Growth hormone transgenesis and feed composition influence growth and protein and amino acid content in transgenic G₃ mutiara catfish (*Clarias gariepinus*)

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Abstract

Clarias gariepinus growth hormone (CgGH) transgenesis was previously used to develop a population of second-generation (G₂) transgenic mutiara catfish (*C. gariepinus*). The third generation of these fish (1-month old fingerlings) had 2–3 times improvement in growth compared to non-transgenic fish in the commercial feed test for 6 weeks of rearing. We assessed the impact of CgGH transgene expression on growth and protein and amino acid content of the G₃ generation of these transgenic mutiara catfish relative to non-transgenic catfish. Since variation in composition of feed mixes can affect protein and amino acid content of fish, we tested three mixtures of commercial feed and boiled tuna (*Euthynnus affinis*): feed A (50:50 feed to tuna), B (65:35), and C (80:20) to transgenic catfish. Feed A* (50:50) was fed as a control to non-transgenic catfish. Feed efficiency, including feed conversion ratio and protein use efficiency (i.e., protein retention and protein productive value), was assessed. Feed efficiency, protein content, and essential amino acid content in G₃ transgenic catfish (feed A and B) were higher than in non-transgenic fish (feed A*). The latter were deficient in lysine and methionine. Transgenic catfish fed with feed C (80:20) showed lysine deficiency and lower growth than fish fed feeds A and B. Feed B (65:35) was the optimal feed mixture utilized; it increased growth, protein levels, and feed conversion efficiency in G₃ catfish. The growth of transgenic fish was higher than non-transgenic fish when supported by feeding with balanced nutrients.

Keywords Amino acids · Catfishes · Gene transfer techniques · Growth hormone transgenic fish · Proximate chemical composition · Growth metrics

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Introduction

As the demand for aquaculture products increases, there is a concurrent need to improve the growth rate of fish in order to increase the efficiency of fish production. Increased production in commercial fish farming can be achieved by genetic improvement of fish growth. For example, insertion of exogenous growth hormone (GH) genes using transgenic technology can produce a 2–3-fold increase in fish growth relative to non-transgenic fish over a few months (Devlin et al. 2004). Studies of salmonids demonstrate that transgenic fish expressing exogenous GH constructs (e.g., OnMTGH1 and CMVOnGH1) display dramatic growth enhancement (Higgs et al. 2009; Pitkanen et al. 1999). Furthermore, *Clarias gariepinus* growth hormone (CgGH) transgenic technology, successfully developed for mutiara catfish (*C. gariepinus*), induces significantly greater growth in transgenic fish; second-generation (G₂) animals showed a 150–200% increase in growth relative to non-transgenic fish (Buwono et al. 2019a). This technology also increased growth in transgenic offspring of other fish species, including G₂ transgenic tilapia (125% increase, Rahman et al. 2001) and G₅ transgenic Atlantic salmon (*Salmo salar*) (100% increase, Levesque et al. 2008). The aim of this study, therefore, was to evaluate mass production of G₃ transgenic mutiara catfish, which requires that levels of expression and transmission of exogenous GH remain high; thus, transgene inheritance must be stable among generations of transgenic fish (Wang et al. 2001). In our previous study, CgGH transmission in the G₂ strain reached 50%, indicating the occurrence of Mendelian transmission and suggesting that these fish can be used to produce G₃ catfish (Buwono et al. 2019b).

Induction of increased growth in transgenic-CgGH catfish is mainly due to increased expression of exogenous GH, which stimulates IGF-1 (insulin-like growth factor 1) production in the liver. Previous studies show that plasma GH and IGF-1 levels in transgenic fish are higher than in non-transgenic fish (Eppler et al. 2007; Raven et al. 2008). Hence, transgenic fish are more responsive to feed (Cook et al. 2000; Raven et al. 2008), and growth phenotype is improved along with feed efficiency (Devlin et al. 2004; Oakes et al. 2007). Furthermore, overexpression of exogenous GH stimulates de novo protein synthesis from precursors available from feed protein (Fauconneau et al. 1996; Mauras and Haymond 2005; Rosa et al. 2008). Enhanced GH secretion plays an important role in stimulating fish growth, improving feed conversion, and increasing protein use efficiency. Thus, GH-transgenic salmon regulate energy metabolism by converting feed carbohydrates into metabolic energy, avoiding the use of protein (protein sparing) to increase protein synthesis (increased protein retention). This increased efficiency in protein use supports more rapid growth (Levesque et al. 2008). Growth hormones, including transgenic GH, also function to promote absorption of amino acids, especially essential amino acids that cannot be synthesized intrinsically in the fish (Farmanfarman and Sun 1999; Li et al. 2009). For example, the content of essential amino acids in fish feed determined protein use efficiency, represented by protein productive value (PPV) to support optimum fish growth in stinging catfish (*Heteropneustes fossilis*) fingerlings (Farhat and Khan 2012).

Therefore, maximum growth potential of transgenic fish depends on the quality of feed protein based on both protein content and amino acid composition (Wilson and Poe 1985; Cowey 1994). Feed composition indirectly influences protein retention, amino acid content, and growth of transgenic fish. Available commercial fish feed might provide transgenic fish with the content protein they need to achieve their growth potential, but alternative fish feeds should also be considered, especially since comparisons of the effects of feeds in transgenic fish growth have rarely been reported.

In the present study, we investigated an alternative feed protein source for transgenic catfish, namely boiled tuna (*Euthynnus affinis*), commonly known as *pindang tongkol* in Indonesia. The fish is prepared by a combination of boiling and salting. *Pindang tongkol* might replace commercial feed since it contains all nutritional elements required by commercial fish species (Uju et al. 2017; Kim et al. 2018). Specifically, we investigated whether overexpression of CgGH, increased levels of protein, and increased amino acid content are reflected in the growth rate of G₃ transgenic mutiara catfish fed mixtures of commercial feed and *pindang tongkol* at different ratios. Finding an optimum balance of feeds would provide important information to help improve feed conversion efficiency and growth.

Materials and methods

Transgene construct and the production of G₂ transgenic broodstock

The transgene constructs designed to produce G₀ transgenic mutiara catfish were pCMV-CgGH (6285 bp) containing CgGH (Buwono et al. 2019b). The pCMV-CgGH plasmid was transferred to mutiara catfish sperm using the Xcell gene pulser electroporator (BioRad Laboratories, Inc., Hercules, CA, USA) by the same procedure as in previous studies (Subyakto et al. 2011). Post electroporation, plasmid-carrying sperm (containing CgGH) was mixed with mutiara catfish eggs to produce G₀ transgenic mutiara catfish.

The production of G₁ transgenic fish was carried out by crossing G₀ transgenic and non-transgenic broodstock, and crossing G₁ transgenic and non-transgenic broodstock was used to produce G₂ transgenic fish. Furthermore, these G₂ transgenic broodstock were used for the production of G₃ transgenic fish.

Validation of the G₂ transgenic mutiara catfish broodstock to produce G₃ transgenic mutiara catfish

G₂ catfish broodstock were derived from a cross from previous studies and showed a CgGH transmission rate of 50% (Buwono et al. 2019b); thus, both male and female catfish required validation for expression of the CgGH gene (GenBank accession number MN 249238.1). Transgene detection used RT-PCR and DNA isolated from a small sample of the catfish tail fin. Once positive G₂ transgenic male and female broodstock were confirmed to carry the CgGH gene (a product of approximately 600 bp using primers GH-F and GH-R; see below), they were used for G₃ transgenic mutiara catfish production. For controls, G₂ non-transgenic broodstock pairs were used to produce G₃ non-transgenic catfish.

G₃ transgenic mutiara catfish were produced by first maintaining G₂ transgenic broodstock until their gonads matured. Maturation of broodstock gonads (5 pairs of broodfish) took place over 1.5 months in a separate rearing tank (2 × 1.5 × 1-m³ round fiberglass). Broodstock were fed a mixture of commercial feed (39% protein content) and *pindang tongkol* (ratio 1:1) twice daily at 3% of their biomass by weight to promote gonad maturity. During this period, water temperature was maintained at 27 °C ± 1 °C using a water heater, aeration was provided to maintain dissolved oxygen, and photoperiod was set to 10:14 h light to dark. Maturity of broodstock gonads was determined by examining the female and male genital papillae; pink genitals are an indicator of sexual maturity (Das Neves et al. 2019). Subsequently, the broodstock was examined to validate G₂ transgenic females and males as follows.

Production of G₃ transgenic mutiara catfish

One pair of transgenic broodstock (Fig. 1) was spawned at around 10-month old (female body weight = 440 g, total length = 40 cm, male body weight = 600 g, total length = 44 cm); the breeding scheme is explained in Fig. 2. Semi-artificial spawning was achieved by an injection of Ovaprim hormone (Syndel Laboratories, Ltd., Vancouver, British Columbia, Canada) (0.5 mL/kg body weight of female catfish; and 0.4 mL/kg body weight of male catfish) combined with the use of *kakaban*, an egg-attachment substrate placed in the bottom of a round fiberglass tank (Buwono 2019b; Kasi et al. 2015). Immediately following injection, the pair was transferred to their spawning tank (1200 L), which was maintained at controlled water temperature (28 ± 1 °C), oxygen level (5 ± 1 ppm), and water volume (400 L). Twelve hours after injection, eggs attached to the *kakaban* substrate were examined. Fertilized eggs were then transferred into a glass aquarium (50 × 30 × 30 cm) filled with approximately 40 L of water maintained at 27 °C $\pm$ 1 °C, with homogenous aeration.

Eighteen hours after spawning, samples ($n = 300$) of fertilized eggs were taken and transferred to another aquarium for hatching. Two days after larvae hatched, they were fed *Artemia nauplii* ad libitum until the age of 14 days, and survival of larvae was calculated (Okomoda et al. 2018). G₃ catfish fingerlings were then fed live *Tubifex* sp. twice a day and maintained until they were 1-month old, when they were used in the growth experiment.

CgGH gene transmission and expression

Inheritance and expression of the CgGH transgene in G₃ catfish fingerlings was measured as an indication of stable Mendelian CgGH gene inheritance. Catfish that carried the CgGH transgene were determined using electrophoresis seeking observation of a 600-bp amplicon following PCR amplification of the introduced transgene construct. CgGH transgene transmission was calculated using 80 catfish fingerlings, with each sample being pooled from small pieces of fin (from four different fins of each fish) of 1-month old fingerlings. CgGH transmission was calculated using the following formula:

$$\text{CgGH transmission (\%)} = \frac{\text{the number of transgenic fingerlings}}{\text{number of test samples}} \times 100$$

The level of CgGH mRNA expression in the G₃ catfish fingerlings was measured with semi-quantitative RT-PCR (sqRT-PCR) with β -actin as an internal expression control (Alimuddin et al. 2008). Total RNA was extracted from larvae and fingerlings using a High Pure RNA Tissue Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Synthesis of cDNA and RT-PCR for CgGH was performed using a My Taq OneStep RT-PCR Mix (Bioline, London, UK) with the following program: 48 °C for 20 min; 40 cycles of 95 °C for

Fig. 1 Second-generation (G₂) transgenic female (a) and male (b) mutiara catfish (*Clarias gariepinus*) broodstock

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Table 1 PCR primers used in the study

Primer	Sequence (5'–3')	Amplicon size (bp)	Reference
GH-F	ATGGCTCGAGTTTGGTGCTGCT	600	Zhang et al. (2009)
GH-R	CTACAGAGTGCAGTTGGAATCCAGGG		
βAct-F	ACCGGAGTCCATCACAATACCAGT	200	Raghuveer and Senthilkumaran (2010)
βAct-R	GAGCTGCGTGTGCCCCCTGAC		

fish were used for proximate analysis of fingerling bodies at the beginning of the experiment. Growth of G₃ transgenic and non-transgenic fingerlings were assessed using mixtures of commercial feed (Prima Feed PF 500) and boiled tuna (*pindang tongkol*) as test feeds. Moist *pindang tongkol* was first mixed with commercial feed pellets, and then, 200 mL of warm water was added for every 1 kg of mixture. The final mixture was converted into pellets (i.e., re-pelleted).

G₃ catfish were gradually adapted to feed mixtures by initially providing a mixed feed (75% commercial feed and 25% *pindang tongkol*) for 1 week while monitoring fish appetites and for 3 days before progressing to a mixture of 50% commercial feed and 50% *pindang tongkol*. Subsequently, the test feed experiment began using the respective mixtures described in Table 2.

Growth performance and amino acid and protein content of G₃ catfish

Approximately 170 test fish (G₃ fingerlings) were obtained from egg samples following assessment of larval survival. Test feeds (A, B, C, and A*, Table 2) were used as treatments, and growth of transgenic and non-transgenic mutiara catfish was evaluated in triplicate. Each replicate included 15 glass aquariums (60 × 50 × 40 cm) with a water height of 30 cm (90 L)

Table 2 Composition of feed mixtures for G₃ transgenic and non-transgenic mutiara catfish (*Clarias gariepinus*)

	Transgenic		Non-transgenic	
	Test feed			
	A	B	C	A*
Commercial feed (g) ^a	500	650	800	500
<i>Pindang tongkol</i> (g) ^b	500	350	200	500
Total mixture (g)	1000	1000	1000	1000
Proximate (%) ^c				
Ash	10.02	11.21	12.41	10.02
Fiber	2.12	2.64	3.25	2.12
Protein	37.40	37.88	38.36	37.40
Lipid	8.52	7.47	6.41	8.52
Carbohydrate	41.94	40.80	39.57	41.94

^a Commercial feed was Prima Feed PF 500, which contains 39% protein

^b *Pindang tongkol* is boiled tuna (*Euthynnus affinis*), which contains 35.80% protein

^c Proximate levels were calculated based on Table 3 [example: protein of feed A = (50/100 × 39/100) + (50/100 × 35.80/100) × 100% = 37.40%]

A* as control treatment

and a fish density of five per aquarium. Catfish were fed twice a day (at 9 am and 5 pm) at a feeding rate of 5% of total biomass. The catfish were reared for 42 days (6 weeks), water was exchanged (50%) every week, water temperature was maintained at $26 \pm 1^\circ\text{C}$ using a heater, and photoperiod was 12:12 h light to dark. Oxygen was supplied by an aeration system and maintained at a level of 5.0 mg L^{-1} . Growth measurements from each treatment were taken weekly in order to adjust the amount of feed based on biomass, while the amount of feed residue in each treatment was also observed. At the end of the experiment, the final weight of fish were measured, and the total amount of feed consumed were determined to calculate feed conversion ratio (FCR). Finally, one catfish was taken from each replicate of each treatment for further analysis (50 g used for proximate analysis of whole body and 0.5 g for analysis of amino acids) (Table 3).

Proximate levels of water, ash, protein, lipids, and carbohydrates in fish followed the methods of AOAC (1990). Water content of a test sample (1 g) was calculated gravimetrically using an analytical balance (Mettler Toledo, Columbus, OH, USA). The sample was weighed, then dried in an oven (Heraeus Instruments Athens, Greece) at 105°C for 4 h, stored in a desiccator, and then re-weighed to determine water content. Ash content (3 g samples) was calculated using dry ashing with a furnace (Thermolyne 1500) at $550\text{--}600^\circ\text{C}$. The protein content (1 g samples) was measured using the Kjeldahl method. Lipid content (5 g samples) was determined using the Soxhlet method. Carbohydrate content (2.5 g samples) was determined using the titration method (Hall 2008).

Feed and protein conversion efficiency during fish growth was evaluated using the weight-gain parameters FCR, protein retention (PR), and protein productive value (PPV). The formulae used were:

$$\text{Average weight gain (g)} = \text{average final weight (g)} - \text{average initial weight (g)}$$

$$\text{FCR} = \text{total amount of feed (g)} / \text{weight gain (g)}$$

$$\text{PR (\%)} = (\text{final amount of stored protein} - \text{initial amount of stored protein}) / \text{amount of feed protein} \times 100$$

where the final amount of stored protein = % final dry weight $\times$ average final weight $\times$ final body protein (g), the initial amount of stored protein = % initial dry weight $\times$ average initial weight $\times$ initial body protein (g), and the amount of feed protein = amount of feed consumed by fish/% mixed protein

Table 3 Proximate composition of commercial feed and *pindang tongkol*

Proximate (%) ^a	Commercial feed (Prima Feed PF 500)	<i>Pindang tongkol</i>
Ash	14	6.04
Fiber	4	0.24
Protein	39	35.80
Lipid	5	12.04
Carbohydrate	38	45.88

^aProximate analysis conducted at Central Laboratory Universitas Padjadjaran following methods of AOAC (1990)

$$\text{PPV} = (\text{average final weight} \times \% \text{final body protein} - \text{average weight initial} \times \% \text{initial body protein}) / \text{total feed} \times \% \text{mixed protein}$$

The amino acid content of G₃ transgenic and non-transgenic catfish was analyzed with 0.5 g of fish muscle tissue at the end of the experiment using ultra-performance liquid chromatography (UPLC; ACQUITY UPLC-H Class, Waters, Milford, MA, USA). Samples were homogenized and hydrolyzed in 5 mL of 6 N HCl and then heated to 110 °C for 22 h. A total of 500 µL of solution were extracted using 0.45-µm filter paper, and 40 µL of alpha aminobutyric acid and 460 µL of AquaBidest were added. Approximately 10 µL of this solution was added to 70 µL of AccQ-Fluor Borate and 20 µL of Fluor A reagent, and total mixtures were vortexed for 1 min. Finally, solutions were incubated at 55 °C for 10 min before being injected into the UPLC system (Rosmawati et al. 2018; Ignatz et al. 2020).

Statistical analysis

Data on weight gain, FCR, protein content, and amino acid content were quantitatively analyzed using one-way ANOVA, with a significance level of $p < 0.05$ used to test differences between the transgenic and non-transgenic fish, and a post hoc Duncan's multiple range test used to assess significance of differences among treatments. The frequency of CgGH of inheritance and CgGH/β-actin expression ratio of G₃ fish were analyzed using comparative descriptions.

Results

Detection of CgGH gene in G₂ transgenic broodstock

RT-PCR analysis positively identified male and female G₂ mutiara catfish broodstock with the CgGH transgene (i.e., exhibiting a PCR amplification products of 600 bp), indicating that both were transgenic and appropriate for G₃ catfish production (Fig. 3).

Fig. 3 Electropherogram for detection of female and male G₂ transgenic mutiara catfish (*Clarias gariepinus*) broodstock. CgGH represents *C. gariepinus* growth hormone (600 bp) and β-actin (200 bp) represents the internal expression control. Tested broodstock: one female and two males. P, pCMV-CgGH plasmid; M1, 1-kb DNA ladder; M2, 100-bp DNA ladder

Transmission and expression of CgGH gene

Transmission of genes in transgenic catfish was expected to be inherited following the Mendelian law based on our previous study (Fig. 2). Observation based on the RT-PCR analysis of G₃ catfish fingerlings showed that inheritance of the CgGH transgene was 70%, an increase over 50% inheritance reported in G₂ transgenic mutiara catfish (Buwono et al. 2019a), therefore crossing between G₂ transgenic broodstock tends to increase the transmission of CgGH in G₃. The level of exogenous GH gene expression also tended to be higher in G₃ than in G₂ catfish (Table 4 and Fig. 4).

Growth performance

After 42 days, body weight measurements and proximate analysis of G₃ catfish showed a tendency for body protein content of transgenic catfish in each treatment to be higher than in non-transgenic catfish (Table 5). A concurrent trend of increased body weight at the end of the experiment was also observed (Table 5 and Fig. 5a). Furthermore, feed conversion rate was higher in transgenic catfish than in non-transgenic catfish (Fig. 5b).

Protein content of G₃ catfish

The protein content of G₃ transgenic mutiara catfish varied depending on the ratio between commercial feed and *pindang tongkol*. The feed composition was predicted to increase the responsiveness of fish to feed, thereby stimulating higher consumption and greater growth and promoted protein accumulation in the fish body (Table 5). Increased protein storage was reflected in PR values, which were typically greater for transgenic catfish than for non-transgenic catfish. The exception was fish fed feed C that showed no increase in PR (Fig. 6a). PPV of transgenic catfish (given feeds A, B, or C) was higher than PPV of non-transgenic catfish (given feed A*, Fig. 6b).

Amino acid content of G₃ fish

Analysis of essential and non-essential amino acid content in G₃ transgenic and non-transgenic mutiara catfish indicated that both groups of amino acids tended to show higher levels in transgenic catfish (Table 6; Fig. 7a and b).

Table 4 Transmission and expression levels of CgGH in G₃ transgenic mutiara catfish (*Clarias gariepinus*) fingerlings

G ₃ transgenic mutiara catfish	T (%)	Transmission and expression levels (CgGH/ β -actin ratio) ^a									
		Lane ^b									
A (sample number 1–10)	70 (28/40)	1	2	3	4	5	6	7	8	9	10
		0	0	0	2.67	2.58	1.87	1.80	2.56	2.14	2.04
B (sample number 11–20)	70 (28/40)	11	12	13	14	15	16	17	18	19	20
		2.71	2.82	0	3.21	2.78	0	0	2.72	2.68	2.80

^a The β -actin gene was used as an internal expression control

^b Each lane reflects four fingerling fin samples

T, transmission; CgGH, *Clarias gariepinus* growth hormone

Fig. 4 Electropherogram of CgGH expression in transgenic mutiara catfish (*Clarias gariepinus*) fingerlings with numbers 1–10 (a) and numbers 11–20 (b). CgGH represents the *C. gariepinus* growth hormone gene (600 bp), and β-actin (200 bp) represents the internal expression control. The numbers 1–20 represent the fish sample number. M1, 1-kb DNA ladder; M2, 100-bp DNA ladder; P, pCMV-CgGH plasmid

Table 5 Measurements of body weight, feed consumed, and proximate analysis for G₃ transgenic and non-transgenic mutiara catfish (*Clarias gariepinus*)

	Transgenic		Non-transgenic	
	Test feed			
	A	B	C	A*
Average Wi (g)	6.09 ± 0.02	6.14 ± 0.02	6.22 ± 0.04	6.19 ± 0.01
Average Wf (g)	105.39 ± 0.55 ^a	130.63 ± 0.38 ^b	87.19 ± 1.97 ^c	41.60 ± 0.06 ^d
Average total feed (g)	446.06 ± 2.79 ^a	488.17 ± 4.09 ^b	406.05 ± 13.75 ^c	240.45 ± 0.64 ^d
Proximate (%)				
Initial				
Water	74.57	74.57	74.57	72.87
Ash	1.74	1.74	1.74	1.96
Lipid	2.97	2.97	2.97	4.90
Protein	11.55	11.55	11.55	10.95
Carbohydrate	9.20	9.20	9.20	9.32
Final				
Water	74.97 ± 0.75 ^a	72.78 ± 0.97 ^a	74.74 ± 0.26 ^a	72.57 ± 0.56 ^a
Ash	1.36 ± 0.03 ^a	1.36 ± 0.04 ^a	1.27 ± 0.06 ^a	1.57 ± 0.09 ^a
Lipid	1.86 ± 0.21 ^a	1.92 ± 0.08 ^a	2.56 ± 0.17 ^b	4.79 ± 0.06 ^c
Protein	15.51 ± 0.61 ^a	17.99 ± 0.27 ^b	13.46 ± 0.17 ^c	11.12 ± 0.11 ^d
Carbohydrate	6.29 ± 0.34 ^a	6.53 ± 0.61 ^a	8.19 ± 0.29 ^b	9.93 ± 0.45 ^c

Data are represented as means ± SEM. Across rows, means followed by the same letter are not significantly different ($p \geq 0.05$). Feeds contained commercial feed and boiled tuna (*Euthynnus affinis*) at the following ratios: A (50:50), B (65:35), C (80:20), and A* (50:50). Feeds A–C were fed to transgenic catfish, while feed A* was fed to non-transgenic catfish. Wi, initial weight; Wf, final weight

Fig. 5 Average weight gain (a) and feed conversion ratio (b) for G_3 transgenic and non-transgenic mutiara catfish (*Clarias gariepinus*). Data are represented as means $\pm$ SEM. Different lowercase letters indicate that the means are significantly different ($p < 0.05$). Feeds contained commercial feed and boiled tuna (*Euthymus affinis*) at the following ratios: A (50:50), B (65:35), C (80:20), and A* (50:50). Feeds A–C were fed to transgenic catfish, while feed A* was fed to non-transgenic catfish

Discussion

Stability of CgGH expression and inheritance in G_3 transgenic mutiara catfish

The transmission of exogenous transgene from one generation to the next depends on stable integration into host genomes. Expression of the exogenous gene may produce a phenotypic improvement of transgenic fish (Chen et al. 2015). A high percentage of transgene transmission is vital for ensuring inheritance of exogenous genes in future generations. CgGH gene inheritance and expression levels in the present study were higher in G_3 transgenic mutiara catfish fingerlings (70%) than in G_2 catfish (50%) in a previous study (Buwono 2019a), as was CgGH/ β -actin expression ratio (1.80–3.21 vs. 0.96–1.37, respectively). We suspected that the increased transmission of CgGH transgene in the third generation was related to the use of G_2 transgenic female broodstock crossed between transgenics in the homozygous state making it possible for more individuals to carry the transgene (Wang et al. 2001; Wu et al. 2003). Based

Fig. 6 Protein retention (a) and protein productive value (b) of G_3 transgenic and non-transgenic mutiara catfish (*Clarias gariepinus*). Different lowercase letters indicate that means are significantly different ($p < 0.05$). Feeds contained commercial feed and boiled tuna (*Euthymus affinis*) at the following ratios: A (50:50), B (65:35), C (80:20), and A* (50:50). Feeds A–C were fed to transgenic catfish, while feed A* was fed to non-transgenic catfish

Table 6 Amino acid content of G₃ transgenic and non-transgenic mutiara catfish (*Clarias gariepinus*)

Treatment	A	B	C	A*	<i>C. gariepinus</i> ¹
Amino acid:					
Essential					
Arginine	1.048 ± 0.001 ^a	1.238 ± 0.006 ^b	1.052 ± 0.025 ^a	0.865 ± 0.023 ^c	0.94 ± 0.08
Histidine	0.469 ± 0.009 ^a	0.597 ± 0.001 ^b	0.385 ± 0.008 ^c	0.364 ± 0.002 ^d	0.39 ± 0.04
Isoleucine	0.820 ± 0.007 ^a	0.880 ± 0.019 ^b	0.756 ± 0.026 ^c	0.708 ± 0.021 ^d	0.64 ± 0.06
Leucine	1.461 ± 0.050 ^a	1.567 ± 0.005 ^a	1.264 ± 0.082 ^b	1.294 ± 0.143 ^b	1.27 ± 0.13
Lysine	1.525 ± 0.008 ^a	1.587 ± 0.009 ^a	1.363 ± 0.055 ^b	1.076 ± 0.053 ^c	1.49 ± 0.12
Methionine	0.457 ± 0.002 ^a	0.573 ± 0.017 ^b	0.379 ± 0.017 ^c	0.272 ± 0.003 ^d	0.35 ± 0.04
Phenylalanine	0.924 ± 0.058 ^a	1.243 ± 0.113 ^b	0.791 ± 0.011 ^c	0.662 ± 0.006 ^d	0.68 ± 0.07
Threonine	1.046 ± 0.001 ^a	1.072 ± 0.002 ^a	0.950 ± 0.025 ^b	0.779 ± 0.089 ^c	0.63 ± 0.06
Valine	0.877 ± 0.003 ^a	0.988 ± 0.009 ^b	0.837 ± 0.021 ^c	0.788 ± 0.019 ^d	0.79 ± 0.09
Non-essential					
Glutamic acid	2.584 ± 0.154 ^a	2.838 ± 0.117 ^b	2.156 ± 0.015 ^a	2.015 ± 0.004 ^c	2.22 ± 0.22
Aspartic acid	1.520 ± 0.062 ^a	1.710 ± 0.052 ^b	1.440 ± 0.015 ^c	1.239 ± 0.013 ^d	1.51 ± 0.17
Glycine	0.907 ± 0.004 ^a	1.538 ± 0.055 ^b	0.832 ± 0.016 ^c	0.744 ± 0.026 ^d	0.73 ± 0.01
Serine	0.753 ± 0.007 ^a	0.850 ± 0.003 ^b	0.709 ± 0.011 ^c	0.662 ± 0.036 ^d	0.56 ± 0.06
Proline	0.610 ± 0.009 ^a	0.839 ± 0.038 ^b	0.558 ± 0.012 ^c	0.490 ± 0.019 ^d	0.75 ± 0.03
Tyrosine	0.643 ± 0.036 ^a	0.868 ± 0.006 ^b	0.537 ± 0.042 ^c	0.417 ± 0.058 ^d	0.56 ± 0.04
Alanine	0.908 ± 0.042 ^a	1.077 ± 0.002 ^b	0.850 ± 0.003 ^c	0.746 ± 0.024 ^d	0.90 ± 0.13
Cysteine	0.077 ± 0.006 ^a	0.087 ± 0.002 ^b	0.074 ± 0.001 ^a	0.062 ± 0.002 ^c	

Data are presented as means ± SD. Across rows, means followed by the same letter are not significantly different ($p \geq 0.05$). Feeds contained commercial feed and boiled tuna (*Euthynnus affinis*) at the following ratios: A (50:50), B (65:35), C (80:20), and A* (50:50). Feeds A–C were fed to transgenic catfish, while feed A* was fed to non-transgenic catfish

¹ Essential and non-essential amino acid contents of *C. gariepinus* (% wet weight) from Rosa et al. (2007)

on that theory, our G₂ transgenic catfish broodstock was potentially possible for G₃ catfish production. It was known that transmission of genes in transgenic fish suggest inheritance in a Mendelian fashion as reported in studies of G₃ transgenic mud loach (*Misgurnus mizolepis*) where 50–53.7% of inheritance was found, suggesting Mendelian inheritance in transgenic mud loach (Nam et al. 1999), while studies in transgenic tilapia also supports this theory (Martinez et al. 1999). Stable inheritance of exogenous GH genes will assure accompanying efficient expression in offspring (Philips and Devlin 2010).

The growth of G₃ mutiara catfish

On average, G₃ transgenic mutiara catfish weighed approximately 2 to 3 times more than non-transgenic fish at the end of the experiment. Significant weight difference was observed in G₃ transgenic catfish compared to non-transgenic (Table 5 and Fig. 8). Higher growth performance in transgenic fish was suggested to be caused by increase in food intake in transgenic fish (Raven et al. 2006; Higgs et al. 2009). Leggatt et al. (2009) suggested that transgenic coho salmon fish had an increased ability to use carbohydrates for energy to spare protein and lipid, hence supporting higher growth. This may explain results in our study where transgenic fish given the mixture of commercial feed with *pindang tongkol* had higher growth and protein content compared to the non-transgenic counterparts. In previous studies, increased growth was reported in transgenic common carp (containing transgene pCagcGH) at G₁ (1.6-fold) (Wang et al. 2001), G₂ (1.8–2.5-fold), and G₃ (1.4–1.9-fold) (Li et al. 2007) relative to non-transgenic fish. Thus, overexpression of exogenous GH gene may result in increased growth of transgenic fish, with increases > 100% possible (Nam et al. 2001).

Fig. 7 Essential (a) and non-essential (b) amino acid content of G₃ transgenic and non-transgenic mutiara catfish. Data are represented as means $\pm$ SD. Different lowercase letters indicate that the means are significantly different ($p < 0.05$). Feeds contained commercial feed and boiled tuna (*Euthynnus affinis*) at the following ratios: A (50:50), B (65:35), C (80:20), and A* (50:50). Feeds A–C were fed to transgenic catfish, while feed A* was fed to non-transgenic catfish

We found that G₃ transgenic mutiara catfish gained the most weight when fed feed B (65% commercial feed and 35% *pindang tongkol*). We suggest that the balanced protein mixture in feed B promoted increased expression of the CgGH gene that stimulates IGF-I production saying that this should be examined in future studies of GH gene expression in this line. IGF-I increases responsiveness to the feed and, in turn, accelerates the conversion of feed into body protein (Cook et al. 2000; Raven et al. 2008). This hypothesis is supported by the increase in catfish body protein levels observed at the end of the present study.

G₃ transgenic mutiara catfish fed with feed B showed both increased body weight and increased conversion of feed protein into body tissue protein. Consequently, these fish had 17.99% body protein content at the end of the experiment, which was $> 2\%$ higher than transgenic catfish fed on any other test feeds. Increased protein levels were shown previously in transgenic common carp, expressing rainbow trout growth hormone gene, with protein levels of 19.48% and 18.02% observed in G₁ and G₂, respectively (Chatakondi et al. 1995; Dunham et al. 2002). GH overexpression was suggested to be general importance for increasing fish growth, improving the efficiency of feed use, and increasing body protein (Oakes et al. 2007). In our study, higher performance was found for feed B. We infer that the nutrient composition in feed B increased the responsiveness of fish to feed compared to other test feeds.

Fig. 8 Weekly observations of average weight during the experiment

Feed conversion ratio

G₃ transgenic catfish showed improved FCR (means 0.78–1.00) relative to non-transgenic catfish (mean 1.36), consistent with increased fish growth. A previous study on transgenic Nile tilapia (*Oreochromis niloticus*) showed a similar FCR value of 0.76 (Rahman et al. 1998). In our study, feed B—with 37.88% protein—led to the lowest FCR, resulting in the greatest growth. The nutritional value of feed is determined by both the amount of protein and the composition of feed protein mixture. The latter is critical for providing essential amino acids (Fu et al. 2000; Wilson 1986; Raven et al. 2006; Rosa et al. 2007).

GH-transgenic fish efficiently convert lipids and carbohydrates in feed into energy for use in routine metabolic activities; therefore, feed protein is spared from the maintenance of basal metabolism. Excess protein is instead used for more rapid growth, as previously observed in GH-transgenic coho salmon (Oakes et al. 2007) and GH-transgenic Nile tilapia (Rahman and Maclean 1999). Protein sparing that promotes growth also was shown in the present study with transgenic catfish. Specifically, catfish were fed feeds A and B that had lipid contents of 1.86% and 1.92%, respectively, and carbohydrate contents of 6.29% and 6.53%, respectively, at the end of the experiment. These were the lowest levels among treatments. Final measurements in transgenic fish showed significantly lower carbohydrate content in transgenic fish samples compared to non-transgenic fish. This suggests the ability of transgenic fish to utilize carbohydrate as an energy source, hence lowering the carbohydrate content in fish. In addition, a protein-sparing action in transgenic fish may also occur causing an increase in protein storage.

Protein retention in G₃ mutiara catfish

We found that PR differed among G₃ transgenic and non-transgenic mutiara catfish according to feed. Feed B, with a commercial feed to *pindang tongkol* ratio of 65:35, increased PR in transgenic catfish relative to that in catfish fed feeds A (50:50 ratio) or C (80:20 ratio). Non-transgenic catfish fed a 50:50 ratio showed the lowest PR. These observations are supported by concurrent increases in catfish body protein content measured at the end of the experiment. The difference between PRs resulting from feeds B and C is perhaps due to different levels of *pindang tongkol* in these feeds. The lower amount of *pindang tongkol* in feed C likely reduced available amino acids that, in turn, reduced body protein content and therefore PR.

Protein stored in the body that includes sufficient essential amino acids to meet metabolic requirements has more influence on growth than high levels of feed protein (Wilson and Poe 1985; Cowey 1994; Fu et al. 2000). In the present study, reduced PR resulted in less average weight gain in G₃ transgenic mutiara catfish fed feed C, and non-transgenic fish fed feed A*. The utilization limit for *pindang tongkol* that resulted in optimum PR was 35%. Using 50% *pindang tongkol* did not result in higher PR in non-transgenic catfish (feed A*); however, PR in transgenic catfish fed feed A, also a 50:50 ratio, was higher than that of non-transgenic catfish. Including 50% *pindang tongkol* in feed did improve PR in transgenic catfish, but using 35% (feed B) provided better results. Overexpression of the CgGH gene thus effectively promoted the conversion of feeds A and B protein into body protein, and we conclude that the balance of essential to non-essential protein in these two feeds was likely sufficient for maximum catfish growth. Overexpression of exogenous GH increased protein levels and PR in transgenic coho salmon (Oakes et al. 2007; Higgs et al. 2009), and increased growth and PR in transgenic Arctic charr (*Salvelinus alpinus*) (Pitkanen et al. 1999) and transgenic Nile tilapia (Kobayashi et al. 2007).

Protein productive value of G₃ catfish

PPV, the ratio of a fish's body protein at the end of an experiment to feed protein consumed (Hepher 1988), can be used to evaluate protein use efficiency. G₃ transgenic mutiara catfish were more efficient at using feed protein than non-transgenic catfish: PPVs for G₃ transgenic mutiara catfish were 6.94–12.3%, but only 4.39% for non-transgenic catfish. Previous studies of GH-transgenic Atlantic salmon (*Salmo salar*) reported a similar PPV, 10% (Cook et al. 2000), whereas PPVs in GH-transgenic Nile tilapia were somewhat higher, 15–20% (Rahman et al. 2001). Our observed increase in PPV is likely due to CgGH gene expression, which increases protein synthesis and, in turn, the protein content of transgenic catfish. Exogenous GH induced glycolytic enzymes in transgenic coho salmon, which caused the catabolism of carbohydrates and lipids to produce metabolic energy and led to increased body protein deposits (Leggatt et al. 2009). Such an increase in protein correlates with the levels of exogenous GH expression observed in our study. Further, higher PPVs of G₃ transgenic mutiara catfish relative to those of non-transgenic catfish also supports these conclusions. Expression of exogenous GH in transgenic fish resulted in GH levels higher than found in non-transgenic individuals in previous studies. These levels led to increased body protein content (Devlin et al. 2000; Dunham et al. 2002; Raven et al. 2008; Higgs et al. 2009). Transgenesis increases the activity of enzymes involved in the Krebs cycle (TCA cycle); these enzymes break down carbohydrates and lipids and allow proteins to feed essential amino acid pools (Melzer 2011; Leggatt et al. 2009).

In our study, PPVs were closely correlated with fish PR values as well as ratios of mixed proteins in feeds. The PPVs of catfish fed feeds B and C were proportional to the increases and decreases, respectively, in the PR of fish that consumed these feeds. In addition, protein-sparing was observed in G₃ transgenic catfish fed feeds A and B, as indicated by the decreased end-of-experiment lipid and carbohydrate levels of catfish that consumed these feeds; this decrease likely caused PR and PPV to be higher in transgenic catfish.

Amino acid content of G₃ transgenic catfish

The protein content in feed can affect the ability of the feed to provide essential amino acids; the content of the amino acids in feed protein determines the balance between essential and non-essential amino acids. An imbalance in essential and non-essential amino acids can lead to amino acid deficiency, which will limit weight gain (Wilson and Poe 1985; Cowey 1994; Ahmed 2012; Cao et al. 2012; Zhang et al. 2017).

G₃ transgenic mutiara catfish had higher essential amino acid content than non-transgenic catfish. The suggested effect of GH was its importance in affecting the breakdown of carbohydrates and lipids through a complex enzymatic pathways into pyruvic acid and then converted to amino acids; hence, both contribute to the increase of amino acid content (Melzer 2011). Feed B led to the highest essential amino acid content among transgenic fish; the content in these fish was greater than the essential amino acid requirement for *C. gariepinus* (Rosa et al. 2007). While feeds A, B, and C increased the weight gain of transgenic fish by 2.8-, 3.5-, and 2.3-fold, respectively, non-transgenic catfish fed feed A* (equivalent to feed A) were deficient in histidine, lysine, and methionine (Rosa et al. 2007). Thus, increasing the amount of essential amino acids in feed above standard requirements can substantially boost weight gain. Other transgenic fish species also showed an increase in essential amino acid content, e.g., GH-transgenic olive flounder (*Paralichthys olivaceus*) when fed commercial feed (Liu et al. 2008) and GH-transgenic *Cyprinus carpio* (Chatakondi et al. 1995; Fu et al. 2000). Essential amino acid content increased in the presence of exogenous GH, which may reflect the role of GH in influence intestinal absorption and renal reabsorption of protein and amino acid metabolism in fish (Farmanfarmaian and Sun 1999).

Deficiency in any one of the three limiting essential amino acids that must be available in fish feed, namely arginine, lysine, and methionine, leads to reduced fish growth (Green and Hardy 2002; Ahmed 2012). These amino acids are thus limiting factors for growth (Wilson and Poe 1985) and must be available in feed at levels sufficient to meet requirements for protein synthesis (Small and Soares 1998; Green and Hardy 2002). Our results showed that arginine, lysine, and methionine were present at increased levels, above the standard requirement for *C. gariepinus* (Rosa et al. 2007), in G₃ catfish fed feeds A and B; however, consuming feed C led to a lysine deficiency and decreased weight gain, PR, and PPV. Non-transgenic catfish having been fed only feed A* were deficient in both lysine and methionine. Feed B increased the content of limiting essential amino acids in transgenic catfish to levels that were sufficient for optimum growth.

Feed B also increased the levels of non-essential amino acids (glutamic acid, aspartic acid, glycine, serine, proline, tyrosine, and alanine) in G₃ transgenic mutiara catfish above those required for *C. gariepinus* (Rosa et al. 2007). In contrast, consuming feed A led to proline deficiency; feed C led to aspartic acid and proline deficiency; and feed A* led to deficiencies in aspartic acid, proline, and tyrosine. Given that consuming feed B led to optimal growth in G₃ transgenic mutiara catfish, these results indicate that enough non-essential amino acids are required in fish feed to support the optimal growth of transgenic catfish.

Conclusions

The effect of CgGH gene transgenesis on growth and protein and amino acid content of G₃ transgenic mutiara catfish was affected by the ratio of commercial feed and *pindang tongkol* in the feed mix. We identified a feed mix that improved body protein content and increased levels of essential and non-essential amino acids of G₃ transgenic mutiara catfish. Using feeds deficient in essential or non-essential or amino acids reduced fish growth, PR, and efficiency of feed protein utilization. By finding an optimum balance of one feed component mixture, we provided important information that can be used to improve growth and feeding efficiency in transgenic catfish.

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Authors' contributions IDB and II conducted the fish trial, reared fish, and collected data; IDB and RG analyzed and interpreted data and wrote the manuscript. The design of the study and data analysis and manuscript formatting involved all authors. All authors also critically reviewed the manuscript for intellectual content and gave final approval for the manuscript to be published.

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Data availability All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical approval This article does not require ethic approval since fish was not listed by the Ethic Commission of Education Ministry and Universitas Padjadjaran Ethic Commission. However, guidelines on fish handling during and after research were based on literature on fish welfare (Terlouw et al. 2008).

Abbreviations

CgGH, *Clarias gariepinus* growth hormone;

CMVOnGHI, cytomegalovirus *Oncorhynchus* growth hormone 1;

FCR, feed conversion ratio;

OnMTGHI, *Oncorhynchus* metallothionein growth hormone 1;

pCAGcGH, plasmid Carp actin grass carp growth hormone;

pCMV-CgGH, plasmid Cytomegalovirus *Clarias gariepinus* growth hormone;

<i>PPV</i> ,	protein productive value;
<i>PR</i> ,	protein retention;
<i>UPLC</i> ,	ultra-performance liquid chromatography

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