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EFFECTS OF DIETARY RECOMBINANT GROWTH HORMONE WITH WATERBORNE CALCIUM CARBONATE SUPPLEMENTATION ON GROWTH PERFORMANCE AND GH–IGF AXIS-RELATED GENE EXPRESSION IN JUVENILE SNAKEHEAD (*Channa striata*)

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ABSTRACT

This study evaluated the effects of dietary recombinant growth hormone (rGH) and waterborne calcium carbonate ($CaCO_3$) supplementation on growth performance, feed utilization, body composition, and growth-related gene expression in snakehead (Channa striata) juveniles. The experiment was conducted for 90 days using four treatments: control (R0C0, without rGH and $CaCO_3$ supplementation), treatment 1 (R5C0, 5 mg kg^{-1} rGH and without $CaCO_3$ supplementation), treatment 2 (R5C25, rGH and 25 mg L^{-1} $CaCO_3$ supplementation), and treatment 3 (R5C50 rGH and 50 mg L^{-1} $CaCO_3$ supplementation), each with six replicates. The results showed that rGH significantly increased final body weight (BWt) and specific growth rate, and reduced feed conversion ratio (FCR) compared with the control. Final body weight was significantly higher in R5C0 and R5C50 than in the control, with no significant difference between these treatments. In contrast, body length increased significantly with increasing $CaCO_3$ dose, with the highest value observed in R5C50. Feed conversion ratio decreased significantly in all rGH treatments but did not differ among R5C0, R5C25, and R5C50. Protein and lipid retention were highest in R5C0 and decreased with $CaCO_3$ supplementation. Body calcium content increased with $CaCO_3$ supplementation, with the highest value observed in R5C50. Gene expression analysis showed that rGH significantly increased GH and IGF-1 expression compared to the control, while no consistent significant differences were observed among combined treatments. These findings suggest that rGH predominantly enhanced weight gain and feed utilization, whereas $CaCO_3$ supplementation under rGH treatment was associated with increased body length. The combined treatments resulted in complementary but parameter-specific effects.

KEYWORDS: Calcium; Growth; Recombinant Growth Hormone; *Channa striata*

INTRODUCTION

Aquaculture has become one of the fastest growing food production sectors worldwide and plays an important role in supporting global food security through the provision of high-quality animal protein. In Southeast Asia, snakehead fish (*Channa striata*) is recognized as a high-value freshwater species due to its strong market demand and nutritional benefits. This species is widely distributed across Asia, including China, India, Sri Lanka, Thailand, Malaysia, Indonesia, and the Philippines (FAO, 2010). In Indonesia, snakehead fish naturally inhabits various freshwater ecosystems such as rivers, swamps, and floodplains in regions including Sumatra, Kalimantan, and Java (Saputra *et al.,* 2021). In addition to its economic value, snakehead fish is also known for its high albumin content, which has been associated with accelerating wound healing and supporting post-surgical recovery (Mustafa *et al.,* 2012).

Despite its high economic potential, the development of snakehead aquaculture remains constrained by relatively slow growth during the juvenile stage. Under conventional culture conditions, snakehead fish may require approximately four to six months to reach marketable size (Gustiano *et al.,* 2019). The prolonged culture period can increase production costs and reduce farming efficiency. Therefore, the development of effective strategies to accelerate growth and improve feed utilization efficiency is essential for enhancing the productivity and sustainability of snakehead aquaculture.

Growth hormone (GH) plays a central role in regulating somatic growth, metabolism, and nutrient utilization in vertebrates. In teleost fish, growth hormone stimulates the production of insulin-like growth

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factor-1 (IGF-1), which mediates somatic growth through the GH-IGF endocrine axis (Reinecke *et al.,* 2005). Advances in biotechnology have enabled the production of recombinant growth hormone (rGH), which has been applied in several aquaculture species to enhance growth performance, improve feed efficiency, and performance, and stimulate physiological growth processes.

In addition to endocrine regulation, mineral availability in the culture environment plays a crucial role in supporting physiological processes related to growth. Calcium is an essential mineral involved in skeletal development, osmoregulation, and enzymatic activity (Lall, 2002). In aquatic organisms, calcium can be absorbed directly from the surrounding water through branchial and epithelial uptake pathways, making water chemistry an important determinant of mineral availability (Guerreiro *et al.,* 2004). The addition of calcium carbonate ($CaCO_3$) is commonly used in aquaculture systems to increase water pH, alkalinity, and hardness, thereby improving water quality and buffering capacity (Cavalcante *et al*., 2009).

In addition to its role in improving water quality, calcium is recognized as an important physiological element in aquatic organisms and may contribute to growth-related physiological processes through its involvement in intracellular signaling and hormonal regulation (Halmos *et al*., 2025; Fuentes *et al*., 2013). Calcium entering the cells through epithelial calcium channels (ECaC) and transported by calbindin-D9k may act as a second messenger involved in hormonal responses (Liao *et al*., 2007; Lin & Hwang, 2016). In aquaculture systems, adequate calcium availability not only supports tissue formation and structural development in fish but also contributes to osmoregulatory processes and the maintenance of water quality stability. Several studies have reported that $CaCO_3$ application in culture media can improve water quality parameters, such as pH and alkalinity, thereby potentially supporting fish growth and survival performance.

Therefore, this study aimed to investigate the combined effects of dietary recombinant growth hormone and calcium carbonate supplementation on growth performance, feed efficiency, body calcium content, body proximate composition, and growth-related gene expression in snakehead juveniles. Understanding these physiological and molecular responses is expected to provide new insights into endocrine and nutritional strategies for improving growth performance and production efficiency in snakehead aquaculture.

MATERIALS AND METHODS

Study Site and Experimental Conditions

This study was conducted from July to October 2025. Fish rearing and gene expression analyses were carried out at the Laboratory of Reproduction and Genetics of Aquatic Organisms, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, Indonesia. Water quality analysis was performed at the Laboratory of Aquatic Productivity and Environment, IPB University. Analysis of calcium content in water and fish body, as well as proximate composition analysis, were conducted at the Advanced Laboratory and Collaborative Center (Biotech Center), IPB University.

Experimental Design

The dosage of $CaCO_3$ in this study was determined based on preliminary testing of calcium (Ca^{2+}) content in the water. The preliminary test was carried out using 15 fish per treatment. Four $CaCO_3$ dose levels were tested, namely 50, 100, 150, and 200 mg L^{-1}. The results indicated that doses ranging from 100 to 200 mg L^{-1} caused mass mortality in the test fish. The preliminary test was carried out using 15 fish per treatment. Fish used in the preliminary test had similar initial body weight (3.77 ± 0.10 g) and total length (5.82 ± 0.06 cm) to those used in the main experiment, and the water conditions were maintained identically to the main culture system to ensure consistency between the preliminary and main experiments.

The main study employed a one-factor treatment design with four treatments groups and six aquaria per treatment, including four aquaria for growth performance and two aquaria for gene expression analysis. The study employed a one-factor treatment design consisting of different $CaCO_3$ supplementation levels under fixed dietary rGH administration, except for the control group without rGH and $CaCO_3$ (Table 1).

Water Stock Preparation

The water used in this study was groundwater that had undergone a sedimentation process prior to use. The $CaCO_3$ used was produced by Takehara Chemical Industry, Japan. Rearing water was prepared in a 200 L container. $CaCO_3$ was added according to the prescribed dosage for each treatment (mg L^{-1}), followed by continuous stirring and aeration to ensure uniform dispersion. The solution was allowed to stand for 24 hours to permit the settling of coarse particles. The sediment accumulated at the bottom of the container was siphoned off prior to the use of the water as a maintenance medium.

After the sedimentation process and removal of coarse particles, water samples from each treatment were collected prior to fish stocking to determine the initial calcium concentration in the stock water following $CaCO_3$ addition. These measurements were conducted before the introduction of fish to distinguish the initial Ca levels from those measured during the culture period. The results are presented in Table 2.

Table 1. Experimental design of the study

Treatment	Description
Control (R0C0)	Feed with rGH 0 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}
T1 (R5C0)	Feed supplemented with rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}
T2 (R5C25)	Feed supplemented with rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}
T3 (R5C50)	Feed supplemented with rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}

Table 2. Initial calcium concentration in stock water before fish stocking

Treatment	Initial Ca concentration (mg L^{-1})
Control (R0C0)	13.27 ± 0.38
T1 (R5C0)	13.27 ± 0.38
T2 (R5C25)	24.58 ± 0.96
T3 (R5C50)	33.14 ± 0.60

Preparation of rGH and Feed

The rGH was obtained from the Freshwater Aquaculture Development Main Center (BBPBAT) Sukabumi, West Java, in powder form produced through a freeze-drying process. Prior to use, the rGH powder was stored at 4°C to maintain its stability and activity. The feed used contained 38–40% crude protein. The rGH protein was dissolved in phosphate-buffered saline (PBS) at 2 mL kg^{-1} feed and further diluted with 50 mL of water prior to application. The solution was evenly sprayed onto the feed according to the designated dosage while continuously mixing to ensure uniform coating. Carboxymethyl cellulose (CMC; 10 g kg^{-1} feed) was used as a binder to improve adhesion of the rGH solution to the feed pellets. Feed for the control treatment followed the same procedure, including PBS and CMC addition, but without rGH supplementation. After spraying, the treated feed was air-dried at room temperature to prevent clumping. The feed was then stored in an airtight container at 4°C. The rGH-supplemented feed was prepared periodically according to daily consumption requirements to maintain hormone stability and activity.

Fish Rearing

Snakehead juveniles used in this study were obtained from a local hatchery and transported to the laboratory. Prior to the experiment, fish were acclimated for two weeks under laboratory conditions. During the acclimation period, fish were fed a commercial diet containing 38–40% crude protein twice daily until apparent satiation. Only healthy fish with relatively uniform body sizes were selected for the feeding trial. At the beginning of the experiment, fish had an average initial body weight of 3.77 ± 0.10 g.

The experiment was conducted in glass aquaria measuring 50 × 60 × 50 cm with a water depth of 15 cm. Each aquarium was stocked with 15 fish. Fish were maintained under static water conditions without additional aeration because snakehead fish possess an accessory respiratory organ that allows them to tolerate low dissolved oxygen concentrations. No additional aeration was applied because snakehead fish possess an accessory respiratory organ (suprabranchial organ), enabling them to utilize atmospheric oxygen and tolerate low dissolved oxygen conditions. This rearing condition was intended to simulate the natural habitat of snakehead and minimize disturbance during culture. Fish were fed twice daily at 08:00 and 16:00 to apparent satiation throughout the 90-day experimental period. Feed was provided gradually until fish ceased active feeding behavior. Uneaten feed was removed by siphoning before the next feeding and was recorded when necessary to maintain water quality and support feed conversion ratio calculations.. To maintain water quality, 30% of the culture water was exchanged daily, and the replacement water was adjusted according to the calcium concentration required for each treatment.

Sampling and Observation

Parameter sampling was conducted periodically during the 90-day maintenance period. Fish body length and weight were measured on days 0, 15, 30, 45, 60, 75, and 90 for all test fish in each experimental unit. Mortality was recorded daily to calculate survival rate. Feed consumption was also recorded daily to determine feed conversion ratio and feed utilization efficiency.

Although body weight and length were recorded periodically during the experiment, statistical comparisons were performed only using final growth parameters (BWt, BLt, SGR, FCR, and SR). Intermediate measurements were used solely to monitor growth progression during the rearing period and were not included in inferential statistical analysis.

Gene expression analysis

Tissue samples were collected on days 0, 3, 15, 30, 60, and 90. Prior to tissue sampling, fish were anesthetized using a commercial fish anesthetic to minimize handling stress. Pituitary organ were collected for GH gene expression analysis, whereas liver were collected for IGF-1 gene expression analysis. Samples were stored in GENEzol^{TM} reagent and kept at -80ºC until analysis using quantitative real-time PCR (qPCR). The qPCR reaction was performed using the MSLPCR05 real-time PCR detection system, with a total reaction volume of 20 µL.

Nucleotide sequences of primers used for qPCR analysis are as presented in Table 3. The specificity of the primer was evaluated based on qPCR amplification performance. The results demonstrated specific amplification of the target gene with no indication of primer-dimer formation, The components of the reaction included 10 µL of SensiFAST SYBR® NO-ROX mix (Bioline, UK), 0.8 µL of primer (10µM), 4 µL of cDNA (50ng µL^{-1}) and 4.4 µL of nuclease-free water. Gene expression levels were normalised to the expression of the â-actin gene, which was used as an internal RNA loading control. Analysis was performed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 3. Nucleotide sequences of primers and qPCR annealing temperatures

Primer	Sequence (5′–3′)	Annealing Temperature (°C)	Reference
GH Fw	CGTACCTGACTGTGGCGAAA	54	
GH Rv	AAGCAGAGTCAGGGGAGTTG	54	
IGF-1 Fw	TCTGTGATGTTGACGAGTGGT	56	Sathishkumar *et al.,* (2024)
IGF-1 Rv	AGCCTGAAATGTTGGGAGTG	56	
β-actin Fw	GCCTTCCTTCCTTGGTATGG	59	Sathishkumar *et al.* (2024)
β-actin Rv	GTGTTGGCGTACAGGTCCTT	59	

Growth Performance Analysis

Fish were weighed at the beginning and at the end of the experimental period to determine growth performance. Weight gain (WG) was calculated as the difference between final body weight (BWt) and initial body weight (W0). Specific growth rate (SGR) was calculated using the formula SGR (% day^{-1}) = [(ln Wt - ln W0) / t] × 100, where t represents the duration of the experimental period in days. Length gain (LG) was calculated as the difference between final body length (BLt) and initial body length (L0). Length-specific growth rate (SGRl) was calculated using the formula: SGRl (% day^{-1}) = [(ln BLt - ln BL0) / t] × 100, where BLt represents final body length, BL0 represents initial body length, and t is the duration of the experimental period in days. Feed conversion ratio (FCR) was calculated as the ratio between total feed intake and total weight gain. Survival rate (SR) was calculated as SR (%) = (Nt / N0) × 100, where N0 represents the initial number of fish and Nt represents the number of fish at the end of the experiment.

Proximate Composition Analysis

Proximate analysis was performed according to AOAC (1995) methods, including moisture content by oven drying at 105°C to constant weight, ash content by dry ashing at 550°C, crude protein by the Kjeldahl method, crude fat by Soxhlet extraction, crude fiber by acid–alkali hydrolysis, and carbohydrate content calculated by difference

Protein retention (PR) and fat retention (FR) were calculated to assess nutrient utilization efficiency using the following equations: PR (%) = [(final body protein - initial body protein) / protein intake] × 100, and FR (%) = [(final body lipid - initial body lipid) / lipid intake] × 100.

Body Calcium Content Analysis

Body calcium content was analyzed at the beginning and at the end of the rearing period using Atomic Absorption Spectrophotometry (AAS) following the methods described Idrus *et al.* (2020). Prior to sample collection, fish were anesthetized using a commercial fish anesthetic to minimize handling stress and ensure humane sampling procedures. Whole-body samples were subsequently collected for calcium analysis. Body calcium concentration was then determined using an atomic absorption spectrophotometer at a wavelength of 422.7 nm (Dewi *et al.,* 2021).

Water Quality Monitoring

Water quality parameters were monitored throughout the experimental period to ensure suitable environmental conditions for fish culture. The parameters measured included temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), alkalinity, total hardness, , and calcium concentration. Temperature, pH, and dissolved oxygen were measured daily, while TAN, alkalinity, hardness, and calcium concentration were measured every 15 days.

Statistical Analysis

Data were analyzed using SPSS software version 26. For growth performance analysis, each aquarium was considered as the experimental unit, whereas individual fish within the same aquarium were treated as subsamples. Although body weight and length were measured periodically throughout the experiment, statistical analyses were performed only using final growth parameters. Normally distributed and homogeneous data were analyzed using analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for multiple comparisons at a 95% confidence level. Data that did not meet the assumptions of normality and/or homogeneity were analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparison. Water quality data were also subjected to statistical analysis. Gene expression data were analyzed descriptively. Differences among treatments were considered statistically significant at $p < 0.05$.

Statement on the Use of AI

In drafting this manuscript, the author used AI technology to a limited extent for improving grammar, sentence structure, and clarity. AI was not used to generate scientific content, data analysis, interpretation of results, or drawing conclusions. The authors remain fully responsible for the content and scientific integrity of this manuscript.

RESULTS AND DISCUSSION

Growth Performance

The survival rate of snakehead juveniles reached 100% across all treatments during the 90-day rearing period, indicating that dietary administration of recombinant growth hormone (rGH) and $CaCO_3$ supplementation up to 50 mg L^{-1} did not result in treatment-related mortality (Table 4). This finding suggests that all treatments remained within the tolerance range of snakehead juveniles. However, physiological stress responses were not directly assessed in the present study; therefore, survival alone cannot be used to confirm the absence of stress(Sulistiyanto *et al.*, 2024; Taruna *et al.*, 2021; Muslim *et al.*, 2021).

Dietary rGH significantly improved final body weight (BWt) and weight-specific growth rate (SGRw) compared to the control ($p < 0.05$). The highest BWt was observed in T1 (22.75 ± 1.52 g), which was not significantly different compared to T3 (21.59 ± 0.62 g), indicating that increasing $CaCO_3$ concentration from 0 to 50 mg L^{-1} was not associated with addi-

Table 4. Survival rate and growth performance of snakehead juveniles fed rGH-supplemented diet and addition of $CaCO_3$ at different doses in the rearing water

Parameters	Control (R0C0)	T1 (R5C0)	T2 (R5C25)	T3 (R5C50)
SR (%)	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
BWt (g)	12.14 ± 0.45^{a}	22.75 ± 1.52^{c}	20.04 ± 0.75^{b}	21.59 ± 0.62^{bc}
SGRw (% day^{-1})	2.50 ± 0.04^{a}	3.11 ± 0.07^{c}	2.98 ± 0.04^{b}	3.06 ± 0.03^{bc}
BLt (cm)	12.40 ± 0.20^{a}	13.71 ± 0.19^{b}	4.14 ± 0.45^{b}	14.88 ± 0.14^{c}
SGRl (% day^{-1})	2.50 ± 0.02^{a}	2.60 ± 0.01^{b}	2.63 ± 0.03^{b}	2.68 ± 0.01^{c}

Note: Control (R0C0 = rGH 0 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T1 (R5C0 = rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T2 (R5C25 = rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}); T3 (R5C50 = rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}). SR = Survival rate. BWt = Final body weight. SGRw = Specific growth rate of body weight. BLt = Final body length. SGRl = Specific growth rate of body length. The mean values in the same rows followed by different superscript letters indicate significant differences at the 5% level of significance ($p < 0.05$).

tional improvement in body weight under rGH administration. Both T1 and T3 showed significantly higher BWt than T2 (20.04 ± 0.75 g) and the control (12.14 ± 0.45 g). A similar pattern was observed in SGRw values. These findings suggest that the observed weight gain may be associated with rGH-mediated regulation involving the GH–IGF axis, which stimulates protein synthesis and somatic tissue accretion (Björnsson, 1998; Reinecke *et al.*, 2005; Fuentes *et al.*, 2013).

In contrast, body length growth responded differently to $CaCO_3$ supplementation. Final body length (BLt) increased significantly with increasing $CaCO_3$ levels ($p < 0.05$), with the highest value recorded in T3 (14.88 ± 0.14 cm), followed by T2 (14.14 ± 0.45 cm), T1 (13.71 ± 0.19 cm), and the control (12.40 ± 0.20 cm). A similar increasing trend was also observed in length-specific growth rate (SGRl).

The increase in length growth without a proportional increase in body weight suggests that calcium availability may have contributed to structural growth processes rather than soft tissue accumulation. The increase in body calcium content observed in $CaCO_3$ -supplemented groups suggests that higher calcium availability was associated with increased calcium accumulation in fish and may be related to the greater body length growth observed in T2 and T3. Although skeletal mineralization was not directly measured in the present study, body calcium accumulation provides indirect supporting evidence that calcium may be involved in structural growth processes. This interpretation is consistent with the known role of calcium in skeletal development and mineralization in fish (Lall, 2002; Lall & Kaushik, 2021). Calcium contributes to biomineralization through the deposition of hydroxyapatite within the collagen matrix of bone tissue (Murshed, 2018).

Feed Efficiency and Body Composition

The feed conversion ratio (FCR) was significantly lower in all rGH-treated groups compared to the control ($p < 0.05$), indicating improved feed utilization efficiency (Figure 1). However, no significant differences were observed among T1, T2, and T3 despite increasing $CaCO_3$ levels. This result suggests that feed efficiency was more closely associated with rGH administration than with $CaCO_3$ supplementation. Growth hormone is known to regulate the GH–IGF axis, thereby improving nutrient utilization efficiency and protein accretion, leading to enhanced growth performance and lower feed conversion values (Fuentes *et al.,* 2013; Pérez-Sánchez *et al.,* 2018), ance and lower feed conversion values (Björnsson, 1998; Perez-Sanchez, 2018).

Whole-body proximate composition showed no significant differences in moisture content among treatments ($p > 0.05$). The detailed results of whole-body proximate composition and nutrient retention parameters are presented in Table 5. However, crude protein content was significantly higher in rGH-treated fish, particularly in T1 (15.38 ± 0.62%), compared with the control. A clearer trend was observed in nutrient retention parameters. Protein retention and fat retention were highest in T1, with values of 22.54 ±

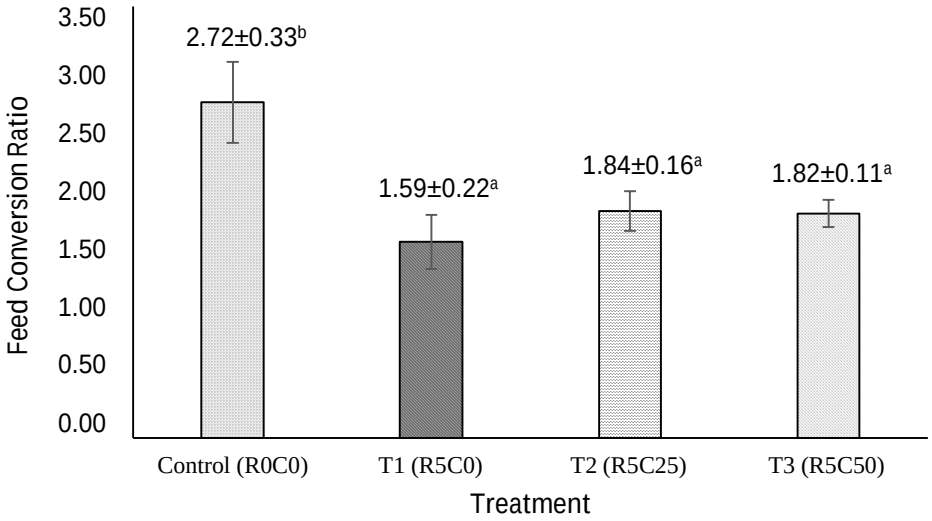

Figure 1. Feed conversion ratio values of snakehead juveniles fed rGH- supplemented diet and addition of $CaCO_3$ at different doses in the rearing water. Control (R0C0 = rGH 0 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T1 (R5C0 = rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T2 (R5C25 = rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}); T3 (R5C50 = rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}). The average values followed by the same superscript letters indicate significantly different at the 5% level of significance ($p<0.05$).

3.88% and 112.15 ± 2.93%, respectively, and were significantly higher than those in T2 and T3 ($p < 0.05$). Meanwhile, T2 and T3 showed lower retention values than T1, while not significantly differences were observed between the two treatments.

The reduction in nutrient retention observed in $CaCO_3$ -supplemented treatments may indicate differences in nutrient utilization patterns compared with fish receiving rGH without $CaCO_3$ supplementation. This interpretation is supported by the higher body length growth observed in T2 and T3. Although skeletal mineralization was not directly assessed in the present study, the growth pattern suggests that $CaCO_3$ supplementation may have contributed to structural growth processes, although skeletal development was not directly evaluated. Therefore, nutrient utilization for protein and lipid accumulation appeared to be relatively more efficient in fish receiving rGH without $CaCO_3$ supplementation.

Fat retention values exceeding 100% may reflect differences in nutrient utilization and energy partitioning, where body lipid accumulation is not solely dependent on dietary lipid intake. Growth hormone has been reported to influence energy utilization and nutrient partitioning by promoting protein-sparing effects and modifying lipid metabolism, which may contribute to changes in body lipid deposition (Kling *et al.*, 2012; Møller & Jørgensen, 2009). Therefore, the high fat retention observed in the present study may be associated with altered nutrient utilization under rGH administration.

Body Calcium Content

The initial body calcium content at the beginning of the experiment was 4.74 ± 0.13 mg 100 g^{-1} body weight. At the end of the experimental period, body calcium content of snakehead juveniles showed significant differences among treatments ($p < 0.05$; Figure 2). Body calcium content increased with increasing $CaCO_3$ supplementation levels. The highest body calcium content ($p < 0.05$) was observed in T3, followed by T2, T1, whereas the lowest value was recorded in the Control treatment. The body calcium content in T3 was 37% higher than that observed in the Control group.

Table 5. Chemical composition, protein retention, and fat content of snakehead juveniles fed rGH-supplemented diet and addition of $CaCO_3$ at different doses in the rearing water

Treatment	Moisture (%)	Crude protein (%)	Fat (%)	Protein retention (%)	Fat retention (%)
Control (R0C0)	70.04 ± 0.78^{a}	13.64 ± 0.23^{a}	4.49 ± 0.17^{a}	9.85 ± 1.05^{a}	51.20 ± 6.39^{a}
T1 (R5C0)	68.92 ± 0.94^{a}	15.38 ± 0.62^{c}	5.22 ± 0.82^{a}	22.54 ± 3.88^{c}	112.15 ± 2.93^{c}
T2 (R5C25)	69.94 ± 0.95^{a}	13.82 ± 0.41^{ab}	4.56 ± 0.37^{a}	16.05 ± 1.70^{b}	79.69 ± 10.91^{b}
T3 (R5C50)	69.68 ± 0.46^{a}	14.99 ± 0.48^{bc}	4.23 ± 0.52^{a}	18.80 ± 0.55^{b}	77.16 ± 9.79^{b}

Note: Control (R0C0 = rGH 0 mg $kg^{-1}$and $CaCO_3$ 0 mg L^{-1}); T1 (R5C0 = rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T2 (R5C25 = rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}); T3 (R5C50 = rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}). The mean values in the same column followed by different superscript letters indicate significant differences at the 5% level of significance ($p < 0.05$).

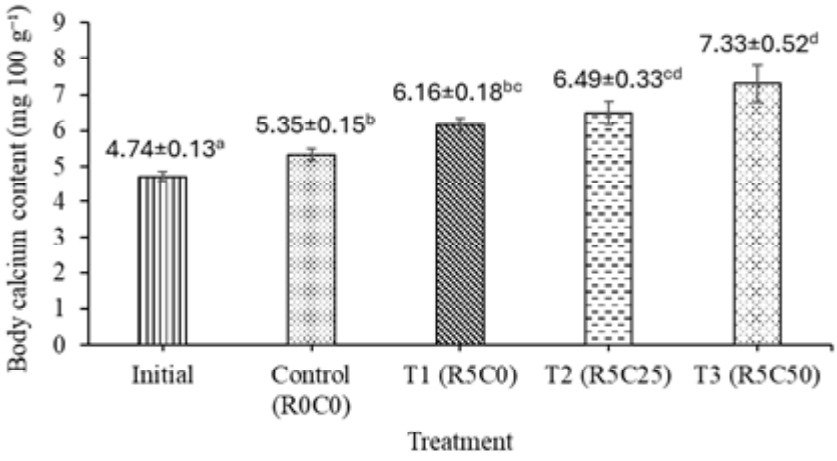

Figure 2. Body calcium content of snakehead juveniles fed rGH- supplemented diet and addition of $CaCO_3$ at different doses in the rearing water. Control (R0C0 = rGH 0 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T1 (R5C0 = rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T2 (R5C25 = rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}); T3 (R5C50 = rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}).

The increase in body calcium content with increasing $CaCO_3$ supplementation suggests increased calcium accumulation under higher mineral availability. This result suggests that $CaCO_3$ supplementation affected mineral status in snakehead juveniles and may partly explain the higher body length growth observed in T2 and T3.

Molecular Responses

Dietary rGH administration resulted in higher GH and IGF-1 gene expression compared to the control, suggesting involvement of the GH–IGF axis during the rearing period (Figure 3 and 4). In Figure 3, GH expression tended to be higher in all rGH-treated groups compared with the control. However, no clear differences were observed among T1, T2, and T3 despite increasing $CaCO_3$ concentrations.

A similar pattern was observed for IGF-1 expression. Fish receiving rGH treatment consistently showed higher IGF-1 expression than the control, whereas differences among $CaCO_3$ supplemented groups were not consistently evident. These results suggest that differences in growth-related gene expression were more closely associated with rGH administration than with $CaCO_3$ supplementation. Recombinant growth hormone has been reported to stimulate the GH–IGF endocrine pathway, which regulates cell proliferation, protein synthesis, and tissue growth (Reinecke *et al*., 2005; Fuentes *et al*., 2013; Yang *et al*., 2021).

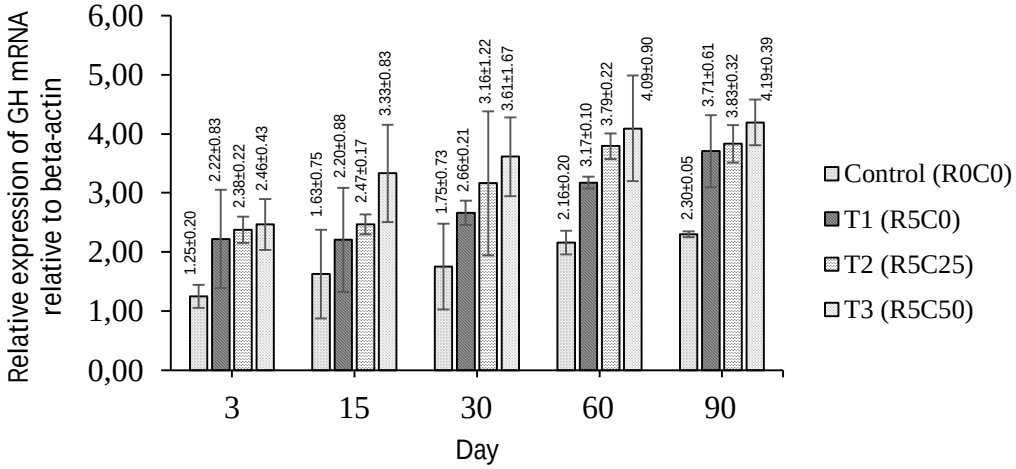

Figure 3. Relative expression levels of GH mRNA against β-actin in snakehead juveniles fed rGH-supplemented diet and addition of $CaCO_3$ at different doses in the rearing water. Control (R0C0 = rGH 0 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T1 (R5C0 = rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T2 (R5C25 = rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}); T3 (R5C50 = rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}).

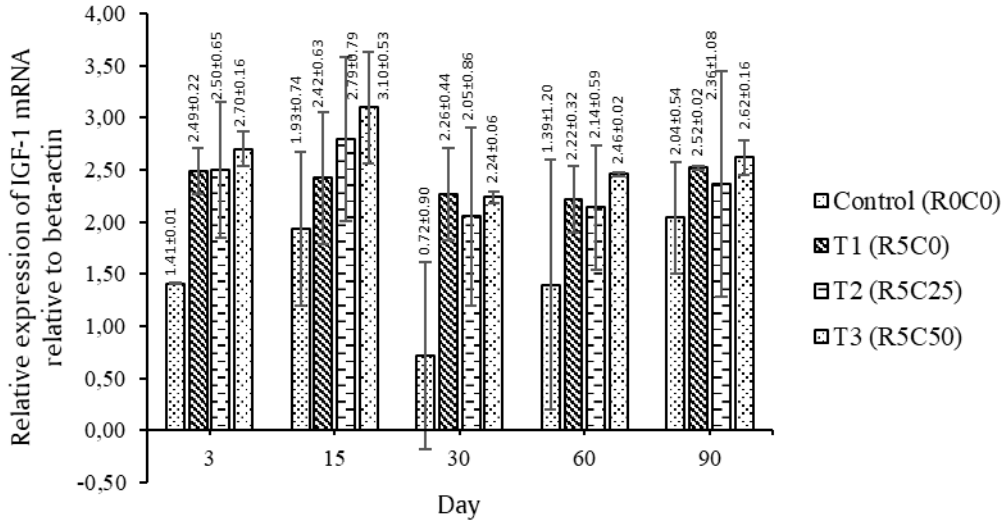

Figure 4. Relative mRNA expression levels of IGF-1 against β-actin in snakehead juveniles fed rGH-supplemented diet and addition of $CaCO_3$ at different doses in the rearing water. Control (R0C0 = rGH 0 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T1 (R5C0 = rGH 5 mg kg^{-1} and $CaCO_3$ 0 mg L^{-1}); T2 (R5C25 = rGH 5 mg kg^{-1} and $CaCO_3$ 25 mg L^{-1}); T3 (R5C50 = rGH 5 mg kg^{-1} and $CaCO_3$ 50 mg L^{-1}).

The activation of the GH–IGF-1 axis plays a central role in regulating cellular growth processes. IGF-1 has been reported to promote cell proliferation, tissue differentiation, and protein synthesis, thereby supporting biomass accumulation during growth (Escobar *et al*., 2011; Yang *et al*., 2021). At the molecular level, IGF-1 signaling activates the PI3K–Akt–mTOR pathway, which enhances protein synthesis while simultaneously inhibiting protein degradation (Yoshida & Delafontaine, 2020; Sakai *et al*., 2021). The activation of these anabolic pathways may contribute to the higher body weight and specific growth rate observed in snakehead juveniles receiving rGH supplementation.

The absence of significant differences in GH and IGF-1 expression among T1, T2, and T3 indicates that no clear transcriptional response was detected under the conditions tested. Instead, $CaCO_3$ appeared to influence growth through mechanisms related to mineral availability and may be associated with structural growth processes.

Rearing Water Quality

All water quality parameters remained within acceptable ranges for snakehead culture throughout the experimental period (Table 6). Temperature, dissolved oxygen, and total ammonia nitrogen remained relatively similar among treatments and did not become limiting factors for fish growth or survival. In contrast, pH increased with increasing $CaCO_3$ supplementation, which may be attributed to the buffering effect of $CaCO_3$ through increased carbonate availability in the water. Alkalinity and hardness also increased significantly with increasing $CaCO_3$ levels ($p < 0.05$), reflecting the effect of $CaCO_3$ addition on water mineral composition.

Table 6. Rearing water quality of snakehead juveniles fed rGH-supplemented diet and addition of $CaCO_3$ at different doses in the water

Parameters	Control (R0C0)	T1 (R5C0)	T2 (R5C25)	T3 (R5C50)	Reference
Temperature (°C)	28.38 ± 0.10^a	28.42 ± 0.13^a	28.47 ± 0.11^a	28.45 ± 0.17^a	26 - 32**
pH	6.90 ± 0.02^a	6.89 ± 0.05^a	7.56 ± 0.08^b	8.26 ± 0.06^c	5.5 – 7.8**
DO (mg L^{-1})	4.07 ± 0.05^a	3.93 ± 0.12^a	3.90 ± 0.05^a	3.98 ± 0.10^a	>2.50*
TAN (mg L^{-1})	0.115 ± 0.019^a	0.332 ± 0.03^b	0.311 ± 0.05^b	0.365 ± 0.025^b	0.089 - 0.609**
Alkalinity (mg $L^{-1}CaCO_3$)	49.99 ± 0.33^a	49.84 ± 0.58^a	57.05 ± 0.78^b	73.10 ± 0.66^c	-
Hardness (mg L^{-1})	62.91 ± 0.49^a	63.04 ± 0.06^a	76.37 ± 0.56^b	90.35 ± 0.75^c	-
Final Ca concentration (mg L^{-1})	21.60 ± 0.18^b	19.78 ± 0.38^a	30.00 ± 0.48^c	38.06 ± 0.19^d	-

*Puspaningsih *et al.,* 2018 ** Meidiana *et al.,* 2022. The mean values in the same rows followed by different superscript letters indicate significant differences at the 5% level of significance ($p < 0.05$).

Despite these changes, the 100% survival observed across treatments suggests that the increase in alkalinity and hardness appeared to remain within conditions tolerated by snakehead juveniles under the experimental conditions. The relatively uniform environmental conditions among treatments may have minimized the influence of environmental variability on growth performance, nutrient retention, and gene expression. Therefore, environmental conditions were unlikely to be the primary factor explaining the observed responses. However, physiological stress responses were not directly evaluated in the present study.

CONCLUSION

Dietary rGH administration improved weight gain and feed utilization in snakehead juveniles. $CaCO_3$ supplementation under rGH treatment was associated with increased body length, increased body calcium content, and altered water mineral characteristics. The increase in body calcium content suggests increased calcium accumulation under higher mineral availability. The results indicate complementary responses between endocrine stimulation and mineral availability under the experimental conditions tested. The results suggest that $CaCO_3$ supplementation under rGH administration may be associated with structural growth, whereas rGH was associated with improved weight gain and nutrient utilization.

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