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A study on induced breeding, embryonic and larval development of *Pangasianodon hypophthalmus* in semi-arid agro-climate

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Abstract

Aim : Breeding performance of *Pangasianodon hypophthalmus* in semi-arid agro-climatic conditions of northern India was evaluated in view of establishing hatchery to supply its seed.

Methodology : *P. hypophthalmus* were induced to breed using carp pituitary gland (PG) extract. Females were administered with priming dose of 2.5 - 3.0 mg kg⁻¹; after 6 hrs, a second resolving dose of 10-12 mg kg⁻¹ was given, while males were administered with single dose of 2.5-3.0 mg kg⁻¹ body weight. Eggs were dry fertilized after 14 hrs of second injection. The fertilized eggs were allowed to incubate in a circular hatchery maintaining a water flow of 30 l min⁻¹. Embryonic and larval developments were assessed by observing eggs at regular interval under a microscope. Optimum water quality parameters were maintained during entire experiment period.

Results : The average fecundity was 57.5 lakh kg⁻¹ of body while fertilization and hatching percentage was 76.50 and 58.20, respectively. After fertilization, eggs became water hardened within 20 min. "Blastodisc stage" appeared after 00:25 hr and "Morula stage" after 03:45 hr of fertilization. "Blastula stage" was observed after 05:30 hr post fertilization. Twitching movement started 24:00 - 26:00 hr post fertilization and hatching started at 34:00-36:00 hr post fertilization. A newly hatched larva measured about 3.80 mm in length with head slightly bent and compact yellowish yolk sac. During the age of 5-10 days of life, pectoral, caudal, ventral and dorsal fins appeared.

Interpretation : Stripped catfish *Pangasianodon hypophthalmus* can be successfully and commercially bred in semi-arid agro-climatic conditions of northern India under controlled environmental conditions using PG as inducing agent. However, females were not observed to ovulate naturally but dry fertilization technique.

Breeding, embryonic and larval development of Asian striped catfish, *Pangasianodon hypophthalmus*

Introduction

Asian striped catfish *Pangasianodon hypophthalmus* is native of Mekong basin of Indochina (Da et al., 2011). It is now recognized as a superior aquaculture species for tropical regions, as well as a major food fish in world market because of its white fine grained sweet flesh (Pal, 2010). It has been introduced for cultivation in different countries of the world (Singapore, Philippines, Taiwan, Malaysia, China, Myanmar, Bangladesh, Nepal and India) because of its high growth, easy culture practice, high disease resistance and high resistance to crowding and low oxygen (Bui et al., 2010; Ahmed et al., 2013; Griffith et al., 2010) hence there is a need to standardize the culture and breeding practices as per the new environment conditions (Begum et al., 2012a). In India it was first introduced in the state of West Bengal through Bangladesh during 1997 (Mukai, 2011). Culture of this species is growing day-by-day in India (Lakra and Singh, 2010; Singh and Lakra, 2012; Kumar et al., 2013). It is extensively being cultured in Andhra Pradesh and West Bengal (Moses et al., 2016). The culture of this species was earlier prohibited in India but now the Government of India has permitted its culture since 2010-2011 (Moses et al., 2016). Fish is cultivated both under monoculture as well as polyculture with Indian major carp. Monoculture of *P. hypophthalmus* fetches better performance and profit to farmers. In India, culture production of *P. hypophthalmus* is 15-20 ton ha⁻¹ yr⁻¹ which is higher than carp production (8-10 ton ha⁻¹ yr⁻¹) in the same area (Singh and Lakra, 2012).

P. hypophthalmus is highly fecund, seasonal spawner and breeds once a year in flooded rivers (Moses et al., 2016). Females fish attain maturity at the end of third year while male mature in two years (Griffith et al., 2010; Vidthayanon and Hogan, 2013). People in Northern India including Punjab, generally prefer fish with less intramuscular spines, hence Pangas catfish has a great potential in diversification as well as vertical and horizontal expansion of aquaculture sectors in this region. For the culture of Pangas at present fish farmers are entirely dependent on other states (mainly West Bengal) for the availability of seed. Transportation of seed increase the risk in the form of high mortality percentage caused due to change in agro-climatic conditions. Moreover, severe cold in northern India restricts the culture of this species in outdoor ponds from April to October only. Thus, brood stock management for seed production requires more attention and special care. Maintaining brood stock in polyhouse during winter season is one of the major practices to save the brood stock for seed production in coming breeding season. Therefore, in view of the increasing aquaculture importance of *P. hypophthalmus* in India, attempts were made to breed the fish under agro-climatic conditions of Punjab for the first time under controlled conditions along with the study on embryonic and larval development.

Materials and Methods

Experimental setup : The study was conducted in the Fish Farm of College of Fisheries, Guru Angad Dev Veterinary and Animal

Sciences University (GADVASU), Ludhiana. The juveniles of *P. hypophthalmus* were procured from Kolkata, West Bengal and raised in the earthen pond of 600 m² size during summer (April to November) and in the poly-houses (4 in number with size of 80 m² each and equipped with electrical heating system) during winter (December to March) for three years 2014-17. Floating feed (28.0% crude protein and 4.5% crude fat on dry weight basis) was formulated with the help of extruder using agro-industrial by-products i.e., rice bran (30%), de-oiled ground nut (30%), de-oiled soybean (25%), fish meal (13%) and commercially available multivitamin and multi-mineral mixture (2%). Fish were fed @ 2% of body weight (two meals equally distributed at 9.00 am, 5.00 pm). Total six numbers of breeding trials were conducted during the month of June to August, 2017. Three-year-old female (1500–2500 g) and male (1200–2000 g) reared in fish farm, College of Fisheries, GADVASU were selected for breeding. Before breeding, mature females were distinguished by their big, soft, distended belly with reddish, swollen ventral genital pore for the present study, while males were identified by their genital papilla which oozes milt with slight pressure on the abdomen. Fish were kept for one day before induced breeding in two different circular cement tanks (3.0 media and 0.75 m depth) for male and female fish with continuous showering for conditioning.

Induced breeding : Pituitary glands of Indian major carps preserved in absolute alcohol (100%) and stored in the refrigerator (at 4°C) were used for extract preparation. At the time of breeding, females were administered an intramuscular priming dose of 2.5 - 3.0 mg kg⁻¹ b.wt. of the pituitary extract (prepared in laboratory). After 6 hrs, a second resolving dose of 10-12 mg kg⁻¹ of b.wt. was given. At the same time, single doses (2.5-3.0 mg kg⁻¹ of b.wt.) of pituitary extract were given to males. Pituitary extract was administered intramuscularly in the region of caudal peduncle above the lateral line of fish. A 2 ml syringe graduated to 0.1 ml division with 22 number needles were used for injection. Immediately after injection male and female fish were kept in separate tanks under continuous showering. Females were found ready for stripping about 12-17 hrs of second injection.

The ready-to-spawn females were taken out and their eggs were stripped on a dry plastic tray by gently pressing their bellies. The males were then stripped and their milt was mixed with eggs with the help of a clean feather and gentle shaking. Milt from one male was sufficient to fertilize the eggs of two to three females. The dry method of egg fertilization was followed. A small amount about 15 ml of distilled water was mixed and stirred for 1–2 min. After thorough mixing, the tray was rinsed with 100–150 ml of water to remove excess milt and mucus. Eggs of this catfish is sticky in nature, they were washed with clay mud water for 3-4 times before transferring it to Chinese circular hatchery. Egg incubation period varied from 36-40 hr at 27.30 ± 0.28°C.

Study of embryonic development : Important parameters of water quality such as water temperature, pH, dissolved oxygen, total alkalinity, hardness, ammonia-nitrogen (NH₃-N), nitrate nitrogen (NO₃-N), nitrite nitrogen (NO₂-N) and phosphate

phosphorous ($\text{PO}_4\text{-P}$) were analyzed following the standard methods (APHA, 2012). Parameters such as fertilization and hatching rate were calculated by the formula given by Gunasekara *et al.* (1996). Fulton's condition factor (K) was calculated according to equation given by Htun-Han (1978). Gonadosomatic Index was calculated by the formula given by Barber and Blake (2008). A few number of egg were collected in petri dish to calculate the rate of fertilization and embryonic development by using florescent microscope Leica DM 3000 LED model. Egg diameter, functional and morphological changes were assessed as per the procedure described by Ferosekhan *et al.* (2015). All the statistical analysis was performed by using software SPSS Ver. 16.

Results and Discussion

Water quality parameters of hatching pool such as temperature, pH and dissolved oxygen were ranged from 26.90-27.90°C, 7.01-7.14, 6.48-7.86 mg l^{-1} , respectively. Similar range of temperature, pH and dissolved oxygen was also recorded by Chand *et al.* (2011) for successful induced breeding of *P. hypophthalmus* where temperature, pH and dissolved oxygen ranged from 28.7–29.3°C, 7.2-7.4, 6.0-6.20 mg l^{-1} , respectively. Whereas, alkalinity and hardness of water ranged higher (286.0-360.0 and 296.0-324.0 mg l^{-1} , respectively) in the present study as compared with the result of Chand *et al.* (2011) (alkalinity : 71-78 mg l^{-1} and hardness 72-76 mg l^{-1} , respectively)

Moses *et al.* (2016) reported May to July as induced breeding season of Pangas in different parts of world such as Vietnam, Bangladesh and Thailand etc. In Punjab, mid of June to mid of August was found suitable for breeding. Delayed maturity

Table 1 : Parameters related with breeding of *P. hypophthalmus*

Hatching time after pituitary injection	12-17 hrs
Average fecundity	57.5 $\pm$ 0.0004 lakh kg^{-1}
Colour of normal egg	Creamish yellow
Colour of fertilized egg	Transparent
Colour of unfertilized egg	Opaque white
Fertilization rate	76.50 $\pm$ 0.60%
Hatching rate	58.20 $\pm$ 1.35%
Hatching time	34.00 $\pm$ 2.00 hr (at 27.33 $\pm$ 0.18°C)
Diameter of egg before fertilization	1.25 $\pm$ 0.03 mm
Diameter of egg after fertilization and water hardening	1.80 $\pm$ 0.05 mm

Values are mean $\pm$ SE

may be attributed to prolong winter and occurrence of late monsoon in Northern India. In general, egg incubation time varied 12-14 hrs (Ferosekhan *et al.*, 2015); however, in the present study it ranged between 12-17 hrs for final ovulation and release of gametes. This could be due to variation in maturity stages and agro-climatic condition. Parameters related with breeding of *P. hypophthalmus* are shown in Table 1. The average fecundity was 57.5 $\pm$ 0.0004 lakh kg^{-1} while fertilization percentage was 76.50 $\pm$ 0.60 kg^{-1} of b.wt., respectively. *P. hypophthalmus* spawns once a year. Fertilized eggs were sticky in nature, transparent, round and increased in diameter from 1.25 $\pm$ 0.03 to 1.80 $\pm$ 0.05 after fertilization and water hardening. Stickiness was removed by treating the eggs with clay mud water. Gonadosomatic index (GSI) of male and female *P. hypophthalmus* during breeding season (June-August) was recorded 7.024 and 10.08, respectively. Condition

Table 2 : Embryonic developmental stages of *P. hypophthalmus*

Stages	Time (hr : min)	Description
Fertilization	0.0 hr	Single cell at animal pole, perivitelline space small
Blastodisc formation	0:25 $\pm$ 0.01 hr	Polar body developed
Two cell	0:35 $\pm$ 0.02 hr	Blastodisc divided to form two cell i.e., first cleavage
Four cell	0:50 $\pm$ 0.04 hr	Second cleavage perpendicular to the 1 st cleavage plane, Four cell formed
Eight cell	1: 00 $\pm$ 0.05 hr	Third cleavage parallel to that of the 1 st cleavage plane, Eight cell formed
Sixteen cell	2:00 $\pm$ 0.08 hr	Fourth cleavage stage parallel to 2 nd cleavage plane, 16 cell formed
Thirty two cell	2:15 $\pm$ 0.10 hr	Fifth cleavage. Blastoderm divided into thirty two cell
Sixty four cell	2:30 $\pm$ 0.12 hr	Sixth cleavage. Sixty four cells in 2-3 layers formed
Morula	3:45 $\pm$ 0.15 hr	Blastodisc consisted of many blastomeres. Numerous cells with flower like appearance of animal pole
Blastula	5:30 $\pm$ 0.24 hr	Blastoderm spreading gradually lowered and flattened. Appearance of epiboly with formation of blastocoel
Gastrula	7:45 $\pm$ 0.30 hr	Blastoderm becomes flattened down onto the yolk sphere. Blastoderm covered almost 3/4th of yolk sac
'C' shape embryo	10.15 $\pm$ 0.50 hr	Embryo looked like letter 'C' over the yolk sphere. Head and tail become clear
Twitching movement	25.00 $\pm$ 1.00 hr	Tail become free from yolk sphere and beating movement to rupture the egg membrane
Hatching	34.00 $\pm$ 2.00 hr	Transparent larvae with straight body and becomes free swimming after a few moments of motionless state

Values are mean $\pm$ SE

Fig. 1 : Embryonic and larval development in *P. hypophthalmus*

Factor (K) ≥ 1 indicates general well-being of fish under the existing water quality. It also indicates health status and physiological activity. In the present study, K value calculated 1.051 ± 0.06 and 1.048 ± 0.15 in male and female fish, respectively indicating that culture environment was suitable for fish.

Average $58.20 \pm 1.35\%$ hatching was achieved under agro-climatic conditions of Punjab with pituitary extract. Successful breeding of *P. sutchi* using same hormone was also reported by Chattopadhyay and Mazumdar (2002) with 77- 90%

fertilization and 87-92% hatching rate, respectively. Relatively less hatching rate in the present study may be due to higher level of alkalinity and hardness in water ($286.0-360.0$ and $296.0-324.0$ mg l^{-1}) which might have deposited over the surface of the eggs restricting oxygen supply to the developing embryo. The developmental stage of eggs was achieved up to C-embryonic stage. The yolk material migrated towards vegetative pole with the progress of embryonic development to provide extra space for division of blastomere at the animal pole. Similar observations with respect to ovarian development was also reported by Moses

et al. (2016) who stated that fertilized eggs of *P. hypophthalmus* were sticky in nature and with the advancement of embryonic development; egg membrane got separate giving birth to the uniform perivitelline space. The yolk sphere migrated towards vegetative pole with further embryonic development.

Embryonic developmental stages of *P. hypophthalmus* is presented in Table 2 and Fig. 1. Cleavage in fertilized eggs occurred only in the blastodisc region. The cleavage was meroblastic type. Cytoplasm of the blastodisc became the embryo, thus meroblastic cleavage was discoidal (Harrison, 2011). About 20 minutes after fertilization, eggs became water hardened. Blastodisc formation started at $0:25 \pm 0.01$ hr (Fig. 1b). The 1st division took place in $0:35 \pm 0.02$ hr. minutes after fertilization (2-celled stage) and the 2nd division within 15 min thereafter (4-celled stage). The 3rd division takes place within 1hr of fertilization (8 celled stages). Sixteen (Fig. 1f), thirty two (Fig. 1g) and sixty four (figure 1 hr.) cell stage appeared at $2:00 \pm 0.08$, $2:15 \pm 0.10$ and $2:30 \pm 0.12$ hr, respectively.

Morula stage appeared at $3:45 \pm 0.15$ hr after fertilization with a ball of cells, clear polar projection and the convex base. The blastomere was divided into many cells at this stage and appeared as a compact mass of cells (Fig. 1i). Blastula stage was observed at $5:30 \pm 0.24$ hr which is characterized by formation of 'blastoderm' and 'periblast'. The embryo was developed from blastoderm, and periblast helped in channelization of yolk materials (Fig. 1j). Gastrulation was characterized by formation of three-layered structure (ectoderm, mesoderm and endoderm) known as gastrula (Harrison, 2011). Gastrula stage appeared at $7:45 \pm 0.30$ h (Fig. 1k) after fertilization. The "C" shaped stage was observed at 10.15 ± 0.50 hr. (Fig. 1l). At this stage, head and tail of the embryo was recognized and further progression of time of another 6-7 hr differentiation of fins, head and tail region were clear.

Time required for development of eggs from fertilization to C-embryonic stage varied. Ferosekhan *et al.* (2015) reported 'C' shape embryonic stage at 09:29 hr. Moses *et al.* (2016) reported the same stage about 12 hr after fertilization for *P. hypophthalmus*. In the present study, time required to reach 'C' shape embryonic stage was about 10.15 hr these differences may be due to variation in age, maturity stages of ova and agro-climatic conditions. Twitching and hatching stage was observed at 21.30 hr of post fertilization. The stage is characterized by yolk attached with head, beating of tail of embryo vigorously for a pan of 5-8 sec with a pause of 2-5 sec. Hatching started at 25.27 hr of post fertilization. Immediate by after hatching, the larvae remained at the bottom and remained motionless. After few moments, activeness of larvae was observed with increasing tail movement (Fig. 1m). Immediate after hatching, larvae measured about 3.80 mm characterized by bent head attached with yolk sac. Mouth was not visible though mouth cleft was visible after 15-17 hr. The ambiguity of mouth formation was also reported by Ferosekhan *et al.* (2015) in *Pangasius pangasius* (varied 12-13 hr) and by Adebisi *et al.* (2013) in *Hemibagrus nemurus* (immediate by after hatching). Eye rudiments, optic and auditory vesicles appeared

around 5.50-6.00 hr of hatching with increasing the size of embryo about 4.75 mm. Alimentary canal was formed about 12 hr of hatching. After 13-15 hrs of post-hatching, pigmentation in eye was recognized. Yolk sac gradually reduced in size and yolk was completely absorbed about 60 hrs after hatching. Islam (2005) observed gradual reduction of yolk sac till 4-7 days after those larvae were ready for accepting external feeding. In *P. sutchi*, first feeding started (diluted and filtered boiled egg yolk enriched with multivitamins) after 40 hrs of hatching. During this time, if enough food particles of suited size were not provided, the larvae did not accustom themselves to external food, became extremely cannibalistic within the next 24 hrs and died after sometime. Cannibalistic nature of young larvae was also reported in *Pangasius sutchi* by Chattopadhyay and Mazumdar (2002) and Islam (2005) whereas in *P. hypophthalmus* by Ferosekhan *et al.* (2015).

Boiled egg-yolk mixed with multivitamins was found suitable as starter feed at around 40 hr after hatching at 3-4 hr interval. Gradual administration of freshly filtered zooplankton along with diluted boiled egg-yolk at 3-4 hr intervals from 60-96 hr after hatching revealed better growth of larvae. Diluted boiled chicken liver enriched with multivitamins were provided four days onward till 12-14 days. Chattopadhyay and Mazumdar (2002) reported lactogen as starter feed (48 hrs after hatching) followed by earthworm dust upto 5-8 days and soybean dust after 10 days onwards as larval feed of *P. sutchi*. Hossain and Rahman (2014) reported boiled fish flesh paste enriched with multivitamins as less preferred starter feed, but diluted boiled egg-yolk was acceptable and helped the larvae to accustom with external feeding habit at around 30 hr after hatching in *P. sutchi*. Administration of a mixture of live but blended *Tubifex* and Artificial plankton *Rotifer* (APR) rather than blended *Tubifex* alone at 3-4 hr intervals during 36-40 hr after hatching was found better with negligible amount of larval mortality. Therefore, it can be concluded that variability in larval feed may be adjusted as per the feed acceptability of larvae and availability of larval feed in particular region and at the same time, time of first acceptance of feed by newly hatched larvae should be observed critically to reduce the mortality due to cannibalism. Successful culture and brood stock development of *P. hypophthalmus* through overwintering under polyhouse condition and successful induced breeding trials in Punjab clearly indicate potentiality of this species for productivity enhancement, as well as vertical and horizontal expansion of aquaculture sector in northern states of India.

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