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**Abstract**

This study presents detailed descriptions of the egg, embryonic, and larval development of the olive barb, *Puntius sarana sarana* (Hamilton), under laboratory conditions. Farm-reared broodfish of *P. sarana sarana* were induced to ovulate with ovatide. The ovulated females were stripped and the eggs were fertilized with sperm by the wet fertilization method. Fully swollen fertilized eggs were yellowish-white with an average diameter of 0.62±0.02 mm. Developing eggs were periodically observed under a microscope and the stages were photographed. Egg and embryonic development proceeded normally, showing distinct blastodisc formation into 2, 4, 8, 16, 32, 64, 128, and 256 cell stages, followed by morula, blastula, early gastrula, middle gastrula, late gastrula, yolk plug, pea shape, bean shape, embryo indication, and advanced embryo stages, up to newly hatched larvae. Newly hatched larvae averaged 1.903±0.002 mm in 26-27°C water, and early larvae development proceeded normally. The yolk sac was completely absorbed 58-72 h after hatching. Details of the embryogenesis and morphogenesis are discussed in the context of artificial spawning in the olive barb.

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Introduction

*Puntius sarana sarana*, a member of the Cyprinidae family, is commonly known as olive barb. The species is distributed throughout India and also found in Nepal, Bangladesh, Bhutan, Afghanistan, Pakistan, and Southeast Asia. It was abundantly available in rivers, streams, ponds, and ditches but natural populations have considerably declined due to anthropogenic activities.

*Puntius sarana* is a potential candidate species for introduction into the Indian major carp polyculture growout system (Jena et al., 2008). Induced breeding techniques using a synthetic inducing agent such as ovaprim and mass scale seed rearing of the species have been standardized (CIFA, 2007). However, the egg and embryonic development of this species was studied in detail and seed survival is poor (Chondar, 1999). Detailed knowledge of embryogenesis/early morphogenesis helps to determine which stages of the embryo or early larva development are critical, and to which the poor seed survival could be attributed.

Developmental studies have been reported for *Barbus lutes* (Hazzaa and Hussein, 2003), *Barilius canarensis* (Sado and Kimura, 2005), *Barbus grypus* (Sahinoz et al., 2007), *Labo bata* (Miah et al., 2009), and *Barbus barbus* (Lugowska, 2009), while more detailed studies have been reported in zebrafish *Danio rerio* (Kimmel et al., 1995) and goldfish *Carassius auratus* (Yamaha et al., 1999). Details of embryogenesis and early morphogenesis described in this study will help to build a framework for the culture of *P. sarana* in captivity. A thorough understanding of the embryonic and larval stages of *P. sarana sarana* will help to plan hatchery requirements that will ensure seed availability and improve fry survival in aquaculture as well as facilitate ranching in selected water bodies for its conservation.

Materials and Methods

The study was conducted at the College of Fisheries, Mangalore, India. Two sets of *P. sarana* (females 120 and 140 g; males 130 and 110 g) were induced to spawn with ovapride at 0.1 ml per female and 0.05 ml per male. The ovulated eggs were stripped, fertilized with normal milt by the wet method, and incubated in glass aquaria with aeration at the ambient temperature of 26-28°C. Development was observed every 10 min until completion of the morula stage and then every 45 min until hatching. Early larvae stages were observed when required. The stages were photographed using a computer assisted stereo microscope (Motic Image Plus, version 2). Before the formation of the embryo, stages were classified based on the number and arrangement of cells (blastomeres) and the extent of epiboly, characterized by the formation of the germ ring and the blastophore. After closure of the blastophore, subsequent stages were classified based on the degree of embryo growth including formation of the pigment, somites, and the separation of the tail from the yolk mass.

Results

**Egg and embryonic development.** The fertilized eggs were small (diameter 0.65±0.05 mm), adhesive, spherical, and transparent. The eggs had a prominent chorion, small perivitelline space, and uniformly distributed yolk (Fig. 1A). The newly fertilized egg remained in the zygote period until the first cleavage. Within 20 min after fertilization, eggs absorbed water, became swollen, increased in size, and attained a diameter of 0.80±0.07 mm.

The blastodisc formation occurred within 30 min (Fig. 1B), showing distinct animal (a) and vegetal (v) poles. Fertilization activated cytoplasmic movement and maternal factor cascades. The polarity of non-yolkly cytoplasm streams defined the animal pole and cleaved the blastodisc into two cells (Fig. 1C). The second cleavage occurred incompletely in a single plane to form four blastomeres after 55 min (Fig. 1D) and the third cleavage started after 65 min in two planes, leading to the formation of eight blastomeres (Fig. 1E). The fourth division occurred within 60-70 min along two planes,
Fig. 1. Stages of egg development in the olive barb, *Puntius sarana sarana* (minutes after fertilization in parentheses): (A) zygote (<30 min), (B) blastodisc (30 min) showing distinct animal (a) and vegetal (v) poles, (C) 2-cell stage (40-45 min), (D) 4-cell stage (55-60 min), (E) 8-cell stage (65 min), (F) 16-cell stage (60-70 min), (G) 32-cell stage (75 min), (H) 64-cell stage (80 min), and (I) 256-cell stage (105 min).

on either side, to produce a 4 x 4 array of cells (Fig. 1F). The fifth cleavage occurred after around 75 min when the blastomeres increased to 32 and were present in a 4 x 8 array (Fig. 1G). Cell division continued and the 64-cell stage formed after 80 min (Fig. 1H). Cells entering this stage were smaller but, from the side, the cell mound looked distinctly higher and, for the first time, some blastomeres completely covered others, forming a second layer above the first. Cleavage continued and a multilayered 256-cell stage occurred 105 min after fertilization (Fig. 1I).

Successive cleavages led to dome formation and the dome stage (early morula) was reached 2 h after fertilization (Fig. 2A). The late morula stage, characterized by less than half the yolk being invaded by the germ layer, was reached within 2.5 h (Fig. 2B). The blastula stage occurred after 4 h, during which the blastoderm became flat and began to spread on the yolk (Fig. 2C). As the thinning and spreading of the yolk syngiotic layer and the blastomeres on the yolk began, the blastoderm assumed the shape of a dome and
the epiboly stage was reached. In the late blastula stage, the blastodermal layer migrated further and reached the 30% epiboly stage during which the formation of the germinal ring was clearly visible (Figs. 2D, 2E).

The gastrula stage was marked by a germ ring (7.5 h) covering more than half the yolk (Fig. 2F). As the epiboly stage continued, the morphogenetic cell movements of extension involution and convergence occurred, producing primary germ layers; 50% epiboly was achieved and a thickened marginal region termed the germ ring appeared almost all around the blastodermal rim. As the convergence movements rapidly continued, cells accumulated near the germ ring (70% epiboly), in which both the epiblast and hypoblast were locally thickened, giving rise to the embryonic shield (Fig. 2G). The epiblast is the surface layer that gives rise to the ectoderm; the yolk syncytial layer is a layer of yolk syncytial nuclei populating the cytoplasm beneath the blastoderm. The hypoblast forms next to the yolk syncytial layer and becomes endoderm and mesoderm. Successive cleavages gave rise to 80% epiboly or the L-stage (Fig. 2H). At about 90% epiboly, a yolk plug was generated by the uncovered yolk cell protruding from the neighborhood of the vegetal pole and was characterized by the complete invasion of the yolk by the germ layer (Fig. 2I). This stage was observed between 10 and 12 h. The dorsal side of the blastoderm appeared thicker than the ventral side.
About 25 min after the yolk plug closure, a tail bud develops on the posterior region of the embryonic axis and protrudes well beyond the yolk (Fig. 3A). About 40 min after the yolk plug stage, a prominent head fold is visible; myomers begin to form and the embryo covers most of the yolk; the near end of the blastophore becomes the tail while the end away from the blastophore becomes the head of the embryo; during this stage,

**Fig. 3.** Stages of egg development in the olive barb, *Puntius sarana sarana* (hours after fertilization in parentheses): (A) two somite stage (11 h), (B) five somite stage showing (11.5 h) head (h) and notochord rudiment (n), (C) eight somite stage (12 h) with optic vesicle (Ov), (D) ten somite stage (12.5 h), (E) thirteen somite (13.5 h) stage with optic primordial (OR) and Kupffer's vesicle (KV), (F) fifteen somite stage (14 h), (G) twenty somite stage with optic placode (OP), (H) twitching embryo (16 h), (I) ready to hatch embryo with vigorous twitching (19-20 h), (J) freshly hatched embryo (22 h).
the number of somites increases from two to five; the notochord rudiment appears on the central axis and the head and tail differentiate (Fig. 3B). In the eight somite stage, the optic vesicle (rudimentary) appears on the head, the embryo assumes a ‘pea shape’, and slight movement starts appearing in the embryo (Fig. 3C). About 12 h after fertilization, the number of somites increases to ten (Fig 3D). The Kupffer’s vesicle is seen on the side of the tail (Fig. 3E). The yolk takes a bulbous shape and there is a further increase in the number of somites (Fig. 3F). Between 12-13 h after fertilization, the posterior part of the yolk starts narrowing and the embryo takes a more or less ‘kidney bean shape’ (Fig. 3G). At 16 h, the tail elongates, the somites increase in number, and occasional faint twitching movements are seen (Fig. 3H). During the next 3-4 h, gill rudiments and fin buds occur and the embryo further increases in size. Within the following hour, the heart appears and the twitching movement becomes rapid (Fig. 3I). The posterior part of the yolk further elongates, the number of somites increases, twitching movements are vigorous, and the embryo is ready for hatching (Fig. 3I). The hatching process is completed by 22 h after fertilization. The yolk is bulbous anteriorly and tubular posteriorly in newly hatched larvae (Fig. 3J).

Larvae development. Morphological changes in the embryo after hatching (ambient temperature 27°C) follow. Newly hatched larvae are slender with an elliptical yolk sac that is highly bulbous in the anterior and a blunt tapering in the posterior end. Hatchlings are almost sessile and seldom move quietly. Freshly hatched larvae are 1.903±0.002 mm in length (Fig. 4A). At 2 h after hatching, there is a slight decrease in the yolk sac, the head is slightly straightened and lifted, and the larva is 1.97±0.002 mm (Fig. 4B). The embryo becomes less transparent and slightly darkened but no melanophores are visible, the yolk sac is partially reduced, and larvae increase to 2.04±0.002 mm (Figs. 4C, 4D). At 7 h after hatching, 2-3 melanophores are visible in the head region, the yolk sac becomes more slender, the eyes and anus are slightly visible, and the embryo becomes stouter on the anterior than on the posterior end (Fig. 4E).

Fig. 4. Stages of larvae development in the olive barb, Puntius sarana sarana (after hatching in parentheses): (A) one min after hatching, (B) larva (2 h), (C) larva (3 h), (D) larva (5 h) (E) larva (7 h), (F) larva (9 h), (G) larva (13 h), (H) larva (15 h), (I) larva (20 h), (J) larva (25 h), (K) larva (32 h), (L) larva (42 h), (M) larva (72 h), (N) fry (15 days), (O) fry (20 days).
At 9 h, the posterior part of the yolk sac is further reduced and ends in a point, chromatophores appear in the eyes, the eyes are clearly visible, a pulsating heart is prominent just beneath the yolk sac, and the larvae reach 2.21±0.004 mm (Fig. 4F). By 13 h, more melanophores appear on the head and trunk, brain lobes are clearly visible, the mouth appears, and the larvae reach 2.59±0.002 mm (Fig. 4G). At 15 h, the yolk sac becomes thinner, the number of melanophores on the head further increases, and reddish coloration can clearly be seen in the opercular area (Fig. 4H). At 20 h, the yolk sac is almost slender, there is huge melanization in the eyes and the periphery of the yolk sac, the pectoral fin buds become longer, and the eyes are prominent (Fig. 4I).

At 25 h, prominent and well-developed pelvic and pectoral fins are present, the eyes increase in size and are darkly pigmented, and the larvae reach 2.95±0.04 mm (Fig.4J). At 32 h, the number of melanophores on the head and trunk further increase and opercula can be seen just a little posterior to the eyes (Fig. 4K). At 42 h, the opercle is prominent, the air bladder is elliptic, a pulsating heart can be seen just beneath the yolk sac, and the larvae reach 3.72±0.12 mm (Fig. 4L). The yolk sac is completely absorbed by the end of the third day and the larvae start feeding on external feed.

At 72 h, the larvae swim actively and the air bladder is conspicuous; the larvae are blackish grey and 4.25±0.04 mm (Fig. 4M). By 15 days, the eyes are well developed and melanophores are distributed throughout the body (Fig. 4N). By 20 days, the larvae have grown in size; the pigmentation intensifies and larvae are black grey (Fig. 4O). The larvae actively consume natural as well as artificial feed and reach maturity at the end of the first year (Fig 5).

Fig. 4 (cont.).

Fig. 5. Six-month old olive barb, *Puntius sarana sarana*. 
Discussion
Hormone induced spawning of *P. Sarana sarana* greatly helped carry out a detailed investigation into the egg, embryonic, and early larval development of the olive barb, *Puntius sarana sarana*. Since olive barb eggs are transparent and have distinct developmental stages and a short incubation period, the stages could be easily detailed.

The diameter of the eggs after fertilization ranged 0.65-0.70 mm, while it ranged 0.7-0.8 mm in *Labeo bata* (Miah et al., 2009) and 4.1-4.8 mm (avg 4.4 mm) in the Indian major carp, rohu *Labeo rohita* (Chakraborty and Murty, 1972). The variations in egg diameter among carp species may be due to the brood size and the species.

The staging of *P. sarana sarana* embryogenesis and morphogenesis could be divided into stages based on morphological changes during development. The morphological changes were comparable with those described for *Danio rerio* (Kimmel et al., 1995), medaka *Oryzias latipes* (Iwamatsu, 2004), *L. bata* (Miah et al., 2009), Cape bonnetmouth *Emmelichthys nitidus* (Neira et al., 2008), and common carp *Cyprinus carpio* (Haniffa et al., 2007).

Early larvae of *P. sarana sarana* possess a large yolk sac, an almost transparent moderately elongated body, no body pigmentation, and prominent eyes until 6-7 h after hatching. The developmental stages followed closely those of *L. bata* (Miah et al., 2009) and the early stages resemble those of zebrafish, except for the time taken to reach each stage of development, which is mostly attributed to a higher ambient temperature.

Morphologically, *P. sarana sarana* larvae show similar morphogenesis as other cyprinids (Hazaa and Hussein, 2003). First feeding began 60 h after hatching, compared to 66 h in *L. bata* (Miah et al., 2009). Commencement of first feeding varies between species depending on the amount of yolk present in a particular species.

The present study provides a detailed description of egg, embryonic, and early larval development of *P. sarana sarana*, an economically important fish species of the Indian sub-continent. The study reveals that the olive barb has short incubation and yolk-sac absorption periods which would help reduce mortality in large scale seed production.

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References


