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ORIGINAL ARTICLE

Proteomic Profiling of Liver from Atlantic Salmon (*Salmo salar*) Fed Genetically Modified Soy Compared to the Near-Isogenic non-GM Line

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Abstract The aim of this study was to investigate potential differences in liver protein expression of Atlantic salmon fed genetically modified (GM) Roundup Ready® soy at a high inclusion level (25% inclusion, constituting 21% of crude protein in the diet) for 7 months or a compositionally similar non-GM diet. The liver was selected as the target organ due to its importance in the general metabolism, and 2D gel electrophoresis used as a screening tool. Samples from 12 individual fish from each diet group were evaluated. Of a total of 781 analysed protein spots, only 36 were significantly different by ANOVA ($p<0.05$) in abundance between the diet groups. All these spots had low fold differences (1.2–1.6) and high false discovery rate ($q=0.44$), indicating minor differences in liver protein synthesis between fish fed GM and non-GM soy. Additionally, low fold differences were observed. Four protein spots were analyzed by liquid chromatography tandem mass spectrometry and identified using a combination of online searches in NCBI and searches in an inhouse database containing salmonid expressed sequence tags and contigs. Follow-up on these proteins by real-time polymerase chain reaction did not identify differences at the transcriptional level.

Keywords Atlantic salmon · Biotechnology · Genetic modification · Liver · Roundup Ready® soybean

Contributions of the authors Sanden, Hemre, and Sissener were part of the planning, execution, and sampling from the feeing trial. Martin and Cash have contributed with planning and help during the proteomics work and data analysis, while Sissener carried out the work itself. Hevrøy contributed with support during primer design and PCR analysis, which Sissener carried out. Sissener was the main author of the paper, but all authors have participated in the writing process.

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Introduction

Aquaculture feed based solely on wild caught fish has been described as unsustainable and ethically questionable (FAO 2005). This, coupled with rising prices of fish meal and oil, has lead to increasing use of plant proteins and oils, and soybean is now a common ingredient in commercial salmonid diets. Globally, around 64% of all soy produced is genetically modified (GM) through modern biotechnology, and Roundup Ready® soy (RRS®) is the most common variety (James 2007). RRS® has been modified to express the gene 5-enolpyruvylshikimate-3-phosphate synthase, conferring resistance to glyphosate, the active ingredient of the herbicide Roundup® (Padgette et al. 1995). Limited availability and added cost of guaranteed non-GM soy is problematic for European fish feed producers, who are currently avoiding GM products (Kaushik and Hemre 2008).

Earlier studies with GM and non-GM soy fed to Atlantic salmon have detected some minor differences between the diet groups, although it is unclear whether these can be

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attributed to the genetic modification (Hemre et al. 2005; Sanden et al. 2005, 2006; Bakke-McKellep et al. 2007, 2008). A review on genetic engineering and aquaculture (Myhr and Dalmo 2005) highlights that there are few peer-reviewed feeding trials and a requirement for long-term studies. Unintended changes in the GM plant can occur as the random insertion of a transgene might disrupt, modify, or silence the expression of endogenous genes (Cellini et al. 2004). A chemical comparison, which is the starting point in GM safety assessment (Kuiper et al. 2001), will often not include all known compounds in the feed ingredient and certainly not unknown compounds; thus, feeding experiments can be useful to detect potential unintended effects.

Methods such as proteomics and microarray have been described as discovery-driven research since they are used to screen for differences in protein expression or mRNA levels in a particular tissue on a global scale (Aebersold et al. 2000). Global protein analysis, comparing tissues from treated and control animals, requires no a priori assumptions regarding which pathways or processes might be affected. Since many proteins are measured simultaneously, the output data could provide indications of affected metabolic pathways that can be tested further by complementary methods or targeted trials.

Changes in the liver proteome have been shown in rainbow trout due to feeding status (Martin et al. 2001) and diet ingredients (Martin et al. 2003; Vilhelmsson et al. 2004). In the two latter studies, up to 10-fold changes in protein abundance were observed as a result of the inclusion of plant proteins in the diets. Comparing soy to other plant ingredients (Martin et al. 2003) and comparing fishmeal with plant proteins (Vilhelmsson et al. 2004) yielded quite different responses, demonstrating the sensitivity of liver protein expression to dietary manipulations. Proteomic profiling has also been used for safety assessment of GM soy (Malatesta et al. 2002), following up observations of nuclear modifications in mice hepatocytes (Malatesta et al. 2002). Livers of mice fed non-GM or GM soy for 2 years displayed up to 4.4-fold changes in proteins involved in metabolism, stress response, and calcium signaling pathways (Malatesta et al. 2002). Increased relative liver size has been observed in Atlantic salmon fed a 30% GM maize diet (Hemre et al. 2007). The liver is a target organ in the study of diet-induced effects, as it is the main metabolic organ and plays an important role in toxicant and immune responses (Morin et al. 1993; Benninghoff and Williams 2008; Tintos et al. 2008).

The aim of this study was to investigate differences in the expressed protein profile in liver of Atlantic salmon fed GM or non-GM soy and follow up findings with transcriptional analysis.

Materials and Methods

Fish Husbandry, Experimental Design, and Sampling

A 7-month feeding trial was conducted on Atlantic salmon at the Institute of Marine Research, Matredal, Norway. The fish had an initial weight of 39.7 g (SD 4.4), underwent the parr–smolt transformation, and were transferred from freshwater to seawater during the course of the trial. Fish were fed two different diets, with four replicate tanks receiving each diet, 120 fish per tank. RRS® was used in one diet (the GM diet) and its near-isogenic maternal line in the other (the non-GM diet; both supplied by the Monsanto Company, St. Louis, MO, USA). The formulation and analyses of the diets are shown in Table 1, and the diets were compositionally similar. In both diets, full fat soybean meal provided 21% of the total protein; the remainder was supplied from fish meal. Further information on feed analysis, growth data, light regime, and other details regarding fish husbandry is described in Sissener et al. (2009). The feeding trial was approved by the National Animal Research Authority in Norway.

Liver samples for proteome and mRNA transcription analysis were collected at the end of the trial. Fish were dip-netted out of the tanks, killed by a blow to the head, measured, and weighed before the liver was quickly dissected out. The fish weighed 189 g (SD 38) and had an average liver index, (liver weight/body weight) × 100, of 1.06 (SD 0.23), with no differences between the diet groups. The liver samples were divided equally between tubes for protein and mRNA analyses, flash frozen in liquid nitrogen, and stored at −80°C.

Proteomic Analyses

Protein Extraction

Frozen tissue pieces were homogenized at room temperature in a 10× volume of lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.3% dithiothreitol (DTT), 5 μl ml^{-1} protease inhibitor cocktail, all reagents from Sigma, Poole, UK) using a Dounce Teflon homogeniser (Polytron, Luzern, Switzerland). The samples were centrifuged for 15 min at 14.5×*g* at room temperature and solubilized proteins in the supernatant retained and stored at −80°C.

Gel Electrophoresis

Proteins samples were initially analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to ensure consistent quality and determine the appropriate loading amount for the subsequent separation in two

Table 1 Formulation and composition of the experimental diets

	non-GM		GM	
	Mean	Range	Mean	Range
Soybean composition				
Protein (%)	36.7	36.6–36.8	38.5	38.2–38.7
Lipids (%)	22.6	22.4–22.9	20.4	20.3–20.4
Starch (%)	1.8	1.7–1.8	1.6	1.6–1.7
Ash (%)	4.8	4.8–4.8	5.1	5.1–5.1
Dry matter (%)	92.6	92.6–92.7	92.9	92.9–93.0
Residue (%)[a]	26.7		27.3	
Feed formulation (g kg^{-1})				
Fishmeal[b]	510		510	
non-GM soy	262[3]		0	
GM soy	0		250[c]	
Fish oil	150		150	
Soy oil	0		8	
Wheat	75		79	
Vitamin/mineral mix[d]	3		3	
Feed composition				
Dry matter (%)	94.0	94.0–94.0	93.8	93.8–93.8
Total protein (%)	46.1	45.9–46.3	45.8	45.7–45.9
Lipids (%)	24.2	24.0–24.3	24.6	24.6–24.7
Ash (%)	9.8	9.8–9.9	9.9	9.8–9.9
Starch (%)	6.1	6.1–6.2	6.2	6.2–6.3
Residue (%)[a]	7.9		7.3	
Vitamin B6 (mg/kg)	14.7	14.4–14.9	14.8	14.5–15.1
Gross energy (kJ/g)[e]	21.5		21.6	

Values are given as the average of two analytical parallels

[a] Residue was calculated as dry matter (protein + lipid + starch + ash)

[b] The fishmeal used was Norse-LT 94 Nordic fishmeal, made from 65% blue whiting, 30% sprat, and 5% cutoffs (fish industry byproducts)

[c] A slightly higher protein and lower lipid content in the GM soy was balanced with using different amounts of soybeans in the two diets and soybean oil, achieving similar diet compositions with the soybeans contributing the same amount of protein in both diets

[d] Vitamins and minerals were added according to NRC (1993)

[e] Gross energy was calculated according to Tacon (1987) using the energy content of 39.5 kJ g^{-1} for lipid, 23.6 kJ g^{-1} for protein, and 17.2 kJ g^{-1} for starch

dimensions with isoelectric focusing preceding SDS-PAGE, as described elsewhere (Cash et al. 1995).

For analysis, 12 gels (samples from three fish of each of four tanks) were run for each diet group. After RNase/DNase treatment of samples (Smith et al. 2005), immobilized pH gradient (IPG) strips (13 cm, pH 4–7, Immobiline™ DryStrips, GE Healthcare, Amersham, UK) were rehydrated with 60 µl sample + 200 µl buffer overnight. Isoelectric focusing was performed on a Multiphor II (GE Healthcare) during three stages with a ramped voltage change (1 min at 300 V, 1.5 h increasing from 300 to 3,500 V, followed by 5 h on 3,500 V). Then, strips were incubated for 30 min with DTT (10 mg/ml in an equilibration stock; 0.05 M Tris, 6 M urea, 30% glycerol, 10% SDS), followed by 30 min with iodoacetamide (25 mg/ml in same equilibration stock), before being transferred to linear gradient 8–16% polyacrylamide gels (15×16 cm; NextGen Sciences, Alconbury, UK). The gels were run in a Protean®II Multi-Cell (Bio-rad Laboratories, Hertfordshire, UK); constant voltage of 100 V was used for 1 h, then 25 mA per gel (voltage limit of 400 V) for 2 h and 20 min. The gels were fixed (50% ethanol, 2% phosphoric acid) overnight, rinsed in MilliQ water, and stained with Coomassie brilliant blue (CBB) G-250 in an equilibration solution (34% methanol, 17% ammonium sulfate, 2% orthophosphoric acid).

Image Analysis

Gels were dried and scanned as 14-bit gray images (Umax Power Look 1120, Umax, UK) and analysis performed in the specialized software Progenesis Samespots, Version 3.1 (Nonlinear Dynamics, Newcastle upon Tyne, UK). A representative reference gel was selected from the non-GM group, and all other gels warped to that image using routines available in the software combined with manual verification and adjustments. The use of identical spot boundaries across all gels, background subtraction, and normalization to total staining intensity in each gel ensured comparable data between all gels. Each spot was manually evaluated using a 3D image display in the software, which allows one to exclude artifacts (distinguished from the protein spots by being spiky or irregularly shaped), split spots if necessary (when a spot clearly contained two peaks), and only include for further analysis those spots deemed to represent real protein spots.

Liquid Chromatography Tandem Mass Spectrometry

Spots to be identified were manually excised from a wet gel and in-gel tryptic digested using an Investigator ProGest robotic workstation (Genomic Solutions Ltd., Huntingdon, UK) based on the method of Shevchenko et al. (1996), modified for CBB-stained samples. Briefly, proteins were reduced with DTT (60°C, 20 min), S-alkylated with iodoacetamide (25°C, 10 min), and digested with trypsin (37°C, 8 h; sequencing grade, modified, Promega, Southampton, UK). The resulting peptide extract was dried by rotary evaporation (SC110 Speedvac; Savant Instruments, Holbrook, NY, USA) and dissolved in 0.1% formic acid for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis on electrospray ionization (ESI)-ion trap instrument.

Liquid chromotography was performed on an UltiMate 3000 LC System (Dionex Ltd., Camberley, Surrey, UK) using a Monolithic Capillary Column (200 µm i.d.×5 cm; Dionex part no. 161409). Eluent A was 3% acetonitrile in water containing 0.05% formic acid, eluent B was 80% acetonitrile in water containing 0.04% formic acid with a gradient of 3–45% B in 12 min at a flow rate of 2.5 µL min^{-1}. Peptide solutions were analyzed in an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Coventry, UK). Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 300–1,500 *m*/*z*, three averages and up to three precursor ions selected from the MS scan 100–2,200 *m*/*z*. Precursors were actively excluded within a 1.0-min window, and all singly charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using the incorporated data analysis software.

Protein Identification

Mass lists in the form of Mascot Generic Files were used as input for Mascot MS/MS ion searches of the NCBI database using the Matrix Science web server (www.matrixscience.com; Perkins et al. 1999). Mascot searches were also performed for all samples on an in-house database consisting of expressed sequence tags (ESTs) and generated contigs of salmonids downloaded from the Genomic research on all salmon consortium (http://web.uvic.ca/grasp/). The search parameters used were: Enzyme = Trypsin, Max. Missed cleavages = 1; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.5 Da; MS/MS tolerance ±0.5 Da; Peptide charge =2+ and 3+; Instrument = ESI-TRAP. If the top match in the salmonid EST/contig database was to unannotated sequence, basic local assignment tool (BLAST) searches were run in the NCBI database for identification.

Transcriptional Analyses

RNA Extraction and Quality Control

Total RNA was purified from frozen tissue using the EZ1 RNA Universal Tissue Kit on the BioRobot® EZ1 (Qiagen, Hilden, Germany), including the optional DNase treatment step. Homogenization in QIAzol lysis reagent from the kit was performed on the bead grinder homogenizer Precellys 24 (Bertin technologie, Montigny-le-Bretonneux, France) for 3×10 s at 6,000 rpm. Quantity and quality of RNA were assessed with Nanodrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA) and the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip® kit (both Agilent Technologies, Pao Alto, USA).

Reverse Transcription and Real-Time PCR

Potential reference genes were chosen from the literature; elongation factor 1-alpha (Moore et al. 2005), beta-actin (Olsvik et al. 2005), and acidic ribosomal phosphoprotein (Hevrøy et al. 2007). For the four target genes, primers were designed using Primer Express® 2.0 (Applied biosystems, Foster city, USA) and tested by one-step reverse transcription (RT)-PCR (Qiagen) and agarose gel electrophoresis. All primers (Invitrogen, Oslo, Norway) are given in Table 2. Constant amounts of 250 ng RNA were reverse transcribed to cDNA on a GeneAmp® PCR 9700 machine (Applied Biosystems) using the TaqMan® Reverse Transcriptase kit with oligo(dT) primers (Applied Biosystems) in 30-µL reactions. Samples from 20 fish (five fish from each of four tanks) from each diet group were run in triplicate wells, and two 96-well plates were used. Each plate contained a dilution curve (from a pooled sample) for determination of amplification efficiency, non-template, and non-amplification controls, as well as three samples for interplate calibration. For real-time PCR, SYBR® Green I Mastermix (Roche), forward and reverse primers, and cDNA were mixed in 96-well plates using a Biomek®3000 Laboratory automation workstation (Beckman Coulter, Fullerton, USA) for each of the seven genes with both of the cDNA plates (14 plates). Thermal cycling was performed on a LightCycler® 480 System (Roche Applied Science, Indianapolis, USA) according to the following protocol; preincubation at 95°C, 45 cycles of amplification with 10, 20, and 30 s at 95°C, 20°C, and 72°C, respectively, and finally, melting curve analysis was carried out between 65°C and 97°C.

Data Analysis

Cycle threshold (Ct) values were calculated using the second maximum derivative method in the Lightcycler® software. Efficiency was determined by the formula $E=10$^(−1/slope), with the slope of the linear curve of Ct values plotted against the log dilution (Higuchi et al. 1993). The stability of the reference genes was evaluated using geNorm VBA applet (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004). The software package GenEx 4.3.5 (MultiD Analyses AB, Gothenburg, Sweden) was used for efficiency correction of the Ct values for all genes, normalization to reference genes, averaging of the RT repeats, interplate calibration, calculation of quantities relative to the average, and log(2) transformation of the numbers.

Statistical Analysis

Statistical comparisons of normalized spot intensities from the 2D gels were performed in Progenesis Samespots,

Table 2 Primers used for real-time PCR

Gene	Forward primer	Reverse primer	Amplicon size	Accession no.
EF1α	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	57	AF321836
ARP	GAAAATCATCCAATTGCTGGATG	CTTCCCACGCAAGGACAGA	106	AY255630
Beta-actin	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	102	BG933897
Calreticulin	CAGGATGCCCGTTTCTATGC	CATATCCGCCGCCACAGT	121	AY372389
α-Enolase	TGGTGCCTCTACTGGTATCTATGAAG	AAGCGCAGGTGCAATAGTTTTAT	121	AY005161
TPP	TGCATCCCTCTGTTCAACCA	AGCAATGACCATGCCGTCTAT	121	BT045576
TPI	CTCCGTCCGCATTATCTATGG	GTCAACAAACTCGGGCTTGAG	121	BT048229

EF1α elongation factor 1α, *APR* acidic ribosomal phosphoprotein, *TPP* thymidine phosphorylase precursor, *TPI* triosephosphate isomerase

yielding fold difference, *p* value by ANOVA, *q* value (a measure of the false discovery rate), and power for each spot. The analysis was first done with the gels divided into groups according to diet, then according to fish tanks. All spots identified as significantly affected by tank were "flagged" to cross-check them for significance in the analysis by diet. Additionally, principal component analysis (PCA) was performed. Significance of the protein hits in the databases was evaluated by the Mowse scores (Pappin et al. 1993; Perkins et al. 1999), and the significance of BLAST hits by expectancy values (Karlin and Altschul 1990). Statistical analysis of the gene expression data was performed in Statistica™ 8.0 (Statsoft Inc., Tulsa, USA) with a nested ANOVA (mixed model ANOVA with diet as fixed effect and tank as random effect) of log(2) transformed values. Normality was assessed by the Kolmogorov–Smirnov test. Additionally, the same data were tested by a pairwise random reallocation test in REST©2005 (Corbett Life Science; Pfaffl et al. 2002) with the Ct values as the input variable.

Results

The gels displayed good resolution of protein spots throughout the pH and size range and were consistent from run to run on visual inspection. Figure 1 shows the reference gel against which the remaining gels were warped. After editing, 781 spots were included in the final dataset. When the diet groups were compared, spot by spot, by ANOVA (in reality independent *t* tests as there were only two groups), 36 spots were significantly different at a threshold level of <0.05. When the same analysis was conducted on the gels divided according to fish tanks, 39 spots were significantly different ($p<0.05$). None of the same spots were significant in both analyses. Principal component analysis of the data showed no discrimination between the diet groups. Further, the fold differences in spot abundances when comparing the diet groups were low; of all the 781 spots, 79% had fold differences of either 1 or 1.1 and the maximum fold difference observed was 1.6.

Four protein spots were selected for identification (Fig. 1, Table 3): three spots which were significantly different between the diet groups and a fourth spot that was equally expressed. The maximum fold difference in spots selected for identification was 1.3, as the spots with higher fold differences were either not significantly different due to high variation or were deemed to not be present in sufficient amounts for MS. The three differentially expressed spots selected had among the lowest *p* values and false discovery rates (*q*), as none of the spots had *p* values below 0.02 or *q* values below 0.44. All four spots were identified with significant hits in both the NCBI and the salmonid EST/contig databases (Table 4), albeit with slightly different results. For the spots 0699, 1036, and 1554, the same protein identities were assigned in both

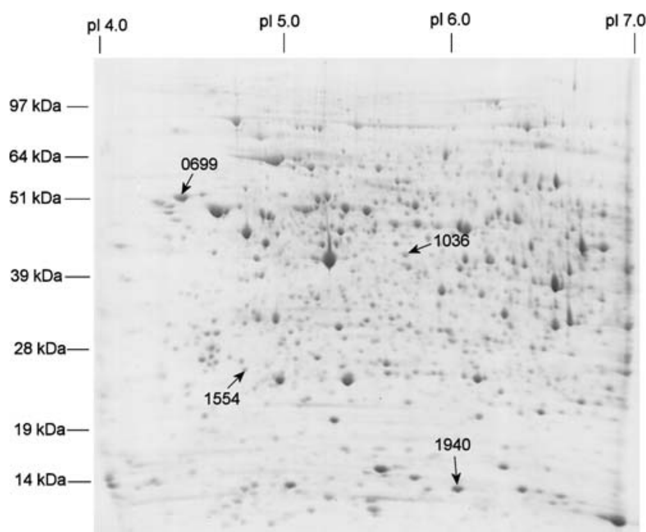

Fig. 1 Representative 2D gel. The position of the spots excised for MS/MS analysis is indicated by *arrows*. Approximate molecular weight (based on molecular weight marker) and pI (based on the linearity of the IPG strip) are given

Table 3 Protein spots selected for identification

Protein spot ID	Fold difference	p Value	q Value	Power
0699	1.3	0.044	0.44	0.094
1036	−1.2	0.022	0.44	0.105
1554	−1.3	0.027	0.44	0.110
1940	1	–	–	–

Spot ID assigned by the software, the fold difference between the GM, and non-GM fed fish (a positive value showing upregulation in the GM group and a negative value showing downregulation in the GM group), p value, q value, and power of each spot

databases. Spot 1036 came up in both databases with significant hits to two different proteins: thymidine phosphorylase precursor and alpha-enolase, the two respective proteins identified by a different set of peptides. For the final protein spot (1940), different results were obtained in the two databases, with a higher Mowse score and sequence coverage for the hit in the salmonid EST/contig database. The predicted mass and isoelectric point (pI) of the identified proteins (Table 4) agreed reasonably well with the migration of the spots in the gel (Fig. 1).

Most search results in the EST/contig database had to be identified through BLAST searches, as the matches were to unannotated sequences. Spot 1036 matched thymidine phosphorlylase precursor (*Salmo salar*) with an expectancy (e) value of $5 \times e^{-141}$ and alpha-enolase (*S. salar*) with an e value of 0, while spot 1554 matched triosephophate isomerase (*S. salar*; e value: $8 * e^{-133}$). The BLAST search of the hit obtained in the EST/contig database for spot 1940 resulted in the top 20 hits on retinol binding protein 2 from various species (e values ranging from $4 \times e^{-58}$–$7 \times e^{-53}$).

The normalized mRNA transcription levels of the four target genes; calreticulin, alpha-enolase, thymidine phosphorylase precursor, and triosephosphate isomerase were not significantly different between the two diet groups (Table 5). The p values obtained by nested ANOVA and by the randomization test in REST© were similar, and no tank effects were observed in the nested ANOVA. Compared to the protein levels, smaller fold differences were observed for the mRNA data, and which diet group had the higher expression level was not consistent between the protein and mRNA data (Table 3, Table 5).

The quality of RNA samples was good, all had RNA integrity values >8, with the average being 8.9, meaning that minimal RNA degradation had occurred. All three of the tested reference genes were included to obtain the most stable normalization index.

Discussion

The fish followed an expected growth pattern throughout the 7 months of feeding the GM and non-GM diets, with no differences in overall performance, feed utilization, haematological, or other health parameters (Sissener et al. 2009).

A total number of 781 spots do not represent the total proteome of the liver. There are probably about 15,000 proteins expressed in rat liver (Gazzana and Borlak 2007). Assuming a similar number in salmon, the proteins investigated here only constitute 5%, probably of the most abundantly expressed proteins. Nevertheless, the analyzed proteins should be sufficient to detect any major metabolic disturbances caused by the diet based on previous results from dietary studies with salmonids (Martin et al. 2001, 2003; Vilhelmsson et al. 2004). The aim of the present

Table 4 Identification of proteins

Spot id	Database	Identity	Mowse score	Species	kDa	pI	Queries matched; sequence coverage	Accession no.
0699	NCBI	Calreticulin	606	*Oncorhynchus mykiss*	48.4	4.39	16 peptides; 41%	AY372389
	EST/contig	Calreticulin	830	*Oncorhynchus mykiss*	48.4	4.39	16 peptides; 41%	AY372389
1036	NCBI	TPP	1205	*Salmo salar*	48.7	5.64	19 peptides, 56%	ACI33838
		α-enolase	255	*Salmo salar*	47.4	5.91	5 peptides; 20%	ACI33096
	EST/contig	TPP	790	*Salmo salar*	n.a.	n.a.	14 peptides; 62%	DW558148
		α-enolase	227	*Salmo salar*	n.a.	n.a.	4 peptides, n.a.	DW552945
1554	NCBI	TPI	1004	*Salmo salar*	26.9	4.91	14 peptides, 74%	ACI68030
	EST/contig	TPI	1062	*Salmo salar*	n.a.	n.a.	13 peptides; 58%	DW576433
1940	NCBI	Ribosomal protein L22	57	*Siniperca chuatsi*	9.2	10.14	1 peptide; 15%	AAY79217
	EST/contig	Retinol-binding prot. 2	785	*Salmo salar*	n.a.	n.a.	12 peptides; 47%	CK887404

The identities obtained both from the NCBI and the EST/contig databases are given. The predicted nominal mass in kDa and the calculated pI from Mascot are not available when the match obtained was not a protein sequence

TPP thymidine phosphorylase precursor, *TPI* triphosphate isomerase, *n.a.* not available

Table 5 mRNA transcription results

Gene	Ct range	non-GM	GM	Fold difference	ANOVA	REST©
Calreticulin	18.13–21.40	1.06 (0.16)	1.19 (0.12)	1.12	ns	ns
α-Enolase	22.30–24.08	1.01 (0.08)	1.01 (0.02)	1	ns	ns
TPP	23.58–25.18	0.99 (0.07)	1.12 (0.08)	1.13	ns	ns
TPI	25.90–28.60	1.07 (0.12)	1.13 (0.02)	1.03	ns	ns

The observed Ct range is given, the mean relative normalized expression (standard error in parenthesis) for each of the two diet groups (non-GM and GM) and the fold difference between the GM and non-GM fed fish. The statistical significance was evaluated by nested ANOVA and in REST© 2005

TPP thymidine phosphorylase precursor, *TPI* triosephosphate isomerase

study was to investigate possible differences between the diet groups with high statistical power, thus, focusing on a high number of biological replicates rather than subfractionation of samples or technical replicates.

The spot-by-spot approach of statistical analysis (univariate analysis) was used to simultaneously test the null hypothesis of no difference for a large number of protein spots, which introduces the problem of false discoveries. At a significance level of $p=0.05$, one would expect 5% of the spots to show significant differences between the diets by chance alone (Chich et al. 2007). The spots that were differentially expressed in the diet groups in this study all had a high false discovery rate ($q=0.44$). With such a high false discovery rate, one cannot support conclusions of diet effects, but the data could be used to identify potentially interesting proteins for further investigation or comparison with other studies. The fold changes observed in the current data were very low compared to other proteomic studies with diet in fish (Martin et al. 2003; Vilhelmsson et al. 2004) and evaluation of GM soy fed to mice (Malatesta et al. 2002). Furthermore, the diet groups were not distinguishable according to the PCA, indicating that other sources of variation, for example random or technical variation, were more prominent than dietary effects. Taken together, these data indicate minor differences at the proteome level between the diet groups.

The four selected protein spots were identified with a high level of confidence, three were matched against salmonid protein sequences in the NCBI database, and all four were matched against salmonid sequences in the EST/contig database (Table 4). This latter database was intended to circumvent the limited availability of protein sequences from fish by taking advantage of nucleotide sequences to identify the proteins. For one protein spot (1940), the results differed between the databases. The match in NCBI had a barely significant Mowse score achieved by a single matching peptide and a predicted pI far outside the pH range of the gel used. The hit obtained against the EST/contig database, however, had a much higher Mowse score and a high sequence coverage (47%) for a salmon sequence, leaving little doubt that this was the correct match. Warnings have been made against automatically accepting significant protein identifications based on a single peptide match (2004), a caution supported by our results. One spot (1036) contained two different proteins based on the recovered peptides. The peptide profile for this spot matched against two salmon proteins with high Mowse scores and high sequences coverage. Further, the theoretical masses and pIs of the two proteins were similar (48.6 and 47.3/5.6 and 5.9), supporting the co-migration of these proteins in the 2DE gel. Consequently, it was not possible to ascertain which of the two proteins was responsible for the significant difference in the spot abundance.

Compared to other proteomic studies in Atlantic salmon and rainbow trout (Martin et al. 2001 , 2003, 2007; Vilhelmsson et al. 2004), this study obtained higher protein identification rate and more confident matches, although the number of proteins selected for identification in this study is low for drawing general conclusions. There were more hits on salmonid proteins, reflecting the rapid development of database resources, and the use of an additional in-house database with salmonid ESTs and contigs improved the identification rate. Additionally, LC-MS/MS is better suited than MALDI-TOF MS (as used in the previous studies) for identifying proteins from less well-characterized organisms and to identify several proteins in a mixture (Aebersold and Goodlett 2001).

The protein spot upregulated in the GM group was calreticulin, and the two downregulated spots were thymidine phosphorylase precursor/ alpha-enolase and triosephosphate isomerase (TPI). TPI was also identified by proteomic analysis as downregulated in liver of mice fed 14% GM soy (Malatesta et al. 2002). The 2.2-fold downregulation observed in that study was greater than the 1.3-fold difference in our data, but this may be due to the species investigated or duration of the trial. This highlights TPI as an interesting protein that should be tested in other studies, with possible cross-species relevance. TPI is a glycolytic enzyme that catalyzes the conversion between glyceraldehydes 3-phosphate and dihydroxyacetone phos-

phate; it is essential for efficient energy production by being involved in several metabolic pathways (Knowles 1991). In an earlier feeding trial with Atlantic salmon, GM soy affected intestinal glucose absorption compared to non-GM (Bakke-McKellep et al. 2008), which may again affect liver glucose metabolism. However, that study used control soy of a different origin and with differences in heat treatment; thus, no clear conclusions can be drawn about the cause of observed effects (Bakke-McKellep et al. 2008).

Analysis of the mRNA coding for the four proteins identified from the three spots that were different between diet groups did not reveal significant differences. A perfect correlation between mRNA and protein levels is unlikely since the protein levels in a cell are determined by degradation and secretion as well as transcription, and there are posttranslational events and time discrepancies between mRNA and protein expression (Pratt et al. 2002; Conrads et al. 2005; Kuo et al. 2005). Discussion of these factors specifically related to the proteins identified in this study is difficult, as limited investigations have been carried out on salmon liver proteins. For example, the degree of glycosylation of calreticulin has been found to be little conserved between species (Michalak et al. 1999), and while there is a potential site for N-glycosylation of this protein in rainbow trout, this appears not to be utilized (Kales et al. 2007). In rainbow trout, calreticulin appears as two separate bands in Western blot, possibly due to differential phosphorylation (Kales et al. 2007), thus, might also have been separated between two spots in our gels if the salmon protein is similar in this regard. Generally, moderate correlation has been observed in studies comparing proteomic and microarray results (Anderson and Seilhammer 1997; Heijne et al. 2003; Link et al. 2006). Studies where PCR was used to confirm proteomics results often show a consistency in directional change, particularly if there are large changes in protein and mRNA levels (Bumke et al. 2003; Martin et al. 2007). However, in our experiment, there were only minimal differences in protein abundance; thus, it was not surprising that we found no correlation.

In conclusion, long-term (7 months) feeding with GM soy had minor effect on abundance of individual proteins in the liver of Atlantic salmon, supporting the previously published data on general performance and health of the fish from this experiment and indicating that GM soy can be used in fish feeds without affecting liver function. However, triosephosphate isomerase was identified as a potentially interesting candidate for further studies.

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