ハタ科交雑魚チャイロマルハタ Epinephelus coioides × アカマグラハタ E. fuscoguttatus の卵発生と仔魚の発育

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Egg and Larval Development of a New Hybrid Orange-spotted Grouper *Epinephelus coioides* × Tiger Grouper *E. fuscoguttatus*

Ivan Chong Chu Koh, Sitti Raehanah Muhd. Shaleh and Shigeharu Senoo

**Abstract:** To establish a seed production technique for a new hybrid orange-spotted grouper *Epinephelus coioides* × tiger grouper *E. fuscoguttatus* the egg and larval development were observed under artificial conditions. Newly ovulated eggs from a female *E. coioides* of 7.5 kg in body weight were measured at 0.81 ± 0.02 mm (mean ± SD) in diameter and weighed 3,505 eggs/g. After fertilization with sperm obtained from a male *E. fuscoguttatus*, its diameter measured 0.83 ± 0.02 mm. The eggs hatched from 17 h 30 min (17:30 h) to 19:00 h after fertilization at 29.0°C at 30.0 ppt salinity. The fertilization and hatching rates were 93.9% and 50.3%, respectively. Newly hatched larvae were 1.52 ± 0.01 mm in total length (TL) and floated motionless at water surface. Larvae commenced feeding at 3 days after hatched (d AH) when mouth and digestive tract were formed and eyes became deeply pigmented. Larvae showed typical early *Epinephelus* type pigmentation and differentiation of second dorsal-fin and pelvic-fin spines was observed prior to 10 d AH and thereafter elongated. Larvae started shifting habitat from pelagic to benthic at 40 d AH. Seven hundred and fifty tails 50 d AH juveniles with mean TL of 22.8 ± 3.6 mm were produced from 21,500 newly hatched larvae.

**Key words:** Hybrid; *Epinephelus coioides*; *Epinephelus fuscoguttatus*; Egg and larval development

*Epinephelus coioides* (Fig. 1A) is a species of Serranidae and 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 is distributed in the Red Sea south to at least Durban, east to the western Pacific, where it ranges from the Ryukyu Islands to Australia and eastwards to Palau and Fiji, Singapore, Hong Kong, Thailand and the Philippines (Heemstra and Randall 1993). The wholesale price of exports from Sabah, Malaysia to Hong Kong is US$ 13 – 20/kg (Personal findings). In Malaysia and most neighboring countries, *E. coioides* is an important commercial food species (Heemstra and Randall 1993), and aquaculture trials have been conducted.

The seed production of *E. coioides* is facing a big problem due to its difficult sperm collection. On the other hand, tiger grouper or brown-marbled grouper, *E. fuscoguttatus* easily produces sperm in captive conditions. In 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. fuscoguttatus* was produced to solve this problem. Hybridization generally comes with hybrid vigor or heterosis which means an increased strength in different characteristics of progeny. *E. fuscoguttatus* (Fig. 1B) was chosen as male owing to its aquaculture advantages. It has good commercial value (US$ 20 – 26/kg in Malaysian seafood restaurants), fast growth rate for groupers, high resistance to diseases (Heemstra and Randall 1993), and is an important food species, being widely cultured in South East Asia (Tucker 1999).

In an attempt to establish a seed production technique for a new hybrid of *E. coioides* × *E. fuscoguttatus* (OGTG), the authors observed the egg development, hatching, and morphological larval development in relation to behavioral changes following artificial fertilization.
Materials and Methods

The experiment was conducted at the fish hatchery of BMRI, UMS. The observation on egg development was carried out in October 2007 followed by the observation on larval development which was conducted in October to December 2007. For the experiment, broodfish of *E. coioides* and *E. fuscoguttatus* that were cultured in the fish hatchery of BMRI, UMS were used. The broodfish were reared for about 3 months prior to the experiment. Broodfish was reared in a cylindrical fiber reinforced plastic tank with 8 m diameter and 3 m height filled with 150 kl of seawater. Broodfish was fed hatchery self-made moist pellets and fish *Sardinella* sp. enriched with cod liver oil till satiation. *E. coioides* and *E. fuscoguttatus* broodfish were anaesthetized with Transmore (alpha-methylquinoline) (Nika) before selection. The selected female *E. coioides* had a soft distended abdomen and a reddish urogenital papilla, and white unripe eggs were obtained through cannulation. Male *E. fuscoguttatus* could ooze milt with gentle pressure near the genital pore. Recorded measurements are shown in Table 1. The female *E. coioides* was treated 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. The water temperature, salinity, dissolved oxygen (DO), and pH during isolation ranged from 28.0 – 29.5°C, 29.0 – 30.0 ppt, 7.0 – 7.8 mg/l and 6.0 – 7.5, respectively.

**Stripping and Fertilization**

At the onset of stripping, eggs oozed out by gentle pressure at abdomen of female *E. coioides*. Female *E. coioides* was anaesthetized before eggs were gently squeezed out into a plastic bowl and obtained eggs were weighed. Sperm was collected from male *E. fuscoguttatus*. Gentle pressure was applied on abdomen of anaesthetized broodfish and sperm was collected using a sperm collector shown in Fig. 2.

![Fig. 1. Broodfish of A, orange-spotted grouper, *Epinephelus coioides*, and B, tiger grouper, *E. fuscoguttatus*; scale is 10 cm.](image1)

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Total Length (cm)</th>
<th>Standard Length (cm)</th>
<th>Head Length (cm)</th>
<th>Body Height (cm)</th>
<th>Body Width (cm)</th>
<th>Body Round (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coioides</em></td>
<td>Female</td>
<td>7.5</td>
<td>73.0</td>
<td>62.0</td>
<td>21.0</td>
<td>22.0</td>
<td>14.0</td>
<td>52.0</td>
</tr>
<tr>
<td><em>E. fuscoguttatus</em></td>
<td>Male</td>
<td>11.0</td>
<td>79.0</td>
<td>65.0</td>
<td>24.0</td>
<td>23.0</td>
<td>14.0</td>
<td>65.0</td>
</tr>
</tbody>
</table>

![Fig. 2. Sperm collection using a sperm collector. A, gentle pressure is applied on abdomen of male *E. fuscoguttatus* to push out sperm, B, the end of sperm collector tube is directed at urogenital papilla, C, plastic container 5 cm in inner diameter is used to collect the sperm, D, suction pressure is produced using sucking by mouth on tube.](image2)
Sperm from *E. fuscoguttatus* was mixed with striped eggs from *E. coioideis*. Eggs and sperm were then gently stirred for 1 minute before fertilization.

**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 eggs released, fertilization and hatching rates were recorded. The sizes of ovulated eggs and fertilized eggs were measured. Forty thousands fertilized eggs were incubated separately in a round 1 kl tank filled with aerated 30.0 ppt salinity seawater. The water temperature, DO and pH during egg incubation ranged from 28.0 - 29.5°C, 7.0 - 7.8 mg/l and 6.0 - 7.5, respectively.

**Rearing for Larval Morphological Observation**

A total of 21,500 tails of OGTG larvae measuring 1.52 ± 0.01 (mean ± SD) mm in total length (TL) hatched from 40,000 eggs at 17 h 30 min (17:30 h) after fertilization (AF) and larvae were reared in the same tank. Different feeds were given at 0800 and 1700 hours *ad libitum* in accordance with the schedule as shown in Fig. 3. *Nannochloropsis* sp. measured about 2 - 4 μm in diameter and were added at 5 × 10⁵ cells/ml. Rotifer Brachionus sp. fed measured about 150 μm in body length. Commercial brine shrimp *Artemia salina* nauplii were fed from 20 day after hatched (d AH). Artificial powder feed, Otohime (Marubeni Nisshin Food) was fed from 25 d AH. A kind of sardine *Sardinella* sp. and squid were minced before feeding.

The rearing water was aerated at one position with 250 - 500 ml/min. Bottom cleaning, tank cleaning and water exchanging (10 - 30%) were carried out daily from 5 d AH till the end of the experiment. The water temperature, salinity, DO and pH during the larval rearing ranged from 27.9 - 29.5°C, 29.5 - 30.0 ppt, 6.5 - 7.5 mg/l, and 7.5 - 7.9, respectively.

**Results**

**Egg Development and Hatching**

Total striped egg mass was shiny white in colour and weighed 245 g (wet weight). Under a microscope the eggs were transparent and had unfixed spherical shape. Unfertilized eggs were measured 0.81 ± 0.02 μm in diameter.
Each egg had an oil globule and a soft covering membrane. One ml of the eggs weighed 1.023 g. The number of eggs was 3,586 eggs/ml or 3,505 eggs/g.

The morphological changes during development are illustrated in Fig. 4A-Q. Immediately after fertilization, the eggs absorbed water and acquired a spherical shape with a hard covering membrane (Fig. 4A). Fertilized eggs increased slightly in diameter and measured 0.83 ± 0.02 mm. Twelve min (0:12 h) after fertilization (AF), the blastodisk appeared and at 0:20 h AF, the first cleavage occurred (Fig. 4B). The fertilization rate at 2-cell stage was 93.8%. The 4-cell stage (Fig. 4C), 8-cell stage (Fig. 4D), and 32-cell stage occurred within 1 h. Then morula (Fig. 4E), blastula, and gastrula (Fig. 4F) developed in that order in 2:05 - 6:20 h AF. At 7:01 h AF, embryo formation commenced (Fig. 4H) and at 10:45 h AF head and 5-myomeres were formed and Kupffer's vesicle appeared (Fig. 4J). Optic vesicles were visible at 11:35 h AF (Fig. 4K) and tail became separated from yolk sac (Fig. 4L). At 14:03 h AF lens vesicles (Fig. 4M) were visible. At 14:38 h AF, the embryo commenced movement (Fig. 4N) and at 15:35 h AF heart was formed (Fig. 4O) and exhibited active movement. At

Fig. 4. Egg development of *E. coioides × E. fuscoguttatus* (OGTG). A, fertilized egg; B, 2 cell stage; C, 4 cell stage; D, 8 cell stage; E, 16 cell stage; F, morula; G, gastrula; H, embryo formation; I, blastopore nearly closed; J, head and 5 myomers formed; K, optic lens appeared; L, tail separated from yolk sac; M, lens vesicle 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 scale (0.5 mm) are all for A to R without M and P.
Fig. 5. Development of *E. coioides* × *E. fuscoguttatus* (OGTG) hybrid larvae. Larval age is shown by hours (h) and days (d) after hatched; scale 1mm.
1:28 h AF, otocyst vesicles appeared (Fig. 4P). Hatching began at 17:30 h AF (Fig. 4Q) and finished at 19:00 h AF. The hatching glands were not observed under the microscope. The hatching rate was 50.3% from initial fertilization. 21,500 OGTG larvae hatched from 40,000 eggs at 19:00 h AF.

**Larval Morphological Development**

The morphological changes of the OGTG larvae are illustrated in Fig. 5. The correspondence between the morphological features and behavioral changes is shown in Table 2.

In newly hatched larvae, mouth and anus were not formed, and the eyes were unpigmented. Newly hatched larvae stay 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 the ingestion of *Nannochloropsis* sp. cells. Larvae were observed to be morphologically prepared for first feeding. However, no rotifer was found in gut of larvae. On 3 d AH, yolk sac and oil globule was totally absorbed in 40.0% of

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<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>Eye slightly pigmented</td>
<td>1</td>
<td>Vertical swimming</td>
</tr>
<tr>
<td>Eyes pigmented, Mouth formed, Intestinal tract and lower jaw moving Black pigmentation on area above intestine Pectoral fin buds appeared</td>
<td>2</td>
<td>Horizontal swimming</td>
</tr>
<tr>
<td>Eye movement Yolk sac totally absorbed Aggregated melanophores on area above the intestine spread gradually, and a spot of melanophore appeared on the intermediate area between anus and tail area Second dorsal and both pelvic-fin spines started to develop Abdominal cavity became heavily pigmented</td>
<td>3</td>
<td>Feeding on Rotifer</td>
</tr>
<tr>
<td>Second dorsal and both pelvic-fin spines elongated</td>
<td>5</td>
<td>Active feeding on Rotifer</td>
</tr>
<tr>
<td>Second dorsal and both pelvic-fin spines completed Anal fin appeared Gut content not visible due to heavy pigmentation Yellow pigmentation on head part and above stomach area Abdominal cavity became silvery coloured Anal, dorsal and caudal fins formed Pelvic fins started forming</td>
<td>10</td>
<td>Aggregation around aeration</td>
</tr>
<tr>
<td>All fin development complete</td>
<td>20</td>
<td>First feeding of <em>Artemia salina</em> nauplii, Larvae started to swim around tank edge.</td>
</tr>
<tr>
<td>Marbled yellow-brown pigmentation Juvenile stage</td>
<td>30</td>
<td>Cannibalism first observed</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Benthic habitat started, First feeding of minced <em>Sardinella</em> sp. and squid</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>All benthic habitat Eating of minced <em>Sardinella</em> sp. and squid</td>
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observed larvae and rotifer were observed in the intestines. Larvae that did not feed on rotifer became inactive and eventually died (Fig. 6). The growth increment was only 1.46 mm in TL during 0.5 d AH (Fig. 7).

On 5 d AH, aggregated melanophores on the area above the intestine spread gradually, and a spot of melanophore appeared on the intermediate area between anus and tail. On 10 d AH second dorsal-fin spine and pelvic-fin spines have differentiated in 40.0% of observed fish (Fig. 8). Whole abdominal cavity became heavily pigmented. Larvae exhibited aggregation near aeration. At 20 d AH, second dorsal-fin spines and pelvic-fin spines were completely elongated (Fig. 9) 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 slightly orange-yellow pigmentation appeared on the head part, area above the intestine and the area between anus and tail 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 point. On 40 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 50 d AH marbled yellow-brown pigmentation was complete and all fin formation was complete. All fishes completed shifting to benthic habitat. Seven hundred and fifty tails 50 d AH juveniles with mean total length of 22.8 ± 3.6 mm were produced from 21,500 newly hatched larvae (Fig. 7).
Egg development

Mean diameter of the fertilized eggs (0.83 ± 0.02 mm) of the new OGTG was similar to that of the other Serranidae of *E. tawina* (0.71–0.90 mm) (Hussain et al. 1975), *E. akaara* (0.71–0.77 mm) (Ukawa et al. 1966) and *Cromileptes altivelis* (0.80–0.86 mm) (Senoo et al. 2002). In this study, hatching time (17:30–19:00 h AF) was significantly earlier than that of the other species of Serranidae. The length of egg development and cumulative hatching are regarded as the first characteristics used to identify hybrids (Chevassus 1983). A previous study in the BMRI fish hatchery of UMS showed that another hybrid, *E. fuscoguttatus × E. lanceolatus*, took only 18:00 h AF for hatching time at 28.5°C, it being apparently earlier compared with *E. fuscoguttatus* of 24:15 h AF at 28.0°C. It was speculated that egg development proceeded more rapidly and hatching occurred earlier than in the parental species.

Fertilization rate was 93.9% in this study. It was higher than that of *E. costae × E. marginatus* (Galamuzina et al. 1999) which was only 50.0%. The combination of OGTG could be considered as a promising hybridization judging from the high fertilization rate. Successful hybridization points to the close evolutionary relationship between crossed species (Hester 1970).

Larval Growth

The newly hatched OGTG larvae were 1.52 ± 0.01 mm in TL. In the other hybrid grouper *E. costae × E. marginatus* (Galamuzina et al. 1999) 0 d AH larval total length (TL) was 1.80 ± 0.09 mm. The other groupers such as nassau grouper (*E. stratus*), brown-spotted grouper (*E. tawina*) and malabar grouper (*E. mala-baricus*) had mean TL of 1.7–1.8 mm (Sadovy and Eklund 1999), 2.25 mm (Nazar and Higuchi 1980) and 1.71–1.84 mm (Yoseda et al. 2005), respectively. Newly hatched OGTG larvae were slightly smaller compared to most of the other grouper species. Larger sized larvae tend to have a larger mouth width and larger amount of endogenous nutrition at onset of first feeding (Kohno 1998). This indirectly improves the larval survival. When compared with its parental species, the OGTG was found to be smaller than orange-spotted grouper (1.69 mm) (Kohno 1998), its female parental species but larger than tiger grouper (1.34 mm) (Kohno 1998), its male parental species. The intermediate size suggests that the OGTG expresses mid-parent hybrid vigor.

However, mean TL of the OGTG larvae was 9.2 mm at 30 d AH and 16.4 mm at 40 d AH. At 40 d AH for *E. stratus*, it reached only 13.5 mm (Sadovy and Eklund 1999); 13.9 mm for *E. morio* at 27 d AH (Colin et al. 1996); and 8.1 mm at 33 d AH for *E. septemfasciatus* (Kitajima et al. 1991). This shows that growth of the OGTG is evidently faster compared to most of the other grouper species.

Growth of the OGTG increased significantly from 30 d AH onwards, and this could be due to introduction of artificial powder feed, Otohime (Marubeni Nisshin Food). Though the larvae preferred live feed such as rotifer in the present experiment, they are nutritionally inadequate. Artificially prepared feeds which are nutritionally enriched could contribute to improvement in larval growth as well as survival. The ability of larvae to be weaned and to consume artificial pellets is of great significance in aquaculture (Lavens et al. 1995).

Larval survival

Generally, most groupers suffer from high mortality at the early larval stage as larvae are small, fragile and have a small mouth at first feeding (Tucker 1999). Tucker (1999) reported that nassau grouper suffered peak mortality at 5–7 d AH when yolk sac and oil globule was exhausted. In this study, the OGTG larval yolk sac and oil globule were already exhausted at around 3 d AH and larval mortality was highest during 5–7 d AH during which survival rate dropped drastically from 70.6% to 35.3%. The OGTG larvae were fed unscreened rotifer for the first feeding; however, larvae with empty
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gut were observed. This suggests that larvae seemed to prefer smaller prey (Duray et al. 1997). Mortality therefore occurred due to starvation, as more energy is exhausted to search for smaller prey (Laurence 1977), making larvae more susceptible to death (Hunter 1980).

At 40 d AH, the OGTG larval survival rate was 7.8% when cultured in 1 kl tank with 30.0 ppt salinity. Newly hatched E. malabaricus (=suillus) larvae had an optimum salinity range 8-24 ppt (Parado-Estapa 1991) and E. taurina late-stage larvae at 25 ppt (Akatsu et al. 1983). Duray et al. (1997) surmised that the effects of salinity may be due to the effects of total osmotic concentration. Lower salinities possibly require less energy cost from larvae for osmoregulation. The low larval survival can also be attributed to tank size and stocking density. Duray et al. (1997) reported mean survival at day 21 or day 24 was significantly higher in 3 kl tanks (19.8%) than in 0.5 kl tanks (7.4%). A previous study (Duray et al. 1995) on E. suillus also reported a higher survival rate when reared in 500 l than those reared in 200 l and 40 l tanks, and at initial stocking density of 20 larvae per l than at 30 larvae per l. Larger tank size would mean less incidence of larvae colliding against the tank wall.

Cannibalism could be considered as a substantial factor of low larval survival rates in advanced larval stage (Tucker 1999). It was regarded as a major cause since dead fish was not observed during daily bottom cleaning. In this study, cannibalism was observed starting from 30 d AH, when larvae first exhibited orange pigmentation. Cannibalism can be minimized by proper feeding, weaning them as soon as possible, and regularly removing extra large fish (Tucker 1999). For the OGTG larvae, sorting is therefore recommended to be carried out as soon as orange pigmentation appears.

Larval Development and Behavior

Morphological development of the OGTG larvae was almost similar to that of the other Epinephelus species (Tucker 1999; Heemstra and Randall 1993). In many marine fish larvae, the eyes, jaw and digestive tracts become functional just before yolk sac absorption, but their locomotory organs are not well developed at the end of the yolk sac stage (Ikewaki and Sawada 1991). This was similar in the OGTG larvae where first feeding commenced at 3 d AH and horizontal swimming ability was observed. However, active feeding was observed much later around 5 d AH.

Grouper larvae have a characteristic pigmentation which begins to develop from early larval stage far before transformation to juvenile stage (Tucker 1999; Heemstra and Randall 1993; Galamuzina et al. 2001). In the OGTG larvae, black pigmentation developed as early around 5 d AH, but orange pigmentation only developed from 30 d AH onwards. The completion of marble brownish colouration, which was similar to that of male parental fish, was only completed at 50 d AH juvenile stage. Groupers shift habitats from pelagic to benthic as they transform from the larval to juvenile stages (Tucker 1999). Groupers also have a characteristic "kite shaped" body, in which second dorsal-fin and pelvic-fin spines greatly elongated at early larval stage, and disappear just before transition to juvenile stage (Heemstra and Randall 1993; Tucker 1999). In the case of OGTG, its behavior in regards to habitat preference and development of spines was similar to those of the other groupers. Larval period of groupers are generally long, lasting from 35 to 70 days (Tucker 1999). Larval period of the OGTG was approximately 50 days and individuals transform 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.

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References


ハタ科交雑魚チャイロマルハタ Epinephelus coioides × アカマダラハタ E. fuscoguttatus の卵発生と仔魚の発育

Ivan Chong Chu KOH ・ Sitti Raehanah Muhd. SHALEH ・瀬尾重治

ハタ科交雑魚チャイロマルハタ Epinephelus coioides × アカマダラハタ Epinephelus fuscoguttatus の種苗生産技術を確立するため、卵発生と仔魚の発育を観察した。体重7.5 kg の雌のチャイロマルハタから採出した卵は、直径0.81 ± 0.02 mm（平均 ± 標準偏差）で、1 g 当たりの卵数は3,505であった。雌のアカマダラハタの精液による受精後、卵の直径は0.83 ± 0.02 mm であった。孵化は、水温29.0℃、塩分30.0 ppt で受精後17時間30分～19時間であった。受精率と孵化率はそれぞれ93.9％と50.3％であった。孵化直後の仔魚は全長1.52 ± 0.01 mm、水面直下で静止状態であった。仔魚は、開口・消化管の形成・眼の黒化した、孵化後3日に初期摂餌を開始した。孵化後10日からハタ科魚類に特有の尾部と肛門の間で体色が黒化し、背鰭および腹鰭の発達が開始した。孵化後40日に、浮遊生活から底続生活へ移行を開始した。21,500尾の孵化仔魚から、750尾の稚魚（孵化後50日、平均全長22.8 ± 3.6 mm）を生産した。