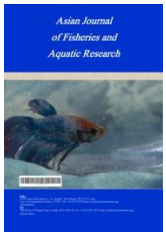

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The Maturity of Female Gonads of G2 Transgenic Mutiara Catfish (*Clarias* sp.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author AP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors IDB and RGH managed the analyses of the study. Author I managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The G2 trangsenic Mutiara catfish (*Clarias* sp.) (MTG) is a Mutiara catfish that is inserted with the CgGH gene (*Clarias gariepinus* Growth Hormone) through the transgenesis. The effect of transgenesis stimulates gonad growth of G2 transgenic Mutiara catfish (*Clarias* sp.) faster than non-transgenic fish. Study aimed to analyze the maturity of the gonads and the spawning ability of female G2 transgenic Mutiara catfish (*Clarias* sp.) to obtain superior broodstock candidates. Experimental method with completely randomized design (pair of parents used as treatment and repeated four times) for spawning was used for this study. Three pairs of parent G2 were crossed semi-artificially as treatment A (female 1 MTG G2 crossed with male 1 MTG G2), B (female 2 MTG G2 crossed with male 2 MTG G2) and C (female 3 MTG G2 crossed with male 3 MTG G2). The results showed that the performance of female G2 transgenic Mutiara catfish (*Clarias* sp.) (treatment A, B and C) was higher given non-transgenic with an average relative fecundity of 82,438 eggs / kg of broodstock, an average egg diameter of 1.76 mm and an average egg weight 1.75 mg. These indications suggest that GH transgenesis increases gonadal maturity. The gonad maturity profile reached the stage of complete maturity (full ripe) compared to Sangkuriang catfish

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(*Clarias gariepinus*) (immature gonads). Therefore it is necessary to compare the reproductive performance of G2 transgenic Mutiara catfish (*Clarias* sp.) with non-transgenic fish (Sangkuriang) as candidates for superior broodstock of catfish.

Keywords: G2 transgenic mutiara catfish; gonad maturity; spawning; transgenesis.

1. INTRODUCTION

Transgenic Mutiara catfish (*Clarias* sp.) is a Mutiara catfish containing the GH (Growth Hormone) gene insertion of Dumbo catfish [1]. The inserted GH gene in Dumbo catfish will be expressed to increase the growth of fish faster than non-transgenic Mutiara catfish so that the broodstock can reach reproductive maturity earlier. Exogenous GH in G1 transgenic Mutiara catfish increases the production of steroid hormones which can stimulate oogenesis and spermatogenesis [2].

Transgenesis is a genetic engineering technique by introducing genes encoding unique characters to provide added value to the target organism [3]. Two gene transfer techniques commonly used in the fish genome are the microinjection method of eggs and electroporation of fish sperm [4]. Electroporation is a suitable gene transfer method for fish, because the transfer system is bulk, considering that large numbers of sperm can be inserted simultaneously with the transgene. The transgene contained in the sperm genome will integrate with the egg genome when fertilization occurs. This allows for gene recombination in the egg embryo genome so that it is hoped that the hatched larvae can express the transgene [5].

The benefits of transgenesis in aquaculture include increasing the growth rate of fish, nutrition, reproduction, increased resistance to cold temperatures and pathogens [6,7]. Transgenic Mutiara catfish has a fast growth compared to non-transgenic Mutiara catfish because Mutiara catfish eggs are fertilized by Mutiara catfish sperm containing GH (Growth Hormone) Dumbo catfish (CgGH) so integrated into the Mutiara catfish genome [1].

Exogenous GH in transgenic fish induces the expression of insulin-like growth factor 1 (IGF-1). Gonadal growth in fish is regulated by IGF-1 which stimulates the production of steroid hormones (estradiol and testosterone) because GH and IGF-1 receptors are bound in gonad cells increasing egg diameter, sperm cell count and larvae production [2].

2. MATERIALS AND METHODS

2.1 Broodstock Maturation

Fifteen pairs of G2 transgenic Mutiara catfish (MTG) broodstock and five pairs of non-transgenic (NTG) catfish were matured for 1 to 1.5 months in four separate fiber basins (one tub of five pairs) (Fig. 1) measuring 1.3 m in diameter and 1 m deep filled water with a height of 50 cm, a heater (model 100w Atman brand) with a temperature of 30ºC is installed. Aeration (model AA-9904 Amara brand) is installed with maximum number to supply oxygen. Broodfish were fed HI-PRO-VITE 781 which was produced by PT Central Proteina Prima Tbk (contains a complete and high quality nutritional composition containing protein (essential and essential amino acids), fat, crude fiber and minerals) and fresh mackerel crumbs.

2.2 Induced Spawning

The treatment of spawning crosses is as follows:

Treatment A: Cross between ♀1_MTG_G2 and ♂1_MTG_G2.
Treatment B: Cross between ♀2_MTG_G2 and ♂2_MTG_G2.
Treatment C: Cross between ♀3_MTG_G2 and ♂3_MTG_G2. (Fig. 2)

The three treatments were repeated four times for parameters of egg diameter, egg weight, fertilization rate (FR), hatching rate (HR) and survival rate (SR).

2.3 Egg Handling and Larvae Rearing

Plastic crates are prepared and filled with water parts, a heater is installed with a temperature of 29-30ºC and install aeration as oxygen supply. Kakaban that have been affixed by eggs (spawned) are put in a plastic box container as a hatchery container. 40 eggs (in each treatment) were taken as a sample to measure their weight and diameter. 300 eggs (for each treatment) were taken and put in a plastic container with a diameter of 22 cm and a height of 14.5 cm as samples to measure FR, HR and SR.

Larval rearing is carried out in plastic containers and plastic jars where the larvae are hatched at a temperature of 28ºC. For two days the larvae are not fed because they still have a yolk sac which is useful as a food reserve for fish larvae. Catfish larvae are fed artemia after two days because it matches their mouth opening. The feeding frequency is 3-5 times so that cannibals do not occur if there is a lack of food, giving artemia to larvae is given until the larva is one week old. The seven day old larvae were fed with tubifex adlibitum twice a day. Tubifex have a protein content of 57%. SR was calculated when the larva was 14-days old.

2.4 Ovary Histology

The preparation of ovarian histological microanatomy preparations was carried out using the paraffin method and HE following the procedures carried out by Wijayanti [8] and carried out at the Central Laboratory of Padjadjaran University including fixation, dehydration, purification, infiltration, embedding, cutting, pasting and staining and shooting.

2.5 Data Analysis

Data were analyzed quantitatively using One Way Anova analysis with the continued test of Duncan's Multiple Range Test (Sigma plot 12) for parameters of egg diameter, egg weight, fertilization rate, hatching rate and survival rate. Ovary histology was analyzed descriptively.

3. RESULTS AND DISCUSSION

3.1 Relative Fecundity

The relative fecundity of female transgenic Mutiara catfish (MTG) G2 treatment A (♀1_MTG_G2 X ♂1_MTG_G2) is 84,622 eggs / kg broodstock, treatment B (♀2_MTG_G2 X ♂2_MTG_G2) is 82,551 items / kg parent and treatment C (♀3_MTG_G2 X ♂3_MTG_G2) of 80,141 eggs / kg parent (Table. 1). This fecundity value is relatively high when compared to sangkuriang catfish, whose fecundity ranges from 40,000 to 60,000 eggs /kg of broodstock [9]. Based on the results of Setyaningrum and Wibowo [10], *Clarias gariepinus* has fecundities ranging from 51,400 to 60,000 grains /kg of broodstock.

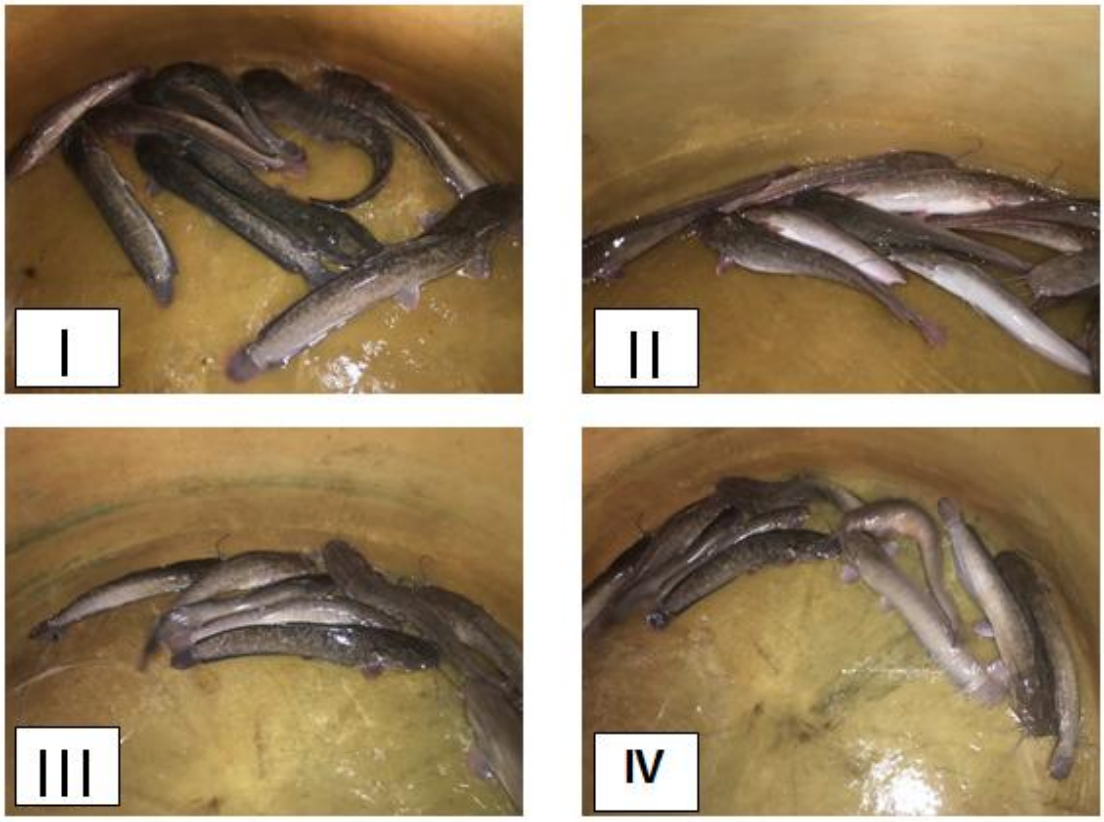

Fig. 1. Broodstock in the Fiber Basins

I : G2 MTG Broodstock A; II : G2 MTG Broodstock B; III : G2 MTG Broodstock C; IV : NTG Broodstock

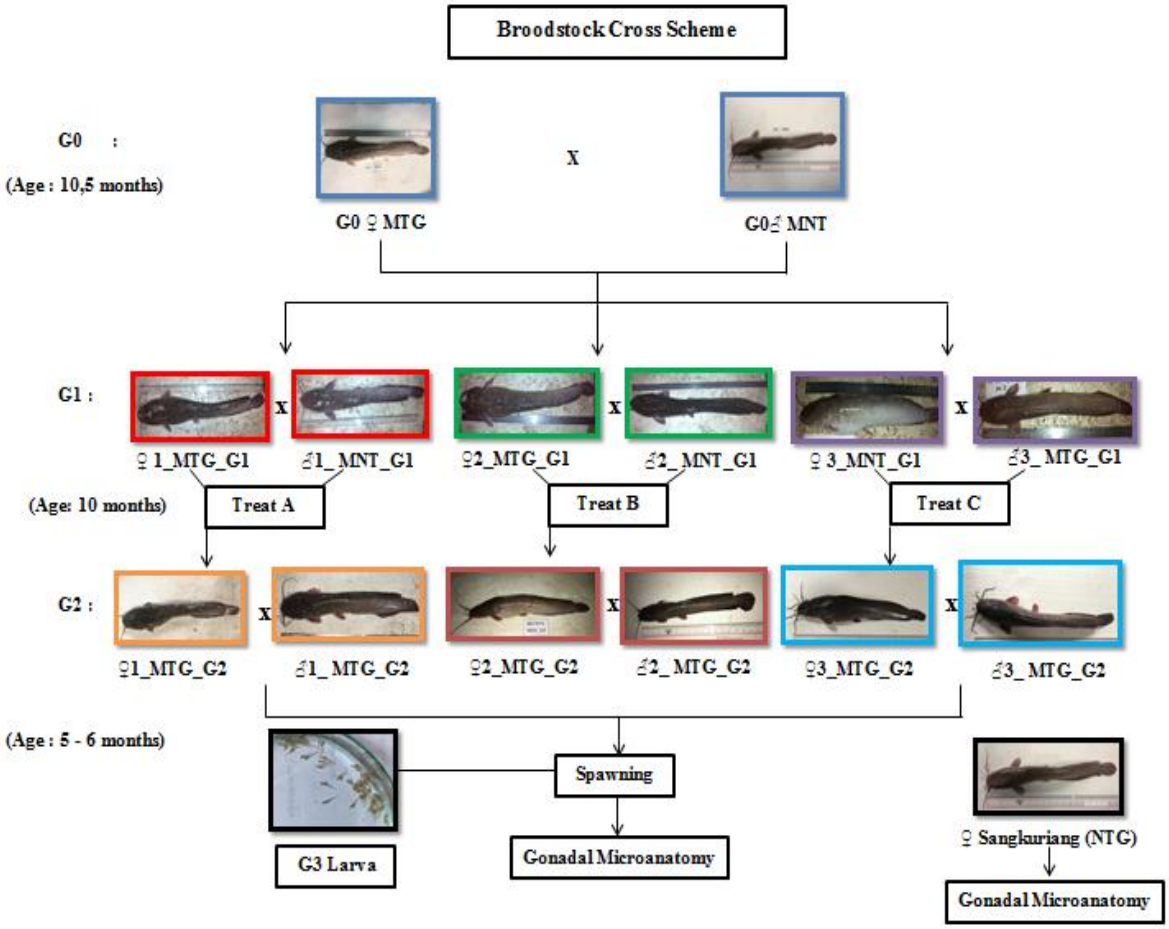

Fig. 2. Broodstock Cross Scheme

♀: Female; ♂ : Male; G_0: founder; G_1 : First Generation; G_2 : Second Generation ; G_3 : Third Generation; MTG : Mutiara Transgenic ; NTG : Non-Transgenic

The high fecundity produced by female MTG G2 catfish is thought to be related to the induction of exogenous GH insertion (CgGH) increasing the number of egg cells [2]. The dumbo catfish GH gene that is inserted will be expressed to increase the growth of fish gonads to mature faster than non-transgenic catfish.

Fecundity of MTG G2 treatment A females was high because the GH gene in transgenic fish promoted oocyte growth and development faster than non-transgenic fish. Haryono [11] stated, the number of eggs produced by the female parent is also influenced by GH, which increases egg fecundity.

3.2 Egg Diameter

The egg diameter produced by MTG G2 females (1.76 mm) was larger than that of non-transgenic females (1.20 mm studied by [12]) (an increase in egg diameter size was 46.7%). According to Iswanto [12], the results of Buwono et al. [2] showed that transgenic female fish had a larger egg diameter than non-transgenic female fish. The high egg diameter size in MTG females can occur due to the contribution of the growth hormone (GH) of transgenic fish, resulting in over-expression of GH and causes the egg diameter to be larger than normal fish. Setyaningrum and Wibowo [10] recorded, non-transgenic *Clarias gariepinus* only has an egg diameter in the range of 0.936-1.033 mm.

The largest average egg diameter was found in female MTG G2 treatment A (♀1_MTG_G2 X ♂1_MTG_G2) which was 1.78 mm, treatment B (♀2_MTG_G2 X ♂2_MTG_G2) was 1.76 mm and treatment C (♀3_MTG_G2 X ♂ 3_MTG_G2) which is 1.73 mm (Fig. 3). There was a significant difference between treatments, but each treatment had a high egg diameter value.

This was due to the Dumbo catfish GH gene in transgenic fish which stimulated an increase in gonadotropin concentrations and triggered an increase in the estrogen production, namely estradiol-17β (E2) and ultimately stimulated ovarian development. Faster than non-transgenic fish. Increased levels of estrogen and vitellogenin will increase the diameter of the oocyte because it is filled with egg yolk mass [13].

Estradiol-17β circulates to the liver, enters the tissue by diffusion and specifically stimulates vitellogenin synthesis. The expression of the hormone CgGH insertion will encourage the vitellogenesis process which stimulates the growth of oocytes. This process is an important aspect of oocyte growth [14].

Transgenesis makes a positive contribution to egg formation so that the egg diameter is larger than normal fish (Fig. 4) This is because the vitellogenin or egg yolk content is more in eggs due to the effect of transgenesis, making larval yolk reserves more than non-transgenic larvae [15], (Loir & Le Gac 1994).

3.3 Egg Weight

The G2 MTG female parent has a large egg weight (Fig. 5), according to Oktanita [16], non-transgenic fish (Sangkuriang) have an egg weight of 1.4 - 1.6 mg. Differences in genetic factors (genes) cause differences in the ability to grow fish. Overexpression of the CgGH gene inserted in transgenic Mutiara catfish affects reproductive performance. The level of gene expression determines GH levels, if overexpressed, it will accelerate the maturity of the gonads or the gonad cells mature more quickly. Exogenous GH was found to affect reproductive performance in several transgenic fish species [17], (Fitzpatrick et al. 2011, Moreau et al. 2011).

The results of statistical tests showed that the weight of eggs in treatment A (♀1_MTG_G2 X ♂1_MTG_G2) which was 1.9 mg have a significant difference with treatment B (♀2_MTG_G2 X ♂2_MTG_G2) which was 1.75 mg and treatment C (♀3_MTG_G2 X ♂3_MTG_G2) which is 1.6 mg. The largest egg weight is found in the female MTG G2 treatment A (♀1_MTG_G2 X ♂1_MTG_G2) which is 1.9 mg. GH-transgenesis in female fish can improve reproductive performance through the effect of FSH (follicle-stimulating hormone) which induces direct and IGF-1 mediated egg proliferation [18]. GH and IGF-1 showed a strong influence in stimulating egg cell production in female broodfish, one of which was increasing egg weight.

3.4 Fertilization Rate (FR)

The FR data diagram for transgenic Mutiara catfish (MTG) G2 (Fig. 6) showing that treatment A had the highest value of 88.35%, followed by treatment B (86.44%) and C (85.58%). The results of Oktanita [16] and Hariz [19] on non-transgenic fish (Sangkuriang), the fertilization rate of transgenic fish is not different from non-transgenic fish. Marnis [20] states that GH transgenesis does not affect the ability of sperm to fertilize an egg, Buwono et al. [2] also stated the same thing in the G1 transgenic Mutiara catfish cross.

The fertilization rate between treatment B and treatment C did not significantly differ, but there was a significant difference between the results of the fertilization rate between treatment A and treatment B and C. This was presumably because there were differences in the maturity level of the parent gonads. However, the results of the hatching rate of MTG G3 catfish eggs are still quite high (an average of 86.3%) because non-transgenic Mutiara catfish only reaches 70% [12].

3.5 Hatching Rate (HR)

The highest hatching rate value is in the results of treatment A (91.18%) followed by treatment B (88.72%) and treatment C (88.52%) (Fig. 7), this value is higher than the hatching degree of catfish. gariepinus used in artificial spawning in Poland ranged from 66.14% -68.50% [21] and in Nigeria, which amounted to 84.15 ± 2.05% [22] and around 60% - 66% [23].

Based on the results of Oktanita's [16] study, the hatching rate of transgenic fish is relatively the same as non-transgenic fish (Sangkuriang). Likewise, the results of Marnis's [20] study showed that the transgenic (PhGH) in transgenic fish did not affect the ability to hatch eggs. The results obtained were not significantly different even though the hatchery rate of transgenic fish was higher than non-transgenic fish. The same thing can be seen in the results of research by Habibullah [24], which shows that transgenic catfish with gene insertion (PhGH) can be inherited in G3 and have a higher hatching rate compared to non-transgenic fish

but it is not very influential (not significantly different).

3.6 Survival Rate (SR)

Each treatment has a high larval survival, treatment A (87.86%), treatment B (86.35%) and C (85.77%) (Fig. 8). The high survival rate in G2 MTG broodstock crosses is one of the advantages of transgenic broodstock. Transgenic fish are thought to have a better immune system and immune system compared to non-transgenic fish. This is consistent with Yada's [25] statement that GH can improve immune system function, including the non-specific immune system, cytotoxic activity, phagocytic, haemolytic, and lysozyme. Trangenic fish have a higher survival rate compared to non-transgenic [20].

3.7 Ovary Histology of Female Transgenic Mutiara Catfish G2

The ovary follicles of transgenic Mutiara catfish in treatment A and B were larger than treatment C (Fig. 9). The maturity level of female gonads (ovaries) of transgenic Mutiara catfish is in the mature category as indicated by the formation of yolk globules, granulosa cells and theca cells (Fig. 9).

The gonad maturity level of treatment A and B female G2 transgenic Mutiara catfish (MTG) was faster than treatment C (Fig. 9). A and B female G2 MTG was categorized in stage III entering the GVBD (Germinal Vesicle Break Down) phase or the fully mature stage (full ripe). GVBD is characterized by the formation of egg yolk granules, granulose and theca cell layers which play a role in the induction of 17α, 20β-dihydroxy-4-Pregnen-3-one (DHP). DHP is stimulated by LH and plays a role in MIH (Hormone-Induced Maturation) [26]. According to Babin et al. [27], LH stimulates granulosa cells in oocytes to produce MIH during the final stages of vitellogenesis until the completion of GVBD.

The gonad maturity of the female parent of C transgenic G2 treatment is in the mature stage of the gonads, although it cannot be said that the maturity is 100% complete (Fig. 9). The yolk globule has been formed and shows the accumulation of a large amount of vitellus (egg yolk precursor), but the granulosa layer has not been formed. However, it has been seen that the formation of theca cell layers has not reached its peak. GVBD process has not occurred, so that It was concluded that the gonad development process of treatment C females began to enter stage III. According to Wallace and Shelman [28], stage III is the oocyte ripening stage. The oocyte moves from the middle position to the edge of the cytoplasm during cooking, then the oocyte nucleus disappears or fuses.

The oocyte size of female fish A and B of transgenic Mutiara (MTG) G2 tended to be larger than that of female C fish, indicated by the increasing number of yolk granules and yolk vesicles (Fig. 9). The histological picture of the ovaries of G2 transgenic fish (females A and B) and females C after spawning showed a mature stage. The mature stage is characterized by thickening of the granulosa cell layer, theca cells, accumulation of yolk granules (yolk granules) in the oocytes [29].

The gonad maturity profile of non-transgenic female broodstock (Fig. 10) is still immature (stage I). Wallace and Shelman [28] described, stage I is developing stage of basic cellular structures including enlargement of the nucleus, formation of nucleoli and subcellular organelles. The yolk globule and yolk vesicle (yolk formation) have not been seen. Theca and granulosa layers are visible which marks the start of egg development and growth. The developing egg oocyte is still in the secondary oocyte stage, characterized by an increase in the diameter of the egg follicle in almost all of the ovary tissue (Fig. 10). Based on Buwono et al. [2] results suggested, transgenic oocyte follicles were observed to be larger than non-transgenic. It can be concluded that, female D (non-transgenic) broodfish is still in the pre-adult period or prospective broodstock stage, and cannot be used as broodfish that are ready to be spawned.

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Table 1. Relative fecundity

Treatment	Relative fecundity (eggs/kg of broodstock)
(A) ♀1_MTG_G2 X ♂1_MTG_G2	84.622
(B) ♀2_MTG_G2 X ♂2_MTG_G2	82.551
(C) ♀3_MTG_G2 X ♂3_MTG_G2	80.141

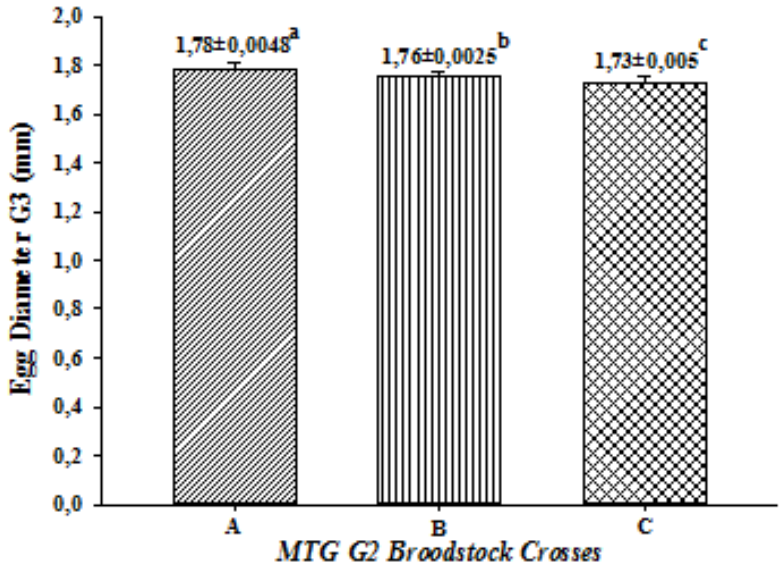

Fig. 3. Egg diameter G3 (mm) graph

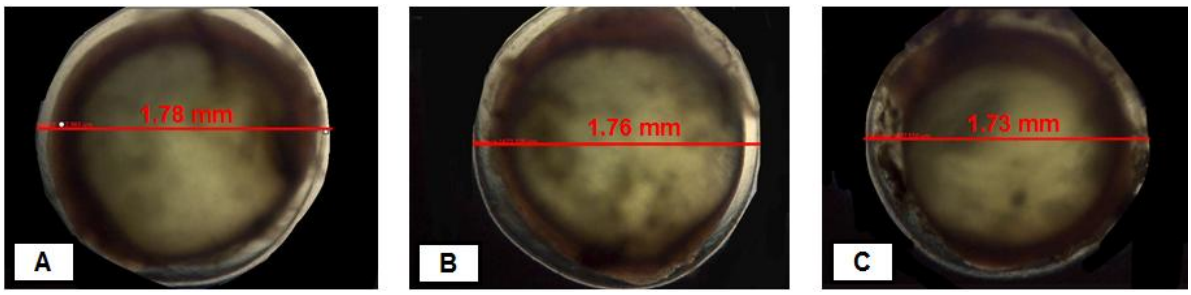

Fig. 4. Transgenic egg diameter
A : Egg Diameter Treatment A; B : Egg Diameter Treatment B; C : Egg Diameter Treatment C

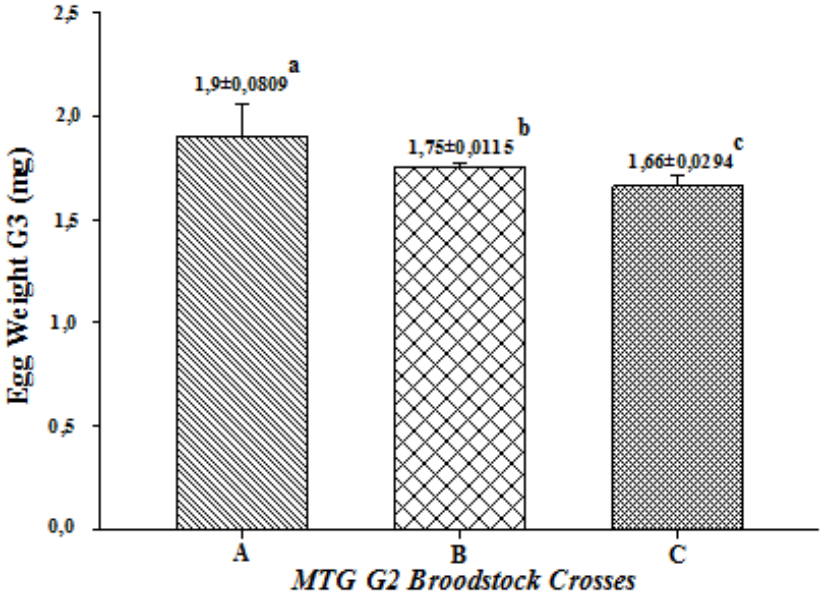

Fig. 5. Egg weight G3 (mg) graph

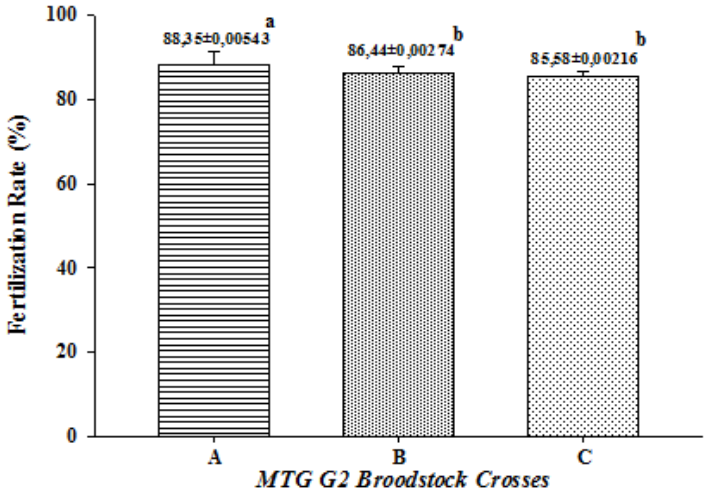

Fig. 6. Fertilization rate graph

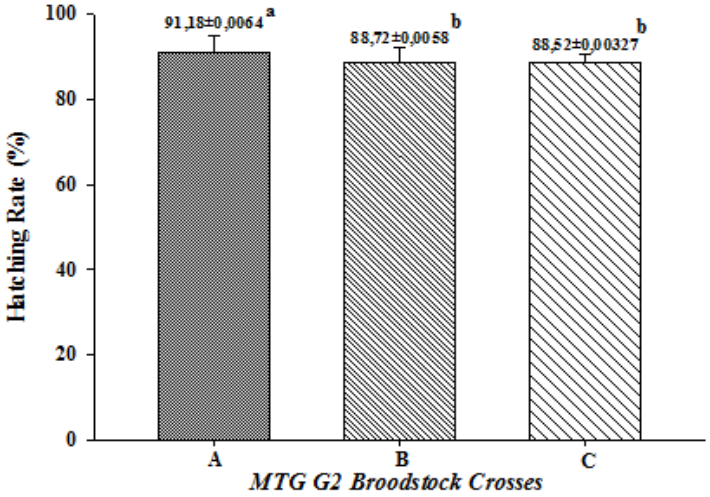

Fig. 7. Hatching rate graph

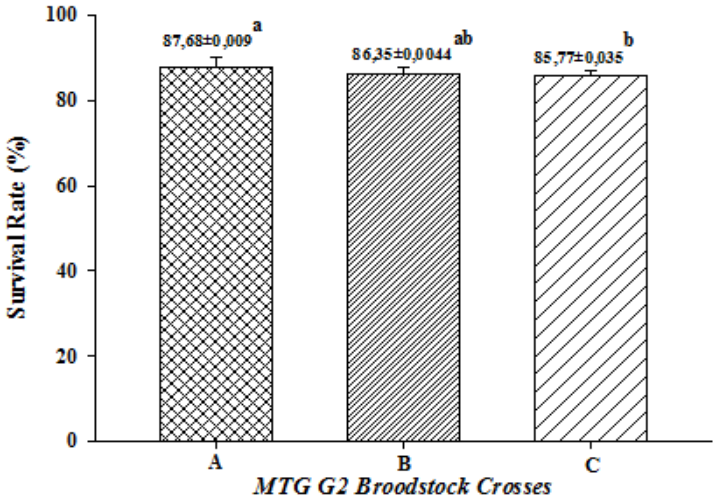

Fig. 8. Survival rate graph

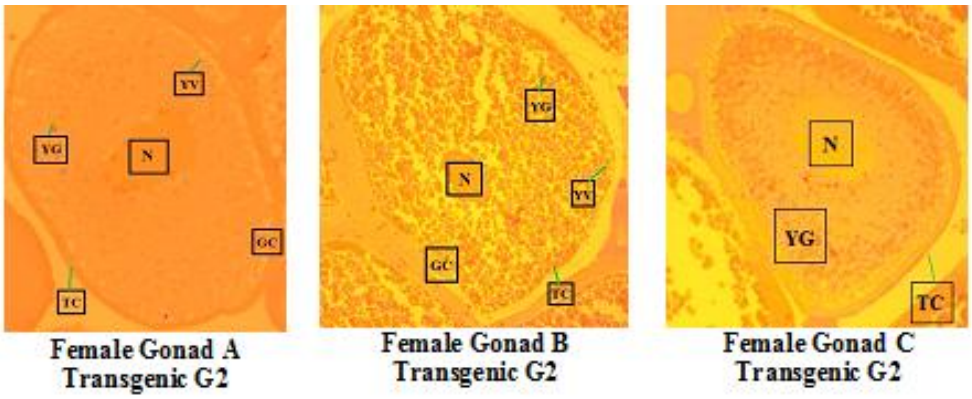

Fig. 9. Ovary histology of MTG G2 catfish
YG : yolk globule; YV : yolk vesicle; N : nucleus; TC : theca cell; GC : granulosa cell.

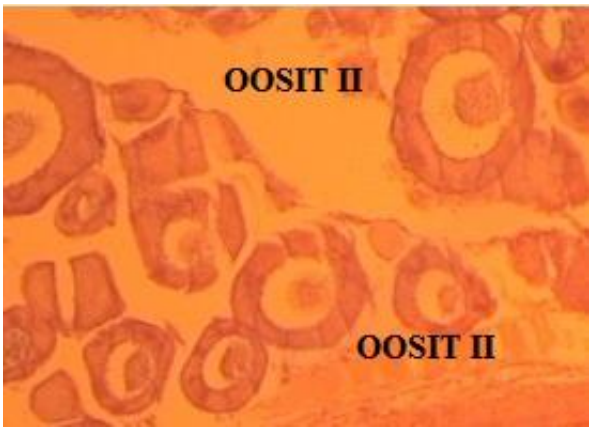

Fig. 10. Ovary histology of non-transgenic

4. CONCLUSION

Transgenic Mutiara catfish can be used as broodstock of superior quality. The results of this study can provide information to breeders or farmers that by selecting parent catfish have perfect gonad maturity can increase seed production which is indicated by high fecundity, hatching rate, and survival rate.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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