



Effect of salinity on embryonic development and hatching of hybrid grouper, *Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*

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Abstract. Groupers are high valued finfish in the Southeast Asian regions. The hybrid grouper *Epinephelus fuscoguttatus* x *E. lanceolatus* (TGGG), is a relatively new hybrid that is in high demand due to its good taste and faster growth compared to tiger grouper *E. fuscoguttatus*. This study establishes and compares the optimum salinity parameters required for successful egg incubation of the two groupers. Eggs were obtained from female *E. fuscoguttatus* through hormone treatment of 500 IU/kg body weight of human chorionic gonadotrophin (hCG) and fertilized with sperm from *E. fuscoguttatus* and *E. lanceolatus*. Eggs were then transferred into incubation aquariums filled with 1 L water at salinities of 15, 20, 25, 30 and 35 ppt. Egg development was observed every hour till hatching occurred. Hatching rates (HR), timing and deformation rate (DR) was recorded. Stages of embryonic development showed no difference in all salinities but differing development speed was observed at certain salinities. Eggs of TGGG hatched faster than tiger grouper at identical salinities. The results indicated that eggs of both groupers had a wide range of salinity tolerance from 25-35 ppt with significantly lower HR and higher DR ($P < 0.05$) at 15 and 20 ppt. The optimal incubation salinity was 30 ppt for TGGG (HR = $70.9 \pm 7.12\%$; DR = $13.3 \pm 2.35\%$) and *E. fuscoguttatus* (HR = $37.3 \pm 6.1\%$; DR = $20.0 \pm 2.9\%$). Occurrence of abnormal larvae significantly increased with suboptimal salinity. The results from this study provide useful information for the successful egg incubation of hybrid TGGG.

Key Words: TGGG hybrid grouper, egg incubation, salinity tolerance, larvae deformation.

Introduction. Groupers are high-value finfish species, belonging to the family Serranidae, which are increasing in importance throughout the Asia-Pacific region, providing a livelihood for small-scale farmers throughout Asia in countries such as Hong Kong, China, Taiwan, Singapore and Malaysia (Johnston & Yeeting 2006). While there are over 159 species worldwide (Heemstra & Randall 1993), the brown marbled grouper, *Epinephelus fuscoguttatus*, orange-spotted grouper, *Epinephelus coioides* and giant grouper *Epinephelus lanceolatus* are three of the most commercially cultured species cultured in Southeast Asia (Moumita et al 2016). Brown marbled grouper, also known as tiger grouper has relatively slow growth rates and is listed as near threatened in the IUCN Red List of Threatened Species (Cornish 2004).

The relative slower growth rates of the tiger grouper resulted in a decline in its popularity and the production of the TGGG hybrid grouper *Epinephelus fuscoguttatus* X *E. lanceolatus* (Senoo 2006; Senoo 2010). First produced at Borneo Marine Research Institute in 2006, the TGGG hybrid showed good taste and faster growth compared to its maternal species, the *E. fuscoguttatus*. As a result, TGGG hybrid grouper has been globally commercialized in Southeast Asia particularly in Hong Kong (Senoo 2010).

However, there is still not much information on the optimal culture of TGGG hybrid. Since its introduction in 2006, only certain aspects such as the egg development and morphology (Ch'ng & Senoo 2008), sexual maturation and gonad development (Luin

et al 2013), genetic markers (Lim et al 2014) and optimal water temperature for juveniles (Moumita et al 2016) have been reported. Information on the optimal egg incubation conditions for TGGG hybrid has been non-existent with most culturist and farmers using to the optimal water conditions for *E. fuscoguttatus* as a reference and guide.

Salinity is one of the most important environmental factors affecting fish hatchery production (Akitas et al 2004). Salinity affects the success of egg incubation and larval rearing of fish (Toledo et al 2004). Unsuitable salinity will lead to poor larvae quality and even hatching of abnormal larvae. However, there is no information in literature on the effect of salinity on the embryonic development and hatching of TGGG hybrid grouper.

The present study examined the effects of salinity (15–35 ppt) on the embryonic development and hatching success hybrid grouper in comparison with *E. fuscoguttatus*. Specifically, the effect of salinity on timing of developmental stages in embryonic phase till hatching, hatching success, and the deformation rates was examined. These experiments will provide basic fundamental knowledge on the optimum salinity needed for successful egg incubation for the hybrid TGGG grouper.

Material and Method. Broodstock were obtained from the hatchery of Pusat Pengeluaran dan Penyelidikan Ikan Laut, Tanjong Demong, located at Besut, Kuala Terengganu, Malaysia. One female *E. fuscoguttatus*, 2 male *E. lanceolatus* and 3 male *E. fuscoguttatus* were used for this study.

Female broodstock of *E. fuscoguttatus* was injected with 500 IU of human chorionic gonadotropin (hCG) per kg body weight. Stripping was done at 36 hours after injection by anesthetizing fish with clove oil. The female broodstock was dried with a towel to avoid of mixing of water and accidental activation of sperm. Hand-stripping method was used and the abdomen of the fish was massaged gently to collect the eggs. The eggs were then weighed and divided into 2 bowls. Milt was collected from males without any prior hormone treatment using a custom built sperm collector. Sperm quality was observed under microscope before fertilization. Dry fertilization method was used; the eggs were mixed with pooled sperm from tiger grouper and giant grouper separately and gently stirred with a feather. Seawater was then added to activate sperm motility and initiate fertilization. After 5 minutes the eggs were then washed thoroughly with seawater and transferred into the experimental aquariums.

Ten g of fertilized eggs were transferred into 20 L incubation aquariums filled with 10 L of seawater at 5 different salinities (15, 20, 25, 30 and 35 ppt). Aeration was provided via single airstone in each aquarium and water temperature, pH and DO was 30-32°C, 7.5 and 5.0-6.0 mg/L respectively. The experiment was conducted in triplicates. Observation of the embryonic development was done by siphoning the eggs from each aquarium onto a petri dish and observing the eggs under a compound microscope for every hour from fertilization. At each observation, 10 eggs from each aquarium were sampled. The stage of development was determined by having at least 50% of the sample. Hatching timing was determined by identifying the time in which at least 50% of the larvae had hatched.

The hatching and deformation rates was conducted by incubating eggs in 2 L aquariums filled with 1 L of seawater at 5 different salinities (15, 20, 25, 30 and 35 ppt). Eggs were stocked at 0.1 g of eggs and determination of hatching rates were done after 100% of the larvae were hatched. The experiment was conducted in triplicates.

The following formulas were used to calculate Hatching rate (HR) and Deformation rate (DR):

$$\text{HR (\%)} = [\text{no. of hatched larvae/no. of egg in aquarium}] \times 100$$
$$\text{DR (\%)} = [\text{no. of deformed larvae/no. of sampled larvae}] \times 100$$

All data were presented as mean \pm standard error of replicate measurements (n = 3). Statistical analyses of data were carried out using SPSS 17.0 software. The differences among salinities were analyzed by using one-way analysis of variance

(ANOVA) while the difference between species was analyzed using t-test. Significance of differences was defined at $p < 0.05$.

Results and Discussion. The effect of salinity on the timing of embryonic development and hatching for *E. fuscoguttatus* and TGGG is shown in Table 1. *E. fuscoguttatus* hatched in 15 hours after fertilization (hAF), with no difference among the salinities. Development of the eggs was also similar at all stages. However, the timing of the embryonic development differed greatly depending on the salinity for TGGG hybrid grouper. The development rate was reflected in the hatching timing, with hatching occurring the fastest in 30 ppt followed by 35 ppt, 20 and 25 ppt and finally in 15 ppt.

Hatching timing was faster in hybrids compared to *E. fuscoguttatus* with 30 ppt hatching beginning at 14 hours and 20 minutes and completion of 100% hatching rate at 16 hours at 30-32°C. This was similar to previous study in which hybrids hatched earlier and finished embryonic development more rapidly compared their parent species (Glamuzina et al 2001). TGGG hatching in this experiment was also faster than previous study by Ch'ng & Senoo (2008) with 18 hAF at 28-30°C which is due to the influence of water temperature (Gracia López et al 2004). Incubation time influences larval size at hatch and also endogenous nutrition (Peterson et al 1977; Gracia-López et al 2004). Grouper larvae are small and fragile with small reserves of endogenous nutrition and a short yolk sac absorption period (Kohno 1998; Ching et al 2012). The increase in larval size and yolk sac would therefore be beneficial towards optimizing larval survival.

Table 1

Time observation on the development of *Epinephelus fuscoguttatus* and TGGG hybrid eggs from spawning to hatching during incubation at different salinities at 30-32°C

Developmental stages	<i>Epinephelus fuscoguttatus</i>					Hybrid grouper				
	Salinity (ppt)									
	15	20	25	30	35	15	20	25	30	35
Cleavage	0:15	0:15	0:15	0:15	0:15	0:15	0:15	0:15	0:15	0:15
Blastula	2:45	2:45	3:02	3:02	2:55	4:00	3:23	02:00	1:30	1:32
Gastrula	5:08	6:08	5:02	5:02	6:07	6:25	5:20	4:40	4:00	4:10
Neurulation	7:08	7:08	7:02	6:45	7:55	12:35	08:30	08:30	6:30	6:40
Organogenesis	9:50	12:08	8:02	7:40	13:55	18:00	12:32	12:32	07:30	07:35
Hatching	15:25	15:20	15:00	15:00	15:05	20:00	15:15	15:15	14:20	14:30

Values represents hours and minutes after fertilization.

Egg development stages were normal in all salinities. Figure 2 shows the embryonic development stages in of TGGG hybrid in 30 ppt. The egg development stages were similar to as previously reported by Ch'ng & Senoo (2008). This showed that the eggs in the study developed normally. Eggs of both *E. fuscoguttatus* ($830 \pm 10 \mu\text{m}$) and TGGG ($830 \pm 10 \mu\text{m}$) were similar to previously reported data of other Epinephelae hybrids, such as TGGG at $840 \pm 30 \mu\text{m}$ (Ch'ng & Senoo 2008); *E. coioides* x *E. fuscoguttatus* at $830 \pm 20 \mu\text{m}$ (Koh et al 2008) and *E. coioides* X *E. lancecolatus* at $836 \pm 10 \mu\text{m}$ (Koh et al 2010).

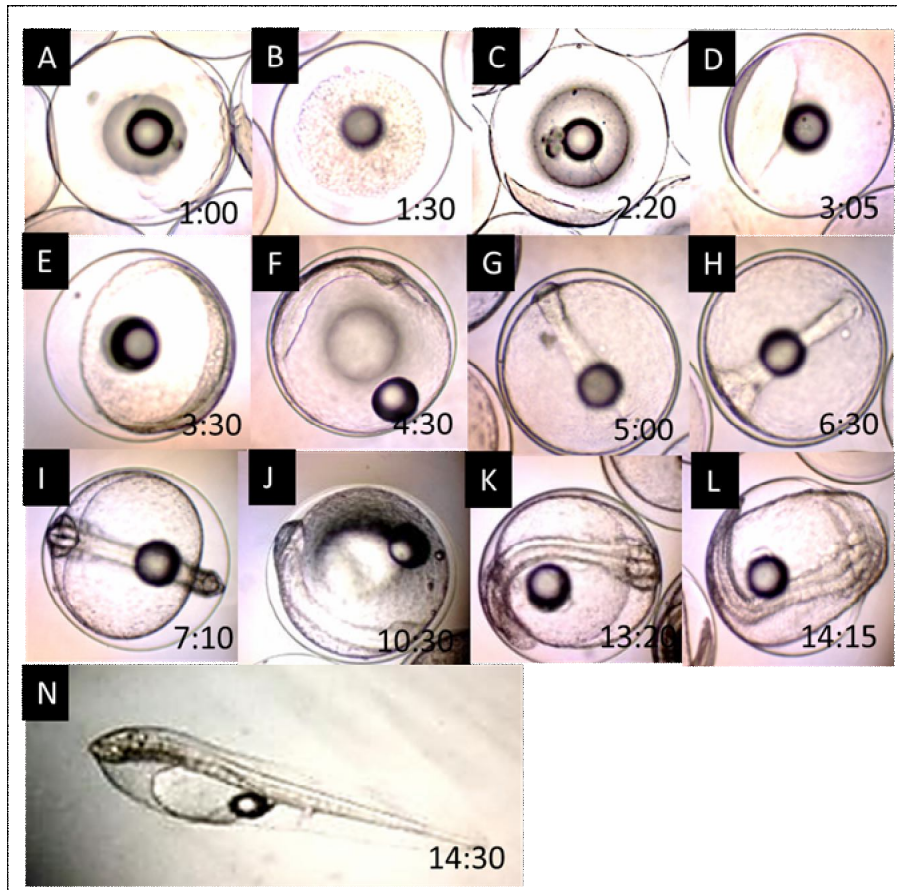


Figure 1. Embryonic development stages from fertilization till hatching at 30 ppt for hybrid grouper (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*) A, 64 cell stage; B, morula stage; C, 10 percent epiboly; D, 40 percent epiboly; E, 80 percent epiboly; F, 90 percent epiboly; G, embryo formation; H head and myomeres formed; I, tail separated from yolk sac; J, Embryo commenced moving; K, heart formed; L, hatching started; M, hatched larvae; number show hours and minutes after fertilization (original).

The results of hatching rates are shown in Figure 2. The highest hatching rate for TGGG hybrid was 30 ppt ($70.9 \pm 7.1\%$) followed by 35 ppt ($52.2 \pm 12.3\%$), 25 ppt ($44.3 \pm 8.0\%$), 20 ppt ($6.9 \pm 1.1\%$) and 15 ppt ($2.8 \pm 0.8\%$). For hatching rates of tiger grouper, highest values were observed in 30 ppt with $37.3 \pm 6.1\%$, followed by 25 ppt ($34.5 \pm 3.0\%$), 35 ppt ($8.7 \pm 1.2\%$), 20 ppt ($6.4 \pm 0.7\%$) and 15 ppt ($5.4 \pm 4.0\%$). Both TGGG hybrid and *E. fuscoguttatus* showed similar patterns with significantly poorer hatching in 15-20 ppt and significantly higher hatching in 25-30 ppt. The hatching rates of hybrids were significantly higher than that of tiger grouper at 30 and 35 ppt. ($p < 0.05$).

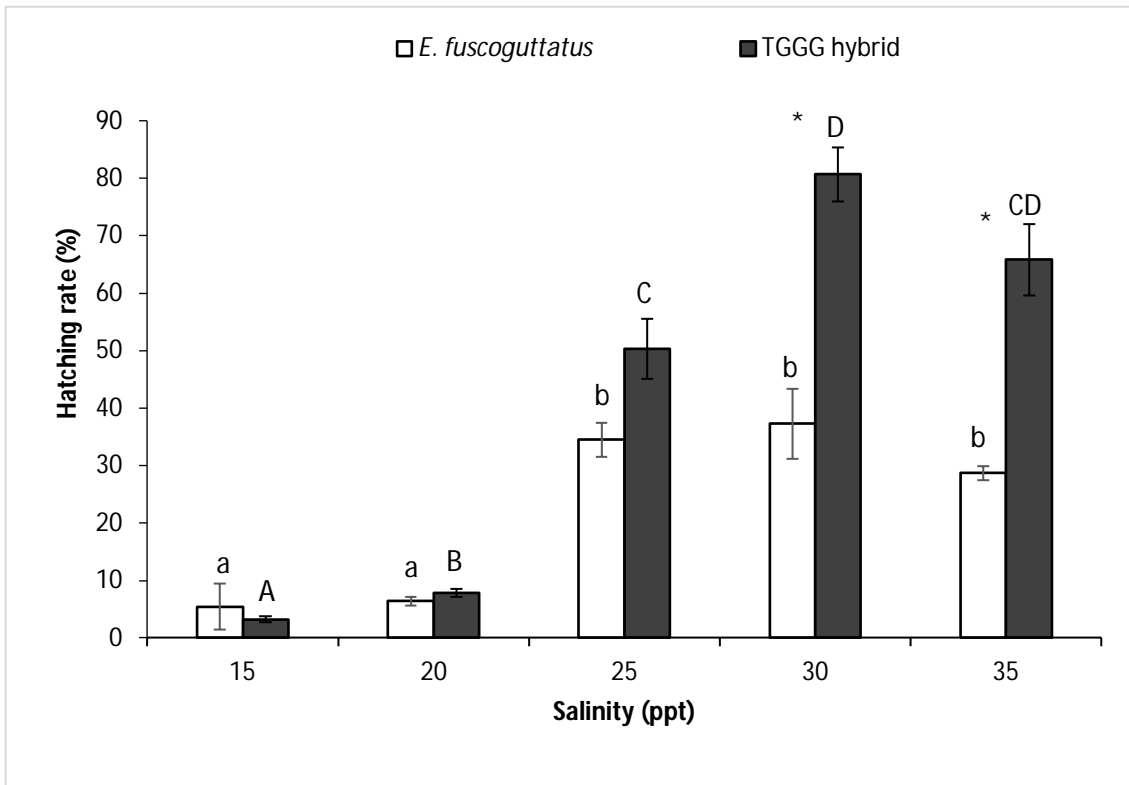


Figure 2. Hatching rates of *Epinephelus fuscoguttatus* and TGGG hybrid at 5 different salinities. Different letters indicate significant differences among salinities of same grouper type ($p < 0.05$). Asterisk denote significant difference among *Epinephelus fuscoguttatus* and TGGG hybrid at identical salinities ($p < 0.05$).

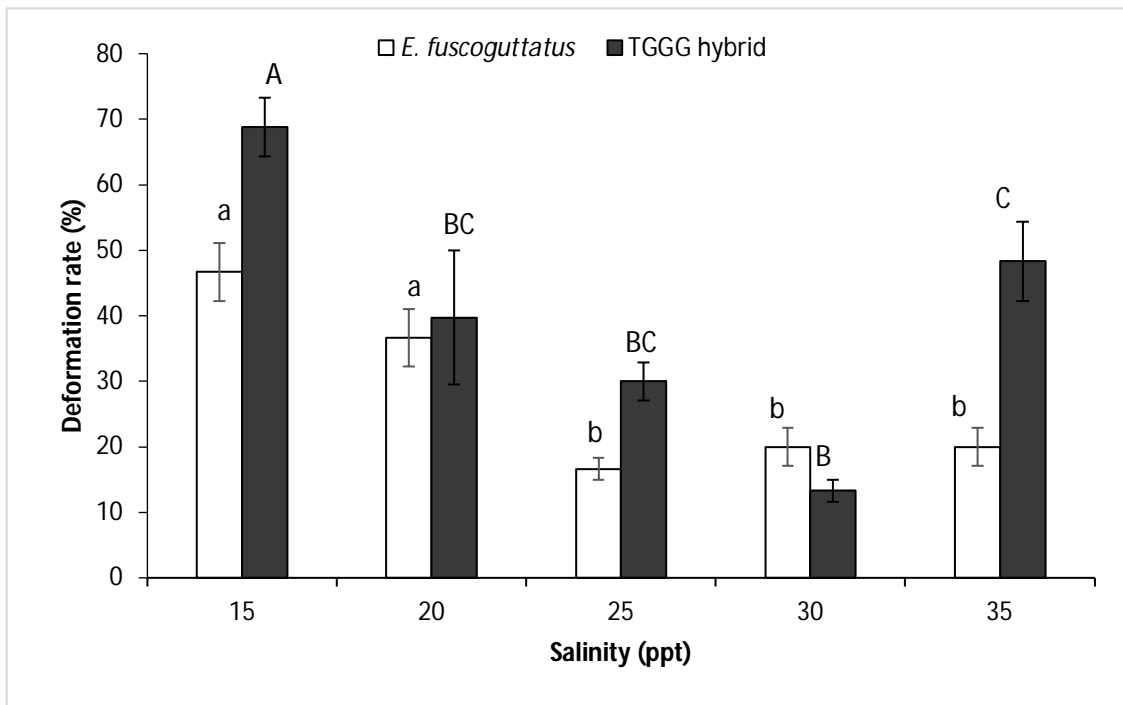


Figure 3. Deformation rates of tiger grouper and hybrid grouper at 5 different salinities. Different letters indicate significant differences among salinities for each grouper type ($p < 0.05$). Asterisk denotes significant difference among hybrid and tiger grouper at identical salinities ($p < 0.05$).

The results of deformation rates are shown in Figure 3. The lowest deformation for TGGG hybrid was found in 30 ppt ($13.3 \pm 2.4\%$), followed by 25 ppt ($30.0 \pm 4.1\%$), 20 ppt ($39.8 \pm 14.4\%$), 35 ppt ($51.7 \pm 8.5\%$) and highest deformation rate in 15 ppt ($68.8 \pm 7.8\%$). 20-30 ppt recorded significantly lower deformation rates compared to other salinities ($p < 0.05$).

The results of this study showed that salinity impacted the hatching timing, hatching rates and larvae deformation in TGGG. The experiment started with eggs in the cleavage stage and showed that they could tolerate the change in salinity and develop well in the salinity ranges of this study (15-30 ppt). This bodes well for transportation at early egg stages followed by transfer into incubation waters with slightly different salinity which will be convenient for farmers. Even if transportation time is lengthy, this would be of no hindrance as eggs are known to have higher tolerance to salinity changes after gastrula stage compared to blastomere (Lee & Menu 1981).

Previous reports on groupers provided varying optimum range of salinity. *Cromileptes altivelis* humpback grouper (34-35 ppt) (Rimmer et al 2004) and *E. fuscoguttatus* (30-32 ppt) (Sugama et al 2012) had very narrow ranges, while *E. coioides* had a wider optimum range (32-42 ppt) (Toledo et al 2004). The optimum salinity for incubation of TGGG eggs in our study was 30 ppt. The highest hatching rates, lowest occurrence of abnormal larvae and also fastest hatching time were all recorded in at 30 ppt which indicated isosmotic condition. At isosmotic condition, energy requirement for osmoregulation is at its lowest (John et al 2012). This allows superfluous energy to be focused on cell division and embryonic development till hatching as water exchanges enters equilibrium.

The abnormal larvae observed had 2 types which were skeletal deformation and stunted larvae which were similar to previous study on *E. coioides* (Rimmer et al 2004). Skeletal deformations are caused by a variety of factors such as swim bladder non-inflation, genetic factors, and also environmental factors and only a small fraction of larvae affected by skeletal malformation survive which causes significant losses to aquaculturists (Andrades et al 1996). Swim bladder inflation occurs at 1-2 days after hatch for groupers, which eliminates the possibility of non-inflation being a factor. Andrades et al (1996) also reported occurrence of deformities before swimbladder inflation and suggested that genetic factor was responsible. Boglione et al (2001) reported that hatchery reared fish had significantly higher skeletal deformities compared to wild caught fish. Occurrence of deformed or abnormal larvae was present in all conditions in our study which agrees with the previous studies. A significant discovery of this study is that sub optimum salinity increased the percentage of abnormal newly hatched larvae. Abnormal larvae per cent were also significantly higher in 35 ppt which had high hatching rates. This might be due to exposure to extreme salinity that resulted in spinal and skeleton curvature (Okamoto et al 2009). Since abnormal larvae were present since hatching, this suggests that the abnormality occurred during embryonic development. Therefore, occurrence of abnormal hatched larvae may be significantly reduced by simple manipulation of water salinity. The result here could be significant to avoid losses for farmers.

Conclusions. The study showed that the egg incubation at 30 ppt optimized the hatching rates and minimized the incubation time and occurrence of abnormal larvae for TGGG. TGGG hybrid also showed stronger salinity tolerance compared to *E. fuscoguttatus*.

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