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Gene Transmission, Growth, and Exogeneous Growth Hormone Expression of G2 Transgenic Betta Fish (*Betta imbellis*)

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Abstract

In our previous research, we had successfully produced G_0 and G_1 *Pangasianodon hypophthalmus* growth hormone (*Ph*GH) transgenic *Betta imbellis*, native ornamental betta from Indonesia, which it's giant- sized variant has valuable price for the breeders. The G_0 and G_1 transgenic (TG) fish showed higher growth rate and body size compared to the non-transgenic (NT) fish. The study was aimed to produce and evaluate the consistencies of transgene transmission and expression in G_2 generation. The growth rate and body size between TG and NT fish was also compared. The G_2 generation was produced using crosses between TG and NT G_1 fish: ♂TG × ♀TG, ♂TG × ♀NT, ♂NT × ♀TG, and ♂NT ×♀ NT. Fish were reared for 12 weeks and transgene detection was performed using the polymerase chain reaction method from the caudal fin clips. The endogenous and exogenous GH expression analysis was conducted using the quantitative real-time PCR (qPCR) method. The results showed that the inheritance of the GH transgene by the G_2 fish was more than 90% in all transgenic crosses. Endogenous GH was expressed at the same levels in the brain of TG and NT fish, but the exogenous GH expression was highly detected only in the TG fish. The G_2 transgenic fish had a higher specific growth rate, up to 31%, compared to the control. The body length of TG crosses were 23-35% higher and had 111-135% higher body weight compared to NT fish. These results showed a promising approached in mass-producing stable lines of giant-sized betta using the GH-transgenic technology.
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1. Introduction

Transgenesis is an important genetic experimental tool used to change or adjust animal traits to be useful in scientific research, biomedical study, and aquaculture by inserting a gene into an organism's genome (Dunham and Winn, 2014). For aquaculture purposes, this gene manipulation has primarily concentrated on elevating the fish growth rates to enhance production efficiency by shortening production times in many species (Devlin, 2011). This technique has shown promising results for increasing fish growth and body size in some fish species, including some with high commercial demand for human consumption, such as carp (Fu *et al*., 2007; Kurdianto *et al*., 2016), salmonid (Devlin *et al*., 2004), loach (Nam *et al*., 2001), and tilapia (Kobayashi *et al*., 2007). Beside the consumable fish, the transgenesis method is also potentially applied in the ornamental fish culture, mostly to enhance its color appearance (Gong *et al*., 2003; Rezaei *et al*., 2019). The application of the GH- transgenesis method in ornamental fish is still scarce.

Betta fish (*Betta* spp.) is an elegant tropical freshwater fish that is popular as a pet and mostly kept in home aquariums. In the wild, this genus is native from Thailand, Cambodia, Indonesia, and also present in other Southeast Asian countries (Kusrini, *et al*., 2016; Panijpan *et al*., 2014). They are domesticated from the wild and desired mostly for their appealing body-color, fin shape, and color pattern (Panijpan *et al*., 2020). Nowadays, giant-sized betta becomes one of the favorable variants, sought by the breeders due to its high price. In Indonesia, the breeders are trying to produce giant-sized betta variants, 1-3 times larger than normal size, through selective breeding. However, there are still some inconsistencies in the fish size and the number of the progenies that reach the giant-size is less than 10% per-spawning cycle (unpublished data). One of the alternative methods to consistently produce giant betta is through the transgenic method and then breeds the germ-line transmitted fish to mass produce the transgenic giant betta progenies. Previous studies have shown that the genetic transformation of fish with the growth hormone transgenic method resulted in significant improvements in weight gain and growth rate of fish. Second-generation of GH-transgenic mud loach *Misgurnus mizolepis* growth was accelerated up to 35 times higher than no-transgenic fish (Nam *et al*., 2001), and about 30 times in Coho salmon *Oncorhynchus kisutch* (Devlin *et al*., 2004). In 16-month-old G_2 transgenic Nile tilapia *Oreochromis niloticu*s, the average growth of the fish weight was 300-500% higher than control with the same rearing time (Kobayashi *et al*., 2007). Growth-hormone transgenic technology also potentially used to increase the growth.

In our recent study, we have successfully produced G_0 and G_1 GH-transgenic fish of the *B. imbellis*, native ornamental betta from Indonesia (Kusrini *et al*., 2018; Kusrini *et al*., 2016a). The gene introduction was conducted using the p*Cc*BA-*Ph*GH vector that consisted of the *P. hypophthalmus* GH cDNA and regulated by *C. carpio* β-actin promoter (Dewi *et al*., 2012). The fish were maintained in the controlled indoor rearing facility in the Research Institute for Ornamental Fish Aquaculture, Depok, West Java, Indonesia. Previously, The G_0 transgenic fish showed 37.8% higher body weight than non-transgenic control after 7 months of rearing, and the *Ph*GH cDNA was detected in the various tissues after PCR amplification (Kusrini *et al*., 2016a; Kusrini *et al*., 2016b). Furthermore, the G_1 transgenic was produced from three different crosses of G_0: transgenic (TG) male and non-transgenic (NT) female, NT male paired with TG female, and NT transgenic fish pair as a control. Transgene transmission in G_1 was about 62.5%. Comparing to the non-transgenic fish, the G_1 transgenic had 47% and 76% higher growth rate, and 32% and 25% higher body length from ♂TG × ♀NT and ♂NT × ♀TG, respectively, at five months old (Kusrini *et al*., 2018).

These previous studies showed promising results regarding the production of the giant variation of betta fish. However, the G_0 fish are mosaics with varying GH expression levels between individuals. G_1 fish also possessed very large variation in expression between individuals even from the same G_0 fish, and relatively low transmission (Kusrini *et al*., 2018). The G_2 fish will probably possessed higher transmission with phenotypes that match the target and had high exogenous GH expression. This G_2 fish are very important to make transgenic homozygote, where these homozygous transgenic fish will be used for mass transgenic fish production (Kurdianto *et al*., 2016). However, the G_2 generation of GH transgenic betta was not produced yet. This study was aimed to produce and evaluate the consistencies of transgene transmission and *Ph*GH expression in G_2 transgenic *B. imbellis* and also compared the growth rate and body length between transgenic and non-transgenic progenies.

2. Materials and Methods

The care and use of experimental animals complied with IPB University and Research Institute for Ornamental Fish Culture animal welfare and experimentation guidelines.

2.1 Material

The G_0 growth hormone transgenic *B. imbellis*

were produced previously by transfection method of pCcBa-PhGH expression vector (Dewi $et~al.$, 2012) into the fertilized eggs of $B.~imbellis$ (Kusrini $et~al.$, 2016). The G_1 progenies were obtained through reciprocal crosses of male/female transgenic G_0 with non-transgenic fish. The G_2 transgenic was produced from reciprocal crosses of G_1 transgenic fish. The fish were obtained from the Research Institute for Ornamental Fish Culture, Depok, West Java, Indonesia, and then reared in the same facility. Other materials were $Artemia$ for feeding (Supreme Plus™, Golden West, USA), GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA), oligonucleotide primers (IDT, Singapore), cDNA synthesis kit Revertra® Ace

qPCR RT Mastermix with gDNA removal (Toyobo, Japan), DNA ladders and loading dye(Invitrogen, USA), Go-Taq® Green Mastermix (Promega, USA), TRI Reagent® (Sigma-Aldrich, USA), nucleases-free water (1st base, Malaysia), alcohol (chloroform, isopropanol, ethanol 96% pro-analyst; Merck, USA), filtered microtips and sterile microtubes (ExtraGene, Taiwan). Equipment used for this study were peqSTAR Thermocycler (VWR, Austria), electrophoresis chamber and UV-illuminator (Bio- Rad's Sub-Cell® System and Power Pac™, Bio-Rad, USA), Rotor-Gene 6000 qPCR machine (Qiagen, USA), refrigerated centrifuge (VWR, Austria), GeneQuant DNA Calculator (Pharmacia Biotech, USA), and various volumes of micropipettes (Axygen, USA).

Table 1. A reciprocal cross between transgenic and non-transgenic B. imbellis to produce G_2 progenies

Male fish (♂)	Female fish (♀)	
	TG	**NT**
TG	♂TG × ♀TG	♂TG × ♀NT
NT	♂NT × ♀TG	♂NT × ♀ NT

Description: TG= transgenic, NT=non-transgenic. Each cross consists of three pairs of fish (n= 3 replication each cross).

Table 2. Primers sequence used in this study

Primer Name	Sequence (5'−3')	*Annealing Temperature* (˚C)	Application
PhGH-Fw PhGH-Rv	TCTTTAGTCAAGGCGCGACATTCGAGA CGATAAGCACGCCGATGCCCATTTTCA	65	PhGH transgene screening
β-actin-Fw β-actin-Rv	TATGAAGGTTATGCTCTGCCC CATACCCAGGAAAGATGGCTG	60	PCR internal control
qBeGH-Fw qBeGH-Rv	TGGAGGTTCTGCTCAAAGATAC CATCTCTACACCATCCTCATTGG	60	qPCR of BeGH
qIGF-1-Fw qIGF-1-Rv	TGTTTGTGTGCGGAG ATAGAG CTCACAGCTCTGGAAGCAG	60	qPCR of IGF-1
qPhGH1-Fv qPhGH1-Rv	CGAGGATTTCTACCAGACCTTG GCTGAGATAGGTCTCCACTTTG	60	qPCR of $PhGH$
EF-1α-Fw EF-1α-Rv	GGTGTGAAGCAGCTCATCGTTGGT AGAGATGGGGACAAAGGCAACAG	60	qPCR normalizator, internal control

Table 3. Transgene transmission in G_1, and G_2 *Betta imbellis*

Crosses	Transgene transmission	
	G_{1*}	**G_2**
♂NT × ♀ NT	0.00	0.00
♂NT × ♀TG	66.67	96.66
♂TG × ♀NT	58.93	90.00
♂TG × ♀TG	n.d	96.66

*from previous study (Kusrini $et~al.$, 2018). Description: n= 30 for each cross. NT= G_1 non-transgenic, TG= G_1 transgenic. N.d= not determined.

2.2 Method

2.2.1 Experimental design and fish rearing

Mature G_1 of transgenic (TG) male (4.7±0.6 cm; 1.2±0.5 g), TG female (4.4±0.3 cm; 1.3±0.3 g), non-transgenic (NT) male (3.17±0.21 cm; 0.79±0.24 g), and NT female (3.40±0.17 cm; 1.04±0.19 g) that were in the same age were used in the reciprocal crossing to produce G_2 progenies. Transgenic crosses were ♂TG × ♀TG, ♂TG × ♀NT, ♂NT × ♀TG, and non-transgenic ♂NT ×♀ NT cross as control (Table 1). The spawning process was conducted in pair (1:1) inside the plastic buckets (d= 40 cm) with 7.5 L of water volume. Eggs were collected and placed in the $50 × 60 × 90$ cm³ tanks. After hatching, G_2 fish were reared for 5 months. The larva was fed with *Artemia* nauplius starting from day 2 until 14 days after hatching (DAH). Then, fish were fed with *Moina* sp. and chopped *Tubifex* sp. until it reached 1 month DAH. *Tubifex* and bloodworm were given until fish reach 3 months DAH. All progenies from each cross were fed with the same amount of feed during the rearing period. Waste was removed daily and 50% water change was done every 3 days. At two months old, sex differentiation was conducted and male fish were reared separately. Male progenies were reared individually (1 fish per-tank), while female progenies were reared in the same rearing tank (10 fish L^{-1}). Fish bodyweight and length were measured every week randomly within the population without prior DNA analysis (n= 40 fish each cross).

2.2.2 Transgene transmission

Fin clips were taken from six weeks old G_2 progenies (n= 30 from each cross) to screen the *Ph*GH transgene within the genome. The screening was done using the polymerase chain reaction (PCR) method. Genomic DNA from the fins was isolated and amplified using the specific *Ph*GH primer that amplifies the exogenous GH cDNA sequence. The primer was consisted of *Ph*GH forward: 5'-TCTTTAGTCAAGGCGCGACATTCGAGA-3' and the reverse sequence was: 5'-CGATAAGCACGCCGATGCCCATTTTCA-3'. The β-actin gene was used for the PCR internal control with forward sequence: 5'-TATGAAGGTTATGCTCTGCCC-3' and reverse sequence: 5'- CATACCCAGGAAAGATGGCTG-3' (Table 2). The PCR program and reaction were based on our previous result (Dewi *et al.*, 2012). The amplification result was visualized under the UV-light after the gel electrophoresis separation. Positive *Ph*GH transgene fish will produce a single band at 334 bp while no-transgene fish will have no observed DNA band after gel electrophoresis

2.2.3 Total RNA extraction and mRNA expression

Total RNA was extracted from the brain of the transgenic progenies from the TG ×TG cross and non-transgenic control from NT × NT cross (n= 3 from each cross). The total RNA was extracted and its concentration was measured using a spectrophotometer at 260 and 280 nm. The cDNA synthesis was conducted from 100 ng µL⁻¹ of total RNA. The qPCR reaction was performed to determine the mRNA expression levels of the growth-related gene. The endogenous *B. imbellis* growth hormone (*Be*GH), and exogenous *Ph*GH expression in transgenic and non-transgenic G_2 progenies were evaluated using the specific primers (Table 2). The qPCR reaction was performed with the total reaction volume was 20 µL, consisted of 10 µL 2× sensiFAST SYBR NO-ROX mix (Bioline, UK), 0.8 µL of each specific primer, 4 µL cDNA, and 4.4 µL NFW (*nucleases-free water*). The amplification program was conducted in the Rotor-Gene 6000 (Corrbet, USA) with Green channel to detect the florescence dye (470 nm for source, and 530 nm for the detector). The qPCR program was set at 95˚C for 3 min, continued with 40 cycles of 95˚C for 10 s, 60˚C for 15s, and 72˚C for 15s. Melting-curve analysis was performed at the end of the amplification reaction to evaluate the specificity of the amplification. The melting curve analysis was performed at 72-95°C with 1°C resolution per-second at Rotor-Gene 6000 machine. All molecular works were performed using the sterile-filtered microtips and tubes (Extra Gene, Taiwan).

2.3 Analysis Data

The transgene transmission data were compared descriptively while the growth performance, growth pattern, and mRNA expression data were compared statistically using the one-way ANOVA analysis followed by Duncan's test at α= 0.05. The expression levels after qPCR analysis were normalized to the elongation factor-1α (EF1α) gene and analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3. Results and Discussion

The growth process in fish is regulated through the growth hormone synthesized in hypophysis and then it is released into the bloodstream. The GH protein will interact with its specific receptors on the cells and altered the intracellular-somatotrophic signaling pathways, with insulin-like growth factor 1 (IGF-1) as the main mediator for cell proliferation and growth (Fuentes *et al.*, 2013; Nipkow *et al.*, 2018). Although not common,

the application of GH transgenesis in ornamental fish is also potential to increase fish growth rate and reduce its production period. In this study, the G_2 generation of GH-transgenic betta is successfully produced using various crosses of TG and NT fish to obtain giant-sized betta fish. PCR analysis confirmed the presence of the transgene in the purified genomic DNA from the G_2 progenies from different crosses groups, except the control (Figure 2). The β-actin as an internal control was amplified in all fish and the non-template control was not contaminated. The amplicon size ofthe PhGH DNA was similar between the plasmid control and sample DNA. The PCR product, corresponding to 334 bp of the specific PhGH cDNA sequence, was detected in 29 of 30 (96.67%) progenies of ♂NT × ♀TG, 27 of 30 (90%) from ♂TG × ♀NT, 29 of 30 (96.67%) from ♂TG × ♀TG, and was not detected in any of the control fish from ♂NT ×♀ NT (Table 3). The inheritance was significantly increased from its G_1 generation. Previously, the gene transmission from the G_0 to the G_1 progenies was 58.89-66.67% (Kusrini et al., 2018). The transgene transmission was more than expected for Mendelian segregation of single locus. This pattern suggested that the transgene DNA might be integrated at multipleloci in founder fish, and thus inherited stably (Chen et al., 2015; Devlin et al., 2004).
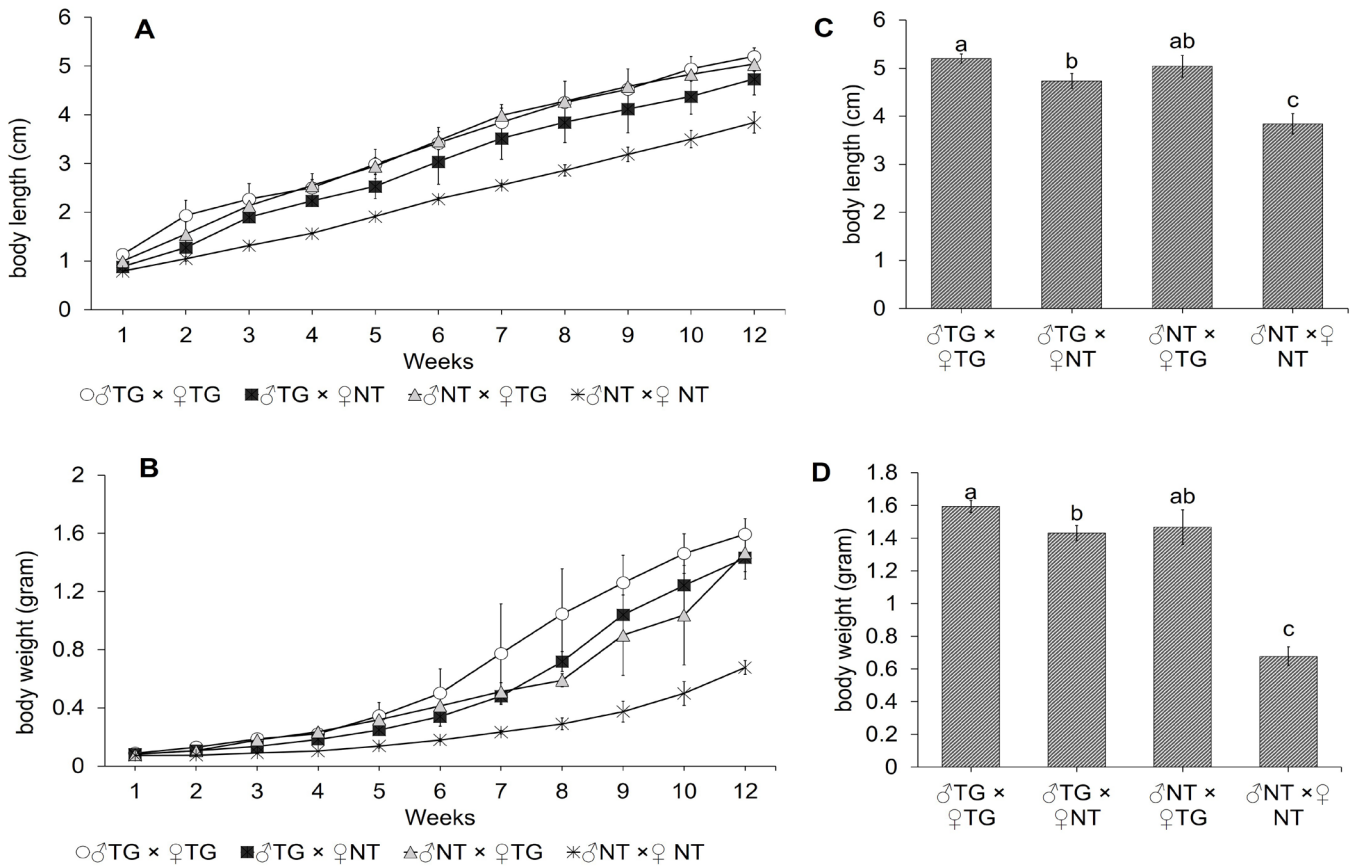
Transgenic
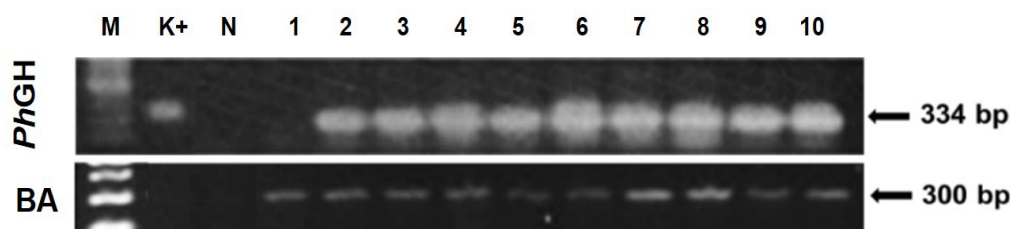
Non-Transgenic
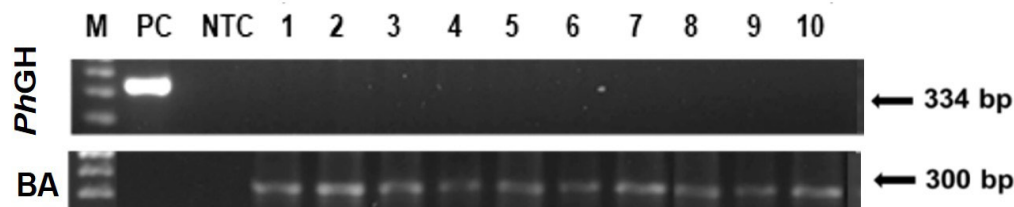
Figure 1. PCR detection of the PhGH gene in the purified genomic DNA from G_2 transgenic and non-transgenic B. imbellis. M= 100 bp DNA ladder, K+= CcBA-PhGH vector, N= no-template control, 1-10= sample number.

Table 4. Growth performance and survival of transgenic G_2 Betta imbelis from different crosses

Parameter	Crosses			
	♂NT ×♀ NT	♂NT × ♀TG	♂TG × ♀NT	♂TG × ♀TG
Body length growth (cm day⁻¹)	0.374±0.02b	0.442±0.03a	0.444±0.278a	0.415±0.032a
Body weight growth (gram day⁻¹)	0.031±0.001b	0.039±0.002a	0.04±0.002a	0.04±0.002a
Specific growth rate (% day⁻¹)	2.689±0.078b	3.5340.169a	3.474±0.142a	3.511±0.101a
Survival (%)	81±2.29a	81±3.46a	79±2.96a	80±2.22a

Description: TG= transgenic G_1; NT= Non-transgenic G_1. Different letters indicate a significant difference between crosses.

Figure 2. Body length and body weight of G_2 transgenic and non-transgenic *B. imbellis*. Bodyweight (A) and body length (B) pattern during 12 weeks of rearing and size comparison of all crosses at the end of the experiment (C and D) are shown. Data were presented as mean ± S.D with the error bar. However, in several points, the error bar was smaller than the marker, thus not shown in the figure. Different letters in the bar graph indicate a significant difference between progenies from different crosses (p<0.05).
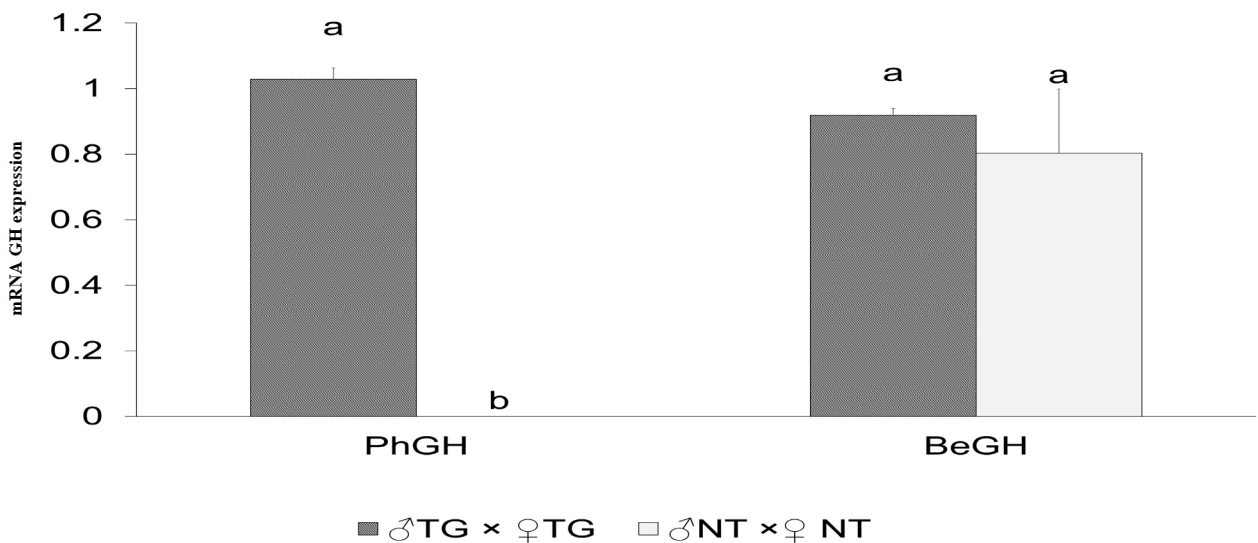
Figure 3. The relative mRNA expression of transgene *Ph*GH and endogeneous *Be*GH of G_2 transgenic and non-transgenic *B. imbellis*. No *Ph*GH mRNA transcript was detected in male NT × female NT non-transgenic control. Data were presented as mean ± SD. Different letters indicate a significant difference in the mRNA expression of the same gene among crosses (p<0.05).

The results showed that the body length and weight growth of all transgenic crosses (♂NT × ♀TG; ♂TG × ♀NT; ♂TG × ♀TG) were significantly higher compared to the non-transgenic control (♂NT ×♀ NT) (Table 4). The progenies from the transgenic crosses also had a higher specific growth rate, up to 31.2%, compared to the control group with no significant difference in the fish survival. The results showed that the progenies from transgenic crosses had a higher average body length and weight during the 12-weeks of rearing compared to the non-transgenic control (Figure 2A and 2B). The body length of fish from transgenic crosses was 23-35% higher than control. The ♂TG × ♀TG progenies had higher body length compared to the ♂TG × ♀NT, but not different than ♂NT × ♀TG (Fig. 2C). Fish body weight showed the same results (Figure 2B). At the end of rearing, the G_2 fish that came from transgenic crosses had a higher average body weight, 111-135% fold compared to NT control. The ♂TG × ♀TG progenies had higher body weight compared to the ♂TG × ♀NT, but not different than ♂NT × ♀TG (Figure 2D). This result was not surprising since it was widely reported in other transgenic fish species that bearing the exogenous GH. In six months old zebrafish *Danio rerio*, GH transgenic fish reached about 155% higher final average mass compared to its non-transgenic siblings (Figueiredo *et al.*, 2007). In the tilapia GH-transgenic Nile tilapia, the body weight of G_2-transgenic fish was 7 times larger compared to the non-transgenic control (Kobayashi *et al.*, 2007). In the G_2 generation of GH-transgenic common carp, specific growth was reported to be 77.4% higher than non-transgenic fish and had 69.2% times higher body weight (Fu *et al.*, 2007).

This significant body weight and length alteration in TG fish might due to the overexpression of exogenous GH-transgene thus affect other growth factors (Triantaphyllopoulos *et al.*, 2020). The *Ph*GH mRNA transcripts were observed in the fish brain and only detected in TG fish (Figure 3). These results indicated that the *Ph*GH DNA was successfully incorporated into the fish genome and undergone the transcription process. The expression of *Ph*GH in the fish brain might have a not significant effect on the transcription of the endogenous *Be*GH since *Be*GH was expressed at the same levels in the brain of transgenic fish and non-transgenic control (Figure 3). However, *in vivo* protein translation of *Ph*GH and *Be*GH was not analyzed in this study and needs to be evaluated in the future. This transcription of *Ph*GH mRNA potentially leads to a significant increase in the GH synthesis, thus resulting in a significant growth rate of TG fish compared to the NT fish (Table 4 and Figure 2). Moreover, compared to the G_1 fish, G_2 betta had a faster growth rate and larger body weight at the same rearing points. The G_1 TG progenies reached 0.4-0.8 g of body weight, 2.7-3.5 cm of body length after 12 weeks (Kusrini *et al.*, 2018), while in G_2 fish it was already reached 0.67-1.59 g for bodyweight and 3.8-5 cm for body length within the same rearing period (Figure 2). Taking together, the G_2 progenies inherited a higher percentage of GH transgene and possessed a higher growth rate compared to its G_1 generation. This might be beneficial for the large and stable production of giant-sized ornamental betta fish. However the commercial use of genetically modified fish in aquaculture is still up for discussion, since the first commercialization of genetically modified fish, GloFish, was in 2003 (Gong *et al.*, 2003), and further, the United States and Canada allowed the commercial processing and selling of the AquAdvantage® Salmon (Ledford, 2015). To prevent potential adverse impact on the environment, biological and environmental containments should be applied in the production of TG betta, for example using the closed containment aquaculture system (Crouse *et al.*, 2021). Furthermore, the transgenic betta should be bred as sterile-triploid fish to avoid reproduction. As example, the transgenic-commercial AquAdvantage® salmon are produced as triploid females, which do not contain fertile gametes and thus have little reproductive potential. They are therefore incompatible with wild diploid fish (Ignatz *et al.*, 2020).

4. Conclusion

The G_2 generation of GH TG betta had been successfully produced, with more than 90% of the inherited transgene. Endogenous GH was expressed at the same levels in the TG and NT, but the exogenous GH was only detected in the TG fish. The growth of G_2 TG fish was up to 31.2% higher than control and TG crosses had significantly higher body weight and length compared to NT. Moreover, G_2 TG fish reached a larger body length and weight in a shorter period compared to G_1. These results showed a promising approached in mass-producing giant-sized betta using the GH-transgenic technology.

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Authors' Contributions

Conflict of Interest

The authors declare that they have no competing interests.

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