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SHORT COMMUNICATION

Natural spawning, embryonic and larval development of F2 hybrid grouper, tiger grouper *Epinephelus fuscoguttatus* × giant grouper *E. lanceolatus*

Fui Fui Ching · Norazmi Othman · Azaharie Anuar · Rossita Shapawi · Shigeharu Senoo

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Abstract This study aims to reveal the first report of the natural spawning of F1 hybrid grouper (TGGG), a crossbreed between the tiger grouper, *Epinephelus fuscoguttatus* × giant grouper, *E. lanceolatus*, since its first production in 2006. This marks the completion of its full cycle after a 10-year period. In order to establish a seed rearing protocol for a novel F2 hybrid TGGG, natural spawning, embryonic and larval developments were thoroughly observed. Five batches of natural spawning were recorded with an average of 1.50–15.3 kg eggs collected, while fertilization and hatching rates were recorded at 85.3–97.6%, and 63.0–98.3%, respectively. F2 larvae hatched out at 17:50 hours with an average body size of 1.74 ± 0.01 mm, and a yolk sac volume of 0.85 ± 0.197 mm^3. The first feeding was initiated 3 days after hatching, which coincided with the onset of functional feeding apparatus and active swimming behavior. Larval dorsal and pelvic spines were formed at 6 days AH coupled with dynamic feeding activity, as more food was found in the digestive tract. Meanwhile, the F2 hybrid grouper shifted habitat from pelagic to benthic as early as 25 days AH, and entered a juvenile stage at 35 days AH, attaining a skin coloration similar to that of the F1 juvenile. This study concluded that naturally spawned eggs of F2 hybrid TGGG were exceptionally high in quality, although larvae were small and fragile, and performed vigorous feeding activities and cannibalistic behavior. Thus, these findings can serve as primary data to further develop the optimal rearing protocol to enhance the overall rearing performance.

Keywords Hybrid grouper · Natural spawning · Embryonic and larval development · Morphology · Sensory organ · Behavior changes

Introduction

Hybridization is a process that involves the crossbreeding of two animals or plants from the different taxa, to produce a new species (Dunham et al. 1987). In aquaculture, hybridization is a powerful tool that offers the hope of producing aquatic organisms with valuable traits, where the offspring carries the characteristics of hybrid vigor or positive heterosis. Generally, the preferred new offspring may result in shorter grow out and production cycle, higher survival, fast growth, better food conversion, enhanced flesh quality, high disease

Fui Fui Ching (✉) · N. Othman · A. Anuar · R. Shapawi
Borneo Marine Research Institute, Universiti Malaysia Sabah, Jln UMS, 88450 Kota Kinabalu, Sabah, Malaysia
e-mail: cfuifui@ums.edu.my

S. Senoo
Aquaculture Technology and Production Center, Kindai University, 3153 Shirahama, Wakayama 649-2211, Japan

resistance as well as the ability to tolerate a wider range of rearing environments, as similarly reported in various marine and freshwater hybrid fish species (Bunlipatanon and U-taynapun 2017; Bartley et al. 1991).

Hybrid groupers have taken the Asian aquaculture industry by storm since 2006, with the first production of hybrid TGGG, a crossbreed between tiger grouper (*Epinephelus fuscoguttatus*) and giant grouper (*E. lanceolatus*). This novel hybrid grouper has gained immediate popularity from aquaculturists and seafood consumers, owing to its production success and premium organoleptic properties, which has led to high commercial value.

To date, there are over ten hybrid groupers that have been produced, among which are crossbreeding in between orange spotted grouper (*E. coioides*), coral grouper (*E. corallicola*), mouse grouper (*Cromileptes altivelis*), camouflage grouper (*E. polyphekadion*), and red spotted grouper (*E. akaara*), among others (Liufu et al. 2007; Addin and Senoo 2011; Huang et al. 2014; Koh et al. 2010). TGGG are widely cited as the most successful hybrid combination, as it is able to grow quickly (Ch'ng and Senoo 2008), has a higher survival and better feeding performance (Othman et al. 2015), and is able to tolerate a wide range of rearing parameters (Shapawi et al. 2018; De et al. 2016).

Luin et al. (2013) studied the complete sexual maturation and gonad development of hybrid TGGG in captivity, and indicated that hybrid TGGG started to mature as female and male at approximately 9.0 and 11.0 kg, respectively. This initial report has opened up the possibility of using the hybrid progeny to develop F2 generation, and perform backcross breeding. In 2016, fertilized eggs were found in hybrid TGGG broodstock tank, and preserved in a hatchery at Borneo Marine Research Institute, Universiti Malaysia Sabah, Malaysia. These matured broodstock of hybrid TGGG had spawned naturally, and produced approximately 20 kg of fertilized eggs within 6 months. This marks the complete closed cycle of hybrid TGGG, after 10 years being introduced into aquaculture (Fig. 1). However, similar to other groupers, high larval mortality is considered the bottleneck that is hindering the establishment of F2 TGGG production.

In an attempt to establish a seed production technique for a new F2 hybrid TGGG, the authors observed the embryonic and larval development in relation to functional sensory organ and behavior changes of this new species. The findings are significantly useful for exploring the ideal rearing protocol, and later further improving the growth performance and overall production.

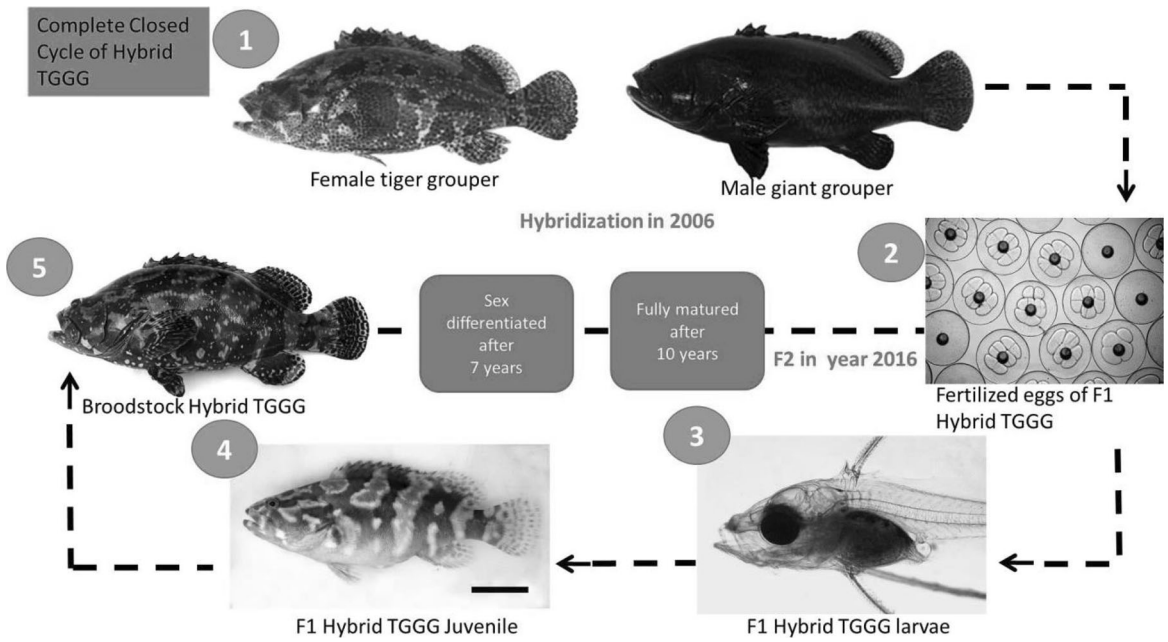

Fig. 1 Complete closed cycle of hybrid tiger grouper, *Epinephelus fuscoguttatus* × giant grouper, *E. lanceolatus* (TGGG)

Materials and methods

Broodstock management

The broodfish of hybrid TGGG used in this study have been reared for a period of 10 years in the hatchery of Borneo Marine Research Institute, Universiti Malaysia Sabah, Malaysia. Broodfish (n = 28) were initially kept in a tank with a capacity of 150 tonnes, and then transferred to one unit of 20 tonnes of cylindrical fiber reinforced plastic (FRP) tank equipped with a recirculation system for experiment purposes. The body weight of the broodfish ranged from 12 to 20 kg. They were fed until satiation daily with enriched prey fish (*Sardinella* sp.) supplemented with pure cod liver oil (Seven seas, Merck Company, United Kingdom), squid oil and vitamin premixes. The water salinity, temperature, dissolved oxygen and pH were maintained at 30–32 ppt, 27.0 ± 1.0 °C, 6.89–7.02 mg L^{-1} and 7.80–8.01, respectively.

Egg collection and larval rearing

Newly fertilized eggs were obtained from broodstock hybrid TGGG that spontaneously spawned between January and April 2016. The eggs were collected around 03:00 hours from a flow-through screen that was initially installed next to a 20 tonne broodstock tank, and were later incubated in a 1000-L FRP tank. Eggs were stocked at about 30 eggs L^{-1}, and gently aerated at 250 mL min^{-1}. The water temperature, pH and dissolved oxygen (DO) in the incubation tank ranged from 29.5 to 30.5 ± 0.5 °C, 6.9–7.2 and 6.8–6.9 mg L^{-1}, respectively.

The first feeding time was commenced at the first hour after the mouth had opened (h AMO), which coincided with the functionality of four feeding-related organs (eyes, mouth, intestine and anus). A 12L:12D (light and dark) light regime was provided by 12 h natural light (640–750 l×) at daytime (06:00–18:00) and 12 h dark (18:00–06:00) at night-time. During the first feeding, rotifer, *Brachionus plicatilis* sp. complex and commercial *Nannochloropsis oculata* (K2, New World Aqua, Korea) were provided to the larvae at a density of 30 individual mL^{-1} and 0.5 × 10^{-6} cells mL^{-1}, respectively.

From 0 h AMO, larvae (n = 10) were sampled from the larval rearing tank to measure total length (TL), yolk sac volume (YV) and oil globule volume (OGV). Larval TL was measured at 6 hourly intervals up to 78 h after hatching (h AH), and continued on a 5-day interval basis. Meanwhile, larval YV and OGV were measured until both yolk sac and oil globule were completely absorbed.

Daily routine regimes include bottom cleaning and water renewal for rearing maintenance purposes. Bottom cleaning was performed to remove debris, excessive feeds and dead larvae. Filtered seawater was later added (10–20%) in order to restore the amount of removed water, which was siphoned out during the bottom cleaning process. In addition, the feeding densities of both rotifers and *N. oculata* in the rearing water were maintained uniformly for the first 20 days of rearing, followed by *Artemia* and mixed species of copepods (80–450 μm) from day 20 to 40 and artificial feed from day 30 onwards. The water quality was monitored twice daily at 09:00 and 16:00 hours.

Sampling I: egg development

Newly fertilized eggs were collected from the incubation tank using a fine scoop net (60 μm mesh size) and transferred to a 500-mL beaker containing filtered seawater. The eggs were taken out and placed on a clean petri dish under a compound microscope (Nikon, Eclipse E600) for observation purposes. The time taken for the eggs to reach different development stages was recorded, and the size of the ovulated eggs was measured. Images of the eggs were captured by a digital camera (Canon, 60D, Japan). The fertilization rate (%) was recorded by calculating the total number of fertilized eggs/total number of eggs × 100. Meanwhile, the hatching rate (%) was determined by calculating total number hatchlings/total number of fertilized eggs × 100.

Sampling II: larval development

Yolk sac and oil globule absorptions Ten newly hatched larvae (n = 10) were sampled at 0 h after hatching (h AH) from the incubation tank. Larvae were positioned on a slide glass, under a profile projector (Mitutoyo, PJ3000, Japan), for measurements of total length (TL), yolk sac and oil globule length (mm) and height (mm), respectively. The yolk sac volume was measured by the formula for a prolate spheroid = $\pi/6\ lh2$, where l represents the yolk sac length and h represents the yolk sac height. The oil globule volume (OGV) was measured by the formula OGV = $\pi/6\ d^3$, where d represents the diameter of oil globule (Bagarinao 1986).

Indication of first feeding time The same specimen used for yolk sac and oil globule absorption was also used to observe larval first feeding time according to the method of Ching et al. (2014), whereby time of the completion of all feeding-related organs was recorded and defined as onset of first feeding time.

Larval morphology, sensory development and behavioral changes Starting from 0 h AH, ten larvae (n = 10) were randomly sampled from the rearing tank at a 6-h interval for the first 48 h. They were alternated with daily sampling from 3 days after hatching (d AH), and the total length and subsequent larval growth stages were carefully measured under a compound microscope and profile projector, respectively. The formation of sense organ was also recorded during the microscopic observation. As for behavioral observation, ten larvae (n = 10) were sampled daily and transferred to a 500-mL beaker, and their swimming behavior was recorded.

Results

Spawning and egg development

Five batches of natural spawning were recorded from January to April 2016 with each batch lasting for 3–5 days. The number of eggs (diameter 860 ± 30 μm) in each spawning ranged from 1.50 to 15.3 kg, with fertilization and hatching rates recorded at 85.3–97.6% and 63.0–98.3%, respectively (Table 1).

Egg development of F2 hybrid TGGG is shown in Fig. 2. At 1 min (0:01 h) after fertilization (AF), 2-cell stage was observed and first cleavage occurred (Fig. 2a). At 1 h 40 min AF, eggs developed to the 4-cell stage (Fig. 2b); and later at 2 h 15 min AF, the eggs developed to the 6-cell stage (Fig. 2c). At 5 h AF, eggs entered into a morula stage (Fig. 2d) and reached the gastrula stage at 8 h AF (Fig. 2e). Meanwhile, at 10 h 45 min AF, the first appearance of Kupffer's vesicles was observed (Fig. 2f).

Later at 11 h 50 min AF, the larval head and myomere were formed (Fig. 2g). At 12 h 35 min AF, the lens vesicle was noticed (Fig. 2h), and at 13 h 30 min AF, the first movement of the embryo was observed (Fig. 2i). The larval heart beat was first detected at 17 h 10 min AF (Fig. 2j), and the hatching started at 17 h 45 min AF (Fig. 2k). Later, newly hatched larvae were seen at 17 h 46 min AF (Fig. 2 l) with an average size of 1.74 ± 0.01 mm.

Larval development

The changes of the larval yolk sac and oil globule volume are shown in Fig. 3. Yolk sac and oil globule volumes were 0.85 ± 0.197 and 0.27 ± 0.05 mm^2, respectively, at 0 h AH (28.5 ± 0.1 °C). The F2 hybrid

Table 1 Natural spawning occurrence number, duration, total eggs, mean fertilization and hatching rates of F2 hybrid TGGG collected from January to April 2016

Batch	Month	Duration (day)	Total eggs (kg)	Fertilization rate (%)	Hatching rate (%)
1	January	3	1.5	97.67 ± 0.6	98.3 ± 0.6
2	February	3	1.6	85.3 ± 0.6	96.0 ± 1.0
3	February	5	1.4	91.3 ± 1.1	74.6 ± 2.5
4	March	5	15.3	92.7 ± 0.6	74.7 ± 2.1
5	April	5	3.64	91.0 ± 1.0	63.0 ± 2.0

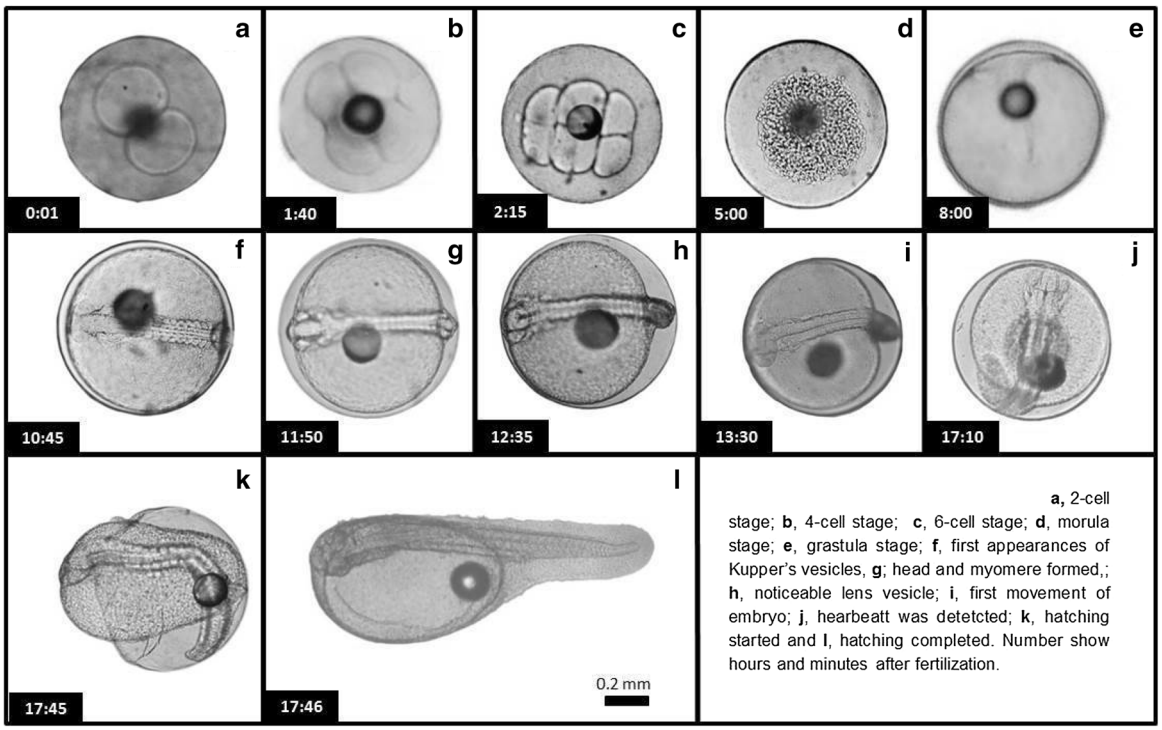

Fig. 2 Embryo development of F2 hybrid TGGG, *E. fuscoguttatus* × *E. lanceolatus*: **a** 2-cell stage, **b** 4-cell stage, **c** 6-cell stage, **d** morula stage, **e** gastrula stage, **f** first appearances of Kupffer's vesicles, **g** head and myomere formed, **h** noticeable lens vesicle, **i** first movement of embryo, **j** heartbeat was detected, **k** hatching started and **i** hatching completed. Numbers show hours and minutes after fertilization

TGGG larvae took approximately 60 and 72 h to absorb both yolk sac and oil globule, respectively. The larval development at an early stage is shown in Fig. 4, and its subsequent growth in Fig. 5. Its correlation to behavioral changes can be seen in Table 2.

Newly hatched larvae were seen floating near the water surface with the head positioned down, and no specific swimming pattern was observed. Larvae also were found with unopened mouth, non-pigmented eyes, closed anus and large yolk sac. The heart beat was observed under a microscope, and the onset of cardiac function in larvae was marked (Fig. 4a). At 6 h AH, the larvae were seen with pigmented eyes (Fig. 4b). At 18 h AH, they were well pigmented with visible pectoral fins, and melanophores can be observed. The anus was seen to be open.

At 24 h AH, the larvae grew with reduced yolk sac (0.08 ± 0.01 mm^3), and started to perform vertical swimming. They were able to avoid any disturbance created surrounding them. Such a reaction is highly associated with the presence of free neuromast and capula. However, during this time, all feeding-related organs were still undeveloped, with no sign of first feeding occurrence.

At 3 days AH, the larvae grew larger and were able to perform both vertical and horizontal swimming patterns, and all four feeding-related organs were well-developed when the larvae have pigmented eyes, an open mouth with a functional lower jaw, peristaltic digestive tract and an open anus (Fig. 4d). Rotifer was first found in the larval digestive tract at this stage. Meanwhile, the yolk sac was absorbed completely, and marked the onset of the larval exogenous feeding phase.

As larvae entered 6 days AH, the dorsal and pelvic spines started to form, and the larvae commenced active swimming behavior coupled with dynamic feeding activities at this stage, as more rotifer were found in the digestive tract. By the age of 25 days AH, both dorsal and pelvic spines were elongated, spiny and covered by melanophore layers. The larvae were seen as more aggressive, particularly in foraging prey and preferred *Artemia* and copepod compared to rotifers. Interestingly, the larvae started to aggregate around the central part of the bottom tank, instead of the other columns of the tank, and eventually switched to the benthic habitat.

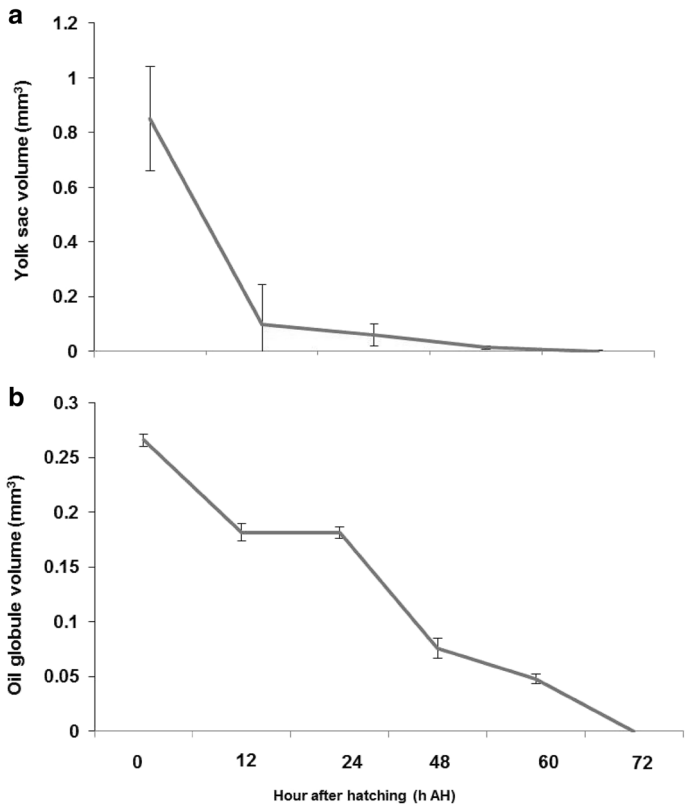

Fig. 3 The changes of yolk sac and oil globule volumes (mm^3) of F2 hybrid TGGG, *E. fuscoguttatus* × *E. lanceolatus*

By the age of 35 days AH, F2 hybrid TGGG entered the juvenile stage, as their morphological characteristics started to resemble those of an adult. The body is no longer transparent with slight pigmentation, and the lateral line can be seen clearly. The stomach was covered with a silver pouch, and was obvious to the naked eyes. At this stage, caudal fins were well-developed, along with the dorsal and pelvic spines. Although juveniles are seen as more aggressive compared to the larval stage, they remain at the bottom, with less swimming activity. However, they performed speedy swimming, particularly when approaching food.

Discussion

Natural spawning

This study revealed the first record of natural spawning in hybrid TGGG in captivity, since its first report on the successful sex differentiation and gonad maturation by Luin et al. (2013). To the best of our knowledge, this is the first report on natural spawning in hybrids among *Epinephelus* sp. and marked the first close cycle of hybrid TGGG. The majority of hybrid fish of various fish species documented were either sterile (Argue and Dunham 1999), low in fertility (Wagner and Oplinger 2013) or functionally fertile (Hodson 1989; Ma and Yamazaki 1986).

Obtaining matured broodstock are among the challenges in aquaculture (Song et al. 2005), and the natural spawning of hybrid TGGG is evidently possible in captivity through sustained efforts on broodstock management, to develop the closed cycle hybrid TGGG. This is beneficial to both the aquaculture and seafood industry, particularly in the Asian region, where hybrid TGGG are prominent (Shapawi et al. 2018).

Spawning triggers are environmental cues that lead the fish to spawn. The sudden changes in the environmental parameters in captivity such as fluctuation of water temperature, salinity, and photoperiod, often trigger several fish to spawn, namely, Yellow perch, *Perca flavescens* (Starzynski and Lauer 2015), striped

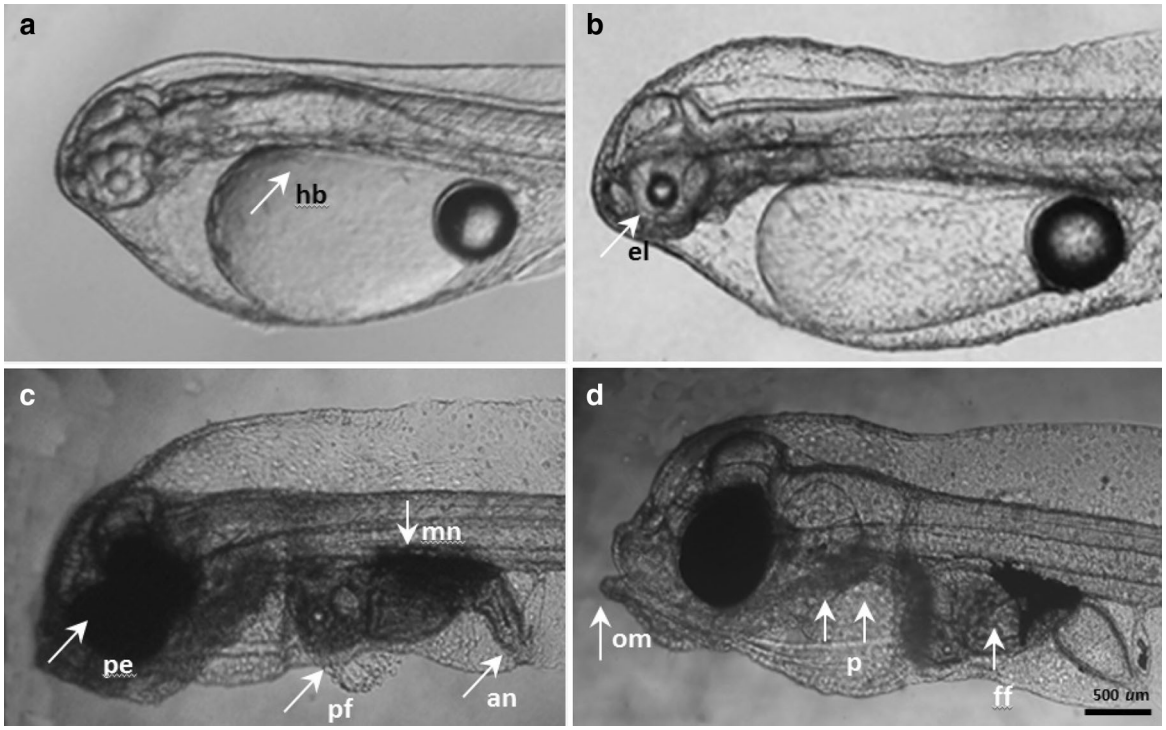

Fig. 4 Early stage larval development of F2 hybrid TGGG, *E. fuscoguttatus* × *E. lanceolatus*. **a** Visible heart beat (hb) detected; **b** onset of pigmentation around eye lens (el); **c** well pigmented eyes (pe), first appearance of pectoral fins (pe), opened anus (an) and visible melanophores; **d** functional and opened mouth (om), peristaltic movement detected in esophagus (p), first feeding detected (ff)

bass, *Morone saxatilis* (Henderson-Arzapalo and Colura 2011), yellowtail, and *Seriola quinqueradiata* (Mushiake et al. 1998), among others. In this study, the natural spawning of F1 hybrid TGGG was triggered by the sudden change of water depth from 3.0 to 1.5 m.

The water level fluctuation is a crucial factor in inducing reproductive adaptations in many fish species, and this study assumes a sudden change of water depth had triggered stress in the matured hybrid TGGG to release hormones, a chemical messenger that travels into the blood and eventually responds in a variety of different ways, including spawning. From an aquaculture point of view, the synchronization of spawning triggered by water depth could allow for some important advantages in hatchery operation. These include less use of costly synthetic hormones, reduced physical injury, and physiological stress of capturing, handling, injecting, and holding broodfish, which can have a greater detrimental effect on spawning success than almost any other factor (Rottmann et al. 1999).

Fertilization and hatching rates

The quality of a fish gamete is often defined as its ability to be fertilized and hatched, and later develops into a normal embryo (Chevassus-au-Louis and Lazard 2009; Bobe and Labbe 2010). Fertilization and hatching rates are powerful indicators that are used to predict the production of high quality fish larvae, larval survivability and economical utilization of overall hatchery operational costs. The fertilization and hatching rate of F2 hybrid TGGG were averaged at 91.5% and 80.7%, respectively. This is considered relatively higher compared to the parental species recorded at 86.8% and 87.2%, respectively, as reported by Ch'ng and Senoo (2008) and other hybrid groupers such as: (1) spotted grouper × tiger grouper (*E. polyphekadion* × *E. fuscoguttatus*) at 51.0% and 38.3%; (2) coral grouper x tiger grouper (*E. corallicola* × *E. fuscoguttatus*) at 75.0% and 35.4%; (3) and orang spotted grouper × giant grouper (*E. coioides* × *E. lanceolatus*) at 91.0 and 33.6%, respectively (Ch'ng and Senoo 2008; Ivan et al. 2008; Addin and Senoo 2011).

The findings reveal the possibility of this new species to be introduced as a suitable candidate of aquaculture species, judging from the high quality of fertilization and hatching rates. The high fertilization and

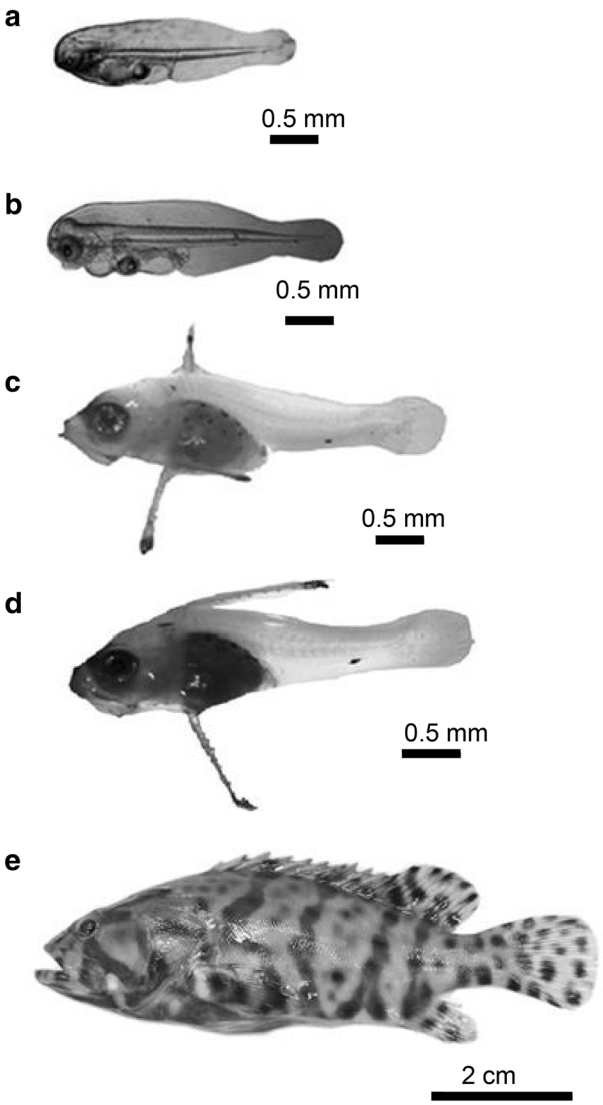

Fig. 5 Morphological changes of F2 hybrid TGGG, *E. fuscoguttatus* × *E. lanceolatus*. **a** 0 h after hatching (h Ah), **b** 12 h AH, **c** 6 days after hatching (d AH), **d** 25 days AH, **e** 35 days AH

hatching rates of F2 hybrid grouper in this study is contributed by the high quality of eggs and milts produced by the fertile hybrid TGGG reared in captivity, where water quality was strictly monitored to the finest level, and the food involved a nutritionally balanced diet.

Broodstock management can be pricy in aquaculture by taking the overall time and cost into consideration. However, given the importance of effective broodstock management in the provision of quality gametes, this critical management should not be neglected, as similarly claimed by Bromage (1998), Izquierdo et al. (2001) and Migaud et al. (2013).

Egg and larval development

F2 hybrid TGGG took 17 h 50 min to hatch, which is slightly shorter compared to the 18-h period taken by F1 hybrid TGGG to hatch, as reported by Ch'ng and Senoo (2008). This is also considered faster than the 19-h period seen in tiger grouper, *E. fuscoguttatus* (Boonlipatanon et al. 2002), the 30-h period seen in the giant grouper, *E. lanceolatus* (Garcia-Otega et al. 2014), the 24-h period seen in the mouse grouper, *C. altivelis* (Senoo et al. 2004), the 20–24 h range seen in some other hybrid groupers (Addin and Senoo 2011; Senoo 2008); and even the 19-h period seen in the backcross hybrid grouper (Gan et al. 2016) (see Table 3).

Table 2 Correlation between morphological and behavioral changes of F2 hybrid TGGG, *E. fuscoguttatus* × *E. lanceolatus*

Morphological features	Days after hatching (d AH)	Behavioral changes
Hatching commenced, unpigmented eyes, heart beat detected, large yolk sac	0	Pelagic behavior, spread all over water column, no swimming activity
Mouth not formed, anus closed, first appearance of eye lens	1	Vertical swimming
Deeply pigmented eyes, peristaltic movement on digestive tract, opened and functional eyes and anus, pectoral fins formed	2	Horizontal swimming, active S-posture , positively phototactic, onset of first feeding
Eye movement detected, yolk sac completely absorbed, air bladder inflated, intestinal tract movement	3	Swimming all water column, actively prey on Brachionus sp.
All fins formed (dorsal and anal), clear pigmentation on head, abdomen and tail	6	Swimming at the center and surface of water.
Spiny spines covered with melanophore, elongated spine.	10	Aggregated around central part of tank, schooling behavior started to form
Slight pigmentation on body, non-transparent, colored abdomen	25	Active and increased swimming speed, prey on artemia and copepode
Well-developed fins, lateral line can be seen, silvered abdomen, enter juvenile stage	35	Benthic habitat. Fed on artificial feed and onset of cannibalism.

Glamuzina et al. (2001) and Sargent et al. (1999) stated that most hybrid fish eggs hatch quicker, and this might be closely related to the adequate supply of all the required nutrients, particularly the good quality of lipid source to maintain energy reserves in eggs for quicker embryogenesis of marine fish. The enriched prey fish diet given to the F1 hybrid TGGG broodstock prior to spawning seems to be responsible for their positive effect on shorter hatching of F2 hybrid TGGG. This is in agreement with a study of Sargent et al. (1989), which stated that prey fish such as *Sardinella* sp. used in this study, store fat or lipid in their muscle mostly as triglycerides, which are an important source of energy to support fish maturation and reproduction.

Similar to other groupers, the larvae of F2 hybrid TGGG are small and fragile upon hatching, and depend exclusively on the nutritive reserves of the yolk sac and oil globules as energy and food sources. A larger yolk sac volume of F2 hybrid TGGG compared to other hybrid groupers reported by Addin and Senoo (2011) seems to be advantageous, as they would have a lengthier preparatory time to gain feeding experience and improve feeding ability, before being entirely reliant on external food sources (Ching et al. 2012). These findings also reveal that F2 hybrid TGGG have 40-h-long nutritional transition period, and this feature makes them stronger to withstand mortality related to starvation in the early stages. Long yolk sac and oil globule absorption duration is widely cited by many as advantageous features to avoid a high larval mortality, due to starvation at early stages in various marine fish larvae species (Pena and Dumas 2005; Yoseda et al. 2006; Ching et al. 2016).

Table 3 Hatching hours (h) of different grouper species

Species	Hatching hour (h)	References
Tiger grouper (TG)	24	Ching et al. (2012)
Mouse grouper (MG)	24	Senoo et al. (2004)
Orange spotted grouper (OG)	20	Kawahara et al. (1997)
Giant grouper (GG)	30	Garcia-Otega et al. (2014)
TG ×GG	23	Ch'ng and Senoo (2008)
OG ×TG	23	Ivan et al. (2008)
OG ×GG	20	Koh et al. (2010)
MG ×TG	23	Senoo (2008)
MG ×GG	23	Senoo (2008)
CG ×TG	19	Addin and Senoo (2011)
CG ×GG	20	Addin and Senoo (2011)
SG ×TG	20	Addin and Senoo (2011)
CG ×TG	20	Addin and Senoo (2011)
TG ×SG	20	Addin and Senoo (2011)
SG ×GG	19	Addin and Senoo (2011)
OGGG ×GG	19	Gan et al. (2016)

Water temperature was recorded at 28–30 °C

F2 hybrid TGGG is morphologically smaller compared to other groupers, and is thus probably difficult to be cultured and potentially display poor growth performances, judging from many cited reports that smaller body size larvae are highly sensitive to various abiotic and biotic factors and vulnerable to mortality (Sakakura et al. 2007; Kohno 1998). Therefore, this study provides substantial information on larval morphological changes in relation to its behavior, as they aged to juveniles to ease F2 hybrid TGGG rearing procedures. The system used can be manipulated to suit its developmental stages to further enhance their overall growth performance.

Morphological development of F2 hybrid grouper is almost similar to that reported in F1 hybrid TGGG by Ch'ng and Senoo (2008), and other groupers (Hussain and Higuchi 1980; Glamuzina et al. 2001). At an early larval stage, the presence of cupula and free neuromast observed in F2 hybrid TGGG as early as 6 h AH indicates their remarkable ability to sense stimuli in water columns. This information is particularly important to develop a handling stress-free rearing technique to culture F2 hybrid TGGG, as both cupula and free neuromast are easily broken by mechanical contact (Mukai and Kobayashi 1992).

In the later stage, the F2 hybrid TGGG, similar to other serranidae fish, goes through metamorphosis processes that represent significant transformation in the post-larvae stage (Park et al. 2014). In this study, the first spinous ray of the ventral fin was observed as early as 10 days after hatching (d AH), indicating their strong swimming capabilities at early stages. This feature is considered beneficial, particularly to coordinate active swimming movement that later contributes to improved feeding performance. However, extra precaution should be given in handling F2 hybrid TGGG at this stage, as larvae start to aggregate and the spine might tangled or broken, causing them to die.

The onset of the first feeding was observed at 3 days AH, as similarly reported in other groupers (Ching et al. 2012; Ch'ng and Senoo 2008; Yoseda et al. 2006). Although F2 hybrid TGGG larvae is characterized by a small mouth size upon hatching, they were able to attain successful initiation of first feeding when *Nannochloropsis* sp. were co-introduced with rotifer into the rearing tank. A similar study reported the use of microalgae to compensate for delays in successful initiation of first feeding for small mouth size marine fish larvae, such as sea bream, *Sparus aurata* (Muller-Feuga et al. 2003), turbot *Scophthalmus maximus* and halibut, *Hippoglossus hippoglossus* (Reitan et al. 1997). In addition, additional microalgae in the tanks created the "green water" rearing condition, thus offering a positive effect on the survival rates of F2 hybrid TGGG at an early stage.

Major morphological deformities including jaw, skeletal, operculum, fin, scale disorientation, and abnormal swimming behavior, among others, were not found throughout the larval to juvenile stages of F2 hybrid TGGG. In general, aquaculture is often threatened by the present susceptibility of cultured fish to disease, low tolerance in a wide range of water qualities, and rearing systems which eventually produce a weak fish with low growth performance. In comparison to the F1 hybrid TGGG, the following generation is characterized by greater hybrid vigor or positive heterosis characteristics (Shapawi et al. 2018). Despite the excellent egg quality and comparable larval growth observed in F2 hybrid TGGG, cannibalism remains the critical factor responsible for the low survival, as similarly observed in other fish species. In the future, further studies on the ideal rearing system for the enhancement of growth performance is investigated to demonstrate the advantages of F2 hybrid TGGG.

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