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Substituting fishmeal with insect protein: Impact on growth and growth hormone gene expression in *Labeo rohita*

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The study investigates the impact of substituting fishmeal with insect protein, specifically black soldier fly (BSF) and mealworm (MW) larvae, on the growth performance and growth hormone (GH) gene expression in *Labeo rohita*. The experiment was conducted in triplicates for 60 days in 30 aquaria with ten fingerlings of *Labeo rohita* in each of them. BSF and MW- based diets were used with the following levels of inclusion ; 0%, 25%, 50%, 75%, and 100%. The results revealed that moderate inclusion levels (75% BSF and 50% MW) enhanced growth performance of *L. rohita*. The 75% BSF diet resulted in the highest weight gain (14.53 ± 0.43 g), final weight (20.08 ± 0.38 g) and specific growth rate (SGR) (1.837 ± 0.038) with the lowest feed conversion ratio (FCR) (1.313 ± 0.071). In contrast, the 100% BSF diet showed reduced weight gain (12.04 ± 0.19 g) and SGR (1.643 ± 0.023). The 50% mealworm based diet achieved the highest weight gain (15.56 ± 0.41 g), final weight (21.90 ± 0.43 g) and SGR (1.763 ± 0.029) with the lowest FCR (1.140 ± 0.070). In comparison, the 100% mealworm diet resulted in the lowest growth performance with a final weight of 19.04 ± 0.44 g, weight gain of 12.68 ± 0.46 g, and SGR of 1.563 ± 0.042. Gene expression analysis showed that BSF inclusion at 75% and mealworm inclusion at 50% upregulated GH and IGF1 expression correlating with improved growth. However, excessive inclusion (100%) of both insect meals led to a decline in gene expression and growth performance. The findings are largely consistent with previous studies but also highlights the species specific differences in the response to insect based proteins as well as the importance of optimizing inclusion levels to avoid metabolic imbalances. Future studies should also attempt to understand the complete lifecycle effect of BSF and MW based diets, including the effect on reproductive success of fish and their capacity to resist disease challenge.

Keywords: lipid metabolism; FCR; SGR; aquafeed; aquaculture; Pakistan.

Introduction

Aquaculture is key contributor to global food security, and reports indicate that the demands for aquaculture products will more than double by the year 2050 (Lock et al., 2016). The increasing demand for fish as source of protein in the context of limited marine resources puts pressure on aquaculture (Cashion et al., 2017). This pressure imposes the challenge of developing sustainable aquafeed ingredients. Conventionally, fishmeal has been used as the primary source of protein in aquafeed due to its nutritional value (Hartviksen et al., 2014). Fishmeal contains the best amino acid composition, essential fatty acids and other micronutrients that are needed in the growth and wellbeing of fish. Now the fishmeal production relies primarily on the wild caught fish and because of this, its production has increasingly become ecologically unsustainable (Hu JunRu et al., 2018). Reasons such as depletion of wild stocks, rise in the cost of fishmeal and overfishing have prompted researchers to seek alternative sources of protein (Mohan et al., 2022). The most promising alternatives include insect based protein such as black soldier fly larvae and mealworm larvae (Henry et al., 2015).

The insects, specifically black soldier fly (BSF) and mealworm (MW) larvae, provide an environmentally sustainable solution to the current issues surrounding conventional proteins (Belghit et al., 2019). Both insect species may be grown on organic byproducts, including food waste, agricultural wastes and even manure (Fawole et al., 2019). These two insects produce high-quality protein, which enables low value organic waste to be converted into a high protein feed ingredient. This qualifies the two insects to be the ideal choice in converting waste into feed ingredients high in protein (Cummins et al., 2017). Moreover, black soldier fly and mealworm larvae have extraordinarily high feed conversion ratios.They do not require as many land, water and energy resources to reach the same consumption levels as livestock or fish on fish farms (Fischer et al., 2022). BSF and MW larvae also have a very good nutritional predictive value when it comes to fish diets, containing a lot of protein (42–45% in BSF and 50% in MW), lipids (35–40% in BSF) and essential amino acids among a diverse array of bio-active compounds, including the incredibly well-known antimicrobial peptides and chitin (Ido et al., 2019).

The possibility of replacing fishmeal in aquafeeds with BSF and MW larvae has been investigated in many studies (Hu et al., 2017). It has been determined that fish with an insect meal- based diet have similar or even greater growth results as compared to fish fed normal fishmeal-based diets (Magalhaes et al., 2017). Besides this, lipids of BSF and the MW larvae are saturated fatty acids like lauric acid that show antimicrobial properties and may hence contribute to developing the immune health of farmed fish (Hu et al., 2019). However, the optimal concentrations of BSF and mealworm in aqua feeds continue to be actively studied, including the substrate of their cultivation as well as their mode of processing (Kroeckel et al., 2012). The inclusion of BSF and mealworm in aquafeeds is capable of reducing the usage of fishmeal without damaging, and even improving, fish health and growth performance (Liu et al., 2017). It is important to understand the optimum levels of BSF and mealworms in aquafeeds so that they are able to supplement fishmeal effectively without affecting fish health or performance (Li et al., 2017).

A critical aspect of understanding the effects of dietary changes on fish growth is the regulation of growth hormone (GH) gene expression. One of the most important regulators of somatic growth is GH, which controls a variety of processes, including protein synthesis, cell proliferation, and the development of tissue in general. In fish, the GH is mainly produced by the pituitary gland and works on different target tissues such as liver, muscles and adipose tissues to influence growth. GH expression and receptor expression are also subject to different factors such as dietary composition, environmental factors, and even hereditary factors (Yossa et al., 2011). Several studies have revealed that the inclusion of various sources of proteins is a factor which influences GH gene expression. As one example, high-quality protein sources (e.g. fishmeal) have been demonstrated to stimulate secretion of GH and facilitate growth. On the other hand, there is an indication that inferior amino acid dietary content can inhibit GH expression, resulting in retarded growth and low feed efficiency (Zheng et al., 2013).

Although the possibility of using BSF and mealworm larvae as fishmeal alternatives is already well-known in the literature, there remain some gaps in the existing body of knowledge (Limbu et al., 2022). BSF and mealworms constitute a number of bioactive compounds such as antimicrobial peptides and chitin that have the potential to affect growth performance. Nevertheless, the effect of the compounds on the gene expression and the metabolic pathways in *Labeo rohita* is unclear. Understanding the molecular interactions between insect components and the fish's biological systems is crucial for optimizing the use of BSF and mealworms in aquafeeds and ensuring the sustainability of aquaculture practices. This study aims to investigate the effects of substituting fishmeal with insect protein, specifically from BSF and mealworm larvae, on growth performance and GH gene expression in *Labeo rohita*.

Materials and methods

Experimental design. Labeo rohita fingerlings were obtained from the Fish Nursery Unit, Head Balloki, Punjab, Pakistan. The fingerlings were transported carefully in the water filled aerated bags. Before the start of the experiment, the fish were immersed in NaCl (5 g/L) solution for few minutes in order to get rid any ectoparasites (Xu et al., 2020). The fingerlings were allowed to acclimatize in laboratory conditions for a period of 2 weeks in glass aquaria. Ten *L. rohita* fingerlings (n = 10) were stocked in each aquarium. During this period, the fish were fed with a basal diet once a day.A recirculating aquaculture system (RAS) was used to maintain water quality parameters. Two trials were carried out separately with fifteen aquaria used for each trial. In the first trial, five diets were used, replacing fish meal with black soldier fly larvae meal. In the second trial, again five diets were used replacing fish meal with mealworm. The composition of both diets is mentioned in Table 1 and 2. Each experiment trial was performed in triplicate for 60 days under completely randomized design.

Table 1
Composition of black soldier fly (BSF)-based diet

Ingredient	0%	25%	50%	75%	100%
Fishmeal	20	15	10	5	0
Soyabean meal	21	21.5	22	22.5	23
Wheat flour	10	8.5	7	6	5
Sunflower meal	8	8	8	8	8
Canola meal	10	10	10	10	10
Corn gluten 30%	15	16	17	17.5	18
Vegetable oil	3	3	3	3	3
Rice polish	10	10	10	10	10
Black soldier fly larvae	0	5	10	15	20
Vitamin premix	1	1	1	1	1
Mineral mixture	1	1	1	1	1
Chromic oxide 1%	1	1	1	1	1
Total	100	100	100	100	100
CP%	32.255	32.330	32.235	32.138	32.040

Table 2
Composition of mealworm (MW) based diet

Ingredient	0%	25%	50%	75%	100%
Fishmeal	20	15	10	5	0
Soyabean meal	21	21	21	21	21
Wheat flour	10	9	8	7.5	7.5
Sunflower meal	8	8	8	8	8
Canola meal	10	10	10	10	10
Corn gluten 30%	15	16	17	17.5	17.5
Vegetable oil	3	3	3	3	3
Rice polish	10	10	10	10	10
Mealworm	0	5	10	15	20
Vitamin premix	1	1	1	1	1
Mineral mixture	1	1	1	1	1
Chromic oxide 1%	1	1	1	1	1
Total	100	100	100	100	100
CP%	32.255	32.330	32.405	32.393	32.293

Growth parameters. The growth performance of *L. rohita* was assessed at the end of each experiment. The following formulas were used to calculate weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) (Muin et al., 2017):

Weight gain (WG) = final mean weight (g) – initial mean weight (g),
Feed conversion ratio (fcr) = feed intake (g) / weight gain (g),
Specific growth rate (SGR) = [(ln final mean weight (g) – ln initial mean weight (g) /days fed) x 100].

Statistical analysis. The data thus obtained were analyzed using one-way ANOVA to assess significant differences between treatments. To compare mean values between treatments, Tukey's Honestly Significant Difference Test was applied at a significance level of $P \leq 0.05$ (Steel et al., 1996). The statistical analysis was done using CoStat software (version 6.303).

Gene expression using ΔCt and ΔΔCt method. After the experiment, the liver and muscle tissues were removed and stored at –80 °C for further analysis. The real time PCR and RNA preparation was done according to the kit manufacturer's protocol. Primers for the target genes, namely the GHR, GH, and IGF1 genes, along with the β-actin gene were selected using the already available literature from the database provided by National Center for Biotechnology Information: www.ncbi.nlm.gov (Table 3). The relative quantification of gene expression was performed using the comparative Ct (ΔΔCt) method following Livak et al. (2001).

ΔCt calculation. The ΔCt value for each sample was calculated by subtracting the Ct value of the reference gene from the Ct value of the target gene:

$$\Delta Ct = Ct_{target} - Ct_{reference}$$

ΔΔCt calculation:

$$\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$$

Fold change calculation:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Table 3
List of primers used for GH, IGF-1 and GHR2 gene in q-PCR

Gene	Sequence (5ʹ-3ʹ)	Size, bp	Tm, °C	Product size
β-Actin-F	ATGAAGATCCTGACCGAGAGA	21	62	105
β-Actin-R	CTCGAAGTCAAGAGCCACATAG	22	62	
IGF-1-F	TGTACTGTGCACCCGTAAAG	20	62	120
IGF-1-R	CCTTACAGGAAGAGTGGCTATG	22	62	
GHR2-F	CGCTGCTGAATGTGAGTTTGAC	22	60.66	216
GHR2-R	CCCGAACCTCGTGATTGATG	20	58.76	
GH-F	ACGCCATCTTTACCAACAGC	20	60.1	157
GH-R	TCCACATCTTGTTTGAAGGTG	21	58.6	

PCR amplification curves of GH, IGF1 and GHR2 gene expression in *Labeo rohita* fed with varying levels of black soldier fly (BSF) and mealworm (MW) based diets are presented in figures 1, 2 and 3.

Results

Growth performance of Labeo rohita. The growth performance of *L. rohita* was assessed by varying levels of the two diet groups, black soldier fly (BSF) larvae and mealworm (MW) larvae, as a replacement for fishmeal. The key growth parameters such as weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR) were measured.

Black soldier fly (BSF) based diet. Table 4 summarizes the growth parameters of *L. rohita* fed on the black soldier fly (BSF)-based diet. The results revealed that a clear trend emerged where both weight gain and final weight generally increased with higher BSF levels up to 75%. However, a slight reduction in growth was observed at 100% BSF level. The initial weight of fish ranged from 5.53 ± 0.04 g to 5.56 ± 0.04 g across all treatment groups (0%, 25%, 50%, 75%, and 100% BSF). The data revealed that a significant increase in final weight was observed with higher BSF addition in the diet. The final weight in the 0%, 25%, 50%, 75%, and 100% BSF groups was 14.11 ± 0.26 g, 15.80 ± 0.70 g, 18.25 ± 0.61 g, 20.08 ± 0.38 g and 17.59 ± 0.16 g, respectively. The weight gain of *L. rohita* fed on varying levels of BSF-based diets showed an increasing trend up to 75% BSF diet as the highest weight gain was observed in the 75% BSF group (14.53 ± 0.43 g). However, the 100% BSF diet group showed a lower weight gain (12.04 ± 0.19 g) as compared to other diet groups. No significant differences were observed between the diet groups in terms of feed intake. The feed intake across different groups ranged from 0.240 ± 0.026 g (0% BSF) to 0.277 ± 0.006 g (75% BSF diet group). The lowest FCR was recorded in the 75% BSF diet group (1.313 ± 0.071 g) while the highest was found in the 0% BSF group (1.987 ± 0.263 g). The SGR increased with higher BSF inclusion, peaking at 1.837 ± 0.038 g in the 75% BSF group. The SGR for the 100% BSF group was slightly lower to 1.643 ± 0.023 g, indicating that the optimal growth occurred at 75% BSF inclusion.

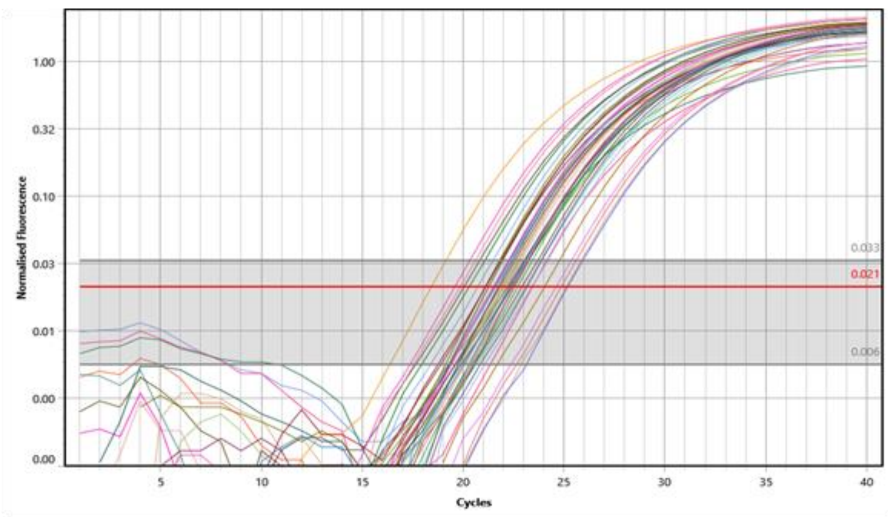

Fig. 1. PCR amplification curves of GH gene expression in *L. rohita* fed with varying levels of black soldier fly (BSF) and mealworm (MW)- based diets

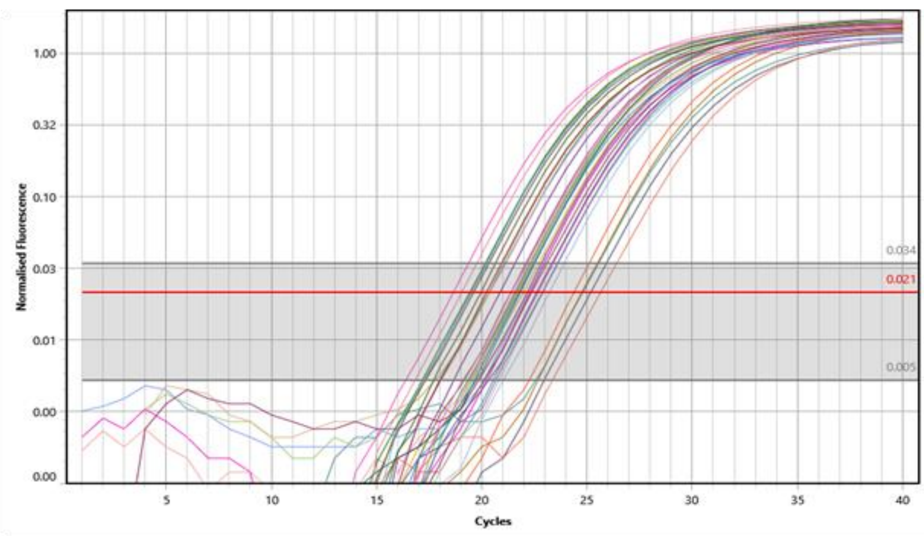

Fig. 2. PCR amplification curves of IGF1 gene expression in *L. rohita* fed with varying levels of black soldier fly (BSF) and mealworm (MW)-based diets

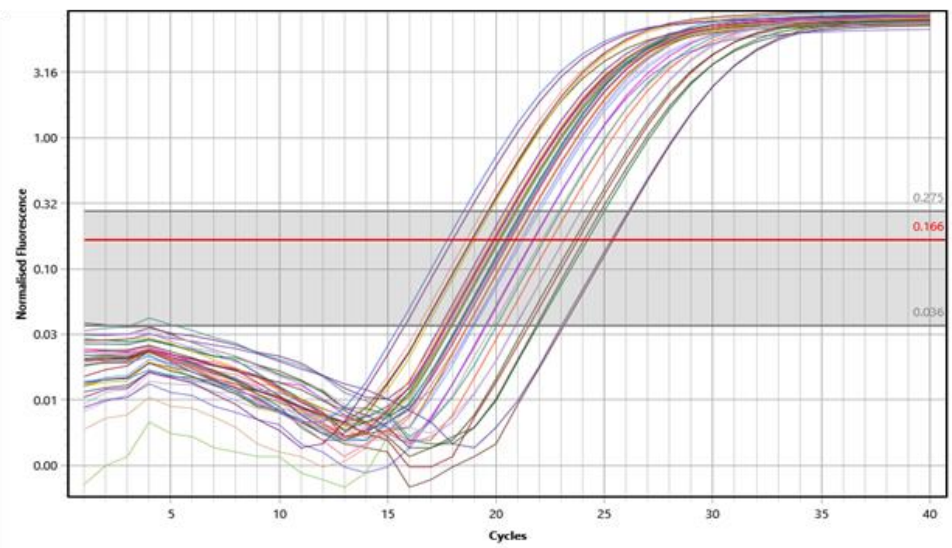

Fig. 3. PCR amplification curves of GHR2 gene expression in *L. rohita* fed with varying levels of black soldier fly (BSF) and mealworm (MW)- based diets

Table 4
Growth performance of *Labeo rohita* fed on black soldier fly (BSF) - based diets (x ± SD, n = 3, duration of experiment – 60 days)

Parameters	0%	25%	50%	75%	100%	P
Initial weight, g	5.56 ± 0.04	5.54 ± 0.04	5.53 ± 0.04	5.54 ± 0.05	5.55 ± 0.04	0.9430
Final weight, g	14.11 ± 0.26[e]	15.80 ± 0.70[d]	18.25 ± 0.61[b]	20.08 ± 0.38[a]	17.59 ± 0.16[c]	0.0000
Weight gain, g	8.55 ± 0.22[e]	10.26 ± 0.74[d]	12.72 ± 0.64[b]	14.53 ± 0.43[a]	12.04 ± 0.19[c]	0.0000
Feed intake	0.240 ± 0.026	0.243 ± 0.012	0.263 ± 0.006	0.277 ± 0.006	0.265 ± 0.009	0.1977
FCR	1.987 ± 0.263[e]	1.690 ± 0.207[d]	1.440 ± 0.095[b]	1.313 ± 0.071[a]	1.540 ± 0.062[c]	0.0038
SGR	1.327 ± 0.021[e]	1.490 ± 0.070[d]	1.703 ± 0.055[b]	1.837 ± 0.038[a]	1.643 ± 0.023[c]	0.0001

Note: means with different superscripts in a row showing statistically significant difference ($P \leq 0.05$).

Mealworm (MW) based diets. The MW based diets showed an increase in weight gain and final weight up to 50% MW inclusion followed by a slight decrease at 75% and 100% MW inclusion. Table 5 summarizes the growth parameters of *Labeo rohita* fed on mealworm (MW)- based diets. The initial weight of *L. rohita* fed on MW-based diets ranged from 6.32 ± 0.17 to 6.35 ± 0.08 g with no significant differences across groups. The data revealed a significant increase in final weight with higher MW addition in diet. The final weight in the 0%, 25%, 50%, 75%, and 100% MW groups was 15.44 ± 0.34, 16.84 ± 0.35, 21.90 ± 0.43, 20.42 ± 0.27 and 19.04 ± 0.44 g, respectively. The data revealed that final weight reached its maximum in the 50% MW group at 21.90 ± 0.43 g. The 100% MW diet group showed a lower final weight of 19.04 ± 0.44 g, suggesting a decline in growth. In terms of weight gain, the highest values was recorded in the 50% MW group (15.56 ± 0.41 g). However, the 100% MW group showed the lowest weight gain (12.68 ± 0.46 g). The 25% and 75% MW diet groups showed intermediate results as 10.50 ± 0.31 and 14.08 ± 0.33 g, respectively. The lowest FCR was recorded in the 50% MW diet group (1.140 ± 0.070) while the highest was found in the 0% MW diet group (1.803 ± 0.131). The SGR was highest in the 50% MW diet group (1.763 ± 0.029). The 75% and 100% MW groups showed a declining trend in SGR with values of 1.670 ± 0.036 and 1.563 ± 0.042, respectively.

Table 5
Growth performance of *Labeo rohita* fed on mealworm (MW) based diets (x ± SD, n = 3, duration of experiment – 60 days)

Parameters	0%	25%	50%	75%	100%	P
Initial weight, g	6.32 ± 0.17	6.34 ± 0.05	6.35 ± 0.08	6.34 ± 0.08	6.35 ± 0.06	0.9965
Final weight, g	15.44 ± 0.34[e]	16.84 ± 0.35[d]	21.90 ± 0.43[a]	20.42 ± 0.27[b]	19.04 ± 0.44[c]	0.0000
Weight gain, g	9.11 ± 0.51[e]	10.50 ± 0.31[d]	15.56 ± 0.41[a]	14.08 ± 0.33[b]	12.68 ± 0.46[c]	0.0000
Feed intake	0.237 ± 0.021	0.247 ± 0.015	0.250 ± 0.020	0.250 ± 0.010	0.260 ± 0.010	0.4366
FCR	1.803 ± 0.131[e]	1.660 ± 0.144[d]	1.140 ± 0.070[a]	1.270 ± 0.020[b]	1.450 ± 0.020[c]	0.0000
SGR	1.270 ± 0.072[e]	1.393 ± 0.015[d]	1.763 ± 0.029[a]	1.670 ± 0.036[b]	1.563 ± 0.042[c]	0.0000

Note: means with different superscripts in a row showing statistically significant difference ($P \leq 0.05$).

Gene expression of growth hormone (GH), growth hormone receptor 2 (GHR2) and insulin-like growth factor 1 (IGF1). The relative gene expression of the GH, GHR2, and IGF1 genes was measured in *L. rohita* fed on BSF and MW- based diets using β-actin as the reference gene. The data was calculated using the ΔCt, ΔΔCt, and fold change methods. The fold change values in gene expression analysis indicates how much the expression of a target gene has increased or decreased in the experimental group relative to the control group and it provides insight into the upregulation or downregulation of three genes within different diet groups.

Black soldier fly (BSF) based diets. Figure 3 shows the Box analysis of the GH, GHR2, and IGF1 genes' fold expression in BSF-based diet groups. There was a notable variations in gene expression across different BSF levels.

The expression of growth hormone (GH) was highest in the 100% BSF group (2.88) followed by 50% BSF (2.53), indicating a positive trend with higher BSF inclusion. The high expression value revealed that BSF had a significant effect on promoting GH production with increased dietary addition, leading to a positive modulation of this growth related gene. However, the 75% BSF group showed a decline in GH expression at 0.87. The value indicates a potential threshold beyond which GH expression diminishes, possibly due to dietary imbalances or the presence of anti-nutritional factors. The highest insulin-like growth factor 1 (IGF1) expression was recorded in the 50% BSF group (8.43) followed by 3.03 in the 100% BSF group, 1.89 in the 25% BSF group and it decreased significantly (0.53) in the 75% BSF diet group. IGF1 is an important mediator of GH signaling and reveals a clear positive response to BSF supplementation particularly at moderate addition levels. Growth hormone receptor 2 (GHR2) expression was relatively stable across varying levels of BSF diets. The highest expression 1.00 was noted in the 0% BSF group followed by 0.98 in the 100% BSF and 0.16 in the 25% BSF group. GHR2 expression is vital for the cellular responses to GH and its relatively constant expression suggested that the receptor sensitivity to GH remained unaffected by varying level BSF - based diets.

Mealworm (MW) based diets. The gene expression data in the MW diet groups showed a contrasting trend. The expression of growth hormone (GH) was highest (1.92) in the 75% MW group and lowest (0.14) in the 100% MW- based diet group. The 50% MW diet group also showed an increase in GH expression at 1.85. The decreasing trend suggested that while moderate inclusion of MW may stimulate GH production, excessive addition may lead to a suppression of GH gene expression potentially due to nutrient imbalances or other factors within the MW. The highest insulin-like growth factor 1 (IGF1) expression decreased progressively with increasing MW inclusion. The highest expression (1.11) was recorded at 0% MW followed by 0.31, 0.38 and 0.16 in the 50%, 75%, and 100% MW diet groups respectively. The findings revealed that while the control group (0% MW) benefited from higher IGF1 expression, increasing the % of MW in the diet suppressed IGF1 expression, possibly due to changes in nutrient availability or amino acid profile in the MW diets. Similar to IGF1, GHR2 expression decreased as MW % increased. The highest expression (1.05) was recorded in the 0% MW group and the lowest value (0.03) was recorded in the 100% MW diet group. This could indicate a reduced ability to respond to GH at higher MW levels, further corroborating the negative impact of excessive MW inclusion (Fig. 5).

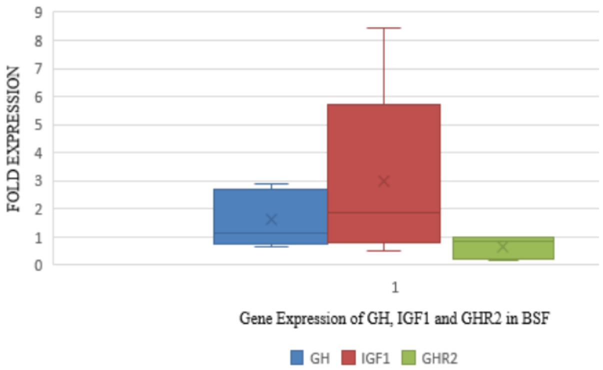

Fig. 4. Fold Expression of GH, IGF1 and GHR2 in black soldier fly (BSF) diet groups

Integrated analysis of growth performance and gene expression. The growth performance of *L. rohita* and the expression of growth associated genes were examined across different diet groups with varying levels of black soldier fly (BSF) and meal-worm (MW) inclusion. The analysis of these parameters provides insights into the molecular reinforcements of growth regulation and the relationship between dietary composition and growth performance.

In the BSF-based diet groups, a notable positive correlation was observed between the fold expression of the GH and IGF1 genes and the growth performance parameters, including weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) The 75% BSF diet group exhibited the highest weight gain (14.53 ± 0.43 g), SGR (1.837 ± 0.038) and the lowest FCR (1.313 ± 0.071 g). These findings corresponded with a significant upregulation of both GH (fold change – 2.53) and IGF1 (fold change – 8.43) expression, indicating that moderate inclusion of BSF enhanced the growth through the activation of growth promoting molecular pathways. The upregulation of GH promotes the secretion of IGF1, which is a key mediator of growth. This in turn positively impacts the growth performance of *L. rohita*. The high IGF1 expression in the 50% BSF diet group is due to enhanced nutrient utilization and anabolic processes, resulting in increased weight gain and growth efficiency. However, the 100% BSF diet, despite showing the highest GH expression (fold change – 2.88), demonstrated reduced weight gain (12.04 ± 0.19 g) and SGR (1.643 ± 0.023), as well as a less favorable FCR (1.540 ± 0.062). This decrease in growth performance correlates with a decline in IGF1 expression (fold change – 3.03) and a reduction in GHR2 (fold change – 0.98) expression. The diminished IGF1 expression in the 100% BSF diet group may indicate that excessive BSF inclusion leads to metabolic inefficiencies, possibly due to imbalances in lipid or amino acid profiles, which hinder the growth-promoting effects of GH signaling. The lower GHR2 expression further suggests a reduced sensitivity to GH, limiting its effectiveness in promoting growth.

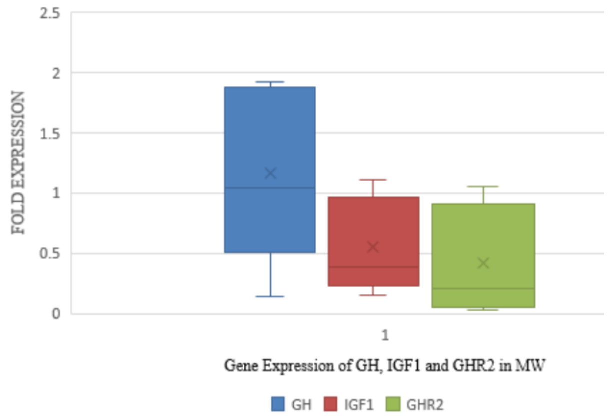

Fig. 5. Fold Expression of GH, IGF1 and GHR2 in mealworm (MW) diet groups

In the MW-based diet groups, a similar trend was observed, albeit with some differences in the magnitude of gene expression and growth performance. The relationship between GH and IGF1 expression and growth parameters was inversely affected by higher inclusion levels of MW. The 50% MW diet group exhibited the highest weight gain (15.56 ± 0.41 g), SGR (1.763 ± 0.036) and the lowest FCR (1.140 ± 0.070), which correlates with the moderate upregulation of GH (fold change – 1.85) and IGF1 (fold change – 0.38). This group showed the optimal balance between GH production and IGF1 activation, supporting efficient growth. The moderate expression of GH and IGF1 suggests that this level of MW inclusion provides sufficient stimulation of growth-promoting pathways without overwhelming the system, leading to optimal nutrient utilization and growth performance. In contrast, the 100% MW diet showed the lowest growth performance with weight gain of 12.68 ± 0.46 g, SGR of 1.563 ± 0.042 g, and FCR of 1.450 ± 0.020. These results correspond to a significant reduction in GH (fold change – 0.14), IGF1 (fold change – 0.16), and GHR2 (fold change – 0.03) expression. The drastic reduction in GH and IGF1 expression indicates a suppression of the growth-related signaling pathways, likely due to nutrient imbalances or the presence of anti-nutritional factors in the MW diet at higher inclusion levels. The reduced GHR2 expression in the 100% MW group suggests impaired receptor sensitivity to GH, further limiting the fish's ability to respond to growth signals.

The correlation between growth data and gene expression data suggests that the molecular regulation of growth in *Labeo rohita* is intricately linked to dietary composition, particularly the inclusion of BSF and MW as fishmeal substitutes. The key genes associated with growth, GH and IGF1, play critical roles in modulating somatic growth, and their expression is positively correlated with growth performance at moderate levels of both insect-based diets. For BSF, 75% inclusion resulted in the most favorable growth performance, coinciding with optimal gene expression of GH and IGF1, thereby suggesting that this diet stimulates the growth pathways most efficiently. At 100% BSF, the decline in both GH and IGF1 expression, coupled with reduced growth performance, indicates that excessive BSF may cause metabolic disruptions that limit growth. For MW, 50% inclusion was similarly associated with optimal growth, reflected by the highest weight gain, SGR, and FCR, alongside moderate GH and IGF1 expression. The inverse trend observed in the 100% MW group, characterized by suppressed GH and IGF1 expression and poor growth, highlights the need for a balanced diet to optimize growth. Excessive MW inclusion appears to impair the molecular mechanisms that regulate growth, potentially due to the cumulative effects of anti-nutritional factors or imbalances in amino acid profiles at higher levels of inclusion.

The data suggests a clear relationship between gene expression particularly GH, IGF1, and GHR2 and growth performance in *L. rohita*. Moderate inclusion of BSF (75%) and MW (50%) in the diet appears to maximize both gene expression and growth performance, supporting the notion that balanced supplementation with insect-based proteins can optimize growth through the modulation of growth-related gene expression pathways. However, exceeding certain inclusion levels leads to a suppression of these molecular pathways, resulting in reduced growth.

Discussion

The current research examined the potential viability of black soldier fly (BSF) and mealworm (MW) larvae- based diets as an economically viable alternative to fishmeal in the dietary management of *Labeo rohita* in terms of growth and growth hormone (GH) gene expression. The results contributed significantly to the knowledge of the nutritional advantages and constraints of such insect-derived proteins, especially concerning growth parameters and gene expression. The results fall in line with majority of the influential works on the matter, providing BSF with favorable justification as an alternative to fishmeal in the composition of fish food. Belghit et al. (2019) further indicated that BSF meal can be utilized to substitute fishmeal by 15 per cent without having any effect on the growth efficiency of Atlantic salmon (*Salmo salar*). The findings revealed that BSF can be a possible source of protein and lipids even to support the same level of growth obtained using the ordinary fishmeal diet. Likewise, Barca et al. (2023) examined the application of BSF meal to substitute fishmeal in the zebra fish diet and found that BSF inclusion had no significant negative impact on growth performances. The findings indicated that there was no significant difference in the weight gains and FCR with diets partially replaced with BSF by up to 25%. These findings are comparable to ours since moderate levels of inclusion of BSF stimulated growth and are also a cost-effective source of protein in aquafeeds.

However, a notable difference arises when BSF inclusion exceeds a certain threshold. According to Ido et al. (2021), an increase in BSF meal (30%) inclusion in *Seriola quinqueradiata* (Japanese amberjack) diets resulted in retarded growth, especially in larvae that were not defatted. It is a vital observation contrasting to our study, in which even at higher levels of BSF inclusion we did not witness any significant growth trade-offs. The difference is probably attributable to the fish species and fat level in the BSF larvae. This carnivorous fish might possibly be more sensitive toward the high-fat diet than *L. rohita*. Ido et al. (2021) suggested that the high fat content of BSF may lead to metabolic stress, affecting growth.

Hu et al. (2017) showed that *Litopenaeus vannamei* grew at the same rate when offered a diet with 10 percent BSF meal substituted in turn for a traditional fishmeal diet. This indicates the flexibility of BSF as a feed ingredient across various species, which validates the conclusion that incorporation of the ingredient can sustain growth performance in both fresh and marine habitats as long as the level of incorporation is optimized. Nevertheless, Zhou et al. (2017) found that *Cyprinus carpio* growth rates were slightly reduced with increasing BSF level (30%) in the diet, reminiscent of the problems with Ido et al. (2021). This reinforces the conclusion that species specific formulations need to be applied in a situation where BSF is employed in aquafeeds. Further, the results of this study are in line with Wang et al. (2019), who demonstrated that BSF meal replaced fishmeal by 20% in tilapia-based diets without influencing growth.

In conclusion, the available literature clearly shows that BSF could be applied as a partial or complete substitute for fishmeal in aquafeed but the optimal levels of addition of BSF are species specific and a close attention should be taken in regard to fat content of BSF to avoid any metabolic perturbations. The effect of BSF on the metabolism of lipids, especially in the context of growth performance also need to be investigated. In particular, fatty acid composition of BSF in combination with lipid metabolism pathways of various fish species is an open question. The presence of lauric acid, which has antimicrobial properties, could offer additional benefits in terms of disease resistance, as discussed by Fischer et al. (2022) and Kroeckel et al. (2012). However, excessive inclusion of BSF without proper fat adjustment may lead to undesirable outcomes.

The gene expression analysis was conducted to explore the molecular mechanisms behind the observed growth patterns and the potential role of insect-based proteins in promoting growth through hormonal pathways. The gene expression patterns observed in this study align with several findings in the literature but also highlighted key differences, particularly in the response of *Labeo rohita* to different insect proteins. The positive relationship that is found between GH and IGF1 expression and growth performance among the BSF diet groups is in line with past findings. According to Belghit et al. (2019), the addition of BSF to fish diet led to GH secretion and improved growth. The current research also reinforced this observation by proving that GH and IGF1 expression at a moderate BSF inclusion level (up to 75%) is the best way to improve growth performance. In a similar manner, Ido et al. (2021) reported that BSF had a positive effect on GH and IGF1 expression in fish especially at moderate incorporation levels. Nevertheless, the results of our research regarding the reduction of GH and IGF1 expression at 100% BSF inclusion are different compared to the study conducted by Hu et al. (2017), which did not indicate a reduction in *Litopenaeus vannamei*, even at increased inclusion of BSF. The difference might exist with respect to fish species or formulation of diet. In our study, metabolic stress could have been initiated by the fat content of BSF at a high inclusion rate, which interfered with the production of GH and IGF1, which previous studies have not examined fully.

The decrease in GH and IGF1 expression with increased mealworm inclusion level is in line with the results reported by Li et al. (2017), who with another study also reported suppressed GH and IGF1 expression when mealworms were incorporated at elevated levels in diets of fish. It shows that the addition of excessive amounts of MW can disturb the molecular pathways of growth, which is probably caused by an imbalance in the amino acids profile, or the release of anti-nutritional factors, which might inhibit the performance of the GH signaling. Research has also demonstrated that activation of insect-based diets related to growth is also possible due to reduced receptor sensitivity towards GH, which is also observed in this study because when the inclusion of higher MW is utilized, there is a corresponding decrease in GHR2 expression (Magalhães et al., 2017). In their study, a reduction in expression of the receptors resulted in reduced efficiency of the response to growth and this also explains their findings, where the inclusion of increased MW was related to reduced expression of GHR2 and inhibition of growth. Studies by Kroeckel et al. (2012) and Fischer et al. (2022) have demonstrated that insect proteins, particularly BSF and MW, can stimulate growth through the upregulation of GH and IGF1. However, excessive inclusion of insect proteins, especially at 100%, appears to impair growth by disrupting GH signaling pathways, as indicated by the reduction in GH, IGF1, and GHR2 expression at higher inclusion levels in this study.

The literature consistently highlights the importance of optimizing inclusion levels to balance nutrient availability and avoid metabolic disturbances (Ido et al., 2021). This study confirms that both BSF and MW can support growth, but exceeding certain thresholds may lead to negative outcomes, as evidenced by the reduced gene expression and growth performance at high inclusion levels.

Conclusion

The current study provides valuable insights into the potential use of BSF and MW as a fishmeal substitute. Moderate inclusion of BSF (75%) and MW (50%) in the diet appears to maximize both gene expression and growth performance, supporting the notion that balanced supplementation with insect-based proteins can optimize growth through the modulation of growth-related gene expression pathways. However, exceeding certain inclusion levels leads to a suppression of these molecular pathways, resulting in reduced growth. Several limitations should be acknowledged on the basis of our results, such as the relatively short duration of the feeding trial (60 days), which means that the long-term effects of insect-based diets on the health and reproduction of fish remain unclear. Moreover, the discrepancy of findings between species indicates that some additional studies must be conducted in order to identify the most effective amounts of inclusion and modes of material processing related to fish of various types. The possible bioaccumulation of heavy metals or other pollutants within insect-based diets must also be studied. Future studies should also attempt to understand the complete lifecycle effect of BSF and MW-based diets, including the effect on reproductive success of fish and their capacity to resist disease. Moreover, the data on gene expression in this study supports the early findings that moderate level of inclusion of BSF and MW in aquaculture diets increases growth by upregulating the growth-related genes such as GH and IGF1. The findings are largely consistent with previous research but also highlight the species-specific differences in the response to insect-based proteins as well as the importance of optimizing inclusion levels to avoid metabolic imbalances.

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