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p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

Embryonic and larval development of lemon fin barb hybrid (♂ *Hypsibarbus wetmorei* × ♀ *Barbonymus gonionotus*)

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Key words

Barbonymus gonionotus
Early larval development
Hypsibarbus wetmorei
Lemon fin barb

Publication Info

Paper received : 22.04.2017
Revised received : 20.06.2017
Re-revised received : 30.07.2017
Accepted : 28.12.2017

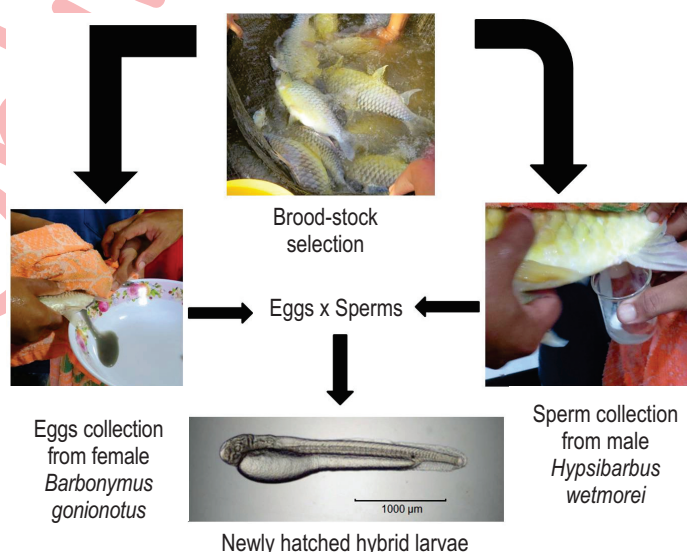
Abstract

Aim : The Lemon fin barb hybrid was developed by crossing Lampam (*Barbonymus gonionotus*) females with Kerai (*Hypsibarbus wetmorei*) males as a potential food source for lower income people in Malaysia due to fast growth and ease of culture. For delicious flesh and high market demand, the farming of this hybrid has subsequently expanded rapidly. Many of the basic biological aspects of this hybrid have not yet been investigated and in this study the embryonic and early larval development were examined.

Methodology : After injecting the brood-stocks with hormones, the matured eggs and sperms were collected by strip spawning. The developing embryonic stages were subsequently observed at 10 min intervals for the first hour, 20 min intervals at the second hour, 30 min for the next hour, and then hourly intervals up to hatching. After hatching, observations continued at 2 hr intervals for the first day and a minimum of 6 hr intervals for the following days.

Results : Results showed that the hatching of this hybrid occurred 14 hrs post-fertilization at a temperature of $24.0 \pm 1^\circ\text{C}$. The hybrid larvae began to actively swim when the yolk sac became absent 46 hrs after hatching, and were considered to be at the early larval stage. No abnormalities were evident and developmental duration and sizes were similar to *B. gonionotus*.

Interpretation : This study represents the first description of the early development stages for Lemon fin barb hybrids that may assist with the establishment of seed production and rearing techniques for aquaculture development in Malaysia.



Introduction

The creation of hybrids in aquaculture is facilitated by the external fertilization strategies that are utilized by the vast majority of fish. The goal is to induce hybrid vigor, also known as heterosis, in which the desirable characteristics from each species are expressed (Bartley *et al.*, 2000). To accomplish this, often hormones are used to allow strip spawning and then different combinations of species, as well as male and females of these species are tried.

Two closely related fish species in Malaysia include the silver barb *Barbonymus gonionotus* (locally known as “keraikunyi”) and lemon fin barb *Hypsibarbus wetmorei* (locally known as “lampam Jawa”). These fish are omnivorous cyprinids that are commercially important throughout South East Asia. It has been reported that *B. gonionotus* can breed easily in captivity and have relatively fast growth rates (Chaudhary *et al.*, 2008; Romano *et al.*, 2017), while *H. wetmorei* have attractive external features and a reportedly sweet taste. For these reasons, the Department of Fisheries (DOF), Malaysia investigated the possibility of heterosis from their hybridization. It was found that the resulting lemon fin barb hybrid obtained by crossing male *H. wetmorei* with female *B. gonionotus*, had external features and taste similar to the former and the fast growth of the latter and, moreover, can produce viable offspring (DOF, 2014).

The lemon fin hybrid, known locally as “kerai lampam” was subsequently promoted by the Malaysian Government as potential food fish for lower income residents of Malaysia. Since the introduction of this hybrid, the aquaculture production has increased three-fold from 29.93 tons in the year 2012 to 151.83 tons in 2014 (DOF, 2017). This is largely due to the ability to reach market size of 500–600 grams within 5–6 months and fetch up to USD 7.45 kg⁻¹ (DOF, 2014). For these reasons, it is expected that the farming of this hybrid will expand. Indeed, several studies have recently examined their nutritional requirements (Suharnili *et al.*, 2015; Ismail *et al.*, 2016), but many aspects of their basic biology are still lacking. This includes information regarding some of their early developmental stages that could be useful to establish breeding and early culture protocols for aquaculture development in Malaysia.

The present study was undertaken to observe the embryonic and larval development of the hybrid lemon fin barb resulting by crossing the male *H. wetmorei* (♂) and female *B. gonionotus* (♀) for stock improvement in aquaculture sector and fisheries management.

Materials and Methods

Source of brood stock and induced breeding : Apparently, healthy brood stocks were collected from ponds at Fisheries Research Institute (FRI), Jelebu, Negeri Sembilan, Malaysia and transferred to the hatchery of FRI in a one ton tank that

received gentle aeration. The brood fishes were acclimatized for at least 8 hrs before being used for breeding. From collected brood stocks, three matured *P. gonionotus* females (0.4 to 0.6 kg) (Fig. 1) and three mature *H. wetmorei* males (1.9 – 2.2 kg) were selected for the study.

The females and males were injected with 0.6 and 0.3 ml kg⁻¹ ovaprim, respectively. After 6 hr, the eggs and sperms were gently strip spawned and collected in separate containers (Fig. 2). These were then gently mixed in a bowl and excess sperm were then removed from the fertilized eggs through several washes. The fertilized eggs were then transferred to a hatching tank for incubation. During the entire experiment of the embryonic and early larval stages, this was performed at ambient water temperature (23.0–25.0°C).

Observations and analysis : A sample of fertilized eggs was collected from the hatching tank by using a glass dropper and after examination under a stereomicroscope (Olympus BX41), it was estimated that more than 80% of eggs were successfully fertilized. From a sample of 10 eggs, the developmental egg stages were observed at 10 min intervals for the first hour, 20 min intervals for the second hour, 30 min for the next four hours and then hourly intervals up to hatching. When hatching was completed, observations continued at 2 hr intervals for the first day and at least 6 hr intervals for the following days. At each stage, ten samples were randomly collected from the hatching tank and directly observed and pictures were taken using a stereomicroscope.

Measurements of embryos and larvae : General morphometric observations of the developing embryos and larvae were made on fresh specimens at a total of four times to identify the developmental stages. The egg developmental stages were studied continuously until the embryo exhibited a twisting movement and fully hatched status occurred. For the larval stages, these were observed from the initial hatching stage until the yolk sac was fully absorbed. The egg diameter was measured using a Keyence Digital Microscope (VHX-500) from the preserved specimens and a total of ten specimens were used to describe each developmental stage.

Results and Discussion

The embryonic and larval development of laboratory-reared Lemon fin barb hybrids exhibited no abnormalities and similar findings have earlier been observed in other fish hybrids. Mia *et al.* (2005) reported that a hybrid from Chinese carp, *Hypophthalmichthys molitrix* and *Aristichthys nobilis* was successfully produced, and would actually sometimes occur naturally in the wild, particularly when mature members of the opposite sex were limited. Meanwhile, cross breeding between *Clarias batrachus* ♀ and *C. gariepinus* ♂ was not only successful, but reportedly led to a higher rate of fertilization, hatching and growth (Rahman *et al.*, 1995).



Fig. 1 : Brood stock fish of *Barbonymus gonionotus*

The measurement of embryonic development phase of this hybrid is briefly described in Table 1. The unfertilized eggs of this hybrid were spherical, demersal, adhesive and bluish in colour (Fig. 3a, 4a) and the average egg diameter was 0.72 ± 0.02 mm. When the fertilized eggs were first laid on the whole surface, they were initially adhesive but this characteristic appeared to be lost upon becoming water-hardened. Fertilized eggs were larger in size than the unfertilized ones, and the vitelline membrane was very close to egg membrane. During this stage the egg was still demersal, adhesive and the yolk was brownish in colour (Fig. 3b, 4b). Several minutes after fertilization, the eggs nearly doubled in size (Fig. 3c, 4c) along with a spot at one pole consisting of a single cell that was readily recognizable with the naked eye.

The first cleavage occurred within 30 min after fertilization, which was either partial or meroblastic, forming a

transitory blastula stage. The blastodisc was divided to form two equal cells and approximately equal blastomeres (Fig. 3d, 4d). The second cleavage occurred 50 min post-fertilization and four blastomeres were clearly observed (Fig. 3e, 4e). The second cleavage was at a right angle to the first. Eight cells were formed at the third cleavage (Fig. 3f and 4f) 1 hr post-fertilization and the cleavage was horizontal, and from this stage onwards the egg diameter size slightly decreased. The fourth cleavage occurred after 1 hr 10 min post fertilization that consisted of sixteen cells (Fig. 3g and 4g) while the fifth cleavage took place 1 hr and 20 min post-fertilization (Fig. 3h, 4h). Blastomeres were divided via meridional cleavage into 32, 64, 128 cells onward geometrically.

After repeated blastomere cleavage, the morula stage (Fig. 3i, 4i) was attained within 2 hrs 30 min post-fertilization. A cap-like structure was seen over the animal pole, which gradually increased in size. The gastrulation stage was observed 4 hrs post-fertilization (Fig. 3j, 4j). The blastoderm cells were spread over from the yolk and the epibolic cells increased overall egg at this stage. The formation of a germinal ring around the yolk was clearly visible at this stage and about half of the yolk was occupied by blastoderm. At the yolk plug stage, the yolk gradually spread over the germ layer (Fig. 3k, 4k).

A rudimentary head and tail appeared and became differentiated 6 hrs post-fertilization, while at 7 hrs post-fertilization, the embryo became elongated and encircled the yolk material during the organogenesis process (Fig. 3l, 4l). Both the head and tail portions of the embryo became differentiated and a heartbeat was visible 8 hrs post-fertilization (Fig. 3m, 4m). After 10 hrs of fertilization, rudiments of the heart and gill appeared and the notochord developed (Fig. 3n, 4n). Meanwhile, the auditory and optic vessels were more visible by the naked eye and nearly before hatching (Fig. 3o, 4o), the embryo occupied the majority of the peripheral space. At this stage, blood circulation was observed. After 12 hr, (Fig. 3p, 4p), the larvae were straight,

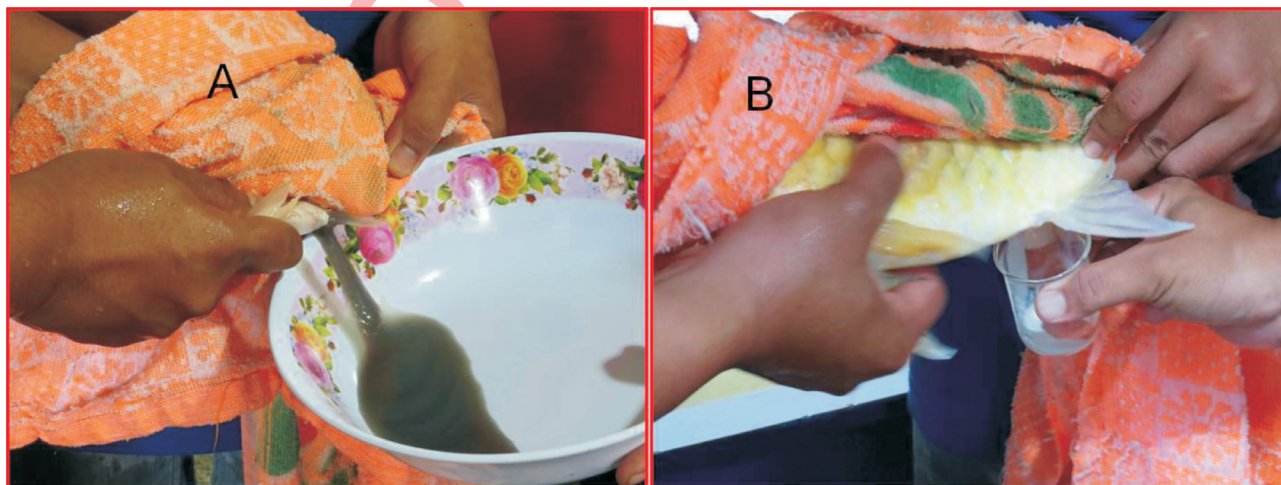


Fig. 2: (A) Collection of eggs from *Barbonymus gonionotus* and (B) sperm from *Hypsibarbus wetmorei*

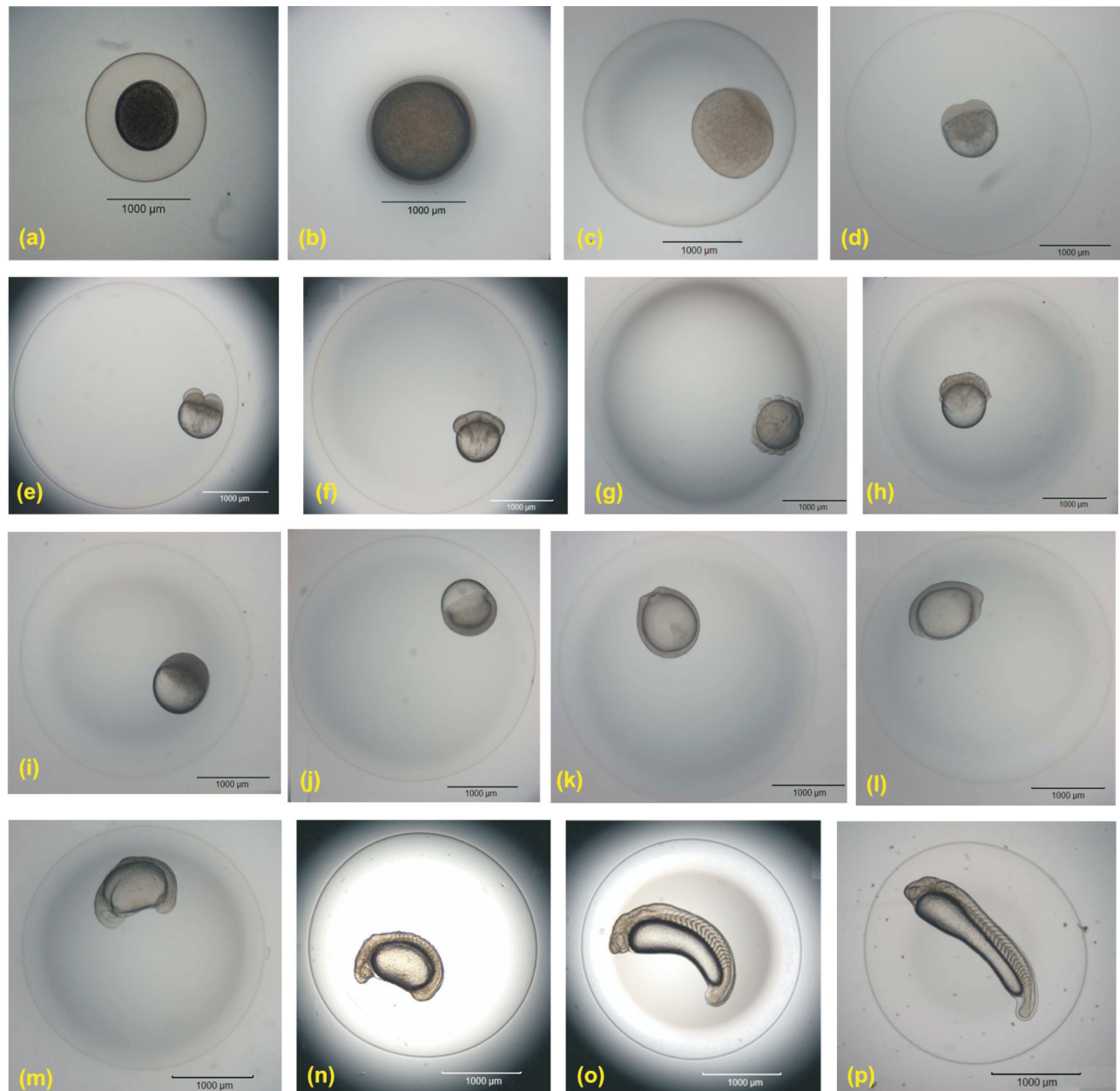


Fig. 3 : Stages in embryonic development process of Lemon fin barb hybrid (2 \times magnification): (a) Unfertilized egg, (b) Fertilized egg, (c) Blastula, (d) 2 celled, (e) 4 celled, (f) 8 celled, (g) 16 celled, (h) Multi-celled, (i) Morula, (j) Gastrula, (k) Yolk-plug, (l) Organogenesis, (m) 8 hrs stage, (n) 10 hrs stage, (o) 11 hrs stage, (p) Just before hatching

slender and the body was transparent including the internal organs. The tail began to move slowly and continuously beating the egg shell with its caudal region.

Hatching occurred 14 hrs post-fertilization and possessed a yolk sac and were initially elongated, and the internal organs were visible (Fig. 5a, 6a). The larvae at 2 hrs (Fig. 5b, 6b) began to swim stronger and developed a more silver color. After 5 hrs of hatching

(Fig. 5c, 6c), the tail became more developed and the movements in this area became more pronounced and while the yolk was still present, it was partially reduced. The eye became more visible, while the head and body was laterally compressed. By 12 hrs, the larvae became more elongated and were more transparent, while the eyes and anus were visible (Fig. 5d, 6d). The intestine was also observed clearly, but the body appeared more silver in color. At 16 hrs of hatching, melanophore bands appeared on the head

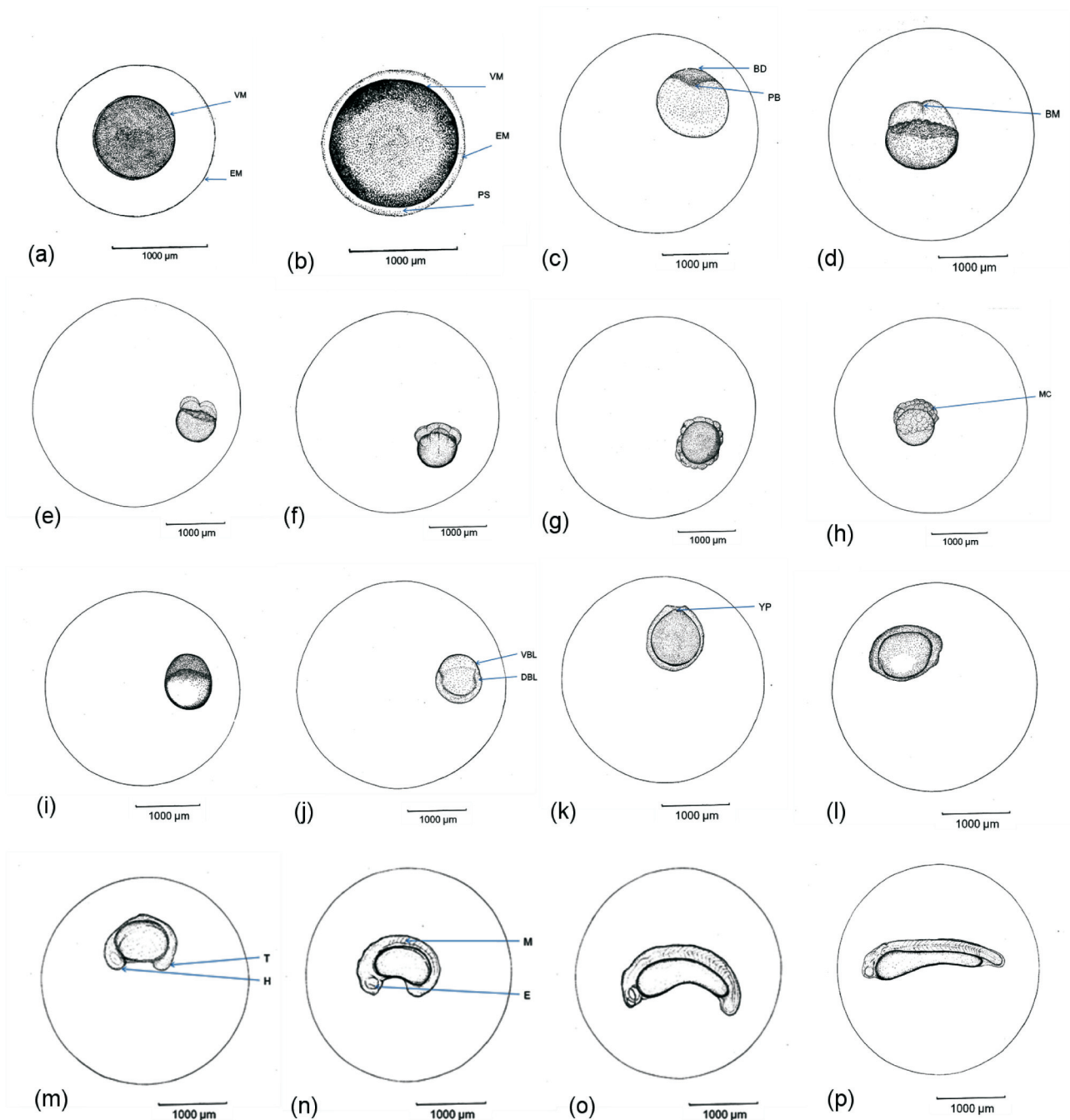


Fig. 4 : Biological sketch of stages in embryonic development process of Lemon fin barb hybrid: (a) Unfertilized egg, (b) Fertilized egg, (c) Blastula, (d) 2 celled, (e) 4 celled, (f) 8 celled, (g) 16 celled, (h) Multi-celled, (i) Morula, (j) Gastrula, (k) Yolk-plug, (l) Organogenesis, (m) 8 hrs stage, (n) 10 hrs stage, (o) 11 hrs stage, (p) Just before hatching. (VM-vitelline membrane, EM-Egg membrane, PS-Perivitelline space, BD-Blastodisc, PB-Periblast, BM-Blastomere, MC-Multi cell, VBL-Ventral blastoporal lip, DBL-Dorsal blastoporal lip, YP-Yolk plug, H-Head portion, T-Tail portion, M-Myomere, E-Eye)

and body, particularly above the eye and around the yolk sac (Fig. 5e, 6e). The pectoral fin bud and mouth cleft formed after 20 hrs of hatching and pigmentation of the eye became pronounced. The heart was distinctly visible, which was located behind the head, and showed regular beats (Fig. 5f, 6f). After 36 hrs of hatching, the

yolk sac was still present, but nearly absorbed, while the eyes were clearly visible as black dots. The body coloration became more silver and yellowish, while the mouth cleft was more distinct and actively moving. Reddish blood was clearly visible around the heart region (Fig. 5g, 6g).



Fig. 5: Stages in larval development of Lemon fin barb hybrid (2× magnification): (a) Newly hatched; (b) 2 hrs stage; (c) 5 hrs stage; (d) 12 hrs stage; (e) 16 hrs stage; (f) 20 hrs stage; (g) 34 hrs stage and (h) 46 hrs stage

After 46 hrs, the yolk sac became completely absorbed, after which they became active swimmers while the tail and head were more defined. The eyeball was dark and prominent, while the mouth cleft was well-formed with a well-developed upper and lower jaw. The pectoral and pelvic fin functioning well with faster movements, while the body color was white and black. The anal aperture and opercula were well-formed and distinct and the larvae were swimming actively and fed exogenously (Fig. 5h, 6h).

The early embryonic developmental duration and hatching of the hybrids was similar to those reported for *B. gonionotus* eggs (Basak *et al.*, 2014). For example, the duration for hatching occurred at 14 hrs in the current study, as well as those reported by Basak *et al.* (2014). However, it should be pointed out that the water temperature for the hybrids was lower at 23 - 25°C compared to 28 - 29°C in the study (Basak *et al.*, 2014). Within their tolerance range, increasing incubation temperatures are well known to accelerate embryonic

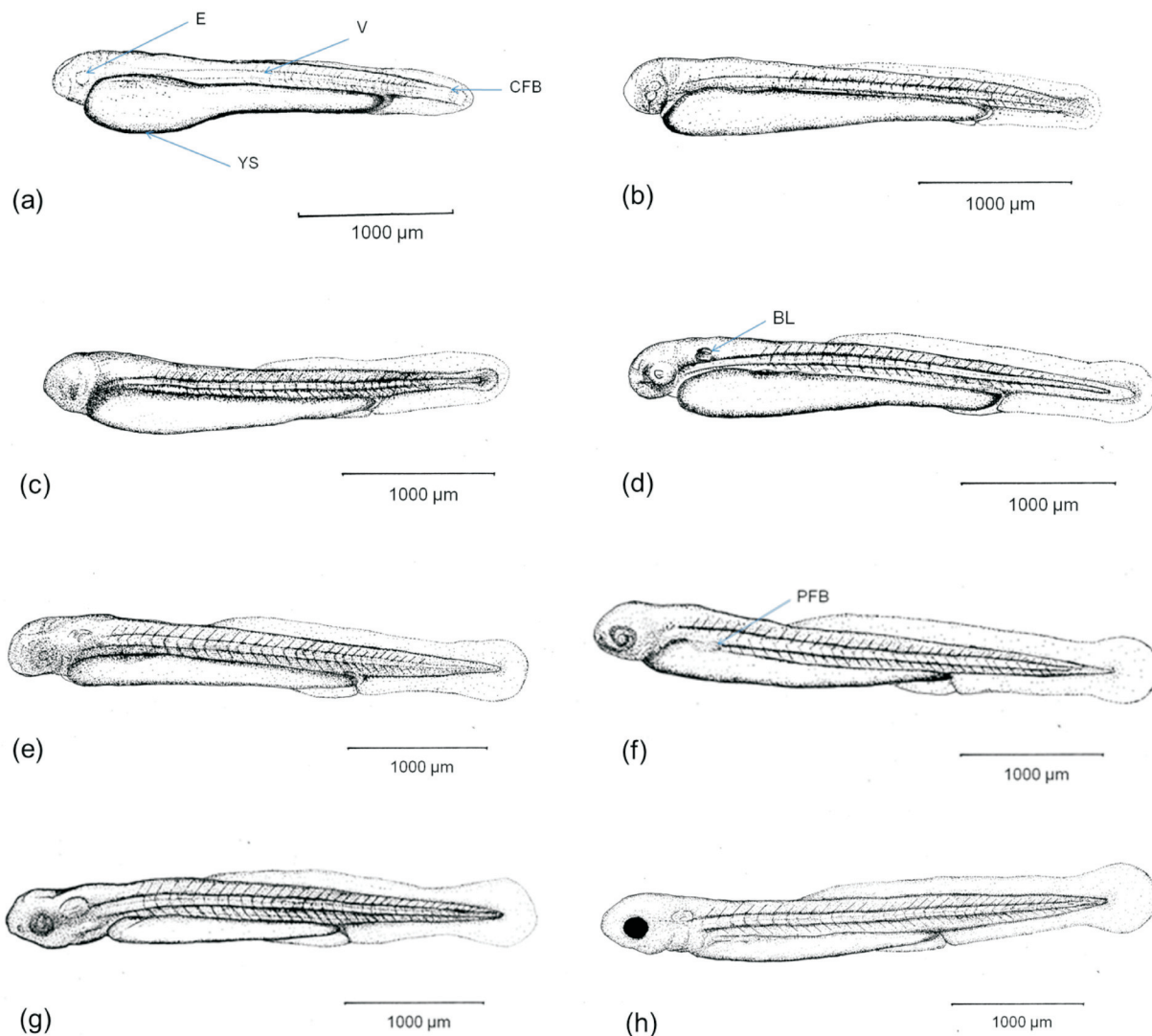


Fig. 6 : Biological sketch of stages larval development of Lemon fin barb hybrid (a) Newly hatched, (b) 2 hrs stage, (c) 5 hrs stage, (d) 12 hrs stage, (e) 16 hrs stage, (f) 20 hrs stage, (g) 34 hrs stage, (h) 46 hrs stage. (E-Eye, V-Vertebra, CFB-Caudal fin bud, YS-Yolk sac, BL-Brain lobe, PFB-Pectoral fin bud)

development (Herzig and Winkler, 1986; Thépot and Jerry, 2015). It is therefore possible that under similar temperature conditions, hatching may be faster for the Lemon fin hybrids. Interestingly, while the unfertilized eggs of the hybrids and those of *B. gonionotus* were similar in size, there was a large discrepancy between the fertilized egg size. In the current study, the egg diameter was 1.08 mm compared to 0.80 mm in *B. gonionotus*. It has been cautioned that egg size is not always a predictor of the overall quality (Migaud *et al.*, 2013), and therefore it cannot be speculated at this time whether heterosis in terms of quality occurred at such an early developmental stage. Moreover, such an increase in size could simply be due to the uptake in water but more research is necessary to determine the cause for such a

size discrepancy. There also appears to be a lack of information regarding egg size, as well as early development in *H. wetmorei*, which limits our interpretation of this finding. Further research in this area, including comparison between the fertilization success, developmental duration and overall hatchability, would better elucidate whether heterosis exists at this early stage of development.

The duration of the embryonic development of these hybrids may be useful for the potential production of triploid fish. After fertilization, the second polar body is extruded during the second meiotic division, and the goal to induce triploidy is to prevent this extrusion by shock (Maxime, 2008). The form of

Table 1 : Description of the phase in embryonic development process of Kerai hybrid

Stage	Phase	Time after fertilization	Mean total diameter (mm)
a	Unfertilized eggs	0 min	0.72 ± 0.02
b	Fertilized eggs	0 min	1.08 ± 0.03
c	Blastula	10 min	2.15 ± 0.13
d	2 cells	30 min	2.79 ± 0.07
e	4 cells	50 min	2.87 ± 0.09
f	8 cells	1 hr	2.90 ± 0.10
g	16 cells	1 hr 10 min	2.59 ± 0.24
h	Multi cells	1 hr 20 min	2.66 ± 0.08
i	Morula	3 hr	2.50 ± 0.24
j	Gastrula	4 hr	2.48 ± 0.11
k	Yolk plug stage	6 hr	2.55 ± 0.17
l	Organogenesis	7 hr	2.44 ± 0.15
m		8 hr	2.09 ± 0.05
n		10 hr	2.26 ± 0.11
o	Beginning hatching stage	11 hr	2.13 ± 0.06
p	Just before hatching	12 hr	2.12 ± 0.12

Values are mean of ten replicates ±SE

shock can be temperature, salinity, pressure or chemicals. Based on when the second meiotic division took place, it can be recommended that a shock be applied between 10 to 30 min post-fertilization. Further research to narrow down this duration, as well as the optimal form of shock should be performed for optimization. It should be noted that often temperature shock is successful at inducing triploidy for freshwater fish (Maxime, 2008).

Upon hatching, the hybrid larvae still possessed their yolk sac, which did not disappear until 48 hrs later. Their initial average total length was 2.6 mm and by 12 hrs, they increased in length to 3.2 mm, which was larger than newly hatched or 12 hrs old *B. gonionotus* larvae at 2.2 and 2.9 mm, respectively (Basak *et al.*, 2014). This finding was obtained despite the temperature was much higher in the study of Basak *et al.* (2014) than in the current study, which likely indicates that the Lemon hybrid grows fast. Such a temperature discrepancy may also explain the shorter duration for complete yolk absorption by *B. gonionotus* after 3 days (Basak *et al.*, 2014), but 4 days was necessary for this to occur in the hybrids. Although feeding was not performed in this study, based on personal observations, this hybrid larvae began consuming newly hatched *Artemia* nauplii within 24 hrs of hatching, despite still possessing their external egg yolk.

The results obtained in this study can provide baseline information for the early culture of this hybrid that is becoming more commercially important within Malaysia. Further research on different incubation temperatures and pH conditions during the embryonic stage, as well as different live food combinations and densities after hatching should be done to optimize the production of this hybrid.

Acknowledgments

This research was supported by Research Grant Scheme by Research Management Centre of Universiti Putra Malaysia, Selangor (Grant No. 01-01-11-1120 RU). Authors would like to express gratitude to the Fisheries Research Institute (FRI), Gelami Lemi at Jelebu, Negeri Sembilan under Department of Fisheries of Malaysia (DOF) for providing the hatchery and laboratory facilities of this research.

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