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RESEARCH **P**APER

Artificial breeding of freshwater catfish *Heteropneustes fossilis* (Bloch, 1794) using synthetic hormone

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The objective of the present study was to study the induced breeding technique and embryonic development of *Heteropneustes fossilis* (Bloch, 1794). The female brooders were injected intramuscularly with hCGhormone (6.95 IU/g dose of body mas). After 12 ± 2 h of latency period, the fertilized eggs were obtained by artificial insemination. The fertilized eggs were demersal, non-adhesive, spherical, brownish green in colour with red cap or blastidosc. The average diameter of the fertilized eggs was 1.5±0.5mm. The different stages of embryonic development were captured under bright field microscope (Olympus Cx 41) using micro publisher 3.3 RTV camera (Qimaging, BC, Canada). The mean fertilization and hatching rate was 98.7±0.5 % and 98.3±2.5 %, respectively. The complete yolk was consumed within 3 days after hatching. The hatching period was 23-24 hr. The newly hatched larvae were 3.0 ±0.2 mm in length.These observations showed that catfish can be bred successfully and this study would help in managing the induced breeding programmes of *H. fossilis*.

Key words : *Heteropneustes fossilis*, hCG, Fertilized egg, Blastodisc, Hatching

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INTRODUCTION

Heteropneustes fossilis (Bloch, 1974) belongs to the family Heteropneustidae is a commercially important and most preferred fish for its taste and good price. This fish is well adapted to adverse ecological conditions in swamps, marshes and derelict. This fish species is considered as an ideal for aquaculture because of its fast growth, tolerance to high stocking densities, ability to survive in low-oxygenwaters, low fat, high protein and iron content and medicinal values (Haniffa and Sivasubbu, 2002). According to Dehadrai *et al.* (1985), this fish can be cultured with a production of 2.0 ton/ha/year. But, the non-availability of sufficient quantity of seed has remained the biggest constraint in expansion of *H. fossilis* aquaculture.

During the past few decades, the induced breeding of catfish started with the use of pituitary gland extract (Kohli and Vidhyarthi, 1990 and Chaturvedi *et al.*, 2001 and 2002). However, the increasing cost of donor pituitary and cumbersome process obliged experts to test alternative hormones for breeding (Zairin and Furukawa, 1992; Fermin, 1992 and Haniffa *et al.*, 1996). Along with, the natural population of this catfish has been rapidly decreasing due to various man-made and natural causes. Khan (1972a and b); Thakur *et al.* (1974 and 1977) and Saha (1998) provided some basic information on the induced breeding of *H. fossilis* with a view to achieve success in mass production of seeds.

Embryogenesis is a basic phenomenon which helps in improving the artificial production of any fish species and also improves their growth rate, culture practice, understand the species adaptations and improve artificial breeding techniques. It will help in understanding the

biology of the species and functional trends and environmental preferences of the different developmental stages (Koumoundouros *et al.,* 2001 and Borcato *et al.,* 2004). The study of embryogenesis of *H. fossilis* is limited except Haniffa and Sivasubbu (2002); Thakur*et al.* (1974) and Kohli and Vidyarthi (1990). However, information on similar catfishes like *Channapunctatus* (Banerji, 1974), *Clariasbatrachus* (Thakur *et al.,* 1974), *Channa stratus* (Marimuthu and Haniffa, 2007) and *Clarias gariepinus* (Osman *et al.,* 2008) are available. Therefore, this attempt has been made to standardize the seed production technology of *H. fossilis* by using synthetic hormone drug (hCG) and to study embryogenesis with the aim of improving their artificial propagation.

RESEARCH METHODOLOGY

Chemicals:

Human chorionic gonadotropin (hCG) was obtained from the local medicinal shop of Lucknow, Uttar Pradesh, India, under the trade name FertiGyn HP 5000 marketed by Sun Pharma Laboratory Limited (Mumbai, India). All other chemicals used for this study were obtained from Merck (Kenilworth, NJ, U.S.A) by local scientific suppliers of Lucknow.

Animal collection and their acclimatization :

The experiment was performed in accordance with local/ national guidelines of ethical committee for experimentation in animals to avoid any type of cruelty.

The healthy brooders of fresh water catfish *H. fossilis* of relatively same length (17±2 cm) and weight (185±20 g) were collected from commercial fisherman. Fish were treated with 0.05 per cent $KMnO_4$ solutions to remove the dermal infection if any. They were acclimatized in 120 l glass aquaria containing water having a pH of ≈ 7.5, dissolved oxygen 5-6 mg/l and a temperature of 24±1°C for one week. Water was renewed daily to remove faecal matter and waste metabolite of fish during acclimatization. During this period, fishes were fed regularly with commercial fish food pellets.

Induced breeding :

For this, five male and five female fish were selected based on the external morphological features. The female fish were artificially induced by Hcghormone (6.95 IU/g dose of body mass) intramuscularly near dorsal fin above the lateral line. Experiment was conducted in five replicates.After inducing, the fish were kept in the same breeding pool. Approximately 12±2 h after the injection, eggs obtained by applying gentle pressure on the female's abdomens were put into fertilization trays and then artificially inseminated with a sperm suspension that was previously obtained by mincing of adult male testes in 0.4 per cent fish saline. Successful fertilization was achieved when the eggs were oriented with the red cap animal pole side up. Thereafter, within a one minute the screening of unfertilized eggs was performed.The fertilized eggs transferred into rectangular hatching trays while taking precaution to avoid damage and fungal/ bacterial contamination during the egg collection process. A continuous flow of water was maintained for aeration to ensure the environmental conditions were optimal for the hatching process.

Data collection:

At the end of exposure time (upto yolk deposition), the number of dead embryos was calculated. For the morphological examination of embryo and hatchlings, the images were captured under bright field microscope (Olympus C x 41) using micropublisher 3.3 RTV camera (Qimaging, BC, Canada) per 24 h interval.

RESEARCH FINDINGS AND ANALYSIS

The latency period of HCG induced catfish *H. fossilis* was 12 ± 2 hr. The mean fertilization rate was 98.7±0.5 % and mean hatching rate was 98.3±2.5 % (Table 1). The fertilized eggs were green with red cap (blastodisc), translucent, round and moderately sticky with a mean diameter of 1.5±0.5mm. The eggs were surrounded by jelly and oily chorion wall (Fig. 1). The chorion wall swelled up and lifted up from the newly fertilized egg (Fig. 1).There was accumulation of cytoplasm at the anterior part to form the animal pole (blastodisc) where all subsequent cell divisions occurred

Table 1: Mean fertilization rate, viability, survival and hatchability of freshwater catfish *Heteropneustes fossilis* (Bloch, 1794)

Observations	Control
Fertilization rate	98.7±0.5
Number of dead embryo at 24 h	3.7±2.1
Hatching rate (%)	98.3±2.5
Survival rate of hatchlings at 24 hr and 48 hr	100

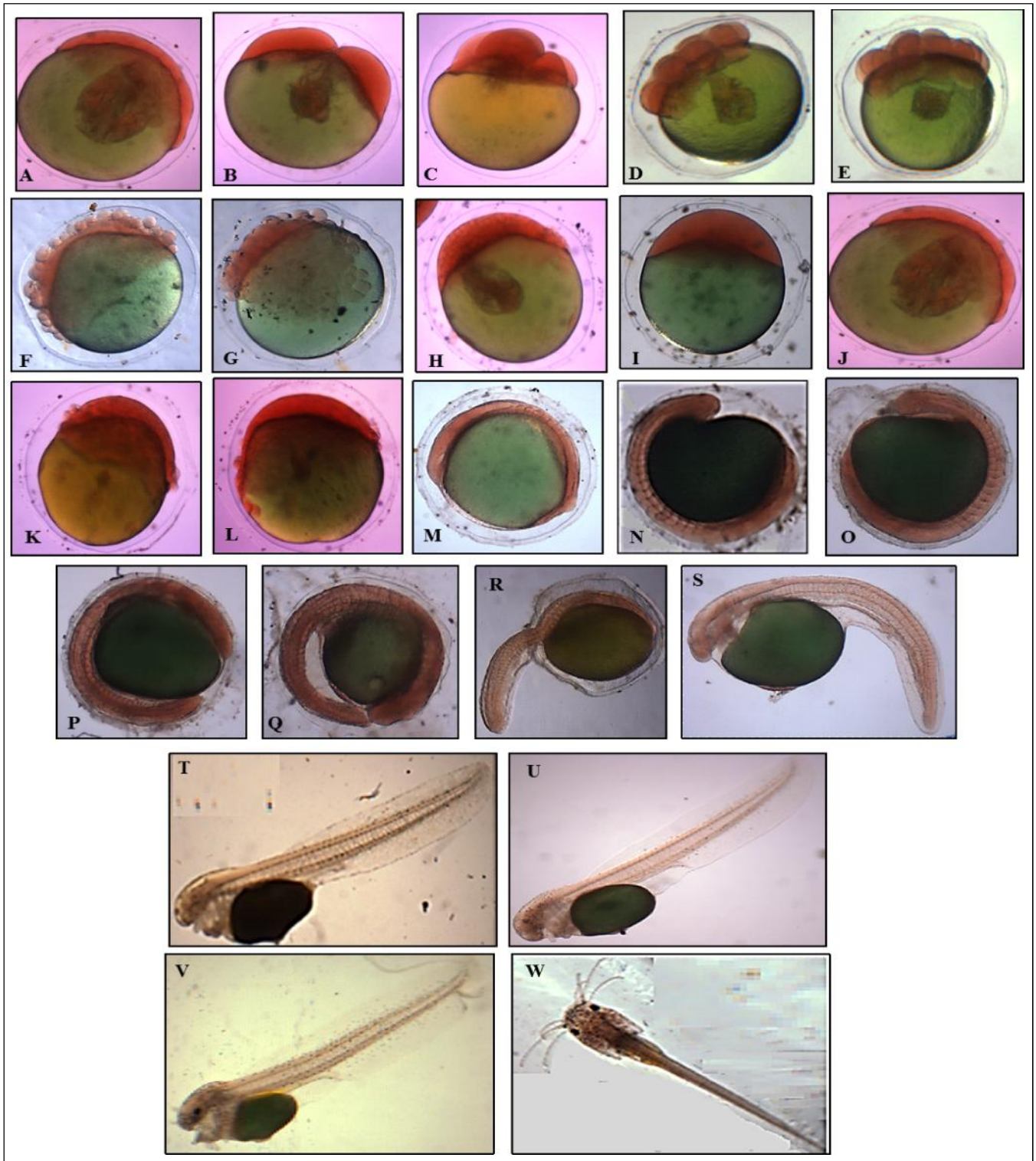

Fig. 1: Stages of embryonic development in *Heteropneustes fossilis* from fertilized egg to fry development. Photographs showing (A): fertilized egg, (B): 2-celled stage, (C): 4-celled stage, (D): 8-celled stage, (E): 16-celled stage, (F): 32-celled stage, (G): 64-celled stage, (H): Morula stage, (I): blastula stage, (J): Gastrula stage, (K): Embryonic shield, (L): 70% epiboly, (M): somite formation:6-somites , (N):15-somites, (O):17-somites, (P): 20-somites, (Q): 25-somites, (R):tail emergence, (S): newly hatched larva, (T): one day old larva, (U): two days old larva, (V): three days old larva, (W): Fry

and nutritive yolk at the posterior part to form the vegetal pole (Fig. 1A). The reddish spot (blastodisc) can be easily seen by naked eye.

Embryo formation*:*

The first cleavage (2-celled stage) was started within 15-18 min post-fertilization and divides blastodisc into two blatomeres (Table 2, Fig. 1A). The segmentation was typically meroblastic. The second cleavage (4-celled stage) appeared 30-35 min after fertilization, which was perpendicular to the first line of cleavage. The 8-celledstage was noticed within 75 min post-fertilization. The 16 and 32-celled stage were noticed at 85 and 120 min. after fertilization. At this stage, the blastomeres were difficult to count as all consecutive divisions led to reduction in cell size. As successive cleavages occurred, the blastomeres were decreased in size and some blastomeres cover each other and making counting of individual cells impossible that called morula stage. Morula stage with elevated rhomboid multicellular blastodisc was reached between 2-2.30 h post fertilization.The blastoderm start spreading over yolk in the form of thin layer, and anterior and posterior ends of embryo differentiated. After 3.30-4 h of fertilization, the blastula stage was observed. The embryonic shield appeared within the next 2 h and by that time more than half a portion of the yolk was invaded and the head and tail ends of the embryo became clearly distinguishable. The gastrula stage was formed after 6.30 h of fertilization, at this stage, formation of germinal ring was occurred. In another 30 min, the yolk invasion was completed and the blastopore was almost closed.

Embryo differentiation:

After 8-8.30 h post-fertilization, the antero-posterior axis was distinguishable, cephalic portion being broader and embryonic rudiment became distinct with 2 somites. The anterior protuberance formed a head fold and the posterior part elongated to form the tail fold. About 6-8 somites were formed after 11 h. In about 12-14 h from fertilization, the number of mesodermal somites gradually increased from 8 to 12 and pigmentation was noticed in the somites and also, the notochord was more clearly seen and the fore, mid and hind brain regions were noticed. At 16 h the embryo occupied the whole space inside the egg, the mesodermal somites ranged from 12-15 in numbers, blood circulation was observed, Ectodermal thickening to form the lens of the eye was indicated, the caudal tail region started to detach from the yolk mass, the embryonic fin fold appears. In the 20 h old embryo, wriggling movement was started in the embryo, 18-20 somites were observed. The embryonic fin fold on the ventral side extended upto the 11th somite. The eye lens was fully formed in the eye and the olfactory placode was indicated. Blood circulation commenced over the yolk into the rudimentary heart lying anterior to the yolk sac. In the 22 h old embryos, the somites number

Table 2: Embryonic development and organogenesis of freshwater catfish *Heteropneustes fossilis* (Bloch, 1794)

Time	Development stages	Description
16±2 hr	Latency period	Ovulation time
30-40 sec	Fertilization or blastodisc formation	Eggs were adhesive, spherical, transparent and greenish in colour and red cap formation
15-18 min	2 celled stage	First cleavage
30-35 min	4 celled stage	Second cleavage
75 min	8 celled stage	Third cleavage
85 min	16 celled stage	Fourth cleavage
120 min	32 celled stage	Fifth cleavage
2-2.30 hr	Morula stage	Blastulation progresses to form a multicellular blastodisc
3.30-4 hr	Blastula stage	A Third of egg space covered by blastoderm cells
6.30 hr	Gastrula stage	Formation of germinal ring
7hr	Yolk plug stage	Yolk invasion complete and cephalic region get thicker in size
20 hr	Advanced gastrula	Formation of 20 somites and wriggling movement started
22hr	Fully active embryo	The egg membrane becomes decomposed
23-24 hr	Hatching	Hatching take place

increased to 22-25 and the yolk was completed encircled by the embryo and the tail end was free from the first 2 somites (Fig. 1R). The olfactory pits and auditory vesicles were prominently visible. The melanophores appeared scattered above the neural chord over the trunk and caudal regions. The heartbeat ranged from 170 to 175 per min and the embryo begins to twist itself, continuously beating against the inside of the eggshell by the caudal region, especially around the middle part of the body. Hatching took place about 23-24 h after fertilization (Fig. 1S). Before hatching, frequent embryonic twitching movements were observed as the embryo tries to rupture the perivitelline membrane. The egg membrane (chorion wall) was broken down from the caudal regionand the hatchling emerged from the egg capsule. The tail was emerged first followed by trunk region. Between this time and the time of hatching, several morphological structures and organs were formed. Body segmentation was completed. Yolk size reduction started few hours after hatching revealing that the embryos have started endogenous feeding which lasted for three days (Fig. 1).

Development of hatchlings:

The newly hatched larvae were transparent and 3.0 ±0.2 mm in length with a laterally compressed body. The hatchlings had un-pigmented eyes and were devoid of distinct mouths and fins. Since the head was very small, it was not distinctly separated from the yolksac. The yolk sac was pale-green and oval in shape with 0.76 mm in diameter. A functional heart with blood circulation was seen. The fin folds were differentiated after 2 to 3 h post hatching. It surrounded the caudal region and extended up to the yolk sac (Fig. 1). The anal fin fold around the tail was continuous. After 8 h of hatching, the hatchling displayed a dorso-ventral unpaired fin and melanophores appeared on the head region, ventral side of the notochord and dorsal side of the body. Internal body organs, like the heart and brain, were clearly distinct. After 24 h, yolk sac was reduced. Dark pigmented and prominent eyespot appeared on the anterior part of the head. The mouth and anal was not yet opened at this stage. The upper and lower jaws were formed. The pectoral fin buds were seen as a small protuberance. The alimentary tract was distinct. The heart was seen in front of the yolk and the blood circulatory system was fully functional. Pigmentation extended to the yolksac both dorsally and ventrally. Barbels appeared in the form of tiny knobs in 1 day old hatchlings. Eyes were dark pigmented and spherical in shape after 36 h old hatchlings. The pectoral fin was oval shaped with a membranous flap and actively used for free movement. The heart was distinctly visible, located behind the head and showed regular beats. The mouth was formed as a terminal opening. Rudimentary gill openings and olfactory pits were differentiated and the yolk reserve was further diminished.

The hatchlings attained 4.8 ±0.2 mm length after 48 h post hatching. The eyeball was dark and prominent, the mouth cleft was well formed with a well-developed lower jaw. The barbels became elongated and prominent around the mouth and the yolk reserve had further reduced in size. The pectoral fin had become paddle shaped. The anal opening and opercula were well formed and distinct. The membranous fin fold surrounds the entire area from behind the head region. Blood circulation was observed in the opercular, heart and tail region. The alimentary canal became short, straight and distinct.

After 72 h post hatching, hatchlings were an average length of 5.5 ±0.2 mm. The body is brownish in colour and the mouth and anus have become fully functional. The size of the stomach is larger than the intestine and the intestine was convoluted ventrally to the stomach. The pectorals had been vascularized with a distinct circular vessel. The head was prominent. Well-developed barbels were noticed at this stage and the size of the barbels had slightly increased from the previous stage. The eyes are further differentiated and pigmentation has appeared around the head and snout. The reserved yolk material has been completely absorbed. Pigmentation were more concentrated in the anterior region, however, the density had decreased gradually. The hatchlings showed vigorous movements.

H. fossilis has an excellent culture potential. The knowledge of early development or embryogenesis is required for expanding its culture practices. Few studies have been reported for its embryogenesis (Thakur *et al.*, 1974; Kohli and Vidyarthi, 1990, Puvaneswari *et al.,* 2009 and Olufeagba *et al.*, 2015). Therefore, the present study was conducted to provide detailed information about the embryonic development of *H. fossilis*. The latency period in this study was 12±2 h reported which was earlier as compared with 18 to 24 hours and 21 to 24 hrs reported for the same species by Haniffa and Sivasubbu (2002) and Roy and Pal (2006), respectively. The recorded mean fertilization rate (98%) was higher than Haniffa and Sivasubbu (2002) (70 to 75%) and Roy and Pal (2006) (93%) for the same species. All the embryonic stages

occurred inside the chorion layer. The egg membrane has a small peri-vitelline space which is filled with fluids which may protect the eggs and the embryo from any external injury (Khan, 1972a). The fertilized eggs of *H. fossilis* became adhesive, representing an adaptation to prevent the eggs flowing in the water currents and provide optimal oxygen supply. Similar finding was observed by Kamler *et al*. (1994); Arockiaraj *et al*. (2003); Islam (2005) and Osman *et al.* (2008), in other catfish. In this spp., the cleavage was discoidal meroblastic, where large yolk restricts cell division at the animal pole (Hall *et al.,* 2004). Same pattern of cleavage was reported by other researchers (Banerji,1974; Thakur *et al.,* 1974; Kohli and Vidyarthi,1990; Kamler *et al.,* 1994; Freund *et al.*, 1995; Kimmel *et al.*, 1995; Arockiaraj *et al.,* 2003; Islam, 2005; Marimuthu and Haniffa, 2007 and Osman *et al.*, 2008). The timing of cleavage in most catfish is also relatively similar as observed by Osman *et al.* (2008) in *Clarias gariepinus*, Marimuthu and Haniffa (2007) in *Channastriatus*. In the present study, the first cleavage occurred within 15-18 min and the 16-cell stage and morula stage was reached in 85 min and 2-2.30 of post-fertilization. Same findings were investigated by Puvaneswari *et al.* (2009) and Olufeagba *et al.* (2015). According to Thakur *et al.* (1974), the first cleavage, 16 cell and morula stages in *H. fossilis* were occurred within 30, 70-80 and 100 min after fertilization. In *Misgurnus mizolepis*, the first cleavage was appeared in 50 min, the 16 cell stage in 1.35 h and the morula stage in 2.20 h of post-fertilization (Kim *et al.,* 1987). In *C. punctatus*, eggs reached the 16-cell stage in 45 min after fertilization (Banerji, 1974). The morula stage in Indian major carps was known to be reached within 2-3.5 h after fertilization (Chondar, 1994). Just 1-2 h before hatching, the embryo of *H. fossilis* showed twisting movements inside the egg envelops. The similar hatching behaviour and early development process was reported in the same fish by Thakur *et al.* (1974), and commonly observed in other catfish species, *C. batrachus* (Thakur, 1980) and *P. sutchi* (Islam, 2005) *C. gariepinus* (Zaki and Abdula, 1983) and *M. montanus* (Arockiaraj *et al.,* 2003).

The mean hatching rate was 95 per cent which was slightly below than Olufeagba *et al.* (2015) result and higher than 60 to 60.5 reported for same species by Haniffa and Sivasubbu (2002). The hatching was occurred at 24 h after fertilization. Same investigation was reported by Freund *et al.* (1995) and Kamler *et al.* (1994) in *H. longifilis* and *C. gariepinus*, respectively. Kohli and Vidyarthi (1990) reported that in *H. fossilis* hatching was occurred at 16-18 h. In *Mystus punctatuts*, the hatching period was 18-24 h (Ramanathan *et al.,* 1985). Banerji (1974) and Marimuthu and Haniffa (2007) found that the hatching of *C. punctatus* and *C. striatus* took place in 24 h.

In the present observation, the newly hatched larva was 3.0±0.2 mm in length which was different from result of Olufeagba *et al*. (2015) reported 2.5mm length. According to Parameshwaran and Kamal (1988), the length of newly hatched larva was 3.88-4.47 mm in *C. marulius*, 2.81-3.22 mm in *C. striatus* and 2.49-2.70 mm in *C. punctatus*, respectively. Ogunji and Rahe (1999) recorded the length of newly hatched larvae of *H. longifilis* to be 4.09-4.9 mm. Mookerjee and Mazumdar (1950) reported a mean length of 5.8 mm in *C. batrachus*, while Bruton (1979) recorded 3.6 mm in *C. gariepinus*. Marimuthu and Haniffa (2007) reported 3.4 mm length of newly hatched *Channastriatus.* It is a known fact that the age and size of broodstock affect the size of eggs and subsequently the size of hatchling. This opinion was established in teleosts by Bagarinao and Chua (1986). This may be the factor majorly responsible for the variation in the mean values of the different authors. In this result, the mouth was open at 36 h after hatching. Ogunji and Rahe (1999) reported the mouth opening at 3-4 h after post-hatching in *H. longifils*. The complete yolk sac absorption of *H. fossilis* was observed on the third day when the larvae measured an average length of 5.5±0.2 mm. The complete disappearance of the yolk materials was observed on the onset of 72 h after hatching. Same results were found in *Clariaslazera* (Pan and Zheng, 1987), in *C. fuscus* (Pan and Zheng, 1982), *C. striatus* (Alikunhi, 1953) and in *Mystus macropterus* (Bleeker) (Wang *et al.,* 1992). In *H. longifilis*, the yolk sac was found to be fully reabsorbed at 55 h after hatching (Ogunji and Rahe, 1999). The culture of the catfish species was not in practices because of the lack of breeding and feeding techniques and also non-availability of seeds from the wild. This study suggests that it is a suitable species for small scale and commercial aquaculture.

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