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Effects of probiotic and Vitamin C on domestication and breeding performance of indigenous koi, *Anabas testudineus* under cage system

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ABSTRACT

An experiment was accomplished on domestication and induced breeding of indigenous climbing perch, *Anabas testudineus*. For domestication, fish were stocked and reared for six months in nine cages (each having 1.0 m^3 in size) set in three ponds (Pond 1, 2 and 3) so that three cages were set up in each pond. Three supplementary feeds such as feed mixed with vitamin C, only supplementary feed and supplementary feed with probiotic (*Bacilus subtilis* and *Aerobacter* sp.) considered as treatment T_1, T_2 and T_3, respectively were provided to triplicate cages in each pond and the cages were considered as replications R_1, R_2, R_3. Highest growth in terms of net length and weight gain was found in T_3 (9.35±2.65 cm and 44.58±4.68 g) followed by T_1 (9.00±2.03 cm and 37.62±3.45 g), and T_2 (6.18±1.57 cm and 34.24±3.08 g), respectively. Likewise, the highest average value of GSI was found in T_3 (7.26±2.12) in the month of April while lowest average value of GSI (0.64±0.21) was found in T_2 in the month of July. Three induced breeding trials were conducted using carp pituitary gland extract (PG) where female broods received 6-8 $mgkg^{-1}$ body weight and males received 2-4 mg kg^{-1} body weight. Fish in T_3 injected with PG 4 $mgkg^{-1}$ for male and 8 $mgkg^{-1}$ for female showed highest result in case of ovulation rate (96.67±5.77), fertilization rate (75.75±2.00%) and hatching rate (90.25±2.18%) followed by T_2 (ovulation, 80±10%, fertilization, 70.25±2.22%, hatching and 85.65±1.80%) and T_1 (ovulation, 73.33±5.77, fertilization, 66.23±1.56% and hatching, 72.75±2.06%).The survival rate of fry was highest in T_3 (65.9±1.09%) followed by T_2 (60.75±2.00%) and T_1 (55.5±2.46%). It was observed that, the mixture of probiotic with feed was more suitable for higher growth and GSI. 2.0 g probiotic (VC-7, Team aqua corporation, Taiwan) and 20 g molasses for 5 L of water were found better result for induced breeding of *A. testudineus*.

Introduction

Among small indigenous species, indigenous climbing perch (*A. testudineus*) is widely distributed in South and South-East Asia. It is mostly found in haor, baor, canals, lakes, ponds and even in swamps areas **(**Siddiqua *et al.*, 2000). It became mature in medium to large size in the rivers, brooks, flooded fields and stagnant water bodies including sluggish flowing canals (Taki, 1978). *A. testudineus*is considered as a valuable item of diet for sick and convalescent persons. According to Saha (1971), it contains high values of physiologically available iron and copper essentially needed for hemoglobin synthesis. It is also becoming highly demandable fish day by day due to its air breathing ability and tolerance against adverse environmental conditions (Alam *et al.*, 2006). Also it is very hardy in nature and can thrive well in low dissolved oxygen and able to aestivate during the dry season (Thakur, 2004). It is believed to have medicinal properties such as disease prevention; and slowing down the ageing process for females (Patowary and Dutta, 2012). Once local koi was abundantly available in almost all freshwater systems of Bangladesh, however, recently population of this fish has been declining because of ecological degradation, indiscriminate fishing, use of pesticides and fertilizers, destruction of habitats, obstruction to breeding migration, management failure etc. International Union of Conservation of Nature (IUCN) enlisted *A. testudineus* as not threatened perch fish in Bangladesh (IUCN, 2000). But due to rough and unplanned water management policy for irrigation, over exploitation, illegal practice of capture fisheries and various ecological changes in its natural habitat; this native species is considered as endangered now (Chakraborty, 2010; Das *et al.*, 2009; Sverdrup, 2002). Considering the importance of this species in nutritional, economics and biodiversity point of view, it is essential to conserve *A. testudineus* in Bangladesh. As indigenous climbing perch has climbing tendency from one pond to another

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pond during rainy season or even breeding season, so domestication of this species might be effective in earthen ponds under cage culture system.

Though, breeding technology of indigenous climbing perch (*A. testudineus*) had been practiced in freshwater station of Bangladesh Fisheries Research Institute (BFRI) but the growth pattern is very slow in culture condition (Kohinoor *et al.*, 1991). Regards, probiotic and vitamin C can be incorporated as dietary supplementation into pond water and supplementary feed to enhance their growth as well as gonadal maturation. As probiotic denoting a beneficial bacterial substance that enhances the growth, immunity and digestibility of fishes. So, incorporation of probiotic along with supplementary feed can be effective for pond aquaculture. Recently, Rahman *et al.* (2018) reported that beneficial effects of probiotic administration into commercial feed additives enhanced the reproductive performance such as GSI, fecundity and larval survival of indigenous species, *Ompok pabda*. In addition, probiotic is useful for enhancing fish growth, reproduction efficiency (especially for females) and development of fish gonad (Rahman *et al.*, 2018). Correspondingly, vitamin C is considered to be an essential component of diet for many teleost species (Dabrowski and Ciereszko, 2001). *Vis-a-vis*, several studies have been undertaken on dietary supplementation of vitamin C into commercial feed that has positive effect on the breeding performance of tilapia (Soliman *et al.*, 1986), Atlantic salmon (Eskelinen, 1989), milkfish (Emata *et al.*, 2000). Previously, induced breeding of indigenous koi has been practiced (Kohinoor *et al.*, 1991; Suraiya *et al.*, 2012) but information on domestication of cage rearing wild perch and its seed production using probiotic and vitamin C are scanty. However, to evaluate the culture and reproductive potentials of *A. testudineus*, information on the domestication and observation on induced breeding are considered essential. Hence, this threatened species needs protection from being endangered through the development of its domestication and induced breeding techniques. Therefore, the present work was planned to domesticate and develop a suitable induced breeding technique of *A. testudieus* under cage culture system using probiotic and vitamin C.

Methodology

Domestication of A. testudineus in earthen ponds using cage system

Pond preparation

Before fish stocking all ponds were dewatered and kept for sun drying for 20-30 days. Dyke was repaired and digging was done into bottom of the pond. Water depth was maintained at 2-3 meter all the year round. Lime was applied 1kg per decimal in three treatments (Pond-1, Pond-2, and Pond-3) to reduce acidity. Cow dung and urea were applied to increase the production of phytoplankton and zooplankton to fulfill the need of nutrition both for growing fish fry and brood fish.

Collection of fish samples

Indigenous climbing perch (*A. testudineus*) were collected from Shamgonj beel, Netrokona district with the help of fishermen. Then they were brought to the Wet Laboratory Complex, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh and kept in fiber tank for acclimatization at 26-28°C through continuous water showering. After 2-3 hours of acclimatization, they were stocked into the cage for rearing.

Stocking of wild perch using cage system in earthen pond

The collected indigenous climbing perch were stocked in nine cages in three different ponds (Pond 1, 2 and 3) each containing three cages of equal size (one cubic meter) (Fig. 1). In each cage 30 fingerlings were stocked and reared them up to their gonadal maturation.

Feed formulation and feeding of stocked fish

Three supplementary feeds such as supplementary feed (35% protein) mixed with vitamin C, only supplementary feed (35% protein), and supplementary feed (35% protein) with probiotic (VC-7) were used and these three feeds were considered as treatment 1, treatment 2 and treatment 3, respectively. As each feed was applied to three cages in each pond, the cages were considered as replication like R_1, R_2 and R_3. Here, vitamin C was mixed with the supplementary feed at the rate of 200 mg kg-1. Similarly, 2.0 g probiotic (VC-7, Team aqua corporation, Taiwan) and 20 g molasses were mixed with 5 L of water that was applied for consecutive three days. Probiotic contained Bacillus subtilis 1×109 CFU/g, *Aerobacter sp.* 1×10^3 CFU/g that was applied in every 15 days interval. Here, molasses was provided for bacterial growth of probiotic. Feeds were applied three times a day (8 am, 1 pm and 4 pm) at the rate of 5% body weight of fish.

Analyses of water quality parameters

Water samples were collected between 09:00 and 10:00 am monthly from each pond surface to a depth of 20 cm. Water quality parameters such as water temperature, dissolve oxygen, pH, concentration of ammonia nitrogen and alkalinity were recorded. Water temperature (°C) from each pond was recorded by using a Celsius thermometer. Dissolved oxygen (mg/l) and pH were measured using a digital DO meter (HANNA, Model-HI 9146, Romania) and a direct reading digital pH meter (Milwaukee PH meter, Model-PH55/PH56, USA), respectively. Total alkalinity (mg/l) was determined titrimetrically according to the standard procedure and

methods (APHA, 1992). Ammonia-nitrogen was measured by a UV VIS Spectrophotometer water analysis kit (DR 6000TM, USA).

Sampling and growth parameter study

Samplings were done in every 15 days' interval during the experimental period. Weight (g) and length (cm) were measured by using a digital electronic weighing scale (TANITA Corporation, China) and a measuring scale in each sampling day during the experimental period. Different growth parameters were calculated using the following formulae:

i) Weight gain (g) = Final body weight – Initial body weight
ii) Length gain (cm) = Final body length – Initial body length
iii) The specific growth rate (SGR % per day) = $[\ln W_2 - \ln W_1 / T_2 - T_1] \times 100$

Where,
W_1 = mean initial weight (g)
W_2 = mean final weight (g)
T_1 = time at the start of the experiment
T_2 = time at the end of the experiment

Calculation of GSI

GSI (Gonado-somatic index) were recorded from January to July, 2018. In each sampling day, only 10 female fishes were selected randomly and the ovaries were dissected out and weighed. GSI was calculated using the following formula:

GSI = [gonad weight / total body weight] ×100

Induction on induced breeding A. tesudineus

Brood fish selection and conditioning

Free oozing broods of *A. testudineus* were selected for induced breeding experiment. In addition, mature female and male broods were identified by observing the secondary sexual characters. Here, females were comparatively larger in size with soft and swollen abdomen and males were comparatively smaller in size and gentle pressing on abdomen released milt.

Breeding trials

Induced breeding trials were performed using pituitary gland extract. Here 1:1 ratio of free oozing male and female broods were selected for induced breeding. Nine breeding trials were conducted under three treatments. From each replication of all treatments, 5 pairs of male and female were selected for induced breeding.

Injecting the PG extract to broods

Just prior to hypophysation, selected females and males were caught from the cistern using a scoop net. During administration of injection, the fish were wrapped by a soft cloth and kept lying on soaked foam. The PG solution was injected intramuscularly at the dorsal side behind the pectoral fin and both male and female fish were kept in the same cistern. Three doses of PG extract were used as inducing agent in all breeding trials for male and females. PG doses of 5.0, 6.0, and 8.0 $mgkg^{-1}$ body weight for female and 2.0 3.0, and 4.0 $mgkg^{-1}$ body weight for male were applied to induce ovulation of fishes by only a single injection for both male and female in T_1, T_2 and T_3, respectively. The ovulation was occurred after 7-8 hours of injection. Eggs released by female fish were allowed to fertilize naturally by the sperm in the same cistern. Fertilized eggs were collected and placed into hatching tank with continuous water flow for proper aeration. The fertilized eggs were incubated at room temperature (26-28 °C). The hatching took place after 18 hours of fertilization.

Determination of ovulation, fertilization and hatching of eggs and survival rate of larvae

For determination of fertilization and hatching rates of eggs and survival of larvae, approximately 100 eggs were placed in a bowl of 2 L capacity with three replications of each treatment. At first, the numbers of fertilized and unfertilized eggs of each bowls were counted with naked eyes and recorded. After approximately 18-20 hours of fertilization, when the hatching completed, the number of hatchlings in each bowl were counted. For determining survivability of larvae, they were reared in hapa for 30 days. During larvae rearing, they were fed Fishtech (BD) Limited feed which contained (37%) crude protein. Hence, breeding parameters like fertilization and hatching rates; and survival rate of larvae were calculated using the following formulae.

i) Ovulation rate (%) = [No. of fish ovulated / Total no. of fish injected] × 100
ii) Fertilization rate (%) = [No. of fertilized egg /Total no. of eggs (fertilized and unfertilized)] × 100
iii) Hatching rate (%) = [No. of hatchling /Total no. of eggs] × 100
iv) Survival rate (%) = [No. of larvae survived /Total no. of larvae] × 100

Statistical analysis

(i) The data obtained in the present experiments were analyzed statistically to see whether there was significant difference or not among the treatments. This was done by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at 95% level of confidence.

Results

Water quality parameters

Mean values of physico-chemical parameters of the experimental ponds of koi are presented in Table 1. The mean values of water temperature were 30.6±1.99, 30.2±1.60 and 28.5±1.18 °C in T_1, T_2, and T_3,

respectively. During experimental period the highest water temperature was recorded 34 °C in June and the lowest temperature 25 °C in January. The values of total alkalinity were estimated as 102±2.99, 115±3.68, and 113±4.11 mgL^{-1} in T_1, T_2, and T_3, respectively. The content of highest alkalinity was recorded 123 mgL^{-1} and lowest 98 mgL^{-1} during experimental period. Among the treatments T_3 showed the significant difference in term of temperature and alkalinity ($P<0.05$). The mean values of pH was 8.70±0.76, 8.40±1.80, and 8.03±1.79 in T_1, T_2, and T_3, respectively. The highest pH value was recorded 9.5 and lowest value 8.0. No significant ($P>0.05$) differences among the treatments were found in the pH value during experimental period. Range of dissolved oxygen in the experimental ponds were recorded 5.60±0.61, 5.50±0.43 and 5.36±0.39 mgL^{-1} in T_1, T_2, and T_3, respectively. During experimental period the highest dissolved oxygen was recorded 6.5 ppm and the lowest 5 ppm. No significant ($P>0.05$) differences among the treatments were found in the DO value during experimental period. During the present experiment the mean value of ammonia-nitrogen was 0.23±0.09, 0.32±0.03 and 0.28±0.07 mgL^{-1} in T_1, T_2, and T_3, respectively. The highest ammonia-nitrogen value was 0.50 in the month of June in T_2 and the lowest value was 0.01 in the month of May in T_3. No significant ($P>0.05$) differences were found among the treatments.

Growth performance and survival rate

After six months of rearing, significantly highest net weight gain and SGR as 44.58±4.68 g and 0.99±0.56%, respectively were observed in T_3 followed by T_1 (37.62±3.45 g, and 0.91±0.023%) and T_2 (34.24±3.08 g, and 0.89±0.034 %), respectively (Table 2). Moreover, highest net length gain were recorded in T_3 (9.35±2.65) followed by T_1 (9.00±2.03) and T_2 (6.18±1.57), respectively (Table 2). Survival rate of fish during the six month domestication period was also significantly higher in T_3 (96%) followed by T_1 (93%) and T_2 (86%).

Gonado-somatic index (GSI) value

Dietary nutrition is very important for gonadal development of fish. The highest value of GSI was found as 7.26±2.12% in the T_3 in April and the lowest value of GSI was 0.64±0.21% in July in T_2 (Fig. 2). In all the three treatments, GSI started to increase from March and reached to pick in April and then decreased again. Therefore, the pick breeding season of climbing perch was seemed to be in April.

Table 1. Average water quality parameters of three different ponds during the experimental period

Treatment	Temperature (°C)	pH	Dissolve Oxygen (mgL^{-1})	Total alkalinity (mgL^{-1})	Ammonia-Nitrogen (mgL^{-1})
1	30.6±1.99[a]	8.70±0.76[a]	5.60±0.61[a]	102±2.99[a]	0.23±0.09[a]
2	30.2±1.60[a]	8.40±1.80[a]	5.50±0.43[a]	115±3.68[b]	0.32±0.03[a]
3	28.5±1.18[b]	8.03±1.79[a]	5.36±0.39[a]	113±4.11[b]	0.28±0.07[a]

Different superscript in the same column indicates significant difference among the value ($p<0.05$)

Table 2. Growth performance and survival rate of indigenous *A. testudenius* (koi) fed with three different supplementary during domestication period

Parameter	T_1 (Supplementary feed with vitamin C)	T_2 (Only supplementary feed)	T_3 (Supplementary feed with probiotic)
Initial weight (g)	8.99±1.30[a]	8.56±0.91[a]	9.01±0.86[a]
Final weight (g)	46.61±4.21[b]	42.80±1.88[c]	53.59±2.46[a]
Net weight gain (gm)	37.62±3.45[b]	34.24±3.08[c]	44.58±4.68[a]
Initial length (cm)	5.89±0.85[a]	5.80±0.73[a]	5.95±0.92[a]
Final length (cm)	14.89±1.23[b]	11.98±1.05[c]	15.30±3.05[a]
Net length gain (cm)	9.00±2.03[a]	6.18±1.57[b]	9.35±2.65[a]
SGR (%/day)	0.91±0.023[b]	0.89±0.034[b]	0.99±0.56[a]
Survival rate (%)	93[a]	86[b]	96[a]

Different superscript in the same row indicates significant difference among the value ($P<0.05$)

Table 3. Observation of induced breeding of indigenous A. *testudineus* using different doses of PG doses

Treatment	Replication	Dose of Injection ($mgkg^{-1}$)		Ovulation rate (%)	Average ovulation rate (%)	Fertilization rate (%)	Average Fertilization rate (%)	Hatching rate (%)	Average hatching rate (%)	Survival rate (%)	Average survival rate (%)
		Male	Female	Combined for male and female							
1	1	2	5	70		65.50±1.88[b]		75.75±1.94[a]		52.25±2.01[b]	55.5±2.46[c]
	2	2	5	80	73.33±5.77[c]	60.75±2.75[c]	66.23±1.56[c]	69.85±2.08[c]	72.75±2.06[c]	57.38±2.21[a]	
	3	2	5	70		72.44±2.36[a]		72.90±2.62[b]		56.87±2.51[a]	
2	1	3	6	90		72.75±2.06[a]		83.25±3.45[b]		63.62±2.13[a]	60.75±2.00[b]
	2	3	6	70	80±10[b]	72.75±2.06[a]	70.25±2.22[b]	89.79±3.12[a]	85.65±1.80[b]	57.78±2.04[c]	
	3	3	6	80		69.75±1.54[b]		83.91±2.56[b]		60.85±2.61b	
3	1	4	8	100		80.45±3.05[a]		93.25±3.93[a]		64.85±2.83[b]	65.9±1.09[a]
	2	4	8	90	96.67±5.77a	71.90±2.12[b]	75.75±2.00[a]	87.65±2.05[b]	90.25±2.18[a]	68.95±3.07[a]	
	3	4	8	100		74.90±2.39[b]		89.85±2.41[b]		63.90±2.87[b]	

Different superscript in the same column indicates significant difference among the value ($P<0.05$).

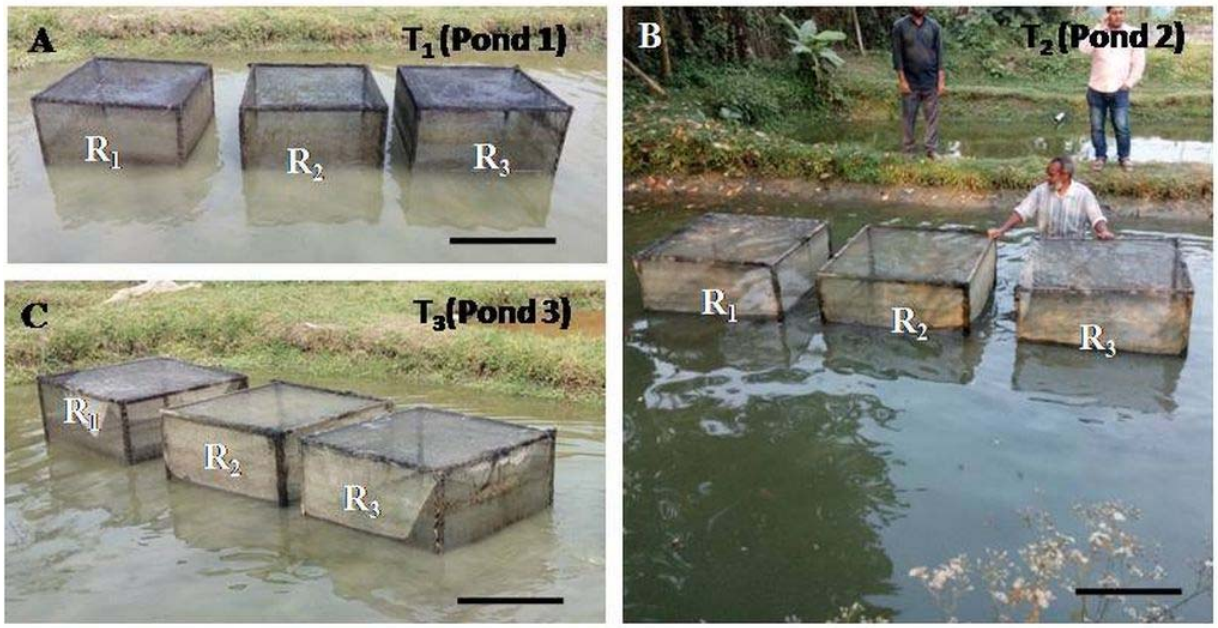

Fig. 1. Brood rearing in earthen pond in three different treatment using cage system (A-C). A, Fishes were fed with commercial supplementary feed with vitamin C; B, only commercial supplementary feed; and C, commercial supplementary feed with probiotic(VC-7)

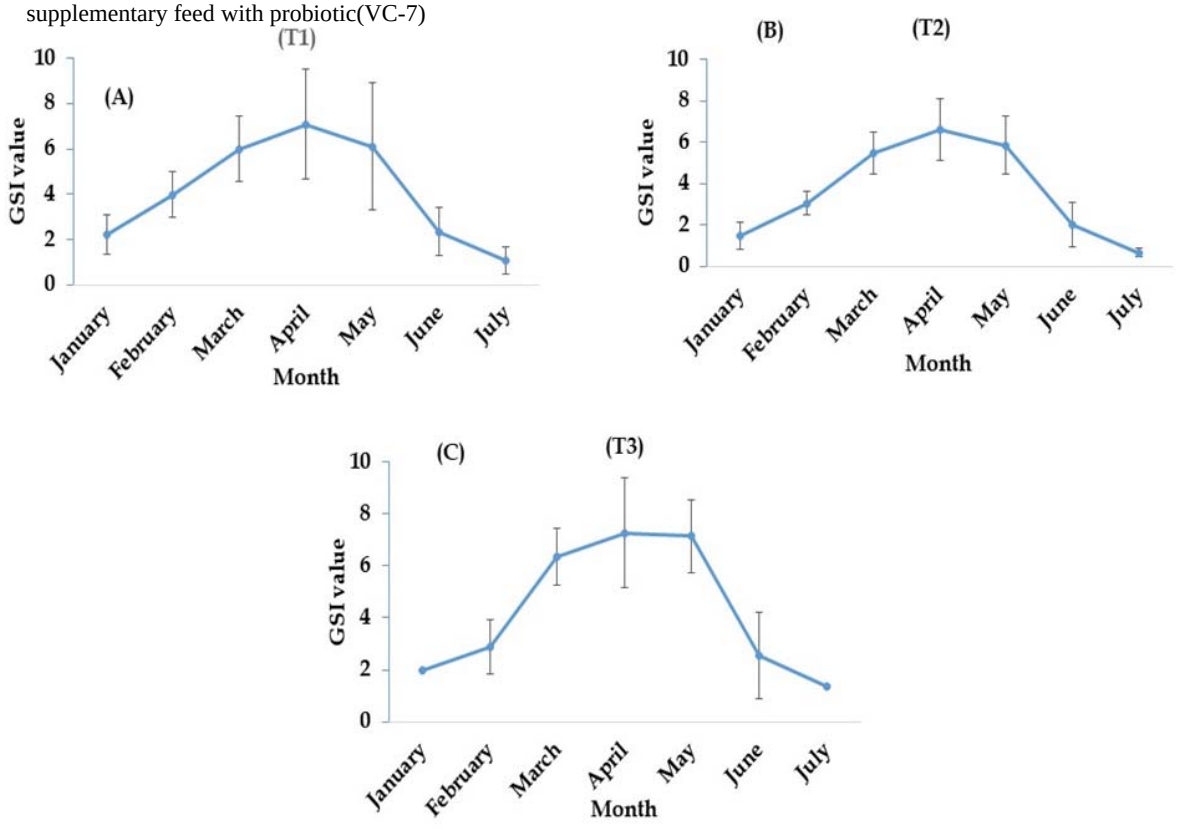

Fig. 2. Monthly variation in mean GSI for females *Anabas testudineus*(Koi) in three treatments (A-C). A, GSI recorded in T_1; B, GSI recorded in T_2 and C, GSI also recorded in T_3.

Breeding performance parameters analysis

The average ovulation rate of females varied among the treatments. Significantly highest average ovulation rate (96.67±5.77%) was recorded in T_3 followed by T_2 (80±10%) and T_1 (73.33±5.77%), respectively (Table 3). Significantly highest fertilization rate was observed in T_3 (75.75±2.00%) followed by T_2 (70.25±2.22%) and T_1 (66.23±1.56%), respectively (Table 3). Significantly highest average hatching rate was recorded

(90.25±2.18%) in T_3 followed by T_2 (85.65±1.80)and T_1 (72.75±2.06), respectively (Table 3). The survival rates of larvae of *A. testudineus* were 55.5±2.46%, 60.75±2.00%, and 65.9±1.09% in T_1, T_2 and T_3, respectively after 30 days of experimental period. The results revealed that a significantly ($P<0.05$) higher survival rate of larvae was observed in T_3 and lowest in T_1 (Table 3).

Discussion

In the present study, mean water quality parameters among the treatments were found within the suitable range for growth of indigenous koi in ponds condition under cage system which was supported by Mondal *et al.* (2010). The value of water temperature in the present study is approximately similar according to Ahmed *et al.* (2014) in case of Vietnam koi in mini pond culture system. However, the level of dissolve oxygen (DO), alkalinity and ammonia appeared to be within the acceptable range similar to the result reported by Chakraborty and Haque (2014) in case of koi under semi-intensive culture system. Higher total alkalinity values might be due to application of lime during pond preparation and monthly interval liming during the experimental period.

Indigenous koi is a wild fish and takes natural food (phytoplankton, zooplankton) more easily than formulated feed. Therefore, we used probiotic in pond, which increased the intensity for taking formulated feed by increasing beneficial bacteria in the intestine (Sugita *et al.,* 1998). Kohinoor *et al.* (2012) reported slightly higher weight gain in case of Thai koi compared to that obtained in the present study. A possible cause of higher weight gain in their study is that Thai koi is a fast growing species. Sugita *et al.* (1998) reported that addition of probiotic bacteria into supplementary feed produced digestive enzymes and essential nutrients that improves feed absorption and resulting increased growth of fish. Sivagami and Kuzhithurai (2016) used probiotic enriched feed in domestication of *Cirrhinus cirrhosis* fingerlings which showed better growth than control groups. This proved the fact that probiotics have direct positive effects on fish growth. Kohinoor *et al.* (2012) observed a high (93%) survival rate in *A. Testudineus* with floating pelleted feed containing 30% crude protein which was relatively lower from the present study. This may be due to supplementation of high protein (35%) containing pelted feed in the present experiment. Rather than this, probiotics and vitamin C was also used which may be a cause of improved survival rate in the present experiment.

Parween *et al.* (1993) reported that the gonado-somatic index increased with the maturation of fish, being maximum during the period of peak maturity and declining abruptly thereafter. Hafijunnahar *et al.* (2016) reported the maximum GSI values in both sexes of Vietnamese koi during the month of May and the minimum in January. Comparing with their experiment our result showed satisfactory range in GSI. Variation in GSI may occur due to the different strain, environmental habitat, nutritional status and genetic changes etc.

Perera *et al.* (2013) demonstrated that highest hatching rate of *A. testudineus* was 87.0±3.9 with the 0.5 ml/kg body weight of Ovaprim hormone. Ahammad *et al.* (2008) reported that fertilization, hatching and survival rates of indigenous koi were 96.87±1.19%, 75.57±1.91% and 71.54±2.23%, respectively using PG dose 6 mg/kg body weight in female and 2 mg/kg body weigh in male. Saha *et al.* (2009) reported that the highest fertilization, hatching and survival rates of Thai koi were 96.33±1.53%, 90±4.35% and 80.33±2.21% respectively for the sex ratio 1:2. Suraiya *et al.* (2012) showed that ovulation fertilization, hatching and survival rate of indigenous climbing perch were 73.33±00%, 85.65±5.78 %, 68.75±7.44% and 63.54±4.25 %, respectively using same PG dose. In the present experiment the ovulation rate was higher, fertilization rate of was little lower that the above-mentioned experiment but satisfactory as it was the first attempt for domestication of indigenous in cage system. Likewise, hatching rate in the present experiment (T_3) was similar with the findings of Saha *et al.* (2009). The survival rate of larvae in the present experiment is satisfactory compared with the findings of Ahammad *et al.* (2008) and Suraiya *et al.* (2012). As well, high survival rate of indigenous climbing perch was reported by Kohinoor *et al.* (2009). These indicated that indigenous climbing perch may be an excellent candidate for aquaculture. As for proper fish culture system, main factors are high growth rate, survival rate, proper stocking of healthy seed, freedom from predation, favorable ecological conditions (physico-chemical factors) and also proper feeding etc. In the present experiment, all the factors were under control and thus it showed satisfactory result. However, to improve the seed quality, growth optimization especially for commercial aquaculture using cage system of this species needs further research to explore it aquaculture potentiality.

Conclusion

The present study revealed that the growth of *A. testudineus* might be satisfactory in cage system when fed with supplementary feed and treated with probiotics in water. However, domestication in cage plays an important role for gonadal maturation, seed production, growth and conservation of *A. testudineus*. Moreover, higher fertilization and hatching rates of indigenous climbing perch can be obtained by using probiotic during brood development. Additionally, present results of the breeding trials will give precise information to the hatchery operators as well as scientific community of the world. Hence, domestication in cage system of *A. testudineus* may be the baseline research for further investigation.

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