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STUDY ON THE EMBRYONIC AND LARVAL DEVELOPMENT OF *CIRRHINA MRIGALA* (F. HAMILTON, 1822) IN RESPONSE OF DIFFERENTIAL DOSAGE OF OVAPRIM

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ABSTRACT

Healthy and mature male and female of *Cirrhina mrigala* were procured from wild habitat and kept in brood ponds to acclimatize according to hatching conditions. Eggs and milts were obtained from absolutely ripe female specimens by treated with doses of 0.3 ml/kg, 0.4 ml/kg and 0.5 ml/kg along with three males @ of 0.2 ml/kg of ovaprim. Both genders were kept with the ratio of 2:1. The spawning was started after eight hours of injection when male chased females. Eggs and milts were collected separately and allow to mix by using a large sized quil followed by a normal washed with normal saline and tennin solution. The non-floating and non adhesive fertilized eggs ranged between 3-4 mm in diameter. The cleavage pattern was started from the onset of fertilization. Two cells, four cells, eight cells and sixteen cells stages were viewed at 0:0, 0:05, 0:10, 0:15, 0:40 hours respectively. Morula and formation of yolk plug were noted after 2:00 and 3:30 hours after fertilization. There was an increased of germ ring appeared after 4:30 hours followed by a series of successive appearance of embryonic streak, somites, optic vesicles on head region and formation of tail with the time duration of 4:30, 6:00, 9:00, 10:00 and 11:00 to 12:00 hours. The immediately hatched larva has total length of 4.2mm without any mouth traces. After 6:00 hours from hatching auditory spots were formed followed by pigmented eye spots and formation of fin folds with an increasing length from 4.4mm to 4.8 mm. The time passed to approach this stage was 12:00 hours. Further remarkable developments were observed after 24, 48 and 72 hours by the formation of mouth opening, intestine, ear bladder, operculum and notochord. The larval size was maximized as much as 5.7 mm after 72 hours. Post larval developments were started at the age of four days with 6mm of total length. Formation of jaws, liver, ventral fin folds, gills, rayed, dorsal and caudal fins, lateral lines and scales over the entire body were observed at the age of 5, 6, 7, 10, 15, 20 and 25 days, respectively. The increments in total length were recorded as 6.5, 6.7, 6.9, 10.3, 16.6, 20.8 and 23 to 35 mm with respect to age. It is concluded that embryonic, larval and post larval developmental cycles are completed from the onset of fertilization within 11 to 12 hours, 72 hours and 25 days.

KEYWORDS: Induced spawning, Carp embryology, Ovaprim, Ovulation response, Carp biology.

INTRODUCTION

The increasing demand of white meat especially from freshwater resources putting an enormous pressure on wild habitat. This approach resulted as a great decline of fish population in lakes and other reservoirs. Therefore growing numbers of fish ponds every year has resulted in an increasing requirements of fish seeds and frys for stocking in both wild and man made water bodies. Induced breeding is the most reliable tool to augment the declining trend of natural stock along with to make sure the sustainable supply of seeds to fish farmers (Blazowska *et al.*, 2009). Among the most demanded Carps, *Cirrhina mrigala* is considered not only for capable of excellent taste with high economic values also to respond against inducing agents (Kaui & Rishi, 2003). By considering this fact it is imperative to concentrate over its early life history because morpho-embryonical changes are very important for the optimization of its large scale seed production, culture and management (Liu *et. al.,* 2000). Keeping it in views the present study was carried out to highlight the detailed aspects of early life history including morphological appearance of eggs, embryonic changes or cleavage pattern of eggs, hatching of eggs, larval development of hatchlings movements and post larval development.

MATERIALS AND METHODS

For understanding the morpho-embryonical aspects of *C. mrigala* an inducing agent i.e. Ovaprim was selected and procured from local scientific store in liquid form (10 ml) contains 20 µg of Salmon GnRHa and 10 mg of doperidon (Brzurka and Adamek, 1999; Afzal (2007). These active ingredients then dissolved in propylene glycol as suggested by Peter *et al*., (1988). Eighteen females of *C. mrigala* (mean weight T1, 3.07 ± 0.208; T2, 3.588 ± 0.166; T3, 3.653 ± 0.185) were divided into three groups as they were tested against three serially arranged doses of Ovaprim i.e. 0.3 ml/kg, earlier tried by Nandeesha *et al*., (1990), 0.4 ml/kg by Das (2004) and 0.5 ml/kg by Nandeesha *et al*. (2009). Side by side six males were also injected at the rate of 0.2 ml/kg of body weight and the ratio of females and males were 2:1 (Naeem *et al.,* 2005).

Method for administration of ovaprim: In common practice there are two modes of administration of Ovaprim i.e., peritoneal (within the body cavity) at the ventral part of the fish body and intramuscular (within the musculature) at the dorsal part of the fish body. The method used by Jhinghran & Pullin (1985) was adopted to inject Ovaprim in present induce breeding trials. The scales of experimental breeders were lifted for the insertion of injection needle. The angle to the base of pectoral fin was between 45^{o} - 90^{o}. A moist towel was also used to wrap the examined fish for further steps.

Morpho-embryonical changes after fertilization: Morphologically the eggs were looked like glass beads with pale brownish yellow yolk contents. These eggs were obtained as cluster with the features of non-floating and non-adhesive. Under examine eggs diameter were ranged between 3 - 4 mm. The fertilized eggs were identified by their appearance i.e. transparent ones while unfertilized or dead eggs were translucent or opaque. Embryonic changes have been examined by taking egg samples in glass vials containing 70% ethanol. The time interval of taking eggs sample was according to Miah *et al*., 2009. Early embryonic developmental stages were studied under a Microscope (Nikon, SMZ 800) with the magnification power of 40 X.

All stages were studied perpetually until the embryo starts twisting movement. The developing individuals were temporarily stained to observe changes with methylene blue. The newly emerged specimens were measured in a Petri dish having 1.0 mm graph paper at the bottom. Photographs were taken by the selection of at least three specimens to describe each stage.

RESULTS

Morpho-embryonic changes after fertilization

Morphological appearance of eggs: Glassy beads like eggs with pale brownish yellow yolk were ready to under go for embryonic development immediately after the fertilization. They were spherical in shape, more or less transparent indicating the power of hatchability. At this stage they were non-adhesive and non-floating. Each egg was measured approximately 1.5 mm in diameter with evenly distributed yolk content (Plate 1a). As immediately eggs were fallen into water it was noted that marked increase in size occurred after passing 15 minutes as ranged between 3 to 4 mm in diameter.

Embryonic changes or cleavage pattern of eggs: As the fertilization has been completed the protoplasm started to concentrate at one side of yolk to form germinal disc or blastodisc (Plate 1b). About five minutes later first activity of cleavage was noticed towards the equatorial plane, which split blastodisc into clearly defined two blastomeres. The duration may vary depending upon the water temperature. It should be ranged 24-31^{o}C (Plate 1c). The cell stage was lasted for about 10 minutes and followed by another cleavage which multiplied two cells into four cells. The direction of second cleavage was at right angle to 1st cleavage (Plate 1d). The third cleavage was initiated after passing 15 minutes and converted four cells into eight blastomeres (Plate 1e). Sixteen blastomeres were resulted by the commencement of 3rd cleavage parallel to 2nd one within 40 minutes of gap (Plate 1f). Subsequently more numbers of cleavage were continued to form 64 cells, 128 cells to form morula. This morula stage was considered as its advanced stage taking two hours of intervals after fertilization (Plate 1g). The yolk content was invaded partially by germ layer. During the study of early embryonic development, it was noticed that all mass of cell condensed as a lens shaped appearance and in advanced embryonic development, it appeared as dome shaped and scattered over the yolk (Plate 1h). Around the margin of blastoderm a thick germ layer was formed which grew larger as the result of growth, germ ring was increased in size to cover yolk sphere. The time duration to cover half portion of yolk sphere was about three and half hours and for completed cover about four and half hours was required. This stage of eggs was suggested as yolk plug stage (Plate 1i). The under developed eggs were changed morphologically as elongated eggs (Plate 1j). The four and half hour old eggs further under go for more advanced stages. A ridge with a streak was appeared called embryonic streak stage and designated as just embryo formation stage. The nearer end from the streak was suggested as tail end while opposite to it as head end of under developed larva. The rounded look of embryo now started to stretch until it was resembled with more or less kidney shaped or pea shaped. The over all time taken to attain pea shaped appearance was six hours from fertilization (Plate 1k). Seven to nine well defined somites were seen in nine hours old embryo (Plate 1l). Just after an hour later 12-15 somites were visible with an optic vesicle on the head end. During the microscopic study, some embryo at this stage exhibits seventeen numbers of somites with well developed head, eyes, a pair of otocysts with two otolith (Plate 1m). With the commencement of 11 to 12 hours, the numbers of somites were counted up to 27 - 30 along with the development of tail. This tail was much elongated and projected beyond the yolk sac after 12–14 hours. The major traces of eye, cellular notochord and embryonic fin folds were also noted in this stage. In 15–16 hours stage simple tubular structure was appeared indicating the emergence of heart. Auditory and optic vesicles were also clearly visible (Plate 1n).


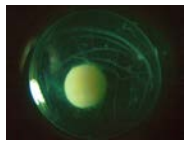

Plate and photograph 1a: Fertilized egg of *Cirrhina mrigala*.


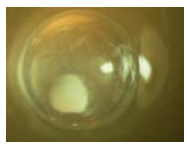

Plate and photograph 1b: Germinal disc formation in fertilized egg of *Cirrhina mrigala*.


Plate and photograph 1c: Two celled stage of egg of *Cirrhina mrigala*.


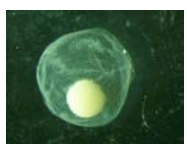

Plate and photograph 1d: Four celled stage of egg of *Cirrhina mrigala*.


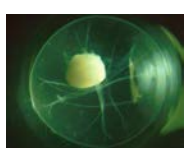

Plate and photograph 1e: Eight celled stage of egg of *Cirrhina mrigala*.


Plate and photograph 1f: sixteen celled stage of egg of *Cirrhina mrigala*.


Plate and photograph 1g: Morula stage of egg of *Cirrhina mrigala*.


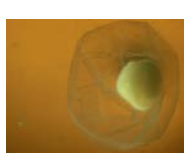

Plate and photograph 1h: Half yolk invasion of egg of *Cirrhina mrigala*.


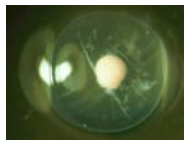

Plate and photograph 1i: Yolk plug stage of egg of *Cirrhina mrigala*.


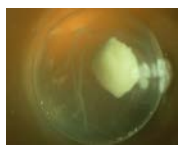

Plate and photograph 1j: Elongation of yolk mass of egg of *Cirrhina mrigala*.


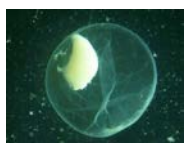

Plate and photograph 1k: Kidney shape of egg of *Cirrhina mrigala*.


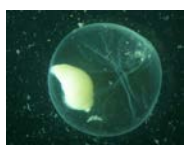

Plate and photograph 1l: Optic vesicle of egg of *Cirrhina mrigala.*


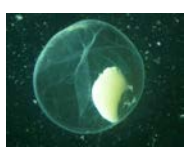

Plate and photograph 1m: Formation of head, eyes, otolith and otocysts of egg of *Cirrhina mrigala*.


Plate and photograph 1n: Formation of tube pore hatching of larva of *Cirrhina mrigala*.


Plate and photograph LD1: Immediately after hatched larva of *Cirrhina mrigala*.


Plate and photograph LD 2: Formation of auditory spot in larva of *Cirrhina mrigala*.

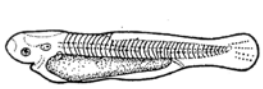


Plate and photograph LD 3: Formation of fin fold and notochord in larva of *Cirrhina mrigala*.

Plate and photograph LD 4: Formation of mouth opening in larva *Cirrhina mrigala*.

Plate and photograph LD 5: Formation of intestine and air bladder in larva of *Cirrhina mrigala*.

Plate and photograph LD 6: Formation of operculum and pigmentation over head in larva of *Cirrhina mrigala*.

Stages of Post larval development of *Cirrhina mrigala*

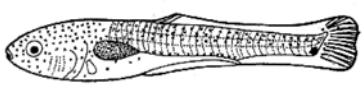

Plate of PL1

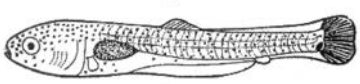

Plate of PL2

Plate of PL 3

Plate of PL 4

Plate of PL 5

Plate of PL 6

Plate of PL 7

Hatching of eggs: The process of hatching was initiated after sixteen hours and was continued up to 19 hours. The tail movement ruptured the egg shell and embryo wriggled out, tail first made contact with surface water by following swirling movement.

Larval development of hatchlings movements: The pictorial presentation of larval development of hatchlings is exhibit in plate LD1 to LD6. Immediately hatched larva was characterized by transparent appearance without mouth. Eyes were lacking with any pigment. Yolk sac placed at lower part consists of two partitions i.e., proximal short bulbus part and distal comparatively elongated part. The traces of musculature also called myotomes were counted as 27 + 10 and the total length was about 4.2 mm (Plate LD1). After passing six hours, newly emerged hatchlings were aged as six hours old hatchlings. The separation between bulbus and elongated partition was vanished followed by two auditory spots appearance in which posterior one was slightly larger. Its total length was about 4.4 mm (Plate LD2). The next observation was taken after twelve hours. Eyes were getting their pigmentation at the centre. The bulbus part of yolk sac started to reduce gradually. Total myotomes were 27 + 14. Fin folds were appeared along with spotted line of notochord. Total length was increased as maximum as 4.8 mm (Plate LD3). Twenty-four hours old hatchlings were become more prominent and visible. Eyes were more pigmented centrally. A well defined slit was observed as mouth opening. Yolk sac was stretched longitudinally. A red or pink spot was seen near the opercular

region. Total length was 5.1 mm (Plate LD4). 48 hours later, mouth was opened, the traces of pectoral fin was more clearly seen along with air bladder. The visibility of intestine was clearly noticed. Dorsal and ventral fin folds were also originated from middle and distal region of air bladder. Gill arches were also seen as prominent lines. Impressions of caudal fin with prominent striations were noted. Chromatophores were appeared on head region. Total length was 5.4 mm (Plate LD5). Completely lost yolk sac indicates the age of 72 hours. Mouth was well developed with greatly increased number of chromatophores at the head region. The necked gill arches covered by operculum. Air bladder changed its shape from rounded to elliptical. Notochord was completely formed with slightly elevated posterior ending out lines of vertebrae were visible. The number of myotomes remains same i.e. 27 + 14 but looked more distinguishable. Total length was 5.7 mm (Plate LD6).

Post larval development: The stages of post larval development are presented in Plate PL1 to PL 8. At the age of four days larval length was 6 mm. Both lips were well developed specially the lower one was thicker and shorter. The ventral fin fold was originated from 4.5 mm of body length while the dorsal fin folds originate just behind the air bladder (Plate PL1).

After fertilization of 5 day, the jaws become elongated comprising of slightly longer upper jaw. Two pigmented striations were appeared at the tip of notochord. Bifurcated caudal fin with twelve fin rays was formed due to a small notch on posterior margin of caudal fin. Total length was increased up to 6.5 mm (Plate PL 2). Six days old larva got more pigments over head and body. Six striations were noted in dorsal fin with a persistence of dorsal fin fold. The separated anal and ventral fin fold was also persisting. 16 fin rays were counted in caudal fin. Notochord was almost bent up word. Liver was easily seen as a red spot near the ear sac. The larva was looked like fry with more yellow golden coloration on anterior and ventral side with pale yellow coloration on the abdominal region. Total length was 6.7 mm (Plate PL 3).

After seven days from the time of fertilization ventral fin fold was appeared. Dorsal fin fold was separated from the fin fold along with eight slightly branched rays. Both jaws become equal in size. Notochord was remaining up turn. 18 fin rays were counted in caudal fin, out of which four on either side unbranched. Total length increased up to 6.9 mm (Plate PL 4). Ten days old larva showed more developed appearance i.e., well developed mouth along with thick jaws. A pair of maxillary barbles was seen by observing under high magnification. Gills were formed completely Anal fin was none distinguish and originate at the anal opening. Fin rays pattern was 2/13 rays in dorsal and 20 rays in caudal fin. Total length increased as much as 10.3 mm (Plate PL 5). More advancement was noted after 15 days. Lower jaws were thicker than upper one. Barbels can be only seen under high magnification. 16 fin rays were counted in dorsal and 22 rays in caudal fin. Caudal peduncle becomes darker due to accumulation of chromatophores. Total length was 16.6 mm (Plate PL 6). At the age of 20 days, maxillary barbells become more clear and visible to normal vision. More or less whole body was pigmented with development of lateral line. Heterocercal appearance of caudal fin observed with a deep forked look. Emersions of scales were started on the posterior region to operculum. Total length was 20.8 mm. (Plate PL 7). As the larvae become 25 days old, scales were formed and covered almost entire body. The position of mouth was inferior. Body got full pigmentation. Triangular bands appeared on caudal peduncles. The fin rays arrangement were found as anal 2/6, ventral 9 caudal 26 rays. The 25 days old fry obtained fresh light green appearance. Total length was 23 – 35 mm (Plate PL 8).

DISCUSSION

For the convenience to understand the morpho-embryonic changes of experimental fish, it is better to discuss these changes in order to cleavage pattern of eggs, larval development and post larval development. As the egg falls in to water a space is created between outer membrane and up coming embryo. The glassy beads like appearance is due entrance of water and protect the delicate egg from external shocks which is also link to the continuous flow of water in ponds and in streams or heavy flow in natural habitat or river (Chakrabarty & Murty, 1972).

Table 1 shows the early embryonic development which starts with the first cleavage. The protoplasm begins to concentrate at one side of yolk to form germinal disc or balstodisc (1b). After five minutes from the onset of fertilization. Formation of two cells occurred (1c). The rate of embryonic development varies with respect to variation of water temperature. This temperature may speed up by means of warming of water by sunlight. (Khan, 1943) This repeated cleavage was observed which divide the two cells into 16 cells and time required for this is 40 minutes. The incubation proceeded from morula to a completion of stage characterized by the formation of tail along with 27–30 somites. The ready to hatch egg concede 11–12 hours of time. (1d–1n). According to Konda Reddy (1977) the time taken for incubation of eggs of major carps ranges from 14 to 18 hours. In present study it was notified that the time for embryonic development was less. Miah *et al*., (2009) studied on the embryonic development of *Labeo rohita* and recorded that early embryonic development was completed with in 18:00 to 20:00 hours. However, Rehman *et al*., (2004) claimed the time required for completion of embryonic development of *Mystus cavasius* was 19:00 hours.

Table 1. Summary of embryonic development of hand stripped eggs of *Cirrhina mrigala*.

Event	Plate No.	Photograph	Stage	Time after fertilization (hours)	Details of event
Fertilized eggs	1a	I	1	0:00	Spherical transparent, non adhesive, none floating
Blastodisc formation	1b	II	2	Onset of fertilization	Cytoplasm concentrated at one side of yolk
Formation of two cells	1c	III	3	0:05	Distribution blstodisc into two blastomere
Formation of four cells	1d	IV	4	0:10	Distribution blstodisc into four blastomere
Formation of eight cells	1e	V	5	0:15	Distribution blstodisc into eight blastomere
Formation of 16 cells	1f	VI	6	0:40	Parallel distribution to form 16 blastomere
Formation of morula	1g	VII	7	2:00	Yolk content invaded parallel by germ layer
Formation of yolk plug	1h	VIII	8	3:30	Condensation of cellular mass as a lens
Increase of germ ring	1i	IX	9	4:30	Formation of germ ring with gradual increase to cover yolk sphere
Formation of embryonic streak, kidney shaped embryo	1j	X	10	6:00	Completion of yolk invasion. Appearance of rudimentary head and tail region
Formation of somites	1l	XI	11	9:00	7-9 well defined somites were formed
Formation of optic vesicle on head	1m	XII	12	10:00	12-15 somites were formed, formation of head, eyes, otolith and otocysts
Formation of tail	1n	XIII	13	11:00-12:00	27-30 somites were formed and tail was more elongated and projected

Table 2 describes shortly the various events of larval development, immediately after hatching. Larval length was 4.2 mm and transparent, without any traces of eyes, mouth and gills. The same observations were earlier studied by Chakrabarty & Murty (1972). Auditory spot was appeared after 6:00 hours and total length was slightly increased (4.4 mm) (LD2). The pigmentations were seen for the first time in 12:00 hours (LD3) as well as the formation of myotomes (27 + 14). A line of notochord was also developed. The gradually increased size of larva was characterized by the formation of mouth opening after 24 hours with more pigmented eye spots (LD4). Intestine was appeared with air bladder after 48 hours but the intestine was empty (LD5). Konda Reddy (1977) reported that intestine was formed after 48 hours old larvae of *Catla catla* with some accumulation of algae and cladocrens. This finding is contradicted to present result. At the age of 72 hours larval length was 5.7 mm with some striations (eight) on caudal fin (LD6). After 72 hours larval stage was stepped ahead towards post larval stages. The same age for completion of larval developments was suggested by Miah *et al.* (2009) for *Labeo bata*.

Table 2. Summary of larval development of hand stripped eggs of *Cirrhina mrigala*.

Event	Plate No.	Photograph No.	Age (hrs)	Total length	Details of Larval development
Partitioning of yolk sac	LD1	LDI	Immediately after hatching	4.2 mm	Transparent, eyes and mouth absent, myotomes 27 + 10
Formation of auditory spot	LD2	LDII	6.0 hours	4.4 mm	Bulbus and elongated partition vanished
Appearance of fin fold and notochord	LD3	LDIII	12.0 hours	4.8 mm	Eyes were pigmented, myotomes 27+14. Spotted line of notochord formed
Formation of mouth opening	LD4	LDIV	24 hours	5.1 mm	Slit like mouth opened, eyes were more pigmented centrally
Formation of intestine and air bladder	LD5	LDV	48 hours	5.4 mm	Development of fin folds, gill arches and accumulation of chromatophores on the head region
Formation of operculum and notochord	LD6	LDVI	72 hours	5.7 mm	Upturned notochord formed 8 striations on caudal fin, more chromatofores on head region

Table 3 summarized the post larval development events. At this stage yolk sac was completely vanished on 4th day. Lips were formed and total length was 6.0 mm (PL1). Similar observations have been notified by Chakrabarty & Murtey (1972). Rudimentary jaws were developed along with bifurcated caudal fins with two rays (PL2). The most important events i.e., the formation of liver as a red spot in post larval development noticed after six days. The over all appearance was yellow golden with total length 6.7 mm (PL3). The larval length was increased day by day with some well defined events like rayed fins of dorsal and caudal region after 15 days of age with 16.6 mm in length (PL6). The most important developments were formation of lateral line and maxillary barbles after 20 days with total length of 20.8 mm (PL7). The short part of maxillary barbles appeared on 12th day in Rohu, claimed by Basavaraju and Varghese (1981). However, Konda & Reddy (1977) observed the appearance of barbells on the 10th day.

Table 3. Summary of post larval development of hand stripped eggs of *Cirrhina mrigala*.

Event	Plate No.	Photograph No.	Age (days)	Total length	Details of post larval development
Formation of lips	PL1	PLI	4	6.0 mm	Lower lip thicker than upper one. Lower and ventral fin folds were formed
Formation of jaws	PL2	PLII	5	6.5 mm	Jaws formed, bifurcated caudal fin with 2 rays was formed
Formation of liver and notochord	PL3	PLIII	6	6.7 mm	Liver and up turned notochord developed, yellow golden in appearance
Formation of ventral fin fold	PL4	PLIV	7	6.9 mm	Jaws become equal, dorsal fin separated from fin fold.
Formation of liver and notochord	PL5	PLV	10	10.3 mm	Development of well developed mouth and thick jaws. Gills formation completed
Formation of rayed dorsal and caudal fin	PL6	PLVI	15	16.6 mm	16 fin rays in dorsal and 22 caudal fins were formed
Development of lateral line and scales on posterior region	PL7	PLVII	20	20.8 mm	Maxillary barbles more developed, Heterocercal tail was formed
Covering of scales over entire body	PL8	PLVIII	25	23 -35 mm	Light green in appearance, body got full pigmentation, body covered completely by scales

The final event is the covering of scale on entire body which was completed after 25 days of age and total length was ranged between 23–35 mm (PL8). The appearance of scale was noted for the first time on 20th day, as claimed by Basavaraju & Varghese (1981). This is contradicting to present observation.

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