交雑魚チャイロマルハタEpinephelus coioides×タマカイE. lanceolatusの卵発生と仔魚の発育

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<td>誌名</td>
<td>水産増殖 = The aquiculture</td>
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<tr>
<td>ISSN</td>
<td>03714217</td>
</tr>
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<td>著者</td>
<td>Koh, I.C.C. Sitti Raehanah, Md.S. 赤沢, 憲明 太田, 康弘 瀬尾, 重治</td>
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<td>巻/号</td>
<td>58巻1号</td>
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<tr>
<td>掲載ページ</td>
<td>p. 1-10</td>
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<tr>
<td>発行年月</td>
<td>2010年3月</td>
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Egg and Larval Development of a New Hybrid Orange-Spotted Grouper *Epinephelus coioides* × Giant Grouper *E. lanceolatus*

**KOH Ivan Chong Chu**, **SITTI RAÆHANAH Muhd. Shaleh**, **Noriaki AKAZAWA**, **Yasuhiro OOTA** and **Shigeharu SEN0**

**Abstract:** To establish a seed production technique for a new hybrid orange-spotted grouper *Epinephelus coioides* × giant grouper *E. lanceolatus*, the egg and larval development were observed. Newly ovulated eggs from a female *E. coioides* of 7.5 kg in body weight were measured 806±20 μm (mean±SD) in diameter and weighed 3.505 eggs/g. After fertilization with sperm obtained from a male *E. lanceolatus*, the diameter of fertilized eggs measured 836±10 μm. The eggs hatched from 17 h 15 min (17:15 h) to 19:20 h after fertilization at 28.0–29.0°C and 30.0 psu salinity. Fertilization and hatched rates were 91.0% and 33.6% respectively. Newly hatched larvae were 1.53±0.01 mm in total length (TL). Larvae commenced feeding at 2 day after hatched (d AH) when the mouth and digestive tract were developed and eyes became deeply pigmented. Larvae showed typical early *Epinephelus* type pigmentation and formation of dorsal and pelvic spines. Cannibalistic behaviour occurred with the appearance of orange pigmentation above the abdominal area at 30 d AH. Deformities were not observed throughout the rearing period. Two hundred and thirty five fishes of 50 d AH juveniles with mean TL of 28.4±2.5 mm were produced from 15,800 newly hatched larvae.

**Key words:** *Epinephelus coioides*; *Epinephelus lanceolatus*; Hybrid; Larval development

Hybridization, or crossbreeding of two different species, has long been used as a tool for improvement of fish stocks (Bartley et al. 2001). Hybridization owing to heterosis may result in hybrid vigor which means an increased strength of different characteristics in progeny (Fosella 2002). In aquaculture this may translate into economic profits. The selection of suitable combinations would therefore result in an aquaculture viable hybrid.

*Epinephelus coioides* (Fig. 1A) is a species of family Serranidae, and is also known as “Orange-spotted Grouper” or “Green Grouper” in English, “Qing Pan” in Chinese, “Chairomaruhata” in Japanese and “Kerapu Hijau” in Malay (Heemstra and Randall 1993). The fish can be found in the Red Sea south to at least Durban, east to the western Pacific, where it ranges from the Ryukyu Islands to Australia and South East Asia (Heemstra and Randall 1993). In Malaysia and most neighboring countries, *E. coioides* is an important commercial food species (Heemstra and Randall 1993) which is also highly fecund and matures as female at around 3 kg, making it a suitable female candidate for hybridization.

*E. lanceolatus* (Fig. 1B) or giant grouper is one of the two largest groupers in the world. It has good commercial value (US$ 20-26/kg in Malaysian seafood restaurants) and very fast growth rate for groupers making it a potential aquaculture candidate (Heemstra and Randall 1993). Giant grouper also easily produces sperm in captive conditions.

At the fish hatchery of Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah (UMS), a hybrid progeny using eggs from a female *E. coioides* and sperm from a male *E. lanceolatus* was produced. In an attempt to
Materials and Methods

Broodfish

The experiment was conducted at the fish hatchery of BMRI, UMS. The observation on egg development was carried out in October 2007, while the observation on larval development which was conducted in October to December 2007.

For the experiment, wild captured broodfish of \textit{E. coioides} and \textit{E. lanceolatus} that had been cultured for over 10 years in the fish hatchery were used. Broodstock was reared in a 150 kl cylindrical fiber reinforced plastic tank (8 m in diameter, 3 m in height) with a water-circulating bio-filtration system. Three months prior to the experiment, broodstock was fed hatchery self-made moist pellets alternating with a fish \textit{Sardinella} sp. enriched with cod liver oil once daily till satiation. Water temperature, salinity, dissolved oxygen (DO), and pH for the culture period ranged from 26.2 – 28.4°C, 31.0 – 32.0 psu, 5.98 – 6.30 mg/l and 6.9 – 7.8, respectively.

\textit{E. coioides} and \textit{E. lanceolatus} broodfish were anaesthetized with alpha-methylquinoline before selection. The selected female \textit{E. coioides} had a soft distended abdomen and a reddish urogenital papilla, and white unripe eggs were obtained through cannulation. Male \textit{E. lanceolatus} could ooze milt with gentle pressure near the genital pore. Recorded measurements are shown in Table 1. Maturation was induced by treatment of the female \textit{E. coioides} with commercial human chorionic gonadotropin (Profasi, Laboratories Serono, Switzerland) at dosage of 500 IU/kg through intraperitoneal injection at the basal part of the pectoral fin. Selected broodfish were isolated in separate net cages (1.5 × 1.5 × 1.5 m) in the culture tank with a re-circulated culture system. Water temperature, salinity, dissolved oxygen (DO), and pH during isolation ranged 28.0 – 29.5°C, 29.0 – 30.0 psu, 7.0 – 7.8 mg/l and 6.0 – 7.5, respectively.

Stripping and fertilization

At the onset of stripping, eggs oozed out into a plastic bowl by gentle pressure at abdomen of female \textit{E. coioides}, anaesthetized with alpha-methylquinoline. Milt was collected from the male \textit{E. lanceolatus} using a milt collector (Senoo et al. 1994; Senoo 2002). Eggs and milt were then mixed and gently stirred for one minute for fertilization (Senoo et al. 1994; Senoo 2002).

Incubation for egg observation

One ml of both newly ovulated eggs and fertilized eggs was taken for measurements. Subsequently, the number of eggs was counted using a profile projector. Ovulation, number of

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Species & Sex & Weight & Total & Standard & Head & Body & Body & Body \\
& & (kg) & Length & Length & Length & Height & Height & Round \\
& & & (cm) & (cm) & (cm) & (cm) & (cm) & (cm) \\
\hline
\textit{E. coioides} & Female & 7.5 & 73.0 & 62.0 & 21.0 & 22.0 & 14.0 & 52.0 \\
\textit{E. lanceolatus} & Male & 35.0 & 113.0 & 96.0 & 38.5 & 39.0 & 27.0 & 87.0 \\
\hline
\end{tabular}
\caption{Species, sex, weight and size of broodfish used in the present experiment}
\end{table}
Egg and Larval Development of a New Hybrid OGGG

Eggs released, fertilization and hatching rates were recorded. The sizes of ovulated eggs and fertilized eggs were measured. Forty thousand fertilized eggs were incubated in a circular tank (110 cm in diameter, 90 cm in height) with 900 kl of 30 psu aerated seawater. The egg development was observed under a microscope and photographs were taken with a digital camera. The water temperature, DO and pH during egg incubation ranged 28.0–29.0°C, 7.0–7.8 mg/l and 6.0–7.5, respectively.

Rearing for larval morphological observation

A total of 15,800 fish of OGGG larvae measuring 1.53 ± 0.01 mm (mean±SD) in total length (TL) hatched at 17 h 30 min (17:30) after fertilization (h AF) and larvae were reared in the same tank. Different feeds were given at 08:00 and 17:00 ad libitum in accordance with the schedule as shown in Fig. 2. Nannochloropsis sp. measured about 150 μm in body length. Commercial brine shrimp Artemia salina nauplii were added at 5 x 10⁵ cells/ml. Rotifer Brachionus sp. measured about 2–4 μm in diameter were added at 25 d AH. Artificial powder feed, Otohime (Marubeni Nisshin Food, Japan) was fed from 25 d AH.

The rearing water was aerated at one position with 250–500 ml/min. Tank cleaning and water exchanging (10–30%) were carried out daily from 5 d AH till the end of the experiment. Water temperature, salinity, DO and pH during the larval rearing ranged from 27.9–29.5°C, 6.5–7.5 mg/l, and 7.5–7.9, respectively.

Estimated Larvae Counting (ELC) method was used to determine the amount of larvae remaining in the tank. The density of the larvae was estimated from five water samples taken from different sections of a tank using a 500 ml glass beaker, to obtain the volume of water sample. Larvae were counted under a stereomicroscope. ELC was computed using the following formula.

\[
ELC = \frac{\text{Number of larvae counted}}{\text{Volume of hatching tank}} \times \text{Volume of water samples}
\]

Survival rate of the fish was then calculated using the following formula.

\[
\text{Survival rate (\%)} = \frac{ELC}{\text{Total amount of larvae on 0 d AH}} \times 100
\]

Results

Egg development and hatching

Total stripped egg mass was shiny white in colour and weighed 245 g. Under the microscope eggs were transparent and had unfixed spherical shape. Stripped eggs were measured 806 ± 20 μm in diameter. Each egg had an oil globule and a soft covering membrane.

The morphological changes during development are shown in Figs. 3A-3Q. Immediately after fertilization, the eggs absorbed water and acquired a spherical shape with a hard covering membrane (Fig. 3A). Fertilized eggs increased slightly in diameter and measured 836 ± 10 μm. At 0:12 h AF, the blastotodisk appeared and at 0:23 h AF, the first cleavage occurred (Fig. 3B). The fertilization rate at 2-cell stage was 91.0%. The 4-cell stage (Fig. 3C), 8-cell stage (Fig. 3D), and 32-cell stage (Fig. 3E) occurred within 1:00 h AF. Then morula (Fig. 3F), blastula, and gastrula (Fig. 3G) developed in that order in 2:48 h – 6:37 h AF. At 7:10 h AF embryo formation commenced (Fig. 3H), and at 10:30 h AF head and myomeres were formed and Kupffer’s vesicle appeared (Fig. 3J). Optic vesicles were visible at 11.10 h AF (Fig. 3K) and caudal fin became separated from yolk sac (Fig. 3L). At

![Fig. 2. Sequence of feeds given during the rearing of E. coioides × E. lanceolatus (OGGG) hybrid larvae.](image-url)
Fig. 3. Egg development of *E. coioides × E. lanceolatus* (OGGG). A, fertilized egg; B, 2-cell stage; C, 4-cell stage; D, 8-cell stage; E, 32-cell stage; F, morula; G, gastrula; H, embryo formation; I, blastopore nearly closed; J, head and myomers formed; K, optic lens appeared; L, tail separated from yolk sac; M, lens vesicles appeared; N, embryo commenced moving; O, heart formed; P, otocyst vesicles appeared; Q, hatching started; R, hatched larva; number shows hours and minutes after fertilization; the scales (0.5 mm) are for A to R without M and P.

13:43 h AF lens vesicles were visible (Fig. 3M). At 14:21 h AF, the embryo commenced movement (Fig. 3N) and at 15:15 h AF heart was formed (Fig. 3O) and exhibited active movement. At 16:17 h AF, otocyst vesicles appeared (Fig. 3P). Hatching began at 17:15 h AF (Fig. 3Q) and finished at 19:20 h AF. The hatching glands were not observed under the microscope. The hatching rate was 33.6% from initial fertilization. 15,800 *E. coioides × E. lanceolatus* larvae hatched from 40,000 eggs at 19:20 h AF.

Larval morphological development

The morphological changes of OGGG larvae are illustrated in Fig. 4. The correspondence between the morphological features and behavioural changes is shown in Table 2.

Larvae started hatching at 17:15 h AF and continued till 19:20 h AF. In newly hatched larvae, mouth and anus were not formed, and the eyes were unpigmented. Newly hatched larvae stayed floating at the water surface without aeration condition. On 2 d AH, the mouth was open, eyes were deeply pigmented, and the lower jaw and intestinal tracts began to move. Black pigmentation also appeared on the area above the intestine. The distal part of the intestinal tract (rectum) was stained green in colour. This could be due to ingestion of *Nannochloropsis* sp. cells. Larvae were observed to be morphologically prepared for first feeding and rotifer were observed in the intestine. On 3 d AH, yolk sac and oil globule was completely absorbed. The growth increment was only 1.26 mm in TL during 0–5 d AH (Fig. 5). Larvae which did not feed on rotifer became inactive and eventually died (Fig. 6).

On 5 d AH, aggregated melanophores on the area above the intestine spread gradually, and a spot of melanophore newly appeared on the intermediate area between anus and caudal fin. On 10 d AH second dorsal-fin spine and pelvic-fin spines have differentiated 60% of observed fish. Whole abdominal cavity became heavily pigmented. Larvae exhibited aggregation near aeration. On 20 d AH, second dorsal-fin spines and pelvic-fin spines were markedly elongated and anal fin began to appear. Gut content was no longer visible due to heavy pigmentation. Larvae started to swim around the tank edge. Black pigmentation became more obvious.

On 30 d AH slight orange-yellow pigmentation appeared on the head part, area above the intestine and the area between anus and caudal fin of fish. Abdominal cavity became silvery coloured. Anal, dorsal and caudal fins were formed. Pelvic fin started to form. Cannibalism was first observed at this time. On 38 d AH, the second dorsal-fin spine started to shorten and pelvic fins were completely developed. Orange-yellowish pigmentation became obvious on head, cheek, above intestine and along lateral line. Larger larvae started to shift their habitat from pelagic to benthic. At 41 d AH, all fin formation was complete and development of yellow-brown colouration with dark stripes started, and it completed together with completion of
Fig. 4. Development of *E. coioideis* × *E. lanceolatus* (OGGG) hybrid larvae. Larval age is shown by hours (h) and days (d) after hatched; scale 1 mm.
Table 2. Correspondence between morphological and behavioural changes in E. coioides × E. lanceolatus (OGGG) larvae with growth

<table>
<thead>
<tr>
<th>Morphological changes</th>
<th>Days after hatching</th>
<th>Behavioural changes</th>
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<tbody>
<tr>
<td>Mouth not formed, Anus closed, Eyes not pigmented</td>
<td>0</td>
<td>Floating at water surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Head down-side posture</td>
</tr>
<tr>
<td>Eye slightly pigmented</td>
<td>1</td>
<td>Vertical swimming</td>
</tr>
<tr>
<td>Eyes pigmented, Mouth formed, Intestinal tract and lower jaw moving</td>
<td>2</td>
<td>Horizontal swimming</td>
</tr>
<tr>
<td>Black pigmentation on area above intestine</td>
<td></td>
<td>Feeding on Brachionus sp.</td>
</tr>
<tr>
<td>Pectoral fin buds appeared</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye movement</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Yolk sac completely absorbed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggregated melanophores on area above the intestine spread gradually, and a spot of</td>
<td>5</td>
<td>Active feeding on Brachionus sp.</td>
</tr>
<tr>
<td>melanophore appeared on the intermediate area between anus and tail area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second dorsal and both pelvic-fin spines started to develop</td>
<td>10</td>
<td>Aggregation around aeration</td>
</tr>
<tr>
<td>Abdominal cavity became heavily pigmented</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second dorsal and both pelvic-fin spines elongated</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Second dorsal and both pelvic-fin spines completed</td>
<td>20</td>
<td>First feeding of Artemia salina nauplii</td>
</tr>
<tr>
<td>Anal fin appeared</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gut content not visible due to heavy pigmentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow pigmentation on head part and above stomach area</td>
<td>30</td>
<td>Larvae started to swim around tank edge</td>
</tr>
<tr>
<td>Abdominal cavity became silvery coloured</td>
<td></td>
<td>Cannibalism first observed</td>
</tr>
<tr>
<td>Anal, dorsal and caudal fins formed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pelvic fins started forming</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal spines started to shorten and pelvic fins formed</td>
<td>38</td>
<td>First feeding of Otohime pellets</td>
</tr>
<tr>
<td>Orange-yellowish pigmentation obvious on head, cheek, above intestine and along lateral line</td>
<td></td>
<td>Benthic habitat started</td>
</tr>
<tr>
<td>All fin formation completed</td>
<td>41</td>
<td>First feeding of minced Sardinella sp. and squid</td>
</tr>
<tr>
<td>Development of yellow-brown colouration with dark stripes started</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Completion of yellow-brown body colouration with dark stripes and spots on dorsal and</td>
<td>50</td>
<td>All benthic habitat</td>
</tr>
<tr>
<td>caudal fins</td>
<td></td>
<td>Eating of minced Sardinella sp. and squid</td>
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Fig. 5. Growth of new hybrid E. coioides × E. lanceolatus (OGGG) larvae. Inserted figure is magnified scale to largely show the early growth; closed circle, mean (n=10).

Fig. 6. Changes in survival rate of new hybrid E. coioides × E. lanceolatus (OGGG) larvae.
Egg development

Mean diameter of fertilized eggs (0.836 ± 0.10 mm) of the new hybrid was similar to those of the other Serranidae of E. tawina (0.71–0.90 mm) (Hussain et al. 1975), E. septemfasciatus (0.82 ± 0.017 mm) (Kitajima et al. 1991), E. aakaara (0.71–0.77 mm) (Ukawa et al. 1966), Cromileptes altivelis (0.80–0.86 mm) (Senoo et al. 2002) and another hybrid grouper, E. coioides × E. fuscoguttatus (0.83 ± 0.02 mm) (Koh et al. 2008). In this study, hatching time, 17:15–19:10 h AF, was similar to two other hybrids, E. coioides × E. fuscoguttatus (Koh et al. 2008) and E. fuscoguttatus × E. lanceolatus (Ch’ng and Senoo 2008), which was 17:30–19:00 h and 18:00 h AF, respectively. This study also found that hatching time was significantly earlier than those of the other pure species of Serranidae. Egg development proceeded more rapidly and hatching occurred earlier than in the parental species.

Fertilization rate was 91.0% in this study. It was higher than E. costae × E. marginatus (Glamuzina et al. 1999) which was 50.0%, respectively. This shows the closeness of evolutionary relationship between crossed species (Hester 1970). The combination of E. coioides × E. lanceolatus could be considered as a promising hybridization judging from the high fertilization rate.

Larval survival

Groupers face high mortalities at the early larval stage since larvae are small, fragile and have a small mouth at first feeding (Tucker 1999). Tucker (1999) and Koh et al. (2008) reported peak mortality at early larval stage when endogenous feed source were exhausted. In this study, hybrid OGGG larval yolk sac and oil globule were already exhausted at around 3 d AH and larval mortality was highest during 3–7 d AH when survival rate dropped drastically from 66.3% to 28.9%. Hatching time of OGGG larvae was in the night. Therefore, even though larvae were morphologically prepared for feeding
At 24 h AH, larvae were unable to consume prey till the following morning since grouper larvae are essentially visual day feeders. A previous study suggested that OGGG larvae seemed to prefer smaller prey (Duray et al. 1997). Larvae were also fed unscreened rotifer which might have been too large for larvae to consume. This suggested that there was a low encounter rate of larvae with suitable sized feed at first feeding. A similar pattern of the survival rate was reported in another hybrid *E. coioides × E. fuscoguttatus* (Koh et al. 2008). Therefore, starvation seemed to be the cause of heavy mortality during early larval stage. (Laurence 1977; Hunter 1980).

In this experiment, survival rate of OGGG larvae at 40 d AH was 2.5% when cultured in a 1 kl tank with 30 psu salinity. This result is much lower than those of the other previous studies on *Epinephelus* type larvae (Doi et al. 1981; Duray et al. 1997). Newly hatched *E. malabaricus* (*E. suillus*) larvae had an optimum salinity range 8–24 psu (Parado-Estapa 1991) and *E. tawina* late-stage larvae at 25 psu (Akatsu et al. 1983). Optimum salinity for the new hybrid OGGG is not known, but it can be surmised that it may be similar to that of the maternal species which is lower than the salinity in this study. Lower salinities possibly require less energy cost from larvae for osmoregulation. (Duray et al. 1997)

In this study, cannibalism was observed starting from 30 d AH, when OGGG larvae first exhibited orange pigmentation. This was peculiar as cannibalism usually occurred simultaneously with settling behavior. Cannibalism can be regarded as the major cause of mortality at advanced larval stage as dead fish were not observed during daily bottom cleaning (Koh et al. 2008; Tucker 1999). For the OGGG larvae, sorting is therefore recommended to be carried out as soon as orange pigmentation appears in order to increase the larval survival.

**Larval development and behaviour**

Hybrid OGGG larvae were morphologically almost similar to those of the other *Epinephelus* species (Tucker 1999; Heemstra and Randall 1993; Koh et al. 2008). The larvae commenced first feeding at 2 d AH when the eyes, jaw and digestive tracts become functional just before yolk sac exhausting and horizontal swimming starting. Ikewaki and Sawada (1991) noted that locomotory organs have not yet been well developed at the end of the yolk sac in marine species.

At early larvae stage, OGGG larvae were unable to be differentiated from other grouper larvae as there are no distinct characteristic for identification. Groupers have similar pigmentation and a characteristic “kite shaped” body during larval stage before developing distinct characteristics that enable species identification just before transformation to juvenile stage (Tucker 1999; Heemstra and Randall 1993; Glamuzina et al. 2001; Koh et al. 2008). In the larvae, the development of distinct yellow-brownish colouration with dark stripes started at 41 d AH. Completion of colouration which was similar to an intermediate pattern of both parental species was only completed at 50 d AH juvenile stage.

Fin development of OGGG larvae was completed as early as 41 d AH which was faster compared to 50 d AH *E. coioides × E. fuscoguttatus* (Koh et al. 2008). The shorter time period suggested that male parental species probably did play a role in affecting the morphological development of hybrids, and that some combinations might be preferred to others. Larval period of the hybrid was approximately 50 days and individuals transformed within a week.

In the present study, we could not compare developmental parameters and survival rate with those of each parental species. In order to evaluate the advantages of this combination, such comparison will be necessary.

**Acknowledgements**

We are grateful to Colonel Professor Datuk Dr. Kamaruzaman Hj. Ampon, Vice Chancellor and Professor Dr. Saleem Mustafa, Director, Borneo Marine Research Institute, Universiti Malaysia Sabah, for their encouragement and support. We thank all the staff of Borneo Marine Research Institute Fish Hatchery, Universiti
Malaysia Sabah for their cooperation in the experiment. We are also grateful to Visiting Professor Dr. Masaru Tanaka, School of Sustainable Agriculture, Universiti Malaysia Sabah for his critical reading and comments on the manuscript.

References


交雑魚チャイロマルハタ Epinephelus coioides×タマカイ E. lanceolatus の卵発生と仔魚の発育

Koh Ivan Chong Chu・Sitti Raehanah Muhd. Shaleh
赤沢憲明・太田康弘・瀬尾重治

ハタ科交雑魚チャイロマルハタ×タマカイの種苗生産技術を確立するために、卵発生と仔魚の発育を観察した。体重7.5 kg のチャイロマルハタから摘出した卵は、直径806±20 μm（平均±標準偏差）であり、1 g 当たりの卵数は3,505であった。タマカイの精液による受精後、卵は直径836±10 μm であった。受精卵は水温28.0-29.5℃、塩分30.0 ppt で受精後17時間15分から19時間20分に孵化し、受精率および孵化率はそれぞれ 91.0％および33.6％であった。孵化直後の仔魚は全長1.53±0.01 mm であった。仔魚は開口し消化管が形成され、眼が黒化した孵化後3日目に摂餌を開始した。孵化後10日からハタ科特有の尾部と肛門の間の体表に黒化部が現れ、第二背鰭および腹鰭の棘が著しく伸張した。孵化後40日より浮遊生活から底生生活へ移行を開始した。21,500尾の孵化仔魚から15,800尾の稚魚（平均全長28.4±2.5 mm、孵化後50日）を生産した。