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Early ontogeny of the Asian catfish Magur, *Clarias batrachus* (Linnaeus, 1758)

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Abstract
The Asian catfish, *Clarias batrachus* (Linnaeus, 1758), known as magur, is a popular food fish of Asian countries for its medicinal value. The major constrain behind culturing this species is scanty knowledge on larval rearing and fourth day mortality syndrome. For mass production of *C. batrachus*, identification of significant developmental events is essential. Here early and post-embryonic stages of *C. batrachus* were described through microphotographs. The first mitotic cleavage occurred at 62 minutes post-fertilization resulting in 4, 8, 16, 32, 64 blastomeres, followed by development of morula, blastula and gastrula stages. The first larva (Total Length 4.5 ± 0.5 mm) emerged after 26 hours at 26 ± 0.5°C. At fourth day post hatching, the larvae (Total Length 9.2 ± 1 mm) commenced fully on exogenous feeding. This study specified the ontogenic changes and commencement of exogenous feeding in *C. batrachus* which would lead to successful larval rearing for mass production.

Keywords: Asian catfish, early life stages, embryonic development, hatching

1. Introduction
The Asian catfish *Clarias batrachus* (Linnaeus, 1758), popularly known as Magur is a potential aquaculture candidate in Asian countries like Thailand, Philippines, Cambodia, Myanmar, China and India for its high growth rate, efficient food conversion and excellent nutritional profile with high consumer preference [1-3]. Out of a total of 33 species of *Clarias*, only seven are found in the Indian sub-continent. Various aspects of the alimentary system, digestive physiologies, foods and feeding habits of *Clarias sp* have been studied in detail [4]. The endocrine regulatory pathways involving reproductive physiology [5] and culture of *C. batrachus* in wild or controlled environment [6] have been described extensively. In search of an effective and economical inducing agent of spawning, arificial spermiation, ovulation and maturation of gamates in *C.batrachus*, a number of modulators & methods have been scanned by several workers [7, 8]. Information is also available on nursery rearing and larval feed management to improve the breeding performance of *Clarias sp* [9]. However, natural population of *C. batrachus* is critically endangered mainly due to intermittent periods of drought and devastation of the natural habitat coupled with uncontrolled introduction of alien species such as *C. gariepinus* [10]. In addition, over-exploitation, reduction in the habitat area, excessive use of pesticides, herbicides and inorganic fertilizers in agricultural farms are also important for depletion of natural stock [10, 11]. Thus large scale captive breeding is essential for conservation of this species for which a fundamental pre-requisite is availability of quality fish seeds (fingerlings). The wild seeds are highly depleted due to factors like overexploitation and pesticidal use in agro ecosystems surrounding aquatic habitats. The uncertainty of captive breeding, poor quality seeds and inconsistent supply of wild seeds are the main bottlenecks for culturing this species [12]. Previously, several works have been done on the spawning behavior [4] and spawning performance [13] of this species to improve breeding performance. However, no detailed study has been performed on the larval development and organogenesis of this species, which is crucial for nursery rearing and feed designing. Chronological organogenesis involves understanding the early embryological and larval development of any culturable species. It gives an insight into incubation temperature and hatching time until complete yolk absorption followed by opening of mouth gape in the developing embryo. Thus, the present study aims to investigate and describe the developmental stages of *Clarias batrachus* during

early ontogeny as a chronological event.

2. Materials and Methods

Healthy and gravid brood fishes (Avg. weight; female 150± 10.5 gm, male 120± 8.5 gm) were collected during the breeding season (July-August) from a local fish farm of West Bengal, India. Fishes were stocked in a pond with a surface area of 0.10 to 0.13 ha and a water depth of 165 to 180 cm. The males were selected on the basis of pointed and reddish genital papilla, while females by a round and reddish papilla, softness of abdomen and uniform size of intra-ovarian oocytes. Artificial breeding was carried out administering Salmon GnRH analogue (APC Nutrients Pvt. Ltd.) intraperitoneally at a dose of 0.02 ml Kg^{-1} body weight of female and 0.15 ml Kg^{-1} body weight of male [14]. After a latency period of 17 hours eggs and sperm were collected from two females and one male brood fish respectively. Sperm was collected by macerating the testis of male fish and diluted in Physiological Saline Solution (0.9% NaCl) [14, 15]. Fertilized eggs were collected by manual stripping and washed with freshwater and transferred to six flow through Glass aquaria (130 cm X 66 cm X 12 cm) of 6 mm thickness and incubation was carried out. The water parameters were studied during the experiment with the values of Dissolved Oxygen 7.8 mg L^{-1}, Total Ammonical Nitrogen 4 mg L^{-1}, pH 6.5 ± 0.5 and temperature 26 ± 0.5°C. From each breeding set around 650± 50 eggs were obtained among which fifty fertilized oocytes were randomly sampled after every ten minutes to study developmental stages starting from unfertilized matured oocyte till hatching under the light microscope (Olympus Trinocular Microscope XSZ156T) mounted with digital camera (Olympus SZ-10 Digital Camera, 18x Optical Zoom).

3. Results and Discussion

The latency period or response time is the time between the first hormonal injection and ovulation indicating the breeding efficiency as the lower or higher doses tend to reduce the egg output during breeding operation [16]. The latency period was found to be 17 hours in *C. batrachus (*at a dose of 0.02 ml Kg^{-1} body weight in female and 0.15 ml Kg^{-1} body weight of male) which is quite longer than the other catfish species like *Heteropneustes fossilis* [17, 18]. This difference may be due to the varied response of different species to the hormone. The unfertilized eggs of *C. batrachus* (1 ± 0.1 mm in diameter) were adhesive, brown in color and slightly smaller while the fertilized eggs were transparent, demersal, spherical and adhesive in nature (Fig. 1a). These could also be seen in *C. gariepinus* [19, 20], *Mystus montanus* [21] and *Mystus vittatus* [22] as a special character. In *C. batrachus*, fertilization stage can be characterized by a slight contraction of the yolk with increased perivitelline space and formation of prominent jelly coat (Fig. 1b). A characteristic red spot in the pigmented animal pole of fertilized egg was also observed (Fig. 1c). The vegetal pole (vitelline part) was largely yolky and translucent. All successive mitotic divisions occurred in the animal pole while the vegetal pole gave rise to nutritive yolk plug (after 26 minutes of fertilization) (Table 1). One cell stage appeared (44 minutes post-fertilization) with a bulging blastomere at the submicropilar area of animal pole. The segregation of the non-yolky cytoplasm at the animal pole was continuous making it highly pigmented separating the blastodisc from the vitelline parts (Fig. 2a). Two cell stage was produced by meroblastic discoidal cleavage. Similar pattern of cleavage occurs in many teleost with telolecithal eggs, containing large amounts of yolk concentrated at the vegetal pole [23, 24]. In our study two equal sized blastomeres were observed at 62 minutes post fertilization which is very close to the observation of Olaniyi and Omitogun [25] in *H. bidorsalis* (69 minutes post fertilization) but much longer compared to *M. vittatus* [22] and *H. fossilis* [26, 27]. Eight cell stage appeared after 82 minutes of fertilization with eight blastomeres at the blastodisc region. The fourth mitotic division resulted in 16 blastomeres after 125 minutes of fertilization (Table 1). The cells were arranged in a 4 × 4 array (Fig. 2c). Thirty-two cell stage could be considered as early morula stage (136 minutes post fertilization) (Fig. 2d) (Table 1). After 162 minutes of fertilization, a mulberry like structure (morula stage) showed numerous compact, elevated and rhomboid blastomeres (Fig. 8) (Table 1). Blastula stage (187 minutes post fertilization) can be described as highly compact cells forming a dome shaped blastoderm (Fig. 3b) (Table 1). During the transitional stage, the condensed cellular materials formed an epithelial layer named Enveloping Layer (EVL) which covered the underlying blastomeres. Adjacent to the blastoderm, acellular structure-Yolk Syncytial Layer (YSL) was formed by the fusion of marginal blastomere layer to the yolk cell. The gastrula was formed after 480 minutes of fertilization (Fig. 4a) (Table 1). The expansion of blastoderm marked the transition of blastula to gastrula. The onset of epiboly was characterized by slow morphogenetic movement involving contraction and expansion of the blastoderm. The characteristic cellular inward movement continued until the blastopore closed which marked the end of epiboly and/or gastrulation. This process led to formation of epiblast and hypoblast. The closure of the blastopore marked the end of morphogenetic movement and the embryonic axis was established (Fig 4b, c). The time of occurrence of morula and gastrula stage in *C. batrachus* were contrary with the results found in *H. bidorsalis* [25] and *H. fossilis* [27] where the first cleavage was observed within 15-20 minute and the 16-cell stage was achieved in 90 min of post fertilization. However in *C. gariepinus*, morula stage occurred at 11 minutes and gastrula stage at 327 minutes post fertilization [20]. Completion of all cleavage stages and hatching within 24 hours to 27 hours was reported in case of *H. longifilis* [28], *H. fossilis* [29] and *C. gariepinus* [17]. The difference in the transition of developmental stages in different catfishes may be due to species specific developmental pattern. The maturation of somites started at 900 minutes post fertilization (Table 1), followed by pigmentation at the cephalic parts of the embryo which later progressed towards the caudal end (Fig. 5a). Somites were noticed cephalocaudally distributed along the back of the embryo forming myotome blocks. The head region (polster) and the bulging tail bud were also noticed significantly. At this stage, the embryos exhibited first sudden muscular contraction (average of 7 movements/min). This contraction gradually became more frequent and stronger leading to vigorous lashings of the caudal part against the chorion (Fig. 5b). Similar hatching behavior was reported by several authors in different catfish species [19, 30]. The tail emerged first, followed by the trunk at 1560 minutes or 26 hours post fertilization (Table 1). The tail bud encircling the yolk sac first got detached from the middle part and later the anterior part of the head lengthened out forming a bulb shaped structure. But in case of *C. gariepinus* the chorion broke down into granules during hatching [15]. During our study period the incubation stage lasted from 26 to 27 hours at 26°C whereas it

was reported to be 23 to 24 hours at 29°C in *H. fossilis* [27]; 21 hours at 27°C for *H. bidorsalis* [25]; 24 hours at 28ºC in *C. punctatus* [31], 23.1hours to 26 hours in *H. longifilis* [32]. The variation in early developmental pattern, hatching and incubation period in fishes is highly temperature dependent and maintains a species specific pattern. The First day old larvae (1 DPH) /alevin stage larvae were translucent, curved and characterized by spreading of chromatophores, onset of circulatory system and optic primordium. At the anterio dorso-cephalic region of the developing embryo, the otic placode developed into otic vesicles with the early emergence of two tiny otoliths. The total length (TL) of the newly hatched larva of this candidate species was found to be 5.5 ± 0.5 mm which was similar to *H. bidorsalis* [25], *M. nemurus* [33] and *C. gariepinus* [20]. But in contrary, the TL was 2.5 to 3.5 mm for *H. fossilis* [26, 27, 34]. The major factor responsible for the difference in TL of the larvae can be the age and size of broodstock which affect the size of eggs and subsequently the size of fry [26]. As development progressed the head started protruding out and body pigmentation darkened. The heart, lens placodes, optic vesicles, olfactory placodes were clearly noticed. The eye pigmentation was apparent but as the eyes were not fully formed, the newly hatched larvae were photophobic. Myotome muscles develop along the length of the body. The early barbells were visible as tiny knots slightly above the heart, which were in front of the yolk sac (Fig. 6a-d). The Second day old larvae measured about 6.01 ± 0.5 mm (TL). The mouth was partially opened with significant development of the cartilage. The epicanthic fold of the eyes was raised, barbell length was increased and the alimentary system developed. The alimentary canal was distinct and had become more pronounced (Fig. 7a-c). Operculum and caudal rays were rudimentarily developed. Third day old larvae measured about 8.4 ± 0.5 mm (TL). Melanophore spreads cephalocaudally covering the body parts but remain heavily concentrated around the cranial region. The eye musculature, operculum and branchial arches were fully developed. Gills were prominently visible at the cephalic region (Fig. 8a&b). The yolk reserve depleted significantly and led to vast swimming ability of the larvae. Very few larvae started exogenous feeding on *Artemia naupli* on third day while the majority started external feeding on 4 DPH. The mean TL of the larva on 4 DPH was 9.2 ± 0.5 mm. The larva became opaque and photophobic. The barbells increased in length and become segmented. About thirteen caudal fin rays were observed in the developing caudal fins. The digestive system was well developed. Our results indicate that larvae of *C. batrachus* were highly photophobic like *H. bidorsalis* [25] and *Steindachneridion parahybae* [35, 36] and very active at night than day time like *H. longifilis* [37]. In the present study mouth gape opened at 4 DPH i.e 96 hours post hatching and exogenous feeding was commenced. But *M. cavasius* were reported to get nutrition from the yolk sac upto 3 days after hatching and exogenous feeding commenced from 3rd days (approximately 70 hours after hatching) [25]. In *H. fossilis*, the mouth was found to be opened at 36 h after hatching [30]. Whereas, in *H. longifilis* [38] the mouth opened at 3-4 hours after post hatching. In *C. batrachus* barbells developed shortly before the larva commenced active feeding like other catfish species [27, 39] and cyprinids [40] which is related to the improved capability of food finding in dark environment.

Table 1: Embryonic developmental stages of *C. batrachus* with respect to 26± 0.5°C

Stages of Development	Time from fertilization (Minutes)	Major descriptions
Fertilized	0	The yolk content starts shrinking away from the perivitelline membrane
Blastodisc stage	26	Animal and vegetal pole can be easily distinguished by appearance of red dots
One cell stage	44	Bulging at the protoplasmic area of animal pole is visible.
Two cell stage	62	First mitotic blastodisc cleavage took place and two equal sized cells are clearly visible. (Fig. 5)
Eight cell stage	81	Presence of eight cells in the blastodisc region as a result of third parallel mitotic cleavage; 2×4 array.
Sixteen cell stage	125	Due to meroblastic type of cleavage blastodisc contains total sixteen cells; 4×4 array.
Thirty-two cell stage	136	Formation of early morula staged cells; 4×8 array.
Morula	162	Formation of a disc of numerous uncountable blastomeres at the animal pole, appearing like "mulberry"
Blastula	187	Formation of a clearly visible dome shaped dark red coloured blastoderm
Gastrula	480	Expansion of blastoderm; randomized transitional movement of cells; initiation of embryonic axis formation.
Somite formation	900	Early somite formation starts cephalocaudally. Head and tail region can be clearly identified.
Hatching	1560 (26 hours)	The embryo comes out of the egg membrane or Chorion; formation of early yolk sac.

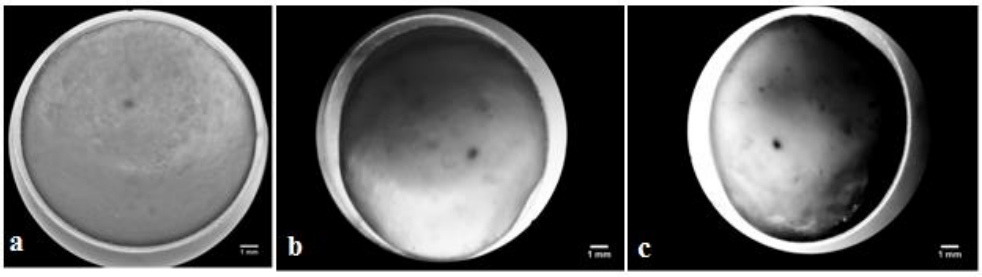

Fig 1: a. Unfertilized egg of *C. batrachus*; **b.** Fertilized egg of *C. batrachus*; **c.** Blastodisc stage of *C. batrachus* embryo. Scale bar 1 mm

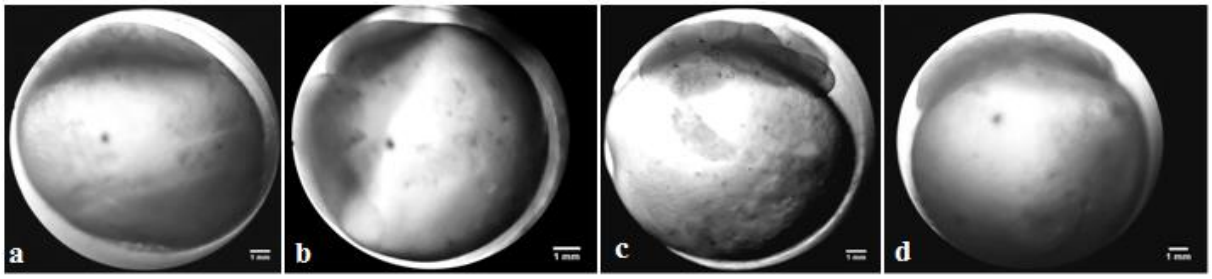

Fig 2: a. One cell stage of *C. batrachus* embryo; **b.** Two cell stage of *C. batrachus* embryo; **c.** Eight cell stage of *C. batrachus* embryo; **d.** Thirty two cell stage of *C. batrachus* embryo. Scale bar 1 mm

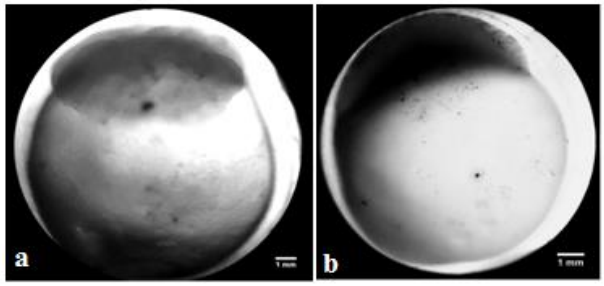

Fig 3: a. Morula stage of *C. batrachus* embryo; **b.** Blastula stage of *C. batrachus* embryo. Scale bar 1 mm

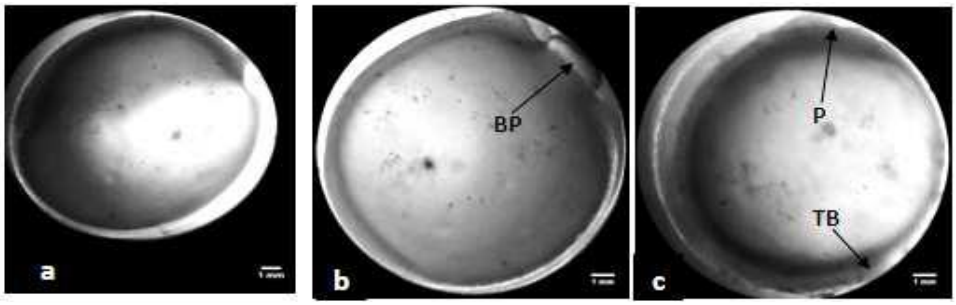

Fig 4: (a- c) Gastrula stage of *C. batrachus* embryo. Scale bar 1 mm. BP, Blastopore; TB, Tailbud; P, Polster.

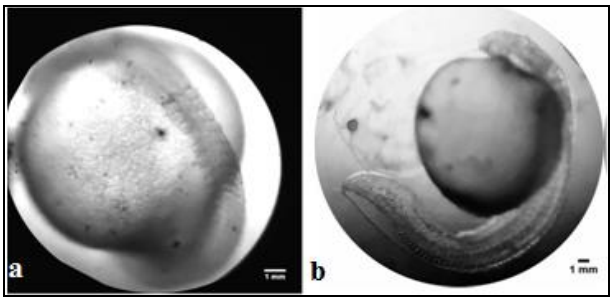

Fig 5: a. Somite stages of *C. batrachus* embryo; **b.** Hatching stage of *C. batrachus* embryo. Scale bar 1 mm. Arrow indicates ruptured chorion. YS, Yolk Sac.

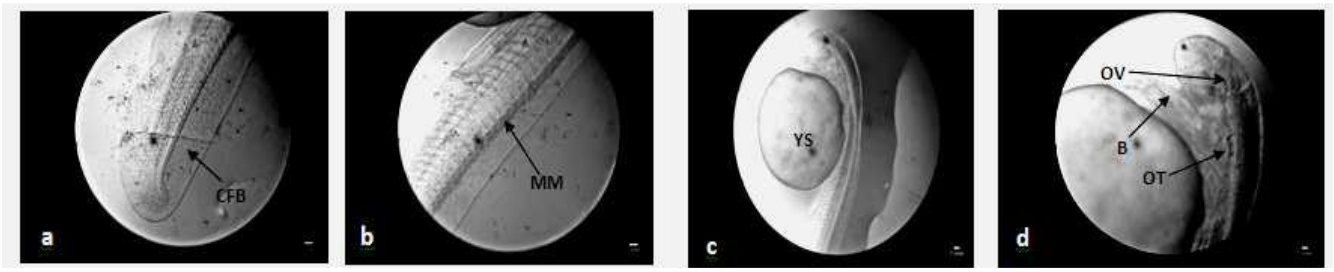

Fig 6: (a- d) 1 DPH (Days Post Hatching) stages of *C. batrachus*. Scale bar 1 mm. CFB, Caudal Fin Bud; OV, Optic Vesicle; YS, Yolk Sac; B, Barbel; OT, Otolith; MM, Myotome Muscle.

Fig 7: (a- c) 2 DPH (Days Post Hatching) stages of *C. batrachus.* Scale bar 1 mm. B, Barbel; AC, Alimentary Canal; YS, Yolk Sac

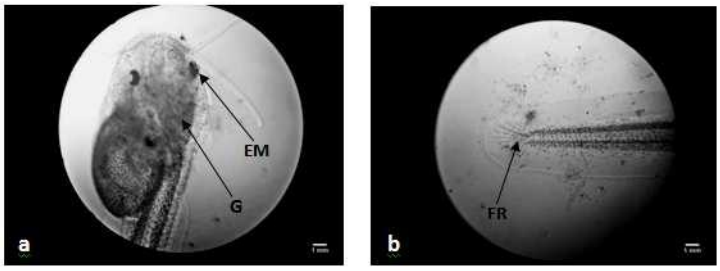

Fig 8: (a, b). 3 DPH (Days Post Hatching) stages of *Clarias batrachus*. Scale bar 1 mm. FR, Fin Rays; EM, Eye Musculature; G, Gill.

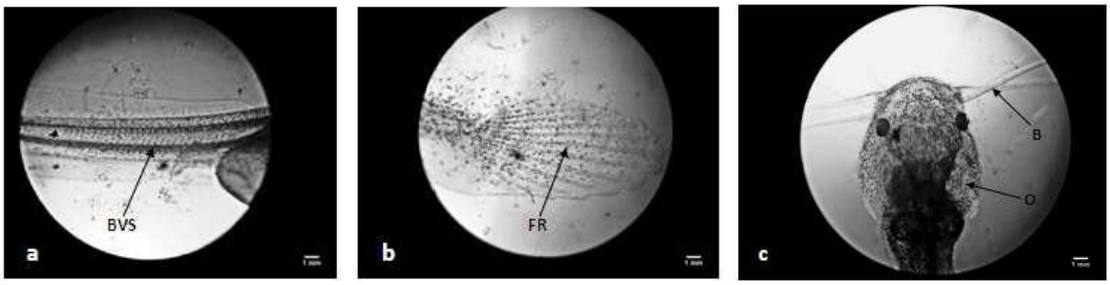

Fig 9: (a- c). 4 DPH (Days Post Hatching) stages of *C. batrachus*. Scale bar 1 mm. BVS, Blood Vascular System; FR, Fin Rays; O, Operculum; B, Barbel.

4. Conclusion

In India, culture *C. batrachus* is restricted due to lack of knowledge on breeding and feeding protocols. Our study has reported some significant morpho-sequential developmental stages in the ontogeny and organogenesis of *C. batrachus* to understand the developmental priorities. Our study has pointed out that the mouth gape opened at 4 DPH and exogenous feeding should be started at this time to avoid "Fouth Day Mortality Syndrome". In addition, the present investigation recommends *ad libitum* feeding under low light or dark environment as appropriate for the photophobic larvae. Thus shorter incubation period, fast development of sensory structures and early commencement of exogenous feeding suggest this species as suitable for small scale and commercial aquaculture.

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