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RESEARCH ARTICLE

Embryonic and larval development of the topmouth gudgeon, *Pseudorasbora parva* (Teleostei: Cyprinidae)

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ABSTRACT. The topmouth gudgeon, *Pseudorasbora parva* (Temminck & Schlegel, 1842), is a small cyprinid fish that inhabits the littoral zones of freshwater habitats throughout Asia and Europe. It is regarded as an invasive species in Europe, but in its native range, in China, as food and as an environmental bio-indicator. In this study, the embryonic and larval development of *P. parva* was investigated for the first time. The eggs of *P. parva* are transparent, adhesive and elliptical. The mean size of fertilized eggs was (1.63×1.31) ± 0.04 mm. From fertilization to hatching, embryonic development could be divided into eight stages and 34 phases, and the incubation period lasted for 109.25 hours at 24 ± 1 °C. Newly hatched larvae were 4.1 ± 3 mm in length, and the yolk absorption was completed within six days after hatching. The first and second swim bladders formed at the third and ninth day, respectively. The pectoral fin formed before the hatching stage, followed by the caudal, dorsal, anal and ventral fin formation after hatching. About 20 days after hatching, the morphology of the fry was similar to the adult fish. These findings provide a basis for determining the complete ontogeny of *P. parva*, as well as facilitate the management and utilization of this fish.

KEY WORDS. Embryogenesis, invasive species, larval development.

INTRODUCTION

The topmouth gudgeon, *Pseudorasbora parva* (Temminck & Schlegel, 1842) is a small cyprinid fish (Gobioninae) native to freshwater habitats throughout China, Japan, Korea and the River Amur basin (Okuda et al. 1996, Priyadarshana et al. 2001). It was accidently introduced to Europe along with the introduction of herbivorous Chinese carps in 1950s and 1960s. This was followed by the rapid and widespread colonization of Europe and North Africa (Boltachev et al. 2006, Britton et al. 2010, Witkowski 2011, Dana et al. 2015, Karabanov et al. 2016). As it can compete for resources with the fry of other fish species and carry parasites, it is generally regarded as an undesirable colonizer, and even a serious ecological threat to the endemic ichthyofauna, in its non-native range (Welcomme 1988, Charrier et al. 2016, Svolikova et al. 2016).

In China, *P. parva* grows quickly, with maximum length up to 11 cm; it is a batch spawner and reaches sexual maturity in the first year of its life (Zhu et al. 2014). Apart from being prey to carnivorous fish, traditionally *P. parva* was also regarded as a delicious and nutritious food for humans (Liang et al. 2010). Nowadays, as a small ornamental fish easily cultivated in both indoor and outdoor environments, it is becoming increasingly popular among aquarium hobbyists (Prinder et al. 2005, Sunardi and Manatunge 2005, Kapusta et al. 2006), as well as a promising environmental bio-indicator widely used in environmental toxicology (Wang et al. 2007, Wu and Ding 2016, Saylor 2016). Although the morphology, nutritional value and propagation techniques of *P. parva* have been studied (Zhang 2009, Zeng 2012, Hu et al. 2017), studies of its embryonic development remain lacking. Therefore, the present investigation was undertaken with the goal to describe the embryonic and larval development of *P. parva* in detail. This will provide a base to determine the complete ontogeny of *P. parva*, as well as reinforce the management and promote the value maximization for this fish.

Figure 1. The adult *Pseudorasbora parva*.

MATERIAL AND METHODS

Sexually mature adult *P. parva* (Fig. 1) were collected from Yezhi Lake (30°03'19"N; 114°26'38"E), Wuhan, Hubei Province, China. Fish were acclimated for more than one week in dechlorinated water in a plastic basin (50 L). Hormonal induction of ovulation was performed using LRH-A_2 and HCG injection. Males had mature sperm and did not require hormonal injections, whereas females were intraperitoneally injected with HCG (2000 U/kg) within 10 h after the LRH-A_2 (5 μg/kg) injection. Eggs were extruded by females 8–9 h after the HCG injection. Extracted eggs were placed in dry transparent culture plates. Sperm was collected by applying abdominal pressure and added to the eggs. After gentle mixing for 20–30 s, 50 mL of water was added to facilitate fertilization. After the fertilization, we added 500 mL of water from a large tank containing continuously oxygenated water.

During the incubation, water was replaced twice daily (8:00 am and 8:00 pm). Water temperature, dissolved oxygen and pH of water were measured by a multiparameter water quality analyzer (YSI Pro Plus, USA) three times a day (8:00 am, 2:00 pm and 8:00 pm), and maintained at 24 ± 1 °C, 7.85–8.03 mg/L and 7.53–7.82, respectively. Egg development was observed at 15 min intervals under a stereoscopic microscope (Olympus SZX-7, 0.8, Japan) equipped with a digital camera. Unfertilized eggs and dead embryos were removed regularly. Embryonic development time was defined as the time when 50% of fertilized eggs were hatched.

After hatching, the larvae were transferred to plastic basins (50 L) filled with aerated water and microscopically analyzed every day until the morphology of the fry was similar to the adult fish (20 days after hatching).

We measured 30 individuals at each developmental stage (embryos, larvae and fry). Statistical analyses were performed using SPSS software (Version 16.0). Data were expressed as mean ± SD.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, the methods were carried out in accordance with the approved guidelines.

RESULTS

Embryonic development

From fertilization to hatching, sequence of the most important events of the embryonic development of *P. parva* is shown in Table 1 and Figs 2–39.

Fertilized egg

The mature eggs of *P. parva* were (1.63×1.31) ± 0.04 mm in size, transparent, adhesive and elliptical (Fig. 2). The membrane composed of two layers contained a large quantity of yolk. About two minutes after fertilization, the egg began to take up water and swell, and then a narrow perivitelline space appeared. The dimensions of fully swollen eggs were about (1.75×1.50) ± 0.04 mm (Fig. 3).

Blastodisc formation stage

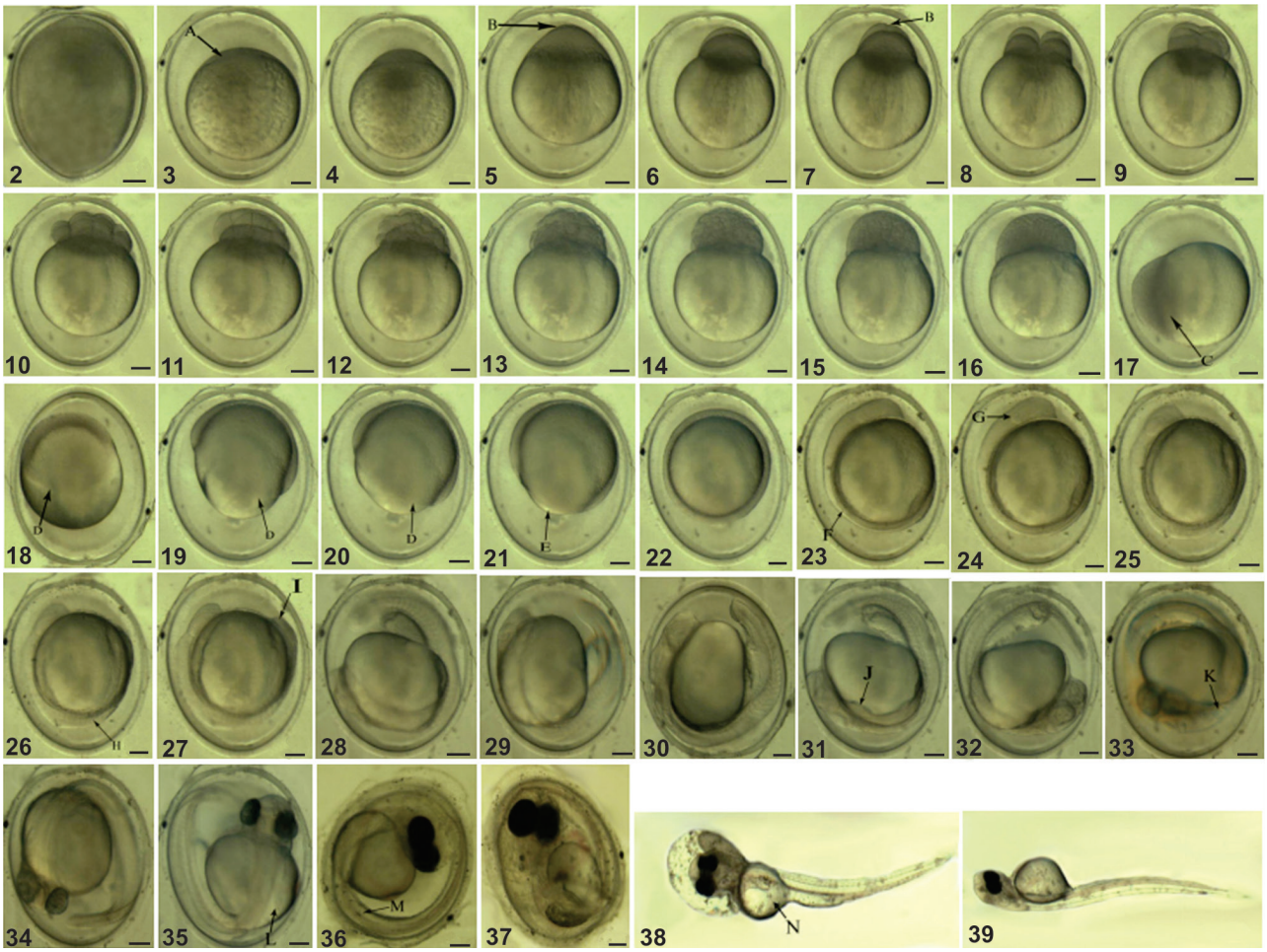
About 34 min after the fertilization, the egg protoplasm moved and aggregated towards the animal pole, forming the primary primordium (Fig. 4). The primordium bulged and formed a blastoderm in the shape of a knoll. The blastoderm became thicker as the protoplasm moved.

Cleavage stage

The cleavage in *P. parva* is meroblastic, as there is plenty of yolk in the egg. About 54 min after fertilization, the first

Table 1. Time and phases of embryonic development of *Pseudorasbora parva* at 24 ± 1 °C.

Serial number	Development stage	Time
1	Fertilized egg	0:00
2	Blastodisc formation	0:34
3	Cleavage stage	0:54 to 3:30
4	Blastula stage	3:44 to 6:49
5	Gastrula stage	8:44 to 11:44
6	Neurulation stage	11:59 to 12:51
7	Organ differentiation stage	18:11 to 95:07
8	Hatching stage	109:15



Figures 2–39. Embryonic development of *Pseudorasbora parva*: (2) Fertilized egg; (3) The fully-swollen egg (A, blastoderm); (4) Blastodisc formation; (5) The first cleavage furrow (B, cleavage furrow); (6) 2-cell phase; (7) The second cleavage furrow (B, cleavage furrow); (8) 4-cell phase; (9) 8-cell phase; (10) 16-cell phase; (11) 32-cell phase; (12) 64-cell phase; (13) Cellulouse phase; (14) Morula phase; (15) Early blastula phase; (16) Mid-blastula phase; (17) Late blastula phase (C, cells epiboly); (18) Early gastrula phase (D, germ ring); (19) Mid-gastrula phase (D, germ ring); (20) Late gastrula phase (D, germ ring); (21) Neural embryo formation (E, blastopore); (22) Blastopore closed phase; (23) Somites appearance (F, somite); (24) Optic vesicle appearance (G, optic vesicle); (25) Optic capsule appearance; (26) Notochord appearance (H, notochord); (27) Tail bud appearance (I, tail bud); (28) Otic vesicle appearance; (29) Crystalline lenses formation; (30) Muscle function phase; (31) Heart bud appearance (J, heart bud); (32) Heartbeat phase; (33) Otolith appearance (K, otolith); (34) Eye pigment appearance; (35) Pectoral fin bud appearance (L, pectoral fin bud); (36) Body pigment appearance (M, body pigment); (37) Hatching prophase; (38) Hatching phase (N, yolk sac); (39) Newly hatched larva. Scale bars: 2–37 = 0.2 mm, 38–39 = 0.5 mm.

cleavage furrow occurred in the topmost blastoderm (Fig. 5). It was divided into two equal blastomeres at 1 h and 11 min after the fertilization (Fig. 6). Due to the pattern of meroblastic cleavage in telolecithal eggs, the second division was meridional and perpendicular to the first cleavage furrow (Fig. 7), resulting in four equal blastomeres about 1 h and 28 min after the fertilization (Fig. 8). The third division was similar and parallel to the first division, eight cells were arranged in two rows of four cells, and the arrangement of the two parallel rows was approximately rectangular (Fig. 9). Two h and 24 min after the fertilization, the fourth division took place: it was perpendicular to the direction of the first division, and resulted in a 16-cell blastomere (Fig. 10). As the division proceeded, the size of the blastomere decreased, while the total volume of the cytoplasm remained unchanged (Figs 11–13). Almost 3 h and 30 min after the fertilization, blastoderms were converted into solid cell clusters and boundary lines among cells became indistinct, which signified the formation of morula (Fig. 14).

Blastula stage

Along with the successive cleavages, there was a progressive increase in the number of cells with a reduction in size. About 3 h and 44 min after the fertilization, the animal pole formed a cap-like structure atop the yolk, and the morula entered the blastula stage. In the early blastula stage, the vegetal pole started to shift slightly (Fig. 15), and the blastocoele formed as the regular movements of the vegetal pole proceeded (Fig. 16). At 6 h and 49 min post-fertilization, blastoderm flattened out and began to wrap around the yolk sac. Thus, blastoderm entered the late blastula stage. When the blastoderm wrapped downward as far as about 1/3 of yolk sac, the embryo began to rotate intermittently in the oolemma (Fig. 17).

Gastrula stage

As a result of epiboly and involution of blastoderm, the germ ring was completed around the margins of blastoderm about 8 h and 44 min after the fertilization, marking the beginning of the gastrula stage (Fig. 18). In the early gastrula stage, the blastoderm enclosed approximately 1/2 of the yolk sac. When the blastoderm expanded to cover 2/3 of the yolk sac, the involution of blastoderm thickened at the margin of the germ ring, and the appearance of the embryonic shield marked the beginning of the middle gastrula stage (Fig. 19). At 11 h and 44 min post-fertilization, blastoderm covered about 3/4 of the yolk sac, thus marking the onset of the late gastrula stage (Fig. 20).

Neurula stage

At 11 h and 59 min post-fertilization, the neural groove began to form in the dorsal midline of the embryo, and the blastoderm entered the neurula stage. During this stage, blastoderm covered about 4/5 of the yolk sac. Simultaneously the blastopore and the yolk plug appeared (Fig. 21). As the epiboly of blastoderm proceeded, the blastopore closed (encircled the yolk) completely 12 h and 51 min after the fertilization (Fig. 22).

Organ differentiation stage

Two somites in the mid-part of the embryo could be observed about 18 h and 11 min after the fertilization (Fig. 23). Very quickly after that, 18 h and 56 min after the fertilization, four to five pairs of somites and an elliptoid optic vesicle could be observed (Fig. 24). About 21 h and 21 min after fertilization a pair of kidney-shaped optic capsules became visible in the optic vesicle. Tail bud also formed, but did not separate from the yolk sac yet (Fig. 25). Right after the notochord formed in the mid-part of the embryonic axis, at 23 h and 34 min after fertilization, tail bud separated from the yolk sac. There were 10–12 pairs of somites in this phase (Figs 26, 27). The length of the tail was about 1/3 of the embryo length, and fin fold could be seen on the surface of the tail. There were 19–20 pairs of somites on the embryonic axis. An otic vesicle behind the optic vesicle became visible approximately 29 h and 20 min after fertilization. At this stage, 3/5 of the V-shaped embryo was encircled with the yolk sac and about 22–23 pairs of somites could be seen (Fig. 28). Crystalline lenses in the optic capsule and 25–26 pairs of somites could be observed 30 h and 25 min after fertilization (Fig. 29). At 31 h and 20 min post-fertilization, the first obvious contraction of somite-derived muscle (myotome) could be observed. Soon, the tail of the embryo began to wriggle intermittently (Fig. 30). At 31 h and 40 min post-fertilization, a tubular rudiment of the heart and 29–30 pairs of somites could be observed (Fig. 31). The heartbeat appeared at 36 h and 30 min post-fertilization. A colorless liquid flowing slowly in the embryo could also be observed at this stage. The rhythm of the heartbeat was about 45–48 beats per minute (Fig. 32). A paired otolith could be observed in the otic vesicle 38 h and 35 min post-fertilization. At this stage, the embryo moved with increased frequency, and the length of the separated tail was about 2/5 of the embryo length (Fig. 33). After 40 h and 15 min post-fertilization, the appearance of pigment could be observed in the crystalline lens, and soon followed by condensing of the color (Fig. 34). Rudimentary crescent pectoral fin appeared simultaneously with the embryo encircling 2/3 of the yolk sac, about 44 h and 35 min after fertilization (Fig. 35). The flow of red blood could be seen in the embryo about 49 h and 35 min after fertilization. At this point, the rhythm of the heartbeat was much more rapid, at 130 beats per minute. After 60 h and 39 min post-fertilization, some stellate pigment spots appeared on the surface of the tail. Later on, the number and size of the spots increased (Fig. 36).

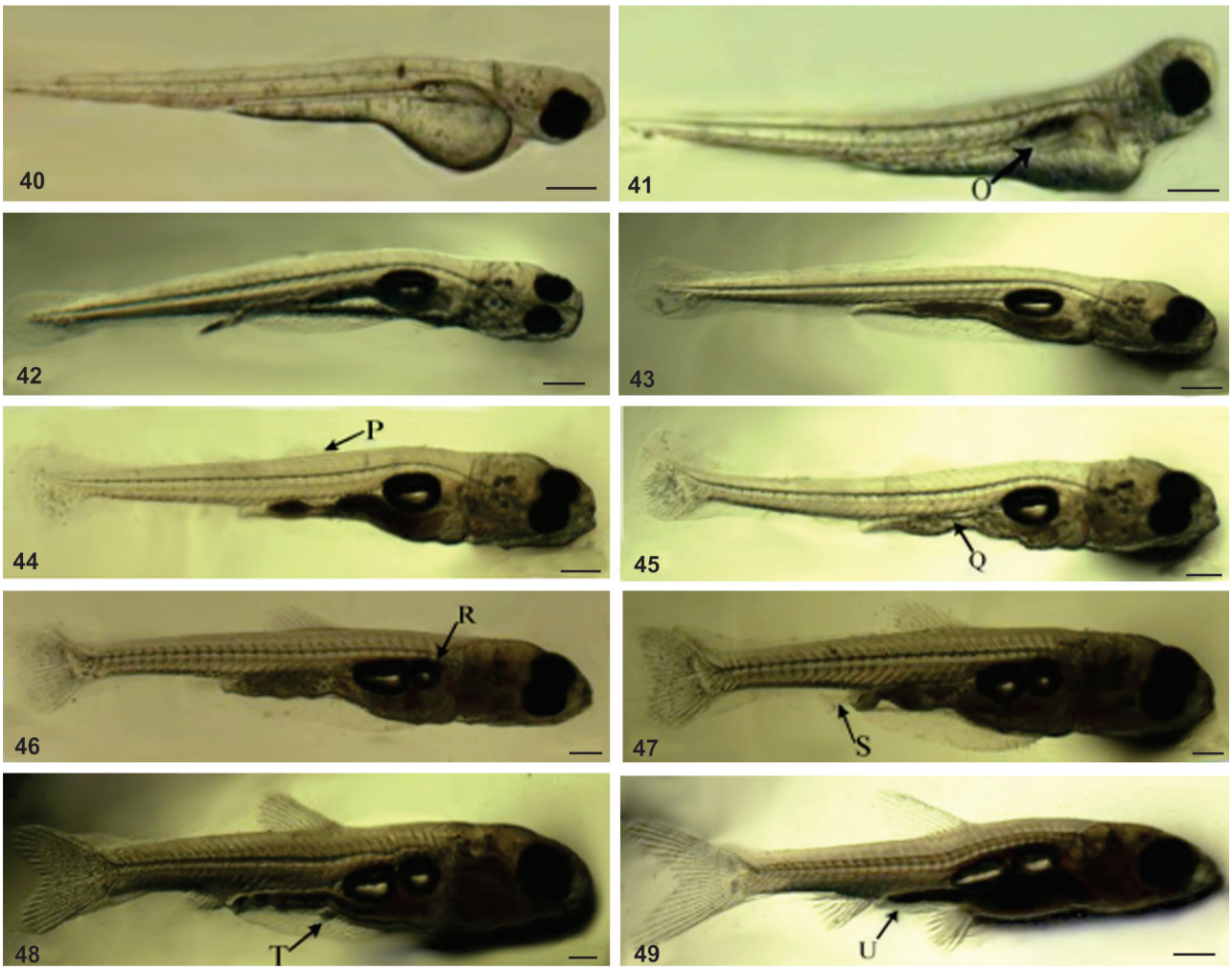
Hatching stage

At 95 h and 7 min post-fertilization, before the hatching stage, the tip of the tail passed over the brain of the embryo and the tail was flattened (Fig. 37). With the acceleration of body movements and increasing pushing against the egg shell, the larvae began to break and shake off the shell about 109 h and 15 min after fertilization. Most of the larvae hatched tail-first, although a proportion hatched head-first. The total length of the newly hatched larvae was 4.1 ± 3 mm (Fig. 38), and most of them remained immobile at the bottom of the tank immediately after hatching (Fig. 39).

Larval development

The pyriform yolk sac of the newly hatched larva was about 1.8 ± 0.12 mm in length, and the head of the larvae was bent toward the yolk sac. One day after the hatching, the larvae were 5.8 ± 0.09 mm in length. At this stage, the mouth was not formed but the larva had a streamlined body. Therefore, the larvae were still completely reliant on endogenous nutrients in this period (Fig. 40).

Two days after hatching, the larvae were about 6.0 ± 0.11 mm in total length, the yolk sac contracted, and the first swim bladder began to aerate. The intestinal tube appeared and larvae's swimming ability improved (Fig. 41).



Figures 40–49. The larval development of *Pseudorasbora parva*: (40) One day after hatching (DAH); (41) Two DAH (O, the first swim bladder); (42) Three DAH; (43) Four DAH; (44) Five DAH (P, dorsal fin); (45) Six DAH (Q, intestinal tube wriggle); (46) Nine DAH (R, the second swim bladder); (47) Eleven DAH (S, anal fin); (48) Thirteen DAH (T, pectoral fin); (49) Twenty DAH (U, fin fold). Scale bars: 40–48 = 0.5 mm, 49 = 1.0 mm.

Three days after hatching, the total length of the larvae was 7.2 ± 0.12 mm and the first swim bladder aerated completely (Fig. 42). In this period, the larvae could swim horizontally for some distance and fed on some algae, protozoa and rotifers. Therefore, larvae entered the transitional yolk-sac larval stage, when they relied both on endogenous and exogenous nutrients.

Four days after hatching, the total length of the larvae was 7.4 ± 0.09 mm, the yolk sac almost disappeared and the liver formed. The pectoral fin fold became easily observable at this stage (Fig. 43).

Five days after hatching, the total length was about 8.3 ± 0.10 mm. The rudiment of the dorsal fin formed, and some osseous fin rays in the under-lobe of the caudal fin appeared (Fig. 44).

Six days after hatching, the yolk sac disappeared completely and the larvae were 8.4 ± 0.07 mm-long (Fig. 45). At this point, the larvae entered the stage of complete reliance on exogenous nutrients, and therefore we refer to them as fry henceforth.

Nine days after hatching, the fry was 9.7 ± 0.08 mm-long. The second swim bladder began to aerate and the dorsal fin separated completely from the fin fold. In addition, the caudal fin bifurcated in this period (Fig. 46).

Eleven days after hatching, the total length of the fry reached 10.0 ± 0.08 mm. In this period, the rudiments of the anal fin appeared and the fry behaved agilely (Fig. 47).

Thirteen days after hatching, the fry was 11.0 ± 0.10 mm-long. The rudiments of the ventral fin were formed, and

the shape of the upper and lower lobes of the caudal fin was almost identical (Fig. 48).

Twenty days after hatching, the total length of the fry reached 13.8 ± 0.07 mm. Only a few fin folds existed on the ventral and caudal peduncle. At this stage, the fry was morphologically similar to adult fish (Fig. 49).

DISCUSSION

The fertilized egg of *P. parva* is a typical telolecithal egg, exhibiting discoidal meroblastic cleavage. The egg size, shape and properties are similar, but not identical, to other Gobioninae species, such as *Saurogobio dabryi* (Bleeker, 1871) (He et al. 1996), *Squalidus argentatus* (Dybowski, 1872) (Sauvage & Dabry de Thiersant, 1874) (Li et al. 2005), *Hemibarbus maculatus* (Bleeker, 1871) (Gu et al. 2006) and *Paracanthobrama guichenoti* (Bleeker, 1865) (Gu et al. 2008a). The perivitelline space in the fertilized egg of *P. parva* is smaller than in four major Chinese carps (Wang and Zhao 2008). It is believed that in some species of fish larger perivitelline space contributes to the defense against environmental adversities and ensures higher survival rates in lotic environments (Lake 1967, Matsuura 1972). Thus, the characteristics of *P. parva* eggs fit this species' preferred reproduction sites: in lakes or ponds with little water flow. The fertilized egg of *P. parva* has two-layered membrane; the outer membrane is the secondary egg envelope which is flexible and adhesive. Therefore, the egg can easily adhere to rocks, gravel and plants in water. At the hatching stage, the outer membrane of the egg becomes thinner until it finally breaks, facilitated by the dissolution by the hatching enzyme and embryonic movements (Xu et al. 2011).

Embryonic development of *P. parva* is similar to other freshwater teleosts. The organs of *P. parva* are almost completely developed before hatching, which is similar to *H. maculatus* (Gu et al. 2006) and *P. guichenoti* (Gu et al. 2008a). It takes about 36 h from fertilization to the heartbeat phase, and 73 h later the hatching begins. A similarly long time, about 75 h, from the heartbeat stage to hatching was also reported in a hybrid between *Siniperca scherzeri* (Steindachner, 1892) and *Siniperca chuatsi* (Basilewsky, 1855) (Mi et al. 2009). From the completion of the organ differentiation to hatching, the embryo remained in the membrane for a long time, which was described as a phenomenon named 'delayed hatching' (Wu and Zou 1993). This phenomenon is likely to be an evolutionary strategy to enhance the ability of the larvae to adapt to their environment immediately after hatching.

The timing of hatching was quite different even among the eggs fertilized at the same time and developed under the same conditions. The interval from the first hatched larva (75.47 h) to the last (116.80 h) was about two days. This phenomenon was also observed in *Esox lucius* Linnaeus, 1758 (Qiao et al. 2005), *H. maculatus* (Gu et al. 2006) and *P. guichenoti* (Gu et al. 2008a). According to previous studies, water temperature, dissolved oxygen, pH, light and hereditary variation can influence the speed of fish embryo development (Cussac et al. 1985, Asoh and Yoshikawa 2002). Influence of external environmental conditions on the embryonic development still needs to act through gene expression and regulation. So, the difference of *P. parva* embryonic development speed may also be regarded as a strategy for the prolongation of the generation.

In addition, during the embryonic development, rotation of embryos was a commonly observed phenomenon. Zhu (1982) found that the embryo rotation of *Clarias fuscus* (Lacepède, 1803) began in the blastula phase, whereas Zhang (2007) reported that the embryo rotation of *Branchiostoma belcheri* (Gray, 1847) began during the neurula phase. Gu et al. (2008b) and He et al. (2014) reported that the embryo rotation in *Erythroculter ilishaeformis* (Bleeker, 1871) and *Botia superciliaris* (Günther, 1892) began in the pre-hatching stage. The rotation of embryos of *P. parva* was observed in the blastula stage in our study. Therefore, the timing of the occurrence of embryonic rotation varies among fishes. There are two possible reasons for the embryonic rotation: 1) depletion of the yolk sac content on the animal pole by the propagating cells has caused a change in the balance of the embryo; 2) the rotation of embryo can increase its utilization of dissolved oxygen, which is conducive to respiration and can promote the hatching.

Newly hatched larvae were about 4.1 ± 3 mm in total length, and remained immobile at the bottom of the tank immediately after hatching. A series of significant changes in the external morphology, as well as some internal organs, could be seen after hatching, during the larval development. During the yolk-sac larva period, only the pectoral fins could be seen, and the larva's ability of swimming and escaping from predators was very limited. The pectoral fins formed before hatching, followed by the caudal fin, dorsal fin and anal fin forming in that order after the hatching, while the ventral fins formed last. The first swimming bladder formed about one day after hatching, and then aerated gradually in subsequent days, while the second swim bladder appeared about nine days after hatching. The complete disappearance of the yolk sac six days after hatching marked the metamorphosis of larvae into fry, which began to feed on algae, protozoa and rotifers. Twenty days after hatching, all fins were formed, and the morphology of the fry was very similar to the adult fish.

In conclusion, the eggs of *P. parva* were transparent, adhesive and elliptical. From fertilization to hatching, the embryonic development lasted for about 109.25 h at 24 ± 1 °C. The newly hatched larvae were 4.1 ± 3 mm in total length. Yolk absorption was completed within six days post-hatching. About 20 days after hatching, the morphology of the fry was similar to the adult fish. These findings provide a basis for determining the complete ontogeny of *P. parva*, as well as facilitate the management and utilization of this fish.

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