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Early embryonic development of the vulnerable Shalyni barb, *Pethia shalynius* (Yazdani & Talukdar, 1975)

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Abstract

The present study provides the first detailed early embryonic development of the Shalyni barb, *Pethia shalynius* (Yazdani & Talukdar, 1975), a vulnerable cyprinid fish occurring in streams and lentic waters of Meghalaya, northeast India. Induced spawning by synthetic hormone injection in May 2019 was conducted to a pair of mature female and male *P. shalynius* under controlled conditions in a well-aerated aquarium. Fertilized eggs were spherical, 0.75–0.80 mm (approx.) in diameter, transparent, unpigmented and non-adhesive. A total of 22 developmental stages could be categorized under seven broad periods, viz. the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatchling. The first cleavage occurred at 15 min post fertilization (mpf), followed by blastulation at 01:23 hr post-fertilization (hpf), gastrulation at 04:20 hpf, initial somite formation at 07:00 hpf, and pharyngula period at 19:20 hpf, respectively. Embryos hatched between 26–27 hpf and the newly-hatched larvae ranged 2.2–2.5 mm in total length. For naturally-declining populations of this vulnerable fish species, inferences drawn from the present study will help provide a baseline data for its conservation and management, and aid the research fields of developmental biology, biotechnology, molecular biology as well as taxonomy of this species.

KEYWORDS

embryogenesis, freshwater barb, induced spawning, northeast India, vulnerable cyprinid

1 | INTRODUCTION

Pethia shalynius (Yazdani & Talukdar, 1975) is a freshwater cyprinid barb, strictly restricted to the streams, rivulets and lentic water bodies of the Khasi and Jaintia Hills in Meghalaya, northeast India (Yazdani & Talukdar, 1975). Additional records show its occurrence along areas of the Assam-Meghalaya border (Sen, 1985). Locally called "Shalyni", *P. shalynius* is categorized as "Vulnerable" due to declining populations, largely resulting from coal mining and quarrying, in the wild (Dahanukar, 2015; D. K. B. Mukhim pers. obs.). Males of *P. shalynius* have a bronze dorsum and belly, and orange-red anal, caudal fins and lateral scales towards caudal peduncle; while females have a bronze dorsum and scales, silvery-white belly and a pale orange caudal fin (Figure 1). The colour on fins and scales of both sexes darken (or attain a peak) during the breeding season. Thus, the Shalyni barb has to be a potential candidate as an ornamental fish species from northeast India. However, this vulnerable species currently has a minor fisheries value (Yazdani & Talukdar, 1975; Froese & Pauly, 2019).

Studies on embryonic development have great significance as they aid towards the conservation and management of fish species by estimating the fertilization efficiencies, possible restocking of threatened fish species and production of juveniles via successful establishment of aquaculture, understanding a species'

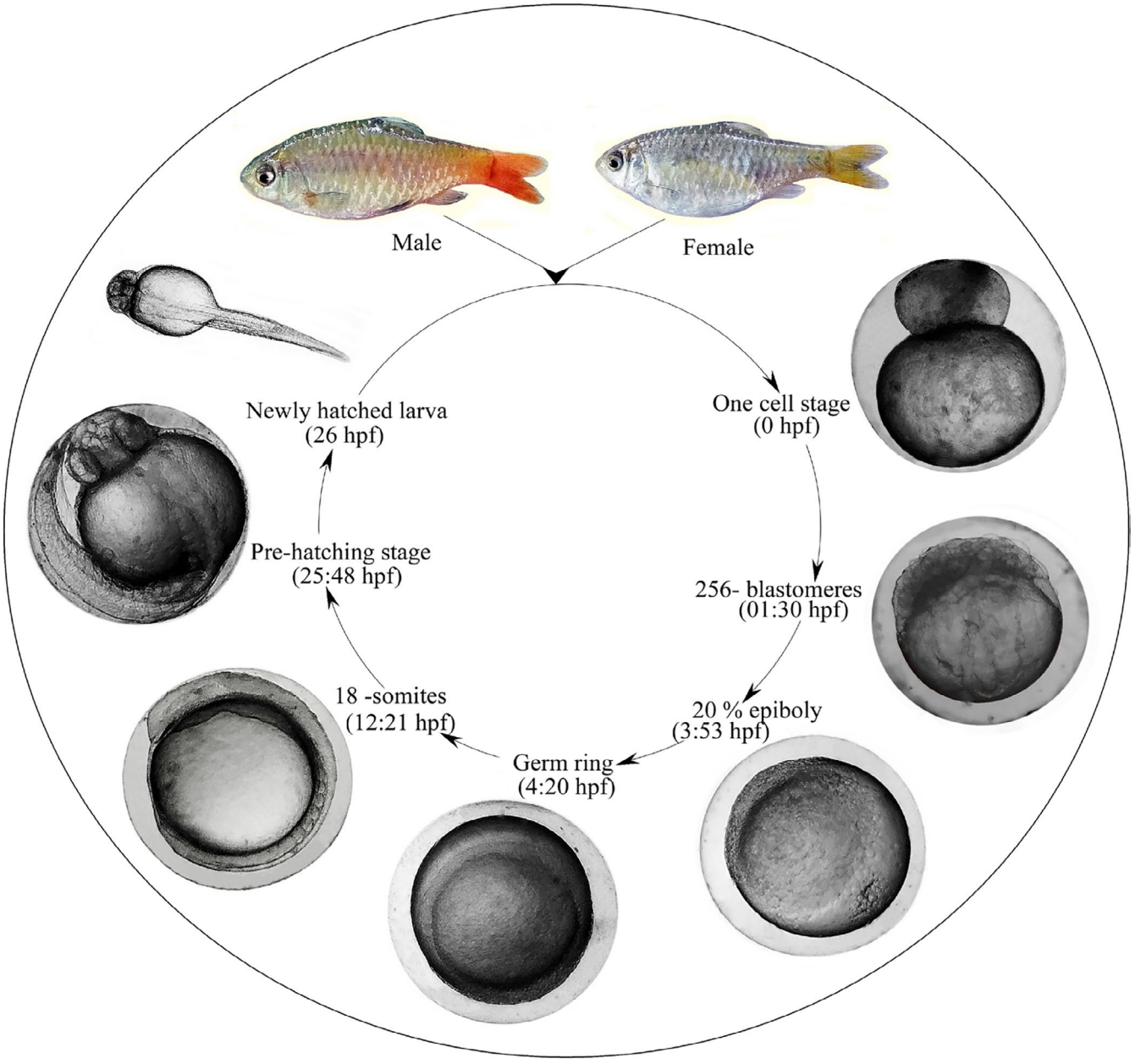

FIGURE 1 Summary of the developmental periods of *Pethia shalynius* (Yazdani & Talukdar, 1975) (hpf, hours post fertilization)

habitat requirements, and/or karyological studies to detect any chance events like polyploidy (Najafpour et al., 2019; Weber & Hostuttler, 2012). Previous studies on *Pethia (=Puntius) shalynius* had primarily focused on the condition factor, morphometry, reproductive cycle, ultrastructure and diet preferences (e.g., Manorama, 2016; Manorama & Ramanujam, 2014, 2017a, 2017b, 2017c). There is no information on the early embryonic development available (Froese & Pauly, 2019). Because of this, the present study was aimed to provide a comprehensive account of the early embryonic developmental stages of the Shalyni barb, covering the critical events of embryogenesis from fertilization to hatching.

2 | MATERIALS AND METHODS

2.1 | Collection of broodstock, maintenance and induced spawning

A total of 48 mature-sized *P. shalynius* (22 males [5.5–6.1 cm total length, 2.6–3.1 g weight] and 26 females [5.7–6.2 cm total length, 2.8–3.3 g weight]) were caught with the help of local fishermen in November 2018 from the Umkhohrah Stream (Barak drainage) in East Khasi Hills, Meghalaya, India (N 25.4°, E 92.0°). They were then transported to the Aquaculture and Biodiversity Centre of Gauhati University, Assam in oxygenated plastic bags with minimal stress.

The fishes were kept in a glass aquarium (121 cm × 45.7 cm × 45.7 cm), and the pH, water temperature,

TABLE 1 Stages of early embryonic development of *Pethia shalynius* (Yazdani & Talukdar, 1975) at 26°C water temperature

Period	Stages	Time (h:m)	Description	Figure
Zygote	1-cell	00:10	Cytoplasm moves toward animal pole	2a
Cleavage	2-cell	00:15	First cleavage form two blastomeres	2b
	4-cell	00:20	Second cleavage forms 4 blastomeres	2c
	8-cell	00:25	Third cleavage forms 8 blastomeres	2d
	16-cell	00:36	Fourth cleavage forms 26 blastomeres	2e
	32-cell	00:50	Fifth cleavage forms 32 blastomere	2f
	64-cell	01:06	Sixth cleavage forms 64 blastomeres	2g
Blastula	256-cell	01:30	Blastulation started (eighth cleavage)	2h
	1k-cell	02:25	Tenth cleavage forms 1k cells, cleavage planes irregular	2i
	20% epiboly	03:53	After the dome shape 20% epiboly stage appears	2j
Gastrula	50% epiboly	04:00	Blastoderm margin arise at 50% of the entire distance between the animal pole and vegetal pole	2k
	Germ ring	04:20	By the thickening of the annulus at the blastoderm margin the germ ring forms	2l
	60% epiboly	04:33	Blastoderm covers 60% of the yolk	3a
	90% epiboly	05:25	Yolk plug develops in the vegetal pole. 90% yolk cell covered by blastoderm	3b
	Early bud	05:35	Blastoderm completely cover the yolk plug	3c
	Late bud	05:48	Tail bud appears in this stage	3d
Segmentation	2-somite	07:00	Somites formation begins, 2 somite were seen	3e
	6-somite	08:12	Appears optic primodium in the head region	3f
	8-somite	09:24	The optic primodium shows a clear horizontal crease	3g
	12-somite	10:29	Twelve somites were clearly visible in this stage	3h
	18-somite	12:21	Tail elongation and contraction of the tail region seen	3i
	23-somite	16:12	Development of the optic vesicle clearly visible	3j
Pharyngula	Pharyngula 1	19:20	Extensions of the tail regions and curved	3k
	Pharyngula 2	25:48	Contraction of the tail and heart beating was seen	3l
Hatching	Hatched larva	26:00	Yolk sac was not absorbed completely	4a
	Dorsal view		Otolith clearly visible	4b

conductivity and dissolved oxygen were maintained as 7.5 ± 0.5, 25 ± 0.8°C, 350–370 μS and 5.8–6.5 mg/L, respectively. The aquarium had dark gravel as a substrate and was illuminated maintaining a 14 hr L/10 hr D cycle. The fishes were fed ad libitum twice a day with live *Tubifex* and chopped earthworms (@ 2%–3% of the body weight) at 10:00 hr and 16:00 hr of Indian Standard Time (IST).

One pair of mature male (weighing 3.0 g) and female (weighing 3.2 g) fishes were artificially spawned by intraperitoneal injection of synthetic hormone (GONOPRO-FH, India) in the month May 2019 (selection was based on the appearance of strong coloration and rapid oozing of ova/milt when gently pressed on the abdomen). The dosage of hormone for both the male and female sexes was 0.016 ml/g of the body weight. Before injection, the fishes were anaesthetized with clove oil (0.1 ml/L). After injection, they were carefully released in a glass aquarium (61 cm × 45.7 cm × 45.7 cm) and monitored for initiation of spawning. The methodology followed in this study was approved by the Institutional Animal Ethical Committee of Gauhati University, Guwahati, Assam (Protocol number: IAEC/Per/2019/PP-IAEC/2019-044).

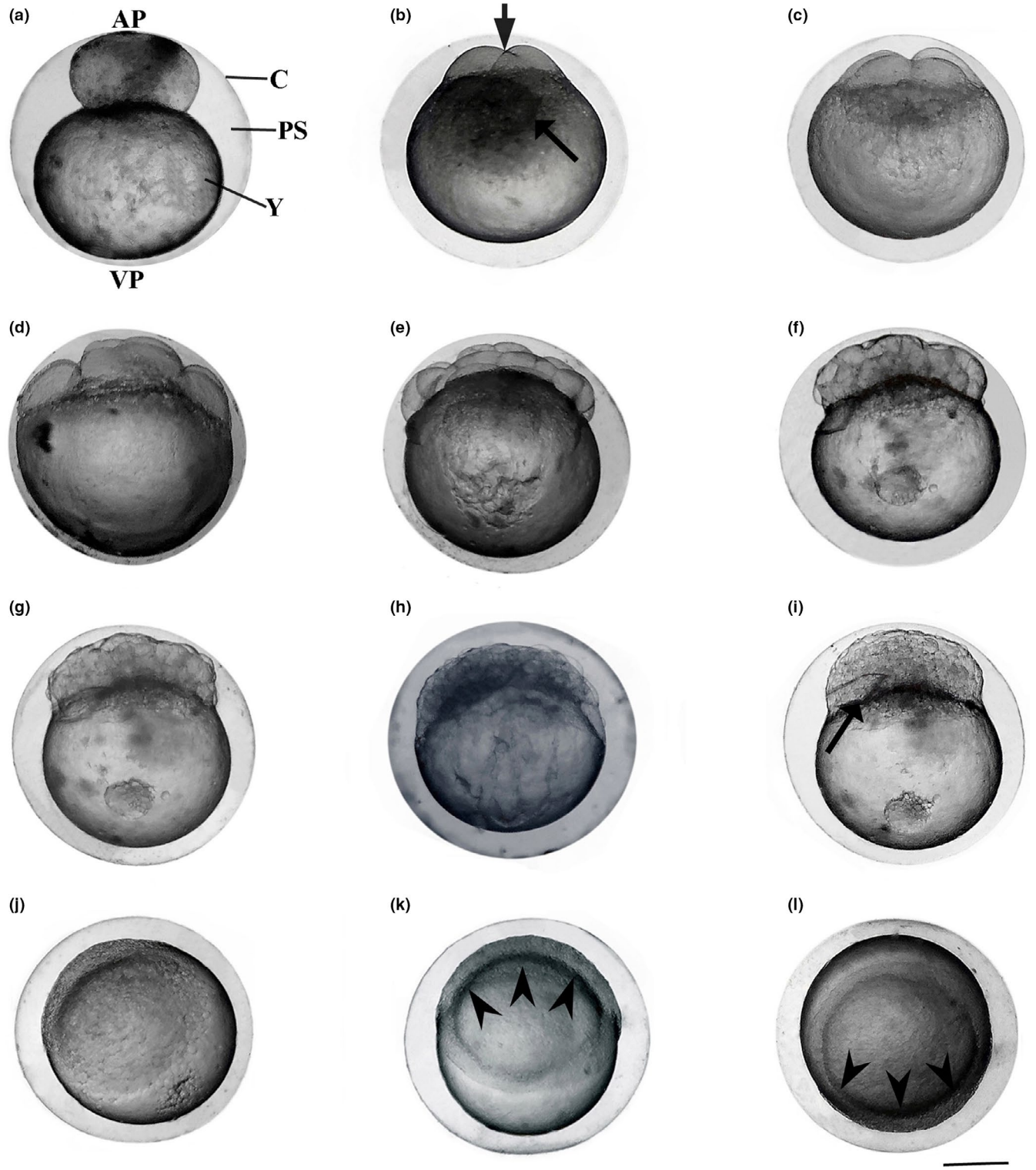

FIGURE 2 Early embryonic development in *Pethia shalynius* (Yazdani & Talukdar, 1975): (a) Zygote (AP, animal pole; C, chorion; PS, perivitelline space; VP, vegetal pole; Y, yolk); (b) 2-cell stage; (c) 4-cell stage; (d) 8-cell stage; (e) 16-cell stage; (f) 32-cell stage; (g) 64-cell stage; (h) 256-cell stage; (i) 1k-cell stage; (j) 30% epiboly; (k) 50% epiboly; (l) Germ ring. Scale bar = 250 μm

2.2 | Study of embryonic development

The fertilized eggs (n = 5) were taken out from the spawning aquarium with the use of a sterilized glass dropper to observe the early embryonic developmental stages. The stages were observed and identified under a Labomed CZM4 stereo-zoom microscope. Measurements of the egg and larval stages were taken using a stage micrometer fitted to the microscope. The photographs were taken with a Sony Cyber-Shot 20.1 MP camera. The various stages

of development were grouped under broader periods, based on the morphological characteristics described by Kimmel et al. (1995).

3 | RESULTS

The female *P. shalynius* released 30 eggs after 10 hr of hormone injection. The fertilized eggs hatched approximately after 26 hr post-fertilization (hpf) at a water temperature of 26 ± 0.4°C. The fertilized eggs were spherical, demersal, non-adhesive, optically-transparent, and measured 0.75–0.80 mm in diameter. The perivitelline space separated the yolk from the chorion.

A total of 22 stages were categorized under seven broader developmental periods, viz. zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching. Figure 1 summarizes the significant developmental periods of the early embryonic development and Table 1 presents the different developmental stages of the early embryonic development of *P. shalynius*.

3.1 | Zygote

The zygote period comprised of a single stage—the one-cell stage at 10 min post fertilization (mpf). This stage was characterized by the accumulation of cytoplasm took place at the animal pole followed by the formation of a blastodisc, signifying the first cell (Figure 2a).

3.2 | Cleavage

The eggs were macrolecithal having large yolk volume and presented discoidal, meroblastic pattern of cleavage. A total of 6 stages could be recognized under the cleavage period—

Two cell stage (15 mpf): The first meroblastic cleavage occurred in the animal pole, vertically along the plane of axis of the blastodisc, and resulting two blastomeres of equal size (Figure 2b).

Four cell stage (20 mpf): The plane of the second cleavage was perpendicular to the first cleavage. As a result, four equal-sized blastomeres were formed (Figure 2c).

Eight cell stage (25 mpf): The third division resulted in eight blastomeres of equal size by cutting the blastoderm vertically along the axis of animal and vegetal poles (Figure 2d).

16 cell stage (36mpf): At this stage, 16 blastomeres (4 × 4 array) were formed by the fourth set of cleavage. This cleavage took place along the two planes, parallel to the second plane of the dividing embryo (Figure 2e).

32 cell stage (50 mpf): This stage was characterized by smaller-sized blastomeres as a result of uninterrupted cell division. It was observed that the 32 blastomeres formed after the 4 × 4 array of cells were horizontally cleaved (Figure 2f).

64 cell stage (01:06 hpf): In this stage, 64 blastomeres were formed by the sixth cleavage, which is the first horizontal division that resulted in two layers each of 32 blastomeres (Figure 2g).

3.3 | Blastula

Three stages could be recognized under this period—

256 cell stage (01:30 hpf): At the start of blastulation, the eighth division formed 256 blastomeres, arranged in numerous rows on the crest of the yolk (Figure 2h).

1k cell stage (02:25 hpf): The tenth cleavage formed approximately 1,000 (1k) blastomeres, which formed an indefinite margin with the yolk (Figure 2i).

20% epiboly (3:53 hpf): In this stage, the margin of the blastoderm extended approximately 20% of the entire distance between the animal pole and the vegetal pole (Figure 2j).

3.4 | Gastrula

Gastrulation period began by flattening of the blastoderm towards the vegetal pole. Gastrula comprised of the following 6 stages—

50% epiboly stage (04:00 hpf): The yolk was observed to attain a dome-like structure, and expansion and spreading of the blastoderm cover 50% the yolk was seen (Figure 2k).

Germ ring (04:20 hpf): This stage was characterized by the development and thickening of the annulus at the blastoderm margin (Figure 2l).

60% epiboly (04:33 hpf): The margin of the blastoderm covered approximately 60% of the distance between the animal and vegetal poles (Figure 3a).

90% epiboly (05:25 hpf): In this stage, almost the full yolk was covered by the blastoderm. Uncovered yolk cell was observed to protrude out from the nearby vegetal pole, known as a yolk plug (Figure 3b).

Early bud stage (05:35 hpf): The yolk plug (YP) was observed to develop further in the vegetal pole (Figure 3c).

Late bud stage (05:48 hpf): The proliferation of the blastoderm across the yolk cell was observed in the late bud stage. The tailbud was observed as an apparent enlargement at the posterior end of the embryonic axis (Figure 3d).

3.5 | Segmentation

In this period, the formation of somites (i.e. somatogenesis) took place along the anterior-posterior axis of the developing embryos. Following the formation of a somatic furrow, development of the first somite took place and the number of somites gradually increased antero-posteriorly. It resulted in elongation of the embryos as development progressed.

Two somite stage (07:00 hpf): Two distinct somites were observed (Figure 3e).

Six somite stage (08:12 hpf): Six somites were observed, and the optic primordium (OP) was seen in the cephalic region (Figure 3f).

Eight somite stage (09:24 hpf): Formation of eight somites was observed along the anterior-posterior axis (Figure 3g).

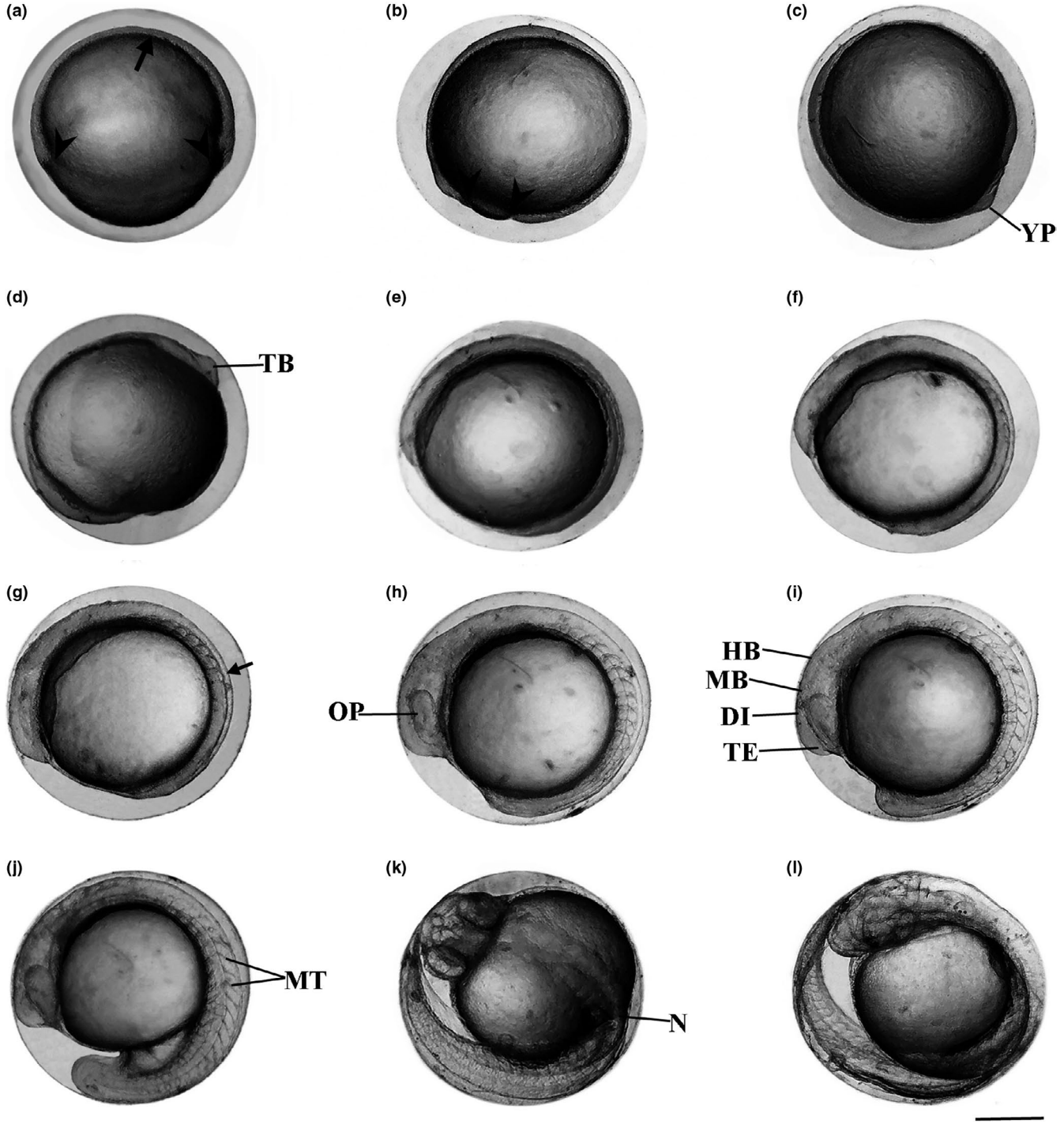

FIGURE 3 Embryonic development in *Pethia shalynius* (Yazdani & Talukdar, 1975): (a) 60% epiboly; (b) 90% epiboly; (c) Early bud (YP, yolk plug); (d) Late bud stage (TB, tail bud); (e) 2-somite stage; (f) 6-somite; (g) 8-somite; (h) 12-somite (OP, optic primordium); (i) 18-somite (DI, diencephalon; HB, hindbrain; MB, midbrain; TE, telencephalon); (j) 23-somite (MT, myotome); (k) pharyngula 1 (N, notochord); (l) Pharyngula 2. Scale bar = 250 μm

12 somite stage (10:29 hpf): OP became more distinct; elongation of the tail region continued; and arranged notochord cells were observed (Figure 3h)

18 somite stage (12:21 hpf): 18 distinct somites were seen, and elongation of the tail was observed. The tail bud obtruded away from the yolk mass. The telencephalon, diencephalon, midbrain, and hindbrain were distinguishable in this stage (Figure 3i).

23 somite stage (16:12 hpf): 23 somites were formed and an extension of the tail was observed. The tail region was separated slightly from the yolky region. Development of the eye was observed and the head portion emerged as well as myotomes were visible (Figure 3j).

3.6 | Pharyngula

Two stages were recognized under this period—

Pharyngula 1 (19:20 hpf): Development of the notochord continued, and the somites reached up to the long post-anal tail region. Blood circulation was seen as the heartbeat started (Figure 3k).

Pharyngula 2 (25:48 hpf): Extensions of muscles could be observed and the larva became more active within the chorion (Figure 3l). The amount of yolk mass reduced as development progressed, and brain development continued.

3.7 | Hatching

The embryos hatched between 26–27 hpf, and the newly-hatched larvae measured 2.2–2.5 mm (for $n = 5$) in length. A newly-hatched larva was unpigmented and could not move freely due to incomplete absorption of the yolk mass (Figure 4a). First pigmentation was, however, observed around the eye area after 2–3 hr post hatching. A distinct pair of otoliths (OT) was also visible through the dorsal view of the larvae (Figure 4b).

4 | DISCUSSION

To our knowledge, the present study provides information on the early embryonic development of *P. shalynius* for the first time. Comparative data on the egg diameter, time of hatching, size of the newly-hatched larvae and incubation temperature of other barb fishes, including *Danio rerio*, with *P. shalynius* is presented in Table 2.

The spherical nature of eggs of *P. shalynius* is similar to that of other barb species like *Puntius binotatus* (Iswahyudi, Marsoedi, & Widodo, 2014), *Sahyadria (= Puntius) denisonii* (Anna Mercy et al., 2015), and *Systomus (= Puntius) sarana* (Ahamad & Basavaraja, 2013). However, the eggs of *P. shalynius* are non-adhesive as opposed to adhesive in the barbs like *P. binotatus* (Iswahyudi et al., 2014), *Barbonymus (= Puntius) gonionotus* (Basak et al., 2014), and *S. denisonii* (Anna Mercy et al., 2015). Non-adhesive eggs are more viable under aquaculture conditions with the advantage of being easily transferable after fertilization (Krise et al., 1986). Also, clumping of eggs can be prevented when incubated at high densities, which will reduce the risks of fungal infections and lessen efforts with egg count or other associated activities (Ringle et al., 1992). Besides, the habitat of *P. shalynius* includes a sandy bottom with dense perennial grass and aquatic macrophytes (D. K. B. Mukhim pers. obs.). Non-adhesiveness is, therefore, favored in the wild too, as there is less chance of adherence to sand particles or other debris.

Information on the characteristics of eggs during the embryonic development is important to judge the fitness of the larvae (Saillant et al., 2001). Egg-size plays a crucial role in larval quality, significantly, in the time of incubation and developmental phases. However, the diameter of eggs of most small-sized ornamental fish species is approximately 0.8 mm (Watson & Chapman, 2002). The diameter of fertilized eggs of *P. shalynius* ranged between 0.75–0.80 mm, which is comparatively similar to that reported in other larger barb fish species like *B. gonionotus* (=0.8 ± 0.05 mm) (Basak et al., 2014), *Pethia conchonius* (=0.75 mm) (Bhattacharya et al., 2005) and *S. sarana* (=0.65–0.70 mm) (Ahamad & Basavaraja, 2013); except smaller than the eggs of *S. denisonii* (1.18–1.31 mm) (Anna Mercy et al., 2015). Similar egg diameter was also reported in *D. rerio* (0.7 mm) by Kimmel et al. (1995). Variations in egg diameter among Cyprinids are species-specific and often related to the brood size (Ahamad & Basavaraja, 2013). However, large egg-size is a reproductive tactic to produce larger larvae with heavier yolks, thereby ensuring better survival in the wild (Watson & Chapman, 2002). As reported by Manorama (2016), mature ova were found between the months March and May (ova diameter = 0.37–0.68 mm), and June and July (=0.90–0.74 mm), respectively, in the ovaries of *P. shalynius*. Our observations additionally support Manorama's (2016) report on gonadal maturity in *P. shalynius*, i.e. the females (including males) attained maturity for induction of spawning in May in captivity as well.

According to Piferrer et al. (2009), any information on the exact timing of first cleavage is crucial for chromosomal manipulations, such as polyploidy, as the process is efficient during this stage. Weber and Hostuttler (2012), too, mentioned the importance of timing of the first cleavage for determining the fertilization efficiency and also to establish the best stages for chromosome manipulations, viz. tetraploidy initiation. In *P. shalynius*, the first cleavage occurred at 15 mpf at a water temperature of 26°C, whereas 45 mpf in *P. conchonius* (Bhattacharya et al., 2005), 40–45 mpf in *S. sarana* (Ahamad & Basavaraja, 2013), 20 mpf in *S. denisonii* (Anna Mercy et al., 2015), 35 mpf in *B. gonionotus* (Basak et al., 2014), and 1 hpf in *P. binotatus* (Iswahyudi et al., 2014). The first cleavage in *D. rerio* reportedly occurs at 45 mpf (Kimmel et al., 1995). Such differences in the initiation of first cleavage are related to differences in the temperatures of incubation.

The larvae of *P. shalynius* started to hatch at 26 hpf, and hatcing was complete by 27 hpf at a water temperature of 26 ± 0.4°C. Larval hatching was reported to start in *D. rerio* at 48 hpf (water temperature 28.5°C) (Kimmel et al., 1995), in *P. conchonius* at 26 hpf (26°C) (Bhattacharya et al., 2005), in *S. denisonii* at 36 hpf (27.5 ± 0.5°C)

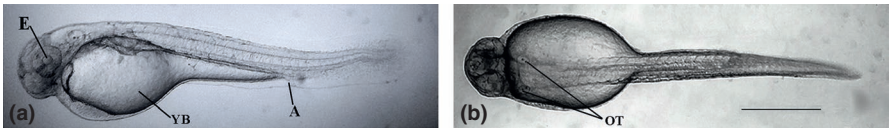

FIGURE 4 Early hatching stages of *Pethia shalynius* (Yazdani & Talukdar, 1975): (a) Lateral view of hatched larva; (b) Dorsal view of hatched larva (A, anus; CFB, caudal fin bud; E, eye; LE, lens; N, notochord; OT, otoliths; P, pigmentation; YB, yolk ball). Scale bar = 500 μm

TABLE 2 Comparisons of developmental aspects of early embryogenesis of *Pethia shalynius* (Yazdani & Talukdar, 1975) with selected Cyprinid fishes

Species name	Egg diameter (in mm)	Time of hatching (hpf)	Hatching temperature (°C)	Larva size (in mm)	References
Pethia shalynius	0.75–0.8	26–27	26	2.32 ± 0.11	Present study
P. conchonius	0.75	26	26	2.75	Bhattacharya et al. (2005)
Systomus sarana	0.65–0.7	26 ± 0.3	27 ± 0.3	1.9 ± 0.003	Ahamad and Basavaraja (2013)
Sahyadria denisonii	1.1–1.3	36–40	27.5 ± 0.5	3.5 ± 0.3	Anna Mercy et al. (2015)
Barbodes gonionotus	0.8 ± 0.05	13.40–14	30.5°C	2.2 ± 0.05	Basak et al. (2014)
Barbus trevelyani	1.5 ± 0.1	67	27–29°C	3.7	Cambray (1985)
Danio rerio	0.70	48	28.5°C	3.3	Kimmel et al. (1995)

(Anna Mercy et al., 2015), in *B. gonionotus* at 13.40 hpf (Basak et al., 2014), and in *P. binotatus* at 24 hpf (28°C) (Iswahyudi et al., 2014), respectively. Therefore, it is clear from the above studies that the time of hatching is temperature-dependent and species-specific, with an optimal hatching temperature between 26°C–30.5°C in Cyprinids.

Pethia shalynius larvae were found to be in the size range of 2.2–2.5 mm (n = 5) in length as compared to 2.75 mm in *P. conchonius* (Bhattacharya et al., 2005), 1.903 ± 0.002 mm in *S. sarana* (Ahamad & Basavaraja, 2013), 3.5 ± 0.2 mm in *S. denisonii* (Anna Mercy et al., 2015), and 2.2 ± 0.05 mm in *B. gonionotus* (Basak et al., 2014), respectively. It needs to be noted here that *P. shalynius* is a small barb with a maximum recorded size of 6 cm in length (Froese & Pauly, 2019). The newly-hatched larvae of *P. shalynius* are therefore relatively larger in size when compared to the larvae of the afore-mentioned larger barb fishes. Large-sized larvae generally tend to have greater yolk reserves and later become efficient swimmers, thereby, benefitting at foraging to accommodate larger prey as well as enhanced capability of escaping from predators in the wild (Bagenal, 1971; Shirota, 1970). Further, it is advantageous in culture conditions to handle them easily and/or to determine suitable feed-size needed for subsequent growth and survival (Joseph, 2001; Kamler, 1992).

In our study, no pigmentation was observed before hatching. Similar findings were also reported by Bhattacharya et al. (2005) in the rosy barb, *P. conchonius*. On the contrary, *D. rerio* larvae attain pigmentation prior-to-hatching (Kelsh et al., 1996). However, melanophore accumulation took place around the eyes of *P. shalynius* at 2–3 hr post hatching. Eye pigmentation is associated with better aid in vision leading to better prey detection and escape predators, and protects the larval fish against harmful ultraviolet radiations, especially in species that dwell in shallow waters (de Carvalho & Caramujo, 2017; Horth, 2006; Kawamura & Washiyama, 1989; Olaniyi & Omitogun, 2014). As the newly-hatched larvae do not have free-swimming capacity owing to heavier yolk reserves, a slower transition from a translucent nature of the larvae to opaque through body pigmentation would be more favoured in the wild as they would less likely be visible to predators.

The present description on the embryological stages of *P. shalynius* was, however, restricted to n = 5 eggs only. Apart from the low number of eggs (=30) released by the female, only 27 eggs were fertilized that eventually hatched and survived through the experiment. As the species is vulnerable and rarely available in the wild, it was necessary to ensure a better hatching rate and successful survival by minimizing any physical/temperature shock to the eggs/hatchlings after inducing spawning of the fish in captivity. Low egg number is perhaps compensated with the production of large-sized eggs and hence larger larvae in the wild. But, further studies are necessary to validate this point for *P. shalynius*.

Embryology and larval development studies of a species have great significance for successful larval rearing for mass production of seed in aquaculture (Borcato et al., 2004; Khan & Mollah, 1998; Koumoundouros et al., 2001; Rahman et al., 2004). The information gathered on the early embryonic development of *P. shalynius*, including the timings of development, incubation, hatching periods and completion of yolk absorption, the size of newly-hatched larva, first exogenous feeding and free swimming behavior, will be helpful for specifying appropriate supervision and management of the species in captivity and optimising different environmental variables. Besides, knowledge on the early life history of the species will make the larval rearing procedure less challenging and thus aid to prevent larval malformations and/or low productivity in culture condition. For naturally-declining populations of the vulnerable *P. shalynius*, inferences drawn from the present study will help to provide a baseline data for its conservation and management, and help the research fields of developmental biology, biotechnology, molecular biology as well as taxonomy of this species. However, future studies based on additional replicates are recommended to estimate the fecundity and rates of fertilization, hatching and survival of the species in culture condition.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated in the present study are included in the manuscript and its figures and tables.

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