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Indigenous Sandy Loaches in Focus: Pioneering Breeding Success of *Acanthocobitis Botia* in Bangladesh

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ABSTRACT

Successful spawning induction occurs when males and females exhibit synchronized behavior. In a previous study, we successfully demonstrated the reproductive biology and induction of spawning in loaches by administering synthetic hormones through injections. Building upon this research, we have applied the same technique to sandy loaches, specifically Acanthocobitis Botia. We injected the GnRH analog synthetic hormone ovaprim into the fish's body in this study. Within 6-8 hours of the injections, ovulation occurred, leading to the male and female engaging in spawning behavior. Our results showed that administering 1ml/kg of ovaprim in T3 yielded the most favorable outcomes in terms of the number of fish laying eggs (100%), total spawning eggs (290±42), fertilization rate (72±4.0), hatching rate (70±4.0), and survival of hatchlings (64±3.60) compared to the 0.75ml/kg in T2 and 0.5ml/kg in T1 treatments. No spawning activity was observed in the control group that did not receive injections. These findings have significant implications for the aquarium industry, as they can help promote the breeding of indigenous small fishes like loaches. Furthermore, this research contributes to the conservation efforts aimed at preserving the vibrant and diverse population of these colorful fishes in their natural habitats.

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Introduction

In our country, the botia puia (Acanthocobitis botia) is known as Natwa, Kholai Muchuri, Biltari, etc. depending on the area. A. botia is found in the hill streams of Sylhet and streams of Dinajpur, Rangpur, and Mymensingh [1]. This species is reported from the upstream Someshwari and Kongsho Rivers of Netrokona, Piyang, and Sari Rivers of Sylhet, Kortoa, Atrai, and Mahananda Rivers of the Northern region and recorded from the Tanguar Haor of Sunamgonj and at the high altitude of Sangu River [2-4]. It occurs in shallow, swift, clear, cool streams and rivers with sandy or gravelly bottoms [1]. It feeds on zoobenthos and insect larvae. It is nocturnal in habit and prefers to hide in the sand and gravel bottoms of hill-stream environment. It protects itself by burying its body in the sand and gravel with great rapidity. Acanthocobitis botia lives in diversified habitats, including hill streams. Inhibits submontane zones [5]. It is also found in canals, bells, and rivers [6]. It is found in hills streams and clear water creeks of Sylhet, streams of Mymensingh, Dinajpur, and Rangpur and recorded from the Chalan Beel and Halti Beel [7-11]. The populations of this species are unlikely to be adversely affected by the threats immediately due to its considerable decline. Moreover, the Extent of Occurrence (1,29,141.05 km2) and area of Previous literature on loaches:

Occupancy (4,123.29 km2) surpasses the threshold values of any threatened category; therefore, this species is assessed as Least Concern. It was considered as Data Deficient [12]. The eyes of this fish are small, and the body color is from olive to yellowish. There is a black spot at the upper corner of the base of the caudal fin. There is a great demand for this fish in the greater Rangpur region. Not only food fishes, but this species also has excellent potential as an ornamental fish. (Galib 2013 mentions that A. botia is one of the most prominent species for indigenous aquarium species due to its outstanding body coloration. The abundance of this fish has significantly decreased daily due to indiscriminate fishing and other environmental reasons. There is no scientific evidence of reproductive biology and induced spawning and fry production of A. botia in Bangladesh. Considering these issues and to protect it from danger, since 2020, the Bangladesh Fisheries Research Institute has started research activities in the ponds of the sub-center by collecting this fish from the Nilphamari district freshwater sub-center, Saidpur to the Chikli river in Rangpur, the Barati, Burikhra, and Teesta rive Later, the sub-station achieved success in producing Botia Puia fish fry through artificial breeding for the first time in the country in 2020-21. This success will ensure and open the door for mass seed production techniques to conserve the species in nature, which are also used as indigenous ornamental fish species in the revolution of the aquarium fish industry.

Materials and Methods

A. botia was domesticated under standard earthen pond conditions. Salmon gonadotropin-releasing hormone analogs (GnRH): Ovaaprim was purchased from Square Pharmaceutical Company, Bangladesh; imported from a metallic tray (2.15m*0.9m*0.30m), were prepared manually.

Site Profile:

The research work was conducted in the hatchery unit of Bangladesh Fisheries Research Institute (BFRI) Freshwater Sub-Station, Saidpur, Nilphamari. The induced breeding trials were done during the peak breeding season from May to August [7].

Collection, Rearing, and Selection of Broodfish for Induced Spawning

A. botia were collected from the greater Rangpur Chikli and Burikhora Rivers. After collection, the fish were transported to the BFRI Sub-Station, Saidpur, with oxygenated polybags adequately packaged. The fishes were domesticated for 2-3 months in a hatchery tank with artificial aeration and proper feeding (mosquito larvae, Brine shrimp, and sometimes zooplankton were supplied) for bloodstock development [13,14]. During the rearing period, standard water quality parameters were strictly maintained. Before peak breeding season, regularly check their gonadal status to ensure they are ready for spawning. Young males and females were identified based on their secondary sexual characteristics. The female has a bulging belly; if pressed on the belly, some mature eggs come out. The male was slender, and if slightly pressed, white milt oozed Srivastava. et al.

Experimental Designs

The study was conducted at the hatchery of the Bangladesh Fisheries Research Institute (BFRI), Fisheries Science Section (FSS) in Saidpur—the experiment aimed to investigate the reproductive response of A. botia using the GnRH analogs ovaprim hormones. Table 1 outlines the various hormones that were administered during the experiment. The male-to-female ratio was carefully maintained at 1:1 for artificial induction. Four experimental groups were established, with a control group for each hormone treatment (control, T1, T2, and T3). The study followed a Complete Randomized Blocked Design (CRD) with three replications of each treatment. Overall, the experiment aimed to analyze the effects of different hormones on the reproductive response of A. botia in a controlled and systematic manner.

Table 1: Application of Hormone Doses of Ovaprim for the Induced Breeding of *A. botia*

Treat	Replication	Sex ratio. ♂:1♀)	Types of hormone	hormone/kg) Male(♂)	Dose (mg/kg) Female♀)
Control		1:1	N/A	-	-
T1	03			0.25	0.5
T2	03	1:1	Ovaprim (ml/kg)	0.37	0.75
T3	03			0.5	1.0

Induced Breeding Protocol

Research has shown that a mature (4-8 grams in weight) was appropriate for induced spawning. (Fig-1). After collecting healthy, strong, mature male and female fish from the research pond during the breeding season, they are kept in an artificial fountain with oxygen flow in a cemented cistern in the hatchery for 5-6 hours. Later, the male and female fish are administrated for hormone injection. Then, synthetic hormone (Ovaprim) is applied to the male and female fish at the rate of 0.5 ml to 1.0 ml per kg, respectively. Spawning hapas were carefully placed inside the cemented cistern tank, ensuring a water depth of 75 cm and providing mild aeration facilities. Intramuscular ovaprim injection was administered to male and female fish, and triplicate samples were taken. A 1:1 ratio and an artificial fountain are arranged. In a hatchery with a metallic tray. The injections were administered just underneath the muscle of the dorsal fin in the evening. Following the hormone injections, the males and females were transferred to separate breeding systems. Each breeding system had a continuous waterfall (shower) and aeration through electronic aerators. This environment was carefully maintained to ensure a quiet and stress-free setting for the fish. A maximum of 5 males and five females were introduced into each breeding hapa measuring 1.2 x 0.9 x 0.9 m. Immediately after hormone administration, the fish were released into the breeding hapas. The tanks contained dechlorinated tap water with quality parameters-maintained water quality parameters (Table 2). After 6-8 hours of applying the hormone injection, the female fish naturally lays eggs. The bred fish are removed from the cage when the egg-laying is complete. After 12-14 hours, the larvae come out of the eggs

Table 2: Water Quality Parameters of the Brood-Rearing Pond of A. botia

Parameters	Value
Air temperature (^{0}C)	28.7±0.36
Water temperature (^{0}C)	26.90±0.50
Dissolved oxygen (mg l^{-1})	7.70±0.34
pH	7.43±0.18
Total ammonia (mg/l)	0.05±0.01

Collection of Breeding Parameters and Analysis

After nine hours of injection, almost all fishes released their eggs, and the broods were removed from the breeding system. During fertilization, approximately 100 eggs were taken in a bowl with three replications and were counted with the naked eye. At first, the numbers of fertilized and unfertilized eggs were counted, and then the fertilization rate was determined. By following the same procedure, the rate of ovulation, hatching, and survival rate was estimated by using the following formula: [15].

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

$$\textbf{spawning rate (\%)} = \frac{\text{NumberNumber of fish released eggs}}{\text{Total NumberNumber of fish injected}} \times 100$$

$$\textbf{Fertilization rate (\%)} = \frac{\text{Number of Fertilized eggs}}{\text{Total Number of eggs}} \times 100$$

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

$$\textbf{survival rate (\%)} = \frac{\text{Number of live embryo}}{\text{Total Number of fish eggs}} \times 100$$

Statistical analysis
All experiments were repeated three times. One-way analysis of variance (ANOVA) was performed using GraphPad Prism (San Diego, CA). P value < 0.05 was considered statistically significant.

Result
This study focuses on the artificial spawning induction of sandy loaches A. botia, which has the epitome of opportunity in a revolution of the indigenous aquarium industry by GnRH analog's synthetic hormone Ovaprim at different doses in hatchery conditions. Results show that GnRH analogs were effective for artificial spawning of A. botia

Induction of Sexual Behavior
Three different doses were utilized to assess the impact of the synthetic hormone GnRH analog ovaprim on the breeding success of B. botia, with no injection administered to the control group of fish. In the initial stages of induced breeding, it was observed that male fish began to pursue females approximately 6-8 hours (Table-3) after hormone injection (referred to as the Latency period). During this time, females also ovulated. Once both male and female fish exhibited their behaviors and engaged in continuous pursuit, the male fish would bend their bodies to hook the female, leading to induced spawning. Following spawning, fertilized eggs were collected from successful pairs. Our hypothesis that higher doses of the synthetic hormone would significantly induce ovulation and spawning behavior was confirmed, as no ovulation or spawning was observed in the control group.

Table. 3: Length and Weight of Experimental Fish A. botia with a Latency Period

Treatment	Body weight(g)		Latency period (hrs)	Incubation Temp. (^{0}C)	Remarks
	M	F			
Control	4.56±0.20	8.34±0.15	-	26-29	No ovulation and spawning
T1	4.68±0.10	7.82±0.11	8-9	27-29	Very few ovulation & spawning
T2	5.04±0.25	7.75±0.21	8-9	28-29	Moderate ovulation and spawning
T3	4.87±0.15	8.12±0.22	6-8	27-28	Good ovulation, fertilization, and hatching

Values are means of data obtained ± Std. Deviation (mean)

Determination of Reproductive Parameters
During the incubation period, the behavior of the brood fish was closely monitored to observe activities such as chasing, hooking, and successful spawning. It was noted that L. guntea typically released eggs 8–9 hours after hormone administration, known as the latency period. The sticky eggs adhered to the hapa, and once spawning was complete, the brood fish were removed and given a brief bath in either a salt solution or a potassium permanganate ($KMnO_4$) solution to prevent infections. They were then transferred to a designated brood fishpond. Hatchlings began to emerge 15–18 hours after spawning to avoid fungal growth; the hatching hapa was replaced with a new one. Egg yolk absorption occurred 60–70 hours after fertilization, signaling the initiation of the first feeding. Our previous research has shown that synthetic hormones can induce ovulation and spawning in other loach species, such as leptocephalus guntea and Somileptes gongota. Table 1 summarizes the average length, weight, and latency period of fish in each treatment group, while Figure 1 illustrates the spawning activity induced by the synthetic hormone ovaprim. The most successful reproductive outcomes, including ovulation, spawning, fertilization, hatching, total spawning eggs, and embryo survival, were achieved with a dose of 1ml/kg of ovaprim, surpassing the results of doses T1 (0.5ml/kg) and T2 (0.75ml/kg). The number of fertilized eggs and the rate of fertilization exhibited a consistent increase with higher hormone doses, and the number of spawning eggs also increase accordingly. (Table-4)

Figure 1: Matured Female of A. botia for Artificial Breeding

Larvae Rearing
The hatchlings were fed semi-boiled egg yolk every six hours for three days, then transitioned to a high-protein feed, Reno Gold, for the subsequent seven days. Due to their small size, L. guntea hatchlings were reared in hatchery conditions for 10–15 days (Fig-3) before being transferred to a fully prepared nursery pond for mass seed production. Once the ovaries were exhausted at 60 hours, boiled egg yolk was provided as food four times a day at six-hour intervals. After 6-7 days in the cage, the larvae were moved to a pre-prepared nursery pond, where suitable larvae were produced within 40-50 days.

Finally, it is concluded that in this study, the successful induced breeding of A. botia mirrors these findings, leading to the exciting discovery of a new method for breeding sandy loaches in hatchery conditions. This innovative approach has implications for the aquarium fish industry in Bangladesh, where there are colorful Indigenous fish species. Artificial reproduction will ensure the supply of larvae, contributing significantly to fish production in the greater Rangpur region and preventing species extinction.

Discussion
This study focused on inducing spawning in A. botia by administering three different doses of the synthetic hormone ovaprim. The results indicated that a dose of 1 ml/kg body weight of ovaprim showed significant breeding performance. Throughout the breeding process, the incubation temperature ranged from 27-30°C, pH levels were 7.3 and 8.5, alkalinity 142-145, and dissolved oxygen levels 6-8 ppm.

Previous research by suggested a breeding temperature of 28°C for L. guntea. Our findings demonstrated that the induction of spawning in T3 (100%) was significantly higher than in T2 (66.67%) and T1 (22%). calculated a female induction rate of 86.7% in zebrafish at 0.1 μM of Org, consistent with their previous report. Additionally, they found that Org also induced male behavior [16,17].

The latency period in our study varied between 6-9 hours after hormone injection. Lower doses required a longer time (8-9 hours) to ovulate, while higher doses required less time (6-8 hours). Smaller fish had a relatively shorter latency period.investigated the latency period of Puntius sophore, which was 6-8 hours, with ovaprim hormone administration, which aligns with our study's results [18].

stated that the latency period of botia dario was 5-6 hours when injected with 0.025ml WOVA-FH per fish. Our study corroborates these findings. botia dario's latency period0.025 ml
The latency period for Puntius sarana was found to be 8 to 9 hours after the administration of the inducing agent. In comparison, Ompok pabda exhibited a latency period of 6 to 8 hours following the administration of Ovatide by [19-21]. The total of spawning eggs, fertilization, and hatching rates were all deemed satisfactory. Notably, higher fertilization and hatching rates were observed in T3 (Figure 2), indicating superior egg quality. This can be attributed to the higher doses of the inducing agent, which stimulated male and female behavior more effectively than lower doses.

Figure 2: Photographs of 3 days Old Hatchlings Produced by Artificial Induction

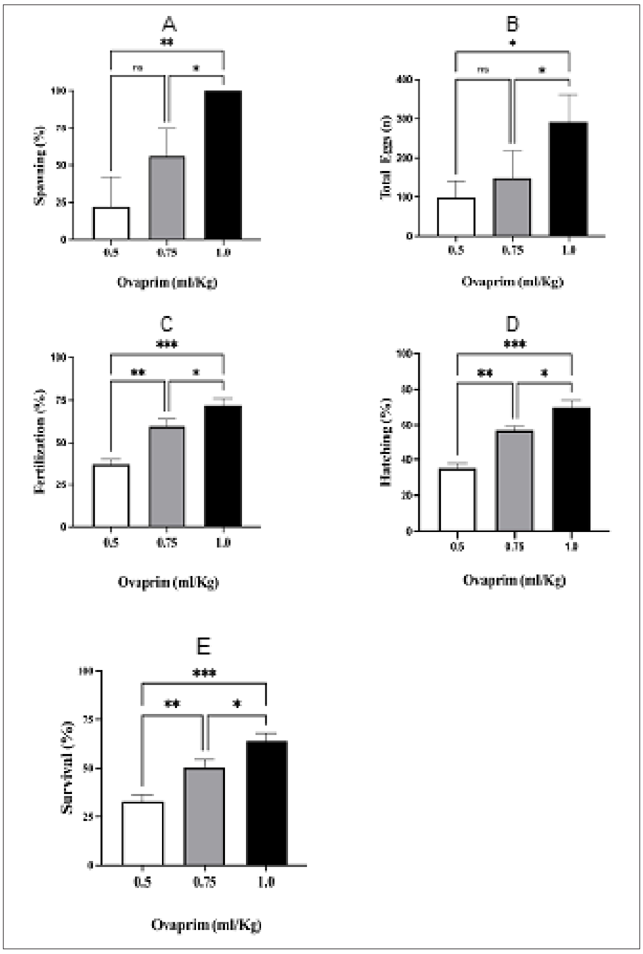

Figure 3: Induced Spawning and Reproductive Output of A. botia by ovaprim

Fishes were treated with the synthetic hormone ovaprim at the indicated concentrations after a 6-9 hrs latency period, and their sexual behavior was evaluated during incubation with females. (A)The fishes that release eggs by mating with male's spawn with many eggs released. Data represent the mean from three trials using different males and females (n=5) with vertical lines showing the standard deviation. (B)After the spawning periods, spawning eggs were counted from different successful females and incubated until hatching. (D). The number of hatchlings produced from fertilized eggs was counted. (E)Survival rate of 3 days old embryo was significantly higher in 1ml/kg hormone dose. A statistical analysis was performed using one-way ANOVA to determine significant differences between the treatment groups. Different stars represent significant differences among the data. *, P value < 0.05, **, P value < 0.01, ***, P value < 0.001, ****, P value < 0.0001. The mark 'no star' indicates that the groups did not differ significantly from the control.

In contrast, lower doses decreased sperm activation, leading to lower total spawning eggs, fertilization, and hatching rates in T1 and T2 compared to T3. While small Indigenous species typically exhibit lower fertilization and hatching rates than Indian carp, our study yielded promising results in breeding parameters using higher doses of the synthetic hormone oval rim.

Table 4: Spawning Response of A. botia using Ovaprim Hormone Under Captive Condition

Trial	Ovaprim (ml/kg ^{1})		Spawning	Fertilization (%)	Hatching (%)	Total Spawning eggs	Survival of Embryo (%)
	M	F					
Control	-	-	-	-	-	-	-
T1	0.25	0.50	22.22±19.24	37.0±2.82	35.33±2.08	99.00±18.0	32.50±3.53
T2	0.37	0.75	55.55±19.24	59.33±4.04	56.33±3.05	147.00±35.0	50.33±3.78
T3	0.5	1.0	100.00±0.0	72.00±4.0	70.00±4.0	290.00±42.0	64.00±3.60

Values are means of data obtained ± Std. Deviation (mean ± SD)

It is important to note that fertilization rates, hatching rates, incubation periods, and survival rates are influenced by various factors, including the doses and types of hormones administered the ratio of hormones used, and water physicochemical parameters [22,23]. These factors play a crucial role in determining the success of breeding programs and should be carefully considered in future research and breeding practices.

Various factors, such as different types of feeds, water quality, and live feed, can impact larval survival rates. Research has shown that larvae fed with egg yolk, specifically Lepidocephalichthys guntea (Hamilton), have demonstrated the highest survival rate of up to 60%, which is consistent with findings by [16]. Successful induced fish breeding is a critical step in practical fish farming, as emphasize [24].

The initial feeding of larvae is crucial for their growth and survival. In our study, we have developed an innovative method for inducing reproductive success in the small loach species L. guntea. This method involves the injection of hormone solutions into the fish's body and integrating supportive breeding media. Our approach has proven highly effective in preserving L. guntea strains and shows excellent potential for being adapted as an artificial egg collection technique for other endangered small fish species. [25-26].

Conclusions

As a delicious food fish and the epitome of color combination, loach fishes play a vital role in promoting the aquarium industry by using small indigenous fishes' sandy loaches. A. botia is a significant aspect of Bangladeshi culture. This pre-rinsing success of induced spawning helps facilitate new thinking to utilize indigenous small fishes. However, more research is needed on mass seed production without injection methods. Moreover, non-invasive methods like these could improve welfare standards in laboratory settings, as a small Indigenous species (SIS) A. botia fry production is crucial by injection method. However, scientists produce zebrafish fry simply by adding an agent into the water with fish by in vivo method with Org-OD and DHP [16]. This innovative approach may suit small fishes like A. botia mass seed production in sustainable aquaculture.

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Competing Interests

The authors have declared that no competing interests exist.

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