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Embryonic and Larval Development of Endangered Temoleh, *Probarbus jullieni* (Sauvage)

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ABSTRACT

The present study was carried out to investigate the embryonic and larval development of the endangered Temoleh, *Probarbus jullieni* in lab-rearing condition. The matured egg and sperm were collected by stripping the bloodstock of Temoleh fish after injecting with ovaprim hormone extract. The samples were collected from hatching tank at every 10 min interval for the first hour, 20 min for the second hour, 30 min for the third hour and then hourly interval up to hatching. After hatching, larvae were observed daily until the complete disappearance of the yolk sacs. The fertilized eggs were spherical, demersal, adhesive and brownish-yellow in colour with a mean diameter of 2316 μm. First cleavage occurred within 10 min post-fertilization at temperature ranged from 26.0 to 28.0°C. Hatching started 22 h post-fertilization and completed within 25 h at the same temperature. The yolk sac was completely absorbed 61 h after hatching. At the same time, the larvae started to swim actively and feed exogenously. This marks the first description on the early life history of *P. jullieni*. The present study will provide some valuable information on the ontogeny, breeding biology and early larval rearing protocol of *P. jullieni* which will ultimately be helpful towards the establishment of large scale seed production technique for conservation and aquaculture production.

Key words: Early development stage, *Probarbus jullieni,* endangered species, aquaculture production, Malaysia

INTRODUCTION

The freshwater fish *P. jullieni* (Sauvage) locally known as "Temoleh" is a commercially important Malaysian wild river carp in Malaysia. The colours of the live specimens are very attractive with 7 narrow longitudinal black stripes from tip of operculum to end of the body trunk. The biggest specimen of *P. jullieni* was 120 cm in total length and 21 kg in weight. It is said to be the largest species of the cyprinids in Peninsular Malaysia (Mohsin and Ambak, 1983). In recent years, the stocks of *P. jullieni* has declined drastically in its natural habitats due to pressures from

unsustainable fishing and habitat degradation, resulting from intensive development activities such as land clearing, deforestation and dam construction (Chew *et al.*, 2010). Total landing has drastically decreased from 350 MT in 2003 to 110 MT in 2007 (Chew *et al.*, 2010). This fish is currently considered as endangered species and recorded in IUCN red list (Hogan *et al.*, 2009; IUCN, 2011).

The importance of early development stages has been described by Rahman *et al.* (2009) for understanding the biological clock and culture techniques of guchibaim (*Mastacembelus pancalus*). Information on early life history 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). In addition, studies on embryonic and early larval development, besides providing interesting information, are imperative and essential to the successful rearing of larvae for large scale seed production and aquaculture (Khan and Mollah, 1998; Rahman *et al.*, 2004).

Considering the enormous importance of *P. jullieni*, the information of the early life history is an important requirement for optimization of the large scale seed production especially for conservation, culture and management. Therefore, an attempt was made to study the detailed embryonic and larval development of the critically endangered *P. jullieni* in a controlled captive condition.

MATERIALS AND METHODS

Broodstock selection: The experiment was conducted during the natural breeding season of *P. jullieni* from November 2011 to February 2012 in the hatchery and laboratory of Fresh Water Aquaculture Expansion Centre at Perlok under the Department of Fisheries of Malaysia. The brood fishes were collected from brood ponds and were transferred to the tank of the hatchery and acclimatized for at least 10 h before used for experiment.

Egg fertilization and incubation phase: Four females and five males with body weights of 5.4-10.6 kg were used for induced breeding. Females were induced to spawn using 0.6 mL kg^{-1} ovaprim hormone whereas males were induced to spawn using 0.3 mL kg^{-1} ovaprim hormone injection. The eggs and sperm were collected from brood fishes and fertilized in one plastic bowl container. Excess sperm were then removed from the fertilized eggs by three consecutive washes with 0.9% NaCl solution. In order to remove stickiness and adhesiveness, the fertilized eggs were washed several times with fresh water and finally transferred to the hatching tray for incubation.

Image observation and analysis: Sample of fertilized eggs was collected very carefully from the hatching tank by using a dropper where they were in constant motion. The developing stages of *P. jullieni* were observed at every ten minutes interval for the first hour, 20 min interval for the second hour, 30 min for the next hour and then every hour interval up to hatching. When hatching was completed, the observations were continued at each 2 h interval for the first day and at least every 6 h interval for the following days to study each developmental stage. For each stage, samples were collected randomly from hatching tank and directly observed under a stereomicroscope (Nikon Eclipse E400). The extra eggs and larvae were preserved in 5% buffered formalin solution and concentrated by settling to the bottom of a vial for further study.

Measurement of embryos and larvae: The morphometric measurements of embryos and larvae were made on freshly prepared specimens, following the method of McEdward (1984) with slight modification. Each sample was observed four times to identify the developmental stages

(Haniffa *et al*., 2003). Developing stages were studied continuously until the embryo started twisting movement and the hatching of fertilized eggs was completed within 25 h. The diameters of the eggs were measured by using Keyence Digital Microscope (VHX-500). Five to ten specimens were used to describe each stage. The whole experiment of the embryonic and larval stages was examined at ambient water temperature (26.0-28.0°C).

RESULTS

Embryonic development: A summary of the embryonic development stages of *P. jullieni* is presented in Table 1. The unfertilized eggs (Fig. 1a) of *P. jullieni* were spherical, adhesive and brownish-yellow in colour. The mean diameter of the unfertilized eggs was 2287.70±24.18 μm with the range from 2148.00 to 2370.00 μm. The newly fertilized eggs were found almost as same as unfertilized eggs but little bit clear in term of colour (Fig. 1b).

After fertilization, the egg diameters were increased immediately. The size of eggs was reduced from 1 h and 20 min until 5 h and 30 min. The fertilized eggs have a reddish spot (blastodisc) as shown in Fig. 1c. The blastodisc was divided into two distinct cells by vertical cleavage and then become four cells (Fig. 1d) within 1 h 40 min after fertilization. Then, the cell in the egg was developed to eight, sixteen and thirty two until multi cells, respectively. The blastodisc was ended by multi cell stage which was appeared within 4 h and 30 min (Fig. 1e).

Table 1: Characteristics of different embryonic development stages of *P. jullieni*

Stage	Phase	Time after fertilization	Mean total diameter (μm)	Characteristic
A	Unfertilized eggs	0 min	2287.70±24.18	Spherical, demersal, adhesive and brownish-yellow in colour
B	Fertilized eggs	0 min	2316.00±36.61	Spherical, demersal, adhesive and brownish-yellow in colour
C	Blastulation	10 min	2695.10±38.39	A red spot on one pole
D		1 h and 40 min	3257.30±21.40	The second division after four blastomeres resulted from two blastomeres
E		4 h and 30 min	3049.80±24.36	Appearance of multiple cells
F	Morula	5 h	3003.00±22.24	Spherical shape, blastomere visible at the animal pole which gradually increased in size over time
G	Early gastrula stage	5 h and 30 min	2997.70±20.20	Appearance of dome-shaped structure
H	Gastrulation	6 h	3266.40±19.03	Blastomere started invading the yolk
I		7 h	3249.20±21.44	Distinct germinal ring occupied a large part of the whole yolk
J		9 h	3290.50±22.24	Blastoderm covered more than half size of the yolk, embryonic shield visible from the animal pole
K	Late gastrula stage	10 h	3266.60±17.51	Blastoderm covered ¾th of the yolk. Embryo shell visible
L	Yolk plug Stage	12 h	3321.70±16.44	Completion of yolk invasion. Head, optic and tail rudiment visible
M	Organogenesis	14 h	3236.50±32.24	Head and tail rudiment more clearly visible
N		18 h	3308.10±26.17	Head become bigger than other part and the optic rudiment clearly visible
O		20 h	3306.10±22.77	Heart rudiment and gill rudiment appeared. Notochord becomes visible, auditory and optic vessels more developed
P	Beginning hatching stage	22 h	3286.30±24.52	Embryo occupied majority of the whole egg peripheral space. Blood circulation was started
Q	Just Before Hatching	24 h	3293.60±25.08	Newly hatched larvae with slow tail movement Continuously beat the egg shell by the caudal region

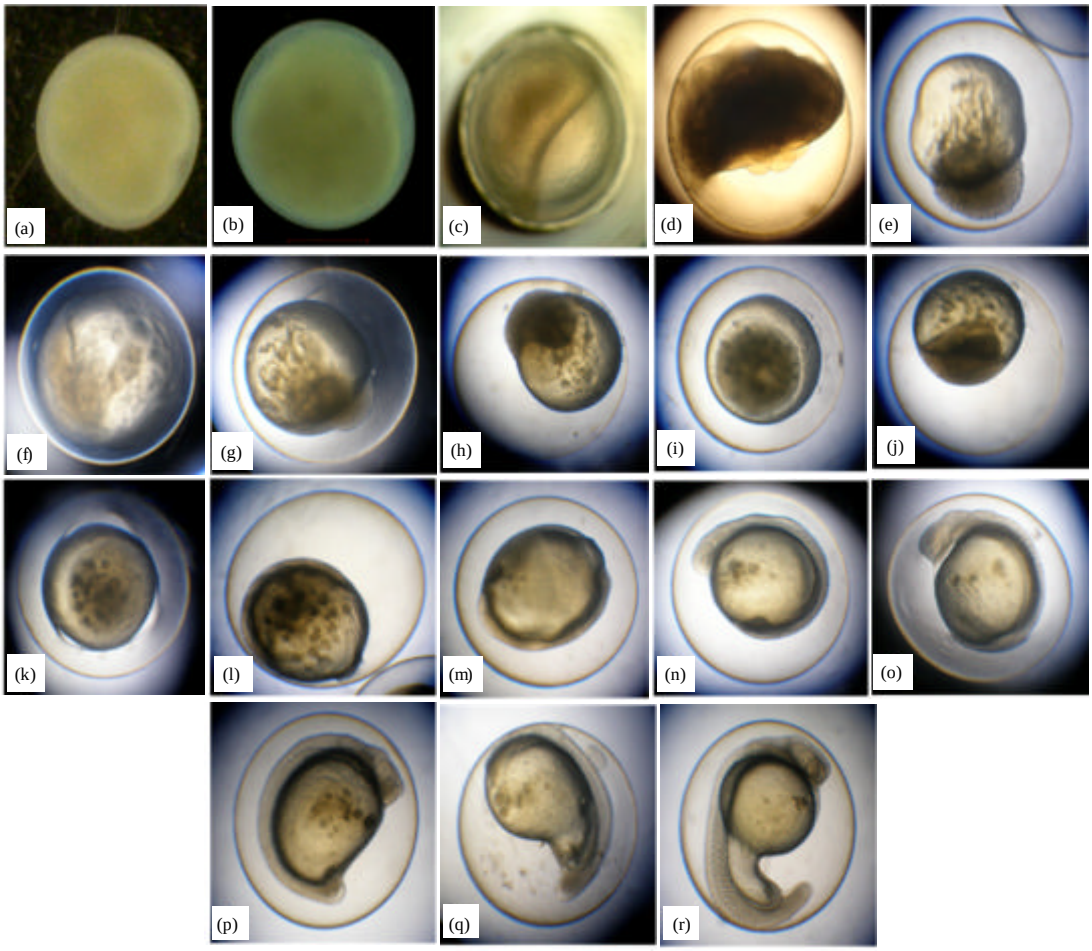

Fig. 1(a-r): Embryonic developmental stages of *P. jullieni*, (a) Unfertilized egg, (b) Newly fertilized egg, (c) Blastulation, (d) 4 cell stage, (e) Multi-cell stage, (f) Morula stage, (g) Early gastrula stage, (h) Gastrulation (i) 7 h old, (j) 9 h old, (k) Late gastrula stage, (l) Yolk plug stage, (m) Organogenesis, (n) 18 h old, (o) 20 h old, (p) Starting hatching stage, (q) Just before hatching and (r) Fully hatching stage

After 5 h of fertilization, the morula stage (Fig. 1f) formed and blastomere became visible at the animal pole which gradually increased in size over time. After 30 min from the morula stage, the dome-shaped structure appeared to become the early gastrulation stage (Fig. 1g) and 30 min thereafter the fully gastrulation stage was formed (Fig. 1h). The gastrulation stage was continuing within their specific physical appearance up to late gastrulation stage within 10 h of fertilization (Fig. 1i-k). The next stage was the yolk plug (Fig. 1l), which was identified by the complete invasion of the yolk 12 h post fertilization. In this stage the head and tail rudiment were visible. The head and tail ends were more clearly differentiated in organogenesis stage within 14 h (Fig. 1m). The head become bigger than other part and the optic rudiment, which clearly become

Table 2: Characteristics of the larval development stages of *P. jullieni*

Stage sample	Age (h)	Characteristics
1	0.00 (hatching stage)	Larvae were straight, slender, transparent showing internal organs, yolk sac attached to the body, tail moved was slowly, head anteriorly and tail posteriorly situated
2	2.00	Larvae move stronger than before, larvae look as slender and silver in colour, yolk sac still remaining but smaller than before
3	6.00	Tail of larvae more clearly observed than before, larvae move the tail to swim, still have yolk egg sac, tail is sharper, the eyes more visible, head and body laterally compressed
4	8.00	Larvae rotate, move more actively, yolk sac partially reduced, body more transparent, eyes and anus slightly visible, intestine also appeared
5	10.00	Larvae swallow nutrient from yolk sac, the body was more silver colour and tail already sharp
6	30.00	Larvae increasing in size, decreasing size of yolk sac, eye can see with black dot colour
7	36.00	Larvae move frequently-almost every second, two black dot of larval eyes obviously be seen through naked eyes, yolk sac decreased by increasing size of body, colour of larvae changed to yellowish silver, mouth cleft formed, pectoral bud also appeared
8	42.00	More aggressive movement, pectoral fin appeared, mouth cleft more prominent, distinct functioned actively, reddish blood was seen around the heart region
9	46.00	Starting to go outside from egg membrane, swimming throngh the running water, yolk sac convex interiorly and air bladder distinct
10	50.00	Larval bodies are more silver in colour, more reddish blood below the yolk sac
11	56.00	The pectoral fin is faster on moving, mouth jaw and eyes started to move, the anus clearly observed, yolk egg sac almost disappeared from the body of larvae
12	61.00	Yolk sac completely absorbed, larvae swim actively and feed exogenously

visible 18 h post fertilization (Fig. 1n). The organogenesis stage ended when the heart rudiment and gill rudiment appeared. At 20 h after fertilization, the notochord becomes visible (Fig. 1o). In this stage, the auditory and optic vessels were also developed.

At 22 h after fertilization, the beginning of hatching stage was found and in this stage the embryo was almost occupied in the egg (Fig. 1p). The blood circulation was evident throughout the whole body. The just before hatching stage (Fig. 1q) was observed within 24 h. In this stage, the embryo was continuously beat the egg membrane by the caudal region with slowly tail movement.

Larval stage: Larval stages were determined by starting the fully hatching stage after 25 h post-fertilization (Fig. 1r) and the larvae can swim actively without having the yolk sac. The detailed larval stages of *P. jullieni* are described in Table 2. The water temperature was kept at 26.0-28.0°C throughout the larval growing stage.

DISCUSSION

The embryonic period was counted from the time when the eggs were fertilized by sperm and ended until the embryo has attained the generalized organ systems as those commonly appeared in other fish species (Mollah *et al*., 2011). There is no comparison to others as no reported similar works are available on *P. jullieni*. The unfertilized eggs of *P. jullieni* (Fig. 1a) ranged between 2.15 and 2.37 mm in diameter which were more or less similar to those reported in *Labeo bata* (Miah *et al*., 2009) and in *Labeo rohita* (Mookerjee, 1945). The diameter of *P. jullieni* eggs from unfertilized to just after fertilization increased from 2.29 to 2.32 mm compared to the diameter of eggs that ranged between 0.7 and 0.8 mm in *L. bate* (Miah *et al*., 2009) and between 4.1 and 4.8 mm in *L. rohita* (Chakraborty and Murty, 1972), respectively. This differentiation may

be occurred due to the differences in species and size of carp broodstocks (Miah *et al*., 2009). In *P. jullieni*, the second cleavage (4 cell stage) occurred at 1 h 40 min, which was much later than that of *L. rohita* and *L. bata* in which 4 cell stage attained within 45 and 55 min, respectively (Mookerjee, 1945; Miah *et al*., 2009). The morula stage formed within 5 h post-fertilization whereas, Miah *et al*. (2009) found this stage in *L. bata* at about 4 h and 30 min after fertilization. On the other hand, Mookerjee (1945) found the same stage in *L. rohita*, which occurred at 5 h and 45 min after fertilization. This differentiation might be due to the differences in species and temperature (Miah *et al*., 2009). In this study, the hatching of *P. jullieni* was observed to be at 24 h after fertilization of egg, which was closer to those observed in *L. bata* (Miah *et al*., 2009) and *Rita rita* (Mollah *et al*., 2011). However, this difference is not significantly affected since the incubation period usually depends on the species and water temperature. The time required for hatching eggs is inversely related to the incubation period (Rana, 1990). Embryonic development and variability of hatching time in the fertilized eggs of majority of the fish are generally influenced by water temperature (Jhingran, 1982).

In larval stage of *P. jullieni*, the egg membrane was not removed until 46 h of hatching stage compared to the other species such as *L. bata* (Miah *et al*., 2009) in which the egg shell of the embryo was ruptured by its tail within 2 h interval post-hatching. Mollah *et al*. (2011) observed that in case of *R. rita*, the egg membrane began to break down within 2 h after hatching. This situation was happened due to the differences is species and water temperature during hatching. The development of the pectoral bud was appeared within 36 h after hatching in *P. jullieni* but in both *L. bata* and *Cirrhinus mrigala*, it occurred at 12 h post-hatching (Miah *et al*., 2009; Khan, 1943). The yolk sac of the *P. jullieni* was completely removed at 61 h after hatching and then the larvae began to swim actively. Miah *et al*. (2009) found this developmental pattern in *L. bata* which occurred within 66 h after hatching whereas, Chakraborty and Murty (1972) observed the same in *C. mrigala* within 72 h post-hatching.

CONCLUSION

The information of the embryonic and larval development of the endangered species of *P. jullieni* is very important to protect this species from extinction as well as to develop their culture technique for aquaculture industry. The present work generated some important information on the early life history, developmental stages and commencement of first feeding time for larval rearing. This study will help the fishery biologist in understanding the biology and ecology of the fish, which might be of great use to take appropriate steps for the sustainable development of the culture, management and biodiversity conservation of *P. jullieni* in captivity.

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