Reproduction performance of transgenic Mutiara catfish (G1) comprising the growth hormone gene

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Recent developments in traditional African catfish aquaculture (Dumbo or Sangkuriang strain) in Indonesia show a decrease in terms of growth and reproduction of the catfish. The technology of growth hormone gene transfer (GH-transgenesis) has been applied to tilapia, salmon, and catfish species with three times higher growth observed in transgenic fish than in nontransgenic fish. This technology has proven to resolve slow growth in catfish aquaculture. The present study aimed to evaluate the reproductive performance of G1 transgenic African catfish (Mutiara strain) comprising the Dumbo catfish growth hormone gene (*Cg*GH). The reproductive performance was compared with G1 nontransgenic Mutiara originated from four crosses of broodstock (A, B, C, and D). Egg diameter, spermatozoa amount, fertilization rate, and hatching rate were evaluated. Estradiol (E2), testosterone (T), and IGF-1 gene expression levels and gonadal histology of G1 Mutiara catfish broodfish were measured to determine the effect of GH-transgenesis on gonadal fertility. The results showed that the number of hatched eggs, spermatozoa, IGF-1 expression level, gonad maturity, E2 and T concentrations were higher in G1 transgenic Mutiara catfish than in G1 nontransgenic catfish. These findings suggest that GH-transgenesis Mutiara catfish broodstock resolves the reduced reproductive performance of catfish in conventional spawning considering that a larger number of larvae were produced.

Keywords: G1 Mutiara catfish; GH-transgenesis; gonad fertility.

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Introduction

The Dumbo strain which is the most widely cultured catfish strain in Indonesia descended from the African catfish which was imported from Taiwan in 1985 [1, 2]. Researchers noted recent decreases in the growth rates of the Dumbo catfish in Indonesia. Therefore, breeding studies were conducted from 2010 to 2014 period at Fish Breeding Research Institute, Sukamandi, West Java, Indonesia [3]. Four strains of African catfish (*Clarias gariepinus*) common to Indonesia (Egyptian catfish, Paiton, Sangkuriang, and Dumbo) were selected as the breeding stocks for the hybridization program [4]. The third generation of this breeding program was named the Mutiara strain and was known to have a higher growth rate than the four foundation strains [5].

The Mutiara catfish was developed from intraspecific hybridization. However, the superiority of their growth was not maintained in subsequent generations [6]. Transgenesis in catfish aquaculture is an alternative method to increase the growth of catfish. This method

involves the transfer of foreign genes into the host genome. So that the host fish can integrate the foreign genes in their gametes and transmit them to their offspring [7]. In this study, the proposed transgenic program of Mutiara transgenic catfish focused on transferring and activating the growth hormone (GH) gene from the Dumbo catfish (*Clarias gariepinus* Growth Hormone, *Cg*GH).

The first generation of transgenic catfish offspring expressing the striped catfish (*Pangasianodonhypophthalmus*) GH had an average weight three times higher than that of nontransgenic fish [8]. Comparisons of growth in the Arctic char (*Salvelinus alpinus* L.) between fish comprising the CMVGH1 gene construct to fish with the OnMTGH1 construct showed that the former had 1.7 times higher growth rate. It was later demonstrated that the growth promotion action was stronger by using the CMV virus promoter [9]. Our previous research in the development of the Mutiara transgenic catfish program showed that the average growth of G2 Mutiara catfish comprising the transgene (CMV-*Cg*GH) derived from mating of G1 broodstock was 3–4 times higher than that of nontransgenic fish [10]. These results indicate that the growth phenotype inherited from the G1 Mutiara catfish broodstock was higher than that inherited from nontransgenic fish.

Biologically, the genetic trait of rapid growth in transgenic fish can be transmitted to the next generation through broodstock management designed to maintain superior characteristics. Fertility factors, such as the ability of fish to produce sperm or egg, remain an important key to evaluate the reproduction of transgenic fish. The broodstock reproductive performance determines the success of transgenic broodstock spawning to produce superior fish in each generation [11]. Previously, it was shown that the random mating between GH-transgenic channel catfish and nontransgenic fish maintained in a single pond was able to show normal reproductive patterns [12]. These results showed that the induction of gonadal growth by exogenous GH in transgenic catfish was able to be maintained until the sexual maturity stage, thus supporting the success of fish spawning. The growth of gonads in fish is regulated by growth hormone-mediated insulin-like growth factor 1 (IGF-1) which stimulates steroid production (e.g., estradiol and testosterone) because GH and IGF-1 receptors are bound to gonadal cells [13, 14].

Generally, fish reproductive performance is represented by fecundity parameters: egg diameter, the number of spermatozoa, the degree of fertilization, and degree of hatching [15]. Overexpression of exogenous GH in transgenic fish induces IGF-1 expression, which increases the egg cell diameter and number of sperm cells and, ultimately, increasing larval production [16, 17]. This study focused on investigating whether GH overexpression affects the reproductive performance of G1 transgenic Mutiara catfish.

Materials and Methods

Production of G0 transgenic Mutiara catfish (germline transmitter)

The sperm and egg of Mutiara catfish (*C. gariepinus*) were obtained from a male broodstock (age 1.5 years, body weight 2.2 kg) and a female broodstock (age 1.5 years, body weight 1.85 kg) both originating from Fish Breeding Research Institute, Sukamandi, West Java, Indonesia. This broodstock was produced by the mass selection method. Induction of spermiation and ovulation on Mutiara catfish broodstock was conducted by using Ovaprim, a synthetic hormone product (Syndel Laboratories Ltd., British Columbia, Canada) with the dose of 0.4 ml/kg body weight (BW) for male fish and 0.5 ml/kg BW for female fish in *Clarias gariepinus* [18]. Male and female broodstock stripping was conducted at 12 h post-injection to collect sperm and eggs.

Transgene constructs were created from the pTarget-CMV expression vector (5,670 bp)

(Promega, Madison, WI, USA) and were used as a vehicle for inserting *Cg*GH sequence (GenBank access number: MN249238)(Figure 1) on the site overhangs of T vector (Figure 2) into the pCMV-*Cg*GH (6,285 bp). Site restriction was conducted to retain the copy of *Cg*GH sequence *in vivo* by using EcoRI (Promega, Madison, WI, USA). Electroporation was carried out by mixing 10 µl of sperm and 25 µl of pCMV-*Cg*GH plasmid (concentration 60 ng/µl) into an electroporation cuvette (0.4 cm). The cuvette was inserted into the gene pulser Xcell electroporator (BioRad, Laboratories, Inc., Hercules, California, USA) with the square wave type: voltage 125 V/cm, number of pulses 3, shock period 30 ms with 0.1 s of shock interval [19, 20]. After electroporation, 265 µl of 0.9% NaCl solution was added to the cuvette to maintain sperm motility. The sperm was mixed with eggs that had been stored in a plastic cup under dry conditions by using 5 ml of distilled water. The motility of the sperm was approximately 90% of normal after electroporation. The fertilization rate, hatching rate, and survival rate of larvae were 95%, 87%, and 87%, respectively.

Rearing of G0 Mutiara catfish larvae until broodfish

Larva rearing culture of G0 Mutiara catfish was performed in fiber tanks until the age of 4 weeks. Catfish fingerlings were transferred to fiberglass tank (water volume 2 m^3) until 11 months old (broodfish candidate). A broodstock candidate Mutiara catfish G0 11-month-old was reared in a cylindrical fiberglass tank (2 m x 1.5 m wide) to induce sexual maturity. During maintenance, the water temperature was regulated at a range of 26°C ± 1°C using a water heater, photoperiods was set as 12 h light and 12 h dark, and an aeration system was applied to maintain dissolved oxygen levels. This protocol induced sexually maturity in the broodfish gonad and was estimated to reach the spawning stage approximately 1 month later. A Hi-Pro-Vite 789 artificial feed (Central Proteina Prima Tbk, Sidoarjo, East Java, Indonesia) was used to feed the broodstock at 3% by weight of biomass twice a day.

Screening of G0 transgenic Mutiara broodstock

PCR identification of G0 transgenic broodstock was performed by using caudal fin clips tissues and primers of GH-F (5'-ATGGCTCGAGTTTTG GTGCTGCT-3') and GH-R (5'-CTACAGAGTGCAGT TGGAATCCAGGG-3') with an amplicon of approximately 600 bp [21]. Two female fish from the three matured gonads G0 broodstock comprising exogenous GH (600 bp) was used for the production of G1 transgenic fish.

Spawning of G0 transgenic Mutiara catfish broodstock for G1 production

Maintenance of three G0 broodstock for gonad maturation (two transgenic females and one non transgenic male) was carried out on a round fiberglass tank (1.3 m diameter, 1 m water depth) filled with aerated water. The water temperature range was adjusted to catfish reproduction at 26°C±1°C by using a water heater [22]. Broodstock was given commercial feed Hi-Pro-Vite 781 (Central Proteina Prima Tbk, Sidoarjo, East Java, Indonesia) (33% protein content) as much as 3% by weight of biomass twice a day. The water in the maintenance tank was changed once a week. Semi-artificial spawning induction was performed by using "kakaban," an egg attachment substrate, which was placed at the base of the maintenance tank. The G0 transgenic female and nontransgenic males broodstock injected with Ovaprim hormone (doses of 0.5 ml/kg BW of female and 0.4 ml/kg BW of male) were added to the fiberglass tank.

Handling of eggs from spawning was conducted in an aquarium with a water heater (27°C±1°C) and continuously aerated water. Eggs which are attached to the kakaban at the bottom of the aquarium hatch approximately 18 h after fertilization. The hatched eggs were removed by using a fine sieve into another aquarium that has been prepared beforehand. Thus, these eggs were categorized as G1 larvae. The G1 transgenic Mutiara catfish larvae were maintained like the G0 transgenic fish larvae until reaching the broodfish stage.

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  1 atggccaggg tgctggtgct gctgagcgtg gtggtgggcg agagcgtgtt cttctaccag
 61 ggcgccacct tcgagaccca gaggctgttc aacaacgccg tgatcagggt gcagcacctg
121 caccagctgg ccgccaagat gatggactac ttccaggagg ccctgctgcc cgaggagagg
181 aagcagctga gcaagatctt ccccctgagc ttctgcaaca gcgacagcat cgaggccccc
241 gccggcaagg acgagaccca gaagagcagc gtgctgaagc tgctgcacac cagctacagg
301 ctgatcgaga gctgggagtt ccccagcaag aacctgggca accctacca catcagcgag
361 aagctggcct acctgaagat gggcatcggc gtgctgatcg agggctgcgt ggacggccag
421 accagcctgg acgagaacca cgccttcgcc ccccccttcg aggacttcta ccagaccctg
481 agcgagggca acctgaggaa gagcttcagg ctgctgagct gcttcaagaa ggacatgcac
541 aaggtggaga cctacctgag cgtggccaag tgcaggagga gcctggacag caactgcacc
601 ctggtggagt actga
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Figure 1. Sequence of *Cg*GH (Dumbo catfish growth hormone gene, 615 bp).

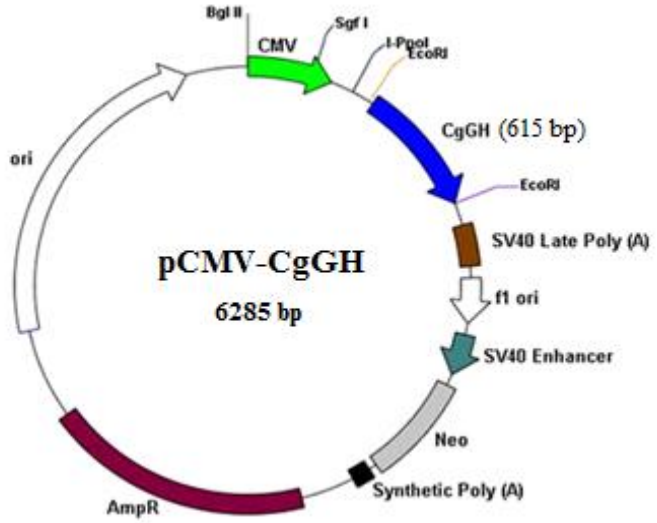

Figure 2. The pCMV-*Cg*GH (6,285 bp) expression vector (designed by using BVTech Plasmid 5.1 (http://www.en.bio-soft.net/plasmid/BVTech).

Screening of G1 transgenic Mutiara catfish broodstock

Seven of G1 broodstock used in this study were derived from the stock of broodstock and were categorized as mature (sexual maturity), which were approximately 1 year old and the weight of 1.0 - 1.6 kg (approximately 12 months or 1 year). Selection of transgenic broodstock was conducted by using RT-PCR method. Template RNA was derived from caudal fish fin (20 mg), which was extracted using High Pure RNA Tissue Kit (Roche, Mannheim, Germany) according to manufacturer's protocol. The cDNA synthesis was performed using MyTaq One-Step RT-PCR Kit (Bioline, London, UK) and *C. gariepinus* GH-F and GH-R primers were used for cDNA amplification [21]. The β-actin gene was used as the control with the primers of βAct-F (5'-ACC GGAGTCCATCACAATACCAGT-3') and βAct-R (5'-GAGCTGCGTGTTGCCCCTGAC-3') [23]. The PCR program was set as follows: 95°C 5 min; 30 cycles of 95°C 30 s, 54°C 30 s, 72°C 1 min; and 72°C 7 min. The amplification product was separated by electrophoresis method on 1% agarose gel. Transgenic positive broodstock was identified by the formation of DNA bands of 600 bp.

Spawning of G1 transgenic Mutiara catfish broodstock

The age of female and male broodstock of transgenic Mutiara catfish and nontransgenic fish was 1 year old. The size of the male and female of G1 fish used for spawning ranged from 1.0 to 1.5 kg (approximately 12 months or 1 year), which was categorized as mature (sexual maturity). Spawning of G1 nontransgenic and transgenic Mutiara catfish broodfish was carried out after the G1 broodfish were found to carry the *Cg*GH gene (600 bp). The spawning procedure of G1 (Table 1) was carried out by following the G0 broodfish spawning procedure.

Table 1. The crossing of G1 broodstock to produce the second generations (G2).

Crossing of G1	Mating of G1 broodstock pairs
A	female-1 transgenic x male-1 nontransgenic
B	female-2 transgenic x male-2 nontransgenic
C	female-3 nontransgenic x male-3 transgenic
D	female-4 control nontransgenic x male-4 control nontransgenic

Reproductive performance of G1 transgenic Mutiara catfish

Egg diameter of each cross of G1 broodstock was calculated after spawning (n=10) by measurements using a microscope equipped with a micrometer (Olympus, Tokyo, Japan). Measurement of egg diameter used the formula of the size of the egg diameter seen under the microscope (µm) x 10 (ocular lens magnification) x 2.5 (objective lens magnification). The relative fecundity of the female broodstock was calculated based on the equation of Legendre [24].

$$RF = (W_0 – W_t) / MW \times EG$$

Where RF is the relative fecundity (%), EG is the egg grains, MW is the mean weight of egg sample (g). W_0 and W_t are the initial and the final weight of female broodstock before and after spawning (g), respectively.

To calculate the number of spermatozoa of G1 male broodstock crossed, 1 µl of fish semen was diluted with 200 µl 0.9% NaCl solution. The number of sperm was counted with the aid of hemocytometer under a microscope with 1,000x magnification. Observation of the degree of fertilization was conducted 20 h after spawning by observing eggs on the spawning substrate (n=300-400 eggs, four replications). The eggs were transferred to the aquarium comprising 60 L finely aerated water with a temperature of 27°C±1°C. Hatching was observed 26 h postspawning (n=300–400 eggs, four replications).

Gonads histology of G1 broodstock

Female (two transgenics, one control nontransgenic) and male (one transgenic and one control nontransgenic) gonads of G1 broodstock were removed surgically and then fixed with the Bouin solution for 12 h. Samples were further processed with paraffin and applied stain with the HE (Hematoxylin Ehrlich-Eosin) staining methods. The gonad tissue was sliced at the thickness of 0.5 µm and soaked in Bouin solution for 12 h. The process of dehydration, clearance, infiltration, planting, cutting, attachment, and staining of HE follows the method described by Schulz [25] and Campbell [26].

Estradiol and testosterone levels of G1 broodstock

The levels of serum estradiol (E2) and testosterone (T) were measured using Enzyme-linked immunosorbent assay (ELISA) method and performed on the G1 broodfish (two transgenics female, one control nontransgenic female, one transgenic male, and one control nontransgenic male) before gonadal surgery. A total of 2 ml of fish blood from each sample was taken from the base of the caudal fin after spawning. Samples were then centrifuged at 3,000 rpm for 20 min. Blood serum was taken and stored at –20°C until analysis. Hormone levels were then analyzed using Fish Estradiol ELISA kit and Testosterone ELISA kit (Bioassay Technology Laboratory, Shanghai, China) following the manufacturer's protocols.

Analysis of IGF-1 mRNA expression in G1 broodstock liver and gonad organs

IGF-1 mRNA expression analysis was performed on liver and gonad tissues of fish by measuring IGF-1: actin expression ratio semi-quantitatively. Expressions were analyzed with the help of Image J software (version 1.33) (National Institutes of Health (NIH), Bethesda, Maryland, USA). A total of 10 mg of liver and gonads from each sample was isolated from the post spawning broodstock and the total RNA was extracted by using High Pure RNA Tissue Kit (Roche, Mannheim, Germany). The expression analysis of IGF-1 gene used the primers designed from exon 1 and exon 2 of fish IGF-1 gene from channel catfish (GenBank access number: NM_001200295.1) with the sequences of IGF-F (5'-CAGTACATCTCGAGTCGTTTCAGC-3') and IGF-R (5'-GGACACGCTGCAGTTTGTGTG-3') while β-actin primers (βAct-F and βAct-R) were used as an internal control. The synthesis of cDNAs of IGF-1 and β-actin was performed by using My Taq One-Step RT-PCR Mix (Bioline, London, UK) with the following program: 48°C for 20 min; pre-denaturation at 95°C for 1 min; 40 cycles of amplification consisted of 95°C 10s, 60°C 30s, and 72°C 30s; and 72°C 5 min.

Statistical analysis

The research design used a completely randomized design with four types of G1 crosses (n=4) to evaluate reproductive performance. Reproductive parameters included egg diameter, relative fecundity, number of spermatozoa, amount of spermatozoa/fish weight, degree of fertilization, and degree of hatching. One-way ANOVA with $p < 0.05$ with Duncan's multiple range test was used to detect significant differences in reproductive performance between transgenic fish and nontransgenic fish between treatments. IGF-1: actin expression ratio, estradiol and testosterone levels, and histology of female and male gonads of transgenic and nontransgenic G1 broodstock were analyzed with comparative description.

Results

Detection of GH exogen from the genome of G0 Mutiara catfish

Utilizing the GH-F and GH-R primers, the PCR analysis result showed that three G0 Mutiara catfish contained the GH exogenous (*Cg*GH, 600 bp), which was confirmed from the DNA sample of the fin from each sample. In addition, the primers also amplified the DNA sequence of the endogen GH Mutiara catfish and resulted in the amplicon of 1,100 bp, which acted as an internal positive control of the PCR reaction (Figure 3).

Identification of G0 transgenic broodstock

Based on PCR analysis of the G0 broodstock, two transgenic females (comprising *Cg*GH) and one nontransgenic male fish were identified (Figure 4). Therefore, the G0 broodstock pairs can be spawned to produce transgenic G1 fish.

Screening of G1 transgenic broodstock

Transgenic positive broodstock was characterized by the presence of *Cg*GH mRNA expression in fins that were the same size as plasmid inserts (600 bp). The verification results for G1 transgenic were conducted before spawning the broodstock and were documented in our previous publication [10].

Reproduction performance of G1 transgenic broodstock

The analysis of reproductive performance on G1 transgenic broodstock is shown in Table 2. The results showed that transgenic female fish had greater egg diameter compared to nontransgenic female fish ($p < 0.05$), but lower fecundity. Meanwhile, the observation of male fish showed that the transgenic fish had a higher sperm amount:weight ratio compared to nontransgenic fish. In addition, the male transgenic fish had higher number of spermatozoa ($p < 0.05$). Post spawning, it was observed that the degree of fertilization and hatching of transgenic fish were higher than that in the control nontransgenic fish ($p < 0.05$).

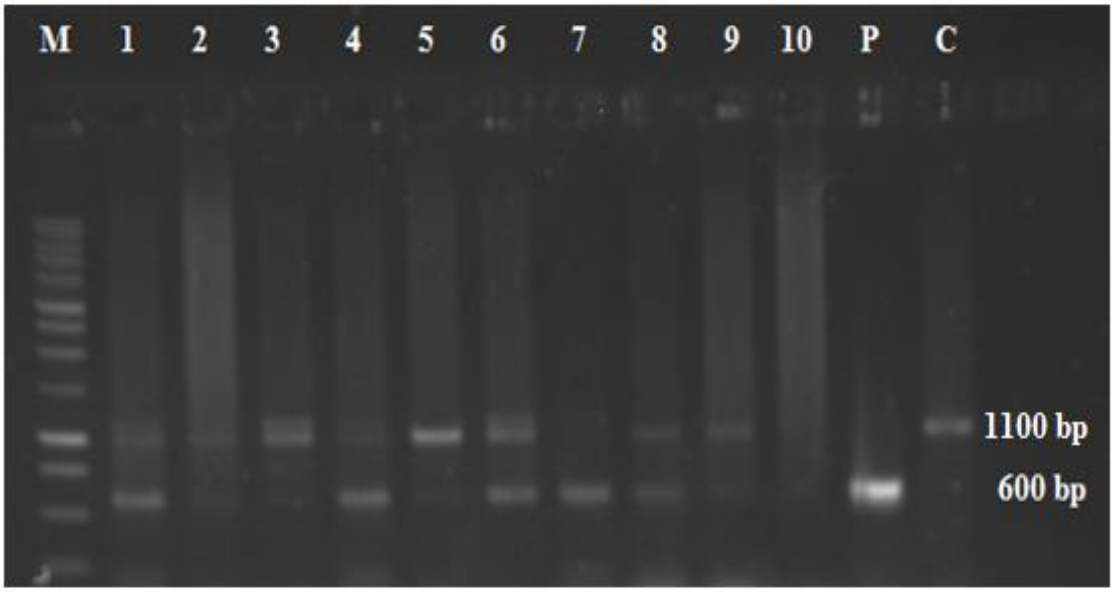

Figure 3. Detection of *Cg*GH transgene in the genome of the Mutiara transgenic G1 catfish (lane number 1, 6, 8), 1 kb DNA ladder (lane M), Mutiara nontransgenic fish (lane number 2, 3, 5, 9), Mutiara transgenic fish (lane 4, 7), Control (lane 10. PCR buffer without DNA template), pCMV-*Cg*GH plasmid (lane P), Control nontransgenic catfish (lane C).

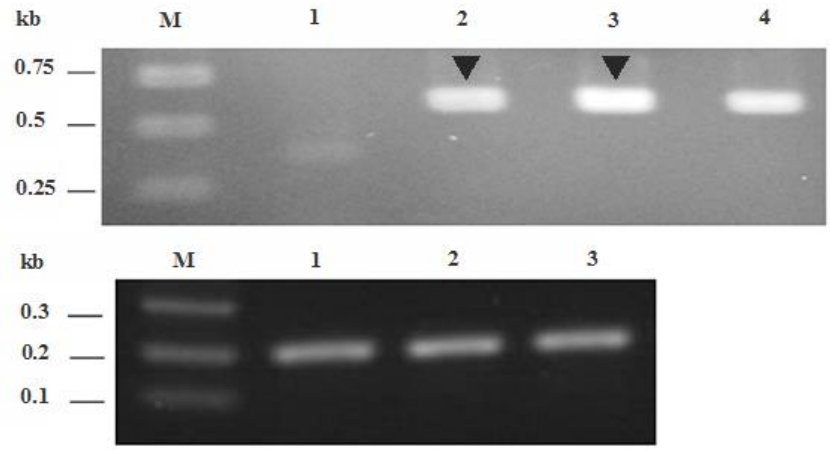

Figure 4. Electropherogram result of the G0 transgenic broodstock identification by PCR method using GH-F and GH-R primers (marked with an arrowhead, above) and β actin (below) as an internal DNA control (lane 1: male, lane 2 and 3: female, lane 4: pCMV-*Cg*GH plasmid, lane M: 1 kb DNA ladder.

The results of male and female gonad measurements and the distribution of male sperm were shown in Figures 5 and 6. The length of the fish gonads was relatively similar, but the gonads of transgenic female fish had relatively larger volumes compared to the nontransgenic females.

Estradiol and testosterone levels of G1 Mutiara catfish broodstock

Based on the measurements of the estradiol and testosterone level, female transgenic fish had higher estradiol levels while transgenic male fish showed higher testosterone levels compared to nontransgenic fish (Table 3).

Table 2. Reproduction performance of transgenic broodstock compared to control non-transgenic fish.

Fish	Parameter	G1 broodstock crosses			
		A	B	C	D (Control)
Female	Egg diameter (mm)	1.82±0.03[a]	1.74±0.01[a]	1.44±0.07[b]	1.45±0.04[b]
	Relative fecundity (eggs.fish/kg)	54,645	45,952	53,334	66,839
Male	Spermatozoa count ($\times 10^6$ sel/ml semen)	6.89±0.13[b]	6.82±0.20[b]	8.60±0.30[a]	5.46±0.34[c]
	Spermatozoa count per weight of fish ($\times 10^6$ sel/ml semen)	6.890	6.044	9.772	6.554
Post-spawning	Fertilization rate (%)	79.36±1.70[b]	76.84±0.54[b]	86.79±1.02[a]	77.16±0.56[b]
	Hatching rate (%)	77.6 ± 072[b]	74.09±1.09[c]	85.67±1.50[a]	70.01±0.57[d]

Note: Data represented as means ± SEM. Means followed by different letters indicated significant difference.

Table 3. Estradiol and testosterone concentrations in the G1 broodfish serum.

Broodstock of G1	Estradiol (ng/L)	Testosterone (nmol/L)
Female A transgenic	102.83 ± 12.34[a]	n.d
Female B transgenic	78.17 ± 0.96[a]	n.d
Female non-transgenic (control)	50.98 ± 5.41[b]	n.d
Male C transgenic	n.d	11.25 ± 0.41[a]
Male non-transgenic	n.d	6.32 ± 0.11[b]

Note: Data represented as means ± SEM. Means followed by different letters indicated significant difference. n.d = not detected.

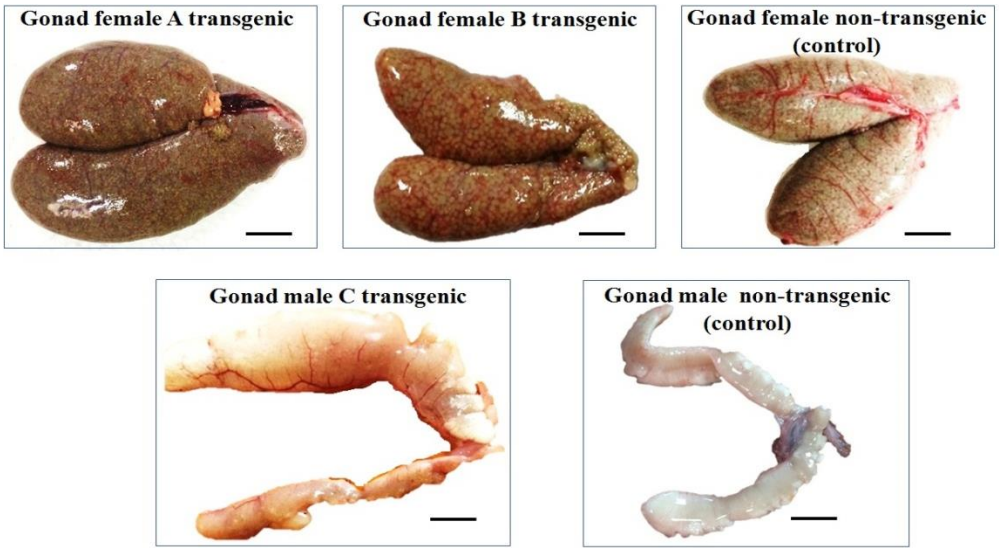

Figure 5. Female (above) and male (below) gonads of the G1 transgenic and non-transgenic catfish. The scale line represents 2 cm.

Ovary and testis histology of G1 Mutiara catfish broodstock

The comparison of G1 nontransgenic and transgenic Mutiara catfish is shown in Figure 6. Transgenic oocyte follicles were observed to be larger than that of nontransgenic. Additionally, transgenic male sperm tended to have higher cell numbers than that of nontransgenic fish.

IGF-1 expression of G1 nontransgenic and transgenic Mutiara catfish broodfish

The results of IGF-1 amplification in the liver

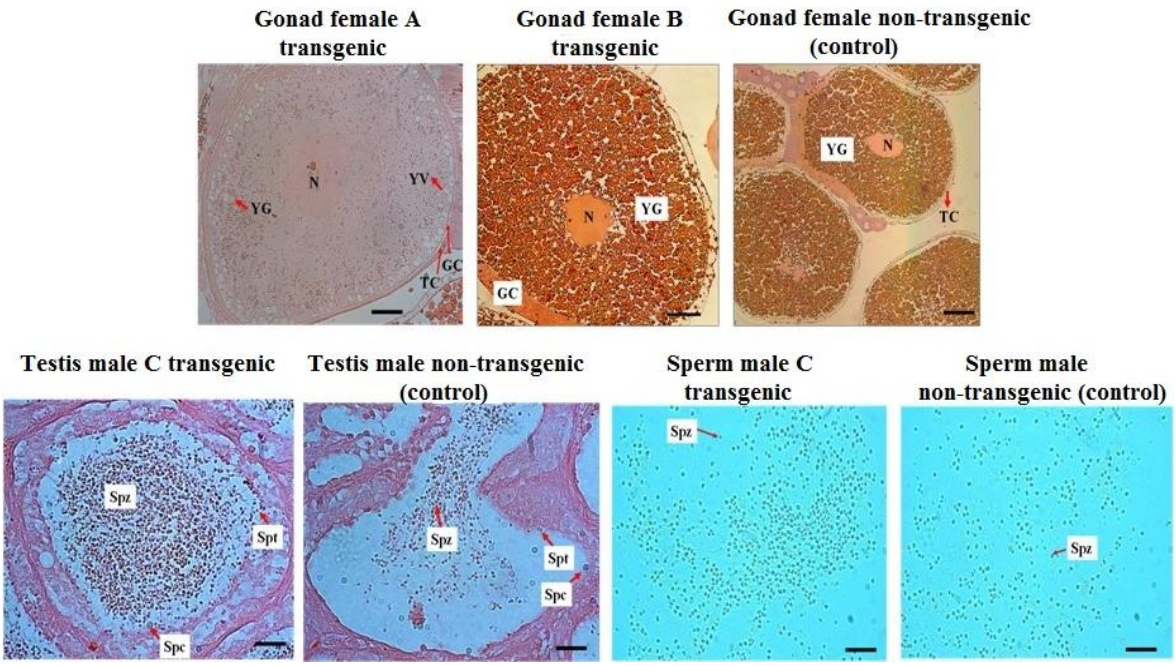

Figure 6. Female (above) and male (below) gonadal histology of the G1 transgenic and nontransgenic catfish, and sperm cells of transgenic and nontransgenic fish postspawning. The scale line represents 2 cm for female gonad and 1 mm for testes and sperm of male fish. YG: yolk globule. YV: yolk vesicle. N: nucleus. TC: theca cell. GC: granulosa cell. Spz: spermatozoa. Spt: spermatid. Spc: spermatocyte.

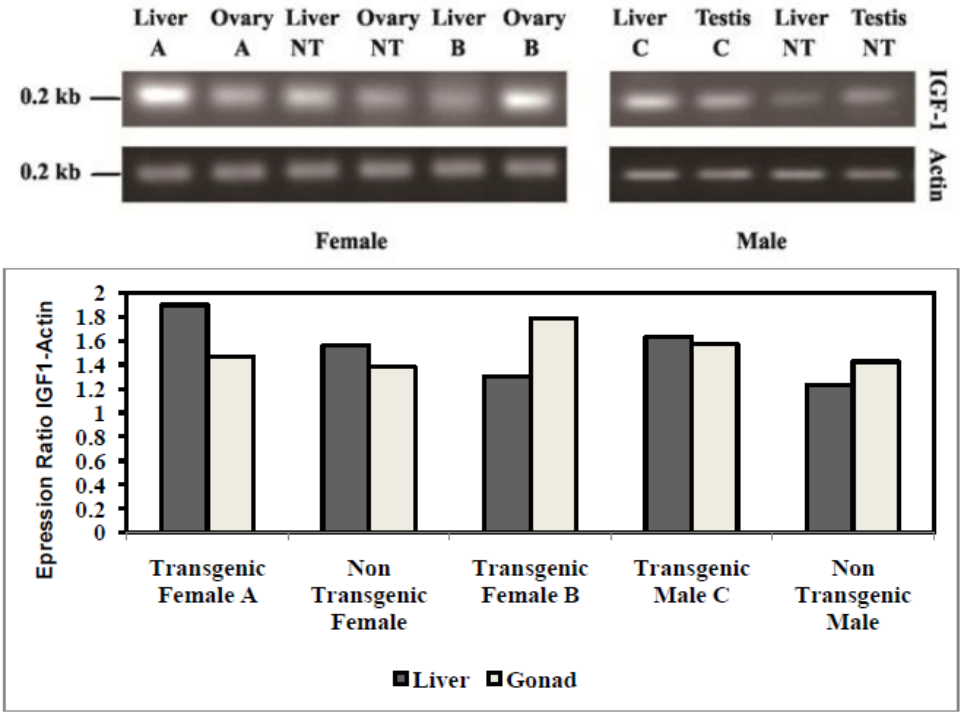

Figure 7. The expression level of IGF-1 mRNA in liver and gonad of GH transgenic (A, B, and C) and nontransgenic (NT) fish post-spawning.

and fish gonad are shown in Figure 7. The results showed the presence of gene expression from all tissue samples. However, IGF-1 gene expression varied in liver and gonad organs.

IGF-1 expression level was generally found to be higher in the liver compared to that in the fish gonads. Expressions in both transgenic and nontransgenic female liver were different, whereas IGF-1 expression was higher in transgenic male than nontransgenic male. Similarly, in gonads, IGF-1 expression in male and female transgenic fish were higher than that in nontransgenic fish.

Discussion

The sequence alignment analysis of the GH Dumbo and Mutiara sequence using the BioEdit (version 7.0) showed a sequence consensus of 53.3%, which confirmed that the catfishes were of different strains. According to the BlastX analysis (https://www.ncbi.nlm.nih.gov/), the sequence alignment of the Dumbo GH and the GH gene of the *C. gariepinus* (GenBank access number: ABN58481.1) showed 97% similarity. However, Mutiara GH sequence and the GH gene of the *C. gariepinus* showed 65% similarity, which indicated that the Dumbo and Mutiara GH sequences were heterologous.

The results of the identification of G0 transgenic Mutiara catfish with PCR showed that two female fishes contained the Dumbo catfish GH gene (*Cg*GH) in the three fishes examined (Figure 4). The research on medaka fish showed that two female broodfishes of transgenic medaka (*Oryzias latipes*) comprising CMV-H2B-GFP (transgenes), which had been identified as G0 (germ-cell transmitter) after crossing with nontransgenic males, produced 15% G1 transgenic [27]. Another research showed that the sperm electroporation could produce 30% G0 transgenic rainbow trout comprising CMV-IgG-cecropin P1 transgenes [28]. Based on these results, the breeding of two female broods of G0 transgenic Mutiara catfish spawned with nontransgenic male broodfish could produce the inheritance of the rapid growth phenotype and G1 transgenic fish production. The transgenic-GH had an advantage in the broodstock selection since it was bigger in size and reached gonadal maturity earlier [29]. The gonadal fertility of transgenic female broodfish remains as an important key in maintaining G1 transgenic offspring production [30, 31].

Screening from seven fishes by PCR showed two female fishes and one male fish positively carrying *Cg*GH (600 bp). Therefore, those fishes were used for four broodstock crosses of G1 transgenic Mutiara catfish. These results indicated that 42.85% (3/7) of the G1 broodstock comprising *Cg*GH were available to be used for future production of transgenic Mutiara catfish. The spawning success of G1 transgenic and nontransgenic Mutiara catfishes shown in Table 1 indicated that GH-transgenesis in G1 transgenic female could trigger fish spawning activity. The overexpression of GH in transgenesis can stimulate broodfish sexual maturity because GH works on gonadal tissue to stimulate ovarian steroid synthesis and spermatogenesis [13, 32].

Overexpression of GH in fish can increase IGF-1 expression. This overexpression leads to an increase in oocyte growth, which leads to an increase in egg diameter [33, 34]. Reproductive performance of G1 transgenic and nontransgenic Mutiara catfish broodstock showed differences from the parameters of the average egg diameter, spermatozoa amount, degree of fertilization, and degree of hatching at each cross (Table 3). The average egg diameter of G1 transgenic female from cross A (1.82 mm) was not different from B (1.74 mm) but was larger than that in nontransgenic fish of cross C (1.44 mm) and nontransgenic control of cross D (1.45 mm) ($p < 0.05$). The diameter increases of these transgenic fish eggs were possible influenced by IGF-1-mediated GH overexpression, which stimulated the growth of oocyte follicles [35, 36]. The average diameter of catfish eggs of normal *C. gariepinus* was 1.46 mm

[37], which was similar to the egg diameter size in the control nontransgenic Mutiara catfish (1.45 mm, cross D) and nontransgenic Mutiara catfish (1.44 mm, cross C). Egg diameters of transgenic Mutiara catfish (A and B cross) increased by 37% and 29% respectively compared to Mutiara catfish of nontransgenic control and tended to be greater than C (Figure 6). However, the larger eggs impacted the relative fecundity of G1 transgenic female broodfish of A and B crosses, which were lower than nontransgenic control of cross C (Table 3).
The increase in sperm amount of G1 transgenic male catfish showed that the role of exogenous GH (*Cg*GH) could stimulate sperm cell proliferation, as described by Miura, *et al.* [32] and also as presented in Table 2 and Figure 6. GH-transgenesis in male fish can enhance reproduction performance through direct follicle stimulating hormone (FSH) effect, which induces sperm cell proliferation mediated by IGF-1 [38]. The GH and IGF-1 showed a strong influence in stimulating the production of Sertoli cell in male broodfish, which indicated that GH played an important role in increasing sperm amount. Based on the broodfish weight (Table 2), it was shown that the sperm amount of G1 transgenic Mutiara catfish (A crosses) was higher than that of nontransgenic fish (crosses C and D).

The difference in egg fertilization rates from G1 broodstock crossing was caused by differences in the number of spermatozoa in male broodfish. The number of spermatozoa of C crossing transgenic fish (8.6 x 10^6 cells) was higher than that of nontransgenic control of D crossing (5.46 x 10^6 cells) and A crossing (6.89 x 10^6 cells) or B (6.82 x 10^6 cells) (Table 2). We showed that the average fertilization rate of the eggs produced by the G1 transgenic female from A crossing did not show a difference compared to transgenic female from B crossing or nontransgenic female from C and control nontransgenic female from D ($p > 0.05$). These results indicated that the overall degree of fertilization of eggs produced by the G1 transgenic Mutiara catfish was significantly different to G1 nontransgenic fish.

The hatching rates of eggs produced from crossing male (85.67%, cross C) or female A (77.61%) and B (74.09%) of G1 transgenic Mutiara catfish were higher than that of the crossing of G1 nontransgenic Mutiara catfish (70.01%, cross D) ($p < 0.05$). The average degree of hatching from the spawning of African catfish (*C. gariepinus*) using Ovaprim ranged from 71.58% to 71.94% [15], which was similar to spawning G1 nontransgenic Mutiara catfish but lower than G1 transgenic Mutiara catfish. This result showed that GH-transgenesis was able to increase the degree of hatched eggs and possibly increased the production of catfish larvae.

In general, IGF-1 expression in female and male broodfish of G1 Mutiara catfish glands in the tissues of liver and gonads was higher than G1 female and male nontransgenic (Figures 6 and 7). The increase of the expression of female or males G1 transgenic associated with overexpression of GH (exogenous GH insertion) in the liver and gonads promote gonadal steroid production, which can eventually stimulate the oogenesis and spermatogenesis [34, 39]. In female gonads of G1 transgenic, IGF-1-induced oocyte follicle growth led to an increase in egg diameter (Table 3) as demonstrated in eel (*Anguila australis*) [16]. Similarly, IGF-1 in the male gonads of G1 transgenic G1-induced mitosis (proliferation) of Sertoli cells, spermatocytes, spermatogonia caused an increase in the number of spermatozoa (Table3), as was reported in tilapia [33, 40]. IGF-1 expression levels in G1 transgenic female were higher than that of G1 nontransgenic, which caused the levels of estradiol (E2) in females A (102.83 ng/ml) and B (78.17 ng/ml) higher than that of G1 nontransgenic (50.98 ng/ml) as shown in Table3. In the ovary of tilapia, the GH (GHr) receptor was located on the immature oocyte, theca cell layer, and granulose cell surrounded the oocyte [41]. The mRNA GHr levels in immature oocytes were known to be higher than that in mature oocytes. The GH receptor is located near the IGF-1 receptor in the ovary, which indicates that GH directly affects the IGF-1 ovary synthesis [42-44]. This explanation

reinforces that overexpression of GH in female transgenic (transgenic female A and B, Figure 6 and 7) caused the level of IGF-1 expression in liver and ovary (A, B) higher than that in the nontransgenic. This finding showed that the GH/gonad-IGF-1 axis was involved in reproduction [44]. The effect resulted the GH gene expression in the pituitary and caused the increased expression of IGF-1 in liver and ovary as shown in transgenic female A and B. The IGF-1 receptor (IGF-R) located in ovary indicated potential function of IGF-1 in the gonad of female fish [39, 45, 46]. The interaction of IGF-1 with the steroid E2 was also shown in the ovary of pre-ovulated Coho salmon, where the IGF-1 induced the granulosa cells to increase the steroid production (E2, DHP) [13]. The increased steroid production was also shown in the results that E2 in female transgenic A and B was higher than that in nontransgenic fish (Table 3), which was caused by the GH overexpression and increased expression level of IGF-1 in liver and ovary (Figure 7).

These findings suggested that GH overexpression by mediating IGF-1 indirectly affected E2 steroid production, the same as the investigation of *Fundulus heteroclitus* [34]. This study indicates the important role of IGF-1 in the fish reproductive system. The IGF-1 increases the secretion of gonadotropin (GTH) that stimulates steroidogenesis of red seabream (*Pagrus major*) granulosa cells [47]. The effect of GH in male catfish (*C. batrachus*) increased the testosterone level produced by the Leydig cell *in vitro*. The steroidogenic action of GH was mediated by the IGF-1 in the testis [48]. GH induced IGF-1, which was produced by liver and stimulated the testis to produce testosterone. According to the reference, it was known that the overexpression of GH in the male Mutiara transgenic catfish caused the expression level of IGF-1 in the testis of C higher than that in nontransgenic fish (NT testis) (Figure 7), and the testosterone production of the male transgenic (male C transgenic) was higher than that of nontransgenic fish (Table 3). Our result also confirmed that the previous study where the increase of IGF-1 expression in G1 transgenic male Mutiara catfish male broodfish caused testosterone (T) levels of G1 transgenic male (12.63 nmol/l) increase higher than it in G1 nontransgenic fish (7.91 nmol/l, in Table 3). The rise of GH levels could increase IGF-1 expression levels in tilapia Leydig cells. Thus, the production of T also augmented [49, 50].

Overall, the results of this study indicated that the reproductive performance of G1 transgenic Mutiara catfish comprising Dumbo *Cg*GH was more effective compared to nontransgenic fish. The production of gametes and catfish larvae from the G1 broodfish spawning of GH-transgenesis could be increased. This method can be used as an improvement from conventional spawning for catfish aquaculture purposes.

Conclusion

GH-transgenesis has a positive effect on the reproduction of G1 transgenic Mutiara catfish and can increase gonadal fertility. The effects on the female broodfish were increased egg size and increased the production of E2, while the male broodfish can increase the number of spermatozoa and production of T. Additionally, GH-transgenesis also increases the fertilization and hatching rates as well as larval production.

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References

1. Yusuf D. 1995. Aquaculture in Indonesia. In Towards sustainable aquaculture in Southeast Asia and Japan: Proceedings of the Seminar-Workshop on Aquaculture Development in Southeast Asia, Iloilo City, Philippines, July 26-28, 1994 (pp. 109-115). Aquaculture Department, Southeast Asian Fisheries Development Center.
2. Iswanto B. 2013. Tracing the identity of Dumbo catfish. Media Akuakultur, 8(2):85-96.
3. Iswanto B, Suprapto R, Marnis H, Imron. 2016. Reproductive performance of Mutiara catfish (*Clarias gariepinus*). Media Akuakultur, 11(1):1–9.
4. Indonesian Fish Breeding Research Centre. 2014. Technical instructions for Mutiara catfish cultivation. Sukamandi, 55p.
5. Dewi SRRPS, Iswanto B, Insan I. 2016. Productivity and profitability of selection of catfish (*Clarias gariepinus*) aquaculture in maintenance in soil ponds. Media Akuakultur, 11(1):11–17.
6. Scribner KT, Page KS, Bartron, ML. 2000. Hybridization in freshwater fishes: a review of case studies and cytonuclear methods of biological inference. Reviews in Fish Biology and Fisheries, 10(3):293-323.
7. Devlin RYT, Donaldson EM, Du SJ, Hew C. 1995. Productionof germ line transgenic Pacific salmonids with dramatical increased growth performance. Can J Fish Aquat Sci, 52:1376–1384.
8. Marnis H, Iswanto B, Suprapto, Imron, Dewi SRRSRP. 2015. Growth and sigosity of transgenic African catfish (*Clarias gariepinus*) F2 carrying the catfish Siamese (*Pangasianodon hypophthalmus*) growth hormone gene. Jurnal Riset Akuakultur, 10(2):161–168.
9. Pitkanen TI, Krasnov A, Teerijoki H, Molsa H. 1999. Transfer of growth hormone (GH) transgenes into Arctic charr (*Salvelinus alpinus* L.). I. Growth responseto various GH construct. Genetic Analysis: Biomolecular Engineering, 15:91-98.
10. Buwono ID, Junianto, J, Iskandar I, Alimuddin A. 2019. Growth and expression level of growth hormone in transgenic mutiara catfish second generation. Journal of Biotech Research, 10:102-109.
11. Fitzpatrick JL, Akbarashandiz H, Sakhrani D, Biagi CA, Pitcher TE, Devlin RH. 2011. Cultured growth hormone transgenic salmon are reproductively out-competed by wild-reared salmon in semi-natural mating arenas. Aquaculture, 312:185–191.
12. Bessey C, Devlin RH, Liley NR, Biagi CA. 2004. Reproductive performance of growth-enhanced transgenic coho salmon. Transactions of the American Fisheries Society, 133(5):1205-1220.
13. Maestro MA, Planas JV, Moriyama S. 1997. Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. General and Comparative Endocrinology, 106:189–201.
14. Berishvili G, Shved N, Eppler E, Clota F, Baroiller JF, Reinecke M. 2006. Organ-specific expression of IGF-I during early development of bony fish as revealed in the tilapia, *Oreochromis niloticus*, by *in situ* hybridization and immunohistochemistry: indication for the particular importance of local IGF-I. Cell and tissue research, 325(2):287-301.
15. Ndimele PE and Owodeinde FG. 2012. Comparative reproductive and growth performance of *Clarias gariepinus* (Burchell 1822) and Its hybrid induced with synthetic hormone and pituitary gland of Clarias gariepinus. Turkish Journal of Fisheries and Aquatic Sciences: 12:619–626.
16. Lokman PM, George KAN, Divers SL, Algie M, Young G. 2007. 11-ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, *in vitro*. Reproduction Research, 133:955–967.
17. Alavi SMH, Rodina M, Gela D, Linhart O. 2012. Sperm biology and control of reproduction in sturgeon: (I) testicular development, sperm maturation and seminal plasma characteristics. Reviews in Fish Biology and Fisheries, 22(3):695-717.
18. Marimuthu K, Satthiyasilan N, Rahman MA, Arshad A, Raj MG, Arockiaraj J. 2015. Induced ovulation and spawning of African catfish *Clarias gariepinus* using ovaprim. Journal of Environment & Biotechnology Research, 1(1):2–9.
19. Faqih AR. 2011. Decreased motility and power of Dumbo catfish sperm fertility (*Clarias* spp.) after stress shock treatment. Exp. Life Sci., 1(2):72–82.
20. Faizal I, Jati SM, Rustidja, Sumantadinata K, Alimuddin, Triastutik G, Subyakto S, Aliah RS. 2002. Comparison of three different techniques of gene transfer in humpback grouper (*Cromileptes altivelis*). Biotropia, 18(1):13–23.
21. Zhang Mao-qun, Cheng-xun C, Guo Y, Jing G, Xiao-mei W. 2009. Cloning and Sequence Analysis of Full-length Growth Hormone cDNA from *Clarias gariepinus*. ACTA Agriculturae Boreali-Sinica, 6.
22. Cek S, Yilmaz E. 2007. Gonad development and sex ratio of sharptooth catfish (*Clarias gariepinus* Burchell 1822) cultured under laboratory conditions. Turkish Journal of Zoology, 31(1):35-46.
23. Raghuveer K and Senthilkumaran B. 2010. Cloning and differential expression pattern of vasa in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comparative Biochemistry & Physiology, Part A, 157(1):79–85.
24. Legendre M, Teugels GG, Cauty C, Jalabert B. 1992. A comparative study on morphology, growth rate and reproduction of *Clarias gariepinus* (Burchell, 1822), *Heterobranchus longifilis* Valenciennes, 1840, and their reciprocal hybrids (*Pisces, Clariidae*). Journal of Fish Biology, 40:59–79.
25. Schulz RW, Dijk W, Van Chaves-pozo E, García-lópez Á, França LRDe, Bogerd J. 2012. Sertoli cellproliferation in theadult testis is induced by unilateral gonadectomy in African catfish. General and Comparative Endocrinology, 177:160–167.
26. Campbell B, Dickey JT, Swanson P. 2003. Endocrine changes during onset of puberty in male spring chinook salmon, *Oncorhynchus tshawytscha* 1. Biology of Reproduction, 69(6):2109-2117.
27. Iwai T, Inoue S, Kotani T, Yamashita M, Iwai T, Inoue S, Yamashita M. 2009. Production of transgenic medaka fish carrying fluorescent nuclei and chromosomes. BioOne Research Evolved, 26(1):9–16.
28. Chiou PP, Chen MJ, Lin C, Khoo J, Larson J, Holt R, Chen TT. 2014. Production of homozygous transgenic rainbow trout with enhanced disease resistance. Marine Biotechnology, 16(3):299-308.
29. Aikio S, Valosaari KR, Kaitala V. 2008. Mating preference inthe invasion of growth enhanced fish. Oikos, 117:406-414.
30. Moreau DTR, Conway C, Fleming, IA. 2011. Reproductive performance of alternative male phenotypes of growth hormone transgenic Atlantic salmon (*Salmo salar*). Evolutionary Applications, 4(6):736-748.
31. Valosaari K, Aikio S, Kaitala V. 2008. Male mating strategy and the introgression of a growth hormone transgene. Evolutionary Applications, 1(4):608-619.

32. Miura C, Shimizu Y, Uehara M, Ozaki Y, Young G, Miura T. 2011. GH is produced by the testis of Japanese eel and stimulates proliferation of spermatogonia. Reproduction Research, 142:869–877.
33. Reinecke M. 2009. Minireview insulin-like growth factors and fish reproduction. Biology of Reproduction, 82:656–661.
34. Negatu Z, Hsiao SM, Wallace RA. 1998. Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. Fish Physiology and Biochemistry 19:13–21.
35. Hull KL and Harvey S. 2001. Growth hormone: roles in female reproduction. Journal of Endocrinology, 168:1–23.
36. Lokman PM, Kazeto Y, Ozaki Y, Ijiri S, Tosaka R, Kohara M, Divers SL, Matsubara H, Moore LG, Adachi S. 2010. Effects of reproductive stage, GH, and 11-ketotestosterone on expression of growth differentiation factor-9 in the ovary of the eel, *Anguilla australis*. Reproduction Research, 139:71–83.
37. Yalçin S, Solak K, Akyurt I. 2001. Certain reproductive characteristics of the catfish (*Clarias gariepinus* Burchell 1822) Living in the River Asi, Turkey. Turk. J. Zool., 25:453–460.
38. Nóbrega, RH, Daltro R, Souza VDe, Crespo D, Waal PPDe, França LRDe, Bogerd J. 2015. Differentiation in zebrafish via Igf3. Endocrinology, 156:3804–3817.
39. Perrot V, Moiseeva EB, Gozes Y, Chan SJ, Funkenstein B. 2000. Insulin-like growth factor receptors and their ligands in gonads of a hermaphroditic species, the gilthead seabream (*Sparus aurata*): Expression and cellular localization 1. Biology of Reproduction, 63:229–241.
40. Berishvili G. 2010. Insulin-like growth factor-3 (IGF-3) in male and female gonads of the tilapia: development and regulation by growth hormone (GH) and 17α-ethinylestradiol (EE2). General and Comparative Endocrinology, 167(1):128-134.
41. Kajimura S, Kawaguchi N, Kaneko T, Kawazoe I, Hirano T, Visitacion N, Grau EG, Aida K. 2004. Identification of the growth hormone receptor in an advanced teleost, the tilapia (*Oreochromis mossambicus*) with special reference to its distinct expression pattern in the ovary. Journal of Endocrinology, 181(1):65-76.
42. Kagawa H, Moriyama S, Kawauchi H. 1995. Immunocytochemical localization of IGF-I in the ovary of the red seabream, *Pagrus major*. General and Comparative Endocrinology, 99(3):307-315.
43. Campbell B, Dickey J, Beckman B, Young G, Pierce A, Fukada H, Swanson P. 2006. Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. Biology of Reproduction, 75(1):34-44.
44. Baroiller JF, D'cotta H, Shved N, Berishvili G, Toguyeni A, Fostier A, Eppler E, Reinecke M. 2014. Oestrogen and insulin-like growth factors during the reproduction and growth of the tilapia *Oreochromis niloticus* and their interactions. General and Comparative Endocrinology, 205:142-150.
45. Wuertz S, Gessner J, Kirschbaum F, Kloas W. 2007. Expression of IGF-I and IGF-I receptor in male and female sterlet, *Acipenser ruthenus*—evidence for an important role in gonad maturation. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 147(1):223-230.
46. Schmid AC, Näf E, Kloas W, Reinecke M. 1999. Insulin-like growth factor-I and-II in the ovary of a bony fish, *Oreochromis mossambicus*, the tilapia: *in situ* hybridisation, immunohistochemical localisation, Northern blot and cDNA sequences. Molecular and Cellular Endocrinology 156(1-2):141-149.
47. Kagawa H, Gen K, Okuzawa K, Tanaka H. 2003. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, Pagrus major 1. Biology of Reproduction, 68(5):1562-1568.
48. nee Pathak, ND, Kumar P, Lal B. 2015. Endocrine regulation of testosterone production by Leydig cells in the catfish, *Clarias batrachus*: Probable mediators of growth hormone. Animal Reproduction Science, 154:158-165.
49. Tokalov SV and Gutzeit HO. 2005. Spermatogenesis in testis primary cell cultures of the tilapia (*Oreochromis niloticus*). Developmental Dynamics, 233:1238–1247.
50. Yamaguchi S, Gen K, Okuzawa K, Matsuyama M, Kagawa H. 2006. Influence of estradiol-17β, testosterone, and 11-ketotestosterone on testicular development, serum steroid hormone, and gonadotropin secretion in male red sea bream Pagrus major. Fisheries Science, 72(4):835-845.