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Effects of lowering dietary fishmeal and crude protein levels on growth performance, body composition, muscle metabolic gene expression, and chronic stress response of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

A study was conducted to evaluate the effects of lowering dietary fishmeal (FM) and crude protein (CP) levels, while maintaining essential amino acid levels, on growth performance, body composition, muscle metabolic gene expression, and chronic stress response of rainbow trout, *Oncorhynchus mykiss*, with and without handling stress. Eight experimental diets (isocaloric) with a 4 × 2 factorial design were formulated to contain two levels of FM (20%, 5%) and four levels of CP (48%, 45%, 42%, 39%). Diets were supplemented with increasing levels of amino acids to maintain dietary essential amino acid (EAA) levels. Trout (34.8 ± 0.3 g) were fed to apparent satiation twice daily for nine weeks to assess growth performance under laboratory rearing conditions, and then for an additional six weeks with and without exposure to handling stress (30 s of chasing followed by 30 s of netted air exposure) twice per week. The 9-week growth trial demonstrated that reducing dietary FM levels from 20% to 5% significantly reduced fish growth and increased feed conversion ratio ($P < .05$). Reducing dietary CP levels from 48% to 42% did not affect trout growth. A dietary FM level of 20% significantly increased whole-body dry matter, CP and total EAAs ($P < .05$) compared to 5% FM inclusion while increasing dietary CP level significantly decreased dry matter, crude fat, and gross energy but increased total EAAs ($P < .05$). Reducing FM and CP levels had no effect on measured stress indices of plasma cortisol, glucose and lysozyme activity ($P > .05$) after 6-weeks of repeated handling stress. The expression of genes in the *gcn2/eif2α/atf4* pathway, triggered in response to protein or amino acid starvation, were evaluated. *General control nonderepressible 2 (gcn2)* decreased with increasing dietary CP level above 42% ($P < .05$), but there were no dietary effects (FM or CP levels) on *eif2α* (*eukaryotic initiation factor 2α*) or *atf4* (*activating transcription factor 4*) expression. In total, gene expression results suggest amino acid limitations on muscle protein metabolism as a result of feeding diets below 42% CP, even when supplemented with synthetic EAA to meeting published dietary requirements. In conclusion, our study demonstrated that 5% dietary FM is insufficient for maximal growth performance, while diets balanced for EAAs show an opportunity to reduce CP level from 48% to 42% without any reduction in growth performance, body composition, metabolic amino acid sufficiency or tolerance to chronic stress. Below 42% CP, reduced growth indices suggest an imbalance in EAA availability.

1. Introduction

Across the aquaculture industry, feed producers have lowered fishmeal (FM) levels in feeds and increased alternative protein sources, especially of plant origin, primarily to cope with volatile increases in FM prices (Burr et al., 2012; Naylor et al., 2009; Gatlin et al., 2007; Cheng et al., 2003). However, the amino acid profiles of plant protein meals differ to that of FM protein (Burr et al., 2012; Cheng et al., 2003), creating certain limitations to their use. Supplemental amino acids have been increasingly used in fish feed formulations based on plant protein feedstuffs to meet physiological requirements for limiting amino acids. In addition to protein source, feed cost is also dictated by the protein level. Commercial grower feeds for rainbow trout, *Oncorhynchus mykiss*, contain crude protein (CP) levels ranging from 42% to 48% depending on fish size (Hardy, 2002). In fact, fish require well-balanced amino acids, not CP, *per se*. Supplemental amino acids also provide an opportunity to minimize the dependency of intact protein sources on meeting the target level of individual amino acids and thus reduce

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dietary CP levels. In this context, a more precise understandings of nutritional requirements of the target species and raw material digestibility play an important role in successfully reducing expensive dietary FM and CP levels and overall feed cost.

While nutritionists use NRC (2011) data as the baseline for nutrient recommendations when formulating feeds, the aquafeed industry has developed somewhat higher parallel recommendations to buffer the requirements of fish under dynamic production conditions. Nutrient requirements of fish under commercial production conditions can be affected by various biotic (e.g., fish density, sex, health conditions) and abiotic (e.g., water quality, feed quality) factors. Stress associated with fish culture practices, such as stocking density, grading, netting, and hauling of fish, may also affect nutrient requirements at fixed feeding rates (Li et al., 2009; Lovell, 2002). Conversely, how fish handle stress following different dietary histories is unclear, especially during periods of chronic stress. There appears to be no effect of varying protein and lipid concentration in rainbow trout diets on baseline cortisol concentrations (Morrow et al., 2004); yet, plant-based diets have been shown to result in a stronger acute, cortisol stress response (Sadoul et al., 2016). The potential for interactions between diet and stress has significant implications for fish wellbeing.

We hypothesized that both dietary CP and FM content could be successfully reduced to maintain growth performance and feed efficiency as long as EAA requirements are met, but that reduced dietary CP and FM content would exacerbate the response to chronic stress. Therefore, the present study evaluated the factorial effects of lowering dietary FM and CP levels, while maintaining dietary EAA concentrations through synthetic amino acid supplementation, on rainbow trout growth performance, nutrient utilization, and response to chronic handling stress.

2. Materials and methods

2.1. Experimental design and diets

The proximate composition and amino acid content of experimental diets are shown in Tables 1 and 2. Diets were formulated to contain two levels of FM (20% or 5%) and within each FM level, varying levels of CP (48%, 45%, 42% or 39%). Diets were supplemented with feed-grade lysine, methionine, threonine, tryptophan, arginine, histidine, isoleucine and valine as needed to match levels in the 20% FM/48% CP diet, while also meeting or exceeding the published EAA requirements of rainbow trout. Experimental feeds were produced by extrusion pelleting, similar to commercial fish feed production technology, at the Bozeman Fisheries Technology Center, Bozeman, MT. All ingredients used in the experimental feeds were ground to a particle size of < 200 μm using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) and processed using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with a ~25 s exposure to 127 °C in the extruder barrel (average across 5 sections). Pellets were dried with a pulse-bed drier (Buhler AG, Uzwil, Switzerland) for 20 min at 102 °C with a 10-minute cooling period, resulting in final moisture levels < 10%. Diets were stored in plastic lined paper bags, shipped to the Hagerman Fish Culture Experiment Station (HFCES), Hagerman, ID and stored at room temperature until fed.

2.2. Fish and feeding trial

Rainbow trout eggs (Troutlodge, Sumner, WA) were hatched and reared using commercial diets for three months at the HFCES. Thirty fish (initial body weight: 35 g) were randomly distributed into each of 24, 145-L tanks. Each tank was supplied with 8–10 L/min of isothermal (15 °C) spring water fed by gravity to the fish rearing system. Each diet was assigned randomly to three tanks in a completely randomized design. Fish were hand-fed to apparent satiation two times per day, six days per week for 15 weeks. Photoperiod was maintained at 14 h light:10 h dark with fluorescent lights controlled by electric timers. Fish were fed for nine weeks to assess growth performance under laboratory rearing conditions, and then the 30 fish per tank were split into two tanks with 15 fish each for an additional six weeks, during which time the fish continued to be reared under similar conditions, with continued feeding of their respective dietary treatments, but either with or without the application of a repeated (2 ×/wk) handling stress, specifically 30-s of chasing, followed by capture in nets, removal from tanks, and 30 s of air exposure.

2.3. Sample collection

At the end of the first nine weeks of feeding, all the fish were counted and weighed to calculate weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and percent survival. Following an additional 6 weeks (15 weeks total on feed) under stressful or control conditions, three fish per tank were euthanized with tricaine methanesulfonate (MS-222,100 mg/L, buffered to pH 7.4) for whole-body analysis. Additionally, two fish per tank were sampled for plasma analysis and muscle gene expression. For measurement of plasma stress indices, fish were sampled 1-hour post-challenge. Blood was collected from the caudal vessels of fish with 3-mL syringes fitted with the heparinized 21G 1-in. needle and centrifuged at 1000 g for 8 min to collect plasma for lysozyme, cortisol and glucose analysis to evaluate the physiological stress response following the additional six weeks. Samples for muscle gene expression were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. All fish handling and sampling, plus the experimental protocols used in this project were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC).

2.4. Sample analysis

Experimental feeds and whole-body fish samples were analyzed for proximate composition and energy content. Fish samples were pooled by tank and homogenized using an industrial food processor. Samples were dried in a convection oven at 105 °C for 12 h to determine moisture level according to AOAC (2000). Dried samples were finely ground by mortar and pestle and analyzed for CP (total nitrogen x 6.25) using combustion method with a nitrogen determinator (TruSpec N, LECO Corporation, St. Joseph, MI). Crude lipid was analyzed by subjecting samples to acid hydrolysis using an ANKOM HCL hydrolysis system (ANKOM Technology, Macedon, NY) and extracting them with petroleum ether using an ANKOM XT15 extractor. Ash was analyzed by incineration at 550 °C in a muffle furnace for 5 h. The energy content of samples was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL).

Amino acid analyses of experimental feeds and ingredients were performed by Evonik Nutrition & Care GmbH. Amino acid composition of whole-body samples was analyzed using a BioChrom 30+ amino acid analyzer (Biochrom Ltd., Cambridge, UK). Lysozyme activity in plasma was analyzed with a lysozyme assay kit (Sigma-Aldrich. St. Louis MO) following the method of Lee et al. (2016) with slight modifications. *Micrococcus lysodeikticus* (0.75 mg mL^{-1}) was suspended in phosphate buffer (0.1 M, pH 6.24), 800 μL of suspension was placed in each well of 48-well plates, and 30 μL plasma was added subsequently. The reduction in absorbance of the samples was recorded at 450 nm after incubation at room temperature for 0 and 30 min in a microplate reader (Infinite® m200 PRO, Tecan Trading AG, Switzerland). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity. Plasma cortisol was analyzed with a cortisol ELISA assay kit (DRG International Inc., Springfield, NJ) validated for rainbow trout (Velasco-Santamaría and Cruz-Casallas, 2007) and plasma glucose with a glucose colorimetric assay kit (Cayman Chemical Inc. Ann Arbor, MI), according to the manufacturer's instructions, respectively. To assay cortisol, 20 μL of either cortisol standard or plasma sample were

Table 1
Ingredient composition of experimental diets.

Ingredients (%)	Diets							
	48CP	45CP	42CP	39CP	48CP	45CP	42CP	39CP
	20FM	20FM	20FM	20FM	5FM	5FM	5FM	5FM
Fishmeal, sardine[a]	20.00	20.00	20.00	20.00	5.00	5.00	5.00	5.00
Poultry by-product meal[a]	14.10	12.49	10.60	8.74	16.50	15.26	13.81	12.38
Blood meal, spray dried[a]	2.00	1.77	1.50	1.24	2.00	1.85	1.67	1.50
Dried distiller's grain with solubles[a]	8.58	6.30	6.02	3.39	11.86	8.71	7.07	2.07
Corn protein concentrate[b]	14.10	12.49	10.60	8.74	16.50	15.26	13.81	12.38
Soybean meal[a]	8.50	7.53	6.39	5.27	4.00	3.70	3.35	3.00
Soy protein concentrate[c]	2.00	1.77	1.50	1.24	12.40	11.04	9.45	7.88
Wheat gluten meal[a]	1.50	1.33	1.13	0.93	1.50	1.39	1.26	1.13
Wheat flour[a]	13.50	18.43	22.09	27.47	11.50	16.91	21.33	28.25
Fish oil[d]	13.12	14.62	16.18	17.79	14.35	15.73	17.25	18.75
DL-Methionine, MetAMINO®[e]	0.00	0.06	0.12	0.20	0.10	0.17	0.23	0.31
L-Lysine sulfate, Biolys®[e]	0.00	0.24	0.49	0.76	0.55	0.81	1.09	1.41
L-Histidine[f]	0.00	0.00	0.00	0.06	0.00	0.00	0.03	0.10
L-Arginine[f]	0.00	0.00	0.05	0.25	0.00	0.00	0.08	0.31
L-Tryptophan[f]	0.18	0.20	0.23	0.26	0.19	0.22	0.25	0.29
L-Threonine[f]	0.00	0.11	0.22	0.34	0.09	0.20	0.32	0.45
L-Isoleucine[f]	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.17
L-Valine[f]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22
Dicalcium phosphate[a]	0.52	0.76	0.98	1.31	1.56	1.85	2.10	2.50
Trace mineral premix[g]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[h]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride[a]	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Stay-C (vitamin C, 35%)[a]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20

[a] Rangen Inc., Buhl, ID, USA.
[b] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[c] Profine VF, The Solae Company, St. Louis, MO, USA.
[d] Skretting USA, Tooele, UT, USA.
[e] Evonik Nutrition and Care GmbH, Hanau, Germany.
[f] Sigma Aldrich, St. Louis MO, USA.
[g] Trace mineral premix supply the following to the diet (mg/kg diet): Zn (as $ZnSO_4$ $7H_2O$), 50; Mn (as $MnSO_4$), 7.5; Cu (as $CuSO_4$ $5H_2O$), 2.5; I (as KIO_3), 1; selenium, 0.05.
[h] Vitamin premix supply the following to the diet (mg/kg diet): D calcium pantothenate, 46.47; pyridoxine (pyridoxine HCl), 13.68; riboflavin, 9.58; niacinamide, 21.78; folic acid, 2.49; thiamine (thiamine mononitrate), 9.1; inositol, 599; biotin, 0.33; vitamin B_{12}, 0.03; menadione sodium bisulfite complex, 1.1; vitamin E (DL α-tocopherol acetate), 131.9 IU; vitamin D_3 (stabilized), 6594 IU; vitamin A (vitamin A palmitate, stabilized), 9641 IU; ethoxyquin, 198.

dispensed into wells of a 96 well plate, followed by 200 µL of cortisol–horseradish peroxidase conjugate. After thoroughly mixing for 10 s, 60 min incubation, and 3 times washing (wash solution provided in the kit), 100 µL of substrate solution containing tetramethylbenzidine were added. Then, the plate was incubated for 15 min at room temperature, the reaction was stopped by adding 100 µL of H_2SO_4 0.5 M, and the optical density was read at 450 nm. For the glucose assay, 85 µL assay buffer and 15 µL of either glucose standard or plasma sample were mixed in each well of a 96 well plate. Then, 100 µL of the enzyme mixture provided was added and incubated for 10 min at 37 °C. The optical density was read at 510 nm.

RNA from each tissue was isolated by homogenizing the tissue in TRIzol (Invitrogen, Carlsbad, CA). The RNeasy 96 QIAcube HT Kit (Qiagen Inc., Venlo, Netherlands) protocol was followed for the rest of the RNA isolation. Quantity and quality of extracted RNA were assessed by Nanodrop ND-1000 spectrophotometer, the 260/280 ratio was > 1.8. Extracted RNA was treated with DNAse, then 1 µg of total RNA was reverse-transcribed using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA). Real-time quantitative PCR was carried out on a CFX96 Real-Time System (BioRad) in a 10 µL total volume reaction using iTaq SYBR Green Supermix (BioRad) and 500 nmol primers according to the protocol provided by the manufacturer. PCR cycling conditions for all genes were as follows: 95 °C for 5 s followed by 55 °C for 30s over 40 cycles with an initial denaturation step of 95 °C for 3 min. For each fish, PCR reactions were run in duplicate on RNA samples. Relative expression values for genes constituting the integrated stress response (ISR), including *eukaryotic initiation factor 2α* (*eif2α*; *de novo* protein synthesis), *activating transcription factor 4* (*atf4*; transcriptional activator), and *general control nonderepressible 2* (*gcn2*; amino acid deficiency), were determined using primers (Table 3) designed from rainbow trout sequences in the NCBI GenBank® database. In addition, a cellular mRNA control was selected from a set of three reference genes (*arp*, *elf1α* and *gapdh*). *Acidic ribosomal protein* (*arp*) was identified as being unaffected by experimental treatments ($P > .05$) and having the least variance within samples, and, thus, was used for normalization of target gene expression in muscle tissue. Primer PCR efficiency was calculated by including five serial dilutions of a standard (pooled from each experimental sample for a given tissue) and utilized for PCR correction for all primer pairs (Pfaffl, 2001). Normalized data were analyzed using the relative quantification method described by Pfaffl (2001).

2.5. Calculation and statistical method

Using the live-weight and feed consumption data, the following indices were calculated.

Weight gain (WG, g/fish) = (g mean final weight–g mean initial weight)

Specific growth rate (SGR,%/d)
= [(ln mean final weight–ln mean initial weight)/number of days]
× 100

Table 2
Analyzed proximate and nutrient levels in experimental diets (% as-fed basis).

Nutrients	Diets							
	48CP	45CP	42CP	39CP	48CP	45CP	42CP	39CP
	20FM	20FM	20FM	20FM	5FM	5FM	5FM	5FM
Dry matter (%)	98.1	97.9	96.8	97.8	98.2	97.4	97.0	97.0
Crude protein (%)	48.5	46.0	42.1	39.4	48.2	45.4	43.0	39.7
Crude fat (%)	16.8	17.4	18.4	18.8	17.8	16.6	16.7	17.8
Ash (%)	8.90	8.48	7.88	7.65	7.80	7.51	7.06	6.67
NFE (%)	22.9	25.0	27.5	31.0	23.3	26.8	29.2	31.8
Gross energy (MJ/kg)	22.7	22.8	22.6	22.8	22.8	22.8	22.4	22.6
EAAs								
Arginine	2.51	2.31	2.18	2.15	2.44	2.30	2.24	2.21
Histidine	1.11	1.01	0.91	0.85	1.05	0.99	0.94	0.84
Isoleucine	1.89	1.77	1.64	1.58	1.83	1.72	1.66	1.62
Leucine	4.53	4.18	3.83	3.41	4.59	4.34	4.09	3.67
Lysine	2.37	2.37	2.28	2.38	2.37	2.37	2.41	2.43
Methionine	1.05	1.03	1.01	1.02	1.01	1.02	1.04	1.04
Phenylalanine	2.28	2.12	1.97	1.77	2.31	2.19	2.05	1.84
Threonine	1.81	1.76	1.75	1.73	1.79	1.78	1.75	1.75
Tryptophan	0.60	0.59	0.60	0.61	0.61	0.60	0.61	0.60
Valine	2.20	2.10	1.90	1.77	2.15	2.03	1.90	1.87
NEAAs								
Alanine	3.16	2.93	2.67	2.42	3.04	2.86	2.64	2.35
Aspartic Acid	3.83	3.56	3.27	3.03	3.67	3.48	3.20	2.87
Cysteine	0.62	0.58	0.53	0.49	0.65	0.62	0.59	0.53
Glutamic Acid	8.09	7.54	7.06	6.33	8.17	7.88	7.47	6.93
Glycine	2.72	2.52	2.32	2.11	2.46	2.34	2.21	1.99
Proline	3.33	3.00	2.85	2.48	3.32	3.20	2.99	2.74
Serine	2.15	1.98	1.83	1.67	2.16	2.07	1.87	1.71
Sum AA	44.2	41.3	38.6	35.8	43.6	41.8	39.7	37.0
Sum EAA	20.3	19.2	18.0	17.3	20.1	19.3	18.7	17.9

Table 3
Primers sequences used in real-time qPCR.

Primers	Component	Sequences (5′-3′)
gcn2[a]	Forward	F - TACAGAACAAGAGCAATGAC
	Reverse	R - ATTGACAGGAAGTTGATGAG
eif2α[b]	Forward	F - TCGGCAAAGTAGATATGTG
	Reverse	R - ACAACAGTGACCTTCTCT
atf4[c]	Forward	ACCAAGATGAAGAGGATGA
	Reverse	GAAGAGGCAGAAGAGTTG
arp[d]	Forward	GAAGGCTGTGGTGCTCAT
	Reverse	CAGGGCAGGGTTGTTCTC
gapdh[e]	Forward	ACTCTGTTGTGTCTTCTG
	Reverse	TTGTCGTTGAAGGAGATG
elf1α[f]	Forward	ACATTAACATTGTGGTCATT
	Reverse	CGCACTTGTAGATCAGAT

[a] *gcn2*: *general control nonderepressible 2.*
[b] *eif2α*: *eukaryotic translation initiation factor 2.*
[c] *atf4*: *activating transcription factor 4.*
[d] arp: *acidic ribosomal phosphoprotein.*
[e] *gapdh*: *glyceraldehyde-3-phosphate dehydrogenase.*
[f] *elf1α*: *elongation factor 1 alpha.*

Survival (%)

= (number of fish at the end of the trial /number of fish at the beginning) × 100

Average feed intake (FI, g/fish)

= g total dry feed intake/number of surviving fish

Feed conversion ratio (FCR)

= g total feed consumed/(g final biomass–g initial biomass + g dead fish weight)

Protein efficiency ratio (PER)

= (g final biomass–g initial biomass + g dead fish weight) /(g total feed consumed × %dietary protein)

Protein retention efficiency (PRE,%)

= g protein gain/g protein consumed × 100

Tank mean values ($n = 3$) were used for all statistical analysis. All data were subjected to multi-factorial ANOVA test using SAS Version 9.4 (SAS Institute, Cary, NC, USA). When a significant main effort or interaction was observed, Tukey's HSD test was used to compare the means. Treatment effects were considered significant at $P < .05$.

3. Results

3.1. Growth performance

Growth performance and feed utilization of rainbow trout juveniles fed diets containing different CP and FM levels for 9 weeks under pre-stress, optimal culture conditions are presented in Table 4. Mortality was low overall, with no significant differences among the treatment groups ($P > .05$). Reducing fishmeal from 20% to 5% significantly reduced final body weight (FBW) and growth rate ($P < .05$). Furthermore, reduction in dietary fishmeal resulted in reduced feed intake and increased FCR as well as reduced PER and PRE ($P < .05$). Reducing dietary CP levels from 48% to 42% did not affect trout weight gain; however, further reduction in CP from 45% to 39% resulted in significantly lower fish weight gain. Reducing dietary CP levels did not affect FCR whereas protein retention significantly improved with decreasing dietary CP. The interaction of the two main factors (FM and CP) significantly impacted feed intake (Fig. 1; $P < .05$); but had no significant effects on growth performance or feed utilization ($P > .05$).

3.2. Whole-body composition

The whole-body proximate composition of rainbow trout juveniles fed the experimental diets for 15 weeks, including a final 6-week stress evaluation, are presented in Table 5. Overall, a reduction in dietary FM (20FM vs. 5FM) level significantly reduced whole-body dry matter (33.3 vs. 32.5%) and crude protein (16.4 vs 15.8%) and increased whole-body ash (2.03 vs. 2.12%) ($P < .05$); while decreasing dietary CP levels significantly increased whole-body dry matter, crude fat and gross energy ($P < .05$). There were no interactions between dietary FM, CP, and stress on whole-body proximate composition ($P > .05$). Stress (non-stress group vs. stress group) significantly increased whole-body dry matter (32.5 vs. 33.5%), crude protein (15.9 vs. 16.2%), crude fat (13.7 vs. 14.6%) and gross energy (9.20 vs. 9.66 MJ/kg) ($P < .05$).

Whole-body amino acid compositions are presented in Table 6. Stress significantly decreased levels of whole-body EAAs, 7.12 vs. 7.48% for stress vs. non-stress group, respectively ($P < .05$). This reduction held true when non-essential amino acids (NEAAs) were included, 14.7 vs.15.1%, respectively, for stress vs. non-stress total whole-body amino acids ($P < .05$). Similarly, whole-body individual essential as well as total amino acids decreased with decreasing dietary CP level ($P < .05$). The interactions between dietary FM, CP, and stress were not significant for whole-body total EAA composition ($P > .05$).

3.3. Biochemical analysis of plasma

The results of the biochemical assessment of plasma components are presented in Table 7. Stress significantly increased plasma

Table 4
Growth performance and feed utilization of rainbow trout juveniles fed experimental diets for 9 weeks.[a]

Diets	Initial weight (g/fish)	FBW (g/fish)	WG (g/fish)	SGR (%/day)	Survival (%)	FI (g,DM/fish)	FCR	PER	PRE (%)
Means of main effects[a]									
Fishmeal									
20FM	34.8	175[a]	141[a]	2.57[a]	99.7	124[a]	0.88[b]	2.61[a]	43.0[a]
5FM	34.8	164[b]	130[b]	2.47[b]	99.7	119[b]	0.92[a]	2.47[b]	39.5[b]
Crude Protein									
48CP	34.8	174[a]	139[a]	2.55[a]	100	125[a]	0.90	2.31[d]	37.8[c]
45CP	34.8	174[a]	139[a]	2.55[a]	99.5	124[a]	0.89	2.46[c]	39.7[bc]
42CP	34.8	168[ab]	134[ab]	2.50[ab]	99.5	120[ab]	0.90	2.61[b]	42.1[b]
39CP	34.8	164[b]	129[b]	2.46[b]	100	117[b]	0.91	2.78[a]	45.3[a]
Pooled SE	0.058	1.645	1.640	0.015	0.188	0.980	0.005	0.039	0.735
Multi-factor ANOVA (P value)									
Fishmeal	0.516	**< 0.001**	**< 0.001**	**< 0.001**	1.000	**0.003**	**< 0.001**	**< 0.001**	**< 0.001**
Protein	0.996	**0.002**	**0.002**	**0.003**	0.585	**< 0.001**	0.102	**< 0.001**	**< 0.001**
Fishmeal × Protein	0.790	0.251	0.246	0.255	0.299	**0.016**	0.222	0.111	0.558

Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold *P values* in the *ANOVA* table.

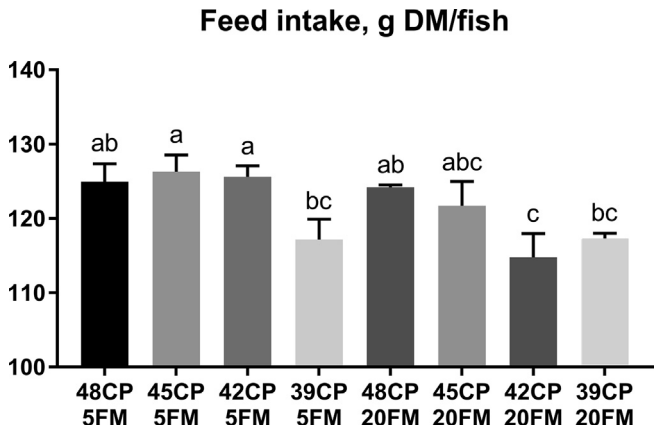

Fig. 1. Feed intake (g DM/fish) of rainbow trout juveniles fed experimental diets for 9 weeks. Bars represent mean ± SE of triplicate samples. Two-way ANOVA indicated a significant CP X FM interaction ($P < .05$). Interaction means having different letters are significantly different by Tukey's HSD test ($P < .05$).

concentrations of cortisol and glucose and increased and lysozyme activity ($P < .05$). However, dietary FM and CP levels had no effect on any plasma biochemical assessment ($P > .05$). The interaction of the main factors (FM and CP) also failed to have any observable effect on any biochemical assessment in the plasma ($P > .05$).

3.4. Gene expression

Relative gene expression in the white muscle of rainbow trout fed experimental diets is presented in Table 8. *gcn2* expression significantly increased with decreasing dietary level of CP ($P < .05$). The main effect of handling stress also significantly increased the expression of *atf4* in muscle but did not affect that of *gcn2*. However, the expressions of *eif2α* and *atf4* were not significantly different among the experimental diets due to FM or CP levels or their interaction ($P > .05$).

4. Discussion

The results of this study validate the use of supplemental amino acids to support growth performance in rainbow trout fed a low crude protein diet. At the same time, fishmeal at a dietary level above 5% was required for maximal growth. After nine weeks of feeding, results indicated that reducing dietary CP levels below 42% reduced growth performance and FI of rainbow trout. Similarly, Gaylord and Barrows (2009) reported no differences in growth of juvenile (initial weight:

Table 5
Whole-body proximate composition (%, wet basis) of rainbow trout juveniles fed experimental diets for 15 weeks.[a]

Diets	Dry matter (%)	Crude protein (%)	Crude fat (%)	Ash (%)	Gross energy (MJ/kg)
Means of main effects[a]					
Stress					
Non-stress group	32.3[b]	15.9[b]	13.7[b]	2.06	9.20[b]
Stress group	33.5[a]	16.2[a]	14.6[a]	2.10	9.66[a]
Fishmeal					
20FM	33.3[a]	16.4[a]	14.4	2.03[b]	9.54
5FM	32.5[b]	15.8[b]	13.9	2.12[a]	9.31
Crude Protein					
48CP	32.0[b]	16.3	13.1[c]	2.04	9.05[b]
45CP	32.7[ab]	16.1	13.9[bc]	2.10	9.36[ab]
42CP	33.3[a]	16.0	14.6[ab]	2.11	9.56[a]
39CP	33.7[a]	15.9	15.0[a]	2.07	9.75[a]
Pooled SE	0.187	0.328	0.314	0.074	0.061
Multi-factor ANOVA (P value)					
Stress	**< 0.001**	**0.048**	**0.009**	0.384	**< 0.001**
Fishmeal	**0.024**	**< 0.001**	0.147	**0.042**	**0.068**
Protein	**0.004**	0.162	**< 0.001**	0.719	**0.002**
Stress × Fishmeal	0.238	0.341	0.528	0.889	0.580
Stress × Protein	0.667	0.371	0.675	0.770	0.722
Fishmeal × Protein	0.785	0.602	0.874	0.116	0.723
Stress × Fishmeal × Protein	0.361	0.381	0.054	0.362	0.306

Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold *P values* in the *ANOVA* table.

20 g) rainbow trout fed 40.9% CP when compared to those fed 49% CP when diets were balanced for amino acids using supplemental sources. Regardless of dietary protein and amino acid content, FM content had an overall negative effect on growth performance when reduced from 20% to 5%. This reduction from 20% to 5% FM is equivalent to a 75% substitution with other protein sources (poultry byproduct meal, DDGS, soy protein concentrate and corn gluten meal). Similar results have also been reported in salmonid diets having a high FM replacement with plant protein, adversely affecting growth indices and FI (Jalili et al., 2013). Antinutritional factors (ANFs), such as phytate, are known to inhibit digestive enzyme activities and reduce digestibility of some nutrients (Robaina et al., 1995) and this likely resulted in lower final average fish weights, feed intake and efficiency. Putative growth promoters in FM, such as hormones and other growth factors, may also

Table 6
Essential amino acid composition (%, wet basis) in whole-body of rainbow trout juveniles fed experimental diets for 15 weeks.[a]

Diets	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Total EAA	Total AA
Means of main effects[a]													
Stress													
Non-stress group	0.97[a]	0.13[a]	0.56[a]	0.69[a]	1.14[a]	1.27[a]	0.50[a]	0.64[a]	0.71[a]	0.08	0.80[a]	7.48[a]	15.5[a]
Stress group	0.92[b]	0.12[b]	0.53[b]	0.66[b]	1.08[b]	1.22[b]	0.47[b]	0.61[b]	0.68[b]	0.08	0.76[b]	7.12[b]	14.7[b]
Fishmeal													
20FM	0.94	0.13	0.54	0.68	1.11	1.26	0.49	0.63	0.69	0.07	0.78	7.31	15.0
5FM	0.95	0.12	0.54	0.68	1.11	1.23	0.49	0.62	0.70	0.08	0.78	7.30	15.2
Crude Protein													
48CP	0.99[a]	0.13[a]	0.58[a]	0.71[a]	1.17[a]	1.29[a]	0.51[a]	0.66[a]	0.73[a]	0.08	0.81[a]	7.66[a]	15.8[a]
45CP	0.93[ab]	0.12[bc]	0.53[b]	0.65[b]	1.08[b]	1.20[b]	0.47[c]	0.60[bc]	0.68[bc]	0.07	0.75[b]	7.08[b]	14.8[bc]
42CP	0.96[ab]	0.13[ab]	0.55[ab]	0.71[a]	1.14[a]	1.29[a]	0.50[ab]	0.65[ab]	0.70[ab]	0.08	0.81[a]	7.51[a]	15.4[ab]
39CP	0.90[b]	0.12[c]	0.50[c]	0.65[b]	1.05[b]	1.19[b]	0.47[bc]	0.60[c]	0.66[c]	0.07	0.73[b]	6.95[b]	14.4[c]
Pooled SE	0.022	0.003	0.015	0.014	0.024	0.031	0.010	0.016	0.016	0.004	0.018	0.157	0.327
Multi-factor ANOVA (P value)													
Stress	**0.007**	**0.004**	**0.001**	**0.001**	**0.002**	**0.005**	**0.002**	**0.018**	**0.003**	0.540	**0.001**	**0.002**	**0.003**
Fishmeal	0.266	0.221	0.462	0.804	0.632	0.150	0.733	0.430	0.509	**0.089**	0.962	0.934	0.571
Protein	**0.003**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	**< 0.001**	0.244	**< 0.001**	**< 0.001**	**< 0.001**
Stress × Fishmeal	0.680	0.740	0.420	0.455	0.435	0.179	0.502	0.625	0.361	0.300	0.555	0.435	0.528
Stress × Protein	0.438	0.581	0.956	0.834	0.844	0.266	0.527	0.418	0.710	0.094	0.737	0.638	0.575
Fishmeal × Protein	0.988	0.214	0.929	0.846	0.696	0.394	0.811	0.997	0.839	0.096	0.896	1.000	0.999
Stress × Fishmeal × Protein	0.842	0.559	0.703	0.321	0.547	0.568	0.487	0.873	0.465	0.432	0.100	0.547	0.513

Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold *P values* in the *ANOVA* table.

Table 7
Plasma lysozyme activity and cortisol and glucose concentrations of on rainbow trout juveniles fed experimental diets for 15 weeks.[a]

Diets	Lysozyme activity (Unit/mL enzyme)	Cortisol (ng/mL)	Glucose (mg/dL)
Means of main effects[a]			
Stress			
Non-stress group	374[b]	6.14[b]	123[b]
Stress group	397[a]	40.5[a]	182[a]
Fishmeal			
20FM	385	22.2	157
5FM	386	24.4	148
Crude Protein			
48CP	395	19.2	141
45CP	376	24.5	147
42CP	384	24.7	156
39CP	387	24.6	168
Pooled SE	5.69	2.03	4.61
Multi-factor ANOVA (P value)			
Stress	**0.047**	**< 0.001**	**< 0.001**
Fishmeal	0.986	0.284	0.200
Protein	0.711	0.172	0.052
Stress × Fishmeal	0.741	0.493	0.498
Stress × Protein	0.879	0.329	0.076
Fishmeal × Protein	0.251	0.836	0.236
Stress × Fishmeal × Protein	0.225	0.963	0.810

Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold *P values* in the *ANOVA* table.

Table 8
Relative mRNA expression of genes (normalized against *arp*) in the muscle of rainbow trout juveniles fed experimental diets for 15 weeks.[a]

Diets	*gcn2*	*eif2α*	*atf4*
Means of main effects[a]			
Stress			
Non-stress group	1.06	1.04	1.01[b]
Stress group	1.05	1.06	1.04[a]
Fishmeal			
20FM	1.06	1.05	1.03
5FM	1.05	1.05	1.02
Crude Protein			
48CP	1.02[c]	1.03	1.01
45CP	1.03[bc]	1.05	1.02
42CP	1.06[b]	1.06	1.03
39CP	1.10[a]	1.07	1.04
Pooled SE	0.006	0.007	0.006
Multi-factor ANOVA (P value)			
Stress	0.768	0.062	**0.038**
Fishmeal	0.916	0.944	0.646
Protein	**< 0.001**	0.231	0.266
Stress × Fishmeal	0.879	0.439	0.881
Stress × Protein	0.332	0.115	0.120
Fishmeal × Protein	0.414	0.991	0.984
Stress × Fishmeal × Protein	0.537	0.210	0.233

Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold *P values* in the *ANOVA* table.

contribute to growth performance at high dietary levels. Unbalanced amino acid concentrations in a diet result in increased protein degradation (Von Der Decken and Lied, 1993; Langar et al., 1993; Kumar et al., 2011) and thereby increased protein turnover. Although low FM diets were balanced to match the profile of high FM diets, while meeting the targeted levels of EAAs, growth performance of fish still dropped. The low biological efficiency of dietary crystalline-amino acids (AAs) compared to protein-bound AAs, such as those in fishmeal, has been reported by several authors (Peres and Oliva-Teles, 2005; Watanabe et al., 2001; Mambrini and Kaushik, 1994). One possible reason for this low bio-efficiency could be the leaching of free AAs from feed. Another is the rapid absorption of crystalline-AAs across the gastrointestinal tract compared to protein-bound AAs, which could accelerate the peak level of plasma AA concentrations and reduce the effectiveness of AA utilization in fish (Peres and Oliva-Teles, 2005; Zarate et al., 1999).

The observed changes in whole-body composition in the present study are in accordance with several previous studies reporting whole-body lipid composition increased as the dietary CP level decreased (Haghparast et al., 2017; Wang et al., 2013; Abdel-Tawwab et al., 2010). Increase in body fat indicates intake of excess dietary energy. Although diets were balanced for digestible energy, diets with decreasing levels of CP contained increasing levels of fat and nitrogen free extract (NFE) which could lead to increased body fat.

As an index of metabolic changes in the muscle, the expression of genes in the *gcn2/eif2α/atf4* pathway, triggered in response to protein or amino acid starvation, were evaluated in the present study. Animal cells have evolved this complex signaling pathway to mediate cellular

responses to environmental stressors such as nutrient deprivation. In this pathway, phosphorylation of *eif2α* initiates a wide range of adaptive mechanisms (Harding et al., 2003; Zinszner et al., 1998). *Eukaryotic initiation factor 2α* phosphorylation occurs via the activation of one of four kinases in response to distinct stressors (Wek et al., 2006; B'chir et al., 2013). Of these kinases, *gcn2* (*general control nonderepressible 2*) drives the integrated stress response (IRS) to amino acid starvation (Sood et al., 2000). In the current study, low dietary CP significantly increased the expression of *gcn2* ($P < .05$). This result is indicative of a limitation of available amino acids, signaling a nutrient stressor in the muscle. *Eukaryotic initiation factor 2α* phosphorylation suppresses general protein synthesis but promotes the translation of specific mRNAs, such as *atf4* (B'chir et al., 2013). *Activating transcription factor 4*, in turn, activates downstream stress-induced genes for protein synthesis, which seek to restore homeostasis (Kilberg et al., 2009; Donnelly et al., 2013). *Activating transcription factor 4* gene expression was upregulated in the stressed group of trout compared to the non-stress group, signifying the systematic activation of the IRS pathway in response to cellular stress. The lack of observed changes in *gcn2* and *eif2α* expression in the stressed fish suggests phosphorylation, rather than expression, may prove to be a better measure of IRS pathway response. However, *gcn2* expression was upregulated with decreasing dietary CP level. Given that *gcn2* is primarily a sensor of amino acid availability, these results suggest that the supplemental amino acids in the low CP diet did not provide the necessary balance of amino acids for efficient muscle protein synthesis.

Many researchers (Pack et al., 1995; Refstie et al., 2000; Cheng et al., 2003) reported that unbalanced diets lower nitrogen retention in salmon and trout because these diets have less digestible energy and an amino acid profile that is suboptimal for muscle growth. In salmonids, increases found in whole-body fat content with the use of different levels of dietary CP were explained by imbalances in amino acid concentrations (Bjerkeng et al., 1997; Kaushik et al., 2004). Another reason could be increased dietary carbohydrate and lipid content as the CP level was reduced. Differences in the source of energy have also been reported to increase fat deposition, especially a high carbohydrate content in trout feeds. (Enes et al., 2008). For example, a high ratio of dietary carbohydrate to protein was observed to increase the deposition of body fat through *de novo* lipogenesis from carbohydrates (Brauge et al., 1994; Fernández et al., 2007; Ozório et al., 2009).

Although there were no interactions between diet (CP or FM) and stress on body composition, adding chronic stress to the fish in the present study increased whole-body lipid ($P < .05$) content. Chronic stress induces an increase in plasma cortisol concentration, which contributes, with other hormones, to the induction of a hypermetabolic state characterized by increased energy expenditure, accelerated net protein breakdown and negative nitrogen balance, and increased gluconeogenesis (Christiansen et al., 2007). At the same time, glucocorticoids, which are stress hormones, can increase fat deposition (Burt et al., 2006). The addition of stress also resulted in an overall increase in whole-body protein ($P < .05$) but a decrease in total amino acid content. Although the difference in whole-body protein concentrations between stressed (16.2%) and non-stressed (15.9%) is small, this suggests an increase in non-protein nitrogen resulting in an artificial increase in calculated protein ([N] x 6.25) relative to the total amino acid content.

Plasma cortisol and glucose are the most commonly measured indicators of stress response in fish (Wendelaar Bonga, 1997). We did not observe significant differences in plasma cortisol levels among the dietary treatments ($P > .05$), but as expected, the stressed group exhibited higher plasma cortisol and glucose levels than the non-stressed group ($P < .05$). Overall, observations of plasma cortisol and glucose changes in stressed and non-stressed fish were similar to previous reports for salmonids (Barton, 2000; Jentoft et al., 2005). The primary stress response involves increases in plasma catecholamines and cortisol (Barton, 2002). These hormones induce secondary stress responses, characterized by increased glucose levels, mobilizing glucose to tissues for homeostasis to cope with energy-demanding processes of restoration (Wendelaar Bonga, 1997; Barton, 2002). As such, stress leads to a reallocation of metabolic energy and negatively impacts other energy-driven physiological processes, such as growth, reproduction and immune function. (Wendelaar Bonga, 1997; Mommsen et al., 1999).

Lysozyme (1,4-β-N-acetylmuramidase) is an enzyme found in fish bodily fluids, including blood, and is known as a non-specific immune trait having bacterial cell wall-degrading capacity, breaking the bond between N-acetyl muramic acid and acetylglucosamine (Samarakoon et al., 2013). Lysozyme has been shown to respond to stressful stimuli (Moeck and Peters, 1990; Røed et al., 1993; Demers and Bayne, 1997). Fevolden and Røed (1993) even suggested that under certain circumstances, lysozyme activity may be a more stable indicator of stress than cortisol in rainbow trout. Similar to the results for cortisol and glucose, dietary levels of CP and FM had no effect on lysozyme activity under stress or non-stress conditions ($P > .05$) in the present study.

Stressors affect fish in all stages of their lives and the stress-specific responses that occur at the biochemical and physiological levels affect the overall health and longevity of the fish. The use of a balanced diet or functional ingredients in diets is thought to positively reduce the physiological stress response and have direct effects on individual health (Gonzalez-Silvera et al., 2018). The push toward more sustainable aquafeeds, often with lower FM and CP, may have the opposite effect. Sadoul et al. (2016) demonstrated a strengthened stress response, behaviorally and physiologically, in rainbow trout fed a plant-based diet for seven months from first feeding then captured and moved to a novel tank. Another study demonstrated no effect of dietary CP or lipid level on baseline cortisol or glucose (Morrow et al., 2004). Together with the present study, these three studies suggest dietary FM and CP composition may have no effect on basal plasma cortisol concentrations, yield a more robust acute cortisol stress response, and have no effect on plasma cortisol concentrations following chronic stress, assuming nutrient requirements are met. Many factors go into the interpretation of these results, such as different diet, fish genotype, and degree and type of stressor. It's clear more research is needed to better understand the effect of dietary history on fish stress and wellbeing.

From this study, we observed that diets balanced for amino acids and other critical nutrients show an opportunity to reduce dietary CP level in feeds for rainbow trout from 48% to 42% without reducing growth performance, feed efficiency, body composition, metabolic amino acid sufficiency or stress tolerance. However, below 42% dietary CP, observations of reduced growth indices suggest an imbalance in EAA availability. Reducing the dietary FM level to 5% also significantly reduced rainbow trout performance, possibly a result of reduced feeding stimulants and/or non-nutritive growth promoters associated with FM; however, neither low dietary CP nor FM impacted the physiological stress response following six weeks of chronic stress. Further studies are needed to better understand the absorption and metabolism of supplemental amino acids, drivers of reduced feed intake, the non-nutritive growth promoting components of FM, and the impact of dietary history of fish wellbeing in order to maximize performance of rainbow trout on low CP-low FM feeds.

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