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DEVELOPMENT BIOLOGY OF *HYPSELOBARBUS KURLALI* (MENON AND REMADEVI, 1995)

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ABSTRACT

Captive breeding of *Hypselobarbus kurali* was performed and the fertilized eggs so got started development into its further level to attain the larval stage. The division was from two celled stage, to four, eight, 16 and so on with the progress of time and then by 12 hour time scale the division turned on to the gastrula stage, then to neurula and blastulation procedures. The organogeny started by the somite stage and the early and late embryonic stage was progressing and by 48 hours the eggs started hatching.

KEYWORDS: *Hypselobarbus kurali*, neurula and blastulation procedures.

INTRODUCTION

Once after fertilization and formation of fertilization membrane the eggs of all species start its development. The embryonic developmental stage begins from fertilized egg membrane formation, cleavage, morula, blastula, gastrula, embryonic body formation, optic vesicle and auditory vesicle formation, blastopore closing, tail formation and hatching stages were observed and examined in general. In the period of larval development after hatching, until the end of the yolk sac absorption period (pre-larvae) and subsequently till the end of metamorphosis (post-larval) the growth is tremendous and is too much complicated (Faruk et al, 2011). In the fish egg, little attention has to been paid to the changes taking place in the egg membrane at the time of fertilization. This may be due to the fact that the egg membrane is already present on the unfertilized egg and is merely elevated upon fertilization (Eizo,

1956). According to the evidence presented by Yamamoto (1939-1954), the cortical alveoli embedded in the cortical layer break down upon fertilization and this change is subsequently followed by the elevation of the egg membrane. The fertilization membrane formation itself is the beginning of development of egg, where the eggs which had undergone the gametic fusion will absorb water to a very high level resulting in the bulging of the cell membrane, which in turn is termed fertilization membrane. The development of zygote to different stages from morula to early embryo, then late embryo and finally hatchling is a time consuming process. The development stages are entirely different at different time scale. Once the male and female gametes fuses and fertilizes the egg soon starts to develop. The development procedure is a complicated sequence of events. For proper development and better survival, the fertilized eggs are incubated, where in they are placed under the favorable conditions for normal development. The fertilization membrane formed during fertilization of eggs will act as the shell for the complete development of embryo. Once the development is completed the larvae will hatch out breaking the external egg shell. In fish eggs, cleavage occurs only in the blastodisc, a thin region of yolk-free cytoplasm at the animal cap of the egg. Most of the egg cell is full of yolk. The cell divisions do not completely divide the egg, so this type of cleavage is called meroblastic. Since only the cytoplasm of the blastodisc becomes the embryo, this type of meroblastic cleavage is called discoidal. Scanning electron micrographs show beautifully the incomplete nature of discoidal meroblastic cleavage in fish eggs. The calcium waves initiated at fertilization stimulate the contraction of the actin cytoskeleton to squeeze non-yolky cytoplasm into the animal pole of the egg. This converts the spherical egg into a more pear-shaped structure, with an apical blastodisc (Leung et al. 1998). Early cleavage divisions follow a highly reproducible pattern of meridional and equatorial cleavages. These divisions are rapid, taking about 15 minutes each. The first 12 divisions occur synchronously, forming a mound of cells that sits at the animal pole of a large yolk cell. These cells constitute the blastoderm. Initially, all the cells maintain some open connection with one another and with the underlying yolk cell so that moderately sized (17-kDa) molecules can pass freely from one blastomere to the next (Kimmel and Law 1985). After fertilization and membrane formation the egg swells in size. When an egg is stimulated by s spermatozoon arriving at the vitelline surface through the micropyle, a transient wave of increase in cytoplasmic free calcium starts at the point of sperm attachment (Gilkey et al., 1978 and Yoshimoto et al., 1986). The cortical alveoli in the vicinity of the micropyle also begin to break down (exocytosis of alveolar contents) about 9 s after sperm attachment (Iwamatsu et al., 1991). The wave of exocytosis begins to propagate over the whole egg

surface and ends at the vegetal pole 154 s after its beginning. As a result of the exocytosis of cortical alveoli into the narrow space between the chorion and the vitellus, the chorion thins and hardens (Ohtsuka, 1960) as it separates from the vitellus to form a wide perivitelline space. Swollen spherical bodies secreted from the cortical alveoli are faintly visible in the perivitelline space. A transient 'contractile wave' of cortical cytoplasmic layer follows the wave of exocytosis (Iwamatsu, 1973 and Iwamatsu and Hirata, 1984). Due to the oscillatory contractions following this distinct contractile wave, the cortical cytoplasm progressively accumulates toward the animal pole to form a thick cytoplasmic layer (Abraham et al., 1993 and Sakai, 1964). At 7–8 min after sperm entry, the second polar body is extruded onto the surface of the cytoplasm at the center of the area where the germinal vesicle broke down during oocyte maturation.

The embryonic development of fishes proceeds under the protection of rigid egg membranes which preserve the embryo from mechanical injury. Salmonid fishes bury their eggs in sandy and stony ground so that they are particularly liable to mechanical damage. This is apparently the reason why the membrane of the salmonid embryo is extremely strong, resisting a load of 3-4 kg/ovum (Gray, 1932; Hayes, 1942, 1949; Hayes and Armstrong, 1942; Zotin, 1953a). This mechanical property of the membrane does not appear immediately after fertilization or activation but is preceded by a whole set of processes which are elicited in the membrane by external factors and by the fertilized or activated egg itself. Thus, according to Manery, Fisher and Moore (1947), hardening of the egg membranes in the speckled trout sets in 2 hours after the release of the egg into water. Fertilized eggs of the lake salmon and trout have been shown by Zotin (1953a, 1958) to secrete substances which are indispensable for the increase of membrane toughness. The secretion of these substances requires a short exposure to water to activate the egg. The activating effect of water upon unfertilized salmon eggs has often been described (Yamamoto, 1951; Soin, 1953; Disler, 1954). The secretion of substances eliciting hardening or solidification of egg membranes after fertilization or activation has been described in some sea-urchin species (Motomura, 1941; Runnstrom, 1947, 1952) and in sturgeons (Zotin, 1953) as well as in the teleostean fish *Oryzias latipes* (Nakano, 1956). Solidification of the membranes of the sea-urchin egg likewise requires the presence of Ca ions (Hobson, 1932). The animal pole rises as a small bulge on the yolk and the colour varies. On the basis of the temperature of water in which the eggs are incubated the eggs starts its cleavage, first cleavage in which the one celled animal pole successively becomes two, then four, eight, sixteen, thirty two and finally sixty four celled stage. This

stage is called morula stage. As the cell division progresses the morula develops to blastoderm, which is a single layer of cell. Later on the blastoderm divides forming several layered structures the blastomeres. As the number of blastomeres increase the size of cells becomes comparatively smaller. After this a segmentation cavity formation occurs in between the yolk and cell mass. The whole structure is thus called the blastula. The blastula cells arrange themselves on the top of the yolk to form a cup like structure. The whole yolk mass will be covered by the developing cells formed as a result of cell divisions. There will be only one opening in the whole blastula, which is the blastopore, which closes later on during different stages of development. This stage is called the transition point to the embryonic development stage from the initial germ cell to the development stages. The developing cells are highly sensitive to even minor shakes during the morula stage which will result in embryonic death.

Later on the cell mass develops and thickens in the form of a semi ring opposite to the blastopore, with a head and tail buds at the two ends. By the time the head and tail becomes clearly visible followed by the clarity of the first segment. 'Optic vesicle' will be formed in place of eyes on the head, eventually the tail bud starts to grow length wise. The progress in growth will be visualized by the appearance of heart, which is beating. This will be followed by the development of a strong blood vessel on the surface of the yolk mass. Here after the embryo gradually starts to twitch its tail occasionally. This will be later on visualized by the twitching of the entire body. Even after this the larvae will revolve in the previtelline space. The revolving and other movements become very vigorous before hatching.

Towards the time of hatching the embryonic metabolites develop some enzymes, which act on the so called shell and dissolve it from inside, there by rendering it weak and enabling the embryo to break it easily and hatch. The time taken by the eggs to develop varies differently among different species. The time is solely depended on the temperature and oxygen supply of the incubating system at which the eggs are incubated at the beginning. A poor supply of oxygen during the latter half of embryonic development, may lead to the death of the embryo. Temperature holds a very significant role in the larval development and hatching. Once if the temperature is high the eggs will hatch out and produce premature or unmatured embryo, which are mostly incapable for survival. If cold waters are provided the development and subsequent hatching will be too slow, which results in the retaining of embryo within the egg shell over a long period up to several days in some cases. Only hatching will be late but the

embryo development still continues within the egg shell. In normal case the fertilized eggs usually develops if all the conditions provided are suitable. During practical observation it's commonly observed that some eggs die after a brief period of development either during the morula stage or before the closing of the blastopore. Oxygen deficiency could be one of the reasons for mortality in certain parts of the incubation like poor water exchange. Temperature which is not suitable for eggs to develop also kills the egg during embryonic development. After fertilization during the beginning of the incubation all the eggs appear to be healthy and well developed. Later on some become white or opaque due to injuries sustained during stripping or artificial fertilization. Another point is the problem with differentiating unfertilized and fertilized eggs in the beginning as both will swell in the same way and polarization also proceeds in the same scenario. But as development ensue the unfertilized egg lag behind with the first cleavage, and the normal development at the animal pole assumes an unusual growth. Fertilized egg subjected to shock, friction or shaking will also result in the improperly development, ultimately resulting in death. The healthy developing eggs are transparent or shining and their contents are clear. They can be clearly distinguished from the bad eggs which are white, opaque and turbid looking. Here in this chapter the development stages of *H.kurali* embryo with respect to time will be explained followed by the hatching and development of larvae.

Developmental stages with respect to time of Hypselobarbus *kurali* embryo.

MATERIALS AND METHODS

The egg after fertilization will be observed through an inverted microscope (model- Leica Galen 111) so as to study the changes and development of embryo with respect to time. The time at which water is added to the egg and milt (sperm) mixture so as to trigger fertilization is considered as the time of fertilization 'zero hour'. Incubation and development is carried out in an experimental set up a one *liter* glass bowl. Water temperature was maintained between 21^0C and 22.5^0C and dissolved oxygen ranged between 4.1 *mg/l* to 5 *mg/l*. the observation are done as per time, and the results are as follows.

RESULTS

The complete development of *H.kurali* eggs taken place in 47 – 50 hours of incubation. About 90% of hatching observed throughout the study was happened by 48 hours. So we could confirm the incubation period of *H.kurali* to be 48 hours. During these 48 hours time

scale the egg travels through several growth stages (PLATE.1). They are explained as follows.

After 20 minutes from the zero hour of fertilization the fertilized egg transformed into its two celled stage. At the animal pole the cells will form as a small cone shaped protrusion, visible to naked eye. On microscopic observation it becomes clearly visible where we could observe two cells on the top of the yolk mass which was the same protrusion to naked eye. These cells are more golden yellowish compared to the yolk mass. This forms the first step of division, which all the fertilized eggs either healthy or damaged will undergo. During this the size of the egg will be 230 μm in size. Once the egg reaches its 25-30 minutes of growth the two celled stage suddenly switches to four celled stage. The four cells on microscopic observation will love like four small cones on the top of a hillock, the yolk mass. These four cells will be adjacent to each other which will be distinctly visible and equally sized. At this point the eggs which had undergone physical stress and shock will start losing its ability to develop and ultimately dies at the stage the egg would assume 250 μm in size. As the development proceeds size of the egg also proceeds but not as fast as internal development. In the next ten minutes that is by 40 minutes time from the zero hours the eggs which had fertilized and are health will enter into its eight celled stage. Here the cells will be seen arranged in two rows. Each cell will be separate and all are equally sized. The total size of the egg assumes 280 μm in size. The cells will be more brightly coloured than the yolk mass. The cells will start occupying the previtelline space.The cells start multiplying faster and occupy the previtelline space with greater number of cells. They seemed like an aggregation of cells, which looks like a cap on the top of the yolk mass. This occurs by 50 minutes time and by the time egg will attain 280 μm in size. Till the time the egg develops a lot. The cell division proceeds to 32 celled, 64 celled and beyond countable numbers of cells. These cells together form a bunch and assume the shape of a mulberry fruit, and so this condition is termed morula stage. Then the development proceeds and the cells turned out into blastomeres and membrane folds. These membrane folds multiplies in number and starts covering the yolk mass leaving a small opening, the blastopore. As its timed quarter the way by 7 hours and 30 minutes the stage is named half yolk invasion stage. Here while observing through microscope we could see the blastomere folds as layers covering the yolk mass half the way. The folds will be differentiated only in layers but no further developments could be visualized. The size of the egg thus will be 310 μm. This stage is also called early gastrula. The development proceeds from early gastrula and reaches a stage were the membrane folds cover the yolk mass towards

3/4th of its total size. It was observed that the germ ring, the embryonic shield and the embryonic axis gets well developed. The blastopore seemed almost closed at this stage and we can see the egg in 340 μm size. From the observed zero hours the present observation was at 12 hour time scale. A great set of development could be observed during this stage. This stage is also termed as the tail bud stage where we could see the head and tail as separated projections. The cephalic region became more prominent and optic vesicles start developing. The auditory vesicles also start developing as narrow strips. On close observation Kupffer's vesicles could be visualized. At this stage the internal yolk mass observed through the microscope assumes the shape of mango. This could be observed in 18 hours of development. The egg thus assumes 380 μm in size. By 24 hours of development the cephalic portion of the developing embryo becomes broader and the embryonic rudiment became distinct with two somites. Myomeres could be visualized prominently. Optic vesicles and auditory vesicles becomes prominent notochord develops visually and the embryo will possess a heart at this stage which starts beating. The egg assumes 430 µm in diameter. The somites become prominent and visible as strips along with the myomeres. Embryo encircles the whole yolk mass. The tail becomes three and starts lashing it within the fertilization membrane. The embryo occupied the whole portion of the egg which is 470 μm in size. The development stage is at the 38 hours of observation. Olfactory vesicle pits, and also auditory vesicles were prominently visible. Before hatching, the whole egg mass will be occupied by the embryo, which twitches within the egg shell, which is the fertilization membrane. The embryo will thus have a prominent head with eyes, auditory vesicle, a well developed two chambered heart and vitelline vessels. The tail will be healthy with notochord extending till the end. The yolk assumes a ball shape in between the head and the tail. The whole egg assumes a 530 µm size. This happens during 44 hours observations. By 48 hours of incubation about 90% of the eggs start hatching. As the twitching becomes vigorous, and due to some enzymes action the external egg shell, the fertilization membrane breaks and the young ones will be released on to the external medium. The larvae hatchlings will be having a long body of nearly 610 μm size and will be provided with a prominent head and a long tail. The yolk sac will be found next to the head. The mouth will be closed connected to the yolk sac. The yolk in the yolk sac serves as the food for the hatching till the mouth opens. The eye will be not that clearly found as black spot.

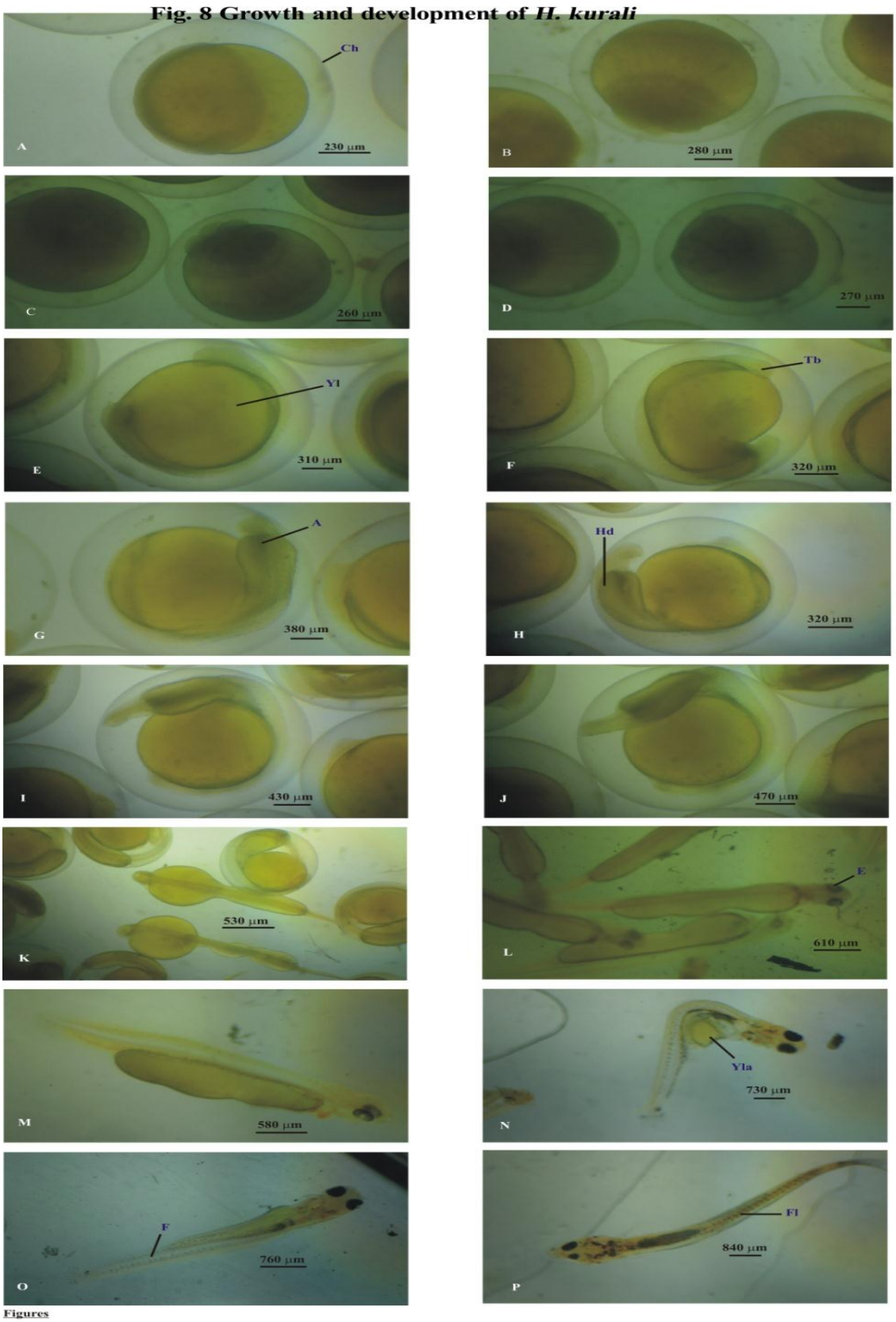

Fig.1. growth and development of *H.kurali*

DISCUSSION

Egg and larval development of cyprinid fishes are well studied and documented (Sado and Kimura 2002, 2005, 2006; Al-Hazzaa and Hussein, 2007), but no studies on the egg development and larval rearing was performed on *H.kurali*. The development of eggs according to time from fertilization time was observed and recorded following Ballon, 1975; Cerny, 1977). In this chapter a detailed description on the development of embryo to larvae and then to fry and fingerling of *H.kurali* are explained. The egg incubation period of *H.kurali* was 48 hours. In general the incubation period for eggs in cyprinids is comparatively shorter. So if the fishes are exposed to warm water before breeding, chances of formation of small sized eggs are about 80% (Kamler, 1992). Ecological niches also contribute to the variation in egg size between species (Calta, 1998). The egg diameter observed for *H.kurali* after fertilization and before fertilization as slightly higher compared to other barbus species (Cambray, 1983, 1985) where as in crucian carps the egg diameter of spawned eggs was 1.45 to 1.52 mm (Schaperclous, 1953). Same time the egg size matches almost with that of *Leuciscus cephalus* and *Cyprinus carpio* (Economou et al, 1991; Hoda and Tsukahara, 1971) and also *Chondrostoma nasus* (Keckeis et al, 2001). Variation in water quality standards like temperature and dissolved oxygen affect the size and incubation time of *H.kurali* eggs. Similar condition prevails among fishes quite commonly and is reported by Almatar et al (2000). The incubation time of crucian carp depends on temperature , four days at 20^0C and two days at 20- 27^0C (Laurila et al, 1987). For ornamental cyprinid like koi carps the temperature and dissolved oxygen observed during the study period conducted by Manikandavelu et al (2009) revealed that the values between tanks had not shown much variation. The temperature ranged from 27.2^0C to 28.7^0C and the dissolved oxygen estimated had shown a range of variation from 5.3 to 5.7 *mg/l*. Up to Manikandavelu et al (2009) the shower arrangement do so as to aerate the set up would have added on to reduce the variation in the water quality range. The incubation time for carp eggs is 14 to 18 hours at a temperature of 24 to 31^0C. It was observed that the incubation time for channa punctatus (Marimuthu et al, 2009) ranged from 24 to 28 hours at the water temperature of 29 ± 1.5^0C. The estimated maximum water temperature for *ex-situ* reproduction of critically endangered cyprinid fish *Achondrostoma occidentale* was reported to be 16 to 22^0C and the dissolved oxygen range between 8.7 to 11.00 mg/l (Gil et al, 2010). It was reported that in *salaria fluviatilis* the embryonic development lasted 12-14 hours at a temperature 20 to 21^0C (Fatima et al, 2010). Kline and Bonar (2009) reported the captive breeding of *Yaqui topminnow* and *Yaqui chub*. Yaqui topminnow held in aquaria produced only a few offspring (< 20

offspring/tank). So for large scale production they designed larger propagation pools. As they reported once the fishes acclimatized to the propagation pools Yaqui topminnow produced large number of offspring as long as the temperature in the pools remained above 21^0C. For the above said work temperature appeared to be the most important factor for triggering spawning. In nature the reproductive activity of Yaqui topminnow as low or not exist at water temperature of 12 to 16^0C, but fish were reproductively active in water that reached 19 to 22^0C (Galat and Robertson, 1992). For the present work, *H.kurali* produced more than 90% hatch at a temperature range of 21.1 to 22.5^0C. Yaqui topminnow also exhibited the closest range where the fishes showed optimal reproductive response at 21^0C or more under laboratory conditions and did not reproduced in the green house set up until the temperature exceeded 21^0C. But for the induced breeding of African mud catfish, *Clarias garipineus* the temperature to be maintained for better spawning and hatching was observed to be 24.5^0C (Olumuji and Mustapha, 2012). It was reported that the captive breeding success of silver sea bream, *Rhabdosargus sarba* relayed on temperature maintained between 21 to 23^0C. Crossland (1977, 1981) reported that snappers are serial spawners and spawn between Octobers to January when the water temperature is from 16 to 21^0C. But studies by Battaglene and Talbot (1992) reported that the eggs of snappers hatched successfully at 21.5 ± 1^0C. It was also reported that the larvae tolerated temperature as high as 31.5^0C. *Horabagrus brachysoma*, the golden catfish spawns and develops at a temperature between 23^0C and 28.4^0C (Padmakumar et al, 2011), which is comparatively higher to that of *H.kurali*. Being a catfish the temperature tolerance may be high, which may be the reason behind the hike in the incubation temperature too.

In the present study induced breeding trials was performed with ovaprim®. The hypophyzation resulted in failure as the fishes became aggressive and caused severe physical damage themselves which resulted in ultimate death. The behaviour of aggression is reported to be the common behaviour of cyprinids (Miller, 1964). Some fishes among cyprinids show there aggressiveness to attract females like that of *Notropis analostanus* , which produces sound by showing aggression which act as a clue for the female to identify the spawning time of male. The significance of sound production during the reproductive behaviour of *Notropis analostanus* (family cyprinidae) studied by John 1963. The sounds produced by fighting males increased the occurrence and average duration of the aggressive behaviour of reproductively mature males. The sounds produced when males court females increased the occurrence of aggressive behaviour between males, but did not increase the average duration

of aggressive behaviour. "Solo-spawning" increased when the courtship sounds were played back, even though no females were present. The courtship sounds when played back to 1 male and 2 females increased the occurrence and average duration, of courtship behaviour. The aggressive behaviour shown by the cyprinid fishes are their adaptation to give intimations to the opposite sex to know the onset of breeding cycle and spawning peak. It is a hormonal controlled act. In the percent study also the aggressive behaviour was a typical family character but as the fishes were induced with gonadotropins the rate of aggressiveness extended beyond the level of control which resulted in ultimate death.

CONCLUSION

Development of fertilized egg to a larvae and hatchling takes place in several steps. The development of fertilized egg starts with the formation of a fertilization membrane which is formed soon after the fusion of gametes. The gametic fusion and formation of a fertilization membrane by intake of water provide space for the internal cell mass along with yolk to develop into the ultimate larvae. The egg division starts from a single cell to a two celled stage which is followed by four celled, six celled and it extents until 64 celled stage. There after the cell division progresses fast with uncountable number of cells this gathers the shape of a mulberry fruit so the stage is named morula stage. After getting into morula stage the division progresses to blastula stage and gastrula stage. There after the germ layer formation takes place which will be further followed by the formation of head and tail buds. With time the optical vesicle and auditory vesicles emerges and develops. The head assumes its shape and so the tail. The whole development takes 48 hours to complete and starts hatching. By 38 hours the embryo heart beats and followed by that the tail starts moving. Later on by 40 hours the internal embryo starts twitching and by 44 hours the larvae use to rotate within the fertilization membrane in the previtelline space and finally by 48 hours the larvae breaks the membrane and jumps out as hatchling which will be 6 to 7 mm in length. The whole process requires 48 hours and the hatchling will be having a prominent head which is continuous with the yolk sac which acts as the feed for the development of the larvae for five to seven more days, which is the basic cyprinid character. The larvae develop day by day and by the seventh day of development the yolk gets completely consumed. The larvae gains the shape of the adult in its miniature size, but even the fins and scales were yet to develop. By the seventh day the larvae reach a length of 12 to 13 mm and after it start consuming live feed. Zooplanktons were provided as feed for the larvae. By the third fingerling stage the larvae gains a length of 29 to 34 mm. The fish larvae turn out to be fingerling which looks similar to

the adult in appearance not in size. The scales, gills, fins etc gets well developed. The tail pigmentation will become more prominent and body assumes silvery white appearance. By this size the fingerlings gets ready to lead an independent life at any provided favorable conditions.

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