. The appearance of tubercles in males was seasonal and disappeared after 3–4 months (Sep-Oct)**.** In addition colour variations were also observed in the breeders. The males were darker in body colour (Plate.1-C) especially in the abdominal region which was white in female. The Pectoral, pelvic and anal fins were also black in colour in males. Females were silvery white with a deep pink lateral band, dorsal and caudal fins being red with black edges, and pectoral, pelvic and anal fins with light black edges. Fully mature *P.pulchellus* females exhibited swollen and Pinkish vent.



1. Photographs of *P. pulchellus*-1A: Male, 1B: Female, 1C: Male and female

1D:Truss analysis, 1E: Ovary, 1F: Spermatozoa of *P.pulchellus*, 1G: Spermatozoa of *L.rohita*



2. Photographs of *P. Pulchellus*-.2A:*P.pulchellus*-Eggs, 2B: Hatchlings, 2C: Larvae(1DAH), 2D: Larvae (4DAH), 2E: Larvae (10DAH), 2F: Fingerlings (52DAH), 2G: Fingerlings (150DAH)

The first photographic evidence for the demarcation of the sexes was brought out in our study. *P.pulchellus* has been described as *Barbus dobsoni* and *Barbus jerdoni* according to the fin and scale description (Day 1878) but was indicated as separate species with the names *Puntius pulchellus* and *Puntius dobsoni* (David *et al.* 1969) and *Hypselobarbus pulchellus* (Basavaraja 2014). These names reportedly refer to a single species due to the wide colour variations not only in juveniles but also between maturing males and females (David and Rahman 1982). Our observations on the different coloration of males and females corroborate the earlier report.

Truss analysis

Many a times an immature male were mistaken for a female because of the absence of the tubercles that appear on the snout only during the breeding season. Hence the method of Truss Network analysis was used for identifying the males from females. Upon drawing the truss lines, the vertical height of the triangle formed by the points T6–T7 and T8 (Plate.1-D) as measured at the perpendicular dropped from the vortex, was higher in males than females ($P<0.0001$, n= 30). Morphometric studies have also been shown to have use beyond traditional applications (Fitzerald *et al.* 2002).

Gonado somatic index and fecundity

The gonado somatic index and fecundity observed from the specimens are given in Table.1. The highest GSI recorded in *P.pulchellus* during July was 10.4. The GSI measured during the month of August ranged from 1.51–

Table 1. Relationship of fish weight and Gonado Somatic Index during, 2006

S.No	Period	Total		Ovary weight (g)	Fecundity	GSI
		Length (mm)	Weight (kg)			
1	July, 2006	390	0.865	90	23652	10.4
2	August, 2006	381	0.840	24	-	2.86
3	"	385	0.996	15	-	1.51
4	"	385	1.011	49	-	4.85
5.	"	399	1.053	28	-	2.66
6	"	390	1.158	28	-	2.42
7	"	422	1.174	80	19284	6.81
8	"	410	1.210	60	-	4.96
9	"	417	1.239	101	28642	8.15
10	"	348	1.449	134	32215	9.25
11	"	396	1.463	183	40129	12.51
12	"	435	1.557	116	31608	7.45
13	"	400	1.627	190	41970	11.68

12.51 in *P. pulchellus.* The highest fecundity was observed in a fish of 400 mm length and 1.627 kg weight while the lowest fecundity was recorded in a fish of 390 mm length and 865 g weight. Interestingly some of the larger sized fish also exhibited low fecundity. The possible size of the fish at first maturity was between 380 mm and 865 g from among the specimens examined.

The GSI is particularly helpful in identifying months and seasons of spawning, as the ovaries of gravid females swiftly increase in size just prior to spawning. An index of 30 just prior to spawning was also observed in the Chinese and Indian Major carps (Jhingran and Pullin 1985). A peak value of 20.149 during June suggested the full development of the ovary which decreased in July to 14.920 indicating that spawning had already commenced. Thus the GSI specifically showed that Catla has single specific spawning (Natarajan and Jhingran 1963). The GSI values of 1.51–12.51 observed in *P.pulchellus.* was similar to the earlier observations recorded in the range of 1.55 to 15.60 over an extended period and some of the fishes with GSI in the range of 9.44 to 15.60 reportedly exuded free flowing eggs upon capture. Specimens showing well developed gonads were also reported from April to July indicating the fish to be having an extended spawning period (David and Rahman 1975).

The gonadosomatic index and fecundity exhibited by *P.pulchellus* generally varied depending upon the length and weight of the fish and ovary. These observations were similar to the results obtained in grass carp *Ctenopharyngodon idella* (Alikunhi and Parameswaran 1963), *Catla catla* (Natarajan and Jhingran 1963) and *Labeo rohita* (Khan and Jhingran 1976). The fecundity of the fish was also observed to be lower when compared with the Indian Major carps (Jhingran and Phullin 1985). The ovary was bilobed with an extended Y shape and orange in colour and measuring 12.5cm in length from proximal to the distal end (Plate. 1-E). The two lobes merged at the distal end and opened through a tube externally as a vent which measured 3.0cm in length. The proximal, mid and distal end of ovaries had a width of 3.5, 3, and 2 cm respectively. The proximal end of ovaries contained eggs in different sizes and stages of maturity. The size of the eggs was observed to be between 0.6 to 2mm in the proximal end and 1 to 2 mm in the mid portion. The distal end contained eggs that were bigger and had a diameter of about 1.5 to 2.0 mm. In this study the released eggs measured 2.0 mm in diameter which upon fertilization increased to 2.75–3mm in diameter. Unlike the Indian Major carps which exhibit a bilateral elongated ovary, the ovary of *P.pulchellus* was bilobed. The lobules projected posterior through a pair of oviducts that connect to the genital papilla which opens to the exterior. The non-uniformity of egg sizes from the proximal to the distal end of ovary in *P.pulchellus* differs from the carps *C. idlella* and *Hypophthalmichthys. molitrix* with large number of uniform sized soft ova which were expelled once (Singh *et al.* 2006). *P.pulchellus* was observed to spawn four times in a year during the breeding season in reservoir, tanks and the rivers. Hence, the number of ova released at each spawning were limited in *P.pulchellus*. The diameter of ova released by fish upon capture was reported to be 2.75 mm and the ova appeared yellowish in colour (David and Rahman, 1975). Generally, the eggs after spawning/stripping are smaller in size and after fertilization and absorption of water they swell increasing in diameter. The diameter of the eggs of *Cyprinous carpio, Labeo rohita, C.Catla and C.mrigala* immediately after spawning were observed to be 1–1.5, 1–1.1, 2–3.2, 1–1.5 mm and increased in size to 2.2–2.5, 3.5–5, 4.4–6.5, and 4.5–6 respectively after water absorption (Singh *et al.* 2006). The increase in size of the eggs in *P.pulchellus* in contrast was very limited (2.0 mm to 2.75–3 mm).

In natural waters, the breeding of *P.pulchellus* was observed to commence soon after the monsoon months from September which continued until April with a peak in September and January. Monsoon rains or floods did not trigger the spawning activity in this species and the breeding also occurred in confined waters such as reservoirs (Anjanapur and Bhadra), tanks (Madaga and Milghatta) and also in the rivers (David and Rahman 1982). However in our breeding experiments conducted during June-July, bright orange eggs could be stripped from fish that had been reared in ponds. Males were also mature as judged by the production of the milt. The spermatozoa of pulchellus (Plate. 1-F) appeared to differ in morphology from that of *Labeo rohita* (Plate. 1-G) and were also observed to be alive up to 8 h at a temperature of 6–8 ºC when suspended in phosphate buffered saline (0.1M, pH 7.2).

Induced breeding of Puntius pulchellus

With the commencement of the monsoon and the decrease in water temperature the breeding experiments were initiated. The mean water temperature observed in the breeding pool during late June – early July was 18.31±0.59 ºC and the pH of the water 7.3±0.5. The average rain fall recorded during late June and early July was 0.925 and 1.2 mm respectively. The water temperature was observed to be playing a significant role in the breeding of *P.pulchellus* as recorded in the case of *C.idella* (Alikunhi *et al.* 1963). The selection of proper breeders needed was of immense significance as the success of the induced breeding experiments mostly depended upon selecting a breeder at its prime stage of maturity. Induced breeding experiments carried out in *P.pulchellus* by earlier workers with the use of pituitary hormones were mostly unsuccessful due to the females not being in prime condition (Sukumaran *et al.* 1987, Ayyar *et al.* 1988).

The Indian major carps and the Chinese carps have similar breeding habits and do not normally breed in confined waters. Hence the induced breeding technique through the use of hormones was adopted. Induced breeding experiments in fish manipulate the fish endocrine system at three levels viz; hypothalamo-pitutitary -gonadal axis. Initial success was achieved by hormonal interventions at the second level following which the induced breeding of

Indian major carps by administration of fish pituitary hormone was successfully established (Chaudhuri and Alikunhi 1957, Chaudhuri 1960, Alikunhi *et al.* 1960) and the technique further refined for attaining commercial production of fish seed of these economic species including Grass carp and silver carp *Hypophthalmichthys molitrix* (Chaudhuri 1963, Alikunhi *et al.* 1962,1963).

With the success achieved in manipulations at the first level i.e hypothalamus (Lin *et al.* 1988, Peter *et al.* 1988), a new method of breeding fishes called "Linpe" method in which a combination of Synthetic Luteinising hormone Releasing hormone analogue (LHRHa) and a dopamine antagonist, domperidone was developed. The induced breeding of *P.pulchellus* was carried out with a combination of pituitary hormones and a hormone preparation. The breeding technique followed for *Puntius pulchellus* is similar to that carried out in Indian major carps (Chaudhuri and Alikunhi, 1957;Chaudhuri, 1960) except for certain minor modifications.

In the present experiment the first injection of hormone preparation (0.5ml/kg b.w) to the fishes of either sex elicited the desired response. After 6–12 h of injection depending upon the conditions of the breeders, males showed signs of expressing milt and the females a soft and swollen belly. At this juncture the second injections of pituitary hormones were given @ 8mg and 2mg per kg b.w. for females and males respectively. The free flowing gametes could be obtained after 6–8 h of the second injection, upon slight pressure on the abdomen. The female released about 2200 eggs which appeared bright orange in colour (Plate.2A). In order to test the extended spawning behavior of the fish, the same breeders were subjected to the injection schedule after a week of acclimatization in pond. It was possible to obtain about 3660 eggs during the second time from the same female fish. This confirmed the capability of a single mature female fish to release eggs more than once reiterating the extended spawning behavior of this species. The breeding schedule was followed subsequently with other matured specimens also resulting in spawning but hatching of the eggs were impaired due to various reasons such as the poor quality of the milt from the males, circulation problems of the eggs and algal contamination of the circulating water in the hatchery.

The upward circulation of the eggs after fertilization appeared to be very crucial for the hatching process as the process prevented the clustering of eggs. The eggs attained a size of about 3mm in 48 hrs. At this stage the eggs became translucent and the twitching larvae were clearly seen when examined with an Inverted Microscope. The egg shell when shed was transparent.

The technique of breeding using pituitary hormones elicited similar response but different results as observed in the Indian Major carps (Chaudhuri and Alikunhi, 1957)., Grass and Silver carp (Chaudhuri, 1963). The administration of the total dose of hormone was complete within 6–8 h. The entire operation of injecting and stripping the spawners usually took eight to fourteen hours (Chaudhuri, 1963). *P.pulchellus* responded to a combination of the hormone preparation (0.5ml/kg b.w) and pituitary hormone extract at a dosage of 8mg and 2mg per kg b.w. for females and males respectively unlike carps wherein two pituitary injections are given.

Embryonic and larval development

The developments of the pulchellus embryos were slow with the elongation of the yolk mass taking about 24 h. Further development such as appearance of the optic cups, elongation of the tail from yolk mass and formation of Kupffer's vesicle (Chakrabarty and Murthy, 1972) was difficult to observe in this case due to the near opaque nature of yellow colored egg mass. Sporadic twitching movement of the embryo was observed only 4 h before the larvae hatched from the egg shell when the egg size had swollen to 3 mm and appeared quite transparent under the microscope. Many eggs were also observed to be in the process of breaking. As the embryo advanced in its development, the movements became more vigorous. The first larva (hatching) appeared after a period of 48 h, with a heavily laden yolk sac (Plate. 2–B). Thus the total time taken from the point of fertilization to hatching was about 48h in the case of *P.pulchellus* .

The hatched larvae were pale orange in colour with a heavy yolk sac, of 4 mm long and weighed 3.4mg. The body was transparent with no chromatophores. Though the embryo had clearly differentiated head and tail regions, they were not yet free from the ovoid yolk mass. The larvae moved sporadically with a propelling movement

After 24h of hatching, eyes were observed to be faintly dark with a central pigmented area surrounded by a colourless rim. Anterior part of yolk sac appeared globular with the narrow end not ending abrubtly. Anal pore appeared as a depression where the yolk sac ends. Total length 5mm.weight:4 mg (Plate. 2–C)

At 48h after hatching, the actively swimming larva measured 7mm in length and weighed 6.9 mg. The lengths of different regions were also measured: Head to Yolk end: 4mm.Yolk end to Tail.2mm. Head length: 1mm (tip or snout to yolk beginning). Yolk was pale orange in colour. The head and tail were colourless (transparent). The mouth was clearly visible with heavily pigmented eyes. Anal depression was well marked. Yolk appeared oblong in shape and ended abrubtly (Plate. 2–D).

The larvae were shifted to an aquarium 8 days after hatching and fed with filtered zooplankton. The larvae after 10 DAH is shown in Plate.2–E.Various stages of the development fingerlings are shown in (Plate. 2–F and G). This is the first report on the embryonic and larval development of this species. The development of the eggs after fertilization followed a path similar to that reported for *Cirrihinus mrigala* (Chakrabarty and Murthy, 1972) except for the total time taken for the hatching of the larvae which was 48 h.

In conclusion, this threatened medium carp could be induced bred through a combination of the hormone

preparation and pituitary hormone successfully. There is immediate need to breed and conserve this fast disappearing species not only due to their low fecundity but also due to the changing environment. Rehabilitation of this species will be an asset to eradicate the weed problem that has been threatening many of the water bodies in the country. As this species is amenable to culture conditions this can be added to increase the culture basket with a new species for propagation commercially. The methodology adopted in identification of male and female and the induced breeding technique may also be useful for induced breeding of other medium carps such as *Puntius carnaticus* and *Puntius kolus.*

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