

Proteomic sensitivity to dietary manipulations in rainbow trout

S.A.M. Martin^{a,*}, O. Vilhelmsson^a, F. Médale^b, P. Watt^c, S. Kaushik^b, D.F. Houlihan^a

^aDepartment of Zoology, School for Biological Sciences, Tillydrone Avenue, University of Aberdeen, Aberdeen, Scotland AB24 2TZ, UK

^bFish Nutrition Laboratory, INRA-IFREMER 64310, Saint Pée sur Nivelle, France

^cDivision of Molecular Physiology, University of Dundee, Dundee, Scotland, UK

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Abstract

Changes in dietary protein sources due to substitution of fish meal by other protein sources can have metabolic consequences in farmed fish. A proteomics approach was used to study the protein profiles of livers of rainbow trout that have been fed two diets containing different proportions of plant ingredients. Both diets control (C) and soy (S) contained fish meal and plant ingredients and synthetic amino acids, but diet S had a greater proportion of soybean meal. A feeding trial was performed for 12 weeks at the end of which, growth and protein metabolism parameters were measured. Protein growth rates were not different in fish fed different diets; however, protein consumption and protein synthesis rates were higher in the fish fed the diet S. Fish fed diet S had lower efficiency of retention of synthesised protein. Ammonia excretion was increased as well as the activities of hepatic glutamate dehydrogenase and aspartate amino transferase (ASAT). No differences were found in free amino acid pools in either liver or muscle between diets. Protein extraction followed by high-resolution two-dimensional electrophoresis, coupled with gel image analysis, allowed identification and expression of hundreds of protein. Individual proteins of interest were then subjected to further analysis leading to protein identification by trypsin digest fingerprinting. During this study, ~ 800 liver proteins were analysed for expression pattern, of which 33 were found to be differentially expressed between diets C and S. Seventeen proteins were positively identified after database searching. Proteins were identified from diverse metabolic pathways, demonstrating the complex nature of gene expression responses to dietary manipulation revealed by proteomic characterisation.

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1. Introduction

Because intensive aquaculture heavily relies upon large inputs of ingredients such as fish meal and fish oil drawn from wild fish as protein and fatty acid sources in the feeds, the sustainability and impact of such activities on world fisheries is questioned [1]. The replacement of fish meal as the major protein source by proteins of plant origin is a major objective for the sustainable development of aquaculture. Progress has been made on the replacement of fish meal with a number of different ingredients, including soybean, lupin, peas and sunflower [2–6]. There are two factors that need to be addressed if successful replacement fish meal is to be achieved: firstly, the amino acid composition of the diets and secondly, the effect of a wide variety

of antinutritional substances that can be present to varying degrees in plant products [7,8].

Most teleost fish species are adapted to use protein as a preferred energy source over carbohydrate and thus require high levels of dietary protein (30–60%) [9]. The essential amino acid requirements of fish correlate well with the amino acid composition of the whole animal and to a certain extent that of the muscle tissue alone [10,11]. Because the amino acid profiles of plant proteins do not meet the essential amino acid requirements of fish [12], supplementation with synthetic amino acids can improve growth and protein utilisation, but only to a limited extent [13,14].

Plant ingredients contain different antinutritional factors (ANFs) of different nature and at different concentrations having adverse effects in fish [8,12,15]. In salmonids, some recent studies have questioned whether the poor performance of fish fed high levels of soybean protein might be related to the presence of some or all heat-stable ANFs [16,17], and existing data suggest that soybean should only be used as a partial replacement for fish meal.

* Corresponding author. Tel.: +44-1224-272867; fax: +44-1224-272396.

E-mail address: sam.martin@abdn.ac.uk (S.A.M. Martin).

However, rainbow trout fed different soy protein products had comparable growth rates between fish fed 100% fish meal diet and those fed a diet containing 100% soy protein concentrate (SPC) [4], having relatively low concentrations of ANFs, notably phytoestrogens. In a subsequent study [14], inclusion of SCP replacing fish meal at levels above 50% led to poor growth, possibly due to high levels of phytoestrogens in the raw material. With these diets, it was also reported that supplementation with amino acids improved protein utilisation by decreasing N excretion rates [18].

We studied parameters of nitrogen metabolism and protein utilisation in groups of rainbow trout fed on diets containing high levels of plant-derived protein, adequately supplemented with amino acids, meeting the protein amino acid profile of fish. Both diets were formulated with practical ingredients that are commonly used in aquaculture fish feeds. One diet (diet S) had high levels of soybean meal believed to induce adverse effects on different metabolic pathways. Parameters of protein metabolism were measured including activities of key enzymes in amino acid oxidation, nitrogen excretion and whole animal protein synthesis. We also used proteomics to identify proteins that are expressed in a differential manner in the liver of fish fed the different diets, to gain insight on the biochemical processes occurring in the liver as a result of altered metabolism due to the diets.

2. Methods

2.1. Fish husbandry and growth

Rainbow trout were maintained at an experimental freshwater fish farm belonging to INRA (Donzacq, Landes, France) under natural photoperiod in a flow-through system. Water was supplied from natural springs at a constant temperature of 17 ± 1 °C. Six groups of 75 fish (initial mean body weight: 14.1 g) were reared in 1 m³ circular glass fibre tanks. Each diet was distributed by hand to triplicate tanks of fish; the fish were fed twice a day to satiation. The feeding trial lasted for 12 weeks (from March 26 to June 15, 2001). Every 3 weeks, fish were weighed after a 24-h starvation period. The diet composition is shown in Table 1. All diets met the amino acid requirements of rainbow trout.

2.2. Enzymes of amino acid catabolism

Six fish from each dietary treatment were sampled at the end of the trial after overnight fasting. Whole liver and a piece of white muscle samples were withdrawn for measurements of the activities of enzymes of amino acid catabolism and for free amino acid composition, respectively. All the tissues were frozen in liquid nitrogen and kept at -80 °C until analysis.

Table 1

Composition of the experimental diets used for rearing rainbow trout

Ingredients (g/kg)	Diet S	Diet C
Fish meal (CP 70%)	389.9	316.4
Wheat gluten (Amylum, Holland)	71.4	0
Extruded whole wheat	135.7	71.8
Extruded peas (Aquatex, France)	215.1	56.8
Soybean meal (CP 42%)	25.3	331.3
Fish oil	101.6	109.5
Binder, Na alginate	10.0	10.0
Mineral mix ^a	10.0	10.0
Vitamin mix ^a	10.0	10.0
CaHPO ₄ ·2H ₂ O (18%)	10.9	16.1
L-amino acid mixture	20.1	68.2
<i>Proximate composition</i>		
Dry matter (DM, %)	90.5	92.2
Proteins (% DM)	46.3	45.1
Lipids (% DM)	16.4	15.6
Gross energy (kJ/g DM)	22.1	22.4

CP: crude protein.

^a According to Ref. [13].

For the determination of enzyme activities, livers were homogenised in 10 volumes of ice-cold buffer (Hepes 30 mM, saccharose 0.25 mM, EDTA 0.5 mM, K₂HPO₄ 5 mM, dithiothreitol 1 mM; pH 7.2). Homogenates were centrifuged at $1000 \times g$ at 4 °C for 10 min. Because glutamate dehydrogenase (GDH EC 1.4.1.3) is a mitochondrial enzyme, supernatants were treated by ultrasounds (100 W, 1 min) to break the mitochondria membranes before the measurements of activity. They were centrifuged at $15,000 \times g$ at 4 °C for 10 min. GDH activity was measured by following the formation of NADH for 10 min after addition of L-glutamic acid to the reaction mixture (Tris 175 mM, semicarbazine 100 mM, NAD 1.1 mM, ADP 1 mM, L-leucine 5 mM; pH 8.5). Activities of alanine amino transferase (ALAT, EC 2.6.1.2) and aspartate amino transferase (ASAT, EC 2.6.1.1) were measured on supernatants obtained after centrifugation at $10,000 \times g$ at 4 °C for 20 min using specific kits (Enzyline, Biomerieux). Soluble protein contents of supernatants were determined by the method of Bradford [19].

2.3. Free amino acid analysis

The amino acid composition of liver and muscle tissue free pools were measured using the method previously described [20,21]. Briefly, tissue free amino acids were extracted from 200 mg tissue by homogenising in 4 ml absolute ethanol in the presence on 50 µM norleucine as an internal standard, samples were made to 5 ml with water and bound and free amino acids separated by centrifugation at $6000 \times g$ for 10 min. Amino acids were detected using an AA analyzer (Applied Biosystems). Results were calculated as micromoles per gram wet weight. Free amino acids were calculated for both muscle and liver for fish fed the different diets.

2.4. Nitrogen metabolism and protein synthesis rates

Twelve rainbow trout from each tank were transferred to smaller tanks (60 l volume) for the study on N metabolism. Hourly ammonia excretion rates were measured following a test meal during two 24-h cycles. Water samples were analysed for nitrogen (N)–ammonia content immediately after collection using the colorimetric method [22]. Measurements of protein synthesis and protein turnover using ^{15}N were performed. For protein synthesis determination, fish were fed a single meal at normal feeding time 09:30–10:30 h consisting of the respective diet enriched with 0.5% ^{15}N -labeled *Spirulina* protein biomass (Martek Biosciences, Columbia, MD, USA). The enrichment of the proteins was >98% ^{15}N . Food consumption was accurately measured by feeding by hand until visual satiety and fish stopped eating. Fish were allowed to settle for 1 h after which the water was thoroughly changed, brought to a 50-l volume and water flow stopped. Oxygen levels were maintained within the normal range for rainbow trout by aeration with supplementary oxygen.

Water samples (4 l) were collected at 6 h following the meal from each experimental tank, the pH of the sampled water was reduced to below pH 2 by addition of HCl. A subsample of 50 ml was taken for determination of ammonia [22] and urea [23] by colorimetric assay. After sampling, the water volume was reduced to 20 l and water flow restored for 10 min to ensure complete change of water. The volume was then returned to 50 l and further samples taken in the same manner at 12, 24, 36 and 48 h following the meal. Samples were maintained at 4 °C until further process. During these time intervals, the nitrogen excretory products that accumulated were measured by colorimetric assays, cumulative N excretion over the experimental period was calculated. Recovery of ^{15}N was achieved by distillation of ammonia into boric acid, the ammonium borate was freeze dried and enrichment of the sample with ^{15}N compared to background ^{15}N determined by mass spectrometry. Full details of methodology are given in Carter et al. [24]

Table 2

Dry matter, crude protein and gross energy intake, growth performance and feed efficiency of the two experimental diets

	Diet C	Diet S	P
Feed intake (g or kJ/kg ABW/day)			
Dry matter	18.6 ± 0.8	20.3 ± 0.5	0.04
Crude proteins	8.4 ± 0.4	9.4 ± 0.2	0.02
Gross energy	416.9 ± 18.9	447.1 ± 11.1	0.08
Initial mean body weight (g)	14.1 ± 0.04	14.1 ± 0.03	
Final mean body weight (g)	90.6 ± 5.0	83.5 ± 4.5	0.14
Specific growth rate (% day ⁻¹)	2.22 ± 0.06	2.12 ± 0.07	0.13
Feed efficiency	0.91 ± 0.03a	0.81 ± 0.04b	0.02
Protein efficiency	2.02 ± 0.07a	1.76 ± 0.08b	0.01

Data are means ($n=3$) ± S.D.

ABW = average body weight.

Feed efficiency = wet weight gain/dry feed intake; Protein efficiency = wet weight gain/crude protein intake. Specific growth rate = $(\text{Ln}(\text{final weight}) - \text{Ln}(\text{initial weight}))/84 \text{ days} \times 100$.

Table 3

Specific activities of enzymes involved in amino acid catabolism in trout fed the two different diets ($n=6$)

	Diet		P
	C	S	
<i>Alanine amino transferase (ALAT)</i>			
IU/g liver	42.1 ± 4.4	54.7 ± 10.2	0.02
IU/g protein	580.3 ± 99.8	668.1 ± 150.6	0.26
<i>Glutamate dehydrogenase (GDH)</i>			
IU/g liver	6.96 ± 1.82	9.22 ± 1.37	0.03
IU/g protein	94.2 ± 18.6	112.3 ± 18.2	0.12
<i>Aspartate amino transferase (ASAT)</i>			
IU/g liver	46.2 ± 7.4	70.6 ± 15.6	0.006
IU/g protein	637.4 ± 131.5	853.7 ± 159.4	0.03

and Fraser et al. [25]. The ammonium borate was frozen and freeze dried (Edwards Super Modulyo Freeze Drier, Edwards, Crawley, UK). Duplicate 25 mg samples of freeze dried ammonium borate were packed in tin capsules (Europa Scientific Ltd., Crewe, UK) for enrichment analysis on an ANC Roboprep-CN linked to a tracer mass isotope ratio mass spectrometer (Europa Scientific Ltd.). The ^{15}N enrichment (atom percent excess, APE) in the samples was known by running samples of known nitrogen enrichment alongside the experimental samples. The enrichment of the experimental diets was diet C, 0.578 APE; diet S, 0.629 APE. The ^{15}N enrichment of ammonia was used to measure the whole animal protein synthesis rates using the end point stochastic model of Waterlow et al. [26] as applied to fish [24,25]. Fractional protein synthesis rates were determined

Table 4

Free amino acids for rainbow trout liver and muscle tissue

Amino acid	Liver		Muscle	
	C	S	C	S
Alanine	5.00 ± 0.21	5.45 ± 0.21	1.20 ± 0.09	1.3 ± 0.16
Arginine	0.50 ± 0.02	0.57 ± 0.03	10.37 ± 0.36	10.26 ± 0.55
Asparagine	0.41 ± 0.06	0.39 ± 0.04	0.83 ± 0.05	0.72 ± 0.09
Aspartic acid	0.88 ± 0.06	1.07 ± 0.09	0.02 ± 0.01	0.07 ± 0.01
Glutamic acid	5.17 ± 0.34	5.24 ± 0.42	0.35 ± 0.03	0.29 ± 0.02
Glutamine	1.81 ± 0.16	1.50 ± 0.09	0.31 ± 0.03	0.20 ± 0.03
Glycine	1.61 ± 0.09	1.45 ± 0.09	10.10 ± 0.80	7.33 ± 1.29
Histidine	1.12 ± 0.06	0.99 ± 0.04	7.20 ± 0.70	5.37 ± 0.45
Hydroxyproline	0.17 ± 0.02	0.17 ± 0.02	0.46 ± 0.06	0.38 ± 0.04
Isoleucine	0.15 ± 0.02	0.17 ± 0.02	0.08 ± 0.02	0.08 ± 0.01
Leucine	0.27 ± 0.03	0.29 ± 0.03	0.16 ± 0.02	0.15 ± 0.01
Lysine	0.42 ± 0.03	0.54 ± 0.04	0.48 ± 0.07	0.64 ± 0.11
Methionine	0.07 ± 0.01	0.04 ± 0.01	<0.01	<0.01
Phenylalanine	0.14 ± 0.01	0.15 ± 0.01	0.10 ± 0.01	0.12 ± 0.01
Proline	0.71 ± 0.03	0.78 ± 0.05	0.52 ± 0.08	0.47 ± 0.03
Serine	0.56 ± 0.03	0.57 ± 0.04	0.42 ± 0.13	0.40 ± 0.10
Taurine	19.26 ± 0.68	18.21 ± 0.66	2.23 ± 0.36	3.28 ± 0.76
Threonine	0.82 ± 0.11	0.86 ± 0.06	0.54 ± 0.06	0.56 ± 0.07
Tryptophan	0.01 ± 0.01	0.01 ± 0.01	nd	nd
Tyrosine	0.23 ± 0.02	0.27 ± 0.04	0.08 ± 0.01	0.11 ± 0.01
Valine	0.44 ± 0.04	0.41 ± 0.03	0.82 ± 0.05	0.80 ± 0.04
Total	36.29 ± 2.94	32.55 ± 2.28	39.75 ± 0.69	38.89 ± 1.07

Amino acids are in micromoles per gram wet tissue (± S.E.).

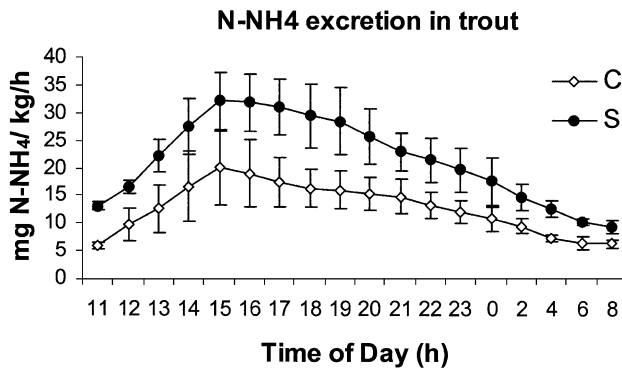


Fig. 1. Post-prandial ammonia excretion in fish fed the experimental diets. Each value is the mean \pm S.E. following a single meal at 10.00. Water samples were collected every 2 h over a 24-h cycle.

(i.e., proportion of protein mass synthesised per day as a percentage; k_s , % day $^{-1}$).

2.5. Protein extraction

Protein extraction was performed as described by Martin et al. [27]; briefly, frozen tissue was homogenised in lysis buffer (9 M urea, 2% (w/v) CHAPS, 25 mM Tris-HCl pH 7.5, 3 mM EDTA, 50 mM KCl, 50 mM DTT, 2% (w/v) Resolyte™ [Merck], 40 μ M leupeptin) at room temperature, using a Dounce teflon homogeniser. Follow-

ing homogenisation, the tissue lysates were centrifuged at 50,000 \times g for 20 min at 15 °C to remove any insoluble particles. The supernatant was then stored at -70 °C until gel electrophoresis was performed. Soluble trout liver proteins (15 μ l) were mixed with 115 μ l re-swelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.3% DTT), and then added to a 7-cm pH 4–7 immobilised pH Gradient strip (IPG) (Amersham-Pharmacia Biotech). Isoelectric focussing was performed in three stages with a ramped voltage change between each step, 200 V for 1 min, 3500 V for 1 h 30 min, 3500 V for 1 h 30 min (all stages at 2 mA and 5 W). For the second dimension electrophoresis, the IPG strip was laid onto a 10–15% gradient polyacrylamide slab gel (8 \times 7 cm) and the proteins electrophoresed at 150 V for 450 V h [28]. The resolved proteins were detected using colloidal coomassie blue G250 staining [29]. Molecular masses of the proteins were determined by coelectrophoresis with standard protein markers. Isoelectric points were determined based on the linearity of the IPG strip.

2.6. Analysis of 2D gels

The gels were scanned at a resolution of 200 dpi using a Hewlett Packard Scanjet 3p flat bed scanner and stored as TIF files. Subsequent analysis of the gel images was performed using the software package *Phoretix 2-D ver-*

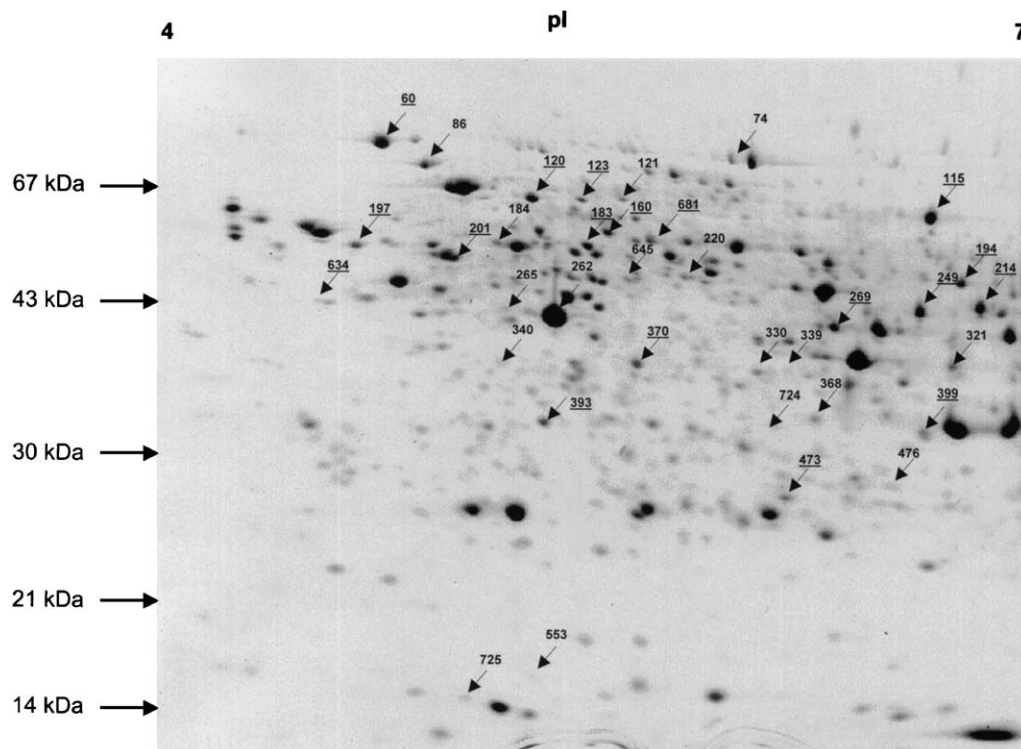


Fig. 2. Two-dimensional gel of rainbow trout liver proteins (fish S3). A total liver protein extract was separated by charge between pI 4 and 7, second dimension was by size on a gradient 10–15% gel. The proteins were located by staining with colloidal coomassie blue G250. Proteins marked by arrows were found to be differentially expressed as a result of dietary manipulation, the corresponding number is the spot reference number. Underlined protein numbers were positively identified by trypsin digest fingerprinting.

sion 5.01 (NonLinear Dynamics, Gateshead, UK). Protein spots were detected using automated routines from the software combined with manual editing to remove artefacts. All protein spots were assigned a spot number, from a reference gel described by Martin et al. [27], *pI* and molecular weights were assigned to all spots. Individual protein spot abundance was determined by the area of the spot multiplied by the density and referred to as the volume. Background was removed and the spot volumes were normalised to the total protein detected on each gel. The normalised spot volume is described as the abundance of a particular protein spot. Three replicate gels were used for each treatment group. Proteins that were found to vary in abundance between the diets were analysed for significance using a Student's *t* test.

2.7. Protein identification by peptide mass mapping

Proteins of interest were excised from the stained gel and subjected to in-gel trypsin digestion [30]. Excised spots were washed, reduced, S-alkylated and digested within the gel using trypsin (sequencing grade modified trypsin; Promega) as described elsewhere [31,32]. An aliquot of the peptide extract produced by this process was passed through a GELoader tip containing a small volume of POROS R2 sorbent (PerSeptive BioSystems, USA) [32]. The adsorbed peptides were eluted in 0.5 μ l saturated solution of α -cyanol-4-hydroxycinnamic acid in 50% acetonitrile/5% formic acid. The mass spectra of the peptide fragments were obtained on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. The instrument was operated in the reflection delayed extraction mode. Spectra were internally calibrated using trypsin auto-digestion products.

For protein identification, trypsin peptide masses were used to search the National Centre for Biotechnology Information (NCBI) non-redundant sequences database using the MASCOT program [33]. The mascot search parameters were as follows: peptide mass accuracy was 50 ppm; protein modifications: cysteine as S-carbamidomethyl derivative and oxidation of methionine allowed. To utilise the EST nucleotide sequences now available for salmonid fish (108,000 sequences), the trypsin digest products were searched in a database containing all salmonid cDNA sequences available (as of 20th Jan 2003) using the Protein-prospector program MS-Fit [34]. This program allowed identities to be made with non-full-length cDNAs from both rainbow trout and Atlantic salmon. Parameters for searching MS-fit were: all six frames to be searched: cysteine as S-carbamidomethyl derivative and oxidation of methionine allowed.

2.8. Gene expression studies

Total RNA isolated from liver and muscle was used as a template for first strand cDNA synthesis, RNA was

reverse transcribed using oligo dT₁₇ primer (100 ng μ l⁻¹) [27]. This was diluted 5-fold to 100 μ l and 2 μ l used as the template for PCR using primers designed against the rainbow trout genes of interest. Primers were β -actin: (AF1 5' ATGGAAGATGAAATCGCC 3', AR1 5' TGCCAGATCTTCTCCATG 3'), aldolase B (ABF1 5' ATGACTACCCAGTTCCCATC 3', ABR1 5' AATG-AATGCAGTTGGGGTCAACAGC 3') and apolipoprotein A I-1 (Apo A I-1) (APF1 5' ATGAAATTCCTGGCTCTCGC 3', APR1 5' GTCAACTGGGGAGCCGAAGGC 3'). PCR conditions were 25 cycles 94 °C, 1 min, 55 °C, 1 min, 72 °C 1 min for all primer sets. Preliminary experiments had shown under these conditions that the amplification had not reached plateau levels and thus could be used for expression analysis. Twenty microlitres of PCR product was separated on 2% agarose gels. The PCR products were normalised to the signal obtained

Table 5
Protein spots that were found to be significantly different in abundance between the diets

Spot	<i>pI</i>	kDa	Diet		<i>P</i>	Change
			C	S		
60	5.1	78.9	1811.6 ± 131.5	464.8 ± 89.6	0.001	C
74	6.2	77.7	331.1 ± 27.2	84.5 ± 22.0	0.002	C
86	5.2	73.8	765.4 ± 62.8	451 ± 40.6	0.014	C
115	6.8	64.3	1287.3 ± 133.5	323.2 ± 118.8	0.006	C
120	5.5	64.2	929.4 ± 117.9	39.3 ± 16.1	0.002	C
121	5.8	64.5	243.9 ± 29.7	97.1 ± 19.6	0.015	C
123	5.7	64.2	246.9 ± 54.3	14.9 ± 4.0	0.013	C
160	5.8	57.2	378.9 ± 86.2	83.2 ± 17.2	0.028	C
183	5.7	55.1	524.9 ± 51.9	240.8 ± 47.0	0.015	C
184	5.4	55.1	185.7 ± 34.1	28.5 ± 3.7	0.038	C
194	6.9	53.9	614.6 ± 35.4	270.3 ± 44.9	0.004	C
201	5.3	52.9	259.7 ± 22.4	147.3 ± 16.5	0.016	C
220	6.1	51.2	231.3 ± 23.3	84.6 ± 37.6	0.03	C
214	6.9	48.7	804.2 ± 191.3	85.0 ± 26.8	0.02	C
249	6.8	47.9	854.2 ± 49.7	375.2 ± 56.7	0.003	C
269	6.5	46.0	506.4 ± 45.3	232.5 ± 29.7	0.007	C
321	6.9	40.6	234.3 ± 23.7	106.3 ± 11.2	0.008	C
393	5.6	34.1	457.9 ± 90.1	97.7 ± 38.5	0.021	C
473	6.4	27.4	213.1 ± 39.9	27.7 ± 1.7	0.01	C
681	6	56.0	277.9 ± 51.3	35.0 ± 20.4	0.012	C
197	5.0	53.5	1113.0 ± 459.8	3231.9 ± 188.8	0.013	S
265	5.5	46.2	31.9 ± 7.5	230.0 ± 63.9	0.04	S
330	6.3	40.2	463.7 ± 243.4	2128.8 ± 122.8	0.004	S
339	6.4	39.9	97.4 ± 41.5	262.0 ± 43.2	0.050	S
340	5.4	39.6	82.2 ± 30.4	249.4 ± 53.4	0.050	S
370	5.7	37.0	95.0 ± 61.3	531.2 ± 35.9	0.004	S
399	6.8	33.2	583.3 ± 244.9	1387.1 ± 150.0	0.049	S
476	6.7	27.3	45.3 ± 20.5	280.0 ± 51.5	0.013	S
553	5.5	16.2	62.7 ± 47.0	366.2 ± 72.8	0.025	S
634	4.8	46.0	121.6 ± 69.7	407.9 ± 36.9	0.037	S
645	5.9	50.8	51.4 ± 35.0	316.1 ± 28.4	0.004	S
724	6.4	33.1	145.9 ± 104.5	614.0 ± 64.3	0.019	S
725	5.2	16.1	41.5 ± 13.0	245.5 ± 57.2	0.025	S

The measured charge (*pI*), molecular weight (kDa) and change are shown (C indicates increase in fish fed C diet, S indicates increase in fish fed diet S). The mean normalised protein abundance (\pm S.E.) is shown for each diet. Data were analysed by Student's *t* test.

Table 6
Results from peptide mass fingerprinting of protein spots excised from the 2DE gels

Reference spot	pI	kDa	Identities by MS-Fit followed by BLASTx				Identities by Mascot				
			Salmonid sequence	MS-Fit Mowse score	Protein	Species identified	Accession no.	Protein	Species identified	Accession no.	Mascot score
60 ^C	4.9	85	BG933954	1.4×10^{-4}	HSP108	<i>Salmo salar</i>	AF387865	HSP108	<i>Gallus gallus</i>	AF387865	201
115 ^C	6.8	67	CA343417	1.6×10^5	Transketolase	<i>Xenopus laevis</i>	AAF67194	N-ethylmaleimide-sensitive factor	<i>Homo sapiens</i>	15314649	82
120 ^C	5.5	66	BX081803	2.3×10^5	HSP70	<i>Oncorhynchus mykiss</i>	P08108	HSP70	<i>Oncorhynchus mykiss</i>		108
123 ^C	5.6	66	CA044261	4.6×10^4	HSP70	<i>Oncorhynchus mykiss</i>	P08108	HSP70	<i>Xiphophorus maculatus</i>		115
160 ^C	5.7	59	AJ295231	1.3×10^4	Nitric oxide synthase	<i>Oncorhynchus mykiss</i>	CAC82808				
183 ^C	5.7	55	AJ272373	3.0×10^{10}	Simple type II Keratin k8	<i>Oncorhynchus mykiss</i>	CAC45060	Simple type II Keratin k8	<i>Oncorhynchus mykiss</i>	CAC45060	88
194 ^C	6.8	54	CA375586	3.3×10^5	Selenium binding protein 2	<i>Rattus norvegicus</i>	NP_543168	Occludin-like protein	<i>Drosophila melanogaster</i>		81
197 ^S	4.8	53	CA386490	7.5×10^3	HSP108	<i>Xenopus laevis</i>	AAO21339	HSP108	<i>Gallus gallus</i>	AF387865	193
201 ^C	5.2	52	CA350990	8.6×10^7	Beta tubulin	<i>Notothenia coriiceps</i>	AAG15317	Beta tubulin	<i>Haliotis discus</i>		95
214 ^C	6.9	51	BX080834	3.5×10^3	Adenosylhomocysteinase 2	<i>Xenopus laevis</i>	O93477	Adenosylhomocysteinase 2	<i>Xenopus laevis</i>	O93477	85
249 ^C	6.7	47	CA363453	6.6×10^7	Homogentisate 1,2-dioxygenase	<i>Mus musculus</i>	XP_147229	–	–		
269 ^C	6.5	45	BG934321	6.4×10^7	Phosphogluconate dehydrogenase	<i>Homo sapiens</i>	AAH00368	–	–		
321 ^C	6.8	39			–	–	–	–	–		
370 ^S	5.6	36	CA039103	5.0×10^4	Hypothetical ORF	<i>Saccharomyces cerevisiae</i>	NP_014422	Protein Phosphatase 2A catalytic chain	<i>Xenopus laevis</i>		114
393 ^C	5.5	33						Apo A I-1	<i>Oncorhynchus mykiss</i>	AAB96972	148
399 ^{SBM}	6.8	33	AF067796	4.8×10^4	Aldolase B	<i>Salmo salar</i>	AAD11573	Aldolase B	<i>Salmo salar</i>	AAD11573	82
330 ^{SBM}	6.2	30	BG933866	2.5×10^{-3}	–	–	–	–	–		
473 ^{FM}	6.4	28	BX076136	2.5×10^4	Hypoxanthine guanine phosphoribosyl transferase	<i>Gallus gallus</i>	AJ697	Hypoxanthine guanine phosphoribosyl transferase	<i>Homo sapiens</i>		75
485	5.4	25	BX074107	2.8×10^4	Apo A I-1	<i>Oncorhynchus mykiss</i>	AAB96972				
487	5.3	25	CA386629	1.6×10^7	Apo A I-2	<i>Oncorhynchus mykiss</i>	AAB96973	Apo A I-2	<i>Oncorhynchus mykiss</i>	AAB96973	87
553 ^{SBM}	5.4	17			–	–	–	–	–		
634 ^{SBM}	4.8	42			–	–	–	Glucose regulated protein precursor (GRP 78)	<i>Gallus gallus</i>	Q90593	447
681 ^{FM}	5.9	57	CA361952	1.0×10^5	Pyruvate kinase	<i>Takifugu rubripes</i>	BAC02918	–	–		

The superscript following the reference spot number indicates if the spot is increased in abundance after being fed the diet. Using MS-Fit, if unannotated cDNA sequences were identified, this sequence was used to search GenBank using BLASTx to show the protein the cDNA encodes, if a significant hit is obtained. All digests were also searched using Mascot search program. (–) indicates no homology for this protein. ^C and ^S indicate which diet the protein is more abundant.

with the β -actin primers. Confirmation that no genomic DNA was present was shown when PCR products amplified using β -actin primers produced only a single band of 240 bp. The actin primers span an intron and the presence of genomic DNA results in a product of approximately 1.1 kbp. No PCR products this size were obtained for any cDNA samples, indicating absence of genomic DNA in the RNA samples.

2.9. Statistical analysis

Data from nitrogen metabolism parameter, proteome analysis and gene expression analysis were performed by independent Student's *t* test. Database searching for protein identities was performed using Mascot [33] and MS-Fit [34]. BLASTx (NCBI) program was used to identify unannotated salmonid EST sequences.

3. Results

3.1. Growth and food utilization

There were no significant differences in specific growth rates of groups of rainbow trout fed the diets soy (S) and control (C) during the feeding trial (Table 2). However, dry matter and protein intakes of fish given diet S were significantly ($P < 0.05$) greater than in those given diet C, leading to significantly lower feed efficiency and protein efficiency (Table 2).

3.2. Enzymes of amino acid catabolism

Activities of GDH, ALAT and of ASAT, expressed as international units per gram liver, were higher in the liver of trout fed diet S ($P < 0.05$) than in those fed diet C (Table 3).

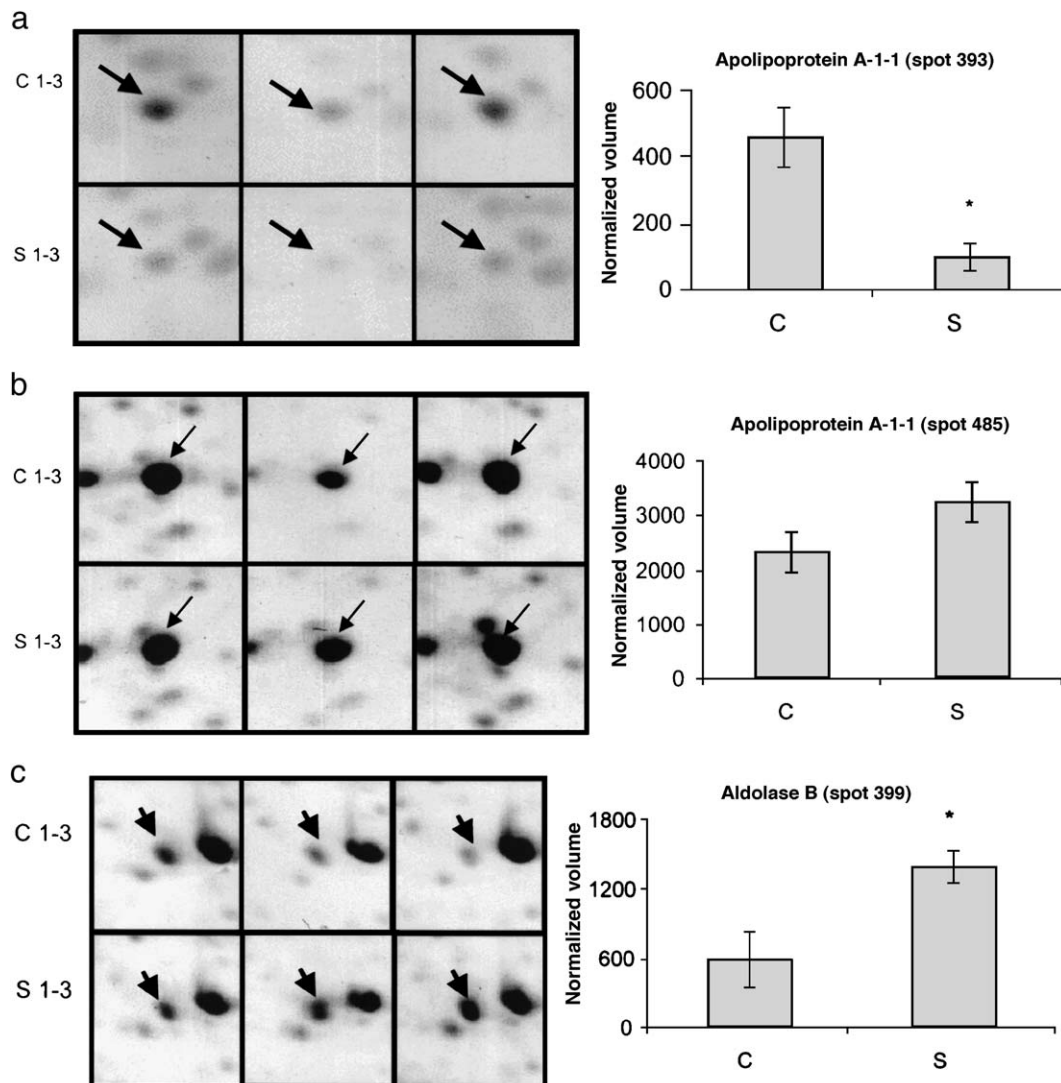


Fig. 3. Changes in abundance of protein spots 393 (a), 485 (b) and 399 (c) in livers of fish fed diets C and S. Total liver protein extracts were prepared from fish fed diet S (S 1–3) and fish fed diet C (C 1–3). Each panel shows an enlarged view of the gel spots from Fig. 2. The positions of spots 393, 485 and 399 are indicated by the arrows.

When the activity was expressed as international units per gram liver, no such differences were found for ALAT and GDH; this is due to differences in soluble protein content between samples.

3.3. Tissue free amino acids

There were no differences between the total free amino acid concentrations between groups in either muscle or liver tissue. There were also no significant differences for individual amino acids. Cysteine was not detected in muscle or liver samples, being below detection levels (Table 4).

3.4. Nitrogen excretion

Ammonia excretion rates reached peak values 5–7 h post-feeding irrespective of diets, suggesting that the rates of dietary protein digestion and amino acids absorption were similar for both diets. The total amount of N–ammonia excretion (mg/kg wet weight) was greater in trout fed the diet S (307.8 ± 70.8) than with fish fed diet C (517.3 ± 71.6) ($P < 0.05$). The cumulative amount of ammonia–N excreted expressed as percentage of N intake was significantly higher in trout fed the diet S $43.7 \pm 0.2\%$ versus $41.9 \pm 0.2\%$ in fish fed diet C ($P < 0.05$). The nitrogen excretion profile for the two diets is shown in Fig. 1.

3.5. Protein synthesis rates

On the day of measurement of protein synthesis rates, food intake represented 7.2 g/kg body weight, with no significant difference between the groups of fish. Urea accounted for a mean of 16.5% of the total nitrogen excretion which was also not significantly different between diets. Protein synthesis rates were calculated based on the fact that the free pool was cleared of ^{15}N amino acids, excretion of ^{15}N was completed by 48 h and that the cumulative excretion of ^{15}N had reached a plateau. Fractional protein synthesis rates were greater ($P < 0.05$) in fish fed diet S (4.47 ± 0.18) than in those fed diet C ($2.36 \pm 0.72\% \text{ day}^{-1}$).

3.6. Detection of differentially expressed proteins

Liver protein extracts from three individual fish for each diet (S 1–3 and C 1–3) were subjected to 2D electrophoresis. The number of protein spots detected on each gel varied from 800 to 1000, and after editing, the total number of spots used for analysis was 750. The abundance of individual spots varied from a maximum of 4.3% to 0.003% of the total protein on the gel. The proteins had molecular weights between 97,400 and 12,400 Da and pI 's between 4 and 7.

The protein profile from fish S3 (Fig. 2) shows a representative example of liver proteins separated by 2DE

and this was chosen as the reference gel for this study; the five other gel profiles were all matched against this reference profile, with each protein assigned a reference number, molecular weight, pI and abundance. Thirty-three protein spots (Table 4) were significantly altered in abundance between the diets ($P < 0.05$), of these 20 were in greater abundance in the fish fed diet C and 13 were greater in fish fed diet S. The proteins that were found to have quantitative changes as result of dietary changes are reported in Table 5.

3.7. Spot identification by mass spectrometry

Twenty-three protein spots were chosen for peptide mass mapping, the abundance of 21 of these proteins was significantly different between dietary treatments. All excised protein spots produced peptide products suitable for database search. Protein identities were found for 19 out of the 23 proteins spots that were excised. Twelve of the proteins were identified by the MS-Fit program with the remaining five identified by the Mascot program. A further two proteins, 485 and 475, are also reported here as they were identified as Apo A I-1 and Apo A I-2. Details of protein homologies for trypsin digest fingerprinting are shown in Table 6. Fig. 3 shows an enlarged area of the six gels and the normalised spot volume for protein spot (393, Fig. 3a, 485, Fig. 3b and 399, Fig. 3c).

3.8. Gene expression analysis

The mRNA expression for aldolase B and Apo A I-1 was analysed by semi-quantitative PCR. The PCR products were

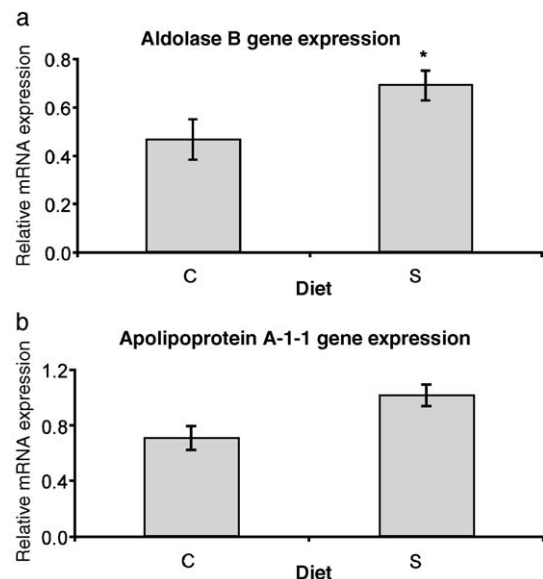


Fig. 4. Relative mRNA expression of Apo A I-1 and aldolase B. PCRs were performed on liver cDNA for five fish fed each diet, the PCR products were separated on 2% agarose gels and stained with ethidium bromide, densitometrical analysis was performed and the Apo A I-1 (a) and aldolase (b) products were normalised to β -actin product.

normalised to β -actin PCR product and the relative mRNA expression is shown (Fig. 4). For Apo A I-1, there was no significant difference in mRNA expression between fish fed the different diets, but the mean relative expression in fish fed diet S was greater than those fed diet C (Fig. 4a). The relative expression of aldolase B is significantly (t test $P < 0.05$) increased in fish fed diet S (Fig. 4b), which correlates with the abundance of the protein spot identified for aldolase B (spot 399). The expression of β -actin did not vary between diets, nor did the abundance of the protein (spot 262, Fig. 2) which was found to be increased in fish fed diet S.

4. Discussion

Growth rates of fish were not altered by the dietary treatments, but protein consumption was greater for fish fed S. We observe that the two diets have different effects on nitrogen metabolism, with changes in protein synthesis rates, and retention of ingested protein. Postprandial patterns of ammonia excretion did not differ between treatments and corresponded to what has been previously described for this species [35,36], but total nitrogen excretion was greater for fish fed diet S. The reduced N retention in the fish fed diet S was a reflection of both increase in N intake and an increase in protein catabolism. In a growing animal, besides the balance between proteins synthesised and protein degradation, the proportion of the synthesised proteins that are maintained as growth is of importance [37,38]. The greater whole animal protein synthesis rates in fish fed diet S than in fish fed diet C indicate greater protein turnover and protein degradation rates in the former. Protein synthesis and retention efficiency are altered by the dietary quality of consumed proteins [39,40]. In addition, activities of GDH and ASAT, two enzymes that are central to amino acid catabolism and indicators of metabolic utilisation of dietary amino acids, were increased in fish fed diet S. A negative correlation has been shown between specific activity of GDH and nitrogen retention [14,41,42]. On the other hand, a relation between activities of the two transaminases (ALAT and ASAT) and dietary protein utilisation has not been established by these authors. We observe here that GDH activity and nitrogen excretion are increased in the fish fed S, similar to that shown by Médale et al. [18].

To further characterize these metabolic changes, proteomic analysis of liver from fish fed the alternative diets was performed. Thirty-two proteins were significantly altered in abundance as a result of the dietary treatment. Twenty-three proteins were excised from gels for identification using peptide mass fingerprinting. Nineteen out of the 23 proteins excised for the gel were positively identified, a larger number than we obtained in a previous study [27] in which 60% of rainbow trout proteins subjected to mass spectrometry were identified. This is also greater than the protein identities obtained by Hogstrand et al. [43] where surface

enhanced laser desorption/ionisation (SELDI) analysis of rainbow trout gill proteins positively identified 12 out of 22 proteins. Since at the time of our previous study [27], the number of sequences has increased dramatically with greater than 108,000 as a result of large-scale EST sequencing efforts for both Atlantic salmon and rainbow trout.

Two structural proteins, keratin II (spot 183) and β -tubulin (spot 201), were down-regulated in fish fed the diet S when compared to those fed the diet C. Although structural proteins such as keratin [44] can have multiple functions, the fact that at least two disparate structural proteins were down-regulated in fish fed diet S indicates a true structural effect. These results may reflect the animals' increased requirement for energy metabolism and, thus, less energy available to synthesis of structural proteins, when fed diet S. Since individual protein levels were normalised with respect to the total protein levels visible on the gels, if a constant level of structural proteins on a per cell basis is assumed, these results can be taken to indicate a lower overall level of non-structural protein expression in fish fed diet C.

Several heat shock proteins (HSPs), including at least two chaperones (HSP70 and HSP78) (spots 120 and 123), were down-regulated in fish fed diet S compared to fish fed diet C. The HSP70 family members are involved in a variety of functions, such as folding of newly synthesised proteins, refolding of incorrectly folded proteins and presenting proteins to the proteasome for degradation [45] and immunoglobulin heavy chain binding activity. Regulation of HSP70 levels in trout hepatocytes has been shown to involve the cytoplasmic glucocorticoid receptor and the proteasome [46]. HSP78 is a homologue of the bacterial protein ClpB which is believed to act in cooperation with the HSP70 system to reactivate aggregated proteins [47]. Recently, however, it has been shown that HSP78 is required for proteolysis of some substrates in yeast mitochondria [48,49], indicating that HSP78 plays an important role in yeast mitochondrial protein turnover.

HSPs are key players in intracellular protein physico-chemistry and may also play an important role in protein turnover. That they are down-regulated in fish fed diet S, rich in soybean meal could potentially have a variety of consequences derived from improper protein maintenance and/or inefficient protein turnover. This may contribute substantially to the fish's ability to grow efficiently.

Another HSP, HSP108 [50], which also has transferrin binding activity [51], was found to be present at high levels in fish fed both diets. However, this protein appears present in at least two isoforms, as a 54- and a 79-kDa protein. In fish fed diet S, the 54-kDa (spot 197) protein was the major species detected, whereas in fish fed diet C, the 79-kDa protein was predominant (spot 60). Members of this protein family are involved in antigen presentation through association with MHC class I molecules [52] and may indicate an immune response in fish fed diet S. Total levels of HSP 108 remained similar in livers of fish fed the two diets.

Several enzymes involved in anabolic metabolism, such as phosphoglucuronate dehydrogenase (spot 269) (a pentose phosphate pathway enzyme), pyruvate kinase (spot 681), adenosylhomocysteinase (spot 214) (involved in biosynthesis of cysteine and phosphatidylcholine) and hypoxanthine–guanine phosphoribosyltransferase (473) (a purine salvage pathway enzyme) were down-regulated in fish fed diet S, rich in soybean meal. This reflects the increased emphases on catabolism relative to anabolism in the fish fed this diet. Decreased activity of the pentose phosphate pathway was observed [53] during starvation experiments in another fish species, the gilthead seabream (*Sparus aurata*). Phosphoglucuronate dehydrogenase is decreased in rainbow trout when there is an absence of carbohydrate in the diet [54], the opposite being the case for sea bream [53]. Pyruvate kinase activity is correlated to glucagon-induced alterations in metabolism [55]. In sea bream, phosphoglucuronate dehydrogenase and pyruvate kinase are both positively correlated with ration size [53].

Transketolase is a thiamine-dependant enzyme that has reduced activity in thiamine-deficient rainbow trout [56] and reflects thiamine deficiency syndrome M74 in Atlantic salmon [57]. It has been suggested that xenobiotics are the driving force behind this thiamine depletion [58]. In our study, fish fed diet S have reduced abundance of transketolase which may reflect dysfunction of thiamine metabolism. Because both diets had adequate vitamin supply, meeting the vitamin requirements of trout [12], whether this possible dysfunction is due to other ANFs present in the soybean meal used here, needs to be analysed. One further protein was identified as a hepatic selenium-binding protein; little is known regarding its physiological role but in rats, this protein has been found to be increased in the presence of aryl hydrocarbons [59].

Several proteins were identified that were more abundant in fish fed the diet S; out of the six spots excised from the gel only three had significant identities, a HSP (spot 197), already described, aldolase B (spot 399) and a protein phosphatase 2A (spot 370). Aldolase B catalyses the reversible cleavage of fructose 1,6-bisphosphate in the glycolytic pathway, forming glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Aldolase B identified here has been cloned and characterised [60] and has been shown to be expressed at high levels in the liver. Aldolase B protein is increased in abundance in the fish fed the diet S. This is most likely a reflection of increased metabolism and general turnover of proteins that is occurring in the fish fed this diet and energy demand in these fish.

The catalytic subunit of serine/threonine protein phosphatase 2A (PP2A) was up-regulated in fish fed soybean-rich diet. PP2A is involved in a wide variety of cellular signaling pathways including those for cell growth. PP2A is a major S/T phosphatase in eukaryotic cells. It is responsible for controlling stimulus-activated protein kinases; among its substrates are protein kinase B, protein kinase C, p70 S6 kinase, calmodulin-dependent kinases, MAP kinases and

cyclin-dependent kinases [61,62]. As it is involved in so many diverse functions, it is not surprising that it is subject to multiple layers of regulation. This regulation includes auto-inhibitory effects on translation of its own mRNA, post-translational modification at its C terminus, protein–protein interactions, among others [62]. That PP2A undergoes post-translational modification suggests that it is likely to be found in more than one 2DE spot.

Two protein spots were identified as apolipoprotein A I-1 (spots 393 and 485), spot 393 was significantly down-regulated in fish fed S, while spot 485 appeared to increase, but not significantly in trout fed soybean meal-rich diets. Protein spot 485 was more than 10 times more abundant than spot 393. Apolipoprotein A I-1 is the major protein in plasma high-density lipoprotein and is a potent activator of the enzyme lecithin/cholesterol acyl transferase, which facilitates the removal of free cholesterol to the liver for excretion [63]. It is of interest that the abundance of this protein has found to be reduced as result of the soybean meal in the diet. Kaushik et al. [4] reported that rainbow trout fed diets containing soy meal had reduced plasma cholesterol levels in comparison to fish fed fish meal diets. In mammals, this hypocholesterimic effect is mediated by apolipoprotein A I. Our results show a decrease in abundance of a minor Apo A I protein spot, which could be interpreted as some form of post-translational modification. In mammals, the expression of Apo A I is affected by both hormonal [64] and nutritional factors [65]. Phytoestrogens present in soy extracts have been shown to increase Apo A I [66], while other factors also present in soy, phorbol esters are known to have the opposite effect where both transcription of Apo A I and secretion is reduced in human liver cells [67]. The regulation of Apo A I has also been shown to be regulated by post-transcriptional mechanism, in particular thyroid hormones [68]. In salmonids, the expression of Apo A I is predominantly in the liver [69,70]. Here we show that the Apo A I mRNA expression is slightly increased in fish fed diet S, which could reflect factors such as phytoestrogens being present in the soybean meal used. Indeed, analyses made by Bennetau-Pelissero et al. [71] show that this sample contained high levels of genistein and daidzein, two major isoflavones having a strong estrogeno-mimetic activity in fish. Another identified Apo, A I-2 (spot 487) protein, was increased only slightly in the fish fed diet S.

5. Conclusions

In this study, we show for the first time, that rainbow trout fed diets containing proteins derived from different plant sources can affect parameters of whole animal nitrogen metabolism and in the same animals changes to the liver proteome are also found. Fish fed a diet containing soy proteins had reduced protein utilisation. Amino acids were

not limiting in the free pool and dietary digestibility of proteins was not different between diets and >90% in both cases. Proteomic analysis revealed proteins expressed in a differential manner as a result of the dietary manipulation. A variety of proteins including heat shock proteins, enzymes, fatty acid binding proteins and structural proteins were differentially regulated. The most likely reason for altered metabolism is the co-purification from soy of antinutritional factors such as phytoestrogens, antigenic agents and many other compounds such as phorbol esters. Similar diverse alterations in gene expression have been shown in rats fed soy protein extracts [72].

The dramatic increase in genomic information now freely available for rainbow trout and Atlantic salmon has enabled much greater success of establishing protein identities, demonstrating that this approach is leading to a more comprehensive understanding of the rainbow trout proteome.

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