

Embryonic and larval development in the freshwater angelfish (*Pterophyllum scalare*)

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Abstract The freshwater angelfish, *Pterophyllum scalare* like many other cichlids show parental care of embryos and larvae. This study was carried out to investigate the embryonic development of *P. scalare*, which shows biparental care and substrate brooding. During the study adult reproductive behavior and parental care was observed. Once the eggs were fertilized upon spawning, the early and later embryonic stages were observed, documented and various embryo length measurements were analyzed to characterize the developmental pattern in this species. Whole mounts and bone and cartilage measurements of acid free double stained larvae were analyzed to further understand the developmental rates in the hatched larvae. The developmental events were compared with those of other documented cichlid species as well as with the zebrafish, *Danio rerio* (Family Cyprinidae), which does not show parental care. After fertilization, cleavage division of the *P. scalare* embryo starts 1.30 hours post fertilization (hpf). On average, cleavage, blastula, gastrula, segmentation and pharyngula periods of embryogenesis are observed for approximately 3 ½, 11 ½, 9 ½, 36 and 12 hours respectively. Ultimately, *P. scalare* embryos hatched around 72 (hpf). Head Length, dorsal, caudal and anal fins show positive allometric growth while body depth and digestive tract show almost isometric growth. The study highlights that similar to a few other studied cichlids, *P. scalare* embryogenesis and larval development occur at a slower rate of development compared to *D. rerio*. In cichlids including *P. scalare*, parental care may allow these embryos the luxury of developing at a slower rate whereas the lack thereof for *D. rerio* embryos may necessitate faster development.

Keywords: *Pterophyllum scalare*, egg, embryo, development, fish larvae

INTRODUCTION

Even though, for many cultured fish, sufficient information on their reproductive behavior is available their embryonic development at often times is ignored. However, an understanding of embryonic development is required not only for captive breeding in aquaculture but also for various experimental studies in areas such as developmental biology, neurobiology, toxicology and pathology (Rahman et al. 2009). For example, the zebrafish, *Danio rerio* is a model organism widely used in vertebrate developmental studies. This species in addition to a few other species such as the rainbow trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*) are among the few species of fish where embryonic development has been studied in detail. Family Cichlidae is one of the largest and one of the most successful groups of bony fish consisting of about 1,300 species (Nelson 2006). Many members

of this family such as freshwater angelfish (*Pterophyllum scalare*), Oscar (*Astronotus ocellatus*), Discus (*Symphysodon* sp.), Blood red parrot fish (Hybrid), Ram cichlid (*Mikrogeophagus ramirezi*), *Cichlasoma dimerus*, Golden eye cichlid (*Nannacara anomala*) and Humped-head cichlid (Hybrid) are widely used in ornamental fish industry and they are among the most popular ornamental fish species cultured in many countries. These fish are relatively cheap and can tolerate a range of physiochemical conditions. Almost all freshwater cichlids provide parental care for their offspring. They display uniparental or biparental care by either mouth brooding or by guarding nests or egg clutches that are attached to external substrates, referred to as substrate brooding (Goodwin et al. 1998).

Among the cichlids, the Freshwater angelfish, a native of tropical South American waters is considered a relatively cheap, ideal community tank fish that has become a popular aquarium fish in



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many countries around the world. This fish comes in several colors and forms such as Cobra angelfish, black zebra angelfish, half black angelfish and Koi angelfish (Padilla and Williams, 2004). The reproductive behavior of this species is relatively well documented. They show biparental care where both parents are involved in substrate brooding. However, similar to many other species their embryonic development is often overlooked (Cacho et al. 2007). Hence, the present study focused on understanding the embryonic and later development of freshwater angelfish as an example of a cichlid that demonstrate both substrate guarding and biparental care.

MATERIALS AND METHODS

Reproductive behavior and embryonic development of freshwater angelfish

After introducing several freshwater angelfish into a 1 x 2 m tank, they were allowed to pair. Once the fish paired, three pairs were used to study their reproductive behavior. Each pair was put into a separate 100 liter tank with gravel-filtered water. The fish were fed twice daily. A clean, longitudinally split PVC tube (about 20 cm long) was placed at an angle of 45° to the floor of the tank as a spawning substrate. Once egg laying was over, a plastic pipette was used to suck several embryos from the PVC surface to study different stages of embryonic development. The embryos were observed under a Leica dissecting microscope and photographed using a Nikon digital camera mounted on the microscope. In addition, embryo measurements were taken using a Zeiss Primo star inverted microscope with the aid of Zen 2012 software. The length and width of the embryo, length and width of the yolk and the height of the blastoderm were measured for all early stage embryos before hatching. Once the embryos hatched the total length, length and width of head and the yolk sac, lengths of tail, trunk, anal fin, caudal fin and dorsal fin were measured. This data was analyzed and compared using MS-Excel 2010 and Minitab 16. The mean, standard deviation, standard errors of the measurements were calculated for each stage.

Embryo fixation

All embryonic stages were fixed in 4% paraformaldehyde for whole mount preparation and bone and cartilage staining. Later developmental stages from seven to 21 days post fertilized were first anesthetized using MS222 before fixing in 4% paraformaldehyde overnight at 4°C. All fixed embryos were stored at 4°C until further analysis.

Embryo Staining

a. Acid free double staining

An acid free double staining procedure based on Walker and Kimmel (2007) was utilized to study cartilage and bone development in later stages. Photographs and measurements of bones and cartilage were taken using a Zeiss Primo star inverted microscope or a Leica Dissecting microscope attached with a Nikon digital camera.

b. Whole mount staining

Whole mount staining with Borax Carmine of later developmental stages was performed to study the morphology. Embryos fixed in 4% paraformaldehyde were transferred into distilled water and 50% alcohol for 15 minutes each to remove excess fixative. Next, embryos were stained in Borax Carmine for 15 minutes and transferred to 50% alcohol for 2 minutes. Embryos were washed in 70% alcohol for 2 minutes and destained in acid alcohol for 2 minutes. Incubation in Ammonia alcohol for 2 minutes was used to stop the reaction. Next, the embryos were dehydrated in 90% alcohol followed by several washes in Absolute alcohol. Finally, the embryos were cleared in clove oil and observed under a light microscope.

DATA ANALYSIS

Larval body measurements were used to determine the pattern of development in relation to body length of *P. scalare*. The allometric patterns of development of *P. scalare* were determined by the growth coefficient using the equation $Y = aX^b$, where Y is the dependent variable/measured character and X is the independent variable/total length (TL), a is the intercept and b is the growth coefficient. When isometric growth occurred, $b = 1$, whereas either positive or negative allometric growth is indicated if $b > 1$ or $b < 1$.

RESULTS

Reproductive behavior and parental care in P. scalare

Before the onset of egg laying, the male and female that were allowed to pair beforehand followed ritualistic pre-mating as well as mating behaviors. Reproductive tubes were clearly visible in both male and female a few days before spawning. Usually, three or four hours before mating the male and female fish cleaned the artificial substrate provided (a PVC tube) using their mouth for nearly 1-2 hours. They spawned approximately between 12.00 noon and 3.00 pm and they were able to spawn regularly at 10-day intervals as long as the previous egg clutch was removed immediately after they end spawning. When the female started egg laying the male followed the female and fertilized the eggs. This was observed for nearly 45 to 60 minutes. Next, the male and female fanned the eggs and removed possibly unfertilized and damaged eggs. Both parents showed parental care. After the embryos hatched, 3-4 day old fries were taken into the mouth of the parents and deposited on nearby aquatic plants.

Early embryonic development

Embryonic stages from the zygote up to the gastrula stage were considered under early embryonic development. As soon as a sperm fertilizes the egg, the 1-cell stage zygote forms and embryonic development starts at the animal pole (AP) of the embryo. The zygote of freshwater angelfish was observed for about 90 minutes after fertilization (Table 1). These eggs are oval-shaped, slightly pale in colour and full of yolk-granules (Figure 1A). The zygote diameter along the horizontal axis is about 1.5 ± 0.05 mm.

Typically, 2-cell stage to 64-cell stage is referred to as the cleavage period. Similar to other fish, *P. scalare* cleavage planes were incomplete, restricted to the animal pole cytoplasm where there is no yolk. The cleavage period in freshwater angelfish embryos was observed for nearly three hours with 30-40 minute intervals between cleavage events (Table 1, Figure 1B-G, Figure 2).

The blastula period of fish generally includes stages from 128-cell to dome stage. During the blastula stage, the number of blastomeres increased but the size of the blastomeres decreased. Resulting blastomeres over laid the yolk at the AP. About 7 hpf, the shape of the blastomere layer changed from “high” to “sphere” to “dome” (Figure 1H-K). The blastula period in *P. scalare* embryos was observed for about 11 ½ hours (Table 1, Figure 1H-K and Figure 2).

Following the blastula stage the embryo undergoes gastrulation. Gastrulation is the period where there is extensive cell movement. In *P. scalare*, the rate of development decreased soon after the “dome” stage, at the beginning of epiboly (Figure 1L, Figure 2) indicating this change. Epiboly is where overlying cells of the blastoderm gradually moves over the yolk towards the vegetal pole (VP) of the embryo. Depending on the percentage of yolk covered, 30%, 50%, 75% and 90% epiboly stages were recorded at 16, 20, 22 and 23 ¾ hpf respectively (Figure 1L-O). After 50% epiboly, the progressing vegetal margin thickened (germ ring formation) suggesting that internal cell movements like involution started around this time. Soon after completion of epiboly, the head and tail buds of the developing embryo were observed (Figure 1P). It takes nearly 9 ½ hours for *P. scalare* to complete gastrulation.

Table 1 Embryogenesis of *P. scalare* at an average temperature of 29° C in comparison to two other cichlids, *O. niloticus* and *C. dimerus* (Korzelecka-Orkisz et al. 2012; Fujimura and Okada 2007; Meijide and Guerrero 2000; Kupren et al. 2014) and the cyprinid model organism, *D. rerio* (Kimmel et al. 1995).

Developmental stages	Period	Hours post fertilization				Distinguishing features of <i>P. scalare</i>
		<i>P. scalare</i>	<i>D. rerio</i>	<i>O. niloticus</i>	<i>C. dimerus</i>	
1-Cell	Zygote	0.00	0.00	0.00	0.00	Cytoplasmic accumulation to form the blastodisc at the animal pole and the appearance of a distinct perivitelline space
2-Cell	Cleavage	1.30	0.45	1.30	1.25	1 st vertical Cleavage
4-Cell		2.00	1.00	2.00	1.45	2 nd vertical Cleavage at right angle to 1 st . Two rows of 2 cells
8-Cell		2.20	1.15	3.00	2.05	Two rows of 4 cells
16-Cell		3.00	1.30	4.00	2.45	Four rows of 4 cells
32-Cell		3.30	1.45	-	-	Four rows of 8 cells
64-Cell	Blastula	4.05	2.00	-	-	Horizontal plane, 2 tires
128-Cell		4.35	2.15	12.00	4.55	Five irregular tires
256-Cell		5.25	2.30	-	5.30	Seven tires
512-Cell		6.05	2.45	-	-	Nine irregular tires
~1000-Cell		6.45	3.00	17.00	-	11 tires, Yolk syncytial layer forms
High		7.32	3.20	-	-	>11 tires, Blastoderm has mound of cells
Oblong		8.00	3.40	-	-	Blastoderm flattened
Sphere		8.35	4.00	-	7.30	Egg became spherical
Dome		10.24	4.20	-	-	Marked beginning of epiboly

Developmental stages	Period	Hours post fertilization				Distinguishing features of <i>P. scalare</i>
		<i>P. scalare</i>	<i>D. rerio</i>	<i>O. niloticus</i>	<i>C. dimerus</i>	
30% epiboly	Gastrula	16.00	4.40	22.00	-	Blastoderm cover 30% of yolk
50% epiboly		20.00	5.15	26.00	-	Blastoderm cover 50% of yolk
Germ ring		21.00	5.40	-	15.30	Local thickening of the blastoderm
Shield		21.35	6.00	-	16.20	-
75% epiboly		22.05	8.00	28.00	21.00	Blastoderm covered 75% of yolk
90% epiboly		23.45	9.00	30.00	22.20	Blastoderm covered 90% of yolk, embryo became thickened at the anterior end, brain started to form in the anterior end of the embryo, yolk plug can be seen at the vegetal pole
Tail bud		24.35	10.00	-	23.00	Tail started to form, 1 st somite appears
3-Somite		25.32	-	40	-	Optic vesicle and otic placode start to form
6- Somite		26.35	-	-	28.00	Head become more visible
8- Somite		-	-	32.00	-	-
10- Somite	Segmentation	28.35	14.00	-	-	-
14- Somite		33.40	16.00	-	-	-
18- Somite		53.30	18.00	38.00	-	Melanophores on the yolk surface of each side of the embryo gradually differentiated
22- Somite		-	-	-	36.00	-
25- Somite		60.00	-	-	-	Pharyngula stage. Tail is completely separated from yolk, Heart started pumping and blood circulation is clearly visible around the yolk and the head region
Hatching		72.00	48.00	90.00	54.00	<i>P. scalare</i> has 26 somites when they hatched

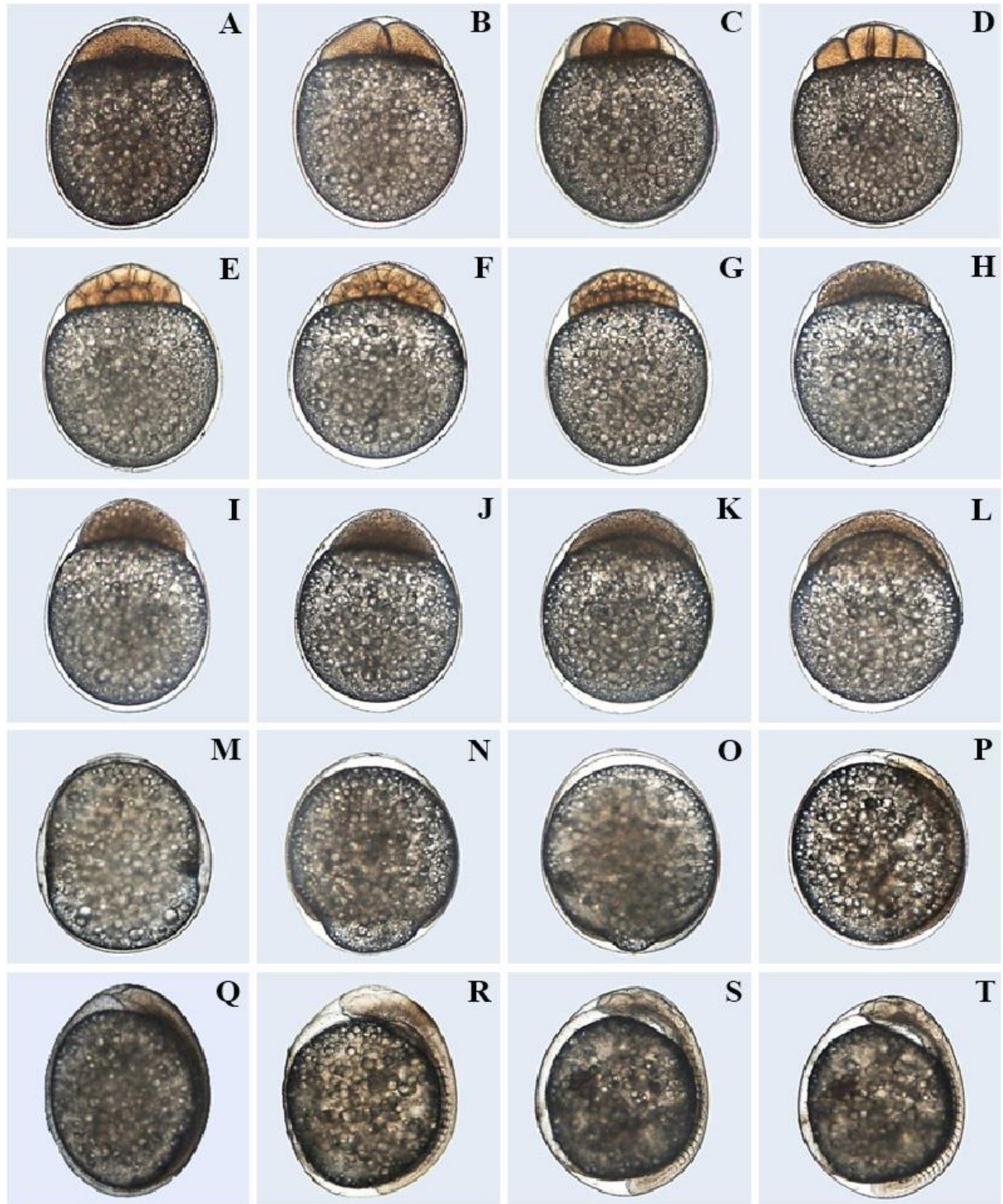


Fig 1 Embryonic stages of *P. scalare*. A) 1-cell stage, B) 2-cell stage, C) 4-cell stage, D) 8-cell stage, E) 16-cell stage, F) 32-cell stage, G) 64-cell stage, H) 128-cell stage, I) 256-cell stage, J) High stage, K) Dome stage, L) 30% epiboly stage, M) 50% epiboly stage, N) 75% epiboly stage, O) 90% epiboly stage, P) Bud stage, Q) 3-Somite stage, R) 12 somite stage, S) 17-somite stage and T) 19-somite stage.

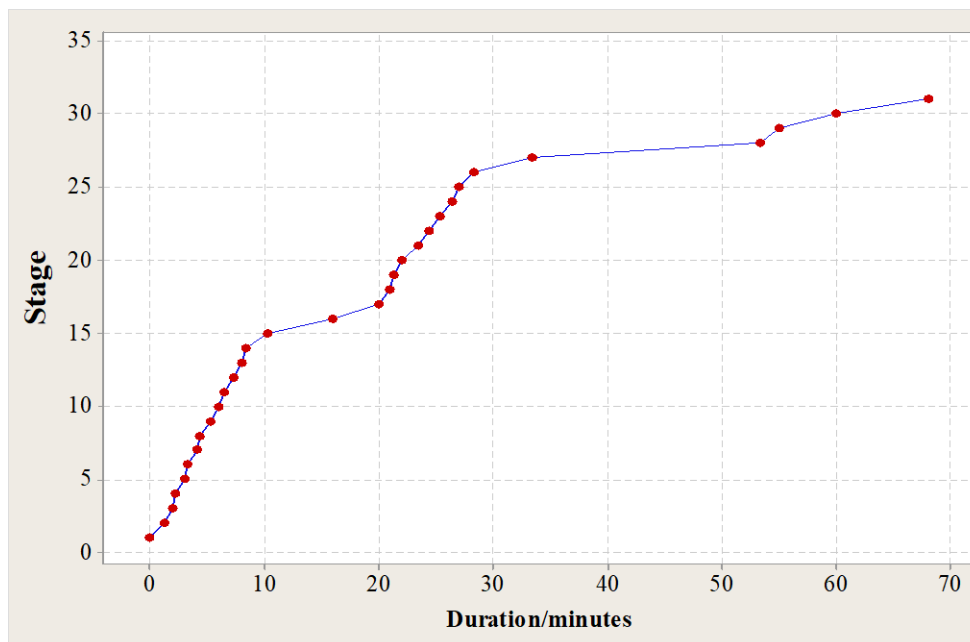


Fig 2 Embryonic developmental rate in *P. scalare* at different stages as a function of hpf. Stages 1-26 in Y-axis denote 1-Cell (zygote) to Hatching. Stages 2 to 7 are cleavage period. From stage 8 to 14 are blastula period. The gastrula period consists of stage 15 to 22 and rest of the stages belongs to segmentation period where stage 31 is hatching.

Later embryonic development

Starting from somite formation up to hatching was considered under later embryonic development. In addition to somitogenesis, head and eye developmental rates, ossification events (development of the upper jaw, lower jaw) and fin development (dorsal, anal, caudal and pectoral fin) were analyzed.

Segmentation period or somitogenesis generally follows at the completion of epiboly and tail bud formation (Kimmel et al., 1995). During this time somites, which will eventually give rise to the dermis, muscle and skeletal elements etc. are sequentially added from the trunk to the tail. The first somite pair was observed at the tail bud stage. Initial somites up until the formation of the 10th somite took place approximately at 30 minute intervals. However, this interval gradually increased as the number of somites increased (Figure 2). The optic vesicle and the auditory placode were visible around the 3rd somite stage (Figure 1Q). At about the time of 5th somite formation, the head

became more defined. Cement glands on the head appeared 50 hpf, approximately at 18-somite stage (Figure 1T).

Pharyngula refers to the stage where the embryo attains the classic vertebrate body organization (Ballard 1981). This stage in *P. scalare* was observed starting at 60 hpf. At this time, the embryo had 25 somites and the tail was completely separated from the yolk. Heart, pigmented blood cells, digestive tract and eyes were well developed. Heart started pumping and blood circulation was clearly visible around the yolk and the head region. *P. scalare* embryos hatched at 72 hpf at which time they had a total number of 26 somites and an average total length of 1.60 ± 0.05 mm (Figure 3A). By 4 days post fertilization (dpf) the larva developed melanocytes in the tail region and yolk sac (Figure 3B). They did not have well developed fins and as a result, the larvae were not able to swim well. Instead, they attached to the spawning substrate using their well-developed dorsal and ventral cement glands (Figure 3C-F).

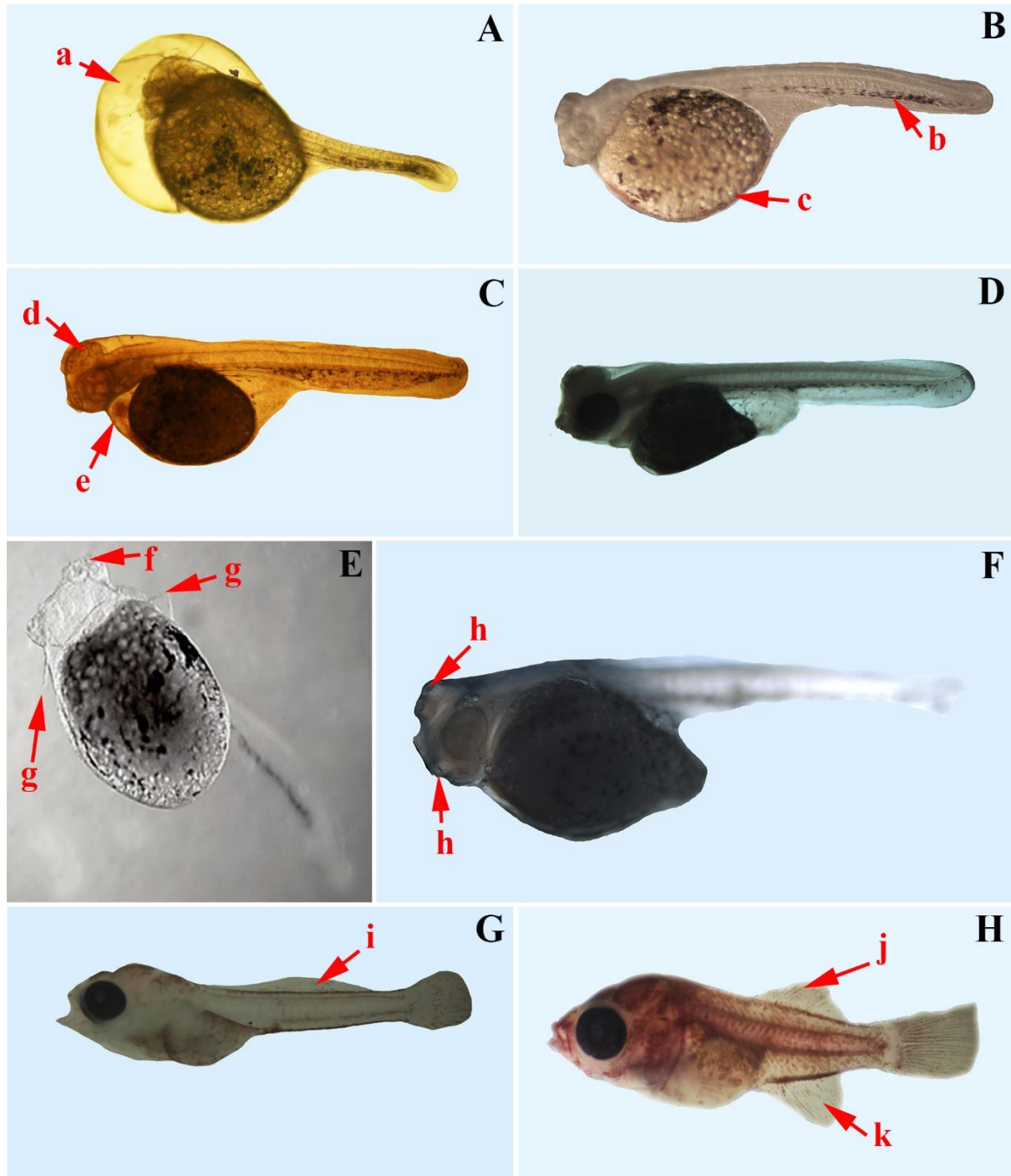


Fig 3 Different larval stages of *P. scalare*. A) Hatching larva 3 dpf (a-Chorion), B) 4 dpf larva (b-Melanocytes on the tail and c-Yolk sac), C) 5 dpf larva (d-Optic cup and e-Heart), D) 6 dpf larva, E) Cement glands on 4 dpf larva (f-Cement gland and g-Pectoral fin buds), F) Cement glands on 7 dpf larva (h-Cement glands), G) 8 dpf larva (i-Slightly developed dorsal fin) and H) 18 dpf larva (j-Dorsal fin and k-Anal fin).

Morphology and developmental rate of the *P. scalare* larvae

P. scalare larval development was observed until 23 to document distinct features in the larval development process. Around 4 dpf, the larvae started to swim and the cement glands started to disappear after 8 dpf. The pigmentation of the eye started at 6 dpf, and continued up to 11 dpf (Figure 3D). The dorsal, anal and caudal fins were continuous at this time (Figure 3G). After 11 dpf, rays were observed on the fins (Figure 3H).

P. scalare larvae exhibited its highest rate of development from 5 dpf to 7 dpf where it increased its body length from 1.7 mm to 2.4 mm. Typically, a relatively constant rate of development in length of the larva is seen from day 12 to 17 dpf. By 23 dpf the average body length of *P. scalare* larvae was 15.04 ± 0.03 mm. The growth or increase in body length in relation to dpf of *P. scalare* larvae

followed an exponential curve (Figure 4). The development of the upper jaw in *P. scalare* occurred at a relatively slower rate until 12 dpf and then rapidly increases from 13th to 21st dpf. On the other hand, the rate of development of lower jaw was initially high during 5th to 10th dpf where it increased from 1.1 mm to 2.39 mm and thereafter it gradually slowed. The fins: dorsal fin ($a = 0.07$, $b = 1.42$, $R^2 = 0.99$), caudal fin ($a = 0.34$, $b = 1.26$, $R^2 = 0.93$) and anal fin ($a = 0.11$, $b = 1.33$, $R^2 = 0.99$), head length ($a = 0.29$, $b = 1.26$, $R^2 = 0.96$) and eye diameter ($a = 0.15$, $b = 1.39$, $R^2 = 0.96$) showed positive allometric growth during larval development indicating that all these structures grow at a faster rate in relation to the body length increment (Figure 5). Meanwhile body depth ($a = 0.29$, $b = 0.96$, $R^2 = 0.92$) and linear length of the digestive tract ($a = 0.45$, $b = 0.98$, $R^2 = 0.87$) showed nearly isometric growth indicating a somewhat slower rate of development in contrast to the body length.

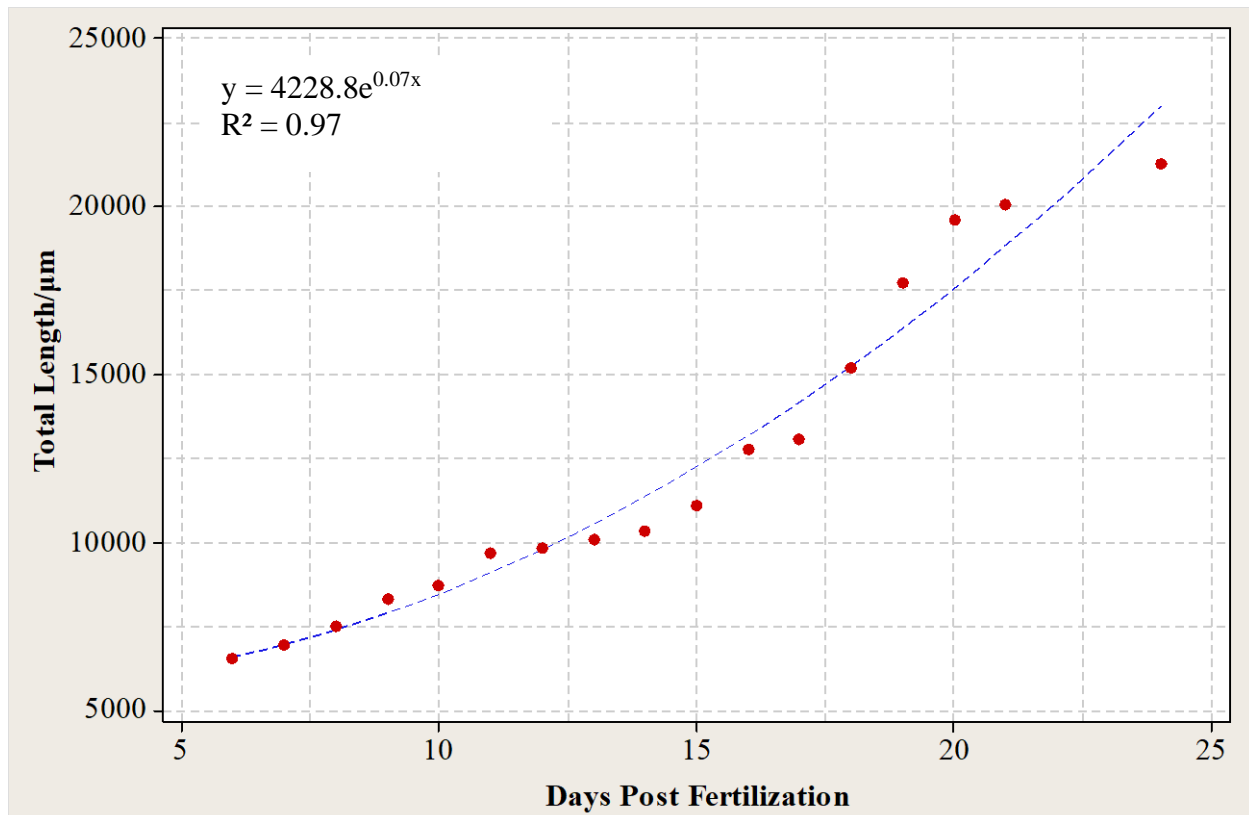


Fig 4 Rate of change in total length of the *P. scalare* larva.

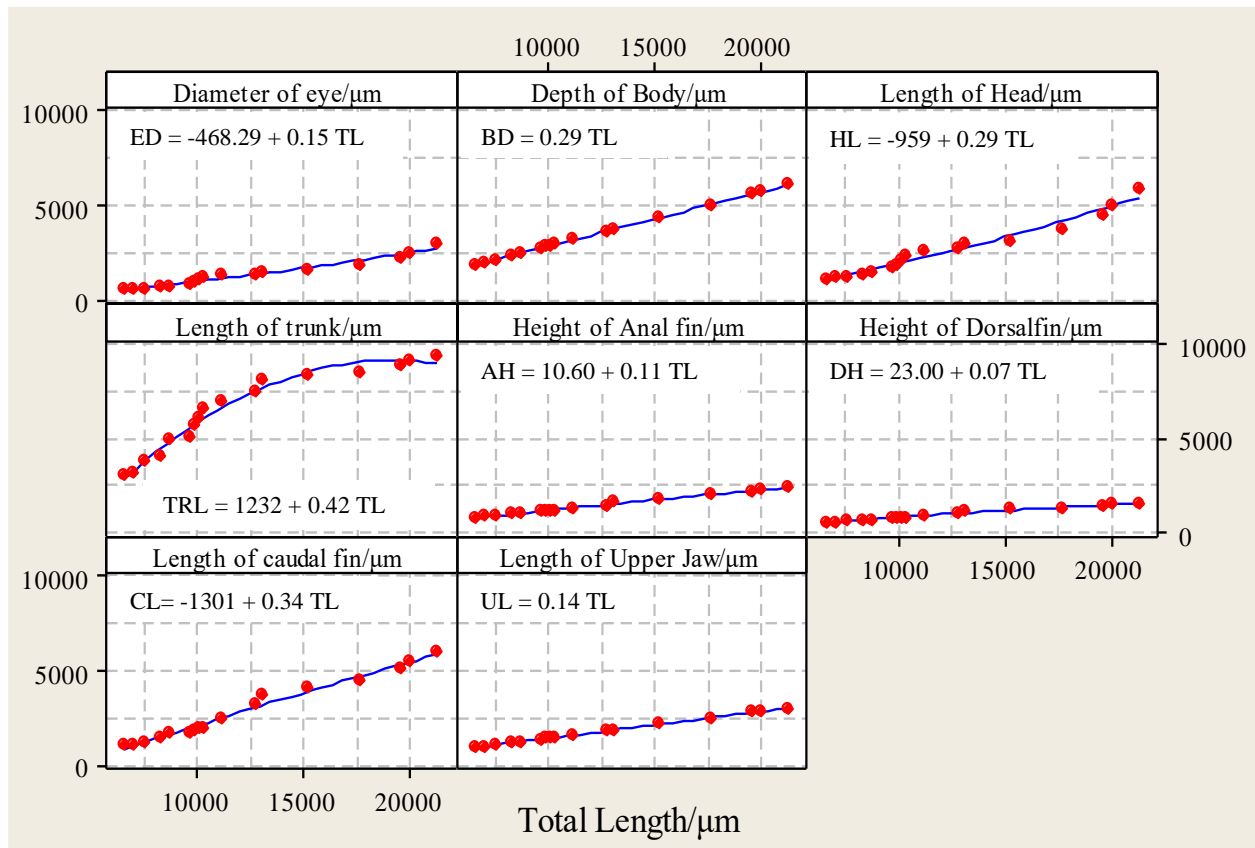


Fig 5 Allometric growth of *P. scalarae* larva. Rate of development in eye diameter, ED ($R^2 = 0.96$, $n = 30$), body depth, BD ($R^2 = 0.99$, $n = 30$), head length, HL ($R^2 = 0.96$, $n = 30$), trunk length, TRL ($R^2 = 0.87$, $n = 30$), anal fin height, AH ($R^2 = 0.99$, $n = 30$), dorsal fin height, DH ($R^2 = 0.98$, $n = 30$), caudal fin length, CL ($R^2 = 0.98$, $n = 30$) and upper jaw length, UL ($R^2 = 0.99$, $n = 30$) of *P. scalare* after hatching.

DISCUSSION

In the animal kingdom, fish show the highest variety of parental care. Parental care ranges from simple burial of eggs to internal gestation and live bearing (Goodwin et al. 1998). It may involve only the male, female, or both parents. Some type of parental care exists in 21% of the families of bony fishes. Among the different types of parental care, guarding behaviour is the predominant type observed among fish. This guarding behaviour includes active chasing of egg or fry predators (Gross and Sargent 1985). When considering male and female involvement in parental care, it is apparent that uniparental care is the most common type of parental care among fishes and male parental care is more common (49%) than female parental care (7%). However, biparental care is found in 13% of the families and it is considered to be the ancestral

form of care to uniparental care (Goodwin et al. 1998; Platania and Altenbach 1998; Nova and Costeira 2007).

Biparental care is commonly seen in cichlids that show substrate guarding. A good example is provided by *P. scalare*. Once a male and a female pair, they remain as a monogamous pair for one to three breeding cycles (Cacho et al. 2007). Next, as was observed during the study, the male and female fan the eggs to supply oxygen for the rapidly developing embryos (Reeb 2001). Because of this parental care, the survivorship of these embryos is high (Korzelecka-Orkisz et al. 2012). However, due to the costs involved in biparental care, the female lays fewer eggs to compensate for this cost (Abadian et al. 2012; Chellappa 1999).

During this study, the embryogenesis and larval development of *P. scalare* was compared with the data available for two other cichlids, *Cichlasoma*

dimerus that shows substrate brooding similar to *P. scalare* and *Oriochromis niloticus* that shows mouth brooding (Table 1). Furthermore, these events were compared to that of the cyprinid, *D. rerio* that does not show any parental care. *P. scalare* eggs are comparable to the eggs of *C. dimerus*, which are 1.65 ± 0.05 mm in diameter along the horizontal axis. The eggs are sticky, which enables them to stick to the substrate and to one another at the time of deposition. This is possibly due to the mucous layer surrounding the smooth translucent chorion analogous to that observed in *C. dimerus* (Meijide and Guerrero 2000).

Most teleost eggs are telolecithal (contains large amount of yolk) and as a result most of the egg cytoplasm is contained in a small area at the AP. Hence, as indicated in *P. scalare* embryos, the ensuing cleavage divisions occur only in this clear cytoplasmic area called the blastodisc (Kimmel et al. 1995; Gilbert 2006). In *D. rerio*, the cleavage stages from 2-cell to 64-cell stage are usually complete within 2 hpf with only 15 minute intervals in between two cleavage events (Kimmel et al. 1995). However, the cleavage period in freshwater angelfish embryos lasts for three hours with 30-40 minute intervals between cleavage events (Table 1 and Figure 1A-G). Similarly, in *C. dimerus* cleavage is observed for 3 ½ hours with nearly 30 minute intervals between consecutive stages (Meijide and Guerrero, 2000) whereas in *O. niloticus* this period is longer and extends for approximately 10 hours (Fujimura and Okada 2007).

The blastula and the gastrula periods of *P. scalare* exhibits a similar developmental pattern to *D. rerio* (Kimmel et al. 1995) albeit at a slower rate similar to *O. niloticus* and *C. dimerus* (Table 1). *P. scalare*, embryos take approximately six hours to complete the blastula stage in contrast to 2 ¼ hours taken for the same stage in Zebrafish embryos (Kimmel et al. 1995). The onset of gastrulation is discernable by the slowing down of the rate of development at the end of the dome stage. Gastrulation is a crucial period during development where there is extensive and coordinate set of cell movements. The overall result of these cell movement events is the production of the three primary germ layers, which are the precursors that will give rise to the different tissues in the body, and the establishment of embryonic axes (Kimmel et al. 1995). Even though it takes approximately five hours to complete gastrulation in *D. rerio*, it takes

about 9 ½ hours in *P. scalare*, similar to *C. dimerus* and *O. niloticus*. Segmentation period or somitogenesis generally follows at the completion of epiboly and tail bud formation (Kimmel et al. 1995). During this time, several body structures like the brain, eyes and the auditory placode become more defined. Though Zebrafish embryos hatch about 48 hpf (Kimmel et al. 1995), the *P. scalare* embryos hatch 72 hpf. They attach to the spawning substrate using well developed dorsal and ventral cement glands which appear at pre-hatching at approximately 18-somite stage (Groppelli et al. 2003). The whole organ is comprised of three pairs of ductless glands, which are conspicuous elevations on the larval head (Groppelli et al. 2003).

P. scalare larvae are referred to as altricial larvae in which, many of the organs develop after the larvae hatch or during or after metamorphosis (Falk-Peterson and Hansen 2001). After these larvae hatched, the yolk sac gradually decreased in size and they undergo drastic metamorphosis where the eyes, jaws, and fins develop rapidly. The rate of lengthening abruptly decreases at the end of tail lengthening at 17.5 dpf similar to what is observed in Zebrafish (Kimmel et al. 1995). According to Çelik et al. (2014), the growth coefficient in *P. scalare* larva change from yolk sac larva to pre-larva to post-larva. However, overall these changes are similar to what is observed in other teleost fishes. The benefits of parental care on the developmental rate of fish embryos, larvae and juveniles have received hardly any attention (Klug et al. 2014). It may be that in cichlids parental care increase the proportion of time spent in embryonic and larval stages while reducing the time spent in juvenile stages. Ultimately, parental care will lead to the increase fitness of both the parents as well as their offspring.

CONCLUSIONS

P. scalare early and later embryonic development as well as its larval development occur at a slower rate similar to those of other studied cichlids but in contrast to that in the cyprinid, *D. rerio*, which develop at a much faster rate. Parental care is likely to influence embryonic and larval development. In cichlids including *P. scalare*, parental care may allow these embryos to develop at a slower rate whereas the lack thereof for *D. rerio* embryos may necessitate faster development.

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