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INDUCED SPAWNING AND LARVAL DEVELOPMENT OF VIETNAMESE KOI, *Anabas testudineus* (Bloch, 1792) USING SALMON GONADOTROPIN RELEASING HORMONE ANALOGUE (S-GnRHA)

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ABSTRACT

The present research work has been carried out to obtain evidence on breeding and embryonic growth of *A. testudineus* by using S-GnRHa. Fish were injected with three different doses (0.25, 0.5, and 1.0 mL/kg body weight) of synthetic hormone S-GnRHa each with three replications, where male brood fish received half of the doses of female. The fecundity of *A. testudineus* was ranged from 47,227 to 77,561 during the study period and except control group all the hormone received group ovulated within 12 hours of hormone injection. Among all groups, the highest fertilization rate (89.33%), highest hatching rate (79.5%) as well as highest survival rate (67.0%) was obtained at 0.5 mL/kg body weight hormone dose recipient group. The fertilized egg's diameter was recorded as 77.59 ± 3.50 µm. The first cleavage had appeared within 18-25 min of fertilization and eventually the morula, blastula, and gastrula stages were observed at 3:10 h, 4 h, and 5:30 h, respectively after fertilization. Larvae with distinguished head, body form and tail appendage spotted between 17-22 h and the larvae started hatching at 19 h after fertilization. The average length of larvae accounted as 105.41 ± 3.73 µm. The findings of present study revealed that 0.5 mL/kg S-GnRHa could be efficient dose for successful induced breeding of *A. testudineus*.

KEYWORDS: S-GnRHa; induced breeding; *Anabas testudineus*; larval development

INTRODUCTION

The climbing perch, *Anabas testudineus* is a well-known airbreathing anabantoidae fish and once it was most abundantly available in Bangladesh. However, due to over exploitation, habitat destruction and lack of seed for commercial culture, the population of this species is declining day by day (Khatun *et al*., 2019). Therefore, Vietnamese strain of koi (*A. testudineus*) was introduced in Bangladesh during the year of 2011 from Vietnam having fast growth rate (Mondal *et al*., 2010), premium nutritive value (Paul *et al*., 2017) and trendy market demand and becomes one of the successful exotic fish for aquaculture in Bangladesh (Faruk *et al*., 2018). The Vietnamese koi have quite different morphometrics in comparing to indigenous strain of Bangladesh (Ara & Nabi, 2018; Helmizuryani *et al*., 2020). The Vietnamese strain in aquaculture of Bangladesh have been reported to be 30 cm in standard length and 198.67 g in body weight, while indigenous strain of reaches only 14.50 cm in length and 63.78 g in weight (Ahamed *et al*., 2018; Hossen *et al*., 2017; Khatun *et al*., 2019).

Various spawning agents have been reported to induce the experimental breeding of *A. testudineus*, such as Wova-FH, Ovatide, Ovaprim, LHRHa, HCG, and PG, pituitary gland (Aktar, 2015; Nabi *et al*., 2020; Sarkar *et al*., 2005; Sukendi *et al*., 2019). It is most common practice to use pituitary gland (PG) and human chorionic gonadotropin (HCG) for hatchery breeding induction in Bangladesh (Amin *et al*., 2016; Kumar *et al*., 2021) but mass production and price range of these agents not handy yet. To overcome above difficulties, complementary agents like salmon gonadotropin releasing hormone analogue, S-GnRHa is launched in Bangladesh to improve production of several aquaculture species (Amin *et al*., 2016; Raze *et al*., 2017). The synthetic analogue of GnRHa is

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cheaper, readily available, and efficient induced hormone being imported in Bangladesh (Mandal *et al*., 2016).

The embryonic development of *A. testudineus* was reported (Zalina *et al*., 2012), and in fish, embryonic development is strongly influenced by temperature, pH, and dissolve oxygen. Research on hormonal induction and associated embryonic development will reveal key information require for mass seed production and post hatching care of this species efficiently. This recent research was aimed at exploring the embryological development of *A. testudineus* for better management, conservation, and commercial seed production in aquafarm using synthetic form of GnRHa.

MATERIALS AND METHODS

Study Area and Duration

The whole experiment has been carried out at commercial hatchery 'Alapur Hatchery and Fisheries' in Bangladesh, Laboratory assays and cytological studies were conducted at laboratory of Fish Biology and Genetics, Bangladesh Agricultural University, Bangladesh and the duration of the hatchery trials was for three months.

Rearing and Selection of Brood Fish

The brood fish was reared in prepared brood rearing ponds (0.08 hectare and water depth 0.9-1.1 meters) at a total of 500 mature fish of both sexes were reared. Before rearing, lime, fertilizer, and cow dung were properly applied. Brood fish were also fed twice daily at 3%-7% body weight with supplementary feed (protein 50%, fat 8%, carbohydrate 20%, ash 15%, fiber 1.5%, calcium 2.1%, and phosphorus 2.5%, vitamins and mineral 1%). Healthy, sexually mature broods were chosen for treatments purpose. Broods were detected by provision of their secondary sexual attributes. The mature male showed constricted abdomens with long projected genital papillae and female are easily spotted due to their however, the bulging abdomen and ringed urogenital papillae. The weight of the mature male and females were ranges from 240-320 g and 344-610 g, respectively.

Induced Breeding

Synthetic hormone S-GnRHa (Ovupin) were injected below the pectoral fin and above the lateral line of fish. Three different doses of S-GnRHa i.e., T1 (0.25 mL/kg BW), T2 (0.5 mL/kg BW), and T3 (1.0 mL/kg BW) have been assigned to 10 pair of fishes in each with three replications and a sex ration of 1:1 maintained for all treatments. Following the hormone injection male and female fishes were held together in 2.1 m x 1.5 m x 1 m sized hapa to monitor their eggs release and spawning after 8-12 hours.

Determination of Ovulation Rate

The rate of ovulation was observed up-to for 12 hours of post injection and results were obtained by using below formula from Legendre (2000).

$$OR\ (\%) = \frac{\text{No.of fish ovulated}}{\text{Total no. of fish injected}} \text{ x } 100$$

information: OR = Ovulation rate

Estimation of Fecundity

The fecundity was calculated by using the formula adopted from Rahman & Samat (2020).

$$F = \frac{\text{No. of eggs in the sample}}{\text{Weight of the sample}} \text{ x Total egg weight}$$

information: F = Fecundity

Determination of Fertilization Rate

A small subsample of fertilized eggs from hatching jar was put on a dish measure fertilization rate to from hatching jar. Following the visual observation, fertilized eggs were identified and counted very well. The rate of fertilization was counted by using below formula.

$$FR\ (\%) = \frac{\text{No. of fertilized eggs}}{\text{No. of (fertilized} + \text{no. of unfertilized)}} \text{ x } 100$$

information: FR = Fertilization rate

Determination of Hatching Rate

It had taken approximate 20 to 24 hours for completion of hatching phase. A subsample was separated on a tray and amounts of the hatchings were identified and calculated by visual aid. The rate of hatching was subjected to calculate by formula (Unuma *et al*., 2004).

$$HR\ (\%) = \frac{\text{No. of eggs hatched}}{\text{Total no. of fertilized eggs}} \text{ x } 100$$

information: HR = Hatching rate

Observation of Embryonic Development Stages

The eggs were observed under microscope until hatched (20-24 h). Observation of embryonic stages were carried out using an OLYMPUS CX21 photo microscope as well as different embryonic development stages were captured by using a camera (Rigla-32, Optikam B3 Digital camara, Italy) attached to the mi-

croscope. By using "Image J" software diameter of different embryonic development stage was measured and analyzed.

Statistical Analysis

Raw data were treated and prepared on excel sheet and further analysis were conducted using IBM SPSS version 26. The one-way analysis of variance (ANOVA) was used to clarify the level of significance ($P < 0.05$) among all the treatments and further significant results ($P < 0.05$) of means were tested with Duncan's New Multiple Range Test (DMRT).

RESULTS AND DISCUSSIONS

It had been noticed very well that dosages of hormone strongly manipulated the percentage of eggs production and the control cluster did not ovulate in experiment. The similar trend in breeding induction were reported with Wova FH (Sarkar *et al*., 2005) and it's with the synthetic Ovaprim (Bhattacharyya & Homechaudhuri, 2009) for *Anabas testudineus*. Among treatments, T2 or 0.5 mL/kg dose received group showed maximum performance in respect of fertilization, hatching, survival rate compared to other hormone received groups (Table 1).

Again, in respect of fertilization rate T_2 group showed the highest (89.33%) followed by 79.33% and 72% in T_3 and T_1, respectively. The hatching rates were 73.58%, 79.5%, and 64.48% in T_1, T_2, and T_3, respectively. The lowest hatching rate was 64.48% in T_3 and highest hatching rate was 79.5% in T_2, respectively, and the difference among three doses were statistically significant ($P < 0.05$). The fecundity of *A. testudineus* was ranged from 47,227 to 77,561 during the study period (Table 2). The significantly highest fecundity was counted in May and the lowest in April (Table 2).

Amin *et al*. (2016) had reported successful induction of breeding in captive reared *A. testudines* with 2 mg/kg for male and 7 mg PG/kg for female with pituitary gland extract and ovaprim injection. A rate of fertilization 98 ± 6.7%, have been reported for same species while inducing with exogenous GnRHa (Mandal *et al*., 2016) and 90%-100% during inducing with 1.5 mL/kg ovaprim (Hasan *et al*., 2018; Kiran *et al*., 2013). Current success in hatching rate appeared to be quite low in contrasted previous research by Sarkar *et al*. (2005) and Mandal *et al*. (2016) who observed a high rate hatching at 90.5 ± 3.65%, 100%, and 99 ± 5.4%, respectively. However, relatively low hatching rate has been reported with injection of commercial SGnRH as 42% (Loh & Ting, 2015), 75% with synthetic hormone WOVA-FH (Behera *et al*., 2016) and 51% in natural condition (Muslimin *et al*., 2018). Such variation could arise due to differences in rearing temperature, water circulation, and quality of selected brood.

The value of fecundity is influenced by a number of issues like size, age, environmental conditions, stocking ration, and dietary constituents (Ganias, 2017; Helmizuryani *et al*., 2020; Tsadik & Bart, 2007). The present findings were differed from (Uddin *et al*., 2017) who reported that the absolute fecundity of Vietnamese koi, *A. testudineus* were 16,832-46,186 for 14-17 cm length and 66-89 g sized fish during his study period. He found the highest absolute fecundity in the month of July and lowest in April. However, Zalina *et al*. (2012) observed fecundity between 2,785 ± 411.9 and 4,851 ± 1,415 eggs/fish for 14-16 cm length and 41-56 g sized female while inducing with LHRHa. This might be due to different geographic, environmental condition, and induction of breeding by using synthetic hormone. Besides, rainfall, physico-chemical parameters of water, age, and size of brood fish might be differed from the present study.

Different Stages of Egg Development

The progress of eggs started with the establishment of blastodisc (Figure 1.A) at room temperature (26 ± 0.5°C). The fertilized ovum of *A. testudineus* were characterized by spherical form, pearl-like in

Table 1. Performance of different treatments of S-GnRHa on *A. testudineus*

Parameters	Treatments		
	T_1 (0.25 mL/kg BW)	T_2 (0.50 mL/kg BW)	T_3 (1.00 mL/kg BW)
Total length (cm)	26.4 ± 1.2	25.5 ± 0.4	25.7 ± 0.6
Body weight (g)	481.0 ± 30.51	475.3 ± 17.9	479.6 ± 8.5
Ovulation rate (%)	100	100	100
Fertilization rate (%)	72.0 ± 2.0^a	89.3 ± 3.2^b	79.3 ± 5.1^a
Hatching rate (%)	73.5 ± 1.8^b	79.5 ± 0.9^c	64.4 ± 4.6^a
Survival rate (%)	50.7 ± 2.31^b	67.0 ± 2.0^c	46.0 ± 1.0^a

Table 2. Fecundity of *A. testudineus* from April to June 2018

Parameters	Month		
	April	May	June
No. of fish examined	10	10	10
Total length (cm)	25.5 ± 1.62[a]	27.8 ± 0.91[b]	26.2 ± 1.70[a]
Body weight (g)	477.9 ± 40.29[a]	545.9 ± 31.81[b]	492.6 ± 42.28[a]
Fecundity	47,227 ± 3423[a]	77,561 ± 5,917[c]	65,295.7 ± 5,608[b]

exterior and equipped with a buoyancy feature. The mean size of egg was measured 77.6 ± 3.5 µm in diameter. The first cleavage appeared at roughly 18-25 min of fertilization (Figure 1.B) and finished around after 40 min to generate the first set of blastomeres (Figure 1.C), and then average diameter was 74.4 ± 4.1 µm. The second cleavage (4 cell stage) was noted at 1 h and 15 min (Figure 1.D-E) and average diameter was 78.3 ± 4.8 µm. third cleavage produced 8 blastomere cells within 1:30 h of post-fertilization and mean diameter was 69.7 ± 8.7 µm (Figure 1.F). Production of blastomere cells increased as time passed and reached 16, 32, 64, 128 cells within 1 h and 55 min, 2 h and 30 min, 2 h and 55 min and 3 h and 10 min respectively after fertilization and mean diameter were observed 79.8 ± 2.8 µm, 79.1 ± 1.30 µm, 81.1 ± 1.6 µm, and 79.1 ± 1.8 µm mm respectively (Figure 1.G, 1.H, and 1.I). The division of embryo remained continued and developed into ball of cells, termed as morula, at about 4 h after fertilization (Figure 1.J) and the blastocoel begins densely invaded by yolk sac growth.

The cell division pattern became poorly synchronized which make cell counting complicated (Figure 1.K). At about 5 h 50 min, the commencement of gastrulation was remarked by resettlement cells (Figure 1.L), and distinction of embryonic tissue started by the end of this phase and three discrete layers were clearly detected (Figure 1.M). The neurula stage developed to be appeared between at 7 h and 40 min – 9 h (Figure 1.N) and organogenesis were clearly noticed at 9 h 30 min (Figure 1.O). The appendage of the larvae was generated at 13 h of fertilization (Figure 1.P). The plasma transmission was distinguished, and the movement of the tail appendage was visibly detectable at 15 h. Larvae with definite head, body and tail surfaced between 17 h – 19 h (Figure 1.Q). The eyes and mouth were detectable but not entirely developed (Figure 1.R). The free moving larvae was appeared between 19 h – 22 h after fertilization (Figure 1.S). The larvae were containing oval yolk sac and some melanophores were spotted throughout the body, head, and somites.

Table 3. Time of different embryonic stages after fertilization in *A. testudineus*

Figure No.	Development stages	Egg size (µm) (mean ± SD)	Development time (h:min)
A	Fertilized egg	77.59 ± 3.50	0 min
B	Blastodisc formation	74.56 ± 5.94	18 –25 min
C	2 – cell	74.36 ± 4.14	30 – 40 min
D	4 – cell	78.30 ± 4.86	1 h and 15 min
E	8 – cell	79.76 ± 8.72	1 h and 30 min
F	16 – cell	79.88 ± 2.85	1 h 45 –1h 55min
G	32 – cell	79.12 ± 1.30	2 h and 30 min
H	64 – cell	81.17 ± 1.62	2 h and 55 min
I	128 – cell	79.12 ± 1.84	3 h and 10 min
J	Morula	77.59 ± 2.30	3 h and 10 min – 4 h
K	Blastula	75.88 ± 2.33	4 h – 5 h 40 h
L–M	Gastrula	74.65 ± 0.92	5 h and 50 min – 7 h and 40 min
N	Neurula	74.59 ± 1.90	7 h and 40 min – 9 h 25 min
O–R	Segmentation	73.00 ± 0.75	9 h and 30 min – 19 h
S	Newly hatched larvae	105.41 ± 3.73	19 h – 22 h

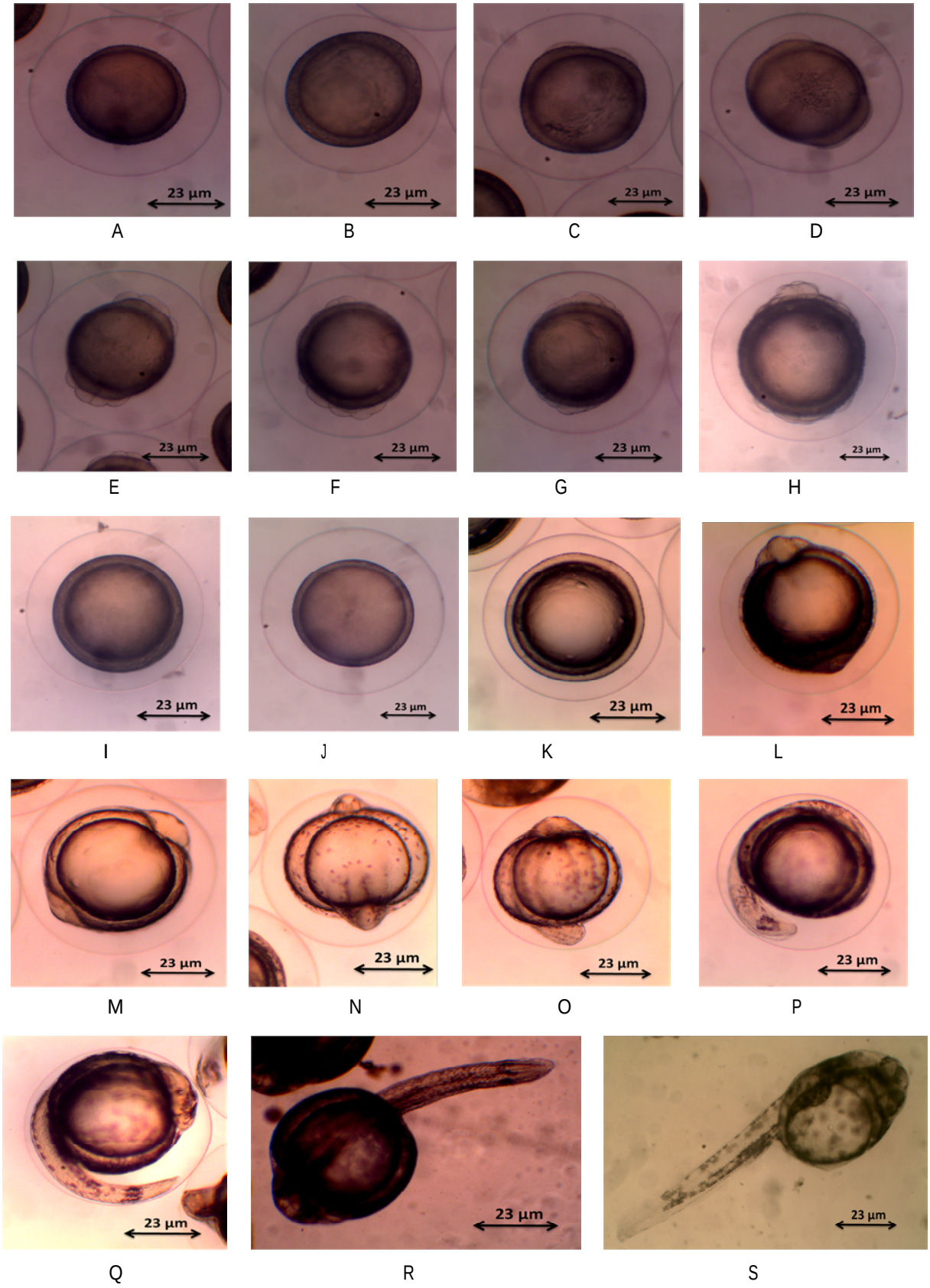

Figure 1. Developmental stages of *A. testudineus*. (a) fertilized egg, (b) blastodisc formation, (c) 2-cell stage, (d) 4-cell stage, (e) 8-cell stage, (f) 16-cell stage, (g) 32-cell stage, (h) 64-cell stage, (i) 128-cell stage, (j) morula, (k) blastula, (l) early gastrula, (m) late gastrula, (n) early neurula, (o)-(r) segmentation, (r) newly hatched larva, (s) 15 h old larva (148.24 µm).

The first cleavage phase had been initiated at almost 18 min after fertilization and finalized after 40 min of fertilization. The second cleavage phase was commented at 1 h and 15 min and the 8, 16, 32, 64, 128 multiplication phases commenced within 3 h and 10 min. The penetration blastoderm onto the yolk was completed after 7 h of spawning. Around 1-2 h prior to hatching, the embryo exhibit curving arrangements towards the egg envelops. The post spawning incubation cycle had lasted for 20 h at a water temperature of 26°C in current study. The newly hatched larva had been measured as 105.41 ± 3.73 µm in length in current research. A measure of 1.8 mm had been observed for newly hatched larvae of the same species (Zalina *et al*., 2012). Several previous noted considerable durations for embryonic development in fish and it depends upon rearing temperature and species as well.

CONCLUSIONS

The present study revealed that 0.5 mL/kg dose of S-GnRHa showed maximum performance in respect of fertilization and hatching rate, survival rate of *A. testudineus* which can be used for successful artificial breeding of this species. The comprehensive knowledge about the embryonic expansion of *A. testudineus* have been recorded in this investigation which will help hatchery owner for efficient and successful breeding.

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