

Sexual Maturation and Gonad Development in Tiger Grouper (*Epinephelus Fuscoguttatus*) X Giant Grouper (*E. Lanceolatus*) Hybrid

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Abstract

The objective of this study is to determine the possibility of sexual maturation of tiger grouper x giant grouper (TGGG) hybrid. Specimens of TGGG were reared in the hatchery for six years in 150-tonne tanks equipped with a water recirculation system. Observations on maturation were conducted. TGGG (49 specimens) were measured for their total length, standard length, head length, body height, body width, body circumference and body weight, which were 73.97 ± 5.69 cm; 62.09 ± 5.10 cm; 22.87 ± 2.06 cm; 22.84 ± 2.42 cm; 13.98 ± 1.74 cm; 58.94 ± 6.18 cm; 9.88 ± 2.46 kg, respectively. Cannulation method could not be done for 80% of the population for TGGG hybrid grouper. The condition factor of TGGG averaged 2.40 ± 0.21 (n=49). Length-weight relationship of TGGG showed a strong correlation ($P > 0.05$) and the equation obtained were: $\log W = -4.3317 + 2.8453 \log L$. The value of regression coefficient (b) equals to 2.8453 and value of correlation coefficient (r) equals to 0.93. In three specimens two pairs of ovaries and one pair of testis were noticed. The values of gonado-somatic index (GSI) were 0.74, 4.05 (female fish) and 1.38 (male fish). It was determined using histology method that the gonad stage was developing stage (Ovary, GSI=0.74) and mature stage (Ovary, GSI=4.05; Testis, GSI=1.38). The average of oocyte cells in each ovary was 83.0 ± 33.0 μ m (n=26; GSI = 0.74) and 238.5 ± 95.4 μ m (n=11; GSI = 4.05). The results suggested that the hybrid gonads have undergone a course of sexual maturation that has never been reported in the past.

Keywords: Tiger Grouper X Giant Grouper, Hybrid, Gonad, Maturation

Introduction

Pioneering work on hybridization of groupers carried out at Borneo Marine Research Institute where the tiger grouper (*Epinephelus fuscoguttatus*) and giant grouper (*E. lanceolatus*) hybrid (named as TGGG hybrid grouper) was first produced [1]. TGGG hybrid grouper shows good taste and faster growth [2]. Due to these advantages, TGGG hybrid grouper become one of the main target species in aquaculture and has been globally commercialized especially in Hong Kong market [3]. Sexual maturation is important because in artificial production, the fish must be in the advanced stage of sexual development and also sexually mature to ensure successful production [4]. Usually, external appearance of brood fish was used to assess the stage of sexual development [4]. According to [5], for grouper species, the female reached maturation stage when characteristics such as swollen abdomen and reddish colour genital papillae were observed. As for male, elongated genital papillae were clearly seen. Still, sometimes the external appearance of fish are extremely subjective and can be misleading [4]. According to [4], by sampling the eggs and sperm of the fish, it eliminates the guess-work and more accurate in determining the sexual maturation.

Hybrids species are sterile species because normally sterile interspecific hybrids cannot produce viable gametes because of the extra chromosomes cannot make a homologous pair at meiosis, meiosis is disrupted, and viable sperm and eggs are not formed [6]. For example, hybrid between gilthead sea bream *Sparus aurata* x red seabream *Pagrus major* was sterile and this is essential characteristic especially in marine aquaculture to reduce the risk of hybrid spawns with local population, which is an escape from aquaculture [7].

Still, there are certain cases of hybrids that show sexual maturation and fertility such as striped bass, *Morone saxatilis* x white bass, *M. chrysops* [8] and Thai walking catfish, *Clarias macrocephalus* x African catfish, *C. gariiepinus* [9]. For these hybrids, the fish shows full maturation especially for the gonads, able to produce both female and male gamete and spawns naturally. For TGGG hybrid grouper case, research concerning the maturation and fertility was not conducted

yet. Plus, in interspecific hybrids, if the hybrids are fertile, other studies concerning the next generation of the hybrids can be available by conducting intracross, backcross or 3-way cross [10]. Plus, repeated backcross of the hybrids may also be able to another alternative to transport some of the characteristics from one species to another. With this, hybrids that mature in only one of the sexes, it is possible to conduct backcrosses with the parental or maternal species or 3-way crosses with other species. The objective of this study is to understand on maturation and fertility of TGGG hybrid grouper, as it is important for the purpose of hybrid seed production.

Materials and Methods

The experiment was conducted at the Hatchery, Borneo Marine Research Institute, Universiti Malaysia Sabah (UMS Hatchery). The observations on the sexual maturation were carried out between December 2011 and March 2012. Total of 49 tails of TGGG hybrid grouper cultured for six years were examined. Rearing was carried out in a 150-tonne cylindrical fiber-reinforced plastic tank (Diameter: 8 m, Height: 3 m) with a water-circulating system equipped with bio-filtration facilities. The water temperature, salinity, dissolved oxygen (DO), and pH during culture ranged from 28.0 – 29.5°C, 29.0 – 30.0 ppt, 7.0 – 7.8 mg/l and 6.0 – 7.5, respectively.

At the time of collecting the test specimens, the water level in the

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brood fish tank of TGGG was reduced to help in the capture operation and reducing stress on the fish. A large hand net and fish bag was used to transfer the captured TGGG to circular 1 tonne fiber-reinforced plastic tanks, each filled with 500 liters of seawater. TGGG were anaesthetized with transmore (alpha-methylquinoline) (Nika). Sexual maturation for TGGG hybrid grouper was determine according to method from [11,12]. Measurements of total length (TL), standard length (SL), head length (HL), body height (BH), body width (BWd), body circumference (BC) and body weight (BW) were carried out. For further observations, cannulation was also performed on TGGG hybrid grouper to determine the egg and sperm maturation. For this step, cannulation of TGGG hybrid grouper was done by using 1 mm diameter plastic tube catheter that was inserted slowly can carefully into the body. Then, suction was applied to draw out a small amount of eggs or sperm from TGGG hybrid grouper. Absolute condition factor, $K = 100 W/L^3$ was computed, where L is the fish length and W is the fish weight. W equals the weight of the fish in grams and L equals the total length of the fish in centimeters [13]. The length-weight relationship (LWR) was estimated by using the equation: $W=aL^b$ where W=weight (g), L=total length (cm), a=constant, b=growth exponent. A logarithmic transformation was used to make the relationship linear $\log W=\log a + \log bL$ [14]. The association degree between length-weight was calculated by the determination coefficient (r^2). The statistical significance level of r^2 and 95% confidence limits of the parameters a and b were estimated [15]. The slope (regression coefficient) of length-weight regressions was

compared to 3 using student's t-test [15] to determine if TGGG hybrid grouper grew isometrically.

Detailed examination of gonad also required dissection and sacrificing of the TGGG hybrid grouper specimens. For this method, TGGG hybrid grouper with distended and soft belly were anaesthetized following measurements of TL, SL, HL, BWd, BC and BW. They were dissected through an incision in the abdominal region. The specimens had to be sacrificed for taking out internal organs for histology observation. Gonads were removed and measured for length and weight. Gonado somatic index was determined according to the formula:

$$GSI = (\text{gonad weight (kg)}/\text{fish body weight (kg)})/100 \text{ [16,17].}$$

Histology observation was done on the gonads. The gonads was fixed in Bouin's solution for 24 hours, embedded in paraffin and was sectioned at 5 μm . The sections were then stained with hematoxylin and eosin. In determining the gonad stage, the classification was followed by [18]. The gonadal stage was classified as one of the three female phase stages (F1, oocytes at the perinucleolus stage (developing stage) and 30 – 110 μm in diameter; F2, cortical alveolar stage (mature stage) and about 220 μm ; F3, vitellogenic stage (spawning stage) with > 400 μm oocyte diameter), and intersex-transitional stage (I, the presence of spermatogonia and/or spermatocytes in the ovarian tissue), or two male phase (M1, the presence of spermatocytes and spermatids (developing stage); M2, with spermatozoa (mature stage)). For female stages, the amount of each oocyte cells were counted and the average of oocyte diameter were calculated to determine the female gonad stage.

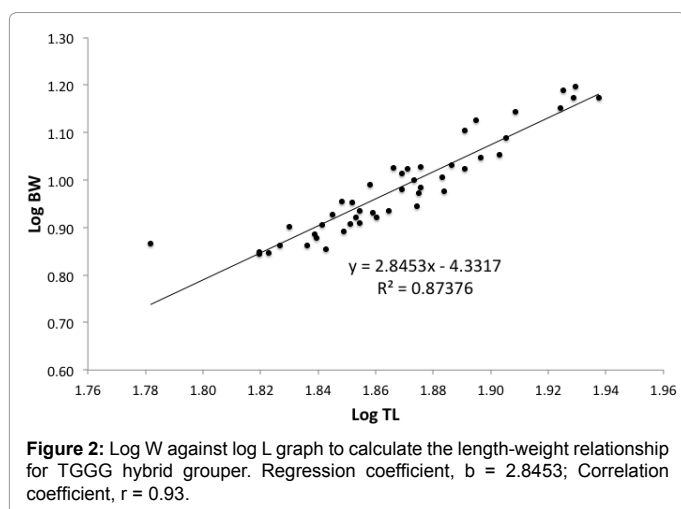
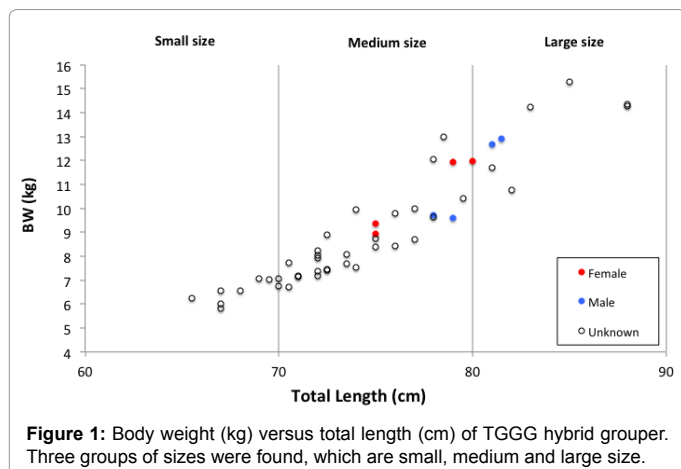
Results

Forty-nine specimens of TGGG hybrid grouper were measured. The average values were: 73.97 ± 5.69 cm (TL); 62.09 ± 5.10 cm (SL); 22.87 ± 2.06 cm (BH); 13.98 ± 1.74 cm (BWd) and 58.94 ± 6.18 cm (BC). As for the body weight (BW), the average was 9.88 ± 2.46 kg.

Out of the 49 specimens examined, 4 were female and 4 were male, which sex of the remaining (41) individuals was undifferentiated, and therefore unknown. Three size groups were found: small, medium and large. The small size group included specimens with body weight and total length in the range of 5-7 kg and 60-10 cm, respectively. The medium size group consisted of specimens with weight and length in the range of 7-12 kg and 70-80 cm, respectively. The large size group comprised specimens measuring 12-16 kg in body weight while 80-90 cm in length. The TGGG females belonged to the medium size group while TGGG males were distributed in both size groups – medium and large (Figure 1). As for cannulation, it was determined that cannulation could not be done for 80% of the population. It was observed that the catheter could not be inserted through the genital opening of TGGG hybrid grouper. (Figure 1)

The condition factor of TGGG averaged 2.40 ± 0.21 (n=49). The length-weight relationship was determined for the entire samples of TGGG hybrid and the equation obtained were: $\log W = -4.3317 + 2.8453 \log L$ (Figure 2). For TGGG hybrid gouper, the regression coefficient value (b) was 2.8453 and correlation coefficient was $r = 0.93$ ($r^2 = 0.87376$). The length-weight relationship showed strong correlation ($P > 0.05$). (Figure 2)

For the dissection, three gonads were found, with two ovaries and one testis respectively. For the first ovary (Ovary 01), the gonad weight was 0.1060 kg and length was 12.5 cm (Figure 3A). As for the second ovary (Ovary 02), the gonad weight was 0.3840 kg and length was 15.5 cm (Figure 3B). For the testis (Testis 01), the gonad weight



Gonad	Body Weight (kg)	Gonad Length (cm)	Gonad Weight (kg)	Gonadosomatic Index (GSI)
Ovary 01	14.26	12.5	0.1060	0.74
Ovary 02	9.46	15.5	0.3840	4.05
Testis 01	10.00	12.0	0.1380	1.38

Table 1: Gonado-somatic index for TGGG hybrid grouper that were found. Body weight (kg), gonad length (cm) and gonad weight (kg) was recorded.

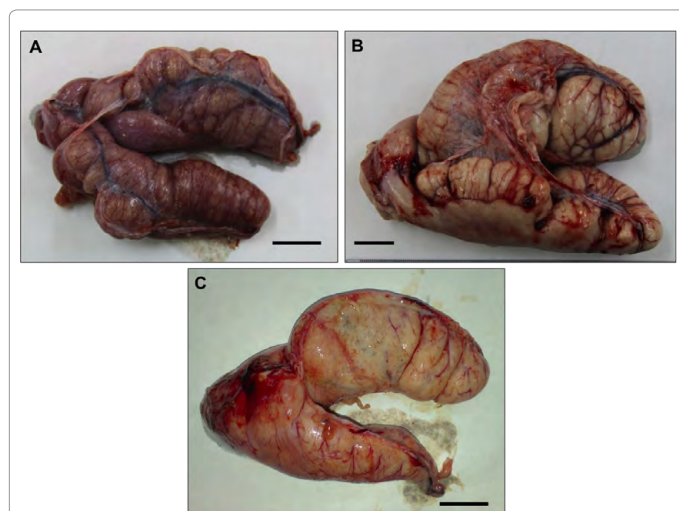


Figure 3: Three gonads were found in three tails TGGG hybrid grouper. Two ovaries and one testis were found respectively. (A) Ovary 01; (B) Ovary 02; (C) Testis 01. Scale = 2 cm.

Gonad	Amount of oocyte cells			Oocyte Diameter (µm)	Gonadal Stage
	Primary oocyte (PO)	Vitellogenic oocyte (VO)	Previtellogenic oocyte (PVO)		
Ovary 01	20	6	-	83.0 ± 33.0 (n=26)	Perinucleolus stage (Developing)
Ovary 02	2	2	7	238.5 ± 95.4 (n=11)	Cortical Alveolar Stage (Mature)

Table 2: Gonadal stage for female TGGG hybrid grouper. Average oocyte diameter was calculated and gonadal stage was determined for each gonad.

0.1380 kg and length was 12.0 cm (Figure 3C). Gonado-somatic index (GSI) values were 0.74 (Ovary 01), 4.05 (Ovary 02) and 1.38 (Testis 01) respectively (Table 1 and Figure 3).

For the histology observation, all three of the oocyte cells, which are primary oocytes (PO), previtellogenic oocyte (PVO) and vitellogenic oocyte (VO) were found for both of the ovaries (Figure 4A, 4B). For Ovary 01, the total amount of PO was 20 and PVO was six. The average of oocyte diameter that was observed in first ovary was 83.0 ± 33.0 µm (n=26) (Table 2). This shows that the gonadal stage for first ovary was perinucleolus stage, which means it is developing stage. As for Ovary 02, the total amount of PO was two; PVO was two and VO was seven (Table 2). The diameter of the oocyte was 238.5 ± 95.4 µm (n=11). This indicates that the gonadal stage of second ovary was cortical alveolar stage, which means it is mature stage. As for Testis 01, spermatozoa were found and it shows that gonadal stage for the testis are in mature stage (Figure 4 and Table 2)

Discussion

In groupers, body weight of female is generally reported to be

smaller than that of the male of the age [19]. It also known that they are protogynous hermaphrodite, which means that the fish first matures as female and some can change sex as the body weight increases [19]. These observations apply for parent (pure) stocks but since TGGG was produced for the first time, there is no published information on it. However, it shares some traits of the parents such as the protogynous hermaphrodite and males growing faster than females. In this study, cannulation method could not be done for 80% of population for TGGG hybrid grouper. In this condition, cannulation was considered difficult for TGGG hybrid grouper. Cannulation requires inserting a catheter through the genital opening to collect egg or sperm [11,12]. The catheter passes through oviduct and when the duct is narrow and immature, forceful application of pressure can lead to its puncture and may cause

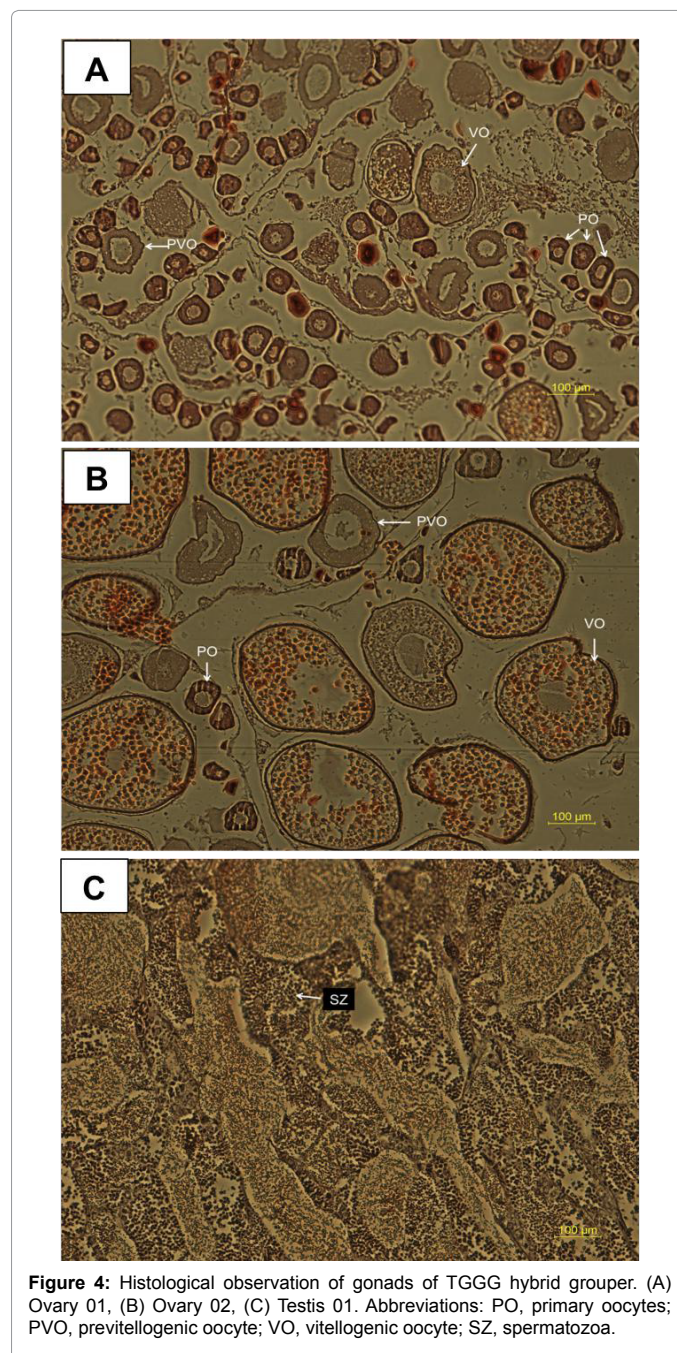


Figure 4: Histological observation of gonads of TGGG hybrid grouper. (A) Ovary 01, (B) Ovary 02, (C) Testis 01. Abbreviations: PO, primary oocytes; PVO, previtellogenic oocyte; VO, vitellogenic oocyte; SZ, spermatozoa.

serious injury to the fish [4]. When that value of regression coefficient $b=3$, indicates that the fish retains the same shape, grows isometrically which means the shape of the fish does not change as the weight and length increases. When value is significantly larger or smaller than 3, it indicates allometric growth. If b is less than 3 ($b<3$), it shows that the fish becomes lighter (negative allometric), if b is greater than 3 ($b>3$), it shows that the fish becomes heavier (positive allometric) for its length as it increase in size [17]. According to [20], value of b depends on the feeding condition, maturing and sex of fish. In this study, regression coefficients (b) of length-weight relationship was $b=2.8453$. Therefore, it is revealed that TGGG hybrid grouper shows negative allometric growth. With this results, suggesting that the progression in growth of the body weight vis-à-vis body length was as expected of a healthy and robust fish.] It was reported that $r = 0.99$ was the highest value linked to the individuals that shows highest weight for a given length [21]. In this current study, the values of correlations coefficient for TGGG hybrid grouper was $r = 0.93$. In conclusion, the relationship between length and weight suggesting a good adjustment in growth for TGGG hybrid grouper.

According to the previous study, the general gonad morphology of fish comprised two hollow-sausage-shaped lobes [22]. These were suspended from the peritoneal wall and joined together posteriorly at the genital pore. There were covered by a thick muscular structure. In contrast with previous study of sexual maturation for hybrid brown trout (*Salmo trutta*) x brook trout (*Salvelinus fontinalis*) [23] the gonads that were found were underdeveloped and deformed. For TGGG hybrid grouper, for the anatomical features, the gonads follow a normal course of development that resembles their parent fish.

Based on [24], there are four stages of sexual maturation for hybrids that are fertility, zygotic sterility, gametic sterility and gonadic sterility. For TGGG hybrid grouper case in this study, it falls under fertility stage that shows gonads are normal in size and structure; gametes are also normal in size structure and finally zygotes are viable [24]. But still, for the current study, only sexual maturation was found and the viability of both oocyte and sperm of TGGG hybrid grouper has not yet been determined. Thus, other studies such as F2 generation [25,26] and backcross experiment [27,28] should be conducted to determine if the oocyte and sperm are viable.

In this study, three tails of TGGG hybrid grouper were dissected and sacrificed in observing the gonad availability. Only three tails of TGGG hybrid grouper were selected to be sacrificed because if by sacrificing the whole population of TGGG hybrid grouper to observe gonad availability, it would considered to be a big loss. These TGGG hybrid grouper is very important and valuable because the current fish that was used for this experiment was the first batch of TGGG hybrid grouper production [1]. Still, other method such as determination of sexual maturation through biopsy (which sacrificing the fish is not necessary) [29] could be done in the future for TGGG hybrid grouper.

Based on the results, for female gonad, all three oocyte cells were found and as for male spermatozoa were found. This indicates that TGGG hybrid grouper shows normal development especially for the cell and gonad. In previous studies, the gonads of roach (*Rutilus rutilus*) x bream (*Abramis brama*) hybrid and gilthead seabream (*Sparus aurata*) x Mediterranean red porgy (*Pagrus pagrus*) hybrid were observed using histology method [30,31]. It can be clearly seen that both of the hybrids gonadal development and gametogenesis progress normally. Still, other studies especially on gonadal development especially on the sex-changing period [29,32] is necessary for TGGG hybrid grouper. With this, female, sex-changing period and male of TGGG hybrid

grouper can be determined. Results of this study suggested that the TGGG hybrid grouper could reach sexual maturation as evident from development of their gonads. This opens up the possibility of using the hybrid progeny to develop F2 generation and performing backcross breeding.

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