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Egg Development of Backcrossed Hybrid Grouper between OGGG (*Epinephelus coioides* × *Epinephelus lanceolatus*) and Giant Grouper (*Epinephelus lanceolatus*)

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Abstract: The hybrid grouper, OGGG is produced from a cross-breed between a female orange-spotted grouper (OG; *Epinephelus coioides*) and a male giant grouper (GG; *E. lanceolatus*). OGGG is an excellent hybrid grouper in Southeast Asia owing to its outstanding organoleptic qualities. GG is the most favorable species in aquaculture industry driven by its notable characteristics. With the purpose of producing a superior aquaculture species, a backcross-breed between OGGGs and GGs was conducted. A sexually matured female OGGG was selected and treated twice with Human Chorionic Gonadotropin (HCG). Subsequently, the stripped eggs were fertilized with preserved sperm of GG and incubated in a circular fiberglass reinforced plastic (FRP) tank. Observation on stripped eggs, egg development and developed time on each egg stage was recorded under microscope. The fertilized eggs were spherical with a mean diameter of 755 ± 28 µm (n = 20). The fertilization rate and hatching rate were 23.3 % and 1.5 %, respectively. The hatching time was commenced from 22:40 to 24:40 hours after hatching (hAF). It was undergone normal egg development and successfully survived up to 11 dAH. It is possible to produce backcrossed OGGG and GG and beneficial to the production of hybrid grouper in aquaculture industry.

Key words: backcross- breed, hybrid grouper OGGG, giant grouper, egg development

Introduction

The most popular cultured groupers in the Southeast Asian region are: the tiger grouper (TG; *Epinephelus fuscoguttatus*), orange-spotted grouper (OG; *Epinephelus coioides*) and giant grouper (GG; *Epinephelus lanceolatus*). They all a part of the Epinephelae family. Among the Epinephelae family, the GG is the most favourable species in the aquaculture industry, driven by its high market value, fast growth and excellent taste (Chan and Johnston, 2007). Similar to other grouper species, the expansion of the GG has often been hindered by the unreliable and limited supply of seeds in grow-outs and ponds (Pomeroy *et al*., 2002, James *et al*., 1999). Currently, most of the seeds are collected from the wild, and are slowly becoming scarce. While in captivity, to mass produce GG is still difficult to achieve as seeing female GG are rarely matured under artificial condition.

In 2006, Universiti Malaysia Sabah (UMS) became the pioneer in hybrid grouper production, and overcame the constraint of producing GG. By seeing the availability of sperm from matured male GG all year round, a total of 5 cross breeds of hybrid groupers were produced, whereby GG sperm was used to fertilize eggs from various female groupers. These hybrid groupers are TGGG (TG, *E. fuscoguttatus* × GG, *E. lanceolatus*); OGGG (OG, *E. coioides* × GG, *E. lanceolatus*); MGGG (Mouse Grouper, *C. altivelis* × GG, *E. lanceolatus*); SGGG (Spotted Grouper, *E. polyphekadion* × GG, *E. lanceolatus*) and CGGG (Coral Grouper, *E. corallicola* × GG, *E. lanceolatus*). This has resulted in the offspring of hybrid groupers carrying 50% of the genetic materials from GG, which are assumed to possess excellent growth and survival performance as pure GG.

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One of the excellent hybrid groupers is the OGGG, which are produced by fertilizing eggs from OG and sperm from GG. The OGGG is among the most valuable and popular hybrid grouper in Southeast Asia, particularly in sea food markets in Hong Kong and China. In 2014, after seven years of rearing in UMS, female hybrid groupers (OGGG) were found to have matured under this artificial condition. This is where the concept of backcross-breed between matured female hybrid groupers (OGGG) and male GGs (Fig. 1) arises in order to produce offspring that may carry 75% genetic material from the GG, and are assumed to perform better than hybrid OGGGs. This attempt was also motivated by the first successful backcross-breed between TGs and TGGG hybrid groupers, reported by Luin *et al*. (in press).

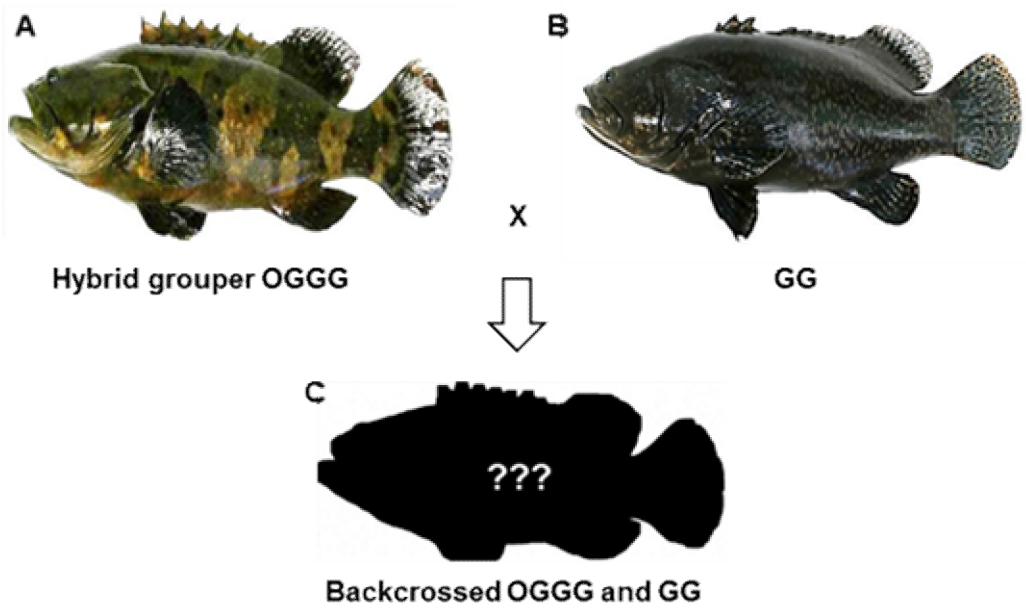

Fig. 2: Characteristics of stripped eggs. A) Shinny white of the stripped egg mass, B) Transparent and uneven stripped eggs under microscope observation

The attempt to backcross-breed OGGGs and GGs is expected to be beneficial for the production of hybrid groupers, as well as the aquaculture industry, by the witness of a superior species which may perform even better growth and have a higher survival than the hybrid OGGGs currently produced. Therefore, in the backcross-breeding between OGGGs and GGs, the egg development was examined in this study, and further compared with other hybrid groupers. The possibility of backcross breeding between OGGGs and GGs is investigated and determined in this study.

Materials and Methods

This study was conducted in the fish hatchery at Universiti Malaysia Sabah (UMS). The brood fish of the hybrid groupers OGGG and GG used in this study have been reared for a period of seven and 14 years, respectively, in 150 tonnes of cylindrical fiber reinforced plastic (FRP; 8 m in diameter and 3 m in height), equipped with a recirculation system. Both types of brood fish were fed with enriched prey fish (*Sardinella* sp.), and supplemented with pure cod liver oil (Seven seas, Merck Company), every two days until satiation. The salinity was maintained at 29.5 to 31.4 ppt; the water temperature was 27.0 to 28.8 ^{o}C; the dissolved oxygen was 6.09 to 7.02 mg/l; and the pH was 7.81 to 8.21.

For egg collection purposes, female hybrid groupers OGGG (weighed at 16.2 kg) were treated with the Human Chorionic Gonadotrophin (HCG) hormone twice in March 2014. The dosage was 250 IU/ kg for each injection. The first injection was given on 26th March 2014, followed by the second injection

24 hours later. The sperm was initially collected with use of a sperm collector based on method by Ch'ng and Senoo (2008). The sperm collectors with the collected sperm were then directly kept in a box of ice of temperature approximately 2-3 ^{o}C, as to preserve the sperm quality for the later use. This sperm preservation method is known as the ice sperm technique. Meanwhile, eggs were collected using the stripping method (Ch'ng and Senoo, 2008), and observation on the characteristics of stripped eggs was performed via a compound microscope (Nikon, Eclipse E600) and direct observation with the naked eye. The weight of the stripped eggs was measured using a weighing scale (A&D, GF3000). Next, 1 ml of stripped eggs was sampled from the egg mass for egg density counting and egg diameter measurement under a profile projector (Mitutoyo, PJ-3000). The other stripped eggs were fertilized by the GG preserved sperm.

Incubation for Egg Observation

The sampling of fertilized eggs was done a few minutes after the stripped eggs were fertilized by the GG sperm for measurements, in terms of egg density and diameter. The rest of the fertilized eggs were then transferred to a 1 tonne circular FRP incubation tank. During the incubation phase, the salinity was maintained at 29.5 to 31.4 ppt; the water temperature was 27.0 to 28.8 ^{o}C; the dissolved oxygen was 6.09 to 7.02 mg/l; and the pH was 7.81 to 8.21. Eggs were sampled regularly from the incubation tank for egg development observation under a compound microscope (Nikon, Eclipse E600). The time for each egg development stage was recorded, and images were captured by a digital camera (Casio, EX-H60). The fertilization and hatching rate (%) was calculated using the following formulae (Miah *et al*., 2008):

-Fertilization rate (%) = (Total fertilized eggs/Total eggs) × 100
-Hatching rate (%) = (Total hatched larvae/Total fertilized eggs) × 100

Upon hatching, measurements were done on the newly hatched larvae, including total length (TL), yolk sac length (L) and height (H), and oil globule diameter (D). The yolk sac volume (YSV) and oil globule volume (OGV) were computed using the following formulae (Bagarinao, 1986):

-Yolk sac volume (YSV) = $\pi/6 \times L \times H^2$
-Oil globule volume (OGV) = $\pi/6 \times D^3$

Results

Characteristics of Unfertilized Eggs

The total stripped egg mass was shiny white in colour and was observed to float on the water surface (Fig. 2A), and weighed 1,070 g with 4,206 eggs/g. A total of 4.5 million eggs were obtained in this study. The eggs were transparent and possessed an uneven spherical shape under microscopic observation (Fig. 2B). Each egg had an oil globule with a diameter of 690 ± 31 µm. Immediately after fertilization, eggs absorbed water, and the diameter was increased to 755 ± 28 µm.

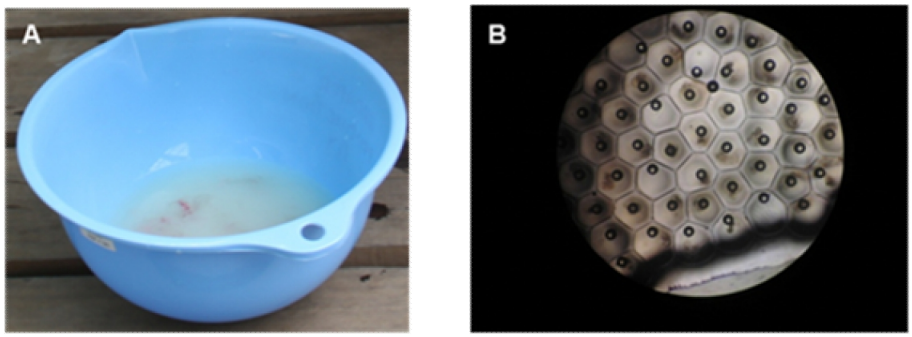

Fig. 2: Characteristics of stripped eggs. A) Shinny white of the stripped egg mass, B) Transparent and uneven stripped eggs under microscope observation

Egg Development, Fertilization Rate and Hatching Rate

The egg development of the backcrossed OGGG and GG is shown in Fig. 3. At 00:10 (hours: mins) hours after hatching (hAF), the first cleavage (Fig. 3A) was observed; this indicated that the fertilization occurred. The fertilization rate was 23.8 %. After that, each cell was divided into a 2-cell stage (Fig. 3B), 4-cell stage (Fig. 3C), 8-cell stage (Fig. 3D), 32-cell stage (Fig. 3E) and finally up to morula (Fig. 3F). It further developed to gastrula (Fig. 3G), and embryo formation commenced (Fig. 3H). The blastopore was witnessed and was nearly closed at 10:30 hAF (Fig. 3I). It completely closed (Fig. 3J) before the head, and 5-myomere (Fig. 3K) was observed. Optic vesicles and the Kupffer's vesicle (Fig. 3L) were visible, followed by tail separation from the yolk sac (Fig. 3M). It was then developed with lens vesicles (Fig. 3N). The hatching process initiated (Fig. 3R) after the embryo commenced moving (Fig. 3O), the otocyst vesicles appeared (Fig. 3P) and heart was formed; heart beats were observed (Fig. 3Q). Finally, the hatched larva (Fig. 3S) was visible, starting at 22:40 hAF and finishing at 24:40 hAF, with a hatching rate of 1.5 %. The egg development of backcrossed OGGG × GG showed similar development with other *Epinephelus* sp.

Characteristics of Hatched Larvae

The first evidence of hatched larva (n=20) was observed at 22:40 hAF, with a total length of 1.67 ± 0.07 mm. An oil globule was located at the vetro-posterior of the yolk sac with YSV of 0.11 ± 0.01 mm^3 and an OGV of 0.004 ± 0.00 mm^3. The newly hatched larvae (0 hAH) was recognized as the yolk sac larva. They hatched with undeveloped organs, including unopened mouth, unopened anus and unpigmented eyes (Fig. 3S). They were suspended and motionless when placed without aeration. The head of the backcrossed OGGG and GG larvae were observed facing downward in the water column. The newly hatched larvae did not swim, nor avoid approaching pipettes. Thus, the newly hatched larvae had no ability to prey on food in a similar way to other marine fish species. Backcrossed OGGG and GG larvae were able to survive for up to 11 days. The egg development up to hatching of the backcrossed OGGG and GG showed development similar with other *Epinephelus* sp.

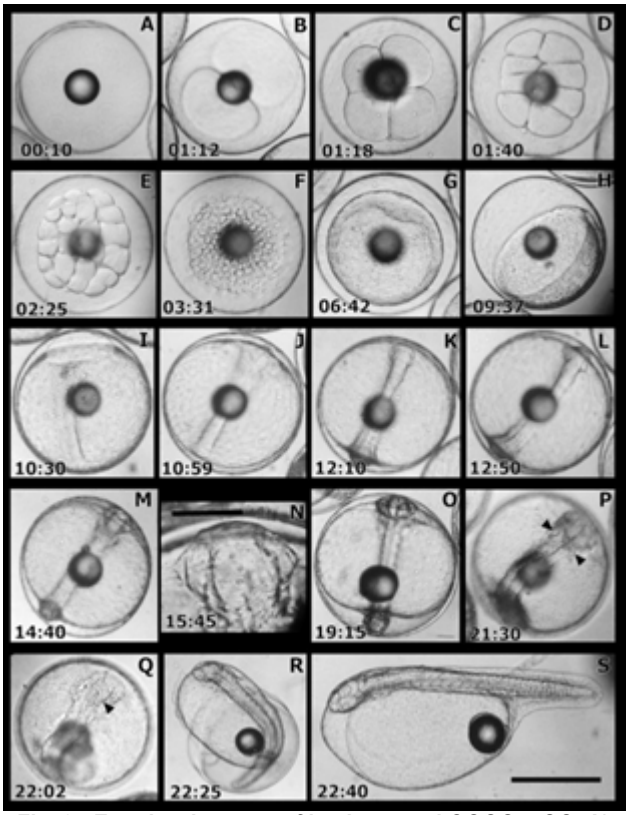

Fig. 3: Egg development of backcrossed OGGG × GG. A) fertilized egg; B) 2-cell stage; C) 4-cell stage; D) 8-cell stage; E) 32-cell stage; F) morula stage; G) gastrula stage; H) embryo formation commenced; I) blastopore nearly closed; J) blastopore completely closed; K) head and 5-myomere formed; L) optic vesicles appeared, Kupffer's vesicle appeared; M) tail separated from yolk sac; N) lens vesicles appeared; O) embryo commenced moving; P) otocyst vesicles appeared; Q) heart formed; R) hatching started; S) hatched larva; number show hours and minutes after fertilization; the scale in N is 0.1 mm while the scale in S is 0.5 mm

Discussion

The egg characteristics and development of backcrossed OGGG and GG are similar to other *Epinephelus* sp. and hybrid groupers. The egg size of backcrossed OGGG and GG was slightly smaller (755 ± 28 µm), compared to other hybrid groupers, including: TGGG at 840 ± 30 µm (Ch'ng and Senoo, 2008); OGTG at 830 ± 20 µm (Koh *et al*., 2008a) and OGGG at 836 ± 10 µm (Koh *et al*., 2008b). However, egg size was found relatively larger than SGTG, at 715 ± 22 µm and CGTG at 738 ± 23 µm (Addin, 2011). The comparison of egg size was crucial to

serve as primary data to indicate egg quality, and later assume its potential survival and growth in subsequent stages (Goncalves *et al*., 2011; Re and Meneses, 2008; Colin *et al*., 1996).

In terms of egg development, a similar pattern was observed between the backcrossed OGGG and GG, and other hybrid groupers, such as TGGG, OGTG and OGGG (Ch'ng and Senoo, 2008; Koh *et al*., 2008a; Koh *et al*., 2008b). However, backcrossed OGGG and GG were observed to have a slightly longer hatching time (22:40 hAF) compared to other hybrid groupers, including: TGGG, at 18:00 to 19:00 hAF (Ch'ng and Senoo, 2008); OGTG, at 17:30 to 19:00 hAF (Koh *et al*., 2008a); OGGG, at 17:15 to 19:20 hAF (Koh *et al*., 2008b); SGTG, at 19:10 to 19:15 hAF; and CGTG, at 19:00 to 19:40 hAF (Addin, 2011). This was because the incubation temperature was slightly lower (27.0 to 28.0 ^{o}C) (Ch'ng and Senoo, 2008; Koh *et al*., 2008a; Koh *et al*., 2008b; Addin, 2011), and thus, causing the hatching time for backcrossed OGGG and GG slightly longer. This data is in agreement with the study reported by Gracia-López *et al*. (2004) and Hart and Purser (1995), whereby hatching time was greatly influenced by the physiochemical properties of water, especially the temperature.

The fertilization rate (23.8 %) and hatching rate (1.5 %) of backcrossed OGGG and GG reported in this study were the lowest compared to other hybrid groupers. The fertilization rates of other hybrid groupers were: 86.8 % for TGGG (Ch'ng and Senoo, 2008); 93.8 % for OGTG (Koh *et al*., 2008a), 91.0 % for OGGG (Koh *et al*., 2008b); 51.0 % for SGTG; and 75.0 % for CGTG (Addin, 2011). Furthermore, the hatching rates were: 87.2 % for TGGG (Ch'ng and Senoo, 2008); 50.3 % for OGTG (Koh *et al*., 2008a); 33.6 % for OGGG (Koh *et al*., 2008b); 38.3 % for SGTG; and 55.4 % for CGTG. Since this was the first attempt to produce offspring by backcross- breeding between OGGG and GG, many factors in controlling and minimizing errors in the egg collection technique need to be improved in order to obtain higher fertilization and hatching rates of this species, as reported by Re and Meneses (2008), and Colin *et al*. (1996). As for the the sperm and the eggs, the quality was assured by both physical observation through naked eyes and also under the compound microscope (Nikon, Eclipse E600). Through these observations, the quality of the fertilized eggs can be determined

The total length of hatched backcrossed OGGG and GG was considered of moderate size. Backcrossed OGGG × GG were slightly smaller compared to TGGG, with 2.00 ± 0.30 mm (Ch'ng and Senoo, 2008), and compared to CGTG, with 1.83 mm (Addin, 2011). However, they were larger than OGTG, with 1.52 ± 0.01 mm (Koh *et al*., 2008a); OGGG, with 1.53 ± 0.01 mm (Koh *et al*., 2008b); and SGTG, with 1.62± 0.00 mm (Addin, 2011). The larger fish that hatched are likely to have advantages in survival and fitness (Hart and Reynolds, 2002).

In the early part of the larval stage, the backcrossed OGGG and GG larvae were undifferentiated from other grouper larvae. No distinct morphological characteristics, such as large yolk sac, head and body shape, were witnessed compared to other hybrid grouper and *Epinephelus* sp. This can be observed in hybrid grouper OGGG (Koh *et al*., 2008b; Glamuzina *et al*., 2001; Kiriyakit *et al*., 2011). The behavior changes of backcrossed OGGG and GG larvae were observed with similar patterns to other hybrid groupers and *Epinephelus* sp.

Conclusion

It is possible to produce backcrossed OGGG and GG; successfully produced larvae in this experiment indicate the first evidence of backcross-breed between OGGG and GG. Egg characteristics were similar to pure and hybrid grouper eggs. This indicates the possibility of production in the future, and further studies should be carried out carried, particularly to enhance the fertilization and hatching rate of this species.

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