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Captive breeding of the stinging catfish, *Heteropneustes fossilis* (Bloch, 1794) found in Brahmaputra River, Assam, India using inducing agent ovasis and its early embryogenesis

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ABSTRACT

Among the freshwater air-breathing fish of southeast Asia, *Heteropneustes fossilis* is considered to be a highly demanded, nutritious and popular fish species. This species was scrutinized in the current study and its embryonic and larval development was assessed.. In addition, the artificial dissemination of stinging catfish (*H. fossilis*) was carried out with different doses of synthetic hormone 'ovasis'. Fertilisation rate was recorded 75 ± 3.1% with a hatching rate of 57 ± 1.9%. Spawning was observed after 26- 30 hours of injection at 23 ± 2˚C. Fertilised eggs were found round in shape, transparent, non-adhesive and greenish in color, with a diameter of 0.8 - 1.0 mm. The incubation period of *H. fossilis* ranged from 27- 30 hours. The hatchlings were transparent and measured 2 - 3 mm in length, with an oval shaped head bearing a green colored yolk sac and a tail. Artificial diet containing 30% protein was supplied to the hatchlings at 5% of their body weight. The bulged yolk was dissolved slowly in successive days of development and completely disappeared on the 5th day. At this stage, most of the larvae were surface dwellers and active swimmers. Environmental factors such as temperature and hardness play a major role in the early development of this species. This study would provide new information which can positively affect the sustainable management and conservation of the species under study.

INTRODUCTION

Heteropneustes fossilis commonly called as stinging catfish or "shingi" is considered to be one of the most ideal fish species in aquaculture, and it is naturally available in the freshwater of mainland southeast Asia and the Indian subcontinent **(Dehadrai *et al.*, 1985; Talwar & Jhingran, 1991; Alok *et al.*, 1993; Vijaykumar *et al.*, 1998**; **Haniffa & Sridhar, 2002)**. The global geographical distribution of *H. fossilis*

is recorded in Bangladesh, Iran, Iraq, Laos, Myanmar, Nepal, Pakistan, Sri Lanka, Thailand, India. While, it is locally available in Assam, Arunachal Pradesh, Manipur, Meghalaya, Tripura, Nagaland, West Bengal and some other parts in India **(Talwar & Jhingran, 1991)**. It commands a good market value in the local market. *H. fossilis*, found in ponds, beels and slow moving rivers belongs to the family Heteropneustidae; this fish species is known as an air breathing fish as it possesses an air sac as an accessory respiratory organ. The species has a fast growth rate, a wide tolerance to high stocking densities and an ability to survive in harsh environmental conditions. It is an excellent source of essential amino acid (EAA) and includes essential fatty acids, monounsaturated fatty acid (MUFA), poly unsaturated fatty acid (PUFA), high iron, important vitamins and other medicinal values **(Sánchez-Alonso *et al.*, 2007; Paul *et al.*, 2016)**. However, a shrinkage of its natural habitats has been reported associated with anthropogenic activities and the degradation of aquatic ecology **(Rahman *et al.*, 2013)**.

Assam, situated in the north-eastern region of India is blessed with a variety of water bodies leading to rich ichthyofaunal diversity. Favourable climatic conditions and topography has made the region a hub of many native fishes. Due to the increasing human population, the demand on fish is increasing day by day. To meet this demand, the state has to import fishes from other states of India. On the other hand, aquaculture is acting as the most feasible alternative to minimize the gap between supply and demand **(Okere *et al.*, 2015)**. However, numerous problems in the aquaculture sector are still unaddressed; one of which is the availability of fish seeds. Many fishes don't breed in the stagnant water which causes a decline in their population. Induced breeding technique has become the best practical method in the region since it assures the timely supply of pure seeds **(Mohapatra *et al.*, 2017)**. Induced breeding is a technique whereby sexually matured breeder fish are stimulated by using reproductive hormone or their synthetic analogs in brood fish through injection or supply in diet which may play significant role to the mass propagation of the desirable fish species and lead to the economic development in the state **(Marimuthu *et al.*, 2000)**. The need of an artificial production of fish seed is tremendously growing due to the decrease of fish seeds in the natural habitats. It gives pure spawn of certain species of fishes under cultivation. Remarkably, spawn collected from natural water is not pure because of some undesirable wild species come with them **(Surnar *et al.*, 2015)**.

Due to high nutritional and medicinal value, the tremendous market demand and the habitat degradation, the population of this fish species is experiencing a declining trend. Moreover, the mass production of *H. fossilis* has not yet been flourished due to lack of fry and fingerling supply on time. To overcome these problems, proper rearing techniques and information on early embryonic, larval development and organogenesis of *H. fossilis* is of utmost importance **(Nesa *et al.*, 2017)**. Therefore, the present study

focused on the captive breeding of *H. fossilis* to propagate its mass production and study the biology of its early embryonic and larval stages and its microhabitat.

MATERIALS AND METHODS

The study was conducted during February-May 2019 at breeding facility, Dibrugarh University Park, (27˚29′ N & 94˚55′ E) (Fig. 1a) Dibrugarh (Assam). Breeding trials of *H. fossilis* were performed during pre-monsoon season.

2.1. Selection of brooders and experimental set-up

Healthy and fully ripe male and female brooders were collected from Brahmaputra basin (Fig. 1b), Dibrugarh and kept quarantine by adding $KMnO_4$ solution in a fibre tank for one week. Fish were transferred into the Aquarium House of Dibrugarh University Park for acclimatisation. Aquaria used in the present study had size dimensions of 120 × 60 × 60cm. Four replica (R_1, R_2, R_3 and R_4) with 10 individuals in each aquarium were used in the present investigation. Favourable conditions were maintained, and the water quality parameters were regularly monitored to simulate their natural environment. Brooders were reared for 3 months (16 February-16 May 2019) on the diet supply of 30% protein twice daily at a rate of 5- 6% of their body weight. The breeding trial was performed on the 17th of May 2019.

Water quality parameters such as dissolved oxygen, alkalinity, hardness, pH and water temperature of the experimental setup were monitored fortnightly i.e. every 14 days by using different protocols **(APHA, 2005)**. 50% of the water of the tanks was replaced at an interval of 6 days.

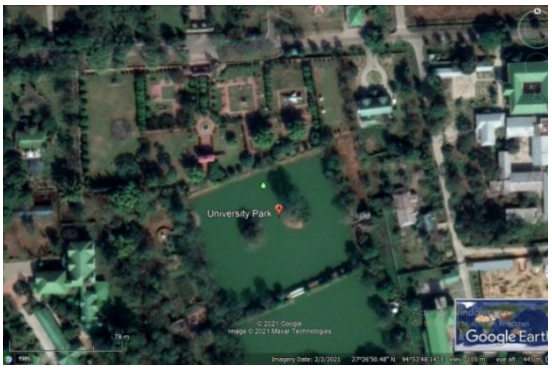


Fig. 1a. Geographical location of the study site

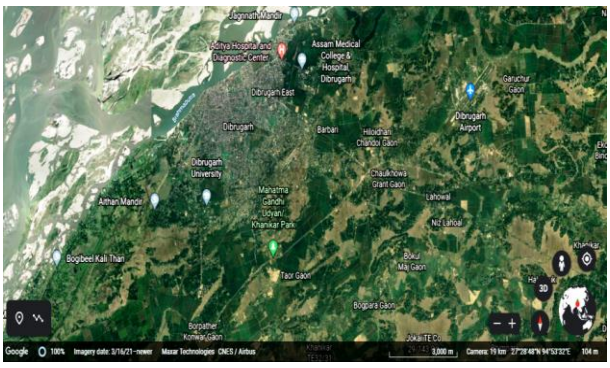


Fig. 1b. Brooders collection site (Brahmaputra River)

Before the brooders were administered with different doses of hormone, the segregation of male and female brooders was done carefully. The male and female brooders were separated 7 days before the treatment and stored them in two different fiber tanks. Males and females were segregated by observing the morphology of the genital papilla. In females, it is rounded and button shaped whereas in males, genital papilla is elongated.

During the trials, a set of brooders (R_1, R_2, R_3 and R_4) was prepared by healthy matured or fully ripe male & female in 2:1 ratio for hormonal injection (Table 1). The selection of brooders for replica was completely done on the basis of their maturity stages. Pressing gently on the abdomen, the healthy bulging abdomen and the flow out ova were the characteristics of the chosen female. While, with a sligh pressure on the abdomen, the healthy males which release milt were the preferred for the experiment.

Table 1. Weight and length of *Heteropneustes fossilis* brooders

Parameter	Sex	Replica			
		R1	**R2**	**R3**	**R4**
Body weight of the brooders before stocking (g)	**Males**	16.0 & 12.7	16.1 & 13.7	15.8 & 12.9	16.2 & 13.2
	Female	16.3	16.5	16.0	16.8
Body weight of the brooders during breeding trial (g)	**Males**	17.12 & 13.67	17.15 & 14.0	16.9 &13.8	17.3 & 13.7
	Female	18.0	17.9	18.5	18.2
Length of the brooders before stocking (cm)	**Males**	13.5 & 12.3	13.8 & 12.3	13.4 & 12.8	14.5 & 14.3
	Female	14.0	15.0	14.5	15.0
Length of the brooders during breeding trial (cm)	**Males**	13.9 & 12.8	14.2 & 13.1	13.8 & 13.0	13.6 & 14.5
	Female	14.5	15.5	15.1	15.9

2.2. Hormonal doses

The synthetic hormone "ovasis" was used as an inducing agent using hypodermal needle. Males were given a slightly lower dose of hormone i.e. 0.3 ml/kg of body weight compared to females (0.5 ml/kg of body weight). They were gently released in the fiber tanks prepared for the breeding trials. Different water quality parameters were strictly monitored during the breeding trials. Appropriate conditions were made to simulate their natural breeding habitats.

3.3. Reproductive parameters

Gonado-somatic index (GSI), fecundity, ovulation rate, spawning rate, fertilization rate, hatching rate, relative length of gut (RLG), gastro somatic index (GaSI) and Condition factor (K) were studied following standard methods. Early embryonic and larval developmental stages at different time intervals were observed and recorded using microscopic studies.

2.3.1. Gonado Somatic Index, GSI (Hopkins, 1979)

$$\textbf{GSI} = \frac{\text{Total weight of the gonads}}{\text{Total body weight}} \times 100$$

2.3.2. Fecundity

Fecundity (Gravimetric method) was studied **Legendre (1986)**.

$$\textbf{Absolute Fecundity} = \textbf{nG /g}$$

Where, n = number of eggs count in the sub sample; G = Total weight of the fish; g= weight of the sub sample

2.3.3. Ovulation rate, fertilisation rate and the hatching rate were calculated following **Bhuiyan *et al.* (2013)** according to the following equations:

$$\textbf{Ovulation Rate (\%)} = \frac{\text{Number of fish ovulated}}{\text{Number of fish injected}} \times 100$$

$$\textbf{Fertilisation Rate (\%)} = \frac{\text{Number of fertilised egg}}{\text{Total number of eggs}} \times 100$$

$$\textbf{Hatching Rate (\%)} = \frac{\text{Number of egg hatched}}{\text{Total number of fertilised eggs}} \times 100$$

2.4. Relative length of gut, RLG (**Al-Hussainy, 1949**):

$$\textbf{RLG} = \frac{\textbf{Gut length of the fish}}{\textbf{Total length of the fish}}$$

2.5. Gastro somatic Index, GaSI (Desai, 1970):

$$\textbf{GaSI} = \frac{\text{Weight of the gut} \times 100}{\text{Total weight of the fish}}$$

2.6. Condition factor (K) or 'Ponderal index':

Condition factor was calculated following **Wooton (1992)**

$$\mathbf{K} = \frac{\boldsymbol{W} \times \mathbf{100}}{\boldsymbol{L^3}}$$

Where, W= Weight of the fish, L= Length of the fish, 10^2 = Factor to bring the K - factor into unit.

RESULTS

All important water quality parameters of different experimental setups during the breeding trials were monitored regularly and also kept in record for future references (**Table 2**).

Table 2. Water quality parameters of breeding setups

Parameters	Number of samples	Aquarium (mean value)	Fibre tank (mean value)
Water temperature (˚C)	6	22.0	23.0
Alkalinity (mg/l)	6	71.5	70.5
Hardness (mg/l)	6	18.3	23.6
DO (mg/l)	6	5.33	5.12
pH	6	7.03	7.10

3.1. Morphological features and feeding parameters

Weight of the population of the *H. fossilis* were ranged from 9.3 - 18.0 (g) and length (cm) were in 11.8 - 16.3. RLG values were found varied from 0.31 - 0.33 with straight and one or two coiled intestine. The gastro somatic index was ranged from 1.24 - 2.23 and mean condition factor (K) was found to be 0.56. (**Table 3).**

Table 3: Length-weight**,** RLG, GaSI and condition factor (K) of *H. fossilis*

Parameters / Replica	Length (cm)		Weight (g)		RLG		GaSI		Condition factor (K)	
	Range	(Mean±SD)	Range	(Mean±SD)	Range	(Mean±SD)	Range	(Mean±SD)	Range	(Mean±SD)
R1 (n=10)	11.9-16.3	13.96±1.650	9.3-18.0	15.37±2.659	0.31-0.33	0.32±0.009	1.24-2.23	1.72±0.404	0.41-0.87	0.58±0.148
R2 (n=10)	12.0-16.1	14.13±1.412	10.7-17.8	15.77±2.039	0.31-0.33	0.32±0.008	1.35-2.14	1.69±0.255	0.42-0.89	0.57±0.138
R3 (n=10)	11.9-16.0	13.99±1.358	10.9-17.5	15.52±1.869	0.31-0.33	0.32±0.006	1.54-2.12	1.83±0.237	0.41-0.87	0.58±0.140
R4 (n=10)	11.8-16.2	14.21±1.286	11.0-17.9	15.34±2.237	0.31-0.33	0.32±0.008	1.44-2.21	1.79±0.318	0.42-0.77	0.54±0.113

3.2. Fecundity, fertilisation and hatching rates

Absolute fecundity of *H. fossilis* was found to be 1840 ± 55. Fertilisation rate of 75 ± 3.1% was recorded with hatching rate of 57 ± 1.9%.

3.3. Embryonic development of the fertilised eggs

3.3.1. Formation of blastodisc

Spawning was observed after 26 - 30 h of injection at 23 ± 2˚C. Newly spawned eggs were round in shape, transparent, greenish in colour with a diameter of 0.8 - 1.0 mm and shrank at the bottom of the fibre tank. The fertilised eggs had a radish spot easily recognisable with the naked eye called blastodisc.

3.3.2. Formation of embryo

First cleavage was observed in 15 - 20 min after fertilisation. It divided the blastodisc in two blastomeres. Second cleavage appeared 40 - 50 min post fertilization. After 2 - 3 h of fertilisation, the size of the blastomeres was decreased. It symbolised the starting of morula stage. At this stage, the anterior and the posterior sides of the embryo were differentiated. After 3 - 4 h of post fertilisation, the blastula stage was recorded (**Fig. 2a**). Gastrulation was in progress approximately after 6.30 h after fertilisation and the blastopore was distinct during this time (**Fig. 2b**).

3.3.3. Differentiation of the embryo

Observation at 8 - 10 h of post fertilisation revealed that anterio-posterior axis was distinguishable, cephalic portion was broader and embryonic rudiment distinct with 2 pairs of somites (**Fig. 2c**). After 13 - 15 h from fertilisation, the embryo preoccupied more than three fourth of the egg peripheral space (**Fig. 2d**). Somites increased into 8 - 12 pairs and the notochord was clearly observed. At 17 h from fertilisation, the developing embryo occupied the whole space inside the egg. At this stage, the mesodermal somites counts increased from 13 to 15 (**Fig. 2e**). The tail part of the embryo started to detach from the yolk. At 20 h old embryo, somites increased to 19 - 20 pairs (**Fig. 2f**).

In 22 h old embryos, 22 - 25 pairs of somites were found and the yolk was completely encircled by the embryo. Hatching took place after 27-30 h of fertilisation. Just before hatching (**Fig. 2g**) embryonic twisting movements were frequently observed as the developing embryo trying to break the perivitteline membrane. During this, the egg membrane was broken down from the tail portion and the hatchling emerged out from the egg capsule.

3.4. Larval development of *H. fossilis*

The incubation period of *H. fossilis* was found to be 27 - 30 h. The newly hatched larvae were transparent, faintly brownish in colour (**Fig. 3a**). The body of the hatchlings were laterally compressed and measured 2 - 3 mm in length. Yolk sac was oval in shape, pale greenish in colour provides nutrient to the body. The head and yolk sac appeared to a bulb like structure. Head being very small in size and cannot be distinctly separated from the yolk sac.

3.4.1. 4 h larvae

The larvae were brownish in colour. At this stage, mouth was not developed. A salient depression indicated the position of the mouth. Eyes were unpigmented and the heart became more recognisable (**Fig. 3b**). Barbels were not noticeable. The larvae were coincided in a clutch and a few of them started swimming.

3.4.2. 8 h old larvae

Bulged yolk gradually reduced and elongated at this stage. Internal body organs like heart and brain became clearly distinct. At this stage, most of the larvae were active and came out in the surface of the water (**Fig. 3c**).

3.4.3. 24 h old larvae

In 24 h old larvae, the yolk moderately reduced, heart was visible in front of the yolk and the blood circulatory system was fully operating. Barbels emerged in the form of tiny knob (**Fig. 3d**).

3.4.4. 36 h old larvae

The eyes were dark pigmented and spherical in shape. Hearts distinctly visible located behind the head. Heart showed regular heart beats. The yolk reserve was further diminished (**Fig. 3e**).

3.4.5. 48 h old larvae

The eyeball was dark and the barbels fully developed and elongated. Yolk reserve further reduced. Pectoral fin has become paddle shaped with an oscillating dorsal margin. Alimentary canal was distinct, short and straight; exogenously feeding started (**Fig. 3f**).

3.4.6. 4 days old larvae

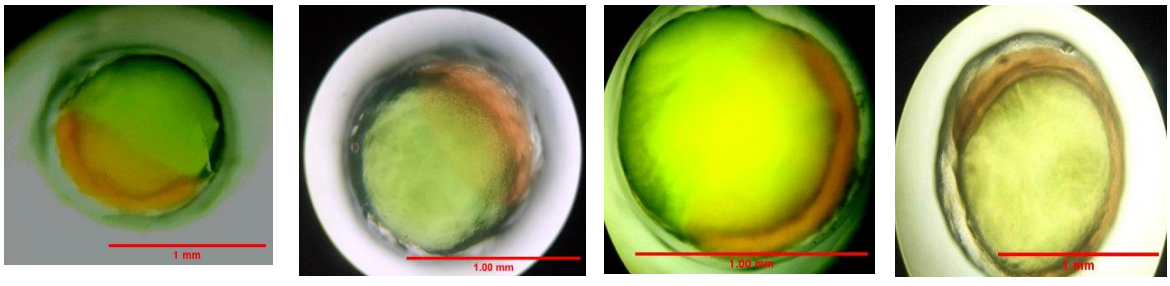
The body became brownish in colour and the mouth became functional on 4 day old larvae. The head was fully prominent and the barbells were slightly increased in size than the previous stage. Four pairs of barbells were observed. The reserved yolk further dissolved (**Fig. 3g**). The larvae have exhibited robust movements to the water surface and occasionally sank to the bottom. The larvae exhibited shoaling behaviour at that stage.

3.4.7. 6 days old larvae

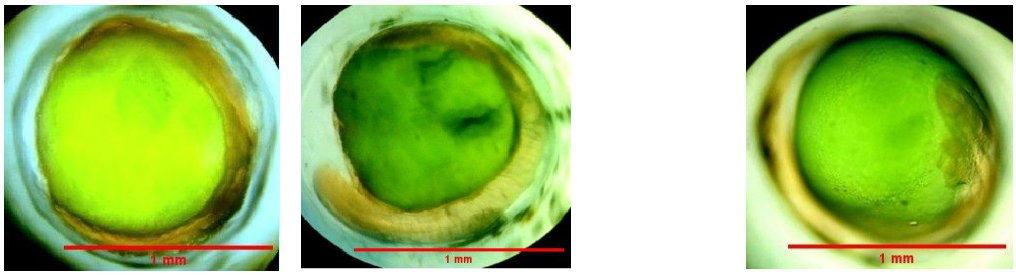
At this stage, the entire body was brownish black in colour. The yolk reserve completely absorbed and the eyeball were large and distinct. The larvae assembled at the bottom of the tank (**Fig. 3h**).

3.4.8. 10 days old post larvae

On 10th day, the larvae exhibited frequent surfacing movements. Dorsal and anal fins were clearly demarcated at this stage (**Fig. 3i**). Active swimming and the foraging behaviour of the larvae were observed. During this stage, the larvae started feed exogenously.



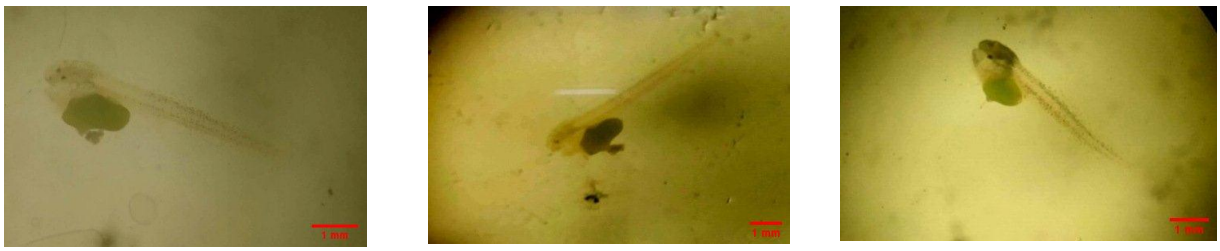
a. blastula **b. gastrula** **c. 10h old embryo** **d. 15h old embryo**



e. 17h old embryo **f. 20h old embryo** **g. just before hatching**

Fig. (2) Embryonic stages of the fertilised egg of *Heteropneustes fossilis* (50 X)

a: Newly hatched larva (100 X) **b: 4 hours old larva (100 X)** **c: 8 hours old larva (100 X)**



d: 24 hours old larva (25 X) **e: 36 hours old larva (25 X)** **f: 48 hours old larva (25 X)**

g: 4-day old larva (50 X) **h: 6-day old larva (25 X)** **i: 10-day old larva (25 X)**

Fig. (3). Early larval developmental stages of *Heteropneustes fossilis*

DISCUSSION

In the present investigation, the fertilisation rate was recorded as 75 ± 3.1% with the hatching rate of 57 ± 1.9%. The current result is also supported by the report of **Haniffa & Sridhar (2002)** on *H. fossilis* where they recorded fertilisation rate of 70% and 75% with 50.5% and 60% hatching rates respectively. **Nesa *et al*. (2017)** reported fertilisation rate of 46.67 ± 5.86% with 41.33 ± 5.69% hatching rate on this fish. However, in some earlier studies, there are reports of higher fertilisation and hatching rate in *H. fossilis* (**Rahman *et al.*, 2013**). The variation in fertilization and hatching rate is might be due to difference in environmental conditions particularly water temperature and precipitation during the study period.

In the present study site, raining was normal during pre-monsoon season. It causes lowering of water temperature. The hatching period of *H. fossilis* was recorded as 27 - 30 h which was slightly longer than the earlier study conducted by **Singh Kohli & Vidyarthi (1990)**. The delay in hatching period may be due to the fluctuation of water temperature and other climatic variability in the study site. Newly hatched eggs were submerged at the bottom of the fibre tank even *Echhornia crassipes* was used as a substratum. Earlier report of **Puvaneswari *et al.* (2009)** revealed that eggs were adhesive and at 1 min after fertilisation, the adhesiveness of the eggs was more perceptible and the eggs adhered to the substratum. This may be due to the higher buoyant tendency of the fertilised eggs of *H. fossilis*. Microscopic study of current investigation revealed that the yolk reserves disappeared on 5th day of hatching which was found to be awhile delayed to the studies of **Puvaneswari *et al.*, (2009)** where the yolk reserves were found to be completely dissolved on 3rd day of hatching. Temperature at 29 ± 1˚C, the yolk sac was completely absorbed within 3 days after hatching (**Miah *et al.*, 2017**) whereas at 22 - 23˚C (our study), yolk sac completely dissolved on 5th day after hatching.

The slow disappearance of the yolk sac may be due to the low hardness i.e. 18.3-25.4 mg/L of the water. **Korzelecka-Orkisz *et al.* (2010)** reported the influence of water hardness on embryonic and larval development in stinging catfish. Some studies also observed that the larvae developed at hardness of 20-50 mg/L had the largest yolk sacs, whereas, in very hard water (121-174 mg/L), larvae were found to develop smaller yolk sacs. The delayed in the development of the larvae in the current study may be due to the impact of ecological factors like water temperature and inadequate nutrient uptake during the rearing period.

CONCLUSION

The study on application of induced breeding technique opens up new scopes and opportunities in aquaculture. Our breeding trials showed success results on the target species, *H. fossilis* with a fertilisation rate of 75 ± 3.1% and hatching rate of 57 ± 1.9%.

Further improvement may be made by taking care of certain limiting factors like temperature, hardness, pH, water circulation, hormonal doses etc. Healthy diets needs to be provided to the brooders. Appropriate environmental conditions should be prepared both for brooders and spawns. The current study will play a crucial role to mitigate the shortage of supply of fish and in proper planning for the conservation of indigenous and economically important fish species in near future.

AUTHOR'S CONTRIBUTION

Chandopal Saikia: Data collection, analysis and manuscript preparation. **Moirangthem Kameshwor Singh:** Experimental design, data analysis, manuscript preparation and overall research supervision. **Susmita Sonowal:** Data collection, analysis and manuscript preparation.

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DATA AVAILABILITY STATEMENT

The data of the current study are available on request from the corresponding author.

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