CHAPTER 5

DEVELOPMENTAL BIOLOGY OF
PUNTIUS POOKODENSIS

5.1 INTRODUCTION

Information on early embryonic and larval development and organogeny is of critical importance in understanding the basic biology of a particular species and their dietary needs and environmental preferences (Koumoundouros et al., 2001; Borcato et al., 2004). Further, studies on embryonic and early larval development are imperative and consequential to the successful rearing of larvae for large scale seed production and aquaculture (Khan and Mollah, 1998).
5.2 MATERIALS AND METHODS

The specimens of *Puntius pookodensis* for the study were obtained from its natural habitats in the Pookode lake, Wayanad district, Kerala. After collection fish were transported to the laboratory in the College of Fisheries, Cochin. The eggs were obtained as a result of different breeding techniques applied as mentioned in chapter 4. The fertilized eggs were collected, using fine tipped droppers, soon after it was spawned. From each clutch 5 to 10 eggs were collected and placed them in small containers of 2 lit volume. Washed thoroughly to remove the debris and other adhered particles and kept in a petri dish containing small quantity of water for further studies. After observations, embryos were placed back into their individual containers. Developing eggs were observed with a trinocular microscope (Labomed) and photographs were taken with SLR camera (Nikon 90 X). The early developments up to hatching of the eggs were done in one hour interval. After hatching the developmental stages were photographed at every 2 hours up to 24 hours and thereafter at every 24 hours up to the juvenile stage. The left sides of the larvae were photographed, to exclude the variations in measurements due to asymmetrical growth. All the measurements were taken under the average room temperature of 28 to 29 °C. The eggs were placed in cavity slides, immersed in water for observations and cavity blocks were used to observe larvae after hatching. The sampled eggs and larvae were fixed in 4 % formalin for further observations.
Measurements

Only a limited set of morphological landmarks were accessible for repeated non-invasive measurements. Here the following measurements like (1) diameter of the fertilized ova (2) total length of the embryo i.e., the length from the snout to the tip of the tail as seen in lateral view (3) number of somites.

In the present study the developmental stages were divided into embryonic development, larval development and post larval development. The embryonic development started inside the chorion and completed at hatching. The larval stage started from hatching and ended by the appearance of fin rays in all fins. After that, the larva was transformed into post larvae. Time course data of the embryonic development is essential for ageing eggs. Given the egg ages, it is possible to estimate not only spawning time but also egg mortality (Uehara and Mitani, 2006). The development of 25 nos on individual embryos was documented right from fertilization.

5.3 RESULTS

5.3.1 Embryonic development

Immediately after fertilization the eggs were swollen up considerably by absorbing water and within five minutes they attained a spherical, transparent and slightly adhesive structure. A streaming movement of the egg protoplasm took place, which resulted in the formation of blastodisc. The fertilized eggs of P. pookodensis were amber coloured en mass, less yolked, glossy, translucent and spherical with an average diameter of 0.62 mm (0.62 ± 0.02 mm). Like most other cyprinids the eggs of P.
*pookodensis* were slightly adhesive, free and demersal. The location of the micropylar region was distinct as a small depression in the animal pole, while it was absent in unfertilized eggs. The yolk which often had a yellowish tinge was coarsely granulated. The eggs were easily collected and transferred for incubation in hatching tanks with continuous oxygenation. The observations revealed that the hatching of eggs accomplished 23 to 27 hrs in the ambient temperature of $28^0\text{C}$ ($28 \pm 2^0\text{C}$). Neutral pH was maintained for the medium throughout the studies. After observations, some of the eggs were preserved in 5% formalin for future studies. All measurements were made from fresh specimens using a calibrated ocular micrometer. They did not have any oil globule. The embryonic stages from fertilized eggs to hatching are summarized in table 5.1.

The fertilized egg was telolecithal and cleavage was meroblastic. The blastoderm formed was restricted to animal pole at the point of entrance of sperm at the level of the micropyle, leaving large yolk mass at the vegetal pole. The first cleavage was meridional and incomplete. The second division was at perpendicular to the first and the third division resulted in the formation of 8 cells. The 4th cleavage resulted in the formation of sixteen celled stage at 1.3 hrs and formation 32 celled stage occurred at about 1.45 hrs. A clear blastocoel began to appear at about 3.3 hrs and the blastula at this stage appeared as a cap of cells over the yolk. By 4 hrs it started to roll over the cytoplasm. After 4.5 h, the blastoderm covered more than half of the yolk surface. At about 5.5 hours the early gastrula stage was reached and an embryonic shield was appeared. Gradually, epibolic germ layers were spread to the equator of the spherical yolk surface and at 6 hrs, the germ ring invaded 3/4th of the yolk surface. At 6.5 hrs,
the neural plate was formed and gradually almost 5/6th of the yolk surfaces become invaded. As the blastopore got closed, yolk plug was projected and the head rudiment was seen lifted up. By 8 hrs, the optic rudiment appeared and gradually by 9.5 hrs it became differentiated into a vesicle. At this stage, the head and tail got differentiated and the myotomes also became clearly visible. At 12 hrs the tail bud was formed and the embryo appeared very much elongated and was seen encircling over the yolk, reaching nearly 3/4th of its circumference. At 15 hrs, caudal fin fold rudiment was drawn out from the yolk and the head region became more and more differentiated. Yolk sac stretches and assumes a characteristic beaked appearance. Tail bud was projected out from the beak like yolk mass distally at around 16 hrs. Paired somites also became distinct at this point of time. At 18 hrs, optic vesicle became conspicuous and the head region got separated; the caudal fin fold became very much elongated and the embryo appeared ‘C’ shaped encircling the yolk. At this period, the muscular somites were seen twitching at intervals. At 21 hrs the heart and optic capsule became conspicuous and embryonic movement became rapid. The heart began to pulsate at 21.5 h. At 22 hrs, tail got free and encircled almost 90 percent of the yolk mass. Gradually the heart pulsation became more rhythmic. The embryo began to roll within the egg case. As the development advanced, the embryo appeared more and more elongated and the tail overlapped the head. The myotomes and auditory vesicle became more prominent and the twitching of embryo started within the cytoplasm. As time passed the twitching movement of the embryo became faster.
Table 5.1

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<th>Time after fertilization (Hours)</th>
<th>Developmental event</th>
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<tr>
<td>01.00</td>
<td>16 celled stage</td>
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<tr>
<td>01.30</td>
<td>32 celled stage</td>
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<tr>
<td>02.00</td>
<td>Early morula</td>
</tr>
<tr>
<td>02.30</td>
<td>Blastulation; Blastodisc formation</td>
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<tr>
<td>04.00</td>
<td>Gastrulation; early gastrula</td>
</tr>
<tr>
<td>05.00</td>
<td>Late gastrula</td>
</tr>
<tr>
<td>05.30</td>
<td>Neurula stage</td>
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<tr>
<td>07.00</td>
<td>Closure of blastopore / Yolk plug</td>
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<tr>
<td>09.30</td>
<td>Optic rudiment appears</td>
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<tr>
<td>12.00</td>
<td>Formation of head and tail, Appearance of myotomes</td>
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<tr>
<td>17.00</td>
<td>Tail region detaches from the yolk</td>
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<tr>
<td>18.00</td>
<td>Twitching</td>
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<tr>
<td>21.30</td>
<td>Heart beat and blood flow starts</td>
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<tr>
<td>23.00 – 24.00</td>
<td>Hatching</td>
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Close to hatching, twitching and lashing of embryo inside the egg capsule became rapid. The egg shell was broken up and the tail emerged out first, followed by the head region. Hatching occurred at around 23.5 hrs. Egg hatching was protracted and the incubation period fluctuated between 23 and 27 hrs post fertilization at the ambient temperature.
5.3.2 Larval and post-larval development

Newly hatched larva (Plate 5.1, Fig.5)

The newly hatched larva appeared to be sluggish and seen attached to any object available in the tank by means of its cement organ. It was transparent without any pigmentation. A continuous fin fold was present starting from the start of the dorsal fin, surrounding the tail and ended in the insertion of the ventral fin. Oral and anal orifices were completely absent. The length of the hatchling at this stage was 2.3 mm.

Hatchling 24 hours old (Plate 5.1, Fig.7)

In 24 hours it attained a mean length of 2.6 mm. Melanophores started to appear on the optic rim and myotomes.

Hatchling 48 hours old

At 48 hours, the larva appeared slender and elongated. The yolk was very much reduced, though not completely exhausted. The hatchlings gradually began to swim up towards the water surface and sometimes found hung up from the water surface. Melanophores became conspicuous on the body surface. Fin rays were started to appear in the pelvic fin and stomach was visible through the transparent body. Above the stomach the gas bladder appeared as a glittering droplet. A small depression was started to appear on the fin fold at the portion of anus.
Hatchling 36 hours old

By the completion of 36 hours the yolk was completely used up and the hatchling started its external mode of feeding. Fully functional mouth was visible. Food particles appeared inside the stomach. Fin rays were appeared in the caudal fin, anal fin and dorsal fin. The distribution of melanophores became more extensive throughout the body.

Hatchling 48 hours old

The larva reached a total length 7.00 mm. Mouth was well developed and lips became distinct. Anterior profile of yolk sac was convex. Dorsal embryonic fin fold commenced from above the constriction of the alimentary canal, anal fin originated below the same level. A single chambered air bladder was visible. A few faint black pigmented spots appeared on the head. Eyes became blackish and pectorals were prominent. Gill arches well developed. Fin rays were present in dorsal and caudal fins.

Hatchling 72 hours old

The larva reached a total length 7.2 mm. They had slightly yellowish colour. Lower lip became mobile. Air bladder became elliptical in outline and blackish. Dorsal and ventral fin folds commenced almost from the same level. Alimentary canal became visible as a straight tube. Black chromatophores were distributed from the inter orbital space up to auditory vesicle. Eyes were deep black in colour. Fin rays were completely grown in all the fins.
Hatchling 96 hours old

By this time the hatchlings entered into the next stage of growth known as post larva. Total length was 7.4 mm. Opercular edge became distinct. Lips were well developed. Prominent black chromatophores became visible on the head and body. Notochord underwent flexion.

Hatchling 7 days old

At this stage the total length was 8 mm. Body colour changed into slightly greenish, lips became thick. A median constriction appeared on the elliptical gas bladder separating it into anterior and posterior lobes, Hind end of the notochord were seen bent upwards. Head became well differentiated. Alimentary canal was started to coil. Prominent black chromatophores were visible on the air bladder and head.

Fingerlings (25.2 mm)

At this point the body colour became greenish yellow with golden reflections on dorsal profile and bright silvery below. Opercles were silvery with a pinkish tinge. Fins were hyaline, scales silvery, minute, prominent, uniform and fully covering the trunk. Minute black chromatophores became visible on the dorsal fin rays.

5.4 DISCUSSION

Nothing is known about the embryology of *Puntius pookodensis*, because it has recently become known to science and this is the pioneer work on it. Although embryonic development of many cultivable and food fishes have been carried out extensively, little attention has been paid to fishes like *P. pookodensis* who showed extremely narrow distribution in their natural habitats. The fish is a recently described
new species (Anna Mercy and Jacob, 2007). In a broad sense the importance of small fishes in an ecosystem has extreme importance. The eradication of such a species from a system could hamper the existence of the whole ecosystem and cause serious injuries. The very limited distribution of the species intended the importance of the particular geographic area thereby by opening scope for further studies. This factor may sometimes play roles in the process of evolution and speciation.

The knowledge on different embryonic stages and its timings of developmental events has importance in developing hatchery techniques of a species. As far as *P. pookodensis* is concerned, because of its special conservation status and limited distribution, defining an effective hatchery technique is tremendously valued. Captive breeding and reintroduction to its natural habitats in one of the steps in conserving a species (Nielsen, 1994). In general, embryonic and larval developmental period of a species of fish consisted of embryonic, larval and juvenile stages. The morphology of larval and juvenile *P. pookodensis* viz., overall appearance, fin ray formation, and pigmentation patterns were similar to that of many small cyprinids as reported by (Jones, 1938; Kimmel *et al.*, 1995; McClure, 1999; Balinisky, 1948).

Many developmental events proved that *P. pookodensis* obeyed the general rules of cypriniform development. Likewise in many cyprinids, *P. pookodensis* developed a specialized cement organ at the tip of the snout, by means of which the hatchlings were attached to the substrata (Balinisky, 1948; Sado and Kimura, 2006; Jenkins and Burkhead, 1994). The eggs were semi adhesive in nature. A bi-lobed gas bladder was also noticed.
The equatorial cleavage appeared to start only at 32 celled stage and the subsequent cleavages resulted in the formation of a multi-celled morula at 2.00 hours. After initial cleavage, the morula entered the blastula stage wherein the central cells formed the blastodisc, while the marginal cells got merged with yolk and formed yolk syncitial layer as reported by (Kimmel et al., 1995). Epiboly was initiated when both the blastodisc and yolk syncitial layers started thinning and expanding, and the blastoderm gradually engulfed the yolk sac (Cardoso et al., 1995). Engulfment of the yolk continued to the gastrulation period during which embryogenesis started. Germ ring was formed as a thickening of the blastoderm margin involuted and differentiated into two layers, the superficial epiblast and the deeper hypoblast. The hypoblast gave rise to notochord and the mesoderm whereas the epiblast differentiated into ectoderm.

In *P. pookodensis* hatching occurred in the 24\textsuperscript{th} hour post fertilization at the ambient temperature of 28 °C. In most cyprinids the incubation period lies in the range of 24 to 36 hours in tropical climate. In many cases it depended largely on the diameter of the egg, ie, the species of fish which produced smaller eggs showed a shorter incubation period compared to the one which had larger eggs. In both the cases, the growth phase after the completion of the organogenesis inside the eggs showed variations. After the completion of organogenesis the embryo increased in volume inside the egg membrane until it was enough to break the shell. The fishes, which have larger eggs it needed longer duration to attain the point of hatching. In *P. pookodensis* the average incubation period was around 24 hours, which showed the fish had a smaller egg than many other cyprinids like *Garra mullya* (Anna Mercy et al., 2005a, b) and
Rasbora dniconius (Premkumar, 1985). The twitching movements of the embryo also caused the egg case to break.

However, the development of the embryo and the variability of hatching time in fertilized egg of most of the fish is generally influenced by the temperature of water (Jhingran, 1983; Rahman, 1975). Evidently, the size of the yolk largely determined the duration of the free living and pre larval periods of fish species (Kamler et al., 1994).

**Larval rearing**

The newly hatched larva of *P. pookodensis* has an average length of 2.3 mm. The size of the newly hatched out larvae showed wide variations among species in the family cyprinidae. It also depended on the size of the egg and length of incubation period. In a cyprinid fish, *Inlecypris auropurpureus*, the newly hatched larva has a length of 3.5 mm and has an incubation period of 49-56 hrs (Sado and Kimura, 2006). While in *Barbus harpeyi*, the incubation period was 70-79 hrs and the length of the newly hatched larva was 4.7 to 5.5 mm in length (Mukhaysin and Jawad, 2012). Larger eggs gave rise to longer larvae. Many factors including species, strain, temperature variations, egg size, fertilization rate and incubation procedures affect the duration of development, survival and initial size and growth of larvae or alevins (Dumas *et al.*, 1995; Jonsson and Svavarsson, 2000). The larval phase began with hatching and lasted until metamorphosis (Helfman *et al.*, 1997) and is regarded as the fundamental stage of life history. However, the innate behaviour of the newly hatched larvae differs widely among species.
The most important bottle neck of larval rearing programme is connected with its exogenous feeding. The point of time when the larva started its exogenous feeding and the optimum size and type of the food it could consume were the main issues (Blaxter and Staines, 1971). The size of the gape of the mouth has great concern. The nutritional requirement of the larva at this stage should match the composition of yolk that caters the pre feeding fish. The gape of the mouth opening of the hatchling apparently determines the size of the food particle accepted by the larvae (Joseph, 2001). The time at which external food is first given to the larvae influenced their subsequent growth and survival (Kamler, 1992).

Unlike some fishes like transparent goby (Gobiopterus chuno), where the ruminence of gas bladder is present in the newly hatched embryo (Sunobe, 1995), while in P. pookodensis the gas bladder made their appearance 48 hours after hatching. It could be probably because of the short incubation period of P. pookodensis compared to the transparent goby, Gobiopterus chuno. While in most cyprinids the gas bladders made their appearance only after a few hours post hatching (Sado and Kimura, 2006). The newly hatched embryo did not possess any melanophore, while it is reported in round herring, Etrumeus teres, before hatching at the 48th hour of development within the egg (Uehara and Mitani, 2006). The shape of the eggs found to be spherical, which is a generalized feature of the cyprinids as it is reported in species like Barbus trevelyani (Cambray, 1985), Danio rerio (Matthiasstrack and Kaischmidt, 2004) and also it implies the free (non or slightly adhesive adhesive) nature of the egg. The oval shape is reported in fishes with strong adhesive property as in Etroplus suratensis (Bindu,
2006), Gobiid fishes (Sunobe, 1995). From the above observations it could be assumed that size of the eggs have positive influence on the length of the incubation period.
### Plate 5.1 Developmental stages of *Puntius pookodensis*

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<td><img src="image1" alt="Fertilized eggs" /></td>
<td><img src="image2" alt="Embryo, 9 hrs after fertilization" /></td>
<td><img src="image3" alt="Embryo, 20 hrs after fertilization" /></td>
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<td><img src="image5" alt="Newly hatched out larva" /></td>
<td><img src="image6" alt="Larva 5 hours post-hatching" /></td>
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<td><img src="image7" alt="Yolk sac larva 24 hrs after hatching" /></td>
<td><img src="image8" alt="Larva one week after hatching" /></td>
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**Fig. 1** Fertilized eggs  **Fig. 2** Embryo, 9 hrs after fertilization  **Fig. 3** Embryo, 20 hrs after fertilization  **Fig. 4** 23 hours old embryo just before hatching  **Fig. 5** Newly hatched out larva  **Fig. 6** Larva 5 hours post-hatching  **Fig. 7** Yolk sac larva 24 hrs after hatching  **Fig. 8** Larva one week after hatching

**Abbreviations:**
- AL: Alimentary canal
- AV: Auditory vesicle
- BR: Brain
- EM: Embryo
- EY: Eye
- FE: Fertilized eggs
- FF: Fin fold
- FR: Fin rays
- GB: Gas bladder
- HE: Heart
- MO: Mouth
- MY: Myomeres
- OG: Oil globule
- YO: Yolk