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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 (*Hermetia illucens*) 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 goldfish (*Carassius auratus*) juveniles. Six isonitrogenous and isoenergetic diets were formulated with BSFLM to substitute FM protein at 0 (T0), 20 (T20), 40 (T40), 60 (T60), and 80 (T80), and 100 % (T100). Each diet was randomly assigned to triplicate groups of 20 fish (Mean weight: 2.8±0.3 g) per aquaria were fed twice a day. Goldfish juveniles fed the T60 diet exhibited maximum growth and feed utilization. However, escalating the percentage of fishmeal substitution with BSFLM above 60 percent led to a significant reduction in growth and feed utilization. The protease activity and the haemoglobin (Hb) value were high in fishes fed with the T60 diet. The MyoD was upregulated in fish fed T40 and T60 diets, while the myostatin was downregulated in fish-fed T40 and T60 diets. Thus, the fishmeal protein substituted at 60% with BSFLM with an inclusion level of 201 g/kg of diet will be suitable for the goldfish juveniles

Introduction

Aquariculture, a pivotal aquaculture component, renders aesthetic value and is an excellent foreign exchange earner. 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 the aquaculture and ornamental fish industry. Fish meal is a primary protein source in fish feed production due to its quality protein and amino acid composition (Tacon et al., (2008) and Metian et al., 2008). Fish meal has been used as an effective feed ingredient up to the inclusion level of 30 percent in the ornamental industry (Mamuad et al., 2021). The upscaling demand and price for fish meal have awakened the need for an alternative protein source for aqua diets. Therefore, many scientific research studies have proposed to find cost-efficient alternative plant-based ingredients for fish meal-free aqua feed production (Diener et al., 2011; Aas et al., 2019, Ytrestøyl et al., 2019; Åsgård et al., 2019). Though several substitute feed ingredients of plant origin are being 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 aquaculture sector's environmental and economic sustainability issues by enabling regulation 893/2017, which allows the utilization of seven different insect species' meals in fish feed. Among the permitted species, the black soldier fly (*Hermetia illucens*) larvae had a tremendous development due to its preference for organic waste as a growth substrate (Sheppard et al., 1934; Meneguz et al., 2018). They can convert low-quality organic material into high-grade protein and fat (Ewald et al., 2020). The black soldier fly larvae have a protein content of about 35-46 % in dry weight basis (Menequz et al., 2018) and lipid content of about 19-37% in dry weight basis (Makkar et al., 2014), and the amino-acid profile and fatty acid profile of black soldier fly larvae make them suitable for inclusion in animal feeds (Makkar et al., 2014). Since the black soldier fly is not a pest, the rearing condition has no specific precautionary protocols and it diminishes the presence of harmful bacteria when compared to other dipteran species thereby reducing the chances of transmitting zoonotic diseases. They also help in reducing the environmental footprint by lessening the greenhouse gas and NH3 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; Sheppard et al., 1981), but insufficient data is available on ornamental fish species. The goldfish, which belongs to the Cyprinidae family, is one of the most substantial ornamental species 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 fulfill the essentiality of sustainable and environmentally friendly goldfish feed. Since the black soldier fly larvae meal is a new venture as a 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.

In fish species, dietary supplementation of BSFLM has been reported to influence the growth performances and relative expressions of muscle growth-related genes, such as MyoD and myostatin (Sudha et al., 2022). In this context, the present study was focused on estimating 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 in juvenile goldfish (*Carassius auratus).*

Muscle growth in fishes is regulated by myogenic factors, such as MyoD and myostatin. MyoD is responsible for satellite cell activation and proliferation during myogenesis (Watabe, 2000). In contrast, myostatin acts as a growth suppresser that inhibits the proliferation and differentiation of satellite cells during muscle growth development (Prabu et al., 2020; Prabu et al., 2021). In fish species, dietary supplementation of BSFLM has been reported to influence the growth performances and relative expressions of muscle growth-related genes, such as MyoD and myostatin (Sudha et al., 2022). In this context, the present study was focused on estimating 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 in juvenile goldfish (*Carassius auratus).*

Materials and Methods

Experimental Fish and Feeding Trial

Goldfish juveniles were procured from Ornamental Fish Trade Centre, Kolathur, Chennai, Tamil Nadu, India. Ostensibly, healthy seeds in the first instance were acclimatized in fiber-reinforced plastic tanks by feeding them with a diet containing 320 g/kg of protein for three weeks. The procured fishes were checked for external symptoms like ulcers, finrot and ecto-parasites, then they were maintained in quarantine facility for 3 weeks. Before the experiment, the fishes were graded to select an individual average weight of 2.8 ± 0.3 g and were stocked in an indoor aquarium facility. Six different experimental groups with different diet concentrations were designed. Each diet was randomly assigned to triplicate groups of twenty fish per aquarium (61cm×30cm×31cm). A total of three hundred and sixty fishes were distributed into 18 aquaria. The aquarium had a water holding capacity of 70 liters 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 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 (APHA, 2005) 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.

Experimental Diets

Six different FM and BSFLM-based isonitrogenous experimental diets were formulated to contain 320 g/kg (Tippayadara et al., 2021) of crude protein (Table 1). All the feed ingredients were procured from a commercial feed ingredient supplier in Chennai, India. The experimental diets were supplemented with black

soldier fly larvae meal (Eco care Agrovet) at levels of 0.0 (T0), 67g/kg (T20) (Sudha et al., 2022), 134g/kg (T40), 201g/kg (T60), 268g/kg (T80), and 355 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 200 g/kg inclusion of fish meal and excluding the addition of BSFLM. The percentage composition of BSFLM is in terms of dry weight basis. The inclusion level of palm oil was adjusted to maintain the isolipidic nature of experimental diets. Dietary ingredients were finely ground, thoroughly mixed using a 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 (DOLLY, Unitech, New Delhi, India). The airtight plastic containers were utilized to store all the experimental diets at room temperature.

Fish Growth Sampling

After 60 days of the feeding trial, all the fish were put down with an overdose of 300 mg/L eugenol and individually counted and weighed to estimate their survival, feed conversion ratio (FCR), the protein efficiency ratio (PER), Thermal-unit growth coefficient (TGC) as follows:

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

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

$$Survival(SR)(\%) = \frac{Total\ number of\ fishes\ survived}{Total\ number\ of\ fishes\ stocked} \times 100$$

$$Feed\ intake\left(\%\frac{BW}{day}\right) = dry\ feed\ intake\frac{(g)}{final} fish\ weight\frac{(g)}{days} feed \times 100$$

$$Feed\ conversion\ ratio(FCR) = \frac{Amount\ of feed\ given(g)}{Weight\ gain(g)}$$

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

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

$$Protein\ retention\ effecieny(PRE)(\%) = \grave{}\frac{Protein\ gain(g)}{Protein\ intake(g)} \times 100$$

Proximate and Amino Acid Analysis

Six fishes from each experimental unit were collected after completion of growth trial to determine whole-body composition, meanwhile the initial proximate composition of the fish was also analyzed. 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) mentioned in Table 2. 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 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%

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 anesthetized using clove oil before collection of blood, and the caudal vein puncture method was used to obtain blood using the 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 a 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. The cyanmethemoglobin method (Drabkin, 1946) was used to analyze the hemoglobin (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:

$$MCV\ (per\ \mu l) = (Ht \times 10)/erythrocytes$$

$$MCH\ (\%) = (Hb \times 10)/erythrocytes$$

$$MCHC\ (g/dl) = (Hb \times 100)/Ht$$

The total serum protein was analyzed 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).

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.

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, 2 µg 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-

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

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.

Statistical Analysis

The experimental data were shown as the mean values ± standard deviation (SD) of three replications. All 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, followed by Tukey's test for multiple comparisons at the significance level of 0.05 was used to compare the differences among the six dietary groups. The software SPSS 20.0 for windows (SPSS) was used to analyze the data statistically.

Results

Growth Performances and Feed Utilization

Growth performances and feed utilization of goldfish juveniles after 60 days of feeding trial are given in Table 4. A 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 were observed at T60 when compared to other experimental diets. The weight gain, SGR, and TGC were found to be highest in the fish-fed T60 diet and lowest in fish-fed control and the T100 diet. Best FCR and PER values were found in the 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 in the fishes fed with control, T80, and T100 diets.

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 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

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	96.66±2.88	96.66±2.88	96.66±5.77	96.66±2.88	0.125
Feed intake (%BW/ day)	1.51±0.005^{b}	1.37±0.005^{d}	1.16±0.005^{f}	1.24±0.005^{e}	1.61±0.005^{a}	1.47±0.007^{c}	<0.000
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
Protein retention efficiency (%)	2.72±0.010	1.45±0.005	3.2±0.005	3.93±0.005	3.61±0.005	3.31±0.007	<0.000
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 aquaria per treatment (n=3) and values with different superscripts indicate significant differences as determined by Tukey's test (p<0.05)

Digestive Enzyme

Dietary supplementation of BSFLM had significant effects on the digestive enzyme activities of goldfish juveniles (Table 6). The amylase activity was found to be highest in fish-fed T40, T80, and T100 diets, while the lowest amylase activity was found in fish-fed T20 diets. The protease activity was found to be highest in fish-fed T60 and T80 diets, whereas the 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.

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 the 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 in the fish-fed T60 diet, while the total cholesterol and triglyceride values were found to be significantly lowest in fish fed T60 diet.

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)

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

Table 7. Hematological 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 aquaria 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.

Muscle-Growth-Related Gene Expression

The goldfish juveniles fed with black soldier fly larvae meal have showcased better growth and feed utilization. The haemato-biochemical indices, mRNA expression of muscle-growth-related genes, and digestive enzyme activities showed that the replacement of FM with BSFLM up to 60 percent is practically feasible without impairing the health status of the fish. However, when the FM protein replacement level exceeds 60 percent, the decrement in growth rate was observed with downregulated relative mRNA expression of Myo D and GH genes. In a nutshell, our study suggests that black soldier fly larvae meal protein up to 60 percent could be a note-worthy replacement for fish meal protein in the diet of juvenile goldfish.

The relative expression of muscle growth-related genes (MyoD, myostatin) and GH gene of goldfish 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.

Discussion

The aquariculture industry has an escalating growth, which requires prompt research to meet the lacunas related to ornamental fish production and feeding (Calado et al., 2017). The main aim of ornamental aquaculture is to introduce more sustainable aquafeed ingredients to contribute to sustainable development goals (Chong et al., 2003; Gasco et al., 2018). The new alternative must enhance fish growth, provide adequate nutrients, and ensure sustainability and economic feasibility (Gasco et al., 2018). Insects are being adjudged to be the best alternative to the fish meal (Belghit et al., 2018). However, only a few types of research have focused on developing insect meal-based diets for commercial ornamental fish species. The growth parameters are vital to evaluating the fish's physical growth and nutritional status. In the present study, the goldfish juveniles fed with 60 percent replacement of BSFLM with FM had good growth performance and feed utilization. In agreement with our study, Sudha et al. (2022) reported that replacing fish meal protein with BSFLM at 60 percent had the highest growth performance significantly and feed utilization of juvenile striped catfish, *Pangasianodon hypophthalmus*. The

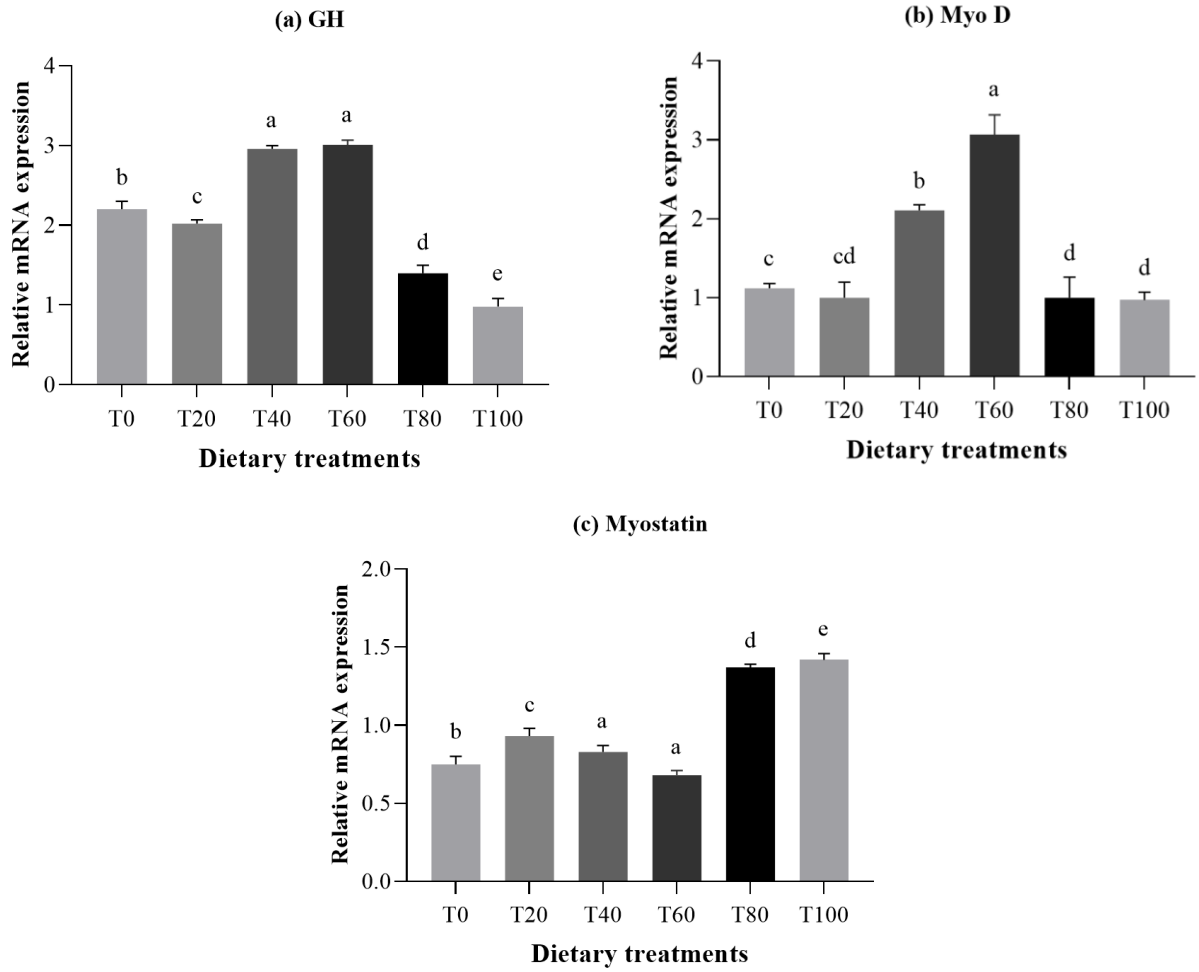

Figure 1. Relative expression of (a) GH, (b) Myo D and (c) Myostatin in the white skeletal muscle tissue of gold fish fed experimental diets.

fishes fed with the T60 diet showcased peaking SGR and decreasing value of FCR, indicating substantial improvement in the growth rate of goldfish at this level of BSFLM inclusion. The PER was found to be greater in T60 bespeaking better protein utilization and turnover. The decline in PER value at T0 portrayed that the fish did not efficiently utilize the protein compared to fish fed other treatment diets. Replacement of fishmeal with black soldier fly larvae meal had no significant influence on the survival of juvenile goldfish. The weight gain was dramatically high in T60 and was observed to be in a declining trend at T80 and T100. In addition to it, when the concentration increases above 60 percent, the growth performances and feed utilization decrease linearly. This may be attributed due to the presence of chitin, which possesses an inhibitory effect on healthy digestion and absorption, ending up in growth retardation. This growth retardation due to chitin was also observed in channel catfish and rainbow trout (St-Hilaire et al., 2007; Anvo et al., 2017).

In this present study, dietary replacement of FM with BSFLM had significant influences on the whole-body moisture, protein, lipid, and ash content of juvenile goldfish, which might be due to the isonitrogenous diets used in this study. Similar to this present study, no significant changes were observed in the whole-body proximate composition of Nile tilapia-fed graded levels of black soldier fly larvae meal, including diets to replace a dietary fish meal (Devic et al., 2018). Belsare et al. (2018) observed significant differences in the whole-body proximate composition of juvenile goldfish fed diets with different protein and lipid levels; this might be due to the variations in the protein and lipid contents of the diet.

A digestive enzyme analysis study is pivotal to knowing the nutrient assimilation of the fish. It provides a critical view on the acceptance of the feed by the fishes. Our results showed that dietary supplementation of BSFLM had a significant effect on the digestive enzyme activities of goldfish juveniles. The protease activity was higher in the T60 diet, wherein amylase activity was more significant in the T40 diet. The fishes fed with T0 exhibited low protease activity. This finding confirms that goldfish juveniles could efficiently utilize the protein when 60 percent fish meal protein was replaced with BSFLM. The intestinal lipase activity was remarkably high in the T100 diet. Fatty acids are found to act as signaling molecules responsible for regulating lipid metabolism pathways. The highest intestinal lipase activity in fish fed the T100 diet might be possible because fatty acids from BSFLM at this inclusion is not conducive to the digestion of goldfish juveniles; thus, the fish must have released additional lipases to aid the digestion of fat. This finding was in line with the study conducted for partial replacement of fish meal with black soldier fly larvae meal in the diet of *Monopterus albus* (Hu et al., 2020).

Hematological and biochemical analysis has been an effective tool in analyzing the effect of an experimental diet on the fish's nutritional status and health status (Shamna et al., 2017; Fazio et al., 2019). In this current study, the blood parameters of goldfish documented are within the justifiable limits of teleost fish (Satheesh Kumar et al., 2012). The highest value of RBC, hematocrit, and hemoglobin was obtained from the fishes fed with a T60 diet. The interrelationship between the RBC and anemia is observed in invertebrates, including fishes (Iheanacho et al., 2017). The MCV, MCH, and MCHC insinuate the hemoglobin content of erythrocytes and reflect the functions of hemoglobin (Iheanacho et al., 2017). Hemoglobin is an important oxygen-carrying protein and plays crucial roles in establishing host resistance against pathogens and in regulating innate immune responses. In the present study, MCHC and MCH were significantly high in the fish-fed T60 diet. These findings show that dietary supplementation of BSFLM improves immunity and promotes fish health. The insect meal contains chitin which acts as an immunopotentiator in animals, including fish and shrimp (Gasco et al., 2018; Xiao et al., 2018; Motte et al., 2019). The biochemical parameters of the blood are affected by a myriad of biotic and abiotic factors, including food, age, temperature, and seasonal pattern (Jawad et al., 2004). In our study, the biochemical parameters, such as serum total protein, albumin, globulin, and A/G ratio, were high in T60 diet-fed fishes. The increased total protein level may be due to the significant depletion of liver glycogen (Ojolick et al., 1995). High globulin levels indicate factors of healthy fish and improved immune system (Kumar et al., 2007; Fawole et al., 2017). The multifunctional protein serum albumin plays a substantial role in the immune system. It transports various biological substances in the fish body, including enzymes, vitamins, and hormones (Punitha et al., 2008). The higher albumin value in T60 indicates the healthy functioning of the immune system. The triglyceride level was minimum at 100 percent concentration, which may be due to the presence of chitin, which has a role in triglyceride hydrolysis (Zhang et al., 2008), by enabling the binding of lipid in the gastrointestinal tract thereby reducing fat absorption.

Myogenesis is a process that involves the formation and expansion of muscle fibers. This process in fish is regulated by several genetic factors, such as MyoD, myostatin, and GH gene (Johnston et al., 2009). The relative mRNA expression of MyoD and myostatin has been affected by the dietary aquafeeds provided. The present study examined the effect of dietary BSFLM protein on the gene expression of MyoD, myostatin, and GH gene. The MyoD showcased an upregulated mRNA expression in goldfish juveniles fed T60 and T40 diets.

In contrast, it downregulated in fish fed T100 diet, which reveals that the dietary supplementation of BSFLM had a positive effect on fish growth performances by enhancing the satellite cell proliferation and differentiation during muscle growth of fishes. The Myostatin value was high in T100, indicating the lower growth performance when 100

percent of fish meal is replaced in the fish diet. Several studies reveal that myostatin negatively influences growth performance, which is in line with the current study. The current study shows that the replacement of FM with BSFLM at 60 percent has increased fish muscle growth. GH serves as an endocrine bioindicator that predicts the growth rate in fishes (Tan et al., 2017). In our present study, BSFLM positively influenced the mRNA expression of the GH gene, which provides a constructive role in the overall growth performance and metabolism of the fishes. The maximum mRNA expression of GH was observed in fish fed with the T60 diet, revealing increased growth performance at this level of BSFLM inclusion in the goldfish diet.

Conclusion

The goldfish juveniles fed with black soldier fly larvae meal have showcased better growth and feed utilization. The haemato-biochemical indices, mRNA expression of muscle-growth-related genes, and digestive enzyme activities showed that the replacement of FM with BSFLM up to 60 percent is practically feasible without impairing the health status of the fish. However, when the FM protein replacement level exceeds 60 percent, the decrement in growth rate was observed with downregulated relative mRNA expression of Myo D and GH genes. In a nutshell, our study suggests that black soldier fly larvae meal protein up to 60 percent could be a note-worthy replacement for fish meal protein in the diet of juvenile goldfish.

Ethical Statement

The experiment was conducted following the procedures of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), The experiment was conducted following the procedures of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Govt. of India on care and use of animals in scientific research. This study was approved by ethical committee of Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam, Tamil Nadu, India

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Author Contribution

A. Kamalii: Conducted the feeding trial, Analyzed 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.

Prabu: Analyzed the data for statistical analysis and corrected the manuscript

Conflict of Interest

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

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