Dietary protein replacement of fish meal with black soldier fly larvae meal: Effects on growth, whole-body composition, digestive enzyme activity, muscle-growth-related gene expression and haemato-biochemical responses of juvenile goldfish, Carassius auratus

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Abstract

A 60-day indoor growth trial was carried out to investigate the effects of dietary replacement of fish meal (FM) protein with black soldier fly larvae meal (BSFLM) at graded concentrations on bio-growth parameters, whole-body chemical composition, digestive enzyme activity, muscle-growth-related gene expression and haemato-biochemical responses of goldish juveniles (*Carassius auratus*). Six isonitrogenous and isoenergetic diets were formulated with BSFLM to substitute FM protein at 0 (T0), 20 (T20), 40 (T40), 60 (T60), 80 (T80) and 100 percent (T100). The aquaria with 20 fishes in each replicate were fed with experimental diet twice a day. Gold fish juveniles fed T60 diet exhibited maximum growth performances and feed utilization. However, escalating the percentage of fishmeal substitution with BSFLM above 60 percentage led to significant reduction in growth and feed utilization of goldfish juveniles. The protease activity was high in T60 and T80, wherein the lipase and amylase activity were high in T100. The relative mRNA expression of GH and MyoD was significantly upregulated in fish fed T40 and T60 diets, while the myostatin was significantly downregulated fish fed T40 and T60 diets. Thus, the fishmeal protein substituted at 60 percent with BSFLM with an inclusion level of 20.1 g/kg of diet will be well suitable for the goldfish juveniles.

1. Introduction

Aquariculture being a pivotal component of aquaculture renders both aesthetic value and is a good earner of foreign- exchange. The ornamental fish industry is an ever-augmenting substantial activity valued at approximately US$15 billion (Rhyne et al., 2017). Feed is the major component in aquaculture and ornamental fish industry. Fish meal is most widely used as a major protein source in the production of fish feed due to its quality protein and amino acid composition. The upscaling demand and price for fish meal has awakened the need of an alternative source of protein for aqua diets. Therefore, many scientific research studies are proposed to find out cost-efficient alternative plant-based ingredients for fish meal free aqua feed production (Diener et al., 2011). Though, several substitute feed ingredients of plant origin have been utilized in the aquafeed formulations (Gatlin et al., 2007), they possess imbalanced amino-acids profiles, low palatability and also contain anti-nutritional factors (ANFs) which confine their use in aquafeed formulations (NRC, 2011).

Currently, insects are earning a flourishing intentness as a principal ingredient for replacing the fish meal in the aquafeeds. The European Food Safety Authority scientific committee (2015) recognized the aforementioned environmental and economic sustainability issues in the aquaculture sector by enabling regulation 893/2017, which allows the utilization of seven different insect species meal in fish feed. Among the permitted species, the black solider fly (*Hermetia illucens*) larvae had a tremendous development due to its preference for organic waste as growth substrate (Sheppard et al., 1934; Meneguz et al., 2018; Vargas et al., 2018). They are able in converting low-quality organic material into high-grade protein and fat (Ewald et al., 2020). The black solider fly larvae having protein content of about 35–46% (Henry et al., 2015; Meneque et al., 2018) and lipid content of about 19–37% (Makkar et al., 2014) and the amino-acid profile and fatty acid profile of black solider fly larvae make them suitable for inclusion in animal feeds (Makkar et al., 2014). BSF larvae meal has very less chances of transmitting zoonotic diseases. They also help in reducing the environmental footprint by lessening the greenhouse gas and NH_3 emissions (Oonincx et al., 2010)

The BSF larvae meal has proven to be a good protein source in some warm water species (Sudha et al., 2022, Bondari et al., 1981 and Sheppard et al., 1981), meanwhile only meagre data is available in ornamental fish species. The goldfish which belongs to Cyprinidae family, is one of the most substantial ornamental species being domesticated in aquaria. It has been cultured worldwide for its aesthetic value and firm inheritance. The present study has been carried out to mitigate the up-scaling prices of ornamental fish feeds and to fulfill the essentiality of sustainable and environmentally friendly goldfish feed. Since, the black soldier fly larvae meal being a new venture as protein swap to fish meal, it is vital to analyze bio-growth parameters, the hematological parameters and digestive enzyme analysis of the fish to confirm the performance of the diets on the experimental fish.

Muscle growth in fishes are regulated by the myogenic factors, such as myoD and myostatin. Myo D responsible for satellite cells activation and proliferation during myogenesis (Watabe, 2000). While, myostatin acts as a growth suppresser that inhibits proliferation and differentiation of satellite cells during muscle growth development. Dietary supplementation of BSFLM has been reported to influence the growth performances and relative expression of muscle growth related genes, such as MyoD and

myostatin in fish species (Sudha et al., 2022). In this context, the present study was focussed to estimate the effects of dietary swap of fish meal with black soldier fly larvae meal on growth performance, muscle growth-related gene expression, whole-body composition and haemato-biochemical responses of juvenile gold fish, *Carrassius auratus*.

2. Materials And Methods

2.1. Ethical statement

This study was conducted based on the approval by the ethical committee of TNJFU, Nagapattinam, Tamil Nadu, India.

2.2. Experimental fish and feeding trial

Gold fish juveniles were procured from Ornamental Fish Trade Centre, Kolathur, Chennai, Tamil Nadu, India. Ostensibly, healthy seeds in the first instance were acclimatized in fibre-reinforced plastic tanks by feeding them with diet containing 320 g/kg of protein over a period of three weeks. Before the experiment, the fishes were graded to select an individual average weight of 2.8 ± 0.3 g and were stocked in indoor aquarium facility. Three hundred and sixty fishes were distributed in 18 groups with twenty fishes in each aquarium. The aquarium had a water holding capacity of 70 litres in which the fishes were stocked for the feeding trial. Satiation feeding was carried out twice a day (09.00 and 17.00 H) for 60 days and the daily feed consumption was noted. Water exchange was carried out at the rate of 10% every three days in each aquarium. Aeration was continuously provided throughout the experimental period using a 5-HP air blower (Everest Pvt). During the growth trial, water quality parameters were monitored daily and the mean values were as follows: water temperature at 30.96±0.54°C, pH at 8.22±0.07, dissolved oxygen at 6.64±0.61 mg/L, ammonia-N at 0.07±0.10 ppm, nitrite-N at 0.76±0.44 ppm, nitrate-N at 0.06±0.10 ppm and hardness at 408.51±68.64 ppm.

2.3. Experimental diets

Six different FM and BSFLM based isonitrogenous experimental diets were formulated to contain 320 g/kg of crude protein (Table 1). The experimental diets were supplemented with black solider fly larvae meal (Eco care Agrovet) at levels of 0.0 (T0), 6.7 (T20), 13.4 (T40), 20.1 (T60), 26.8 (T80) and 33.5 g/kg (T100) to replace fish meal protein at 0, 20, 40, 60, 80 and 100 percent, respectively. The experimental diet T0 was observed as a control diet with 20 g/kg inclusion of fish meal and excluding the addition of BSFLM. The inclusion level of palm oil was adjusted to maintain isolipidic nature of experimental diets. Dietary ingredients were finely ground, thoroughly mixed using vertical ingredient mixer (Jinan Sunpring Machinery) and then extruded at 60–70°C to prepare 1.5-mm floating pellets using a single screw extruder (Unitech). The air tight plastic containers were utilized to store all the experimental diets at room temperature.

TABLE 1: Formulation and chemical composition of the experimental diets (g/kg of diet)

Ingredients	T0	T20	T40	T60	T80	T100
Black soldier fly larvae meal [a, f]	0	67	134	201	268	335
Fish meal [b]	200	160	120	80	40	0
Soybean meal [b]	270	270	270	270	270	270
Groundnut oil cake [b]	80	80	80	80	80	80
Corn flour [b]	266	246	226	206	185	165
Rice bran [b]	100	100	100	100	100	100
Cassava starch [b]	30	30	30	30	30	30
Palm oil [c]	34	27	20	13	07	0
Dicalcium phosphate [b]	10	10	10	10	10	10
Vitamin premix [d]	5	5	5	5	5	5
Mineral premix [e]	5	5	5	5	5	5
Proximate composition (g/kg dry matter)						
Crude protein	329.4	322.8	325.7	323.2	320.9	323.4
Crude lipid	70.1	71.2	71.6	70.4	71.1	70.9
Crude fibre	32.2	33.6	33.8	33.7	33.9	33.8
Ash	83.9	83.5	84.3	83.6	83.4	83.8

[a]Eco care agrovet, Puducherry, India

[b]National co-operative consumers 'federation of India, Chennai, India

[c]Local market, Chennai, India

[d]Composition of vitamin premix (quantity/kg): Vit. A—10,000,000 IU, Vit.B1—5,000 mg, Vit.B2—5,000 mg, Vit.B3—6,000 mg, Vit.B5—6,000 mg, Vit.B6—6,000 mg, Vit.C—60,000 mg, Vit.D3— 2,000,000 IU, Vit. E—10,000 EU, Vit. H—200 mg.

[e]Composition of mineral premix (quantity/kg): magnesium—2,800 mg, iodine—7.4 mg, iron—7,400 mg, copper—1,200 mg, manganese—11,600 mg, zinc—9,800 mg, chlorides cobalt—4 mg, potassium—100 mg, selenium—4 mg, calcium carbonate—27.25%, phosphorous—7.45 mg, sulphur—0.7 mg, sodium—6 mg, Calpan—200 mg, aluminium—
1,500 mg and choline chloride— 10,000 mg

[f]Proximate of BSFLM – crude protein-42.27 %, crude lipid-18%, crude fibre- 8.73%, ash- 24.16%, moisture- 20.3%

2.4. Fish growth sampling

After 60 days of feeding trial, all the fish were put down with an overdose of tricaine methane sulphonate (MS-222; Sigma-Aldrich) and individually counted and weighed to estimate their survival, feed conversion ratio (FCR), protein efficiency ratio (PER), Thermal-unit growth coefficient (TGC) as follows:

$$Weight\ gain\ (g) = Final\ body\ weight(g) - Initial\ body\ weight(g)$$

$$Daily\ weight\ gain(DWG)(g) = \frac{(Final\ weig\square t - Initial\ weig\square t)}{Duration\ of\ rearing\ periods(Days)}$$

$$Survival(SR)(\%) = \frac{Total\ numberof\ fis\square es\ survived}{Total\ number\ of\ fis\square es\ stocked} \times 100$$

$$Specific\ growt\square\ rate(SGR\ \%\ per\ day) = \frac{[(\ln(Final\ weight - \ln(Initial\ weight)]}{duration\ of\ rearing\ period(days)} \times 100$$

$$Feed\ conversion\ ratio(FCR) = \frac{Amount\ offeed\ given(g)}{Weig\square t\ gain(g)}$$

$$Protein\ effeciency\ Ratio(PER) = \frac{Weight\ gain(g)}{Total\ protein\ fed(g)}$$

$$Thermal\ growth\ coefficient(TGC)$$

$$= \frac{\left[Final\ weight\text{^}\frac{1}{3} - Initial\ weight\text{^}\frac{1}{3})\right]}{[Mean\ water\ Temperature\ (°C)X\ duration\ of\ days)]} \times 100$$

2.5. Proximate and amino acid analysis

Six fishes from each experimental unit were collected after completion of growth trial to determine whole-body composition. The proximate composition such as moisture, crude protein, crude lipid and ash contents of experimental diets as well as whole body was estimated following standard protocols (AOAC, 2010). The amino acid composition of control and treatment diets were estimated using ultra-pressure liquid chromatography (UPLC; Model—Waters ACQUITY-UPLC, Waters), following the method described by Ishida et al. (1981).

TABLE 2: Amino acid composition of the experimental diets (g/kg of diet)

	T0	T20	T40	T60	T80	T100
Essential amino acids						
Arginine	25.6	25.2	24.9	23.6	23.2	23.4
Histidine	12.5	11.7	11.8	12.4	12.6	12.5
Isoleucine	18	17.4	17.5	18.6	18.3	18.1
Leucine	26.1	25.8	25.5	25.7	26.1	25.6
Lysine	19.5	19.3	19.2	18.8	18.6	18.3
Methionine	5.9	5.7	5.4	5.2	5.2	50.1
Phenylalanine	18.8	19.2	19.4	18.7	18.4	18.7
Threonine	14.6	14.3	14.5	14.2	14.1	14.1
Tryptophan	3.1	2.8	2.7	2.4	2.3	2.4
Valine	19.6	19	19.1	19.5	19.4	19.6
Non-essential amino acids						
Cysteine	5.3	5.4	5.4	5.2	5.3	5.1
Tyrosine	13.3	13.5	13.5	13.3	13.3	13.3
Glutamic acid	61.7	63.1	64.6	65.2	66.1	66.6
Aspartic acid	43.4	43.2	42.7	46.4	44.8	44.4
Glycine	19.5	19.5	19.5	20.7	20.3	20.2
Serine	18.2	18.2	18.3	18.6	18.5	18.6
Alanine	18.8	17.2	17.4	17.8	18.5	19

2.6. Haemato-biochemical assay

The blood samples were obtained from three individual fishes from each replication of aquaria to analyze the hematological and serum biochemical parameters at the end of the feeding trial. The fishes were anaesthetized using clove oil before collection of blood and caudal vein puncture method was used to obtain blood using 1-ml syringe. The collected blood samples were expelled into heparinized and non-heparinized tubes and stored immediately on ice. The serum was obtained by keeping the non-heparinized tubes in slant position for 2 hours and then centrifugation at 3,500 rpm for 25min at 4°C in a refrigerated centrifuge (Eppendorf Centrifuge 5804 R) was done. Neubauer hemocytometer was used to determine the RBC (red blood cell) counts. Cyanmethemoglobin method (Drabkin, 1946) was used to analyze the haemoglobin (Hb) contents whereas the microhematocrit method (Nelson & Morris, 1979) was used to determine the hematocrit (Ht). Erythrocyte indices, such as MCH, MCV and MCHC were calculated according to the equation given by Wintrobe (1934). The equations are as follows:

MCV (per μl) = (Ht × 10)/erythrocytes

MCH (%) = (Hb × 10)/erythrocytes

MCHC (g/dl) = (Hb × 100)/Ht

The total serum protein was analysed following the Biuret method (Reinhold, 1953). The bromocresol green binding method (Doumas et al., 1971) was utilized to calculate the albumin content. The globulin value is derived by deducting the albumin values from the total serum protein. A/G ratio is calculated by dividing the albumin value and globulin value. The method of

Parekh and Jung (1970) was followed to calculate Serum cholesterol (CHO). Triglyceride (TG) levels were estimated following the protocol of Rice (1970).

2.7. Digestive enzyme analysis

At the end of the feeding trial, three fish (n=3 triplicate per treatment) from each treatment were randomly selected, and intestine samples were collected by dissecting it on chilled condition. The sample was then homogenized with cold phosphate buffer (pH 7.8) and centrifuged at 4500 rpm for 5minutes. The supernatant was kept at -20°C until the enzyme assay was carried out.

Quantifying of amylase activity was fulfilled using 3.5 dinitro salicylic acid colorimetric technique based on the method illustrated by Clark (1964). Cherry and Crandel (1932) method was used to determine the lipase activity by measuring the fatty acid release caused by enzyme hydrolysis of olive oil. The protease activity was quantified using Lowry, Rosebrough and Farr technique. The enzyme activity was observed based on the changes in absorbance using a spectrophotometer (Lamba 25UV Win Lab V 6.0). One unit of enzyme activity was expressed as 1µg of maltose, fatty acid and tyrosine released per minute.

2.8. Quantitative real-time PCR (qRT-PCR)

Sequentially, after the end of the feeding trial, the skeletal muscle (for *MyoD* and *myostatin* gene) and pituitary gland (for GH gene; *n* = 3 fish per aquaria) was collected to extract the total RNA as per the manufacturer's instruction using RNA iso-plus (Takara Bio). Then, 2g of total RNA was reverse-transcribed to cDNA according to the manufacturer's instruction. The protocols of Prabu et al. (2021) were utilized for relative gene expression studies. The gene-specific primers of MyoD, myostatin, 18sRNA, GH gene and β actin were shown in Table 3. The quantitative real-time-polymerase chain reaction (qRT-PCR) consisted of 20 ng of cDNA template, 10 µM of each primer (forward and reverse), and 1× SYBR Green PCR Master Mix Kit (Takara Bio), in a 20 µL of total volume. The qRT-PCR was performed in a C1000 Touch thermal cycler-CFX96 Real-time PCR (Bio- Rad). The PCR cycling profiles were carried out programmed with an initial denaturation at 95°C for 10 min, along with 40 cycles of 15 s denaturation at 95°C, annealing at 60– 62°C (depends on the target genes) for 30 s, extension at 72°C for 30 s and ended with dissolution curve. The threshold cycle values of the qRT-PCR performed in triplicates were calculated and from that the relative expression level of specific gene was presented as $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The genes *18S rDNA* and *β*-Actin were utilized as an internal control gene to collate the relative expression levels of the genes.

TABLE 3: Primers used for qRT-PCR analysis

Gene name	GenBank number	Primer sequence (5´-3´)
Myogenic Factor (*MyoD*)	GU246722	Forward: CCACCTGTCAGACAACCAGA Reverse: ACTGCGTTCGCTCTTCAGAC
Myostatin 1	FJ972683	Forward: TCCACATGACCCTGCAGAC Reverse: TGCACCACACATACTCCTCATC
18Sribosomal DNA (*18SrDNA)*	JF698683	Forward: GGACACGGAAAGGATTGACAG Reverse: GTTCGTTATCGGAATTAACCAGAC
Pituitary growth hormone	XM_003442542	Forward: TCGGTTGTGTGTTTGGGCGTCTC Reverse: GTGCAGGTGCGTGACTCTGTTGA
β-Actin	EU887951.1	Forward: CCACACAGTGCCCATCTACGA Reverse: CCACGCTCTGTCAGGATCTTCA

2.9. Statistical analysis

All the experimental data were shown as the mean values ± standard deviation (SD) of three replications. Each and every data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) and transformed when the data did

not show normal distribution. One-way ANOVA was performed to test the significant differences, wherein the Tukey's test was used to find out the significant differences between experimental groups. Differences were considered significant at $p < 0.05$. The software SPSS 20.0 for windows (SPSS) was used to statistically analyze the data.

3. Results

3.1. Growth performances and feed utilization:

Growth performances and feed utilization of goldfish juveniles after 60 days of feeding trial are given in Table 4. Significant difference was observed in weight gain, FCR, PER, SGR and TGC of fishes fed with BSFLM supplemented diets and control diet. The highest growth performance and feed utilization was observed at T60 when compared to other experimental diets. The weight gain, SGR and TGC were found to be highest in fish fed T60 diet and lowest at fish fed control and T100 diet. Best FCR and PER values were found in fish fed T60 diet. No significant difference was observed in the survival of the fishes fed with different experimental diets. The growth performance and feed utilization were significantly low at the fishes fed with control, T80 and T100 diets.

TABLE 4: Effect of replacing FM with BSFLM on growth performance and feed utilization of goldfish juveniles

	T0	T20	T40	T60	T80	T100	*p* value
Initial body weight (g)	2.92±0.09	2.66±0.18	2.87±0.06	2.68±0.14	2.82±0.22	2.85±0.07	0.22
Final body weight (g)	$4.20±0.07^{c}$	$4.21±0.18^{c}$	$4.64±0.06^{b}$	$5.09±0.16^{a}$	$4.12±0.20^{c}$	$4.11±0.08^{c}$	<0.001
Weight gain (g)	$1.28 ±0.15^{c}$	$1.55±0.21^{bc}$	$1.77±0.12^{b}$	$2.40±0.20^{a}$	$1.30±0.10^{c}$	$1.25±0.14^{c}$	<0.001
Survival (%)	95.57±3.54	96.66±2.88	88.33±2.88	96.66±2.88	96.66±5.77	96.66±2.88	0.125
Specific Growth Rate (% per day)	$0.60±0.07^{b}$	$0.76±0.11^{b}$	$0.79±0.05^{b}$	$1.06±0.09^{a}$	$0.63±0.07^{b}$	$0.60±0.07^{b}$	<0.001
ADG (g/fish)	$0.021±0.002^{c}$	$0.025±0.003^{bc}$	$0.029±0.002^{b}$	$0.040±0.003^{a}$	$0.021±0.001^{c}$	$0.020±0.002^{c}$	<0.001
FCR	$2.59±0.14^{ab}$	$2.52±0.16^{ab}$	$2.28±0.10^{b}$	$1.88±0.14^{c}$	$2.56±0.17^{ab}$	$2.79±0.18^{a}$	<0.001
PER	$0.37±0.06^{c}$	$0.45±0.05^{ab}$	$0.52±0.03^{b}$	$0.78±0.07^{a}$	$0.38±0.01^{ab}$	$0.36±0.02^{c}$	<0.001
Thermal Growth Coefficient	$0.009±0.001^{b}$	$0.012±0.001^{b}$	$0.013±0.00^{b}$	$0.017±0.001^{a}$	$0.010±0.001^{b}$	$0.009±0.001^{b}$	<0.001

Note: Values were expressed as means ± SD of three replicate cage per treatment (n=3) and values with different superscripts indicate significant differences as determined by Tukey's test ($p < 0.05$)

3.2. Whole body composition

The whole-body composition of fish fed with control and graded levels of BSFLM included diets is presented in Table 5. Dietary replacement of fish meal with black soldier fly larvae meal had no significant differences in whole-body moisture, protein, lipid and ash contents of juvenile gold fish.

TABLE 5: Whole-body chemical composition (g/kg of wet weight) of goldfish juveniles fed experimental diets

	Initial	T0	T20	T40	T60	T80	T100	*p* value
Moisture	758.32	743.033 ± 3.45	741.83 ± 5.03	741.83±8.52	738.23±3.99	744.91±3.12	738.26±7.32	0.654
Crude protein	124.32	133.33±3.32	129.06±5.43	134.77±8.17	137.06±2.73	135.92±2.38	135.07±3.09	0.415
Crude fat	64.35	72.77±5.51	77.73±4.53	77.14±4.93	76.33±3.56	73.03±1.46	7.5967±5.78	0.541
Ash	27.54	23.23±1.83	26.17±4.51	24.23±1.12	22.82±.1.96	26.37±2.73	22.03±2.02	0.245

Values are means ± SD (n = 3). Values in the same line with different superscript letters are significantly different ($p < .05$)

3.3. Digestive enzyme

Digestive enzyme activity of goldfish juveniles fed experimental diets are represented in Table 6. Dietary supplementation of BSFLM had significant effects on the digestive enzyme activities of gold fish juveniles. The amylase activity was found to be highest in fish fed T40, T80 and T100 diets, while lowest amylase activity was found in fish fed T20 diet. The protease activity was found to be highest in fish fed T60 and T80 diets, whereas lowest protease activity was found in fish fed T0 and T20 diets. Significantly highest and lowest lipase activity was found in fish fed with T100 and T0 diets, respectively.

TABLE 6: Digestive enzyme activity of goldfish juveniles fed different experimental diets

	T0	T20	T40	T60	T80	T100	*p* value
Amylase	$0.433±0.04^{b}$	$0.373±0.01^{c}$	$0.480±0.01^{a}$	$0.420±0.01^{b}$	$0.483±0.04^{a}$	$0.466±0.02^{a}$	<0.001
Protease	$3.03±0.02^{d}$	$3.07±0.02^{d}$	$3.54±0.04^{b}$	$3.88±0.01^{a}$	$3.81±0.00^{a}$	$3.47±0.01^{c}$	<0.001
Lipase	$0.49±0.01^{e}$	$0.69±0.00^{d}$	$0.70±0.01^{d}$	$0.89±0.01^{c}$	$1.49±0.00^{b}$	$1.89±0.00^{a}$	<0.001
Amylase as micromole of maltose released min−1 mg−1 protein							
Protease as micromole of tyrosine released min−1 mg−1 protein							
Lipase as units mg−1 protein							

3.4. Hematology and serum biochemical parameter

Hb, Ht, WBC, RBC, MCV, MCH and MCHC levels were significantly different among the dietary groups (Table 7). The Hb value was found to be significantly highest in fish fed T60 diet compared to fish fed other experimental diets. The red blood cell indices (MCV, MCH, MCHC) were negligibly low at fish fed T100 diet. The biochemical parameters, such as total protein, albumin, globulin and A/G ratio values were significantly high at fish fed T60 diet, while the total cholesterol and triglyceride values were found to be significantly lowest in fish fed T60 diet.

TABLE 7: Haematological and biochemical parameters of goldfish juveniles fed different experimental diets

	T0	T20	T40	T60	T80	T100	*p* value
Haematological parameters							
Hb (g/dl)	4.30±0.36^{b}	4.33±0.05^{b}	4.33±0.05^{b}	4.93±0.05^{a}	4.06±0.05^{b}	4.23±0.05^{b}	<0.001
RBC (million/cu mm)	0.53±0.00^{b}	0.48±0.01^{c}	0.54±0.00^{b}	0.57±0.01^{a}	0.48±0.01^{c}	0.43±0.0^{d}	<0.001
PCV	7.5±0.20^{c}	7.23±0.05^{d}	7.9±0.10^{b}	8.3±0.10^{a}	6.43±0.05^{c}	6.16±0.05^{e}	<0.001
MCV	125.3±1.65^{f}	145.5±0.15^{c}	153.8±0.52^{a}	151.6±0.10^{b}	133.5±0.10^{d}	129.5±0.05^{e}	<0.001
MCHC	59.6±0.05^{d}	60.1±0.05^{c}	59.5±0.10^{d}	62.8±0.10^{a}	61.5±0.43^{b}	55.6±0.05^{e}	<0.001
MCH	84.7±0.10^{c}	87.6±0.10^{b}	91.53±0.15^{a}	91.66±0.05^{a}	82.5±0.10^{d}	80.46±0.05^{e}	<0.001
Polymorphs	3.10±0.10^{c}	3.13±0.05^{c}	3.13±0.05^{c}	3.43±0.05^{b}	2.66±0.15^{d}	14.2±0.05^{a}	<0.001
Lymphocytes	96.2±0.10^{b}	95.26±0.05^{c}	96.5±0.10^{a}	94.93±0.05^{d}	96.1±0.10^{b}	80.53±0.15^{e}	<0.001
Eosinophils	0.43±0.05^{e}	0.80±0.10^{d}	0.46±0.05^{e}	1.73±0.05^{b}	1.06±0.11^{c}	4.93±0.05^{a}	<0.001
Monocytes	0.26±0.05^{b}	0.56±0.15^{a}	0.13±0.05^{bc}	0.33±0.05^{c}	0.33±0.05^{c}	0.03±0.05^{c}	<0.001
Biochemical parameters							
TP	3.28±0.01^{e}	4.54±0.05^{b}	3.71±0.00^{c}	5.29±0.01^{a}	3.47±0.01^{d}	3.68±0.07^{c}	<0.001
Albumin	0.84±0.15^{f}	1.05±0.01^{d}	1.02±0.15^{e}	2.36±0.010^{a}	1.36±0.015^{b}	1.17±0.005^{e}	<0.001
Globulin	2.41±0.005^{d}	2.57±0.01^{c}	2.66±0.01^{b}	2.92±0.005^{a}	2.12±0.01^{e}	2.53±0.02^{c}	<0.001
A/G Ratio	0.34±0.01^{c}	0.41±0.01^{bc}	0.37±0.01^{bc}	0.70±0.10^{a}	0.63±0.005^{a}	0.45±0.005^{b}	<0.001
TCHO	111.0±1^{b}	119.0±1^{a}	116.0±1^{a}	75±1^{d}	115.6±0.57^{a}	96.6±1.15^{c}	<0.001
Triglycerides	101.6±1.5^{d}	169±1^{a}	132±0^{b}	99±1^{d}	110.6±0.57^{c}	84.6±1.52^{e}	<0.001

Values were expressed as means ± SD of three replicate cages per treatment (n=3), and values with different superscripts indicate significant differences as determined by Tukey's test ($p < 0.05$).

Abbreviations: TCHO, total cholesterol, haemoglobin; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; TP, total protein; PCV, packed cell volume.

3.5. Muscle-growth-related gene expression

The relative expression of muscle growth-related-genes (MyoD, myostatin) and GH gene of gold fish juveniles were illustrated in figure 1. The relative expression of GH was significantly upregulated in fish fed T40 and T60 diets compared to fish fed other experimental diets. The mRNA expression of MyoD was significantly upregulated and downregulated in fish fed T60 and T100 diets, respectively. However, the relative mRNA expression of myostatin was significantly downregulated in fish fed T40 and T60 diets.

Declarations

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE:

This study was conducted based on the approval by the ethical committee of TNJFU,

Nagapattinam, Tamil Nadu, India.

HUMAN AND ANIMAL ETHICS:

The experimental procedure involving animal (fish) was approved by the ethical committee of TNJFU, Nagapattinam, Tamil Nadu, India.

CONSENT FOR PUBLICATION:

The authors agree to publish the article under the springer publication.

DATA AVAILABILITY STATEMENT:

The data that support the findings of this study are available within the article.

COMPETING INTERESTS:

The authors declare that they have no relevant financial or non-financial interests to disclose.

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AUTHOR'S CONTRIBUTION

A. Kamalii: conducted the feeding trial, analysed the data and drafted the manuscript.

Cheryl Antony: conceptualized and designed the study, and corrected the manuscript.

B. Ahilan: formulated the experimental diets and corrected the manuscript.

A. Uma: carried out the gene expression analysis part and corrected the manuscript.

E. Prabu: analysed the data for statistical analysis and corrected the manuscript.

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Figures

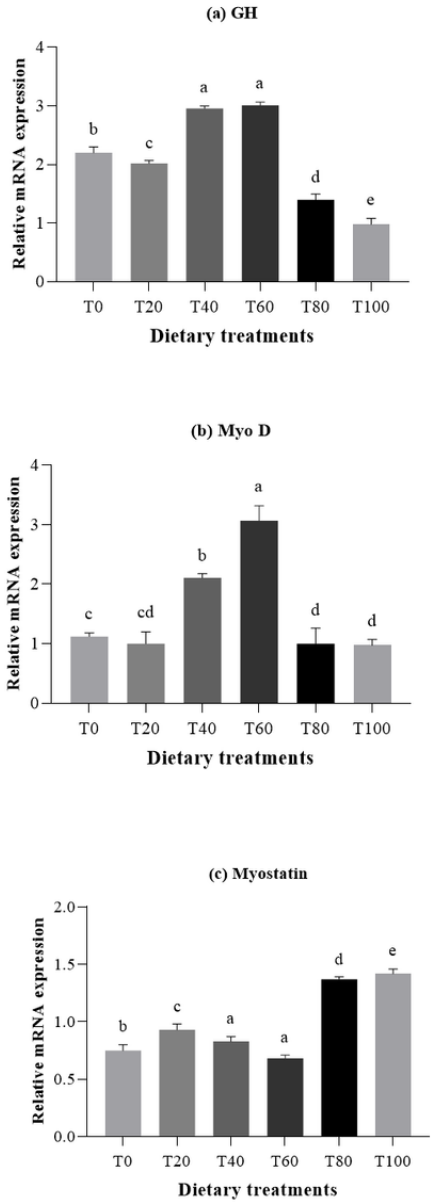

Figure 1

Relative expression of (a) GH, (b) Myo D and (c) Myostatin in the white skeletal muscle tissue of gold fish fed experimental diets. Values are mean ± SD represented by vertical error bars for each treatment (n=9). Different letters indicate significant ($p < .05$) differences among treatments determined by Tukey's test.