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Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (*Oncorhynchus mykiss*)

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The high dietary protein requirements of salmonid fish are met with fishmeal-based feed in commercial aquaculture. The sustainability of this practice is questionable and, therefore, the feasibility of substituting fishmeal with plant-based products needs to be investigated. We investigated growth and metabolism in rainbow trout (*Oncorhynchus mykiss*) fed a diet composed of a mixture of plant proteins compared with those fed a fishmeal-based diet. Using two-dimensional gel electrophoresis of liver protein extracts, we showed that the liver protein profile changed in response to the alteration in the diet. A number of metabolic pathways were identified as sensitive to the protein source substitution. These included pathways involved in primary energy generation, maintenance of reducing potential, bile acid synthesis, and transport and cellular protein degradation. Interestingly, the pathways shown to be affected in the present study were somewhat different from those identified in our previous work with soyabean-based-protein replacement of fishmeal, with the effects on the abundance of several stress response proteins notably absent. We conclude, therefore, that the metabolic effects of plant protein replacement in aquaculture feed varies with plant-protein source.

Proteome: Mass spectrometry: Two-dimensional gel electrophoresis: Plant protein

Most teleost fish species are adapted to use amino acids as the preferred energy source over carbohydrate, and thus require high levels of dietary amino acids (300–600 g/kg; Cowey, 1995). In commercial aquaculture, this requirement is met with fishmeal-based feed. The sustainability of this practice, which requires large inputs of wild fish for feed, has been questioned (Naylor *et al.* 2000). Thus, the replacement of fishmeal as the major protein source with proteins of plant origin is a major objective for sustainable aquaculture in the future. Progress has been made on the replacement of fishmeal with a number of different ingredients, including soyabean, lupin, peas and sunflower (Gomes *et al.* 1995*a*,*b*; Kaushik *et al.* 1995; Burel *et al.* 2000; Carter & Hauler, 2000). When formulating a replacement for fishmeal, the amino acid composition, especially of the ten essential amino acids for teleost fish, has been shown to be an important consideration (Gomes *et al.* 1995*b*; M Mambrini and S Kaushik, unpublished results). 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 (Wilson & Cowey, 1985; Cowey, 1995). No single agricultural crop source will yield feed of a suitable amino acid composition (Kaushik, 1990). Supplementation of agricultural crop sources with synthetic amino acids can improve growth and protein utilisation, but this requires precise knowledge of the ideal protein composition (Médale *et al.* 1998; Yamamoto *et al.* 2002; Cheng *et al.* 2003).

A second consideration is the presence of anti-nutritional factors, such as protease inhibitors, lectins, antigenic proteins, phenolic compounds, oligosaccharides and phytates, which are present to varying degrees in plant products such as soyabean meal (Kaushik, 1990; Davies & Morris, 1997; Francis *et al.* 2001). Anti-nutritional factors are known to affect performance of salmonid fish, with decreased digestion and reduced utilisation of proteins leading to decreased growth rates (Moyano *et al.* 1991; Krogdahl *et al.* 1994; Vielma *et al.* 2000). However, when rainbow trout were fed soyabean-protein concentrate containing comparatively low concentrations of anti-nutritional factors, comparable growth rates were observed with fish fed a fishmeal-based diet (Kaushik *et al.* 1995; Mambrini *et al.* 1999). Recently, it was found that rainbow trout fed a diet containing soyabean protein at 30 % total protein content had a substantially altered liver protein profile when compared with those fed a diet where 30 % total protein was derived from non-soyabean plant sources (Martin

Abbreviations: FM, fishmeal-based diet; PP100, plant-protein-based diet; 2DE, two-dimensional gel electrophoresis.

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et al. 2003). These differences included altered levels of several known stress proteins, indicating an effect on the fish's stress response mechanism, presumably in response to the presence of anti-nutritional factors (Martin *et al.* 2003).

The liver is a important organ for the metabolism of N and other metabolites, making it an obvious choice of focus. Not surprisingly, considering the numerous metabolic pathways and other functions taking place in the liver, it expresses thousands of gene products, many in comparatively high abundance. For characterising the effects of a dietary change at a global level, methods that focus on single or a few enzymes or metabolic pathways at a time are clearly impractical. A proteomic approach, based on two-dimensional gel electrophoresis (2DE) of crude liver tissue extracts, was therefore taken. This approach gives us the ability to analyse up- and downregulation of different, possibly unrelated, metabolic pathways without making any assumptions regarding which types of pathways are likely to be affected. Until recently, proteomic research on salmonid fish was hampered by the limited availability of salmonid protein data in the public databases such as SWISS-PROT or the non-redundant GenBank database (Martin *et al.* 2001; Hogstrand *et al.* 2002). However, the recent availability of a large number of salmonid expressed sequences (about 169 000) within GenBank has rendered proteomic research on salmonids much more practical. In recent work in our laboratory, we have been able to match peptide mass spectra against a much larger database than before, yielding a protein identification success rate $>70\,\%$ (Martin *et al.* 2003).

In the present study, we investigated growth variables, efficiency of protein deposition and liver protein profiles of rainbow trout (*Oncorhynchus mykiss*); the trout were fed a diet where fishmeal was substituted with a formulation that met the amino acid requirements and contained maize gluten meal, wheat gluten, extruded peas, rapeseed meal and extruded whole wheat.

Table 1. Experimental diets for rainbow trout (*Oncorhynchus mykiss*)

Diet...	FM	PP100
Ingredients (g/kg)		
Fish meal	638·0	0
Maize gluten meal	0	232·4
Wheat gluten	0	200·0
Extruded peas*	0	163·3
Rapeseed meal†	0	100·0
Extruded whole wheat	203·4	0
Fish oil	128·7	158·7
Binder (sodium alginate)	10·0	10·0
Mineral premix‡	10·0	10·0
Vitamin premix‡	10·0	10·0
$CaHPO_4.2H_2O$§	0	40·0
L-amino acid mixture	0	75·7
Approximate composition		
DM (g/kg)	944	916
Protein (g/kg DM)	515	486
Lipid (g/kg DM)	197	192
Gross energy (kJ/g DM)	22·7	23·6
Amino acids (g/kg DM)		
Arg	35·9	33·2
Lys	40·3	35·1
His	9·2	12·6
Ile	18·5	22·6
Leu	33·9	42·0
Val	21·8	28·6
Met	12·1	9·9
Phe	16·8	20·4
Thr	24·1	23·2
Tyr	14·0	15·7
Asp	42·7	21·0
Glu	74·6	128·9
Ser	21·3	21·1
Pro	21·1	33·1
Gly	31·2	14·1
Ala	29·8	20·4
Sum amino acid	447·3	481·5

* Aquatex, Sopreche, Boulogne sur Mer, France.
† Primor 00, Sopreche, Boulogne sur Mer, France.
‡ National Research Council (1993).
§ 180 g P/kg.

Methods

Fish husbandry and growth

Rainbow trout were grown as described previously (Martin *et al.* 2003) at an experimental freshwater fish farm belonging to INRA (Donzacq, Landes, France) under a natural photoperiod in a flow-through system at 17 ± 1°C. The feeding trial lasted for 12 weeks (21 March to 7 June 2002). For each diet, four groups of seventy-five fish each (initial mean body weight 19 g) were reared into $1\,m^3$ circular glass-fibre tanks. Every 3 weeks, fish were counted and weighed in groups after a 24 h starvation period. The diet composition is shown in Table 1. Both diets, the plant-protein-based diet (PP100) and fishmeal-based diet (FM), met the amino acid requirements of rainbow trout (National Research Council, 1993). Feed was analysed using standard procedures: DM content after drying at 110°C for 24 h, fat content after light petroleum (40–60°C) extraction, crude protein ($N\times6{\cdot}25$) content by the Kjeldahl method after acid digestion, and energy content after combustion in an adiabatic bomb calorimeter.

Two-dimensional separation of proteins

Liver sampling and protein extraction was performed as described by Martin *et al.* (2001). Briefly, fish were killed by benzocaine overdose followed by decapitation. Livers were removed, segmented, frozen in liquid N_2 and stored at -70°C. Frozen tissue was homogenised in lysis buffer (9 M-urea, 3-((3-cholamidopropyl)dimethylammonioniol)-1-propane-sulfonate (20 /l), 25 mM-Tris-HCl pH 7·5, 3 mM-EDTA, 50 mM-KCl, 50 mM-1,4-dithiothreitol, Resolyte™ (Merck, Whitehouse Station, NJ, USA; 20 ml/l), 40 μM-leupeptin) at room temperature using a Dounce Teflon homogeniser (Polytron, Luzern, Switzerland). Following homogenisation, the tissue lysates were centrifuged at 30 000 ***g*** for 20 min at 15°C to remove any insoluble particles. The supernatant fraction was then stored at -70°C until 2DE was performed. Soluble trout liver proteins (15 μl) were mixed with 115 μl re-swelling buffer (7 M-urea, 2 M-thiourea, 3-((3-cholamidopropyl)dimethylammonioniol)-1-propane-sulfonate (40 ml/l), 1,4-dithiothreitol (3 ml/l)), and then added to a 70 mm pH 4–7 immobilised pH

gradient strip (Amersham-Pharmacia Biotech, Amersham, Bucks, UK). Isoelectric focusing was performed at 200 V for 1 min and 3500 V for 1 h 30 min (at 2 mA and 5 W). For the 2DE the immobilised pH gradient strip was laid onto a 10–1 % gradient polyacrylamide slab gel (80 × 70 mm) and the proteins electrophoresed at 150 V for 450 Volt hours (Cash *et al.* 1999). The resolved proteins were detected using colloidal Coomassie Blue G250 staining (Anderson *et al.* 1991). Molecular masses of the proteins were determined by co-electrophoresis with standard protein markers. Isoelectric points were determined based on the linearity of the immobilised pH gradient strip.

Analysis of two-dimensional 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 (version 5.1; NonLinear Dynamics, Gateshead, Tyne & Wear, UK). Protein spots were detected using automated routines from the software combined with manual editing to remove artifacts. The spots were matched against 559 reference spots, numbered from 24 to 773 using the reference gel described by Martin *et al.* (2001). Matching was performed using a combination of seed-matching and automatic matching by the Phoretix program (Nonlinear Dynamics). All matches were verified by eye. The proteins had molecular masses of approximately 10–100 kDa and isoelectric point 4–7. 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 volume of all proteins detected on each gel. The normalised spot volume is described as the abundance of a particular protein spot relative to the total. Five replicate gels were analysed for each treatment group. Proteins that were found to vary more than twofold in abundance between the diets were analysed for significance using Student's *t* test.

Protein identification by peptide mass mapping

Proteins were excised from stained gels representing fish fed on FM and PP100 and subjected to in-gel trypsin digestion (Jensen *et al.* 1999). Excised spots were washed, reduced, *S*-alkylated and digested within the gel using trypsin (sequencing grade modified trypsin; Promega, Southampton, Hants., UK) as described elsewhere (Shevchenko *et al.* 1996; Wilm *et al.* 1996). A portion of the peptide extract produced by this process was passed through a GELoader tip containing a small volume of POROS R2 sorbent (PerSeptive BioSystems, Foster City, CA, USA) (Wilm *et al.* 1996). The adsorbed peptides were eluted in 0·5 μl saturated solution of α-cyanol-4-hydroxycinnamic acid in acetonitrile–formic acid (50:5, v/v). The mass spectra of the peptide fragments were obtained on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF MS. The instrument was operated in the reflection delayed extraction mode. Spectra were internally calibrated using trypsin auto-digestion products.

For protein identification, peptide masses from trypsin digests were used to search against the National Centre for Biotechnology Information non-redundant sequences database and an in-house generated database containing all salmonid cDNA sequences available (as of 14 July 2003; 169 000 sequences). Search programs used were Mascot (Perkins *et al.* 1999) and Protein Prospector MS-Fit (Clauser *et al.* 1999). The Mascot search variables were: (1) peptide mass accuracy 50 μg/l; (2) protein modifications: cysteine as *S*-carbamidomethyl-derivative, oxidation of methionine allowed. Variables for searching MS-Fit were: all six frames to be searched; cysteine as *S*-carbamidomethyl-derivative; oxidation of methionine allowed.

Results

Growth trial

Fish fed on FM grew to a significantly larger ($P<0·05$) than those fed on PP100 (Table 2). Intake of digestible energy and digestible N were not found to vary significantly

Table 2. Growth performance, feed intake and feed efficiency of rainbow trout (*Oncorhynchus mykiss*) fed the experimental diets for 12 weeks†

(Mean values with their standard errors for four fish per group)

Diet...	FM		PP100	
	Mean	SE	Mean	SE
Growth:				
Initial body weight (g)	19·2	0·2	19·2	0·1
Final body weight (g)	147·1	1·8	111·1*	2·5
SGR (%)	2·61	0·03	2·25*	0·02
Feed intake:				
Digestible energy intake (kJ/kg per d)	354·3	2·9	354·8	2·8
Digestible N intake (mg N/kg ABW per d)	1305·9	8·7	1320·8	8·6
Feed efficiency‡	1·33	0·03	1·17*	0·01
Protein efficiency ratio§	2·58	0·05	2·42*	0·01

FM, fishmeal-based diet; PP100, plant-protein based diet; SGR, specific growth rate (((ln (final weight) − ln (initial weight))/84(d)) × 100; Ricker, 1979; ABW, average body weight ((final weight + initial weight)/2).
Mean values were significantly different from those for the FM group (Student's *t* test): *$P<0·05$.
† For details of diets and procedures, see Table 1 and p. 72.
‡ Feed efficiency = wet weight gain/dry feed intake.
§ Protein efficiency ratio = wet weight gain/crude protein (N × 6·25) intake.

between diets. Feed efficiency and protein efficiency were significantly decreased in fish fed on PP100 (Table 2).

Proteomics

Liver protein extracts from five individual fish for each diet (PP100 1–5 and FM 1–5) were subjected to 2DE, yielding 2DE gels each containing from 694 to 980 protein spots, varying in abundance from 0·006 to 8·100 % total protein on the gel.

The protein profile in Fig. 1 shows a representative example of liver proteins separated by 2DE. The reference gel for the present study, against which the ten gel profiles were matched, was the same as in our previous study (Martin *et al.* 2003). The same reference numbers were used, with new numbers added for protein spots not reported earlier.

Abundance levels of individual spots varied considerably among samples, as may be seen from the error margins (± 1 SE) presented in Table 3, in some cases confounding analysis of whether abundance was affected by the diet. However, thirty protein spots (Table 3, Fig. 1) were consistently and significantly (more than twofold difference in abundance, $P<0{\cdot}05$) altered in abundance between the diets. Of these, one (no. 764) was only present in fish fed on PP100, twenty-three others were present in greater abundance in the fish fed on PP100 (upregulated by intake of plant protein), and six were in greater abundance in fish fed on FM (downregulated by the presence of plant protein).

Of the thirty protein spots affected by the diet, twenty-three were deemed to be of sufficient abundance (>0·05 % total gel volume) to permit peptide mass fingerprinting. They were excised from the gels and subjected to trypsin digestion and MS. The peptide fragments produced were matched against predicted peptide mass fingerprints derived from public databases (Table 4). Of the twenty-three mass fingerprints obtained, fourteen yielded significant protein identities, whereof six were obtained by searching against a publicly available protein database and thirteen by searching against a database created from 169 000 salmonid nucleotide sequences (Table 4). In all five cases where both search methods gave positive hits, the same identity was obtained. For further corroboration, molecular mass and isoelectric point values observed on the 2DE gels were compared with those predicted by ExPASy (http://ca.expasy.org/tools/pi_tool.html) for the matched polypeptides (Table 5).

Discussion

Trout fed on PP100 grew more slowly than those fed on FM (Table 2). This was not a consequence of reduced feed intake, as both groups of fish consumed similar amounts of energy and N. However, there was a significant difference in protein efficiency ratios between the two groups and, hence, the difference was likely to be a result of poorer utilisation of consumed N. Among possible nutritional causes for reduced protein utilisation are the presence of anti-nutritional factors (Francis *et al.* 2001) and limited availability of essential amino acids (Cowey & Walton, 1989). Although care was taken that the amino acid composition of PP100 closely matched that of the control diet, levels of three essential amino acids (arginine, lysine and

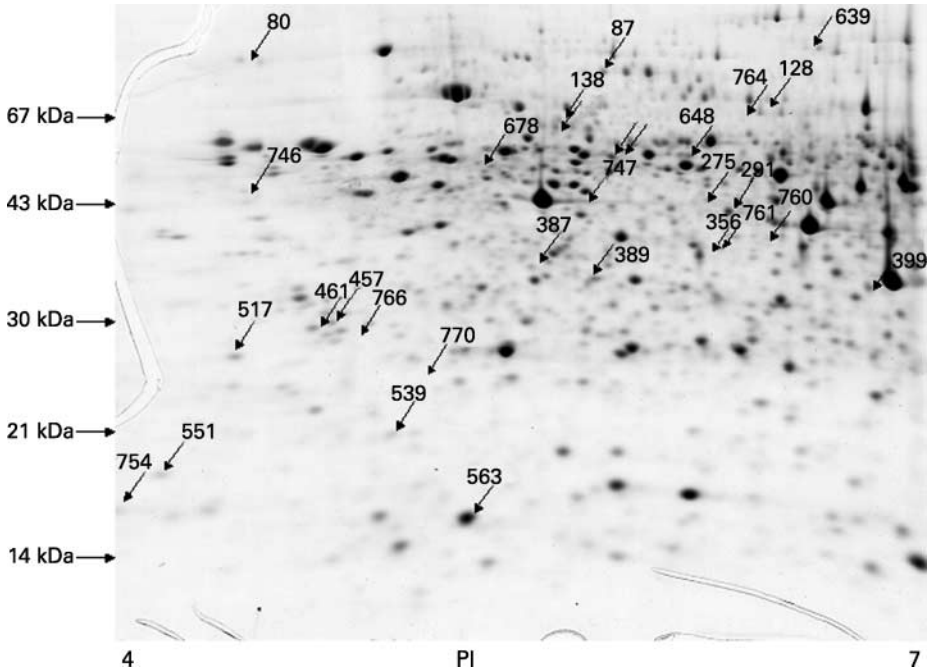

Fig. 1. A representative two-dimensional gel of rainbow trout (*Oncorhynchus mykiss*) liver proteins. PI, isoelectric point. A total liver protein extract was separated by charge between PI 4 and 7, second dimension was by size on a 10–15 % gradient gel. The protein spots were stained with colloidal Coomassie Blue G250. Protein spots (↓) were found to increase or decrease in abundance in response to dietary protein source substitution. For details of procedures, see p. 72.

Table 3. Protein spots affected by dietary plant protein substitution*
(Mean values with their standard errors for five determinations)

Spot reference no.	PI	MM (kDa)	Normalised volume diet FM†		Normalised volume diet PP100†		Fold difference	Statistical significance of effect: *P*‡
			Mean	SE	Mean	SE		
Downregulated:								
128	6·3	66	303	57	60	19	5·1	0·026
291	6·4	42	521	37	273	30	2·0	0·004
356	6·3	38	161	37	44	19	3·6	0·031
747	5·6	43	101	19	19	11	2·2	0·040
760	6·3	39	41	6	21	5	2·0	0·040
766	4·8	27	12	1	6	1	2·1	0·004
Upregulated:								
80	4·4	82	9	4	47	8	5·0	0·007
87	5·7	75	58	14	262	21	4·5	<0·001
138	5·5	67	99	16	267	39	2·7	0·009
144	5·4	63	26	6	265	66	10·2	0·018
190	5·9	54	6	2	50	9	8·5	0·018
199	5·9	53	60	16	156	13	2·6	0·020
275	6·1	45	1	0·6	11	0·6	9·1	<0·001
387	5·6	35	97	3	251	49	2·6	0·035
389	5·8	35	192	45	414	54	2·2	0·027
399	6·8	33	59	12	130	10	2·2	0·028
457	4·7	29	26	7	57	5	2·2	0·021
461	4·7	27	75	9	190	12	2·5	0·004
517	4·4	22	15	6	135	29	9·0	0·013
539	4·9	19	7	3	18	3	2·5	0·033
551	4·1	17	40	11	143	28	3·6	0·018
563	5·2	15	814	198	3762	984	4·6	0·039
639	6·4	84	10	6	28	5	2·9	0·047
648	6·1	55	17	5	154	46	8·9	0·040
678	5·3	48	26	7	69	15	2·7	0·044
746	4·4	46	45	13	107	15	2·4	0·012
754	4·1	15	6	2	36	4	6·5	<0·001
761	6·1	36	44	21	204	34	4·6	0·006
764	6·2	65	0		102	17	N/A	N/A
770	5·0	21	4	1	18	4	4·4	0·026

PI, isoelectric point; MM, molecular mass; FM, fishmeal-based diet; PP100, plant-protein-based diet.
* For details of diets and procedures, see Table 1 and p. 72.
† Values are mean normalised protein abundance.
‡ Student's *t* test.

methionine) were slightly lower (results not shown), but high enough to meet the requirements of rainbow trout (National Research Council, 1993).

Proteomics

The identities of the proteins observed to be up- or down-regulated (Table 4) by the plant-protein substitution indicate that several processes are affected, most notably those of primary energy metabolism, as discussed later. The protein identities obtained are presented with good confidence, since Mowse and Mascot scores are generally high, expectancy values for BLASTx searches of expressed sequence tags are low (Table 4), and calculated molecular mass and isoelectric point values (Table 5) display an acceptable level of agreement with the observed values, considering that post-translational modifications may significantly affect these values and that some of the identities are based on proteins from distantly related species (Table 4). Furthermore, all of the identities are proteins that would be expected to be present in the liver in detectable quantities.

Enzymes affecting NADPH levels

Interestingly, two isoforms of transaldolase were detected in the present study, an acidic one (spot 356, isoelectric point 6·2) and a less acidic one (spot 761, isoelectric point 6·3). Abundance of both was affected by the diet (Fig. 2, Table 3). Presumably, spot 356 represents a phosphorylated form of this protein. Transaldolase possesses several putative phosphorylation sites (Banki *et al.* 1994) and its activity correlates with phosphorylation state (Lachaise *et al.* 2001). Furthermore, it has been shown that phosphorylated and non-phosphorylated forms of transaldolase can be distinguished by 2DE (Lachaise *et al.* 2001). The less acidic (non-phosphorylated) form is upregulated when fish are fed the plant-protein-based diet, whereas the more acidic (phosphorylated) form is downregulated. However, total transaldolase levels (spot 356 + spot 761) remained essentially unaltered (Table 4), suggesting that transaldolase gene expression was unaffected. Assuming, therefore, that the more acidic form represents the active enzyme, our present results indicate that transaldolase activity may be lower in livers of fish fed PP100. Transaldolase is a key enzyme of primary

Table 4. Identities of protein spots based on peptide mass fingerprinting*

			Identities by MS-Fit followed by BLASTx†						Identities by Mascot‡			
Spot reference no.	PI	MM (kDa)	Salmonid sequence	MS-Fit Mowse score	Protein	Species	Accession no.	E	Protein	Species	Accession no.	Mascot score
Downregulated:												
128	6·3	66	CA057756	$6·7 \times 10^{5}$	(No significant homology)				(No significant hits)			
291	6·4	42	24595286	$2·9 \times 10^{5}$	β-Ureidopropionase	*Homo sapiens*	NP_057411	$5·0 \times 10^{-91}$	(No significant hits)			
356	6·3	38	24591427	$5·5 \times 10^{5}$	Transaldolase	*Cricetulus griseus*	AAL55523	$1·0 \times 10^{-104}$	(No significant hits)			
Upregulated:												
80	4·4	82	BX087381	$2·4 \times 10^{5}$	(No significant homology)				(No significant hits)			
87	5·7	75	D89083	$3·7 \times 10^{9}$	Transferrin	*Oncorhynchus mykiss*	Q9PT13	0·0	Transferrin	*Oncorhynchus mykiss*	Q9PT13	114
138	5·5	67	CA057507	$1·2 \times 10^{4}$	(No significant homology)				(No significant hits)			
144	5·4	63	CA376060	$1·4 \times 10^{9}$	L-plastin	*Homo sapiens*	AAB02845	$1·0 \times 10^{-109}$	(No significant hits)			
190	5·9	54	27750546	$2·1 \times 10^{4}$	Malate dehydrogenase	*Homo sapiens*	JC4160	$5·0 \times 10^{-88}$	(No significant hits)			
199	5·9	53	CA362730	$7·8 \times 10^{5}$	(No significant homology)				(No significant hits)			
275	6·1	45	BX318659	$1·6 \times 10^{5}$	(No significant homology)				(No significant hits)			
387	5·6	35	24669112	$8·7 \times 10^{6}$	Electron transfer flavoprotein, α subunit	*Danio rerio*	AAH47845	$3·0 \times 10^{-99}$	Electron transfer flavoprotein, α subunit	*Danio rerio*	AAH47845	84
389	5·8	35	24669112	$6·1 \times 10^{4}$	Electron transfer flavoprotein, α subunit	*Danio rerio*	AAH47845	$3·0 \times 10^{-99}$	Electron transfer flavoprotein, α subunit	*Danio rerio*	AAH47845	87
399	6·8	33	AF067796	$4·8 \times 10^{4}$	Aldolase B	*Salmo salar*	AAD11573	0	Aldolase B	*Salmo salar*	AAD11573	82
457	4·7	29	CB499007	$3·2 \times 10^{3}$	(No significant homology)				(No significant hits)			
461	4·7	27	29324514	$8·9 \times 10^{3}$	Proteasome subunit α 2	*Carassius auratus*	BAA25915	$1·0 \times 10^{-101}$	(No significant hits)			
517	4·4	22	(No significant hits)						Cytochrome *c* oxidase	*Sus scrofa*	A00007	121
563	5·2	15	CA384491	$1·0 \times 10^{7}$	H-fatty acid binding protein	*Oncorhynchus mykiss*	O13008	$9·0 \times 10^{-66}$	(No significant hits)			
648	6·1	55	CA354171	$9·5 \times 10^{3}$	Cytosolic HMG-CoA synthase	*Gallus gallus*	P23228	$3·0 \times 10^{-99}$	(No significant hits)			
678	5·3	48	24591229	$9·4 \times 10^{5}$	Proteasome 26S ATPase subunit 4	*Homo sapiens*	NP_694546	$1·0 \times 10^{-120}$	Proteasome 26S ATPase subunit 4	*Mus musculus*	Q8BKU2	117
746	4·4	46	BX087531	$4·9 \times 10^{3}$	(No significant homology)				(No significant hits)			
761	6·1	36	CA346256	$4·9 \times 10^{5}$	Transaldolase	*Cricetulus griseus*	AAL55523	$1·0 \times 10^{-104}$	(No significant hits)			
764	6·2	65	BX085804	$6·7 \times 10^{5}$	(No significant homology)				(No significant hits)			

PI, isoelectric point; E, expectancy; HMG, hydroxymethylglutaryl.
*For details of procedures, see p. 72.
†Clauser *et al.* (1999).
‡Perkins *et al.* (1999).

Table 5. Observed *v.* predicted molecular masses and isoelectric points of identified proteins*

	Observed			Predicted	
Reference spot no.	PI	MM (kDa)		PI	MM (kDa)
Downregulated:					
291	6·4	42	β-Ureidopropionase	6·1	43
356	6·3	38	Transaldolase	7·0	37
Upregulated:					
87	5·7	75	Transferrin	5·9	75
144	5·4	63	L-plastin	5·4	64
190	5·9	54	Malate dehydrogenase	6·4	64
387	5·6	35	Electron transfer flavoprotein, α subunit	6·9	39
389	5·8	35	Electron transfer flavoprotein, α subunit	6·9	39
399	6·8	33	Aldolase B	8·0	39
461	4·7	27	Proteasome subunit α 2	6·0	26
517	4·4	22	Cytochrome *c* oxidase	4·7	26
563	5·2	15	Fatty acid binding protein H	5·8	14
648	6·1	55	HMGCoA synthase	5·4	58
678	5·3	48	Proteasome 26S ATPase subunit 4	6·3	58
761	6·1	36	Transaldolase	7·0	37

PI, isoelectric point; MM, molecular mass; HMG, hydroxymethylglutaryl.
* For details of procedures, see p. 72.

metabolism, controlling the balance between the oxidative and non-oxidative branches of the pentose phosphate pathway and, thus, the output of NADPH and ribose 5-phosphate (Banki *et al.* 1996; Kruger & von Schaewen, 2003).

In a parallel study using the same diets, glucose-6-phosphate dehydrogenase activity was increased in fish fed on PP100 (F Médale, unpublished results). Taken together, increased glucose-6-phosphate dehydrogenase activity and decreased transaldolase activity indicate that while the oxidative phase of the pentose phosphate pathway is increased in activity, that of the non-oxidative phase is decreased. This will generate high levels of NADPH, while the resulting pentose sugar intermediates can be reconverted to glucose 6-phosphate for repeated passage through the oxidative phase or entry into the glycolytic pathway. Indeed, abundance of aldolase B (spot 399), an enzyme of the glycolytic pathway, was also increased (Table 3).

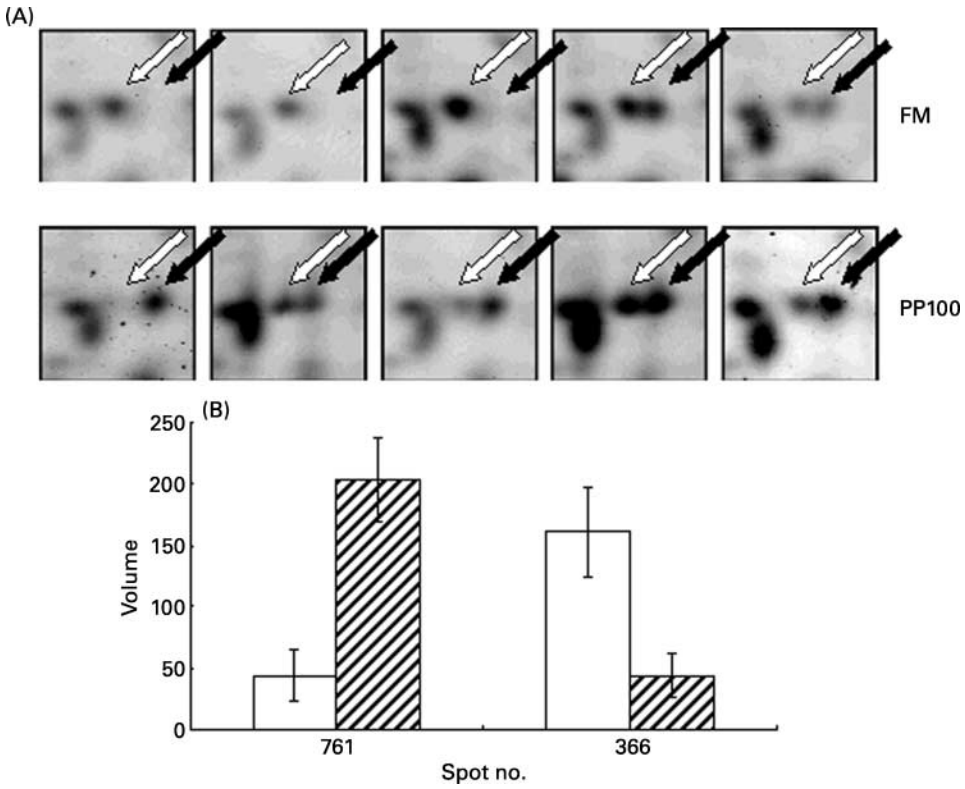

Fig. 2. (A), changes in abundance of the two protein spots (356 and 761) representing transaldolase. ⇓, Spot 356; ↓, spot 761; FM, fish-meal-based diet; PP100, plant-protein-based diet. (B), abundance of the two transaldolase spots was compared between fish fed the two diets. □, FM; ▨, PP100. Values are means with their standard errors shown by vertical bars. For details of diets and procedures, see Table 1 and p. 72.

Further evidence of increased NADPH production in fish fed on PP100 can be seen in that abundance of malate dehydrogenase (malic enzyme; spot 190) was increased in fish fed PP100 (Table 3), as was its activity (F Médale, unpublished results). Taken together, this strongly indicates that trout fed on PP100 generate a greater amount of NADPH than do trout fed on FM, which may affect lipogenic pathways and ATP production.

Other enzymes of energy metabolism

Two protein spots identified as the α subunit of electron-transferring flavoprotein (spots 387 and 389) were upregulated in trout fed PP100 (Table 3). Electron-transferring flavoprotein is involved in the early part of β-oxidation of fatty acids, receiving electrons from fatty acyl-CoA dehydrogenase and transferring them to ubiquinone (Eaton *et al.* 1996). The electrons are then carried down the respiratory chain, which includes cytochrome *c* oxidase (spot 517), whose levels are also increased in fish fed on PP100 (Table 3). It thus appears that livers of trout fed on PP100 have a greater capacity for fatty acid breakdown, presumably to supply ATP and acetyl-CoA for energy and biosynthesis.

Biosynthetic enzymes

Hydroxymethylglutaryl-CoA synthase (spot 648) was increased in abundance in fish fed on PP100 (Table 3). It catalyses the condensation of acetoacetyl-CoA and acetyl-CoA to form hydroxymethylglutaryl-CoA and free CoA. While the mitochondrial isoform of this enzyme is involved in ketogenesis (Hegardt, 1999), the cytosolic isoform works with hydroxymethylglutaryl-CoA reductase to generate mevalonate and thus start the cholesterol-generating isoprenoid pathway (Kattar-Cooley *et al.* 1990; Sato & Takano, 1995; Hegardt, 1999). The protein extracts used in the present study were expected to comprise mainly cytosolic proteins, and the trypsin digest fingerprint of protein 648 showed the greatest similarity to a cytosolic hydroxymethylglutaryl-CoA synthase, indicating that sterol biosynthesis may be increased in fish fed on PP100. Furthermore, the H-fatty acid binding protein (spot 563), involved in transport of taurine-conjugated bile salts (Dietrich *et al.* 1995), was also increased in abundance in fish fed on PP100. Taken together, these results suggest the possibility that the increased production of NADPH, ATP and acetyl-CoA, discussed earlier, is used partly for increased bile production.

Nitrogen metabolism

Previous studies on plant-protein substitution in feed for rainbow trout have shown an increase in activity of enzymes involved in amino acid metabolism (Moyano *et al.* 1991; Martin *et al.* 2003). In the present study, however, we did not observe any changes in abundance of known amino acid metabolising enzymes and, in a parallel study (F Médale, unpublished results) where the same diets were used as in the present study, no appreciable difference was observed in the activities of three key enzymes of amino acid metabolism. Recent work on the gilthead sea bream (*Sparus aurata*) indicated that effects of protein source substitution on amino acid metabolising enzymes may be in part due to the essential:non-essential amino acid ratio in the diet (Gomez-Requeni *et al.* 2003). It seems plausible, therefore, that the lack of impact on amino acid metabolism in the present study reflects the similar amino acid composition of the two diets (Table 1).

Proteolysis

Two subunits of the proteasome (spots 461 and 678), a major protein degradation engine, were observed to be increased in abundance in the PP100-fed fish (Table 3). Interestingly, starved fish were also found to have increased protein degradation compared with fed fish, but through an increase in the lysosome-associated protease cathepsin D (Martin *et al.* 2001). While proteasome activity increases under conditions of starvation in warm-blooded animals (Medina *et al.* 1995; Wing *et al.* 1995), in rainbow trout it is decreased (Martin *et al.* 2002). Recent work in our laboratory has shown a negative correlation between growth efficiency and proteasome activity in the liver (Dobly *et al.* 2004). The fish fed on PP100 had a lower growth efficiency than the control fish, confirming this finding.

Comparison with a previous study

Martin *et al.* (2003), studying partial substitution of fishmeal with soyabean meal, found thirty-three proteins up- or downregulated in response to the diet change, seventeen of which were identified. We note that there are surprisingly few similarities to the present study. Most of the seventeen identified proteins affected by the dietary presence of soyabean meal were not affected in the present study (Table 6). Only the downregulation of transaldolase and upregulation of aldolase B were observed in both studies. In the previous study, rainbow trout fed on a diet where 30 % of the fishmeal was substituted with soyabean meal had altered levels of several stress proteins, e.g. HSP70, HSP108 and aryl sulfotransferase (Martin *et al.* 2003), indicating a stress response that was not observed in the present study. Indeed, most of the proteins whose levels were altered compared with fish fed the control diet were proteins directly involved in primary energy metabolism (Table 4). Thus, the 'stressful' effects of the soyabean-meal diet are not experienced by fish fed on PP100, even though it contained no fishmeal at all.

In conclusion, the fish fed on PP100 did not perform as well as the fish fed on FM. The majority of the proteins affected by the diet are involved in primary functions, such as maintenance of reducing potential and energy generation. From this, we conclude that rainbow trout fed a diet containing plant proteins have higher energy demands than those fed fishmeal-based diets, a conclusion supported by a parallel study where the heat increment of feeding was found to be significantly higher in fish fed on PP100 than in those fed on FM (F Médale, unpublished results). These

Table 6. Proteins affected by soyabean-protein substitution but not by the mixed plant protein substitution diet*
(Mean values and standard errors for five determinations)

Spot reference no.		Normalised volume diet FM†		Normalised volume diet PP100†		Fold difference	Statistical significance of effect: *P*‡
		Mean	SE	Mean	SE		
60	HSP 108	2199	484	3109	576	1·4	0·262
115	Transketolase	2704	379	2998	363	1·1	0·591
120	HSP 70	1488	231	1216	74	1·2	0·315
123	HSP 70	480	87	371	48	1·3	0·313
160	NO synthase	415	41	523	87	1·3	0·308
183	Keratin II k8a	970	137	870	204	1·1	0·696
194	Se BP 2	160	76	178	61	1·1	0·885
197	HSP 108	146	33	185	43	1·3	0·500
201	β-Tubulin	296	38	203	28	1·5	0·088
214	Adenosylhomocysteinase	158	36	134	64	1·2	0·754
249	Homogentisate dioxygenase	1466	424	735	200	2·0	0·172
269	Phosphogluconate dehydrogenase	455	64	432	127	1·1	0·875
330	Hydroxyphenylpyruvate dioxygenase	42	10	32	7	1·3	0·436
370	Ser/Thr phosphatase 2A	82	33	58	19	1·4	0·562
393	ApoA-I 1	764	234	629	60	1·2	0·603
473	HxG phosphoribosyl transferase	38	12	46	14	1·2	0·689
553	Trout EST BX082497	15	7	38	13	2·6	0·149
634	Glc-regulated protein	19	7	38	18	2·0	0·430
681	Pyruvate kinase	447	102	215	51	2·1	0·088

FM, fishmeal-based diet; PP100, plant-protein-based diet; HxG, hypoxanthine guanine; EST, expressed sequence tags; glc, glucose.
* For details of diets and procedures, see Table 1 and p. 72.
† Values are mean normalised protein abundance.
‡ Student's *t* test.

findings constitute the first ever demonstration of multiple metabolic consequences of changes in dietary protein source in fish. Further insight on such considerations will be of great value when formulating feed from plant-derived proteins as substitutes for fishmeal for the sustainable development of aquaculture.

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