Ronald Hardy

I am providing several references to provide background and support for my comments regarding feeds for salmon and trout, digestibility of feeds, progress towards development of more efficient and sustainable feeds for trout. I list several here that provide background and data on digestibility of feeds and feed ingredients by trout and have attached other peer-reviewed papers published in international scientific journals that provide a broader context on the topics.

National Research Council (NRC) 2011. Nutrient Requirements of Fish and Shrimp. National Academy Press, Washington, D.C.

USDA-ARS Trout-Grains Project. Database of nutrient digestibility of traditional and novel feed ingredients for trout and hybrid striped bass.
https://www.ars.usda.gov/pacific-west-area/aberdeen-id/small-grains-and-potato-germplasm-research/docs/fish-ingredient-database/

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Improved performance of a rainbow trout selected strain is associated with protein digestion rates and synchronization of amino acid absorption

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Replacement of fishmeal in feeds is critical for sustainable aquaculture growth. However, replacement with plant protein concentrates reduces fish performance. A rainbow trout strain selected for high performance on a plant protein diet was compared to a non-selected strain to identify physiological mechanisms associated with improved performance. Nutrient digestibility in fishmeal and plant protein diets was assessed and no strain differences were found. Levels of amino acids in the hepatic portal vein and caudal vein were measured at intervals after a single force-feeding of fishmeal, four plant protein concentrates, and a mixture of the concentrates with or without supplementation of three limiting amino acids. Each ingredient affected plasma amino acid levels in a singular manner when fed individually but without predictable additive effects when fed as a mixture. Amino acid supplementation altered uptake and plasma concentrations of all the essential amino acids. The selected trout strain fed the plant protein mixture with amino acids showed a synchronous and homogenous pattern for essential amino acids over time in the hepatic portal vein in contrast to that of the non-selected strain. The results demonstrate that selection favorably altered temporal dynamics of plant protein digestion.

Replacement of fishmeal as the major protein source in feeds is critical for continued growth of the aquaculture industry as well as advancement of sustainable aquaculture[1–3]. Plant protein concentrates produced from grains, oilseeds and pulses are the leading alternative protein sources to replace fishmeal in fish feeds. However, numerous studies have shown suboptimal fish growth performance and reduced protein retention efficiency when carnivorous fish species are fed low-fishmeal, high-plant protein feeds even when all known dietary essential nutrients, including amino acids, are present above required levels[4–8]. There are several factors blamed for reduced growth of carnivorous fish fed plant protein-based diets, including reduced feed intake, antinutrients in plant products, differences in levels of biologically significant components, such as anabolic steroids or phytoestrogens in plant proteins compared to fishmeal, unidentified nutrient deficiencies and an imbalance of essential amino acids[9–11] In the case of the later, plant proteins generally have less lysine, methionine and threonine compared to fishmeal and are often deficient compared to dietary requirements of fish[3]. To correct deficiencies in plant protein-based diets, amino acid supplements are added[3]. However, evidence suggests that this approach may cause an imbalance of amino acids in blood plasma associated with rapid uptake of supplemented amino acids and delayed digestion and absorption of amino acids of plant origin compared to fishmeal[12–14]. Protein synthesis in cells requires that all essential amino acids are available at the moment proteins are synthesized; if one essential amino acid is not present in sufficient amounts, the remaining amino acids are alternatively metabolized for energy[3]. This may result in lower protein retention efficiency and increased protein turnover, a common observation when fish are fed plant-based feeds[5–7,13].

A rainbow trout strain (ARS/KO) has been developed using selective breeding over 12 years (six generations) based on growth performance when fed an all-plant protein feed at the University of Idaho in collaboration

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Ingredient	Plant meal Diet	Fishmeal Diet
Soy protein concentrate	25.63	15.00
Soybean meal	19.55	
Corn protein concentrate	17.54	10.00
Wheat gluten meal	4.07	7.00
Wheat starch	8.81	18.00
Fish meal		33.00
Fish oil	15.70	14.00
L-Lysine	1.40	
DL-Methionine	0.38	
Threonine	0.20	
Taurine	0.50	
Dicalcium phosphate	3.33	1.20
Potassium chloride	0.56	
Sodium chloride	0.28	
Magnesium oxide	0.05	
Stay-C (35% ascorbate)	0.20	0.20
Choline chloride	0.60	0.60
Yttrium oxide	0.10	0.10
Trace mineral premix[a]	0.10	0.10
Vitamin premix 702[b]	1.00	0.80

Table 1. Composition of the experimental diets (g/100 g). [a]Supplied the following per kg diet: copper, 3 mg as copper sulfate pentahydrate; manganese, 10 mg as manganese sulfate monohydrate; iodine, 5 mg as potassium iodide; sodium selenate 0.960 mg; and zinc, 37 mg as zinc sulfate, heptahydrate. [b]Supplied the following per kg diet: vitamin A palmitate, 9650 IU; cholecalciferol, 6600 IU; DL-tocopheryl acetate, 132 IU; menadione sodium bisulfate 1.1 mg; thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine HCl 13.7 mg; DL-calcium pantothenate, 46.5 mg; cyanocobalamine 0.03 mg, nicotinic acid, 21.8 mg; D-biotin, 0.34 mg; folic acid 2.5; and inositol, 600 mg.

with the US Department of Agriculture's Agricultural Research Service. The selected strain grows rapidly and efficiently when fed all plant-protein feeds containing 45% soy products, unlike non-selected trout that exhibit 10–15% lower growth and feed efficiencies than selected trout[15]. The selected strain can be considered as a model to explore and identify physiological parameters associated with improved plant protein utilization in carnivorous fish.

The goal of the present study was to identify digestive mechanisms associated with improved performance of the selected strain when fed an all-plant protein, soy-based diet. To explore digestive mechanisms, we performed two series of experiments. In the first experiment we investigated if selection improved nutrient digestibility by comparing the selected strain with a non-selected fast-growing strain of rainbow trout when fed a fishmeal-based diet and the selection diet (all-plant protein soy-based). In the second experiment, both rainbow trout strains were fed five practical ingredients (fishmeal and four plant protein concentrates) individually or as mixture of the plant protein concentrates with or without amino acid supplementation. Plasma amino acid patterns were measured over time after a single feeding to investigate if plasma amino acid temporal patterns at the absorption site, the hepatic portal vein (HPV), and from the systemic blood, the caudal vein (CV), could reveal differences between the two trout strains associated with growth performance and be used to assess alternate ingredients. Further, we investigated if results obtained with single feed ingredients could predict nutritional value when the alternate ingredients were combined and if supplementing a plant protein mixture with limiting amino acids influenced absorption and apparent utilization of other amino acids. Finally, we tested the hypothesis that the improved protein utilization and growth demonstrated by the selected strain when fed an all-plant protein soy-based diet was associated with synchronized amino acid uptake.

Materials and Methods

Experiment 1: Digestibility trial. *Experimental diets.* *In vivo* digestibility values for fishmeal (FMD) and plant meal-based (PMD) diets in two different strains were determined by feeding selected and non-selected rainbow trout groups the experimental diets containing 0.1% yttrium oxide as an indigestible inert marker (Table 1). Diets were cold-pelleted at the Hagerman Fish Culture Experiment Station (HFCES) using a California pellet mill fitted with a 4.0 mm die. The pellets were forced-air dried at 37 °C for 48 h to less than 10% moisture. Samples of each diet were collected for analysis.

Fish and feeding. Rainbow trout from broodstock (House Creek and ARS/KO strains) maintained at HFCES were used in the digestibility study. Eight groups of 35 fish (average body weight 228 g) were stocked into eight 450 l tanks supplied with 25 l min^{-1} of constant temperature spring water (15 °C). Each diet was randomly assigned to two replicate tanks of fish per strain. Fish were fed their respective diets twice daily to apparent

Ingredient	Diet Minus	Diet Plus
Soy protein concentrate	38.37	37.27
Soybean meal	29.27	28.43
Corn protein concentrate	26.26	25.51
Wheat gluten meal	6.09	5.92
L-Lysine		2.04
DL-Methionine		0.55
Threonine		0.29

Table 2. Composition of the experimental plant-protein mixtures (g/100 g).

satiation for eight days. Photoperiod was maintained at a constant 14 h light: 10 h dark with a timer controlling fluorescent lights. Fish were acclimated to the experimental diets for four days, and then on days five and nine, all the fish from each tank were lightly anaesthetized using tricaine methanosulfonate (MS-222, 100 mg/L, buffered to pH 7.0), removed from water, and feces were gently expelled using light pressure on the abdomen near the vent, a process called stripping. Care was taken to avoid contamination of feces with urine from the fish. Fecal samples were collected in aluminum pans, pooled by tank and were frozen between strippings. Two strippings generated 71–112 g wet fecal samples which yielded 11–14 g dry feces per tank, sufficient material for subsequent chemical analysis. All protocols used in the digestibility trial were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee in accordance with relevant guidelines and regulations.

Chemical analysis. Experimental feeds and fecal samples from both strains were analyzed for proximate composition, mineral and amino acid levels and energy content[16]. Samples were dried in a convection oven at 105 °C for 12 h to determine moisture level according to AOAC[17]. Dried samples were finely ground by mortar and pestle and analyzed for crude protein (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. Energy content of samples was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL). Yttrium was measured in feeds and feces samples from the digestibility trial by inductively-coupled plasma (ICP) analysis at the University of Idaho Analytical Sciences Laboratory, Moscow, ID. Amino acid levels in feeds and fecal samples were hydrolyzed and analyzed with an amino acid analyzer (Hitachi Amino Acid Analyzer L-8800) by the University of Missouri's Agricultural Experiment Station Chemical Laboratories, Columbia, MO. Chemical analyses were done in duplicate.

Apparent Digestibility Coefficients (ADCs) for dry matter, organic matter, protein, amino acids, lipid and energy, were calculated using the following equation described by Bureau *et al.*[18].

$$\text{ADC diets} = 1 - [(F/D) \times (Di/Fi)]$$

where D = % nutrient of diet, F = % nutrient of feces, Di = % digestion indicator of diet, Fi = % digestion indicator of feces.

Experiment 2. *Experimental fish and dietary treatments.* Two strains of rainbow trout were used, the selected strain (ARS/KO strain) and a non-selected (House Creek strain)[15]. Both strains originated from broodstock held at the HFCES. Three hundred and fifty individuals (175/strain) with an average weight 580 ± 209 g were distributed randomly in 70 tanks (5 individuals/strain/tank). The tank water volume was 144 l and each tank was supplied with 12 l min^{-1} of constant temperature spring water (15 °C) under a controlled photoperiod (14 h light: 10 h dark). The study was conducted over a period of three weeks such that for every day of sampling 10 tanks per test diet were used. Prior to experimental use, the fish were hand fed to apparent satiation with a commercial diet (Skretting, USA), then fasted for two days. All protocols used in the trial were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee in accordance with relevant guidelines and regulations.

Diets. Five practical, high-protein ingredients were used in the feeding experiment, anchovy fish meal (FM); corn protein concentrate (CPC); soybean meal (SBM); soy protein concentrate (SPC) and wheat gluten meal (WGM). A plant-protein soy-based mixture was prepared from the ingredients to duplicate the protein composition of the selection diet used to develop the ARS/KO trout strain. The mixture was either supplemented with crystalline amino acids (lysine, methionine and threonine) in proportions equal to those used in the selection diet (Diet Plus) or without amino acid supplementation (Diet Minus) (Table 2).

Force feeding. The force-feeding procedure followed that of Ambardekar *et al.*[13] with minor modifications. After a period of 48 h fasting and prior to gavage, trout were lightly anesthetized (40 mg/l MS-222, buffered to pH 7.0) and weighed. Each of the test ingredients was mixed with two parts water to create a slurry and delivered to the fish by stomach intubation at 0.5% of live body weight (ratio of dry ingredient or blend to wet body weight). Each anesthetized fish was forced fed the diet slurry with a 60 ml syringe attached to a piece of Tygon tubing long enough to reach the stomach of the fish. After feeding, fish were placed in a vigorously aerated freshwater rinse tank for several minutes and then were returned to their holding tank.

Blood sampling. Blood samples were only taken from individuals that did not show signs of slurry regurgitation. Blood sampling points were set at 3, 6, 12, 18 and 24 h post force-feeding (for each time point a different tank with fish was used). Approximately 5 min before blood sampling, each fish was anesthetized in 100 mg/l buffered MS – 222 to heavy sedation, i.e., stage 4 when gill operculum movement slowed. The abdomen was opened, and blood was collected (0.2 to 0.3 ml) from the hepatic portal vein (HPV) with a heparinized winged infusion set (butterfly needle; 12-inch tubing, 23 G and 3/4-inch ultra-thin wall needle) connected to a 1 ml syringe. After gently inverting the syringe 3–4 times for proper mixing, the blood was transferred to a 0.6 ml conical Eppendorf tube on ice. Next, blood was collected (1 to 1.5 ml) from the caudal vein (CV) using a 3-ml heparinized syringe with 22 G 1.5-inch needle. After gently inverting the syringe 3–4 times for proper mixing, the blood was transferred to a 2 ml round bottom Eppendorf tube on ice. Blood samples were centrifuged at 2,000 g for 5 min at 4 °C, and the upper plasma layer collected without red blood cells or buffy coat (white blood cells). Plasma proteins were precipitated by adding 13 µl of sulphosalicylic acid into 130 µl of plasma and mixing by gentle vortex for 5 sec. The samples were then incubated at 4 °C for 20 min and then centrifuged at 16,000 g at 4 °C for 15 min. Deproteinized plasma (105 µl) was then mixed with 30 µl of 0.3 M NaOH. Finally, 28 µl of internal standard (2.5 mM norleucine) and 117 µl sodium citrate loading buffer (pH 2.2) were added and mixed by vortex for 5 sec and then transferred to a spinX 0.2 µm filter tube and centrifuged for 2 min at 15,000 g at room temperature. The retained filtered sample was then analyzed using a Biochrom 30 amino acid analyser (Biochrom LTD Cambridge, UK) according to the manufacturer's protocol.

Statistical analysis. *Experiment 1.* Apparent digestibility coefficient values were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). The interaction of strain and diet effects on dry matter, crude protein, lipid, organic matter, energy and amino acid digestibility were analyzed by two-way ANOVA at a 5% level of significance ($\alpha \leq 0.05$). *Post-hoc* tests (Tukey's HSD test) were performed to identify treatments that differed significantly. Statistical analysis was conducted using Statistica (StatSoft, Tulsa OK, USA).

Experiment 2. The five practical ingredients were tested for significant interaction effects of strain and time on plasma amino acid levels. Regarding the plant-protein mixtures with and without amino acid supplementation, we tested if there were significant interaction effects of strain, diet and time on plasma amino acids. Plasma amino acid concentration values were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). When the assumptions for normality and homoscedasticity were met, multifactorial analysis of variance (ANOVA) was performed using Statistica (StatSoft, Tulsa OK, USA). In the case when data were violating the assumptions, a permutational multivariate analysis of variance (PERMANOVA) was performed using Primer 7 (Primer-E Ltd, Plymouth, UK). *Post-hoc* tests (Student-Newman-Keuls test) were performed to identify treatments that significantly differed. Plasma amino acid concentrations were expressed as the mean of three replicate measurements.

Results

Apparent digestibility coefficients (ADCs). No significant interaction effect between diet and strain for apparent digestibility was detected (Table 3). A statistically significant diet effect was detected for dry matter and crude protein with higher levels reflected in the groups fed the plant-protein based diet. The same pattern was also observed with all individual amino acids (IAA and DAA) and the sums (sum of IAAs and TAAs). The only significant strain effect was proline digestibility ($p < 0.05$).

Plasma amino acids. Fishmeal. In the HPV, there were significant ($P < 0.05$) strain and time interactions on Met, Val, Ile and Leu plasma concentrations with both reaching peak levels for the selected strain at 12 h post-prandially (Fig. 1a,c; Table S1). A time effect was significant ($P < 0.01$) on Thr, Phe, His, Lys and Arg; all peaked also at 12 h post-prandially. In the CV, no interaction was found. Significant differences ($P < 0.05$) were detected regarding time (all the amino acids) and strain (only His) main effects ((Fig. 1b,d); Table S2). Thr, Met, Val Ile and Leu peaked at 18 h post-prandially while His, Lys and Arg peaked 12 h and Phe at 6 h. However, Val, Ile, Leu, Met, Thr and His recorded significant higher concentrations between 12 and 18 h, while Lys and Arg reached peak levels between 6 and 12 h. Finally, His was significantly higher for the non-selected strain.

Soy protein concentrate. In the HPV, fish fed SPC showed a significant ($P < 0.01$) peak at 6 h for Thr, Val, Ile, Leu, Met, Phe Lys and Arg (Fig. 2a,c; Table S3). At 18 h a second peak was reached ($P < 0.01$) for Thr, Val, Ile and Leu. In the CV an interaction was observed regarding Arg ($P < 0.05$) at 12 h the selected showed significantly lower concentration (Table S4). The time main effect was significant ($P < 0.05$) for all the amino acids except His. A plateau was observed for all amino acids between 3 and 18 h (except Thr which reached a plateau between 6–18 h) and by 24 h concentrations were below initial baseline levels (Fig. 2b,d).

Soybean meal. In the HPV, significant interactions ($P < 0.01$) were detected for all amino acids except Thr and Met (Fig. 3a,c; Table S5). All plasma amino acid concentrations peaked at 12 h in the selected strain and higher compared to the non-selected strain. In addition, the non-selected strain showed a peak at 18 h for Phe, His and Arg higher compared to selected strain. A time effect was observed regarding Met, showing constant levels between 3 and 12 h and dropping later on, reaching its lowest concentration at 24 h post-prandial ($P < 0.01$). In the CV no interaction was observed (Fig. 3b,d; Table S6). A significant ($P < 0.05$) time effect was observed. Phe and Arg concentrations at 6 h were significantly lower compared to concentrations between 12 and 24 h, while the concentrations of Ile and Leu at 6 h were significantly lower compared to all other time points. In contrast, Met was the only plasma amino acid that showed the highest concentration only at 3 h compared to the whole monitored period.

Corn protein concentrate. In the HPV, significant ($P < 0.05$) interactions observed regarding Thr and Leu (Fig. 4a,c; Table S7). The plasma Thr and Leu concentrations in the selected strain peaked at 18 h and were

	Plant Based Diet		Fishmeal Based Diet		DIET x STRAIN	DIET	STRAIN
	SEL	NON SEL	SEL	NON SEL	P-value	P-value	P-value
Dry Matter	76.4 ± 0.2	77.6 ± 0.3	73.2 ± 1.2	74.2 ± 1.6	ns	P < 0.05	ns
Crude Protein	93.6 ± 0.1	93.5 ± 0.4	85.3 ± 0.7	86.5 ± 0.7	ns	P < 0.001	ns
Lipid	98.0 ± 0.2	97.3 ± 0.1	98.0 ± 0.5	96.1 ± 0.8	ns	ns	ns
Organic Matter	80.2 ± 0.2	81.3 ± 0.4	79.6 ± 1.1	80.5 ± 1.4	ns	ns	ns
Energy	84.1 ± 0.1	84.9 ± 0.3	83.0 ± 1.0	82.9 ± 1.5	ns	ns	ns
Alanine	96.2 ± 0.1	95.6 ± 0.4	90.6 ± 0.5	90.6 ± 0.7	ns	P < 0.001	ns
Arginine	98.4 ± 0.1	98.3 ± 0.2	92.8 ± 0.5	93.2 ± 0.6	ns	P < 0.001	ns
ASX[a]	93.3 ± 0.3	93.0 ± 0.3	86.0 ± 0.8	87.6 ± 0.7	ns	P < 0.001	ns
Cysteine	91.2 ± 0.7	91.8 ± 0.1	81.2 ± 1.0	84.4 ± 1.3	ns	P < 0.001	ns
GLX[b]	97.3 ± 0.1	96.6 ± 0.3	92.3 ± 0.4	92.4 ± 0.6	ns	P < 0.001	ns
Glycine	92.1 ± 0.2	91.4 ± 0.4	82.2 ± 0.7	82.1 ± 1.0	ns	P < 0.001	ns
Histidine	96.5 ± 0.2	96.2 ± 0.2	93.2 ± 0.4	93.6 ± 0.5	ns	P < 0.01	ns
Isoleucine	95.7 ± 0.1	95.1 ± 0.3	91.1 ± 0.5	91.3 ± 0.7	ns	P < 0.001	ns
Leucine	96.9 ± 0.1	96.3 ± 0.3	93.7 ± 0.4	93.9 ± 0.5	ns	P < 0.01	ns
Lysine	97.3 ± 0.2	97.4 ± 0.1	92.4 ± 0.3	93.6 ± 0.5	ns	P < 0.001	ns
Methionine	97.4 ± 0.2	97.4 ± 0.1	91.1 ± 0.3	92.2 ± 0.5	ns	P < 0.001	ns
Phenylalanine	96.8 ± 0.0	96.7 ± 0.1	93.2 ± 0.4	93.2 ± 0.6	ns	P < 0.001	ns
Proline	95.3 ± 0.2	93.0 ± 0.4	87.8 ± 0.2	86.8 ± 0.7	ns	P < 0.001	P < 0.05
Serine	95.7 ± 0.2	95.0 ± 0.6	89.1 ± 0.6	89.5 ± 0.8	ns	P < 0.001	ns
Threonine	92.9 ± 0.3	92.0 ± 0.3	88.1 ± 0.4	88.8 ± 0.8	ns	P < 0.01	ns
Tryptophan	97.0 ± 0.0	97.1 ± 0.0	95.3 ± 0.8	95.4 ± 0.3	ns	P < 0.05	ns
Tyrosine	96.8 ± 0.1	96.4 ± 0.1	92.2 ± 0.6	92.2 ± 0.6	ns	P < 0.001	ns
Valine	94.2 ± 0.1	93.3 ± 0.5	90.4 ± 0.5	90.8 ± 0.7	ns	P < 0.01	ns
Sum AA	95.7 ± 0.2	95.2 ± 0.3	89.7 ± 0.5	90.1 ± 0.7	ns	P < 0.001	ns
Sum EAA (10)	96.5 ± 0.1	96.1 ± 0.2	92.1 ± 0.4	92.6 ± 0.6	ns	P < 0.001	ns

Table 3. Apparent digestibility coefficients. [a]ASX = aspartic acid + asparagine. [b]GLX = glutamic acid + glutamine.

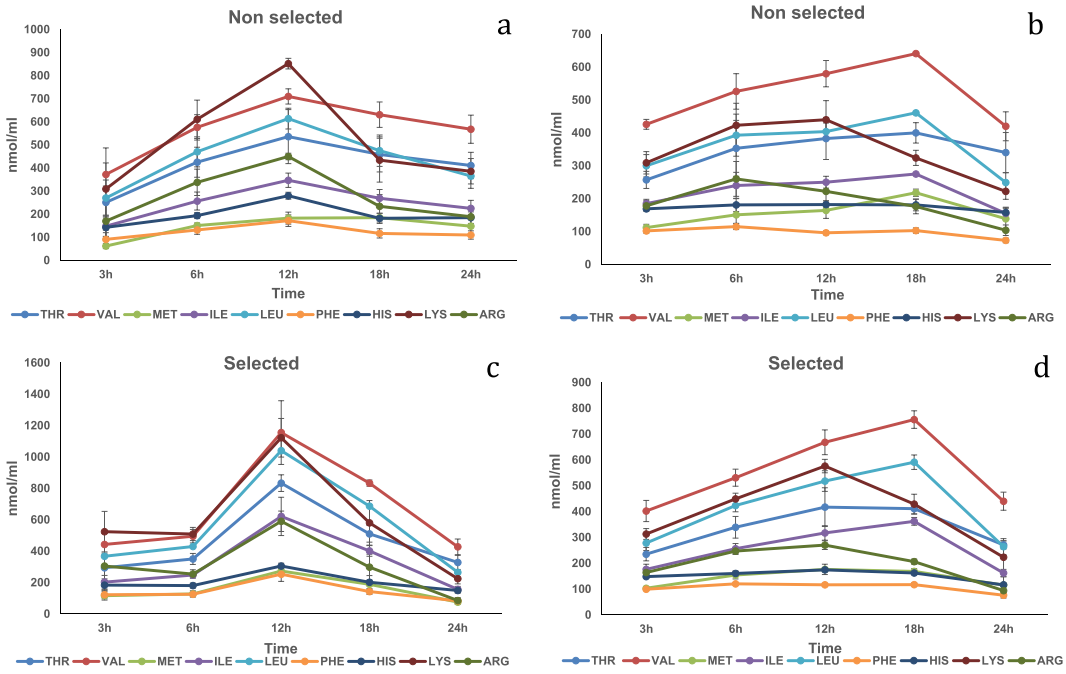

Figure 1. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of fishmeal.

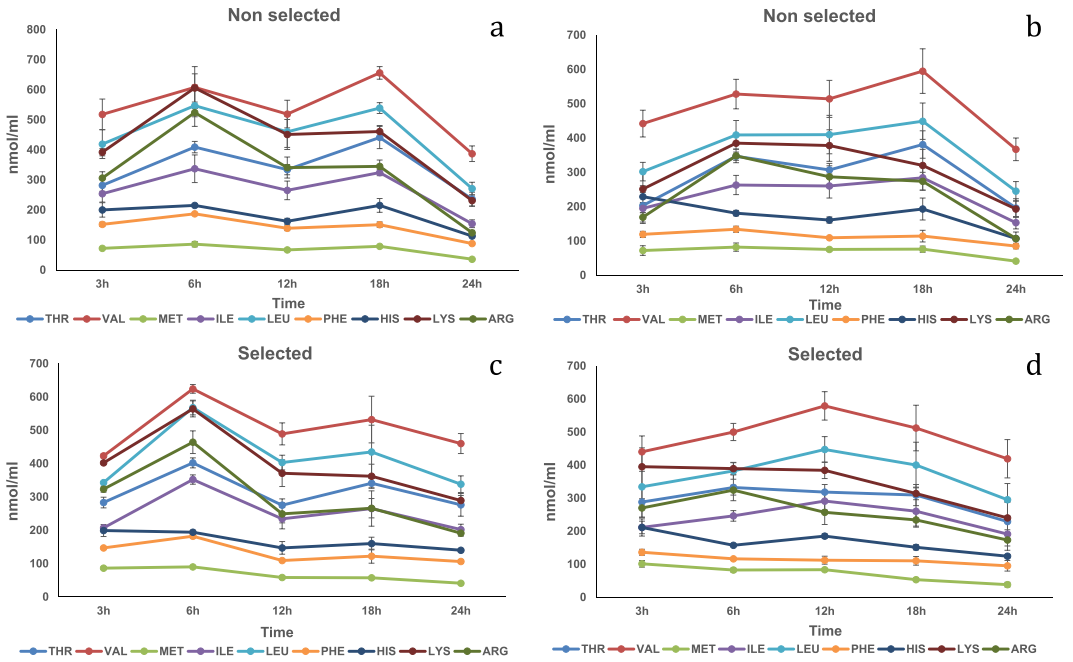

Figure 2. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of soy protein concentrate.

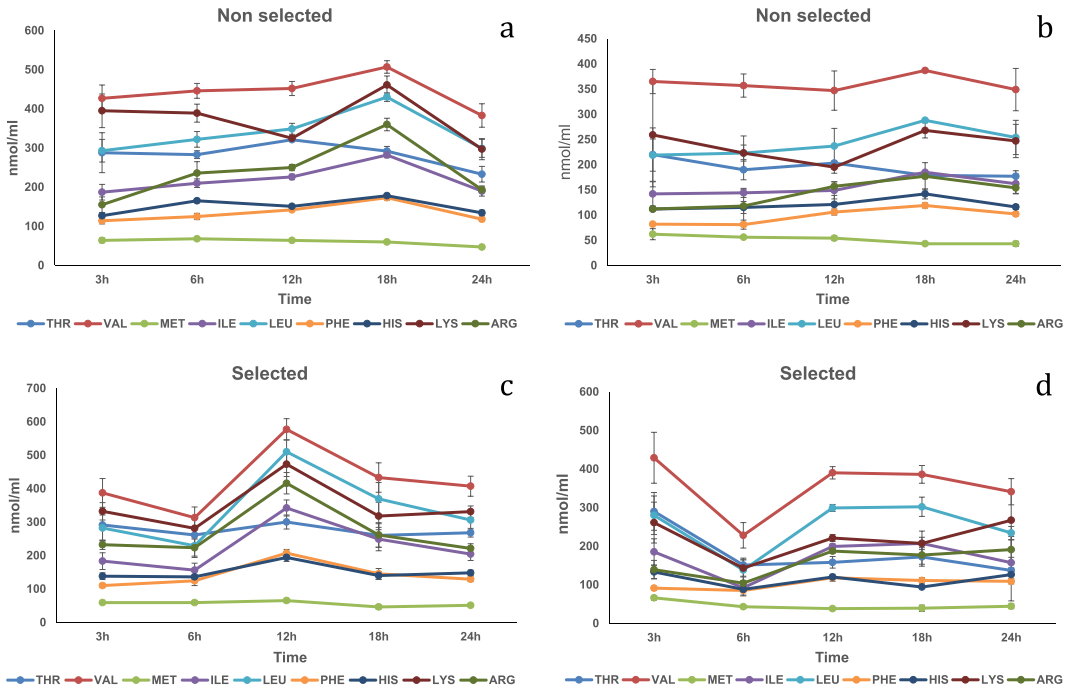

Figure 3. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of soybean meal.

significantly higher compared to the non-selected strain. Time had a significant ($P < 0.05$) effect on Val, Ile, Leu and Phe. Val and Ile plasma concentrations dropped significantly at 24 h. In contrast, the plasma concentration of Phe at 18 h was significantly higher compared to the 3 h and 6 h time points. A significant main effect ($P < 0.05$) was observed in Val and Lys concentrations with lower values in the selected strain compared to the non-selected strain. In the CV, no interactions were observed (Fig. 4b,d; Table S8). A significant time effect ($P < 0.05$) was observed regarding plasma concentrations of Met, Leu and Phe. All the three amino acids showed a significant

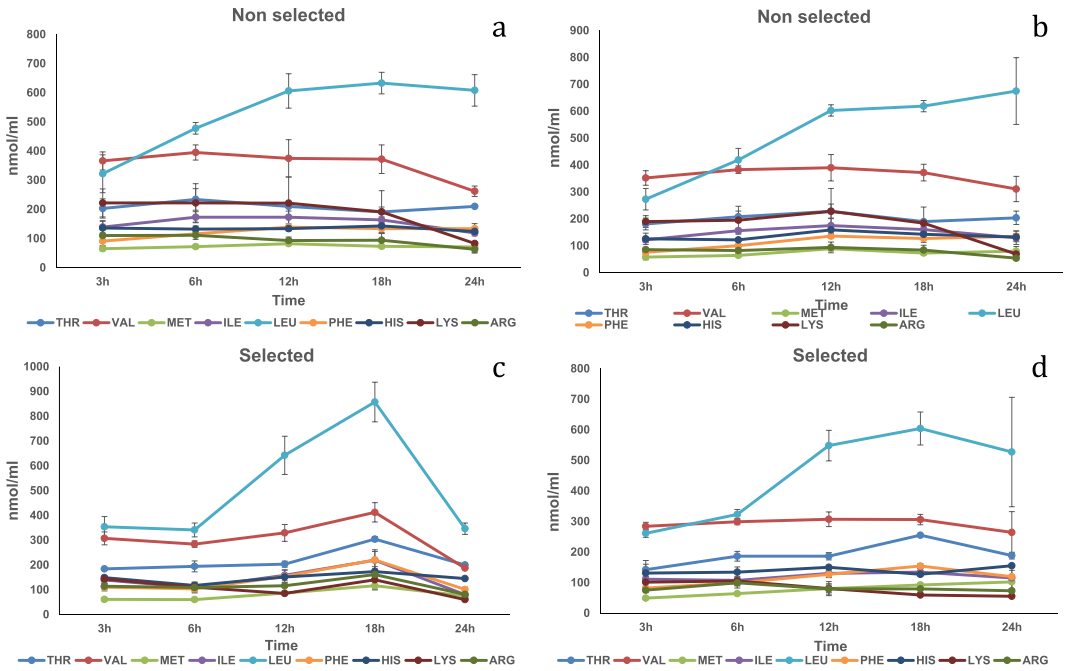

Figure 4. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of corn protein concentrate.

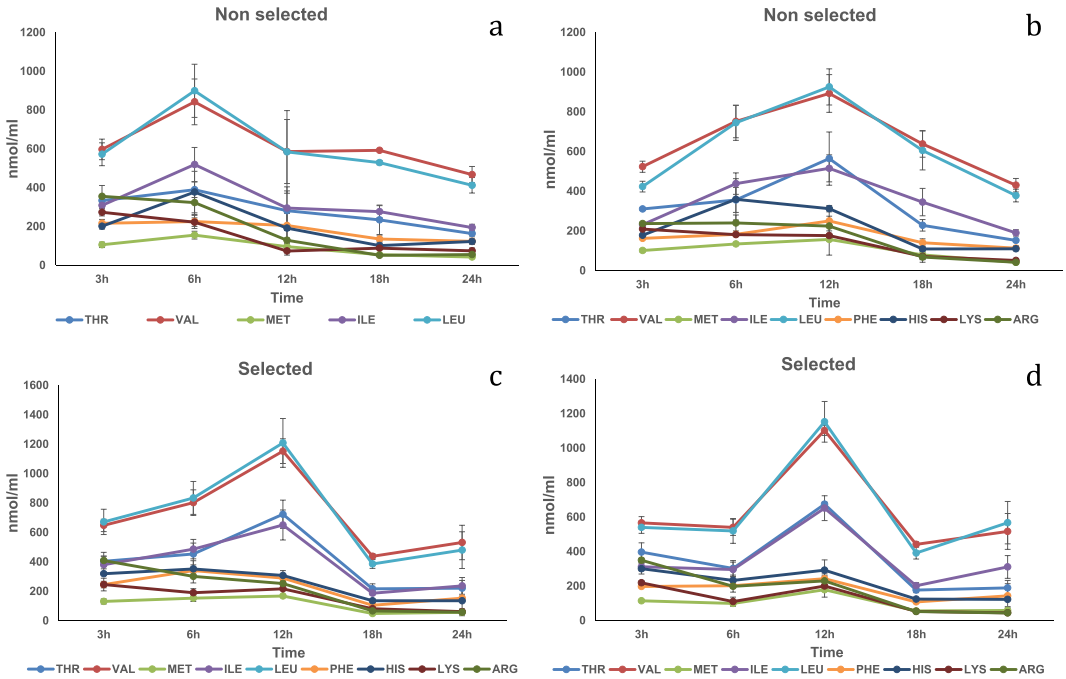

Figure 5. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of wheat gluten meal.

increase in their concentrations at 12 h. Strain had a significant effect ($P < 0.05$) on Val, Ile and Lys plasma concentrations being lower in the selected strain.

Wheat gluten meal. In the HPV, significant interaction effects ($P < 0.05$) were observed for Thr, Val, Ile, Leu and Lys (Fig. 5a,c; Table S9). All the concentrations of the earlier mentioned amino acids showed higher values at 12 h in the plasma of the selected strain compared to the non-selected strain. Time had a significant effect ($P < 0.05$) on the concentration of Met, Phe, His and Arg, showing a drop of their levels at 18 h post-prandially.

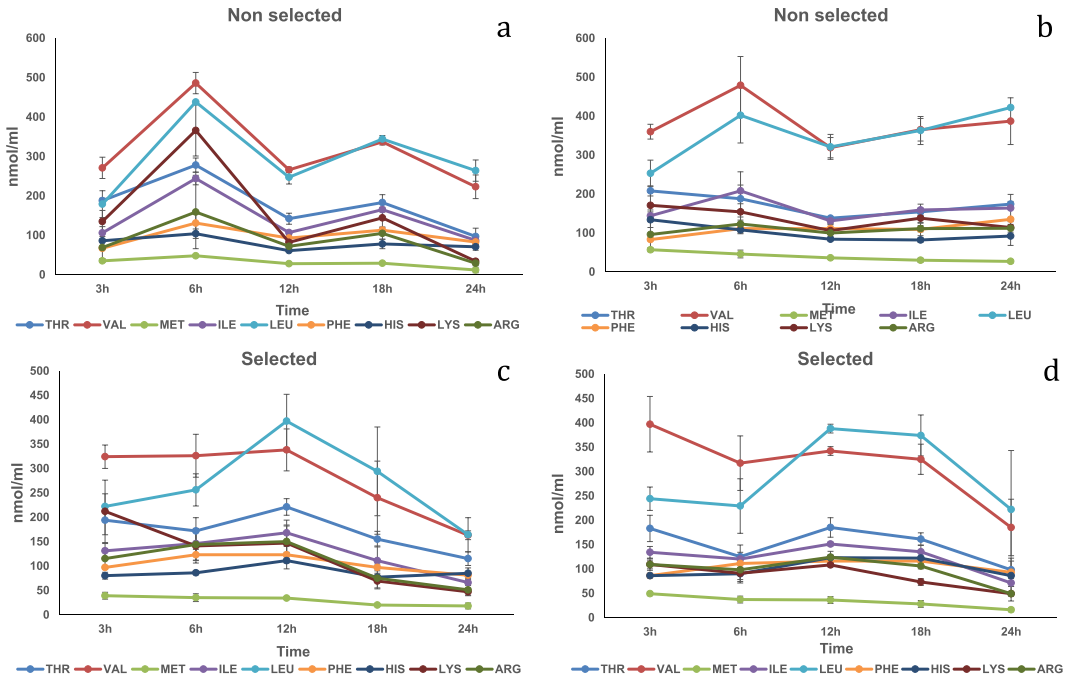

Figure 6. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of a plant protein mixture without amino acid supplementation.

Moreover, a significant strain effect ($P < 0.01$) was observed for His, with the selected strain having higher concentration levels compared to the non-selected strain. In the CV, significant interaction ($P < 0.05$) was observed with Val (Fig. 5b,d; Table S10). Val plasma concentration at 12 h post-prandially was higher in the selected strain compared to the non-selected strain. Regarding Thr, Met, Ile and Leu, their plasma concentrations peaked at 12 h post prandially. His, Lys and Arg concentrations dropped significantly at 18 h.

Plant protein mixtures. In the HPV, a diet by strain by time interaction was significant ($P < 0.05$) for Thr, Leu, Phe, His and Lys plasma concentrations (Table S11). At 3 h the selected strain fed Diet Plus showed significantly higher concentrations of Thr and His compared to the other treatments, while Phe and Lys showed significantly higher concentrations compared to the non-selected strain fed Diet Minus (Fig. 6a,c). Also, at 3 h the selected strain fed Diet Plus showed significantly higher concentration for Leu compared either to the selected and non-selected strains fed Diet Minus. At 6 h the non-selected strain fed Diet Minus showed significantly higher Leu concentration compared to the selected strain fed Diet Minus and to the non-selected strain fed Diet Plus. At 12 h the non-selected strain showed significantly higher Thr concentration compared to the other groups while the selected strain fed Diet Minus showed higher Leu concentration compared to the non-selected fed Diet Minus. At 24 h the non-selected strain fed Diet Plus showed significantly higher Lys concentration compared to the other groups. A strain by time significant interaction ($P < 0.05$) was detected for plasma concentrations of Val and Met. At 3 h, the selected strain showed a significantly higher concentration of Met compared to the non-selected strain. At 18 h the non-selected strain showed significantly higher plasma concentration for Val compared to the selected strain. A diet by time significant effect ($P < 0.05$) was detected for Val, at 3 h post-prandially in both fish strains fed Diet Plus compared to fish fed Diet Minus. Time had a significant effect ($P < 0.01$) on Arg and Ile plasma concentrations. At 24 h post-prandially, both amino acids reached the lowest concentrations (Fig. 7a,c). Finally, a significant diet ($P < 0.01$) effect was observed regarding Met and Arg plasma concentrations with the fish fed Diet Plus showing significantly higher concentrations compared fish fed Diet Minus.

In the CV, a significant diet by strain by time interaction ($P < 0.05$) was detected for Thr, Val, Met, Ile, Leu, His and Lys plasma concentrations (Table S12). At 3 h the selected strain fed Diet Plus showed significantly higher concentrations of Thr and His compared to the other treatments, and Lys was higher compared to the selected and non-selected groups fed Diet Minus (Fig. 6b,d). At 6 h the selected strain fed Diet Minus showed lower Leu concentration compared to the non-selected strain. At 6 h the selected strain fed Diet Plus had higher concentration of Lys compared to the selected strain fed Diet Minus. At 18 h the non-selected strain fed Diet Plus showed significantly higher plasma concentrations of Thr and Lys compared to all the other groups, while Ile plasma concentration was higher compared to the selected strain fed Diet Plus (Fig. 7b,d). At 24 h the non-selected strain fed Diet Plus showed significantly higher concentrations for Thr and Lys compared to the other groups and for His, Val and Ile compared to the selected strain fed Diet Minus. For Leu, the selected strain fed Diet Minus showed significantly lower values compared to the non-selected strain fed either Diet Plus or Diet Minus. Time had a significant effect ($P < 0.01$) on Arg plasma concentration with the 24 h being significantly lower than the 6 and 3 h. A diet effect also was detected for Arg with the fish fed Diet Plus showing significantly higher plasma concentrations compared to fish fed Diet Minus ($P < 0.001$).

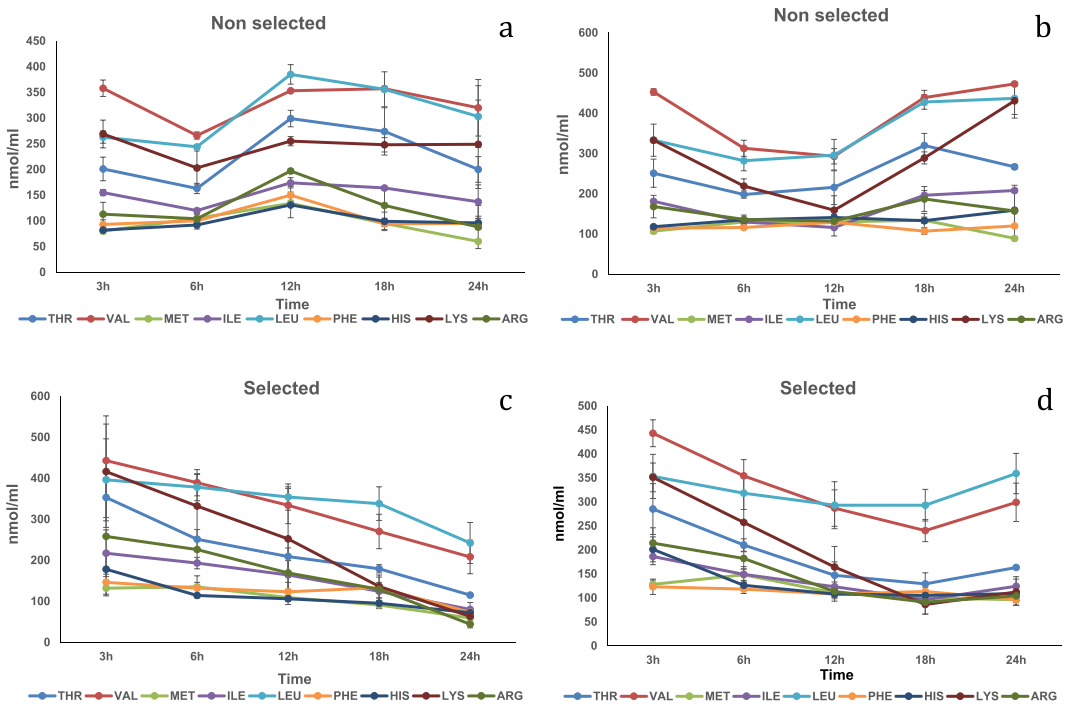

Figure 7. Free essential amino acid (except tryptophan) mean concentrations n = 3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein (**a**,**c**) and caudal vein (**b**,**d**) of two strains of rainbow trout during a 24 h period after force feeding of plant protein mixture with amino acid supplementation.

Discussion

To our knowledge this is the first study to explore and provide novel insights into the physiological mechanisms that allow a carnivorous fish species to thrive when fed an all-plant protein diet. Apparent digestibility coefficient (ADC) results demonstrate that selection for improved growth and plant protein utilization in rainbow trout does not affect apparent digestibility of nutrients. Our results showed a diet effect with the all-plant protein-based diet having higher protein digestibility which is in accordance with other studies[19,20]. Callet *et al.*[20] compared a rainbow trout strain after three generations of selection on a plant-based diet with a control line strain in a 2×2 factorial design (strain by diet) and did not detect any interaction. However, they found a significant increase in ADC of protein and decreased values for energy, lipid, moisture and starch when fish were fed an all plant-based diet compared to a fishmeal-based diet. Although measuring apparent digestibility of nutrients remains an important tool for evaluating feed ingredient quality, it cannot be considered sufficient to assess metabolic utilization of amino acids because it does not provide information regarding specific rates of nutrient absorption and metabolism[21].

Concentrations of plasma amino acids collected in the HPV were elevated compared to the systemic blood amino acid concentrations levels found in samples from the CV. In a study conducted by Karlsson *et al.*[21] using cannulated rainbow trout and force-fed 1% body weight, similar differences in amino acid concentrations between HPV and dorsal aorta samples were found. These authors postulated that blood returning to the sinus venosus from the hepatic circulation is diluted by other systemic venous return in direct proportion to the relative proportion of hepatic blood flow[21]. In the present study, plasma amino acid profiles were strongly affected by dietary source and reflected the amino acid composition of ingredients while also maintaining their relative ratios over time. This agrees with other studies on rainbow trout[14,22–25]. We found significant interactions of strain by time in all the tested ingredients in the HPV, except for soy protein concentrate. In contrast, in the CV there was no interaction found across ingredients, except for valine in wheat gluten meal. Plasma amino acid measurements from the CV provides less resolution regarding protein digestion rates compared to the HPV and this may be related to hepatic and post-hepatic metabolism in contrast to intestinal uptake[21].

Fishmeal is defatted, dried meal and the protein source of choice in feeds because carnivorous fish are piscivorous. Thus, the postprandrial pattern of plasma amino acids in fish fed fishmeal is likely to closely match that of fish consuming natural prey. The fish force-fed fishmeal showed a peak in the HPV for all the plasma amino acids at 12 h postprandial, with the selected strain reaching higher levels compared to the non-selected strain, while in the CV the plasma amino acids peaked at 18 h. Fishmeal, as expected, showed an overall homogeneous pattern for all the amino acids, similar to findings in other studies[14]. Replacing dietary fishmeal with plant protein concentrates results in a shift in postprandial plasma amino acid temporal profiles and synchronization[14,23]. This shift is assumed to be caused by antinutritional factors, protein solubility differences, and gastric evacuation rate differences which ultimately affect the digestion rate of plant proteins[12,24,26]. In our study, the non-selected strain showed marked differences in plasma amino acid concentrations when force-fed plant protein concentrates compared to fishmeal. Comparing strains fed individual protein concentrates, the selected strain showed higher

peaks in the HPV at 12 h postprandially when force-fed either soybean meal or wheat gluten meal compared to the non-selected strain which showed a later peak when fed soybean at 18 h and an earlier peak at 6 h when force-fed wheat gluten meal. In the CV, plasma amino acid concentrations did not differ significantly for the two strains fed either ingredient. These results suggest that selection has altered the temporal dynamics of plant protein digestion and absorption, thus providing an explanation for the higher growth and protein retention observed in the selected strain fed plant protein-based diets. Wheat gluten was the only ingredient for which both the non-selected and selected strains exhibited similar amino acid profiles at 12 h postprandially regarding the caudal vein. Wheat gluten meal postprandial amino acid patterns for the selected strain were homogeneous and similar to fishmeal patterns, except for lysine content, which was lower and in agreement with previous studies[23,27]. Not surprisingly, wheat gluten is considered comparable to fishmeal when supplemented with amino acids and research has shown that wheat gluten can replace fishmeal in rainbow trout diets[28]. An interaction between strain and time for threonine and leucine was detected in the HPV of fish fed corn protein concentrate with the selected strain showing peaks at 18 h postprandially. Other amino acids, although not significantly different between strains, were higher in concentration at 18 h in the selected strain in the HPV. In contrast, in the CV, the non-selected strain showed significantly higher plasma amino acid concentrations compared to the selected strain. For corn protein concentrate the concentrations of most of the plasma amino acids measured in the hepatic or caudal veins were lower compared to the other plant protein ingredients with the exception of soybean meal in the caudal vein, despite the fact that the protein content of corn protein concentrate is relatively high (75%). Finally, soy protein concentrate was the only ingredient for which interactions were not found and further, no strain effect was detected. The only significant effect found was related to time with two major peaks found in the HPV at 6 and 18 h postprandial, while in the CV a plateau for almost all the amino acids was observed between 6 and 18 h. Soy protein concentrate is considered one of the most promising plant protein sources to replace fishmeal due to its high protein content and lower antinutritional factor levels. Several studies have reported that high inclusion levels showed comparable results to a fishmeal-based diets[29–32].

No significant interactions were found between the selected and non-selected strains when fed amino acid supplemented or non-supplemented protein concentrate mixtures. In the HPV, balancing the plant protein mixture with supplemental amino acids (Diet Plus) not only increased concentrations of all the essential amino acids but notably had an effect on plasma amino acids temporal behavior. The selected strain fed Diet Plus showed a peak in amino acid uptake at 3 h postprandially in contrast to the other treatments. However, supplementation with amino acids generally led to an alteration of all dietary essential amino acid uptake in both strains compared to the non-supplemented mixture. Moreover, the selected strain fed Diet Plus showed a noteworthy difference compared to the non-selected strain, specifically, a synchronous and homogenous decreasing pattern for all the essential amino acids over time. Further, significant interactions were detected in CV samples for most of the plasma amino acids with the selected strain maintaining the same synchronized plasma amino acid decreasing pattern as was showed in the HPV. In contrast, the non-selected strain showed significantly higher concentrations at 24 h postprandially for arginine, threonine, valine, leucine and very high concentration of lysine compared to the other treatments. The interactions found in the CV demonstrate the strong effect that an all-plant protein mixture can have on the digestive physiology of a carnivorous fish species. Studies using rainbow trout showed that feeding a plant-protein mixture leads to much less synchronous amino acid uptake compared to when fishmeal is replaced by a single plant protein source, suggesting that different plant-based protein ingredients are diverse in the way they affect the uptake of dietary amino acids[14]. Research in swine has demonstrated asynchronous nutrient absorption patterns can be induced by formulating diets using ingredients with different digestion and absorption kinetics[33]. Furthermore, in the present study, the addition of crystalline amino acids into the all-plant protein mixture affected the plasma concentrations of all amino acids as it did for uptake reflected in the HPV. Rolland *et al.*[34] showed that supplementing a diet with methionine as a single amino acid influenced the plasma profiles and concentrations of other essential amino acids. However, in the present study the selected strain showed a remarkably synchronized dietary amino acid uptake pattern which influenced the pattern of postprandial appearance of free amino acids in the systemic blood over time. We assume that the fast and homogeneous dietary amino acid uptake in the HPV and the fast-postprandial plasma amino acid disappearance are results of selection for growth on and tolerance of an all-plant protein diet. The selected strain has more rapid growth (~10%) and higher protein retention efficiency (~15%) when fed an all-plant protein diet compared to a non-selected rainbow trout strain fed a fishmeal-based diet[15]. For optimal amino acid utilization to occur, postprandial plasma amino acid appearance rates should not exceed net protein synthesis capacity. Compared to mammals, the amino acid pool in fish available for protein synthesis derived from intracellular protein degradation is much less[35]; implying that a transient amino acid imbalance would have negative effects on muscle protein turnover. We hypothesize that plasma amino acid synchronization and increased genetic potential for growth of the selected strain can explain the postprandial plasma amino acid disappearance rate. Our findings are in accordance with a study in growing pigs which was designed to evaluate the effects of synchronized amino acid availability on protein metabolism[33]. The researchers reported a reduction in protein retention from 57% to 47% in pigs fed a balanced diet characterized by asynchronous temporally amino acid availability.

Conclusion

This is the first study to explore and provide novel insights on digestive physiology of a carnivorous fish strain genetically selected over six generations for improved plant protein utilization efficiency and growth. Our findings demonstrated that improved performance of the selected strain is associated with a synchronous protein digestion of the plant protein mixture and synchronization of amino acid absorption leading to improved availability and utilization. Protein and amino acid digestibility, though a useful quality assessment tool, does not provide information related to temporal nutrient absorption. In contrast, monitoring temporal plasma amino acid patterns allows assessment of absorption rates and overall metabolic utilization of amino acids. However,

temporal plasma amino acid patterns of single ingredients cannot be used to predict amino acid dynamics when ingredients are combined in a blend. Most importantly, the results showed that supplementation of amino acids to a plant protein concentrate mixture affects the digestion process of the diet in terms of uptake and utilization of all essential amino acids. These insights were made possible by using the selected rainbow trout strain as a unique model to pursue the discovery of physiological mechanisms associated with increasing use of plant proteins in sustainable fish feeds.

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Author contributions

A.B. and R.W.H. designed the experiments and planned the statistical analysis. A.B. performed the experiments under supervision of R.W.H. A.B. performed the statistical analyses. A.B. was primarily responsible for drafting the manuscript with critical insights and editing from R.W.H.

Competing interests

The authors declare no competing interests.

Additional information

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Replacing fishmeal with blends of alternative proteins on growth performance of rainbow trout (*Oncorhynchus mykiss*), and early or late stage juvenile Atlantic salmon (*Salmo salar*)

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ABSTRACT

The purpose of this study was to evaluate blends of alternate proteins as replacements for fishmeal in diets for rainbow trout (*Oncorhynchus mykiss*) and to use the results to develop and test alternate protein blends in diets for juvenile Atlantic salmon (*Salmo salar*). Nine experimental diets in which protein blends replaced 63%, 82% or 100% of fishmeal in the formulation (20, 10, and 0% fishmeal) were fed to rainbow trout (initial weight 19.5 g) for 12 weeks. Weight gains of trout fed diets containing the soy protein concentrate-based blend and the fishmeal control diet were similar, except at the 100% fishmeal replacement level, and significantly higher than that of trout fed diets containing the other blends. The soy protein blend and another based on wheat gluten meal were modified slightly and evaluated in early stage Atlantic salmon juveniles (initial weight 5.5 g). Protein blends replaced 50%, 66% or 84% of fishmeal (30, 20 or 10% fishmeal). Weight gains of early stage juvenile salmon after 18 weeks of feeding were significantly lower and feed conversion ratios higher when fed diets containing either blend compared to the fishmeal control diet, and gains decreased as level of fishmeal replacement increased. Blends were then modified further and tested in advance stage salmon juveniles (initial weight 31.5 g). These blends were solely either all-plant protein-based or contained poultry by-product meal. Both blends were evaluated with or without addition of *Spirulina* algae meal. Alternate protein blends completely replaced fishmeal in experimental diets. After 12 weeks of feeding, no differences in weight gain or feed conversion ratios were measured among groups fed experimental diets containing protein blends or the fishmeal control diet. Replacement of fishmeal with alternative protein blends in diets for early stage juvenile salmon is not recommended and the penalty in growth is severe. Fishmeal can be completely replaced in diets for late stage salmon over 30 g without compromising fish performance or using land animal protein ingredients in feed formulations.

1. Introduction

Commercial fisheries landings for both direct consumption and for fishmeal production have not increased for over a decade (FAO, 2010). The demand for seafood continues to increase, and aquaculture production has filled the shortfall associated with static wild fish landings (FishStat Plus, 2010). In fact, in 2012 aquaculture production is expected to exceed capture fisheries as a source of finfish products for consumption (FAO, 2010). Aquaculture production is expected to increase further and this will require higher production of aquafeeds. The inclusion of plant-protein sources in aquafeeds has increased due to the limited amount and increasing cost of fishmeal available for production of animal feeds (e.g., Gatlin et al., 2007; Glencross et al., 2005; Naylor et al., 2009). One of the greatest challenges for the aquafeed industry is to reduce fishmeal levels in feed further and increase the amount of plant protein and ingredient diversity in diets of carnivorous fishes.

The search for fishmeal replacements in rainbow trout (*Oncorhynchus mykiss*) Atlantic salmon (*Salmo salar*) feeds has been ongoing for many years and lately has received more attention as fishmeal prices and aquaculture production have increased (Gatlin et al., 2007). Many different plant-protein sources have been examined including plant-protein meals and plant-protein concentrates (Lim et al., 2008). Generally, levels of plant meals in salmon and trout feed formulations are limited in salmon and trout formulations by their composition (relatively low crude protein and high crude fiber content) and by the presence of anti-nutritional factors and non-soluble carbohydrates (Krogdahl et al., 2009; Lim et al., 2008; NRC, 2011). Plant-protein concentrates are more promising ingredients to replace fishmeal in aquafeeds than are plant meals. Canola, soy, pea,

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barley, rice protein concentrates, along with wheat gluten meal, have all been tested as fishmeal replacements with varying degrees of success (Forster et al., 1999; Thiesses et al. 2003; Barrows et al., 2007; Lim et al., 2008; Gaylord and Barrows, 2009). Protein digestibility of most plant proteins for salmon is generally similar to or higher than that of fishmeal; except for bacterial protein meal, extracted soybean, oat, rapeseed (canola), and sunflower meals (Storebakken et al., 2000; Glencross et al., 2004; Aas et al., 2006; Refstie et al., 2006; Aslaksen et al., 2007; Denstadli et al., 2007; Kraugerud et al., 2007). Amino acid profiles, however, are inferior to fishmeal and amino acid supplementation is needed to maintain growth performance of fish fed diets containing high levels of plant-protein concentrates (Gaylord and Barrows, 2009; Lim et al., 2008).

Commercial diet formulations use a combination of alternate protein sources to replace fishmeal to better balance amino acid levels, but many of the studies evaluating ingredients to replace fishmeal involve single ingredient substitutions (Gatlin et al., 2007; Lim et al., 2008. The purpose of this study was to test protein blends as replacements for fishmeal in diets for rainbow trout and to use these results to develop and test protein blends in diets for early and late stage juvenile Atlantic salmon.

2. Methods and materials

2.1. Rainbow trout trial

The rainbow trout trial tested three protein blends at three levels of substitution for fishmeal. The protein blends were formulated around three plant-protein concentrates, soy protein concentrate (SPC), corn gluten meal (CGM) and barley protein concentrate (BPC) (Table 1). SPC, BPC and CGM were combined with other protein ingredients and supplements to produce protein blends that contained digestible protein levels similar to menhaden fishmeal (Select grade). The rationale behind the protein-blend approach was that in commercial feed formulations, reducing fishmeal levels is best accomplished by combining several alternate protein sources to approximate the amino acid profile of fishmeal. Supplementing protein blends with amino acids further improves the nutritional profiles of blends in comparison with that of fishmeal (Cheng et al., 2003). Minerals shown to be important when feeding fishmeal-free diets were supplemented to each of the three blends (Barrows et al., 2010). Each of the three protein blends was included in experimental feeds at three dietary levels (nine experimental diets) such that 63%, 82% and 100% of the fishmeal was replaced (Table 2). A fishmeal control diet was also fed. The ten experimental diets were formulated to contain 39% digestible protein and 19% crude lipid, similar to commercial trout diets that contain 44% protein of which 87% is digestible. Vitamin and mineral premix levels remained constant in the feed formulations. Fish oil levels varied depending upon the lipid content of the blends. The feeds all contained levels of essential amino acids above minimum dietary requirements for rainbow trout (NRC, 2011).

Table 1
Protein-blend formulations (g kg^{-1}) used as fishmeal replacements in rainbow trout feeds.

Ingredient	SPC	CGM	BPC
Soy protein concentrate (SPC)[a]	241.2	–	–
Corn gluten meal (CGM)[b]	258.8	295.2	245.2
Barley protein concentrate (BPC)[c]	–	–	258.4
Poultry by-product meal[d]	143.5	279.5	233.5
Blood meal[e]	91.2	149.8	119.8
Soybean meal[f]	181.7	190.6	52.1
Lysine	29.3	32.3	36.9
Methionine	7.1	7.9	8.2
Threonine	7.1	8.9	10.1
Taurine	7.9	7.9	7.9
Mono-dicalcium phosphate	23.3	19.0	21.0
Sodium chloride	2.8	2.8	2.8
Potassium chloride	5.6	5.6	5.6
Magnesium oxide	0.5	0.5	0.5
Calculated composition, as-is basis			
Crude protein	629	637	630
Fat	26.5	46.1	38.8
Total phosphorus	12.1	14.2	13.5
Lysine	55.1	56.1	56.3
Methionine	17.6	18.3	18.1
Cystine	9.3	9.2	6.8
Threonine	27.6	27.0	27.3

[a] Solae, Pro-Fine VF, 693 g/kg crude protein.
[b] Cargill, 639.00 g/kg protein.
[c] Montana Microbial Products, 520 g/kg protein.
[d] Griffin Industries, 600 g/kg protein.
[e] IDF Inc., 832 g/kg protein.
[f] ADM Inc., 476 g/kg protein.

All of the diets were produced using commercial manufacturing technology at the U.S. Fish & Wildlife Service Bozeman Fish Technology Center, Bozeman, MT, USA. All ingredients were ground to a particle size of <200 μm using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN). The diets were 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 less than 10%. All added fish oil was top-coated after the pellets were cooled using a vacuum-coater (AJ Mixing, Ontario, CA). Diets were stored in plastic lined paper bags at room temperature until fed. Feeds were then shipped to the University of Idaho's Hagerman Station where they were analyzed to confirm that calculated proximate compositions were achieved and where the rainbow trout feeding trial was conducted. Diets were fed within four months of manufacture.

Juvenile rainbow trout (House Creek strain) from the University of Idaho broodstock were used in the feeding trial. The fish averaged 19.5 g at the start of the trial. Fish were stocked into 145 L tanks supplied with 4 L min^{-1} of constant temperature (14.5 C) spring water supplied by gravity in a single-pass water system. Water flow to each tank was increased to 8 L min^{-1} as the feeding trial progressed. Each tank contained 30 fish and each experimental diet was fed to three replicate tanks, arranged in a completely randomized design within the indoor fish rearing system. Fish were fed three times per day by hand to apparent satiation, and feed consumption was recorded. Photoperiod was maintained at a constant 14 h light:10 h dark with automatic timers. Fish in each tank were bulk-weighed and counted every three weeks during the 12-week trial. Average fish weight gain, feed intake per fish, percent feed intake, feed conversion ratio (FCR), thermal growth coefficients (TGC), and productive protein value (PPV) were calculated over the entire study. All experimental protocols involving rainbow trout rearing and sampling were approved by the University of Idaho's Institutional Animal Care and Use Committee.

2.2. Atlantic salmon trials

2.2.1. Early stage juvenile trial

Based upon the results of the trial with rainbow trout, a feeding trial with early stage juvenile Atlantic salmon (5.5 g initial weight) was designed. Two protein blends were formulated using SPC, CGM and wheat gluten meal (WGM) to be equivalent to menhaden fishmeal (Select grade) in digestible protein content (Table 3). Amino acids were supplemented to ensure adequate levels of the three essential amino acids that were most limiting in the protein blends, lysine, methionine and threonine (NRC, 2011). Blends were also supplemented with minerals. The blends were then included in six experimental diets and a fishmeal control diet to replace 50%, 66% and 87% of fishmeal in the diets (Table 4). The experimental diets were manufactured as described above and shipped to the USDA, Agricultural Research Service National Cold Water Marine Aquaculture

Table 2
Diet formulations (g kg^{-1}) used in the protein-blend evaluation using juvenile rainbow trout (initial weight 19.5 g). Number after protein blend refers to percentage of fishmeal replaced.

Ingredient	FM control	SPC63	SPC83	SPC100	BPC63	BPC82	BPC100	CGM63	CGM82	CGM100
Menhaden fishmeal[a]	529.6	198.5	99.3	–	198.5	99.3	–	198.5	99.3	–
Blend 1 SPC	–	390.3	503.0	615.7	–	–	–	–	–	–
Blend 2 BPC	–	–	–	–	–	–	–	434.6	56.16	668.7
Blend 4 CGM	–	–	–	–	434.5	551.6	668.7		–	–
Wheat flour[b]	314.4	229.2	208.7	187.8	192.3	168.4	145.1	189.1	155.2	141.5
Menhaden fish oil[c]	136.0	162.0	169.0	176.5	154.7	160.7	166.2	157.8	163.9	169.8
Vitamin premix[d]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Choline chloride	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Stay-C (35% ascorbate)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Trace mineral premix[e]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Analyzed composition (as-is basis)										
Crude protein	429	453	455	451	483	482	498	493	471	481
Fat	174	196	185	178	151	165	160	169	180	150
Ash	93	70	62	52	75	68	63	587	61	50
Gross energy (joules/g)_	22.3	22.9	23.0	23.4	23.1	23.1	23.5	23.8	23.6	23.8

[a] Omega Proteins, Menhaden Special Select, 628 g/kg protein.
[b] Manildra Milling, 12 g/kg protein.
[c] Omega Proteins.
[d] Supplied the following per kg diet: vitamin A palmitate, 9650 IU; cholecalciferol, 6600 IU; DL-tocopheryl acetate, 132 IU; menadione sodium bisulfate 1.1 mg; thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine HCl 13.7 mg; DL-calcium pantothenate, 46.5 mg; cyanocobalamine 0.03 mg, nicotinic acid, 21.8 mg; D-biotin, 0.34 mg; folic acid 2.5; and inositol, 600 mg.
[e] Supplied the following per kg diet: copper, 3 mg as copper sulfate pentahydrate; manganese, 10 mg as manganese sulfate, monohydrate; iodine, 5 mg as potassium iodide; sodium selenate 0.960 g; and zinc, 37 mg as zinc sulfate, heptahydrate.

Center in Franklin, ME. The fishmeal control diet was fed to three replicate tanks and each experimental diet was fed to duplicate tanks of fish.

Early stage juvenile Atlantic salmon (St. John's strain) from the USDA ARS National Cold Water Marine Aquaculture Center's breeding program were used in the feeding trial. Fifty fish (mean weight 5.46 ± 0.06 g) were stocked into each 265 L tank supplied with 8 L min^{-1} of oxygen-saturated water from a recirculating biological filtration system at 2.0–3.0 g L^{-1} salinity. Dissolved oxygen and temperature were monitored continuously and ammonia, nitrite, nitrate, carbon dioxide, and pH monitored weekly to insure optimal water quality conditions. Fish were fed a commercial feed for one

Table 3
Protein-blend formulations (g kg^{-1}) used as fishmeal replacements in diets for early stage juvenile Atlantic salmon (initial weight 5.5 g).

Ingredient	Menhaden fishmeal	SPC	WGM
Menhaden fishmeal[a]	1000	–	–
Soy protein concentrate[b]	–	218.6	–
Corn gluten meal[c]	–	268.8	331.9
Wheat gluten meal[d]		95.4	185.4
Poultry by-product meal[e]	–	263.5	319.2
Blood meal[f]	–	61.2	59.7
Lysine	–	34.3	42.9
Methionine	–	7.1	7.1
Threonine	–	7.9	10.9
Taurine	–	15.0	15.0
Mono-dicalcium phosphate	–	19.3	19.0
Sodium chloride	–	2.8	2.8
Potassium chloride	–	5.6	5.6
Magnesium oxide	–	0.5	0.5
Calculated composition			
Crude protein	678.0	664.5	665.4
Fat	96.0	32.4	39.8
Total phosphorus	24.3	14.1	14.4
Methionine	19.9	18.4	18.5
Cystine	6.0	10.8	11.2
Lysine	50.4	53.8	54.1
Threonine	28.2	30.0	30.4

[a] Omega Proteins, Menhaden Special Select, 628 g/kg protein.
[b] Solae, Pro-Fine VF, 693 g/kg crude protein.
[c] Cargill, 639.00 g/kg protein.
[d] Manildra Milling, 753 g/kg protein.
[e] IDF Inc., 832 g/kg protein.
[f] Griffin Industries, 600 g/kg protein.

Table 4
Diet formulations (g kg^{-1}) used in the protein-blend evaluation with early stage juvenile Atlantic salmon (5.5 g initial weight). Number after protein blend refers to percentage of fishmeal replaced.

Ingredient	FM control	SPC50	SPC66	SPC87	WGM50	WGM66	WGM87
Menhaden fishmeal[a]	600.9	300.0	200.0	100.0	300.0	200.0	100.0
Soy protein blend	–	312.5	420.4	528.3	–	–	–
Wheat gluten blend	–	–	–	–	321.5	428.2	534.9
Wheat flour[b]	242.9	202.0	182.9	163.7	196.6	179.6	163.0
Menhaden fish oil[c]	136.2	153.0	158.5	164.5	150.2	155.1	159.9
Mono-dicalcium phosphate	–	12.5	18.2	23.5	11.7	17.1	22.2
Vitamin premix 702[d]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Choline chloride	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Stay-C (35% ascorbate)	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Trace mineral premix[e]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Calculated composition							
Crude protein	400	417	425	432	425	433	442
Fat	190	190	190	190	190	190	190
Total phosphorus	16	15	15	15	15	15	15
Methionine	9.7	10.6	11.0	11.3	10.8	11.1	11.5
Cystine	3.1	4.9	5.6	6.2	5.1	5.8	6.5
Lysine	26.1	29.9	31.3	32.8	30.4	31.9	33.3
Threonine	13.8	16.3	17.2	18.2	16.7	17.6	18.6

[a]Omega Proteins, Menhaden Special Select, 628 g/kg protein.
[b]Manildra Milling, 12 g/kg protein.
[c]Omega Proteins.
[d]Supplied the following per kg diet: vitamin A palmitate, 9650 IU; cholecalciferol, 6600 IU; DL-tocopheryl acetate, 132 IU; menadione sodium bisulfate 1.1 mg; thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine HCl 13.7 mg; DL-calcium pantothenate, 46.5 mg; cyanocobalamine 0.03 mg, nicotinic acid, 21.8 mg; D-biotin, 0.34 mg; folic acid 2.5; and inositol, 600 mg.
[e]Supplied the following per kg diet: copper, 3 mg as copper sulfate pentahydrate; manganese, 10 mg as manganese sulfate, monohydrate; iodine, 5 mg as potassium iodide; sodium selenate 0.960 g; and zinc, 37 mg as zinc sulfate, heptahydrate.

week after stocking prior to the start of the 18-week diet study. A natural photoperiod (14L: 10D initial, 9L:15D final) and ambient water temperatures (decreasing from 14.1 °C to 8.3 °C) were followed during the study. Fish were fed using automatic feeders such that feed was supplied at 110% of maximum expected intake to ensure that growth was not feed-limited. Fish in each tank were bulk-weighed and counted every four weeks during the trial. Average fish weight gain, feed intake per fish, percent feed intake, FCR, TGC, and PPV were calculated for the 18 week feeding period.

2.2.2. Late stage juvenile trial

A follow-up feeding trial with late stage juvenile Atlantic salmon (31.5 g initial weight) was designed based upon the results of the trials with early stage juvenile Atlantic. Protein blends were formulated using SPC, CGM, WGM, poultry by-product meal and *Spirulina* algae meal to be equivalent to menhaden fishmeal (Select grade) in digestible protein content (Table 5). Amino acids and minerals were supplemented as in the previous trial. The blends were then included in four experimental diets to completely replace fishmeal (Table 5). The experimental diets and a fishmeal control diet were manufactured as described above and shipped to the USDA, Agricultural Research Service National Cold Water Marine Aquaculture Center in Franklin, ME. Each of the five diets was randomly assigned and fed to three replicate tanks.

Atlantic salmon juveniles (St. John's strain) from the USDA ARS National Cold Water Marine Aquaculture Center's breeding program were used in the feeding trial. One hundred and twelve fish (mean weight 31.5 ± 2.9 g) were stocked into each 265 L tank supplied with 8 L min^{-1} of oxygen-saturated water from a recirculation, biological filtration system at 2.0–3.0 g L^{-1} salinity. Water quality was monitored as described above. Rearing conditions were the same as described above for small juvenile Atlantic salmon except that photoperiod (11.5 h light and 12.5 dark initially to 14 h light and 10 h dark, final) and ambient water temperature (8.3 °C to14.1 °C) increased during the study. Fish were fed using automatic feeders such that feed was supplied at 100% of maximum expected intake. Fish in each tank were bulk-weighed and counted every four weeks during the 12-week trial. Average fish weight gain, feed intake per fish, percent feed intake, FCR and TGC were calculated for the entire study. Samples of fish from the treatment groups were not taken at the end of this study because the fish were needed for a follow-up study designed to evaluate the performance of family groups fed the fishmeal control diet and the plant protein-based diet. All experimental protocols involving Atlantic salmon were approved by the National Cold Water Marine Aquaculture Center's Animal Care and Use Committee.

Table 5
Diet formulations (g kg^{-1}) used in the protein-blend evaluation with late stage juvenile Atlantic salmon fingerlings (31.5 g initial weight). FM = fishmeal, LAP = land animal protein, PP = plant protein and S = *Spirulina* algae meal supplementation.

Ingredient	FM control	LAP blend	LAP-S blend3	PP blend	PP-S blend5
Menhaden fishmeal[a]	386.2	–	–	–	–
Poultry-by-product meal[b]	–	319.2	258.1	–	–
Corn protein concentrate[d]	160.2	160.2	129.5	273.4	238.1
Soy protein concentrate[e]	–	–	–	216.4	149.4
Wheat gluten meal[h]	45.0	45.0	45.0	–	–
Spirulina[g]	–	–	112.5	–	112.5
Wheat starch[f]	127.5	156.1	118.3	88.0	73.7
Menhaden fish oil[c]	226.2	198.5	203.5	258.0	251.5
Vitamin premix 702[i]	15.0	15.0	15.0	15.0	15.0
Choline chloride	6.0	6.0	6.0	6.0	6.0
Stay-C (35% ascorbate)	3.0	3.0	3.0	3.0	3.0
Taurine	–	15.0	15.0	5.0	5.0
L-Lysine	14.5	12.5	16.9	45.0	25.7
DL-methionine	–	2.2	4.0	3.6	4.8
Threonine	–	0.8	3.0	2.1	4.9
Mono-dicalcium phosphate	14.8	56.0	59.4	53.1	55.9
Potassium chloride	–	5.6	5.6	5.6	5.6
Sodium chloride	–	2.8	2.8	2.8	2.8
Magnesium oxide	–	0.5	0.5	0.5	0.5
Trace mineral premix[j]	1.0	1.0	1.0	1.0	1.0
Astaxanthin	0.6	0.6	0.6	0.6	0.6
Calculated composition					
Crude protein	406	413	421	407	413
Fat	261	261	260	261	260
Total phosphorus	14	14	15	14	16
Methionine	10	12	12	12	12
Cystine	5.2	3.2	2.7	7.4	6.1
Lysine	31	32	32	32	32
Threonine	3.5	1.2	1.1	4.1	3.3

[a] Omega Proteins, Menhaden Special Select, 628 g/kg protein.
[b] IDF Inc., 832 g/kg protein.
[c] Omega Proteins.
[d] Cargill, Empyreal 75, 748 g/kg protein.
[e] Solae, Pro-Fine VF, 693 g/kg crude protein.
[f] Manildra Milling, 40 g/kg protein.
[g] Earthrise Nutritional Products, 727 g/kg protein.
[h] Manildra Milling, 753 g/kg protein.
[i] Supplied the following per kg diet: vitamin A palmitate, 9650 IU; cholecalciferol, 6600 IU; DL-tocopheryl acetate, 132 IU; menadione sodium bisulfate 1.1 mg; thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine HCl 13.7 mg; DL-calcium pantothenate, 46.5 mg; cyanocobalamine 0.03 mg, nicotinic acid, 21.8 mg; D-biotin, 0.34 mg; folic acid 2.5; and inositol, 600 mg.
[j] Supplied the following per kg diet: copper, 3 mg as copper sulfate pentahydrate; manganese, 10 mg as manganese sulfate, monohydrate; iodine, 5 mg as potassium iodide; sodium selenate 0.960 g; and zinc, 37 mg as zinc sulfate, heptahydrate.

2.3. Sample analysis

Feed samples and fish tissue samples were analyzed for proximate composition using AOAC (1990) methods. Frozen whole fish samples were partially thawed, then ground in an industrial food processor (Robot Coupe R2, Ridgeway, MS). Samples were dried in a convection oven at 105 °C for 8 h to determine moisture levels. Dried samples were finely ground by mortar and pestle and analyzed for nitrogen (N) using a LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, MI). Crude protein (CP) was calculated from sample N content (total nitrogen × 6.25 = CP). Crude fat was analyzed using an ANKOM XT15 extraction system (ANKOM Technology, Macedon NY) with petroleum ether as the extracting solvent, and ash was determined by incineration at 550 °C in a muffle furnace.

2.4. Statistics

Weight gain, FCR, TGC and PPV were subjected to one-way Analysis of Variance and Tukey's HSD Test for comparison of treatment mean values, using the Statistical Analysis System (SAS, 1985). Statistical significance was set at $P \leq 0.05$.

3. Results

Trout fed the fishmeal diet weighed 257 g at the end of the feeding trial, and had a FCR of 0.85 and a TGC of 0.287 over the course of the feeding trial (Table 6). Weight gains of trout fed the SPC blend diets at the 63% and 82% replacement levels were statistically similar to weight gain of trout fed the fishmeal diet (247 and 252 g, respectively), but performance (final weight, and TGC values) of trout fed the diet with 100% fishmeal replacement with the SPC blend was significantly lower (Table 6). Trout fed the BPC blend diets at 33% and 66% replacement levels exhibited significantly lower weight gain and TGC values than fish fed the fishmeal control diet not significantly different from values of fish fed the SPC blends at the same dietary level. Trout fed diets in which fishmeal was totally replaced with either the BPC or the CGM blends had significantly lower weight gain (15% lower), lower TGCs and higher FCRs than fish fed diets with lower levels of fishmeal replacement. Feed intake progressively decreased as replacement levels of fishmeal increased in the BPC group but was variable in the CGM groups, exhibiting no progressive decline (Table 6). There were no fish mortalities during the trial. Proximate composition of fish at the end of the study did not vary among dietary treatment groups (data not shown).

Early stage Atlantic salmon juveniles fed the fishmeal diet had significantly higher final weight and TGC values at the conclusion of the trial than fish fed diets containing either alternative protein blend at all levels of replacement. Fish fed diets containing protein blends weighed significantly less, 21.7 g for fish fed the SPC blend and 21.4 g for the WGM blend (Table 7). There were no significant differences in weight gain between juveniles fed diets containing either protein blend or among different levels of the protein blends, although fish fed diets with the highest level of protein blends gained the least weight (Table 7). FCR values were higher in all groups fed protein-blend diets compared to the fishmeal control group. Proximate composition of fish at the end of the study did not differ among dietary treatment group (data not shown) but PPV values were lower in all treatment groups containing protein blends compared to the PPV value of the fishmeal control treatment.

In contrast to results with early stage Atlantic salmon juvenile fish, late stage juveniles fed diets containing protein blends with no fishmeal did not differ significantly in final weight, feed intake, FCR, TGC or percent survival after 12 weeks of feeding from the fishmeal control diet (Table 8). Fish tripled their initial average weight over the course of the study. Addition of *Spirulina* algae meal to the diets had no statistical effect on fish performance. Notably, performance of fish fed diets lacking any animal or fish protein was statistically similar to that of fish fed diets containing land animal protein ingredients.

4. Discussion

The results of this study demonstrated differences in fish growth performance between rainbow trout and juvenile Atlantic salmon fed diets in which alternative protein blends provided most of the dietary protein, and also demonstrated that performance of juvenile Atlantic salmon fed diets in which protein was supplied from alternative protein blends improved with fish size/age. The effects of plant-protein meals and concentrates on growth of rainbow trout have been well studied (Gatlin et al., 2007; Lee et al., 2006; Lim et al., 2008). In most studies, rainbow trout fed diets containing a single alternate protein source or blends of protein sources exhibited reduced growth at high levels of replacement unless the diets contained 20–30% fishmeal (Adelizi et al., 1998; Glencross et al., 2010; Gomes et al., 1995). This is thought to be associated with differences between fishmeal and alternate protein sources in amino acid profile and availability, and, in the case of plant-protein ingredients, the absence of other essential nutrients and compounds such as macrominerals, trace minerals, sterols and taurine. In the present study, rainbow trout fed a diet in which the SPC blend replaced 63 and 87% of the fishmeal in the formulation grew as well as fish fed the fishmeal control diet (53% fishmeal). However, at 100% fishmeal replacement, fish weight gain was significantly reduced, despite the fact that limiting amino acids, macrominerals, trace minerals and taurine were supplemented to meet reported dietary requirements of the fish (NRC, 2011), Differences in feed intake account for a portion of the reduced fish weight gain, but differences in FCR and PPV suggest that other essential nutrients may be

Table 6
Final weight, feed conversion ratio (FCR), thermal growth coefficient (TGC) and productive protein value (PPV) for rainbow trout fed experimental diets for 12 weeks.[1]

Diet[2]	Final Weight (g)[3]	Total feed intake (g feed/fish)[3]	FCR[3,4]	TGC[3,4]	PPV[4]
FM control	257 ± 4.7^{A}	202 ± 4^{AB}	0.85 ± 0.01^{E}	0.287 ± 0.003^{A}	0.381 ± 0.015^{AB}
SPC63	247 ± 6.7^{ABC}	188 ± 5^{D}	0.83 ± 0.01^{E}	0.280 ± 0.004^{ABC}	0.393 ± 0.008^{A}
SPC82	252 ± 0.6^{AB}	192 ± 2^{CD}	0.83 ± 0.01^{E}	0.284 ± 0.000^{AB}	0.396 ± 0.029^{A}
SPC100	240 ± 3.6^{BC}	199 ± 3^{CB}	0.90 ± 0.01^{C}	0.277 ± 0.002^{ABC}	0.341 ± 0.007^{ABC}
BPC63	239 ± 2.3^{BC}	190 ± 1^{CD}	0.87 ± 0.01^{CDE}	0.275 ± 0.002^{BC}	0.329 ± 0.008^{ABCD}
BPC82	240 ± 3.2^{BC}	188 ± 4^{D}	0.85 ± 0.01^{E}	0.277 ± 0.002^{ABC}	0.362 ± 0.011^{ABC}
BPC100	218 ± 2.4^{D}	178 ± 2^{E}	0.90 ± 0.00^{CD}	0.261 ± 0.002^{D}	0.305 ± 0.005^{BCD}
CGM63	230 ± 4.8^{CD}	206 ± 2^{AB}	0.98 ± 0.03^{B}	0.270 ± 0.003^{CD}	0.296 ± 0.022^{CD}
CGM82	234 ± 0.5^{CD}	184 ± 1^{DE}	0.86 ± 0.01^{CDE}	0.272 ± 0.001^{CD}	0.396 ± 0.017^{ABC}
CGM100	220 ± 2.1^{D}	209 ± 5^{A}	1.04 ± 0.02^{A}	0.263 ± 0.002^{D}	0.258 ± 0.015^{D}

[1] Mean of three replicate tanks ± SEM. Initial fish weight was 19.5 g.
[2] FM is the fishmeal control diet. SPC, BPC and CGM are the soy protein concentrate, barley protein concentrate and corn gluten meal blends, respectively. Numbers denote the percentage of fishmeal replaced with the alternate protein blend.
[3] Values within columns having a common superscript letter do not differ significantly ($P > 0.05$).
[4] FCR = feed fed (g)/weight gain (g); TGC = (Final fish weight $^{1/3}$ – initial fish weight $^{1/3}$/degree days) * 100; PPV = (protein intake per fish/protein gain per fish).

Table 7
Final weight, feed intake, feed conversion ratio (FCR), thermal growth coefficient (TGC) and productive protein value (PPV) for early stage juvenile Atlantic salmon fed experimental diets for 18 weeks.[1]

Diet[2]	Final weight (g)[3]	Total feed fed (g/ fish)[3]	FCR[3,4]	TGC[3,4]	PPV[3, 4]
FM control	27.29 ± 0.16^{A}	40.18 ± 0.55	1.85 ± 0.03^{C}	0.075 ± 0.001^{A}	0.236 ± 0.002^{A}
SPC50	21.83 ± 2.17^{B}	37.25 ± 2.31	2.31 ± 0.14^{B}	0.062 ± 0.006^{BC}	0.185 ± 0.009^{BC}
SPC66	22.90 ± 0.17^{B}	37.61 ± 0.41	2.18 ± 0.63^{B}	0.065 ± 0.002^{B}	0.195 ± 0.003^{B}
SPC87	20.31 ± 1.48^{B}	35.74 ± 1.44	2.42 ± 0.12^{AB}	0.059 ± 0.005^{BC}	0.173 ± 0.007^{BC}
WGM50	22.11 ± 2.31^{B}	35.31 ± 3.60	2.17 ± 0.09^{B}	0.062 ± 0.008^{BC}	0.196 ± 0.004^{BC}
WGM66	22.97 ± 1.51^{B}	37.92 ± 0.88	2.22 ± 0.13^{B}	0.064 ± 0.004^{BC}	0.186 ± 0.008^{BC}
WGM87	19.23 ± 0.87^{B}	35.44 ± 0.96	2.63 ± 0.06^{A}	0.054 ± 0.002^{C}	0.156 ± 0.004^{C}

[1] Means of two replicate tanks ± SEM for plant-protein diet; three replicate tanks for the FM control treatment group. Initial average weight of the fish was 5.5 g.
[2] FM is the fishmeal control diet. SPC and WGM are the soy protein concentrate and wheat gluten meal blends. The number denotes the percentage of fishmeal replaced with the protein blend.
[3] Values within columns having a common superscript letter do not differ significantly ($P > 0.05$).
[4] FCR = feed fed (g) / weight gain (g); TGC = (Final fish weight $^{1/3}$ – initial fish weight $^{1/3}$/degree days) * 100; PPV = Productive protein value (protein intake/protein gain).

limiting or that utilization of dietary protein and/or protein turnover may be altered in trout fed diets containing the highest levels of plant-protein blend. Further research is required to identify the factor(s) responsible for these observations.

This is the first study to our knowledge that compared the effects of feeding diets with alternative protein blends on the performance of early stage Atlantic salmon juveniles with that of late stage juveniles. The results show that growth was reduced in early stage juvenile salmon even when the fish were fed diets containing 30% fishmeal despite the fact that blends contained approximately one-third land animal proteins (poultry by-product meal and blood meal) and met or exceeded the dietary requirements of the fish (NRC, 2011). The early stage juvenile salmon were purposely overfed at 110% of their expected intake so that feed availability would not limit fish growth. This resulted in higher FCRs than those reported in other studies and made it impossible to judge the effects of diet formulation on feed intake. PPV values were much lower in the early stage juvenile salmon than in the rainbow trout study. Growth rates of early stage juveniles in the present study were two to three times lower than rates reported in the literature (Austreng et al., 1987; Berge and Storebakken, 1996). This can likely be attributed to the fact that the published studies were conducted in the spring and summer where photoperiod was increasing whereas our study was conducted in during fall and early winter months as photoperiod and water temperatures were decreasing. Rainbow trout have been shown to have over a four-fold reduction in growth rate (from 2.0%/day to >0.5%/day) when the photoperiod was reduced to 8L:16D which was similar to the photoperiod at the end of our study (Taylor et al., 2005). Juvenile Atlantic salmon are similarly affected by photoperiod changes.

Late stage Atlantic salmon juveniles (31.5 g initial weight) in the second salmon study responded differently than early stage juvenile salmon, having similar weight gains and FCR values in all dietary treatment groups, including the fishmeal control group. The growth rates of the late stage juvenile salmon in the present study were equivalent to those reported by Austreng et al. (1987) and remained steady throughout the study. The late stage juvenile salmon in the second salmon feeding trial were exposed to an increasing photoperiod because the study started in late winter and concluded in late spring. This likely increased feed consumption, leading to faster growth in the second study compared to the first.

Our results suggest that even when fishmeal is included at 30% of the diet, early stage juvenile Atlantic salmon (5 g) will not grow as fast as fish fed a diet containing 60% fishmeal. However, growth performance of late stage juvenile Atlantic salmon fed fishmeal-free diets was equivalent to fish fed a fishmeal control diet. This results is in contrast to earlier published studies in which larger juvenile Atlantic salmon exhibited reduced growth when fed diets containing less than 30% fishmeal and high levels of plant proteins, supplied as blends or as single protein sources. Although Refstie and Tiekstra (2003) and Øverland et al. (2009) found that feeding Atlantic salmon (82 g or 160 g respectively) diets that contain at least 30% fishmeal resulted in similar growth to fish fed 60% fishmeal diets, Mundheim et al. (2004) found that growth was reduced when fishmeal constituted 29% or 19% of the diet for 130 g Atlantic salmon. Drew et al. (2007) and Torstensen et al. (2008) found similar results for Atlantic salmon with initial weights of 48 g and 350 g respectively. Refstie et al. (2001) reported that growth of Atlantic salmon fed diets containing soybean meal or soy protein concentrate was not significantly different from salmon fed a fishmeal diet; however, fishmeal levels their diets exceeded 36%. All of these studies used fish that were at least smolt size at the beginning of feeding trials, with the smallest fish in the studies initially weighing 48 g.

Post-juvenile Atlantic salmon with an initial weight of approximately 950 g did not exhibit any differences in growth when fishmeal was replaced with soybean meal (12.7% of the diet) or wheat gluten (19.7%) (Storebakken et al., 2000). However, fishmeal levels were either 43.4% (soybean meal diet) or 32.1% (wheat gluten diet). The effects of a plant-protein blend containing sunflower expeller, corn gluten meal, soy protein concentrate, and wheat gluten meal on growth and lipid composition of Atlantic salmon were examined by Pratoomyot et al. (2011). This study found that sub-adult salmon (1.3 kg initial weight) grown to harvest weight could tolerate this plant-protein blend as long as fishmeal was at least 25% of the diet. Fish growth was significantly lower for fish fed diets that contained 18, 11 or 5% fishmeal (Pratoomyot et al., 2011). When fishmeal was completely replaced in post-juvenile Atlantic salmon (330 g initial weight), the fish exhibited significantly lower weight gains compared to fish fed a diet containing 49% fishmeal (Espe et al., 2006).

Our results are the first to show that late stage juvenile Atlantic salmon fed diets containing alternate protein blends in place of fishmeal grow as fast as fish fed a fishmeal-based diet, whether or not the alternate protein blends contained land animal proteins or *Spirulina*, an beneficial ingredient in feeding trials with juvenile

Table 8
Final weight, apparent feed intake, feed conversion ratio (FCR) and thermal growth coefficient (TGC) for late stage juvenile Atlantic salmon fed the experimental diets for 12 weeks.[a].

Diet[b]	Final weight (g)	Total feed fed (g/ fish)	FCR[c]	TGC[c]	Survival (%)
FM control	106.3 ± 6.8	74.9 ± 5.8	0.86 ± 0.02	0.128 ± 0.011	94.3 ± 0.3
LAP blend	103.2 ± 6.0	75.8 ± 2.8	0.95 ± 0.02	0.120 ± 0.013	94.8 ± 1.5
LAP-S blend	109.9 ± 4.0	70.0 ± 3.7	0.94 ± 0.04	0.133 ± 0.015	96.3 ± 2.4
PP blend	109.7 ± 3.7	74.9 ± 5.8	0.86 ± 0.02	0.135 ± 0.011	94.3 ± 0.3
PP-S blend	101.7 ± 4.3	75.9 ± 2.6	0.93 ± 0.01	0.120 ± 0.005	92.4 ± 1.1

[a] Means of three replicate tanks ± SEM. Initial average fish weight was 31.5 g.
[b] FM is the fishmeal control diet. LAP is the land animal–plant-protein blend and PP blend is the plant-protein blend. Addition of *Spirulina* algae to the blend is designated by S.
[c] FCR = feed fed (g) / weight gain (g); TGC = (Final fish weight $^{1/3}$ – initial fish weight $^{1/3}$/degree days) * 100.

marine fish (R. Barrows, unpublished data). A likely explanation for the positive results found in the present study using fishmeal-free diets compared to results of other published research is that in the present study, diets were supplemented with essential amino acids, minerals and other compounds, including a vitamin premix specifically developed for use in plant-based feeds for salmonids (Barrows et al., 2010).

5. Conclusions

Rainbow trout fingerlings fed a diet in which up to 87% of fishmeal was replaced with an alternate protein blend containing SPC had similar growth to fingerlings fed a fishmeal diet, but trout fed other protein blends based on CGM or BPC had lower weight gains. Early stage juvenile Atlantic salmon (5 g) grew poorly when fed diets containing alternate protein blends compared to growth rates of fish fed a fishmeal control diet even when fishmeal was included at 30% of the diet. However, late stage juvenile Atlantic salmon (initial weight 31.5 g) grew well on diets containing plant-protein blends, even when fishmeal and land animal protein ingredients were completely replaced in the diet. These results demonstrate that advance stage juvenile Atlantic salmon can be reared using fishmeal-free diets, although further testing in commercial settings should be undertaken.

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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.

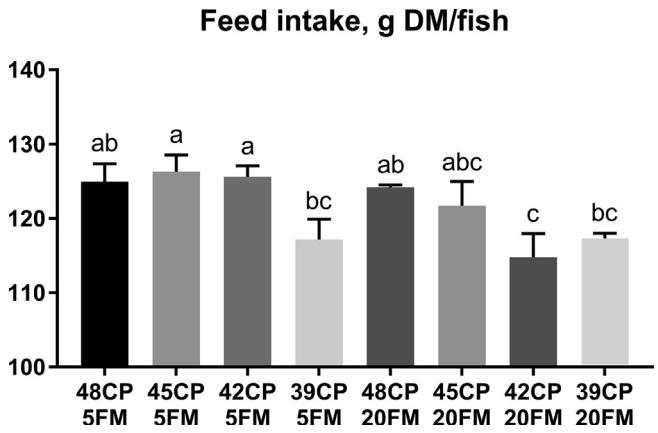

Fig. 1. Feed intake (g DM/fish) of rainbow trout juveniles fed experimental diets for 9 weeks. Bars represent mean ± SE of triplicate samples. Two-way ANOVA indicated a significant CP X FM interaction ($P < .05$). Interaction means having different letters are significantly different by Tukey's HSD test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

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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Effect and interaction of rainbow trout strain (*Oncorhynchus mykiss*) and diet type on growth and nutrient retention

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Abstract

Eight strains of rainbow trout were introgressed to develop a single strain (H-ARS) that was selected for faster growth when fed a fishmeal-free, plant-based diet (Selection Diet). For four generations, families from these crosses were fed the Selection Diet and selected for increased weight gain. Growth and nutrient retention were compared among H-ARS and two parental strains, the House Creek (HSC) and Fish Lake (FL) fed either a fish meal or Selection diet for 12 weeks. There was a significant effect of strain ($P < 0.01$), but not diet on weight gain, and a significant interaction of strain by diet ($P < 0.05$). The H-ARS trout gained more weight averaged across diet (991% of initial wt.) than the HC (924%) or FL trout (483%). The FL trout fed the fish meal diet gained more weight than FL trout fed the selection diet (510% vs 456%). Conversely, H-ARS trout fed the plant-based diet gained more weight than those fed the fish meal diet (1009% vs 974%). HSC trout had similar weight gain fed either diet (922% vs 926%). A significant effect of strain on protein retention ($P < 0.01$) was observed, along with a significant strain by diet interaction ($P < 0.02$). The results demonstrate that rainbow trout can be selectively improved to grow on a plant-based diet.

Keywords: rainbow trout, plant-based diet, selection, growth

Introduction

Aquaculture production is expanding significantly around the world. One of the major impediments to both current and developing aquaculture production is the availability of sustainable sources of feed (Naylor, Hardy, Bureau, Chlu, Elliott, Farrell, Forster, Gatlin, Goldburgh, Hua & Nichols 2009). Currently, fishmeal is the major protein source utilized in a number of aquaculture diets, especially for piscivorous and carnivorous species. Current use of fishmeal is nearing 70% of total production with predictions that global aquaculture demands will exceed availability within the next 10 years (Food & Agriculture Organization of the United Nations 2009). For decades, researchers have been evaluating alternative protein sources to replace fishmeal as the major protein component in aquaculture diets (Robinson & Meng 1994; Skonberg, Hardy, Barrows & Dong 1998; Torstensen, Espe, Sanden, Stubhaug, Waagbe, Hemre, Fontanillas, Nordgarden, Hevroy, Olsvik & Berntssen 2008). The use of formulated plant-based diets has been reported for many different species (Papatryphon 2001; Fontainhas-Fernandes, Gomes, Reis-Henriques & Coimbra 2009). However, there have been no reports of a piscivorous species of fish demonstrating better growth on diet formulated with the fishmeal protein completely replaced with plant protein.

To effectively replace as much fishmeal as possible with protein from plant sources, different methods have been employed to increase the protein availability of these products. These approaches include different processing methods used to increase protein levels, enhance positive material aspects and reduce anti-nutritional factor levels in plant-based fishmeal replacement diets (Gatlin, Barrows, Brown, Dabrowski, Gaylord, Hardy,

Herman, Hu, Krogdahl, Nelson, Overturf, Rust, Sealey, Skonberg, Souza, Stone, Wilson & Wurtele 2007). Most reports showing growth comparisons between fish reared on standard fish meal diets compared with diets where fishmeal is replaced with alternative protein ingredients, typically plant, report a significant reduction in growth in the fish reared on the diets containing alternative protein sources (Kaushik, Cravedi, Sumpter, Fauconneau & Laroche 1995; Cheng & Hardy 2003; De Francesco, Parisi, Medale, Lupi, Kaushik & Poli 2004). Genetic enhancement of existing piscivorous and carnivorous stocks of fish is another approach for improving growth and utilization of plant-based feeds. Genetic variation has been found, and in some cases, used to improve traits such as disease resistance, growth rate, feed efficiency, offspring size, quality traits, morphology and others in agricultural animals (Mrode & Kennedy 1993; Heringstad, Klemetsdal & Ruane 2000; Baeza, Dessay, Wacrenier, Marche & Listrat 2003). Meanwhile, in aquaculture, selective breeding has been shown to improve salinity tolerance, growth and disease resistance (Dunham, Brady & Vinitnantharat 1994; Rezk, Smitherman, Williams, Nichols, Kucuktas & Dunham 2003; Kamal & Mair 2005).

Research has been conducted to determine the relative genetic potential for improving growth of certain fish species fed specific plant-based feeds. Palti (Palti, Silverstein, Wieman, Phillips, Barrows & Parson 2006) found no change in the rankings of 20 full-sib families of rainbow trout for growth when the fish were fed a fishmeal or a plant protein-based diet. However, the plant-based diet used in Palti's study contained 10% krill. However, a later study using whitefish (*Coregonus lavaretus*) found substantial genetic variation for growth traits between families fed either a fishmeal diet or a diet with 50% of the protein replaced with soybean meal (Quinton, Kause, Koskela & Ritola 2007). Furthermore, a more recent study using diets where fishmeal was completely replaced found significant genetic variation in growth for salmonids (*Oncorhynchus mykiss*) fed plant-based feeds (Pierce, Palti, Silverstein, Barrows, Hallerman & Parsons 2008). Similar results comparing marine ingredient-based diets against diets where the fishmeal and fish oil is completely replaced by plant products were found in sea bream (Le Boucher, Vandeputte, Dupont-Nivet, Quillet, Mazurais, Robin, Vergnet, Medale, Kaushik & Chatain 2011).

Although these and other studies were done to determine genetic variance and the potential heritability for growth and utilization of a plant-based diet, no studies have been reported using fish that had been selected for growth on a fishmeal-free, plant-based feed for several generations. From variability previously detected in studies in rainbow trout, and the need for improved strains, a selection programme was initiated in 2000 to generate a strain of rainbow trout with improved growth characteristics when reared on a fishmeal-free, plant protein-based diet. Reported here is the growth performance of this selected strain of rainbow trout after four generations of selection in comparison to two of the parental stocks, a fast growing domesticated strain and a slower growing conservation strain.

Materials and methods

Fish stocks

Fish strains used in this study consisted of a domesticated strain selected for growth for several generations (Housecreek) (Overturf, Casten, LaPatra, Rexroad & Hardy 2003), a strain reared by US Fish and Wildlife Service (Ennis, MT) and used for stocking in streams and lakes (FishLake) and a strain generated by introgression and selection (Hagerman-ARS). The Hagerman-ARS (H-ARS) strain was introgressed with the following fish strains; Oregon and Housecreek (HSC) from the College of Southern Idaho Hatchery in Twin Falls, Idaho; R9 and Kamloops from the Idaho State Fish Game Hatchery in Hayspur, Idaho; Fish Lake (FL), Shasta, and Arlee from the US Fish and Wildlife Hatchery in Ennis, Montana; and the Donaldson strain from the University of Washington, Seattle, Washington. The H-ARS stock has been selected for growth on a plant-based diet (Table 1) containing fish oil, but without fishmeal. Selection pressure on the H-ARS stock has been maintained for four generations by retaining the top 15% (as assessed by weight) of the fish from the 40 best performing families for each year class(as assessed by family average for specific growth rate and feed conversion ratio at 6 months post feeding) as broodstock. Fish rearing was carried out at the University of Idaho's Hagerman Fish Culture Experiment Station. Experiments were carried out in 140 L tanks supplied with constant 15°C temperature spring

Table 1 Ingredient and nutrient composition of standard control fishmeal diet and plant meal selection diet

	g kg^{-1}	
Ingredient	**Fish meal**	**Plant meal**
Soy protein concentrate*	—	256.3
Corn protein concentrate†	61.6	175.4
Wheat gluten‡	—	4.1
Soybean meal§	158.4	19.6
Fish meal¶	336.0	—
Wheat starch**	262.3	891.0
Blood meal††	58.5	—
Poultry meal‡‡	46.5	—
Menahaden oil§§	120.6	157.0
Vitamin premix***	10.0	10.0
Methionine	2.9	3.8
Taurine	5.0	5.0
Dicalcium phosphate	—	33.3
Trace min. premix¶¶	1.0	1.0
Choline CL	1.1	6.0
Stay-C	0.3	2.0
Potassium chloride	—	5.6
Magnesium oxide	—	0.6
Sodium chloride	—	3.8
	Calculated composition, as is basis	
Crude protein, g kg^{-1}	444.0	444.4
Crude fat, g kg^{-1}	160.8	160.7
Phosphorus, g kg^{-1}	11.0	11.3

*Solae, Pro-Fine VF, 693 g kg^{-1} crude protein.
†Cargill, Empyreal 75, 761.0 g kg^{-1} protein.
‡Manildra Milling, 750 g kg^{-1} protein.
§ADM Inc., 480 g kg^{-1} protein.
¶Omega Proteins, Menhanden Special Select, 628 g kg^{-1} protein.
**Manildra Milling, 4 g kg^{-1} protein.
††IDF Inc., 832 g kg^{-1} protein.
‡‡American Dehydrated Foods, 734 g kg^{-1} protein.
§§Omega Proteins Inc.
¶¶Contributed in mg kg^{-1} of diet; zinc 40; manganese 13; iodine 5; copper 9.
***Contributed, per kg diet; vitamin A 9650 IU; vitamin D 6600 IU; vitamin E 132 IU; vitamin K3 1.1 gm: thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine hydrochloride 13.7 mg; pantothenate DL-calcium 46.5; cyancobalamin 0.03 mg; nicotinic acid 21.8 mg; biotin 0.34 mg; folic acid 2.5; inostitol 600.

water at a flow rate of approximately 11.5 L min^{-1}. Photoperiod was maintained at 14 h light:10 h dark. The composition of the two diet formulations, the fishmeal control and the plant protein selection diet, are shown in Table 1. Fish were handled and treated according to the guidelines of the University of Idaho's Institutional Animal Care and Use Committee.

Experimental setup and sampling

Three strains of rainbow trout, Housecreek (HSC), FL and H-ARS, were stocked into 18 140 L tanks (six tanks per strain) at 35 fish per tank. The average fish weight of the fish was 30 ± 1.6 g. The fish strains were stocked randomly among tanks and three tanks of each strain of fish were fed either the fishmeal control diet (FM) or the plant-based selection diet (PB) (Table 1). The fish were fed to apparent satiation twice daily, 6 days a week, for 12 weeks. The fish from each tank were bulk-weighed and counted every 4 weeks, and the amount of feed fed to each tank was recorded daily throughout the experiment. Whole body samples were taken (five fish per tank) every 4 weeks throughout the experiment.

Diet preparation

Both the fishmeal control diet and the selection diet were produced with commercial manufacturing methods using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) at the Bozeman Fish Technology Center, Bozeman, MT. Diet mash was exposed to an average of 114°C for 18-s in five barrel sections, and the last section was water cooled to an average temperature of 83°C. Pressure at the die head was approximately 450 psi. The pellets were then dried in a pulse bed drier (Buhler AG) for 25 min at 102°C and cooled at ambient air temperatures to reach final moisture levels of < 10%. Fish oil was top-dressed using vacuum coating (A.J Flauer Mixing, Ontario Canada) after the pellets were cooled. Diets were stored in plastic lined paper bags at room temperature until used. All diets were fed within 4 months of manufacture.

Proximate analysis

Whole fish were pooled by tank and ground for homogeneity prior to analyses. Whole fish samples, individual fillet and diet samples were analysed in duplicate assays using standard AOAC (1995) methods for proximate composition. Dry matter and ash analysis was performed on a LECO thermogravimetric analyser (TGA701, LECO Corporation, St. Joseph, MI, USA). Protein (N × 6.25) was determined using the Dumas method (AOAC 1995) on a LECO nitrogen determinator (TruSpec N, LECO Corporation, St. Joseph, MI, USA) and

was measured using a Foss Tecator Soxtec HT Solvent Extractor, Model Soxtec HT6 (Höganäs, Höganäs, Sweden). Total energy was determined using adiabatic bomb calorimetry (Parr 6300, Parr Instrument Company, Moline, IL, USA). Protein retention and energy retention efficiencies were calculated as follows:

$$\begin{aligned}&\text{Protein retention efficiency (PRE)}\\&= (\text{protein per gram of final fish weight}\\&- \text{protein per gram of initial weight})\\&\quad \text{protein gain} \times 100/\text{protein fed}\end{aligned}$$

$$\begin{aligned}&\text{Energy retention efficiency (ERE)}\\&= (\text{calculated energy of final fish tank weight}\\&- \text{energy of initial fish tank weight})\\&\times 100/\text{energy fed. Muscle ratios were}\\&\text{calculated by (fillet weight/body weight)}\\&\times 100.\end{aligned}$$

$$\begin{aligned}\text{Muscle Ratio} &= \text{Muscle Ratio (MR)}\\&= \text{fillet mass with ribs (g)}\\&\quad \times 100/\text{fish mass (g)}\end{aligned}$$

Statistical analysis

Microsoft Excel was used to graphically represent the data. Fish performance, nutrient retention and carcass composition data were analysed with the general linear models procedure using a factorial treatment design and the Statistical Analysis System (SAS 9.2; SAS Institute 2008, Cary, NC, USA). Tank mean values were considered units of observation for statistical tests, and mean values were considered significantly different when $P < 0.05$. Any value expressed as a percentage was arcsine transformed prior to analysis (Sokal & Rohlf 1981).

Results

Strain and dietary effects for growth and feed conversion

A significant effect on growth was seen for strain, but not diet (Fig. 1). Both the H-ARS and HSC strains of fish exhibited higher performance than the FL strain when fed the fishmeal control diet as evidenced by significantly higher final weights, weight gain, specific growth rate (SGR) and per cent weight gain. However, there were no significant interactions found in growth between the

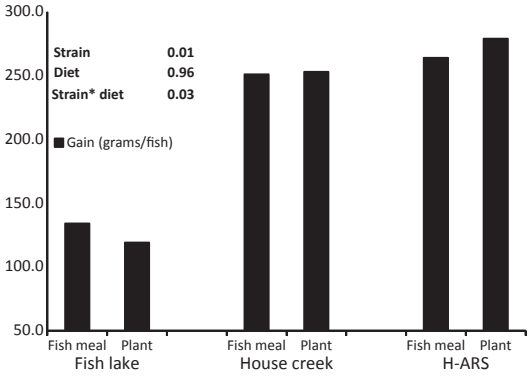

Figure 1 Comparison of weight gain for the Fish Lake, Housecreek, and H-ARS strains of fish on either a fishmeal or plant-based diet with significant interactions shown in legend.

H-ARS and HSC strain fed the fishmeal control diet. On the plant-based selection diet, the H-ARS strain significantly outperformed both the HSC and FL strains in terms of weight gain, SGR and per cent weight gain, whereas the HSC strain showed significant improvement for all measured growth parameters over the FL strain when fed the plant-based selection diet. Comparison between diets for each strain showed that the FL strain grew significantly better when fed fishmeal control diet than when fed the plant-based selection diet. The HSC strain showed no significant differences in growth between the fishmeal and the plant-based diet. The H-ARS strain showed significant improvements in growth on the plant-based selection diet over the fishmeal feed (Table 2).

There was a significant strain interaction on feed intake with both the H-ARS and HSC strains having a significantly higher feed intake than the FL strain on either of the diets. For FCR, there was a significant strain by diet interaction where FL was found to have a higher FCR on the fishmeal diet compared with the HSC and H-ARS strains, but showed no difference between the diets. The HSC strain showed significant differences for FCR between the diets with lower levels found on the fishmeal control diet. The H-ARS strain had no significant difference in FCR between the two diets (Table 2).

Dietary and strain effect on body composition and nutrient retention

No significant interactions were found within or between the strains and diets for protein, fat and

Table 2 The effect of diet and strain on growth and feed parameters of selected versus non-selected rainbow trout reared on either a fish meal or plant meal-based diet

Strain	Diet	Final weight, g fish^{-1}	Gain		Gain, % initial	Feed Intake	
			g fish^{-1}	SGR		% body wt.	FCR
Fish Lake	Fish meal	167	134.0	2.26	510	1.67	0.90
	Plant-based	152	119.0	2.11	456	1.61	0.91
House Creek	Fish meal	282	251.0	3.09	922	1.80	0.81
	Plant-based	283	253.0	3.09	926	2.08	0.93
H-ARS	Fish meal	294	264.0	3.16	974	1.95	0.86
	Plant-based	309	279.0	3.20	1009	2.03	0.89
	Probability of > F value						
Model		0.01	0.01	0.01	0.01	0.01	0.13
	Strain	0.01	0.01	0.01	0.01	0.01	0.48
	Diet	0.86	0.94	0.19	0.72	0.07	0.04
	Strain × diet	0.03	0.03	0.02	0.05	0.07	0.20
	CV	3.44	3.95	1.94	3.50	5.89	5.92
	R-square	0.98	0.99	0.99	0.99	0.79	0.47

SGR, specific growth rate; FCR, feed conversion ratio.

Table 3 The effect of diet and strain on body composition and nutrient retention of selected and non-selected rainbow trout fed either a standard fishmeal control or plant-based selection diet

Strain	Diet	Moisture	Body composition				PRE	ERE
			Protein	Fat	Ash	cal g^{-1}		
Fish lake	Fish meal	68.2	16.8	13.1	1.90	6741	20.2	40.9
	Plant-based	67.4	17.1	12.8	2.10	6744	17.1	40.7
House creek	Fish meal	68.9	16.4	12.7	1.90	6727	41.2	40.0
	Plant-based	67.5	16.6	13.6	2.10	6848	42.7	41.7
H-ARS	Fish meal	68.6	16.9	12.5	2.00	6704	45.6	40.2
	Plant-based	67.7	16.5	13.4	2.00	6828	47.3	41.3
	Probability of > F value							
Model		0.13	0.38	0.70	0.49	0.20	0.01	0.47
	Strain	0.83	0.83	0.41	0.93	0.61	0.01	0.99
	Diet	0.01	0.14	0.81	0.07	0.04	0.94	0.12
	Strain × diet	0.51	0.26	0.62	0.74	0.33	0.02	0.38
	CV	0.96	5.37	3.44	8.37	1.14	4.03	2.75
	R-square	0.47	0.33	0.20	0.28	0.42	0.99	0.29

Cal g^{-1}, calories per gram; PRE, protein retention efficiency; ERE, energy retention efficiency.

ash content. However, there was a significant interaction for moisture between the diets. No significant interactions were found for energy retention. However, there were significant strain and diet-by-strain interactions found for protein retention efficiency (Table 3). The H-ARS and HFS showed significantly higher protein retention efficiencies than the FL strain for both diets. Furthermore, the H-ARS strain showed higher protein retention efficiencies than the HSC strain for both diets (Fig. 2). No significant interactions were found in muscle ratios between any of the strains of fish for either diet.

Discussion

Production of commercial aquaculture diets utilizing protein from sustainable sources will constrain rising feed costs and maintain more stable feed prices in the future. With this in mind, there has been extensive research effort invested in improving the formulation of diets to enhance growth

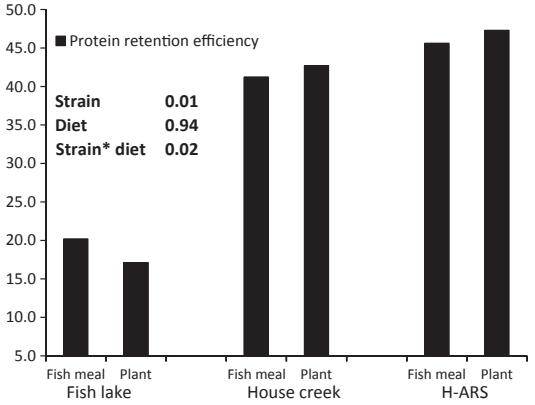

Figure 2 Comparison in protein retention efficiency for the Fish Lake, Housecreek, and H-ARS strains of fish on either a fishmeal or plant-based diet with significant interactions shown in legend.

and utilization and to test and develop sustainable economical feeds (Gatlin *et al.* 2007). Further research has been done to evaluate and determine the potential to improve the performance of fish using selective breeding, so that they grow more efficiently and utilize feeds containing sustainable protein sources (Quinton *et al.* 2007; Pierce *et al.* 2008). Although many different feeds incorporating almost every conceivable plant product have been tested, with many providing reasonable growth and FCRs, researchers have not yet been able to equal the growth found with fishmeal-based diets for piscivorous and carnivorous species. There are multiple reasons why a feed containing plant protein instead of fishmeal would reduce growth, including the presence of saponins, protease inhibitors, lectins and other anti-nutritional factors, improper amino acid balance, lack of steroids present in fish meal, reduced availability of certain minerals and vitamins and reduced palatability (Francis, Makkar & Becker 2001; Gatlin *et al.* 2007; Glencross, Booth & Allan 2007). However, the record on selecting carnivorous fish to grow more efficiently on these plant-based diets is mixed. A number of studies evaluating genetic variation in aquaculture species have determined both positive and negative potential for certain species on different formulated fishmeal diets (Palti *et al.* 2006; Pierce *et al.* 2008; Le Boucher *et al.* 2011; Quinton *et al.* 2007).

A major issue of improving growth on a fishmeal replacement diet is lack of knowledge on what physiological or metabolic changes result in reaction to specific plant nutrient components. There are several physiological changes that could be taking place independently or interactively depending on the dietary makeup of the feed. Selection might involve improving tolerance to anti-nutritional factors, increasing overall feed intake, palatability senses, amino acid sensing and regulation or metabolic regulation involving vitamins and minerals, or a possible combination of these different actions. Furthermore, compounding the complexity is how different diet formulations or nutrient components might affect each of these components and how variations in fish strains play a role. Alternatively, the deleterious overabundance or lack of one or more specific nutrients might obscure positive genetic variation for the growth and utilization of other dietary components. Previous studies have all differed in their findings and this could most likely be attributed to differences in the diets, dietary components and the stocks of fish used.

In the present work, families from several rainbow trout strains were first tested for variation in growth when fed a plant-based diet versus a standard fish meal diet. After it was established that sufficient variation was present, a selection programme was instigated involving the introgression of eight fish stocks reared on an economically feasible plant-based feed. The fish used in the current study had been under selection for growth for four generations, and over this period, their average weight when grown on the plant-based selection diet increased from an average of 178 to 256 g at 5 months from first feeding with an average realized heritability of 0.42. From other unreported studies, we have observed that these fish under selection perform better on high carbohydrate diets (35%), have increased tolerance for low palatability feeds and improved intestinal health when fed plant-based feeds when compared with non-selected fish (unreported data). In this study, we compared the growth of these 4th generation selected stocks to two initial strains of fish, a fast growing domesticated strain and a strain used in conservation stocking, that were part of the initial introgressed stocks used in development of the H-ARS strain of trout. Previous studies have reported that even with the best formulated plant-based feeds, trout and salmon weight gain was typically at least 10% reduced compared with practical fishmeal based feeds (Carter & Hauler 2000; Cheng & Hardy 2003). In the present study, selected fish showed no significant difference in weight gain or

SGR with the fast growing domesticated strain on the control fishmeal diet, but had significantly better SGR and weight gains and showed improved protein retention efficiency on the plant-based diet used in selection. This is the first report whereby a stock of fish has been shown to have grown faster when fed an all-plant-based feed compared with a fishmeal based feed.

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REVIEW ARTICLE

Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal

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Abstract

Aquafeed ingredients are global commodities used in livestock, poultry and companion animal feeds. Cost and availability are ditated less by demand from the aquafeed sector than by demand from other animal feed sectors and global production of grains and oilseeds. The exceptions are fishmeal and fish oil; use patterns have shifted over the past two decades resulting in nearly exclusive use of these products in aquafeeds. Supplies of fishmeal and oil are finite, making it necessary for the aquafeed sector to seek alternative ingredients from plant sources whose global production is sufficient to supply the needs of aquafeeds for the foreseeable future. Significant progress has been made over the past decade in reducing levels of fishmeal in commercial feeds for farmed fish. Despite these advances, the quantity of fishmeal used by the aquafeed sector has increased as aquaculture production has expanded. Thus, further reduction in percentages of fishmeal in aquafeeds will be necessary. For some species of farmed fish, continued reduction in fishmeal and fish oil levels is likely; complete replacement of fishmeal has been achieved in research studies. However, complete replacement of fishmeal in feeds for marine species is more difficult and will require further research efforts to attain.

Keywords: aquafeeds, plant protein, alternative protein, fishmeal

Introduction

Sustainable aquaculture seems like an oxymoron; how can aquaculture be sustainable when it requires more inputs that it yields in outputs? The same is true for any form of livestock or poultry production. The problem is in the definition of sustainable. For the purposes of this paper, sustainable is defined in relative terms that address the issues associated with the perception that aquaculture, at least of carnivorous fish species, is not sustainable. The main sustainability issue is use of marine resources, e.g., fishmeal and fish oil, in aquafeeds. If aquaculture consumes wild fish in the form of fishmeal and fish oil at higher amounts than what is produced, then aquaculture is a net consumer of fish, not a net producer. If the reverse is true, then aquaculture is a net producer of fish. However, this does not address sustainability because fishmeal and fish oil production is finite, and at current rates of use in aquafeeds and expected growth rates of aquaculture production, eventually aquaculture's demand for fishmeal and oil will exceed annual fishmeal and fish oil production. The answer to this problem is to replace fishmeal and fish oil with alternative ingredients derived from crops such as soybeans, wheat, corn or rice.

Fishmeal and fish oil

Global fishmeal and oil production averaged 6.5 and 1.3 million metric tonnes (mmt), respectively, over the past 20 years. However, in some years production is higher and in others lower. Variability in production is associated with variability in landings of fish used to make fishmeal. The most important source of variability in landings is associated with El Niño events in the eastern Pacific Ocean that affect landings of anchoveta (*Engraulis ringens*) in Peru and, to a lesser extent, northern Chile. Landings in this area can decrease by 4–5 mmt, leading to a decrease of fishmeal production of 1 000 000 metric tonnes (mt) or more in an El Niño year. For example, in 2006,

fishmeal production was 5 460 000 mt, about 1 mmt lower than the 20-year average. Consequently, aquaculture used a higher percentage of fishmeal production in 2006 than will be the case in average years. Overall, however, the percentage of annual global production of fishmeal and oil being utilized in aquafeeds has increased steadily over the past 20 years from approximately 15% to 65% and 85% for fishmeal and oil respectively (Tacon & Metian 2008). In 2006, 27% of the fishmeal used in the aquafeed sector went into feeds for marine shrimp (Table 1). Feeds for marine fish utilized 18% and salmon feeds 15% of the fishmeal used in aquafeeds. Overall, 45% of the fishmeal use in aquafeeds in 2006 was used in feeds for carnivorous fish species such as salmon, trout, sea bass, sea bream, yellowtail and other species. Surprisingly, 21% was used in feeds for fry and fingerling carp, tilapia, catfish and other omnivorous species. The situation with fish oil was even more dramatic; 88.5% of fish oil production in 2006 was used in aquafeeds (835 000 mt). The leading consumer of fish oil in 2006 was salmon feeds, utilizing 38% of global production (Table 2). Marine fish, trout and marine shrimp feeds used much of the remaining fish oil.

Global fishmeal and oil production is unlikely to increase beyond current levels, although with increasing recovery and utilization of seafood processing waste, global production could increase by 15–20%. Nevertheless, continued growth of aquaculture production is fundamentally unsustainable if fishmeal and fish oil remain the primary protein and oil sources used in aquafeeds. Sooner or later, supplies will be insufficient. However, alternatives to fishmeal and fish oil are available from other sources, mainly grains/oilseeds and material recovered from livestock and poultry processing (rendered or slaughter byproducts). For aquaculture to be sustainable from the feed input side, these alternatives must be further developed and used. The main drivers of change in aquafeed formulations are price of fishmeal and oil relative to alternative ingredients, and insufficient information on the nutritional requirements of major farmed species and bioavailability of essential nutrients that is needed to formulate feeds containing alternative ingredients.

Table 1 Estimated fishmeal use in feeds for selected species groups in 2006*

Species group	Metric tonnes (mt)	Per cent aquafeed use	Per cent total production
Marine shrimp	1 005 480	27	18
Marine fish	670 320	18	12
Salmon	558 600	15	10
Chinese carps	409 640	11	8
Trout	223 440	6	4
Eel	223 440	6	4
Catfish	186 200	5	3
Tilapia	186 200	5	3
Freshwater crustaceans	148 960	4	3
Miscellaneous freshwater carnivores	111 720	3	2
Total	3 724 000	100	68.2

*Adapted from Tacon and Metian (2008). Total fishmeal production in 2006 was 5 460 410 mt, below the 20-year average due to El Niño.

Table 2 Estimated fish oil use in feeds for selected species groups in 2006*

Species group	Metric tonnes (mt)	Per cent aquafeed use	Per cent total production
Marine shrimp	100 200	12	10.6
Marine fish	167 000	20	17.7
Salmon	359 050	43	38.1
Chinese carps	0	0	0
Trout	108 550	13	11.5
Eel	16 700	2	1.8
Catfish	33 400	4	3.5
Tilapia	16 700	2	1.8
Freshwater crustaceans	16 700	2	1.8
Miscellaneous freshwater carnivores	8350	1	0.9
Total	835 000	100	88.2

*Adapted from Tacon and Metian (2008). Total fish oil production in 2006 was 943 500 mt, below the 20-year average due to El Niño.

Aquafeeds for both carnivores and omnivores fish species have always contained fishmeal because until 2005, fishmeal protein was the most cost-effective protein source available. Over the previous 30+ years, the price of fishmeal remained within a trading range of US$400 to US$900 per mt, varying in price in relation to global supply and demand. However, in 2006, the price of fishmeal increased significantly to over US$1500 per mt and since then, prices have remained above US$1100, suggesting that a new trading range has been established. This has increased pressure to replace fishmeal with plant protein ingredients.

Production of protein and oil from grains and oilseeds

In contrast to fishmeal and fish oil, world production of grains and oilseeds has increased over the past two

decades as a result of higher yields and increased plantings. In 2007, global production values for maize (corn), wheat and soybeans were 785, 607 and 216 mmt respectively (http://faostat.fao.org/site/526/default.aspx). The yield of soybean meal from crushing for oil production is approximately 2/3, making soybean meal production approximately 145 mmt, 20 times the annual production of fish meal. Plant oil production is likewise much higher than fish oil production. In 2007, palm oil was the top product at 39.3 mmt, followed by soybean oil (35.6 mmt), rapeseed oil (16.8 mmt) and corn oil (15.2 mmt). This compares to 0.98 mmt of fish oil. Yields per hectare for soybeans in the United States have progressively increased from 386 kg ha^{-1} in 1993 to 474 kg ha^{-1} in 2007, an average gain in yield of slightly over 6 kg year^{-1}. Yields are increased by more efficient use of fertilizer and water and gains due to plant breeding. Higher grain and oilseed production is also likely from higher plantings. Most arable land in the world is already being cultivated, but opportunities to expand exist in several areas, such as the Commonwealth of Independent States, an entity comprised of 11 former Soviet republics. This area has 13% of the world's arable land but produces just 6% of the world's crops.

Although world grain production has increased, consumption has also increased, often to levels in excess of production. This has lowered the quantity of grain reserves carried over from year to year. However, the economic downturn has changed consumption patterns by reducing consumption of soybean meal by the livestock sector, particularly in China. The outlook for aquafeeds is promising, especially in light of the fact that aquafeeds comprise $<4\%$ of total global livestock feeds. Availability of plant protein ingredients for use in aquafeeds is not an issue.

Progress with replacing fishmeal with plant proteins

Before 2006, many advances had been made in replacing portions of fishmeal in aquafeeds with alternative protein sources and the percentages of fishmeal in feeds for salmon, trout, sea bream and sea bass, all carnivores species, had decreased by 25–50%, depending on species and life-history stage. Similarly, the percentage of fishmeal in feeds for omnivorous fish species also declined, especially in grow-out feeds. However, fishmeal use by the aquafeed sector continued to increase because aquaculture production and therefore production of aquafeeds increased. In the early 1980s, for example, aquafeeds used approximately 10% of annual fishmeal production. By 1995 and 2005, aquafeeds used nearly 29% and 50%, respectively, of annual fishmeal production. During the same period, use in poultry and swine feeds decreased by an equal amount because less expensive alternatives, such as soybean meal and corn gluten meal, were increasingly used. Similar but less dramatic substitutions of fishmeal by soybean meal and corn gluten meal occurred in salmon and trout feed. Despite changes in feed formulations for farmed fish, the dramatic increase in fishmeal prices in 2006 and the sustained higher trading range that followed increased feed prices and costs of production. Although prices have declined, the most pressing problem facing the aquaculture industry remains the cost of feed, and there is substantial pressure on feed companies to develop less expensive formulations that maintain efficient growth at lower cost per unit gain. The conventional wisdom is that this goal can only be achieved by lowering fishmeal levels in feeds further. Substituting plant protein ingredients for fishmeal to supply approximately half of dietary protein has been relatively easy but replacing higher percentages of fishmeal is difficult. There are a number of challenges that must be overcome to maintain rapid growth rates and feed efficiency values at higher levels of substitution of fishmeal.

Challenges associated with replacing fishmeal with plant proteins

The first is the cost per kilogram protein from plant protein concentrates compared with fishmeal. Until 2006, fishmeal protein was much less expensive than protein from soy or wheat concentrates, e.g., soy protein concentrate or wheat gluten meal. Although the run-up in fishmeal price made the plant proteins more competitively priced after 2006, in 2007 commodity prices increased dramatically, again making protein concentrates less competitive. Prices increased as a result of increasing demand for their use in feeds, foods, and in the case of corn, as starting material for ethanol production. For example, corn averaged US$2 per bushel for a 30-year period until 2007, when it began to increase in price outside of its normal trading range. Between mid-2007 and mid-2008, the cost of number 2 corn in Chicago increased from US$2.09 per bushel to US$5.87 per bushel. Soybeans saw a similar increase, from US$5.83 per bushel in May of

2007 to US$13.28 per bushel in May of 2008. Wheat jumped from US$5.27 per bushel to US$12.99 per bushel over the same period. Not surprisingly, prices for protein concentrates from corn, soybeans and wheat also increased. In the case of corn gluten meal (60% crude protein), the price jumped from US$257 per tonne to US$575, while soybean meal (48% crude protein) increased from US$179 to US$335. However, despite those rapid increases in prices, the cost per unit protein for plant protein sources remained lower than that of fishmeal protein, about US$7–10 per protein unit compared with US$14 for fishmeal.

Commodity prices as well as fishmeal prices declined in late 2008, but they did not return to their pre-2007/07 levels. It remains to be seen if the pricing relationships between fishmeal and plant protein concentrates will adjust to favour plant proteins, or if demand for fishmeal will result in higher prices, driving a switch to higher plant protein concentrate use in aquafeeds. Other plant-derived protein ingredients, such as lupin and rapeseed/canola protein concentrates, have been developed and researched as potential fishmeal substitutes, but there is no significant production of any alternative protein concentrate other than those from soy or wheat.

Grain and oilseed prices increased unexpectedly and dramatically over 2007/08, primarily because, on a macro-economic scale, demand increased faster than supply. But what drove demand? Certainly, in the United States, demand for corn as a seed stock for ethanol production was a factor. Brazil, the European Union (EU) and the United States produce 90% of global ethanol for biofuels use. Producing a litre of ethanol requires 2.56 kg of corn; ethanol capacity in 2008 in the United States was 7.1 billion litres requiring 61 580 000 mt of corn. Legislation in the US mandated production of 36 billion litres by 2022. In 2007, 92.9 million acres of corn were planted, up 14.6 million acres from 2006 and the highest since 1944. Of the corn produced in 2007, 26.6% was destine for ethanol production. By 2016, 109 226 040 mt of corn will be used to produce ethanol in the United States unless legislation mandating higher production of ethanol is changed. Global grain production hit record levels of 2 095 000 000 000 mt in 2007, yet supplies were barely adequate to meet demand. This supply–demand relationship was partially responsible for the high prices now seen for corn, plus increased acreage devoted to corn production in the United States came at the expense of soybean and wheat production, resulting in record prices due to demand exceeding supplies. Increasing wheat prices were also driven by lower production in Australia as a result of a multi-year drought. However, other drivers also caused corn, soy and wheat prices to increase. Demand for livestock feed increased, especially in China. In 2008, China fed 600 million swine, compared with 108 million for the United States and 240 million for the EU. China was increasing its hog population by 8–10% per year. To put that in perspective, the annual increase in hog production in China was almost half of the entire hog population in the United States. China has neither the water or aerable land to produce the grain needed to feed its hogs and is not inclined to import meat; therefore it has been and will continue to be a huge importer of soybeans and grains. Aquaculture production has increased tremendously over the past 15 years, as has aquafeed production from approximately 13 mmt to over 30 mmt. Nevertheless, aquafeed production is $< 5\%$ of annual global livestock feed production and therefore not a factor in grain or oilseed demand. Prices for commodities were also driven by speculation as commodity trading, especially in futures, was very active until the economic collapse of late 2008. The economic contraction experienced throughout the world in 2008/09 reduced demand for grains and oilseeds, but other disruptions continued to confound estimates of grain and oilseed supply/demand relationships.

The second challenge facing the aquafeed industry as it moves to substitute higher amounts of fishmeal with plant proteins pertains to the known nutritional limitations of plant proteins. Corn gluten meal is an important alternate protein source already in widespread use in aquafeeds, but corn gluten meal has limitations as a fishmeal substitute associated with its amino acid profile and non-soluble carbohydrate content. Corn protein is highly digestible to fish, but corn is deficient in lysine, making it necessary to supplement feeds containing high amounts of corn gluten meal with synthetic lysine, or blend corn gluten meal with soy or wheat protein concentrates to produce a mixture with an amino acid profile more suited for fish. Unlike proteins from oilseeds, such as soy or rapeseed/canola, corn protein concentrates do not contain anti-nutrients that limit its use in feeds. However, the crude protein content of corn gluten meal is slightly over its 60% guaranteed minimum level. This means that 40% of corn gluten meal is composed of non-protein material, mainly non-soluble carbohydrates. Non-soluble carbohydrates are of little nutritional value to fish (Stone 2003). Corn gluten meal

can be produced to contain higher protein levels if non-soluble carbohydrates are not added back to the protein fraction during manufacturing, but this practice leaves manufacturers with no outlet for the non-soluble carbohydrate fraction.

Soybean meal use is limited in feeds for salmonids and perhaps other species because of its relatively low protein content and also due to intestinal enteritis that occurs in some fish species from prolonged use of feeds containing over 30% soybean meal (Rumsey, Siwicki, Anderson & Bowser 1994; Krogdahl, Bakke-McKellep & Baeverfjord 2003). Soybean meal contains only 48% crude protein, much lower than fishmeal or plant protein concentrates, such as soy protein concentrate (~ 75% crude protein) or wheat gluten meal (~ 75–80% crude protein). The relatively low protein content of soybean meal restricts its use in high-energy diets because there is little room in formulations for ingredients that are not somewhat purified. The same holds true for distiller's dried grains with soluble (DDGS). Conventional DDGS contains 28–32% crude protein, insufficient to be considered a protein concentrate. New technologies are being used to remove fiber from DDGS, thus increasing its protein content to 40% or more. This approach makes high-protein DDGS a suitable ingredient for use in feeds for omnivorous fish species but not for carnivorous fish species requiring high-protein or high-energy feeds for optimum growth and health.

The most promising alternate protein sources to use in aquafeeds are high-protein concentrates produced from soy, wheat and other grains or oilseeds. Soy protein concentrate does not cause intestinal enteritis in salmonids and can replace up to 75% of fishmeal in feeds for salmonid species (Kaushik, Cravedi, Lalles, Sumpter, Fauconneau & Laroche 1995; Stickney, Hardy, Koch, Harrold, Seawright & Massee 1996; Refstie, Korsoen, Storebakken, Baeverfjord, Lein & Roem 2000; Storebakken, Refstie & Ruyter 2000; Refstie, Storebakken, Baeverfjord & Roem 2001). Worldwide, about 500 000 mt of soy protein concentrate is made, and about 70% is used in human food applications; the balance is used in pet foods and milk replacers for calves and piglets. Production could easily double to meet current and expected demand, but even at this level of production, the quantities would be insufficient to meet the expected demand in aquafeeds for 1.5–2.0 mmt of fishmeal substitution by 2015. However, ethanol production in the United States had the unexpected effect of reducing the acreage of soybean plantings, as farmers switched from planting soybeans to planting corn. Thus, emphasis on ethanol production from corn lowered US soybean production. Increased production from Brazil and Argentina made up some of the shortfall in US production. Wheat and rapeseed are the other main crops which are produced in sufficient quantity to be potential sources of protein concentrates for use in aquafeeds. Rapeseed is produced for its oil, leaving the protein-rich residue available for other uses. Rapeseed/canola protein concentrates have been evaluated as fishmeal substitutes with relatively good results, providing that measures are taken to enhance feed palatability and minimize the effects of glucosinolates which affect thyroid function (Higgs, McBride, Markert, Dosanjh & Plotnikoff 1982). Wheat protein concentrate is already widely produced and sold as wheat gluten meal, but nearly all of current production is used in human food applications.

The third challenge facing the aquafeed industry as it moves higher substitution of fishmeal with plant proteins pertains to speculative and unknown nutritional limitations of plant proteins compared with fishmeal. Fishmeal is a complicated product containing essential nutrients as well as a large number of compounds that are biologically active. Feed formulators blend plant protein concentrates and supplement amino acids to ensure that the amino acid content of feeds in which fishmeal levels are reduced meets or exceeds the amino acid requirements of farmed fish. They may also supplement feeds with mineral supplements such as dicalcium phosphate or double the trace mineral premix to boost feed calcium, phosphorus and trace mineral levels when fishmeal is removed from fish feed formulations. However, this may not be enough to overcome other deficiencies or imbalances that arise when fishmeal levels are lowered in feeds. This challenge is similar to that facing the poultry feed industry 20–30 years ago. At that time, a small percentage of fishmeal was routinely added to poultry feeds; without it, growth performance was reduced. Fishmeal was said to contain unidentified growth factors that were necessary for optimum growth and efficiency. Over time, researchers identified a number of dietary constituents that were supplemented into poultry feeds, allowing formulators to lower and finally eliminate fishmeal as a feed ingredient. The unidentified growth factors were primarily trace and ultra-trace elements. While the situation in aquafeeds in analogous, it is not identical because the unidentified growth factors required for fish are less likely to be trace elements and more likely to be amines, such as taurine, and possibly steroids.

Imbalances in macro and trace minerals cannot, however, be eliminated as nutritional concerns in all-plant feeds. Fishmeal is rich in macro and trace elements, in contrast to plant proteins. Research is needed to identify optimum levels of required minerals and to demonstrate potential antagonistic interactions among ingredients that lower mineral bioavailability. Research is also needed to identify and test 'semi-essential' nutrients and other biologically active materials in fishmeal.

The fourth challenge associated with replacing fishmeal with plant protein concentrates is associated with anti-nutritional compounds in plant proteins. Plant protein concentrates present a mixed picture concerning anti-nutrients (Francis, Makkar & Becker 2001). Proteins produced from oilseeds, in general, contain more anti-nutrients of concern for fish than do proteins produced from grains. However, many are destroyed or inactivated by processes involved with product manufacture or during extrusion pelleting. For example, soybean meal contains compounds that cause distal enteritis in the intestinal of salmonids. However, soy protein concentrate does not cause intestinal enteritis in salmonids. The factor(s) in soybean meal responsible for enteritis is evidently removed or deactivated during the processing involved with extracting carbohydrates from soybean meal to make soy protein concentrate or soy isolates.

Other anti-nutrients in plant proteins of concern in fish nutrition are not destroyed by processing or pelleting and therefore must be mitigated by supplementation. Anti-nutrients in this category include phytic acid glucosinolates, saponins, tannins, soluble non-starch polysaccharides and gossypol. Phytic acid (myo-inositol hexakis dihydrogen phosphate) is a six-carbon sugar which contains six phosphate groups, and is the storage form of phosphorus in seeds. The phosphorus in phytic acid is not available to monogastric animals, such as humans or fish, and passes through the gastro-intestinal tract. In fish farms, this can enrich ponds or rivers into which farm effluent water is discharged, contributing to eutrophication. Phytic acid also ties up divalent cations under certain conditions, making them unavailable to fish. Thus, fish can become deficient in essential minerals, especially zinc, when the phytic acid level in feeds is high, unless the diet is fortified with extra zinc. Phytic acid is present in all plant protein ingredients, and is much higher in protein concentrates, such as soy protein concentrate, than in soybeans or soybean meal. Glucosinolates are present in rapeseed (canola) products and interfere with thyroid function by inhibiting the organic binding of iodine. Their effects on fish cannot be overcome by supplementing iodine to the diet, but they can be overcome by dietary supplementation with triiodothyronine (Higgs *et al*. 1982). Saponins are found in soybean meal and are reported to lower feed intake in salmonids (Bureau, Harris & Cho 1996, 1998). Gossypol is a constituent of cottonseed meal that is well known to cause reproductive problems in livestock and fish, including reduced growth and low haematocrit (Hendricks 2002). Non-starch polysaccharides are not toxins, but they are poorly digested by fish and may interfere with uptake of proteins and lipids. Supplementing feeds with exogenous enzymes reduces this problem but may cause another by the breakdown products from non-starch polysaccharides, namely galaxies and xylems, are poorly tolerated by fish (Stone 2003).

Phytoestrogens are another constituent of some plant proteins that may be problematic in fish feeds, although this is not clearly established. Phytoestrogens commonly detected in fish feeds are genistein, formononetin, equol and coumestrol (Matsumoto, Kobayashi, Moriwaki, Kawai & Watabe 2004). The effects of phytoestrogens in fish feeds are more likely to affect male reproduction than that of females (Inudo, Ishibashi, Matsumura, Matsuoka, Mori, Taniyama Kadokami, Koga, Shinohara, Hutchinson, Iguchi & Arizona 2004), but some evidence suggests that exposure to dietary phytoestrogens at the fry stage when sexual differentiation occurs may alter sex ratio (Green & Kelly 2008).

The final challenge associated with replacing fishmeal with plant proteins is the potential to increase the effects of aquaculture on the aquatic environment. As mentioned above, most plant protein ingredients contain non-protein fractions that are poorly digested, such as phytic acid, non-soluble carbohydrates and fibre. These materials pass through the digestive tract of fish and are excreted as feces. In freshwater farming systems, these materials may stay in ponds or be discharged into streams or rivers in flow-through farming systems. In the marine environment, they pass through pens into surrounding waters. Nutritional strategies must be developed to minimize this potential problem, along the lines of strategies developed to lower phosphorus discharges from freshwater fish farms (Gatlin III & Hardy 2002).

Summary

As research findings that allow higher levels of plant proteins to be substituted for fishmeal in aquafeeds to

be made, new challenges are likely to emerge. These challenges may be related to the effects of replacing fishmeal in aquafeeds on product quality, environmental impacts of aquaculture or the economics of production. Each of these challenges could affect the rate at which the aquafeed industry moves towards the use of more sustainable aquafeeds that contain less and less fishmeal. At present, fishmeal remains the primary protein source in aquafeeds for marine species and others at the fry or fingerling stages. Fishmeal now shares the role as primary protein source in feeds for salmon and trout, and is only a minor protein source in grow-out feeds for omnivorous fish species. Depending on research findings and economics, in the near future fishmeal will no longer be the primary protein source in aquafeeds for carnivorous fish species, but rather be a specialty ingredient added to enhance palatability, balance dietary amino acids, supply other essential nutrients and biologically active compounds or enhance product quality.

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