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Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets

Stéphane Panserat[1]*, Cathy Kolditz[1], Nadège Richard[1], Elisabeth Plagnes-Juan[1], Francois Piumi[2], Diane Esquerré[2,3], Francoise Médale[1], Genevieve Corraze[1] and Sadavisam Kaushik[1]

[1]*INRA, UMR1067 Nutrition Aquaculture and Genomics, Pôle d'Hydrobiologie, CD 918, F-64310 Saint-Pée-sur-Nivelle, France*
[2]*INRA, DGA, UMR 314, Laboratoire de Radiobiologie et d'Etude du Génome, CRB GADIE, F-78350 Jouy-en-Josas, France*
[3]*CEA, DSV, IRCM, SREIT, Laboratoire de Radiobiologie et d'Etude du Génome, F-78352 Jouy-en-Josas, France*

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Reducing the reliance on fishery by-products as amino acid and fatty acid sources in feeds for farmed fish is a major objective today. We evaluated the effect of dietary fish oil or dietary fishmeal replacement by vegetable oils and plant proteins respectively through analysis of hepatic transcriptomes in rainbow trout (*Oncorhynchus mykiss*). Fish were fed right from first feeding with diets based on plant by-products before being killed. We analysed the hepatic gene profile using trout cDNA microarrays (9K). Our data showed that seventy-one and seventy-five genes were affected after fish oil and fishmeal replacement respectively. The major part of modified gene expression coding for proteins of the metabolic pathways was as follows: (i) a lower level of expression for genes of energy metabolism found in fish after fishmeal and fish oil replacement; (ii) a lower level of gene expression for fatty acid metabolism (biosynthesis) in fish fed with vegetable oils; (iii) a differential expression of actors of detoxification metabolism in trout fed with vegetable oils; (iv) a lower level of expression of genes involved in protein metabolism in fish fed with plant proteins. Overall, our data suggest that dietary fish oil replacement is linked to a decreased capacity of fatty acid biosynthesis (fatty acid synthase) and variation of detoxification metabolism (cytochrome P450s) whereas dietary fishmeal replacement may depress protein metabolism in the liver as reflected by glutamine synthetase.

Fish nutrition: Plant products: Fish oil: Fishmeal: Liver: Transcriptomics: Rainbow trout

Feed for intensively farmed fish still relies heavily on feedstuffs of marine origin, fishmeal and fish oil. This impairs the sustainability of fish production, while aquaculture should be a solution to the generally observed decline in fishery resources[1]. Research is intense for finding ways to replace marine feedstuffs (fishmeal and fish oil) by plant feedstuffs[2]. In the past 20 years, fish feeds have included large amounts of fish oil, given the beneficial effects on N utilisation and environmental load[3,4]. Partial and total replacement of fishmeal by vegetable protein sources is similarly the object of several studies in almost all species[2,5].

Efforts towards replacement of fishmeal by other alternative protein sources have been undertaken for more than two decades and there is a vast amount of literature on partial replacement of fishmeal by plant feedstuffs[6]. A number of disadvantages have been ascribed to the use of plant protein sources: relatively low protein content, amino acid imbalance, low palatability, presence of endogenous anti-nutritional factors and large amounts of carbohydrates[2,7,8]. Attempts have been made to develop fishmeal-free diets for different species including salmonids either with single ingredients duly supplemented with amino acids[9] or using a mixture of different protein sources[10–13]. It is clear that a substantial reduction in the dietary levels of fishmeal can be achieved although total replacement of fishmeal by plant ingredients is still not common in salmonids. Some earlier studies have shown that total replacement of fishmeal by plant proteins leads to decreased growth of rainbow trout possibly linked to a modification of a number of hepatic metabolic pathways[14].

Several studies with salmonids (rainbow trout, brown trout, Atlantic salmon, Pacific salmons) have shown that it is possible to replace fish oil by a single vegetable oil or mixture of vegetable oils without affecting growth or feed efficiencies[4,15]. Since the flesh fatty acid composition is known to be affected by the dietary fatty acid profiles, it is also known that once the fish are grown with vegetable oils over the major part of the life cycle, a finishing diet based on fish oil as the major lipid source can be used to tailor the final flesh fatty acid composition with the levels of *n*-3 PUFA (EPA and DHA) ideally suited for human nutrition and health[15–17]. The metabolic consequences are also numerous, mediated by a number of interacting pathways.

Abbreviations: FAS, fatty acid synthase; INRA, French National Institute for Agricultural Research; REST, Relative Expression Software tool; TMEV, TIGR Multiple Experiment Viewer.

* **Corresponding author:** Dr Stephane Panserat, fax +33 5 59 54 51 52, email panserat@st-pee.inra.fr

The objective of the present study was to analyse the hepatic gene expression profile in rainbow trout (*Oncorhynchus mykiss*) fed over a long period diets with or without either fish oil or fishmeal, replaced respectively by a mixture of plant oils or plant proteins. We analysed specifically the liver since this is the main organ involved in nutrient utilisation as the centre of intermediary metabolism in animals. After transcriptomic analysis, differentially expressed genes were identified and some were specifically studied in rainbow trout liver following the plant-based diet intake.

Experimental methods

Feeds, fish rearing and sampling

Triploid rainbow trout were reared in the French National Institute for Agricultural Research (INRA) experimental fish farm at constant water temperature (17 ± 1°C) and under natural photoperiod conditions (Donzacq, Landes, France). To test the effect of fish oil replacement, fish were fed from first feeding to commercial size during 62 weeks with two isoproteic (51 % crude protein), isolipidic (30 % crude fat) and isoenergetic (26 kJ/g) diets, differing only by the lipid source, i.e. either fish oil or a mixture of vegetable oils (30 % palm, 15 % linseed, 55 % rapeseed) as previously described[18] (Table 1).

To test the effect of fishmeal replacement, fish were fed from first feeding to commercial size during 52 weeks with two isonitrogenous, isolipidic and isoenergetic diets, differing only by the protein source, i.e. either fishmeal or a mixture of plant proteins (Table 2). These diets were produced by feed manufacturers (Nutreco, Stavanger, Norway and Le Gouessant Aquaculture, Lamballe, France, respectively).

Fish were randomly distributed into triplicate tanks per dietary treatment. Each diet was distributed by hand to visual satiation 6 d over 7 d and feed consumption was recorded every week. At the end of the growth trial, six fish from each group (two per tank) were randomly sampled 24 h after the last meal in order to have data following the long-term plant-diet adaptation. Fish were killed by a sharp blow to the head. Livers were weighed and immediately frozen in liquid N_2 and kept at −80°C pending analyses.

Table 1. Composition of the diets used in the fish oil replacement studies*

	FO diet	VO diet
Ingredients (g/kg feed)		
Fishmeal (Scandinavian LT-fish meal; Norsildmel, Norway)	466·9	466·9
Maize gluten meal (Cargill, Staley, NC, USA)	135·2	135·2
Soyabean meal (Denofa, Fredrikstad, Norway)	100·0	100·0
Whole wheat (Statkorn, Oslo, Norway)	46·1	46·1
Capelin oil (Nordsildmel, Norway)	226·8	–
Rapeseed oil (Oelmuhle, Hamburg, Germany)	–	124·7
Palm oil (Denofa, Fredrikstad, Norway)	–	68·1
Linseed oil (Oliefabriek, Lictervelde, Belgium)	–	34·0
Vitamin and mineral premixes†	25·0	25·0
Analytical composition		
DM (%)	92·2	92·5
CP (% DM)	50·9	51·3
Crude fat (% DM)	29·7	29·5
Gross energy (kJ/g DM)	26·4	26·1
Fatty acid composition (g/100 g total fatty acids)		
14 : 0	9·9	2·0
16 : 0	13·4	19·5
18 : 0	1·5	2·7
Total saturates	26·0	25·4
16 : 1	10·0	1·6
18 : 1	14·4	39·9
20 : 1	16·6	2·0
22 : 1	10·9	1·6
Total monounsaturates	52·5	45·2
18 : 2*n*-6	3·6	13·7
20 : 2*n*-6	0·2	0·06
20 : 4*n*-6	0·2	0·06
Total *n*-6 PUFA	6·5	14·4
18 : 3*n*-3	0·8	10·0
18 : 4*n*-3	2·3	0·7
20 : 4*n*-3	0·3	0·1
20 : 5*n*-3	4·5	1·5
22 : 5*n*-3	0·3	0·1
22 : 6*n*-3	3·9	1·6
Total *n*-3 PUFA	12·2	14·0

FO diet, fish oil diet; VO, vegetable oil diet; CP, crude protein.
* For further details, see Richard *et al.*[18]. Diets were produced at Nutreco Technology Centre, Norway as extruded pellets.
† Mineral and vitamin premix according to National Research Council recommendations.

Chemical composition of the diets

The experimental diets were analysed using the following procedures. DM was determined after drying at 105°C for 24 h. Gross energy was determined using an adiabatic bomb calorimeter (IKA; Heitersheim Gribheimer, Germany). Protein content (N × 6·25) was determined by the Kjeldahl method after acid digestion. Total lipid content was determined by the method of Folch *et al.*[19], after extraction by dichloromethane rather than chloroform. Fatty acid composition of the diets (and the whole body of fish fed with or without fish oil) was determined in the total lipid extract after acid-catalysed transmethylation as previously described[18]. Amino acid composition was determined after acid hydrolysis: amino acids were separated by ion-exchange chromatography using pH gradient elution followed by post-column derivatisation ninhydrin according to the method of Moore & Stein[20].

cDNA microarrays

Nylon microarrays were obtained from INRA-GADIE biological resources centre (Jouy-en-Josas, France; http://www-crb.jouy.inra.fr/). A total of 9023 rainbow trout cDNA originating from a pooled-tissue library[21] plus 193 controls were spotted after PCR amplification. PCR products were spotted onto Hybond N + membranes as described by Nguyen *et al.*[22]. Positive (plant luciferase cDNA depot) and negative (water depot) controls were also spotted on each microarray.

Hybridisation, scanning and quantification of microarrays

Total RNA were extracted from rainbow trout liver using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Four hepatic RNA samples corresponding to four individuals per

Table 2. Composition of the diets used in the fishmeal replacement studies*

	FM diet	PP diet
Ingredients (g/kg feed)		
Fishmeal (LT 94, Norway, CP 70 %)	472·20	–
White lupin (Cana, France, CP 41 %)	–	100·00
Maize gluten meal (CP 60 %)	–	150·00
Wheat gluten (CP 81 %)	–	250·40
Extruded whole wheat	361·00	94·00
Dehulled peas (Aquatex, France, CP 22 %)	–	25·00
Soyabean meal (CP 46 %)	–	120·00
L-Lysine (liquid form, Eurolysine, France)	–	17·20
Calcium monophosphate	–	35·70
Fish oil (Feedoil, Sopropèche, France)	159·50	200·50
Astaxanthin (Carophyll pink, DSM, France)	0·25	0·25
Vitamin and mineral premix (INRA, France)†	6·55	6·55
Betaine HCl	0·50	0·50
Analytical composition		
DM(%)	94·4	95·9
CP (% DM)	39·1	41·9
Crude fat (% DM)	26·5	28·4
Gross energy (kJ/g DM)	22·8	24·5
Amino acid composition (g/16 g N)		
Arginine	7·1	5·4
Histidine	2·6	2·3
Isoleucine	3·8	4·1
Leucine	7·3	9·1
Lysine	6·9	5·3
Methionine + cystine	3·8	3·7
Phenylalanine + tyrosine	7·3	8·6
Threonine	4·0	3·4
Tryptophan	1·1	0·9
Valine	4·7	4·5

FM diet, fishmeal diet; PP diet, plant-protein diet; CP, crude protein.
* Diets were produced at Le Gouessant Aquaculture, France as extruded pellets.
† Mineral and vitamin premix according to National Research Council recommendations.

dietary group were used for microarray hybridisation at INRA UMR1067 transcriptomic facility (St-Pée-sur-Nivelle, France) according to the following procedure. RNA quality was determined using an Agilent bioanalyser. A first hybridisation was performed at 42°C for 48 h using γ^{33}P-labelled T7 promoter oligonucleotide (5′-CACTATAGGGAATTTGGCC-3′) to estimate the amount of cDNA in each spot. After stripping (3 h at 68°C, 0.1X SSC, 0·2 % SDS), hybridisations with hepatic cDNA were performed. Microarrays were prehybridised for 1 h at 65°C in hybridisation buffer (5X Denhardt, 5X SSC, 0·5 % SDS). Labelled cDNA were prepared from 5 μg RNA by simultaneous reverse transcription and labelling for 1 h at 42°C in the presence of 1·85 MBq (50 μCi) [α- 33P] dCTP, 5 μM-cold dCTP and 800 μM each of dATP, dGTP and dTTP and 200 units SuperScript™ III RT (Invitrogen) in 30 μl final volume. A positive control corresponding to the luciferase mRNA (20 ng) (Promega, Madison, WI, USA) was simultaneously prepared. RNA was degraded by treatment at 68°C for 30 min with 10 % SDS (1 μl), 0·5 M-EDTA (1 μl) and 3 M-NaOH (3 μl) and then equilibrated at room temperature for 15 min. Neutralisation was done by adding 1 M-2-amino-2-hydroxymethyl-propane-1,3-diol-HCl (10 μl) and 2 M-HCl (3 μl). Microarrays were then incubated with the corresponding denatured labelled cDNA for 48 h at 65°C in hybridisation solution. After three washes (1 h at 68°C with 0.1X SSC, 0·2 % SDS), microarrays were exposed for 65 h to phosphor-imaging plates that were scanned using a Fuji BAS-5000 (Fuji, Tokyo, Japan). Signal intensities were quantified using AGScan software (bioinformatic platform Sigenae; INRA; http://www.sigenae.org/)[23,24].

Microarray data analysis

Data microarrays were deposited in BioArray Software Environment (BASE) database[25], a 'minimum information about a microarray experiment' (MIAME) supportive customisable database available at the bioinformatic platform Sigenae. Signal processing was performed using vector oligonucleotide data to correct the relative amount of DNA present in each spot. At this step, low nucleotide signals (less than three times the background level) were excluded from the analysis. After correction, the signal was normalised by dividing each gene expression by the median value of the array before log transformation. Data were subsequently analysed using statistical TIGR Multiple Experiment Viewer software (TMEV; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA), which is a suite of microarray data analysis applications. Variation of gene expressions between two dietary treatments was termed significant when the P value was $<0{\cdot}01$ using the two-sample t test for microarrays[26] and followed by supervised hierarchical clustering for significant genes only. Organisation of genes for biological interpretation in the context of gene ontology was performed using GoMiner software (Genomics and Bioinformatics Group, National Institutes of Health, Bethesda, MD, USA; http://discover.nci.nih.gov/gominer/)[27].

Data mining

Rainbow trout sequences originating from INRA Agenae[21] and the US Department of Agriculture[28] and expressed sequence tag (EST) sequencing programs were used to generate publicly available contigs (http://public-contigbrowser.sigenae.org:9090/index.html). The 4th version (om.4) was used for BlastX (version 4 (om.4); Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/) comparison. The score of each alignment was retrieved after performing a BlastX comparison.

Real-time RT-PCR

Gene expression levels were determined by real-time RT-PCR using six RNA including those used for microarray analysis. Total RNA (1 μg) was reverse transcribed to cDNA with the Superscript™ III RNAse H RT kit (Invitrogen, Carlsbad, CA, USA) using oligo dT primers. Real-time PCR was performed in the iCycler iQ™ (Bio-Rad, Hercules, CA, USA). Quantitative PCR analyses for gene expressions were performed on 10 μl of the RT reaction mixture using the iQ™ SYBR® Green Supermix (Bio-Rad). The total volume of the PCR reaction was 25 μl containing 200 nM of each primer. Primers were designed so that they were overlapping an intron when it was possible (Primer3 software; http://biotools.umassmed.edu/bioapps/primer3_www.cgi) using known sequences in nucleotide databases (Table 3).

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Table 3. Primer pairs for analysis by real-time PCR of selected genes*

Gene	Name	5′–3′ forward primer	Name	5′–3′ reverse primer
Elongation factor 1α	EF17/8	5′-TCCTCTTGGTCGTTTCGCTG-3′	EF19	5′-ACCCGAGGGACATCCTGTG-3′
Experiment with fish oil replacement				
Cathepsin B	CATB-3	5′-TGGGCCTGTAGAGGGTGCTT-3′	CATB-4	5′-CCACCATCTCCGACTCGATG-3′
Fatty acid synthase	FAS-1	5′-TGATCTGAAGGCCCGTGTCA-3′	FAS-2	5′-GGGTGACGTTGCCGTGGTAT-3′
Ubiquinol cytochrome c-reductase protein 2†	UCR-2	5′-TGGGCCTGTAGAGGGTGCTT-3′	UCR-2	5′-AGGGGATTCGCGAATAAGT-3′
CYP1A3/A1	1A3-3	5′-TTTGTCATGGGCTGTTGTGT-3′	1A3-4	5′-CGGAAGATCTCCAGGATGAA-3′
CYP3A27	3A27-3	5′-ATCTTCGCCGGCTACGAGAC-3′	3A27-4	5′-CGTCTTCTTGGCGACCCTCT-3′
Experiment with fishmeal replacement				
Ribosomal protein 60S L27	RL27-1	5′-CACAACCATGGGCAAGAAGA-3′	RL27-2	5′-TCAGGGCAGGGTCTCTGAAG-3′
Ribosomal protein 40S L35	RL35-1	5′-TCGCAAGTTCGCTGTCAAGG-3′	RL35-2	5′-ACGGTGTACCGCGTTCCAAT-3′
Ribosomal protein 40S S6	RS16-3	5′-TTTCAGGTGGCGAAACATGC-3′	RS16-4	5′-GGGGTCTGCCATTCACCTTG-3′
Ribosomal protein 40S S7	RPS-1	5′-GCGAAAAGCCAGATGAGTTC-3′	RPS-3	5′-CCGCTGAACTTCTTTTCCAG-3′
Glutamine synthetase	GLNA-5	5′-AACGCAAGCCTGCAGAAAC-3′	GLNA-6	5′-GGGACCAGGGAAGCCGTTAG-3′
Glutamine synthetase GS01 (AF390021)	GS01-1	5′-CTGCAGTCTGTGTGTTCAGGGTAGA-3′	GS01-2	5′-CATCTGTCTGGAATTGTTAAGTCCATA-3′
Glutamine synthetase GS02 (AF390022)	GS02-1	5′-GGCAGTGTCTTTAAATGGCAACA-3′	GS02-2	5′-ACGCTACAATTGGCAAGACTGA-3′
Glutamine synthetase GS03 (AF390023)	GS03-1	5′-GTGTATCAATTTGCTACTCATGTTTAACAT-3′	GS03-2	5′-AAAATGGGTTCTTGATACAACTTCTACTAA-3′

*Nucleotide sequences were extracted from the Sigenae database (http://www.sigenae.org/) except for GS01, GS02 and GS03 from Genbank[45].
†Reverse transcription with random primers.

Thermal cycling was initiated with the incubation at 95°C for 90 s for hot-start iTaq™ DNA polymerase activation. Thirty-five steps of PCR were performed, each one consisting of heating at 95°C for 20 s for denaturing, and at 59°C for 30 s for annealing and extension. Following the final cycle of the PCR, melting curves were systematically monitored (with a gradient of 0·5°C per 10 s from 55 to 94°C) to ensure that only one fragment was amplified. Samples without RT and samples without RNA were run for each reaction as negative controls.

Data analysis

Data are presented as mean values and standard deviations. We analysed the effects of the different diets with an unpaired two-tailed Student's *t* test (Systat 9 software products; SPSS, Inc., Chicago, IL, USA), except for microarray data (see before) and quantitative RT-PCR data. For the latter, significant differences were considered at $P<0{\cdot}05$. Relative quantification of the target gene transcript with the *ef1α* reference gene transcript[29] was made following the Pfaffl method with the Relative Expression Software tool (REST©)[30,31]. This mathematical algorithm computes an expression ratio, based on real-time PCR efficiency and the crossing point deviation of the unknown sample *v.* a control group:

$$R = ((E_{\text{target gene}})^{\Delta CT(\text{mean control}-\text{mean unknown sample})}_{\text{target gene}})/$$

$$((E_{EF1\alpha})^{\Delta CT(\text{mean control}-\text{mean unknown sample})}_{EF1\alpha}),$$

where E is PCR efficiency determined by standard curves using serial dilution of cDNA (cDNA dilutions from 1/16 up to 1/512), ΔCT being the crossing point deviation of an unknown sample *v.* a control. Statistical differences in gene expression between control and sample were evaluated in group means by randomisation tests[31] using REST© software. A total of 2000 random allocations were performed and significant differences were considered at $P<0{\cdot}05$.

Results

Fish rearing and endpoint analysis: growth rate, feed efficiencies and whole-body composition

In the fish fed with fish oil and vegetable oils, growth performance was similar throughout the long-term study irrespective of dietary treatment; at the end of the growth study the two groups reached a final body weight of about 1 kg (see Richard *et al.*[18] for further details). No differences were found in feed efficiency, protein feed efficiency and feed intake (Table 4). As it is well known that replacement of fish oil by vegetable oils may produce major changes in fatty acid composition of fish, we also analysed the whole-body fatty acid composition for the fish oil- and vegetable oil-fed fishes (Table 5); the whole-body fatty acid composition reflected largely the composition of the diet (Table 1). The fish fed vegetable oils exhibited the highest levels of 18 : 1, 18 : 2*n*-6 and 18 : 3*n*-3, whereas the fish fed the fish oil diet had a very high percentage of long-chain MUFA (14·1 % for 20 : 1 and 6·9 % for 22 : 1) and also the highest proportion of EPA and DHA (2·4 and 5·2 % respectively). In contrast to the fish fed with vegetable oils, trout fed with plant proteins had

Table 4. Effects of fish oil replacement on growth performance and feed efficiency in juvenile rainbow trout over 62 weeks (initial body weight (IBW) 0·120 g)*

(Mean values and standard deviations for three tanks)

	FO diet		VO diet	
	Mean	SD	Mean	SD
FBW (g)	1011	40	1019	63
Daily growth index†	1·85	0·08	1·85	0·15
Feed efficiency‡	0·95	0·04	0·95	0·08
Protein efficiency ratio§	1·86	0·07	1·86	0·15

FO diet, fish oil diet; VO, vegetable oil diet; FBW, final body weight.
* There was no significant difference between the groups ($P>0·05$; Student's t test).
† Daily growth index = $100 \times (FBW^{1/3} - IBW^{1/3})$/duration (49 d).
‡ Feed efficiency = wet weight gain (g)/dry feed intake (g).
§ Protein efficiency ratio = wet weight gain (g)/crude protein intake (g).

Table 6. Effects of fishmeal replacement on growth performance and feed efficiency in juvenile rainbow trout over 52 weeks (initial body weight (IBW) 0·215 g)

(Mean values and standard deviations for three tanks)

	FM diet		PP diet		
	Mean	SD	Mean	SD	P*
FBW (g)	775	26	659	24	0·005
Daily growth index†	2·35	0·02	2·22	0·03	0·003
Feed efficiency‡	1·06	0·02	0·95	0·03	0·01
Protein efficiency ratio§	2·71	0·06	2·28	0·07	0·002

FM diet, fishmeal diet; PP diet, plant-protein diet; FBW, final body weight.
* By Student's t test.
† Daily growth index= $100 \times (FBW^{1/3} - IBW^{1/3})$/duration (49 d).
‡ Feed efficiency = wet weight gain (g)/dry feed intake (g).
§ Protein efficiency ratio = wet weight gain (g)/crude protein intake (g).

significantly lower growth rates, lower feed efficiency and lower protein feed efficiency than fish fed with fishmeal ($P<0·05$; Student's t test) (Table 6), even though the feed intake was higher in the fish fed vegetable proteins. These data suggest a low capacity of metabolic adaptation of the fish fed with plant proteins from first feeding.

Differentially expressed genes in the liver of rainbow trout linked to diet composition

Analysis of microarray data showed that almost 0·8 % of genes were differentially expressed in our two experimental comparisons: (i) between the fish fed fish oil and vegetable oils, and (ii) between the fish fed fishmeal and plant proteins (Tables 7–10) ($P<0·01$, t test; TMEV). Among the seventy-one genes differentially expressed between the fish fed with fish oil and those fed with vegetable oils, sixteen and fifty-five hepatic transcripts exhibited, in the fish fed with vegetable oils, higher and lower abundance respectively (Tables 7 and 8). Moreover, among the seventy-five genes differentially expressed between the fish fed with fishmeal and those fed with plant protein, fifteen and sixty hepatic transcripts exhibited respectively higher or lower abundance in the fish fed with plant proteins (Tables 9 and 10).

Table 5. Effect of fish oil replacement on mean fatty acid composition of whole-body lipids after feeding the experimental diets for 62 weeks

Fatty acid composition (g/100 g total fatty acids)	FO diet (n 3)	VO diet (n 3)
14 : 0	7·93	1·80*
16 : 0	16·03	17·26
18 : 0	2·08	2·91
Total saturates	26·97	22·70*
16 : 1	9·45	2·41*
18 : 1	21·56	41·41*
20 : 1	14·11	3·39*
22 : 1	6·85	1·63*
Total monounsaturates	52·26	48·93*
18 : 2*n*-6	4·48	12·78*
20 : 2*n*-6	0·29	0·60
20 : 4*n*-6	0·16	0·16
Total *n*-6 PUFA	5·04	13·72*
18 : 3*n*-3	1·03	6·97*
18 : 4*n*-3	1·12	0·75
20 : 4*n*-3	0·70	0·54
20 : 5*n*-3	2·38	0·97*
22 : 5*n*-3	0·64	0·36
22 : 6*n*-3	5·16	3·43*
Total *n*-3 PUFA	11·20	13·57*

FO diet, fish oil diet; VO, vegetable oil diet.
* Mean value was significantly different from that of the fish fed the FO diet ($P<0·05$).

Biological significance of the results: data clustering and gene ontology

We researched the significance of our data analysing some genes expressed in the clusters linked to specific biological process (Figs. 1 and 2). First, for the fish fed with or without fish oil, we observed three main clusters after analysing gene expression (Fig. 1 (a)). Cluster I was composed of numerous genes playing key roles in lysosomal and proteasomal proteolysis (cathepsins B and D, subunit of proteasomes) which were more expressed in the fish fed with vegetable oils ($P<0·01$, t test; TMEV). We found also in this cluster one gene, CYP1A3, involved in xenobiotic metabolism. Cluster II was found to be mainly composed of genes involved in intermediary metabolism especially energy metabolism (succinate dehydrogenase, ubiquinol dehydrogenase) and lipid metabolism (fatty acid synthase (FAS) and long-chain fatty acid elongase). In this cluster, some genes also play roles in mRNA processing (splicing, maturation) (for details see Fig. 1 (b)). All the genes in cluster II were expressed at a lower level in the fish fed with vegetable oils ($P<0·01$, t test; TMEV). Finally, cluster III was mainly composed of genes involved in cell growth and maintenance (keratin, kindlin, actin): they were expressed at a higher level in the fish fed with vegetable oils than in those fed the fish oil-based diet ($P<0·01$, t test; TMEV). Overall, after dietary fish oil replacement by a blend of vegetable oils, a number of genes involved in lipid metabolism (lipogenesis, steroid synthesis, xenobiotic detoxification), protein catabolism, and transcription regulation were detected by gene ontology analysis (Table 11).

Second, for the fish fed with plant proteins and fishmeal, four clusters were detected (Fig. 2 (a)). Whereas cluster I was composed of miscellaneous genes, cluster III was linked

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Table 7. Hepatic transcripts exhibiting higher abundance in fish fed with vegetable oils after microarray analysis*

Clone name	Sigenae contig†	Swissprot-hit description	*P*
tcay0031b.c.17	tcay0025b.p.18_3.1.om.4	FXN2_HUMAN Human T-cell leukemia virus enhancer factor	$6{\cdot}9 \times 10^{-4}$
tcba0021c.m.22	tcay0011b.k.02_5.1.om.4	ZN41_HUMAN Zinc finger protein 41	0·001
1RT149O15_A_H08	15021467.1.om.4	SNXN_HUMAN Sorting nexin 23	0·002
tcay0011b.d.05	15029633.1.om.4	AR1A_HUMAN Actin-related protein 2/3 complex subunit 1A	0·004
tcak0001a.e.11	tcad0004a.b.23_3.1.om.4	IF39_HUMAN Eukaryotic translation initiation factor 3 subunit 9	0·006
tcbk0041c.m.02	tcbk0041c.m.02_5.1.om.4	DLX6_BRARE Homeobox protein DLX-6	0·006
tcac0002c.h.01	AF267126.1.om.4	CP3R_ONCMY Cytochrome P450 3A27 (*EC* 1.14.14.1)	0·007
tcay0038b.c.08	tcay0036b.l.02_3.1.om.4	CSP7_HUMAN Cofactor required for Sp1 transcriptional activation subunit 7	0·007
1RT88C17_A_B09	15026549.1.om.4	MPP5_HUMAN MAGUK p55 subfamily member 5	0·007
tcbk0019c.d.07	tcbk0019c.d.07_5.1.om.4	UNKNOWN	0·007
tcbk0036c.i.08	tcay0036b.p.22_5.1.om.4	LPB1_HUMAN Liprin-beta 1	0·007
tcad0001a.b.13	tcad0001a.b.13_5.1.om.4	CRBA_DROME Cyclic-AMP response element binding protein A	0·008
tcbk0026c.f.15	tcay0015b.j.03_3.1.om.4	K1CR_XENLA Keratin	0·008
tcbk0003c.k.15	tcbk0003c.k.15_5.1.om.4	YB85_YEAST Hypothetical 124·0 kDa protein in PBP2-ABD1 intergenic region	0·009
tcay0031b.d.23	tcay0031b.d.23_3.1.om.4	URP1_MOUSE Unc-112 related protein 1 (Kindlin-1) (Fragment)	0·009
tcbk0026c.d.10	17236272.2.om.4	MUSC_HUMAN Musculin	0·009

* Where $P<0{\cdot}01$ by *t* test (TIGR Multiple Experiment Viewer software; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA). The sixteen genes are classified following the *P* values.
† http://public-contigbrowser.sigenae.org:9090/index.html

to intermediary metabolism, especially energy metabolism (quinone oxidoreductase, ubiquinol reductase), amino acid metabolism (arginase) and amino acid transport. Cluster II and cluster IV, even though also associated with metabolism, were focused on protein metabolism respectively with proteolytic (cathepsin, proteasome) and proteosynthetic (eight ribosomal proteins) pathways (for details, see Fig. 2 (b)). Clusters II, III and IV were composed of genes that were expressed much less in the fish fed with plant proteins ($P<0{\cdot}01$, *t* test; TMEV). Globally, the replacement of fishmeal by the plant protein sources indeed leads to a large number of genes involved in protein and amino acid metabolism being revealed by the gene ontology analysis (Table 12).

Focus on specific differentially expressed genes

We focused the present study on specific genes that can illustrate the major pathways modified by the diet variation on rainbow trout liver. In the context of dietary fish oil replacement, five genes involved in proteolysis (cathepsin), energy metabolism in mitochondria (ubiquinol cytochrome c reductase), lipid metabolism (FAS and long-chain fatty acid CoA ligase) and detoxification metabolism (cytochromes P450) have been selected (Table 13). Using six individuals per dietary group and the quantitative RT-PCR, except for the cathepsin D, we confirmed that cathepsin B, ubiquinol cytochrome c reductase, FAS and cytochrome P4501A3 were expressed less in the fish fed with vegetable oils ($P<0{\cdot}05$; REST test). Moreover, we also checked that the cytochrome P4503A4 was more highly expressed in the liver of the fish fed with vegetable oils ($P<0{\cdot}05$; REST test). Also, in the context of dietary fishmeal replacement, genes involved in proteosynthesis (ribosomal proteins) and amino acid metabolism (glutamine synthetase) were selected to be analysed by quantitative RT-PCR (Table 14). We confirmed by quantitative RT-PCR that the expression of the glutamine synthetase gene was lower in the livers of the fish fed with plant proteins than in those fed fishmeal ($P<0{\cdot}05$; REST test): we analysed more precisely three glutamine synthase paralogous genes by specific amplification of the isoforms and we confirmed that GS01 and GS03 gene expressions were depressed by almost 7-fold in these fish. However, all the analysed genes coding for ribosomal proteins (four genes coding for ribosomal proteins of the 40S (6S and 7S) and 60S (L27 and L35) subunits) were not differentially expressed ($P>0{\cdot}05$; REST test): these latter results did not confirm the microarray data.

Discussion

Overview

In the present study, the substitution of dietary fishmeal by vegetable proteins seems to have some adverse consequences on fish growth whereas no differences were found in fish fed vegetable oils as previously observed[13,14,18]. This is the first ever set of nutrigenomics data in fish fed diets devoid of either fish oil or fishmeal. Analysis of the hepatic transcriptomes revealed that less than 100 genes were differentially expressed between all the nutritional conditions ($<1\,\%$), which is a relatively low number of differentially expressed genes. Two possible explanations are (i) that the cDNA microarray used in the present study is a generic rainbow trout cDNA tool (issue from 9023 cDNA extracted from different tissues at different developmental stages[21]), and not a specific rainbow trout liver cDNA microarray; and (ii) that although the diets differed in terms of ingredients (marine *v.* plant origins), they were not drastically different in terms of proximate composition (Tables 1–3). It is also interesting to note that when we analysed globally the data about differentially expressed genes, we observed no common genes between the two sets of experiments, with enrichment of differentially expressed genes in lipid metabolism and protein metabolism after fish oil and fishmeal replacements respectively. This suggests that the modification of liver transcriptomes was highly dependent of the origin of the ingredient, i.e. vegetable oil and plant proteins. Thus, in the following discussion, we will analyse separately the data from the two nutritional experiments by focusing on specific molecular actors.

Table 8. Hepatic transcripts exhibiting lower abundance in fish fed with vegetable oils after microarray analysis*

Clone name	Sigenae contig†	Swissprot-hit description	*P*
1RT121N04_D_G02	15060218.1.om.4	MAFK_MOUSE Transcription factor MafK	$1{\cdot}36 \times 10^{-4}$
1RT156K02_C_F01	15078821.1.om.4	ROAA_MOUSE Heterogeneous nuclear ribonucleoprotein A/B	$2{\cdot}07 \times 10^{-4}$
1RT67M18_C_G09	tcad0003a.n.17_3.1.om.4	DHSB_HUMAN Succinate dehydrogenase [ubiquinone] iron-sulfur protein	$3{\cdot}26 \times 10^{-4}$
tcba0008c.g.18	tcba0008c.g.18_5.1.om.4	SYN_HUMAN Asparaginyl-tRNA synthetase	$3{\cdot}84 \times 10^{-4}$
tcba0003c.g.01	tcba0003c.g.01_5.1.om.4	TERA_RAT Transitional endoplasmic reticulum ATPase	$4{\cdot}03 \times 10^{-4}$
1RT77M23_A_G12	15025059.1.om.4	SGK3_HUMAN Serine/threonine-protein kinase Sgk3 (*EC* 2.7.1.37)	$6{\cdot}54 \times 10^{-4}$
1RT80C06_C_B03	15026055.1.om.4	ANXD_HUMAN Annexin A13	0·001
tcag0002b.n.03	AF059711.1.om.4	CP13_ONCMY Cytochrome P450 1A3 (*EC* 1.14.14.1)	0·001
tcad0006a.f.22	tcad0006a.f.22_3.1.om.4	PRSX_HUMAN 26S protease regulatory subunit S10B (Proteasome subunit p42)	0·002
tcba0014c.h.05	tcay0018b.k.17_3.1.om.4	HS47_CHICK 47 kDa heat shock protein precursor (Collagen-binding protein 1)	0·002
tcab0001c.e.06	tcab0001c.e.06_5.1.om.4	FAS_CHICK Fatty acid synthase (*EC* 2.3.1.85)	0·002
1RT121J10_D_E05	15018967.1.om.4	UNKNOWN	0·003
1RT122N23_B_G12	15017668.1.om.4	LCF4_RAT Long-chain-fatty-acid–CoA ligase 4 (*EC* 6.2.1.3)	0·003
tcba0018c.g.23	tcba0018c.g.23_5.1.om.4	UNKNOWN	0·003
1RT108I09_A_E05	15061441.1.om.4	EGL1_HUMAN Egl nine homolog 1 (*EC* 1.14.11.-)	0·003
1RT34K04_C_F02	15076953.1.om.4	ELK3_MOUSE ETS-domain protein Elk-3	0·003
1RT114N22_D_G11	15066941.1.om.4	SARA_HUMAN GTP-binding protein SAR1a	0·004
1RT54E07_A_C04	15022193.1.om.4	UNKNOWN	0·004
1RT65M18_C_G09	15023915.1.om.4	T9S3_HUMAN Transmembrane 9 superfamily protein member 3 precursor	0·004
1RT106E04_C_C02	tcad0002a.a.16_3.1.om.4	PR39_YEAST Pre-mRNA processing protein PRP39	0·004
1RT158E21_A_C11	15089300.1.om.4	PUB1_SCHPO Ubiquitin–protein ligase pub1 (*EC* 6.3.2.-)	0·004
tcay0009b.b.09	tcay0023b.g.13_3.1.om.4	YCD1_HUMAN Hypothetical protein CGI-131	0·005
1RT160F10_D_C05	tcad0003a.l.14_5.1.om.4	KCY_PIG UMP-CMP kinase (*EC* 2.7.4.14)	0·005
1RT49M06_C_G03	15080981.1.om.4	YMN0_YEAST Hypothetical 65·0 kDa protein in COX14-COS3 intergenic region precursor	0·005
1RT77N13_B_G07	15025133.1.om.4	PGS2_HORSE Decorin precursor	0·005
1RT78F08_D_C04	15025579.1.om.4	UNKNOWN	0·005
1RT85P10_D_H05	15015655.1.om.4	TBA_ONCKE Tubulin alpha chain	0·005
1RT121P18_D_H09	tcad0002a.f.16_3.1.om.4	NFX1_HUMAN Transcriptional repressor NF-X1 (*EC* 6.3.2.-)	0·005
1RT121E14_C_C07	15063507.1.om.4	THA1_MOUSE THAP domain protein 1	0·006
1RT146D15_B_B08	tcay0029b.b.09_5.1.om.4	HS47_CHICK 47 kDa heat shock protein precursor	0·006
1RT116G12_C_D06	15026944.1.om.4	SMD2_HUMAN Small nuclear ribonucleoprotein Sm D2	0·006
1RT95I04_C_E02	tcad0007a.c.03_3.1.om.4	VATL_HUMAN Vacuolar ATP synthase 16 kDa proteolipid subunit (*EC* 3.6.3.14)	0·006
tcbk0025c.b.12	tcbk0004c.f.11_5.1.om.4	FAS_ANSAN Fatty acid synthase (*EC* 2.3.1.85)	0·006
1RT130F18_D_C09	6187098.1.om.4	TCPB_HUMAN T-complex protein 1	0·006
tcay0001b.e.11	tcay0028b.l.18_3.1.om.4	DD15_MOUSE Putative pre-mRNA splicing factor RNA helicase	0·006
1RT80E07_A_C04	tcav0003c.j.08_3.1.om.4	YE6A_SCHPO Hypothetical protein C6G10.10c in chromosome I	0·006
1RT135H16_D_D08	15029400.1.om.4	PNPO_MOUSE Pyridoxine-5′-phosphate oxidase (*EC* 1.4.3.5)	0·006
tcad0001a.i.06	tcad0001a.i.06_3.1.om.4	RSP6_CHLRE Flagellar radial spoke protein 6	0·007
tcad0006a.g.21	tcad0006a.g.21_3.1.om.4	GCSP_MOUSE Glycine dehydrogenase	0·007
1RT101L17_B_F09	tcay0032b.m.12_3.1.om.4	NEK1_HUMAN Serine/threonine-protein kinase Nek1 (*EC* 2.7.1.37)	0·007
1RT138J19_B_E10	15064382.1.om.4	HP28_RAT 28 kDa heat- and acid-stable phosphoprotein	0·007
1RT103A16_C_A08	tcad0003a.m.01_3.1.om.4	CATD_CLUHA Cathepsin D precursor (*EC* 3.4.23.5)	0·007
1RT126E19_A_C10	15019695.1.om.4	KLF4_MOUSE Kruppel-like factor 4	0·008
1RT148G07_A_D04	15015121.1.om.4	SYQ_HUMAN Glutaminyl-tRNA synthetase (*EC* 6.1.1.18)	0·008
1RT151H09_B_D05	15021715.1.om.4	ACS1_RHIME Acetyl-coenzyme A synthetase 1 (*EC* 6.2.1.1)	0·008
1RT132N07_B_G04	tcay0027b.l.03_3.1.om.4	PGBM_HUMAN Basement membrane-specific heparan sulfate proteoglycan core protein precursor	0·008
1RT140L18_D_F09	15020168.1.om.4	SHO2_HUMAN Leucine-rich repeat protein SHOC-2	0·008
1RT75D05_B_B03	15024751.1.om.4	G2A_MOUSE Lysophosphatidylcholine receptor G2A	0·009
tcbk0032c.i.16	tcav0001c.d.12_5.1.om.4	UCR2_HUMAN Ubiquinol-cytochrome c reductase complex core protein 2	0·009
1RT108E12_C_C06	15020717.1.om.4	UNKNOWN	0·009
1RT116C04_C_B02	tcad0001a.p.06_3.1.om.4	CATB_CHICK Cathepsin B precursor (*EC* 3.4.22.1)	0·009
tcay0027b.m.22	tcay0020b.d.06_3.1.om.4	ACTT_FUGRU Actin	0·01
1RT62D24_D_B12	15023633.1.om.4	UNKNOWN	0·01
1RT41M15_A_G08	15077566.1.om.4	PUM1_MOUSE Pumilio homolog 1	0·01
tcac0002c.p.03	15014607.1.om.4	LCB1_HUMAN Serine palmitoyltransferase 1 (*EC* 2.3.1.50)	0·01

* Where $P < 0{\cdot}01$ by *t* test (TIGR Multiple Experiment Viewer software; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA). The fifty-five genes are classified following the *P* values.

† http://public-contigbrowser.sigenae.org:9090/index.html

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Table 9. Hepatic transcripts exhibiting higher abundance in fish fed with vegetable proteins after microarray analysis*

Clone name	Sigenae contig†	Swissprot-hit description	*P*
tcay0001b.g.18	AF281350.1.om.4	UNKNOWN	0·001
1RT120N05_B_G03	15062971.1.om.4	DTD_HUMAN Sulfate transporter	0·002
tcbk0031c.h.14	15015934.1.om.4	BRD2_HUMAN Bromodomain-containing protein 2	0·002
1RT120C23_A_B12	15062875.1.om.4	YQIK_BACSU Hypothetical protein yqiK	0·002
tcba0020c.o.23	tcba0020c.o.23_5.1.om.4	UNKNOWN	0·003
tcbk0052c.c.07	tcbk0052c.c.07_5.1.om.4	SMF1_HUMAN SWI/SNF-related	0·004
1RT151D10_D_B05	tcay0015b.p.08_3.1.om.4	PTPA_RABIT Protein phosphatase 2A	0·007
1RT90J08_D_E04	15016677.1.om.4	RBB5_HUMAN Retinoblastoma-binding protein 5	0·007
1RT42N11_B_G06	15079057.1.om.4	AGM1_HUMAN Phosphoacetylglucosamine mutase (*EC* 5.4.2.3)	0·009
1RT162N09_B_G05	15083591.1.om.4	LAT2_RAT Large neutral amino acids transporter small subunit 2	0·009
1RT35I10_C_E05	15077084.1.om.4	DYN1_HUMAN Dynamin-1 (*EC* 3.6.5.5)	0·009
tcba0023c.h.11	tcba0023c.h.11_5.1.om.4	UNKNOWN	0·009
tcbk0016c.a.07	tcbk0016c.a.07_5.1.om.4	ACTM_APLCA Actin	0·009
tcbk0021c.h.05	tcbk0021c.h.05_5.1.om.4	KELC_DROME Ring canal kelch protein	0·009
tcac0003c.h.09	tcac0003c.h.09_3.1.om.4	PAB5_ARATH Polyadenylate-binding protein 5	0·009

*Where $P<0·01$ by *t* test (TIGR Multiple Experiment Viewer software; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA). The fifteen genes are classified following the *P* values.
† http://public-contigbrowser.sigenae.org:9090/index.html

Gene profiling after fish oil replacement by vegetable oil mixture: focus on specific genes

We found that replacement of fish oil by vegetable oils has a major impact on lipid, energy and xenobiotic metabolism.

As regards key actors involved in intermediary metabolism, we analysed FAS, which is the key enzyme of fatty acid biosynthesis *in vivo*; this metabolic pathway is highly active in rainbow trout liver[32]. Two FAS cDNA were spotted on the microarrays and both of them were detected (and clustered) to be down-regulated in the fish fed with vegetable oils and confirmed by quantitative RT-PCR. FAS gene expression was 10-fold lower in the fish fed with vegetable oils. The lower level of hepatic FAS gene expression in the trout fed with vegetable oils could be explained by the diets based on vegetable oils having higher levels of linoleic (18 : 2*n*-6) and linolenic (18 : 3*n*-3) acids than the fish oil[18] and by linolenic acid known to decrease FAS mRNA levels[33]. The unambiguous finding in the present study of a significantly lower (10-fold) FAS gene expression in the fish fed with vegetable oils was not linked with a decrease of liver FAS activity in the same fish. This was even though FAS activity (IU/g liver) tended to be lower ($P=0·051$) for the fish fed vegetable oils (4·0 (SD 0·9)) compared with fish fed fish oil (6·1 (SD 1·8)) (see Richard *et al.* [18]). It cannot also explain the modification of whole-body fatty acid composition which reflected mainly the composition of the diets (Tables 1 and 5). Our data about FAS mRNA levels suggest once more that molecular data (measures of gene expression level) are not always associated with significant effects at the protein-metabolic pathway level.

The second actor is one involved in lysosomal proteolysis, which includes proteases such as cathepsin B[34]. Cell proteins are always in a dynamic equilibrium between synthesis and degradation depending on nutritional status[35]. Lower expression of the CATB gene in the trout fed with vegetable oils cannot be presently explained and it is difficult to provide any putative biological consequence of this observation. The third gene is the ubiquinol cytochrome c reductase (UCR) which is involved in mitochondrial metabolism, i.e. oxidative phosphorylation. We found that the expression of UCR was down-regulated in the fish fed with vegetable oils. This is in agreement with the data of Barzanti *et al.* [36] in rats, which showed a putative modification of UCR gene expression by dietary lipids.

Part of our analysis in the fish fed with vegetable oils dealt with the hepatic detoxification metabolism which is catalysed by a multi-enzyme family, namely the cytochromes P450s in rainbow trout[37]. These enzymes have generally a large spectrum of endogenous as well as exogenous substrates, and CYP1A members catalyse the biotransformation of environmental disruptors or pollutants such as polychlorinated biphenyls in the liver before their elimination. Because CYP1A genes are induced by the presence of their substrates[37], the lower expression of the CYP1A3 gene in the liver of the trout fed diets with vegetable oils could be due to the lower levels of pollutants such as dioxins in these diets. This result is not surprising given that the fish oil is susceptible to contamination with lipophilic organic chemicals that are now ubiquitous in the marine ecosystems and consequently in aquaculture systems[38,39]. Our own results (G Corraze, unpublished results) show that the muscle levels of dioxins and polychlorinated biphenyls are reduced in trout fed vegetable oils compared with those fed fish oils (WHO toxic equivalent: 1·96 pg/g and 1·08 pg/g for fish oil and vegetable oil groups, respectively). The CYP3A27 in rainbow trout which metabolises testosterone can be reduced by phyto-oestrogen; however, this cannot presently easily explain the higher CYP3A27 gene expression in the fish fed vegetable oils. Moreover, CYP3A27 also has some similarities with the human CYP3A4[40] which can convert cholesterol to 4-β-hydroxycholestrol before its elimination in bile salts[41]. Thus, the higher expression of the CYP3A27 gene in the rainbow trout fed with vegetable oils can be related to the lower level of plasma cholesterol observed in these fish (6·45 (SD 1·07) *v.* 3·97 (SD 0·23) g/l for fish fed fish oil and vegetable oils respectively; see Richard *et al.* [18]), suggesting that higher CYP3A27 activities can be the cause of the lower level of cholesterol in plasma. This needs further study to confirm and understand the link between fish oil replacement and the higher level of the CYP3A27 gene. Overall, the two hepatic

Table 10. Hepatic transcripts exhibiting lower abundance in fish fed with vegetable proteins after microarray analysis*

Clone name	Sigenae contig†	Swissprot-hit description	*P*
tcba0020c.j.10	tcav0004c.f.03_5.1.om.4	MTL2_MOUSE Methyltransferase-like protein 2 (*EC* 2.1.1.-)	$9{\cdot}7 \times 10^{-4}$
tcad0009a.k.02	tcad0009a.k.02_3.1.om.4	CRB1_CHICK Beta crystallin B1	$3{\cdot}2 \times 10^{-4}$
tcay0021b.b.07	tcay0021b.b.07_3.1.om.4	CPM1_ONCMY Cytochrome P450 2M1 (*EC* 1.14.14.1)	$3{\cdot}4 \times 10^{-4}$
tcay0011b.k.04	tcay0011b.k.04_5.1.om.4	CAO3_HUMAN Acyl-coenzyme A oxidase 3	$3{\cdot}4 \times 10^{-4}$
tcbi0009d.l.22	tcbi0009d.l.22_5.1.om.4	RL13_BRARE 60S ribosomal protein L13	0·001
tcay0023b.k.09	tcay0023b.k.09_3.1.om.4	MEPD_PIG Thimet oligopeptidase (*EC* 3.4.24.15) (Endopeptidase 24.15)	0·001
1RT148H10_D_D05	tcay0038b.p.14_5.1.om.4	SN24_HUMAN Possible global transcription activator SNF2L4	0·001
tcbk0002c.e.02	15012882.1.om.4	RL35_HUMAN 60S ribosomal protein L35	0·002
tcad0004a.f.19	tcad0004a.f.19_3.1.om.4	NIFU_RICPR NifU-like protein	0·002
1RT165A11_A_A06	tcaj0001a.a.12_5.1.om.4	U520_HUMAN U5 small nuclear ribonucleo protein 200 kDa helicase (*EC* 3.6.1.-)	0·002
tcba0008c.m.21	tcay0022b.h.16_3.1.om.4	SEP8_HUMAN Septin 8	0·003
tcac0003c.f.09	15077731.1.om.4	SFR2_MOUSE Splicing factor	0·003
tcba0011c.d.17	tcba0011c.d.17_5.1.om.4	KAP2_BOVIN cAMP-dependent protein kinase type II-alpha regulatory chain	0·003
tcac0002c.a.04	AF390023.1.om.4	GLNA_SQUAC Glutamine synthetase	0·003
tcba0018c.b.23	tcay0014b.h.22_3.1.om.4	UCR1_MOUSE Ubiquinol-cytochrome c reductase complex core protein I	0·003
tcab0002c.l.09	tcab0002c.l.09_5.1.om.4	RL27_HUMAN 60S ribosomal protein L27	0·004
tcbk0020c.f.18	tcay0014b.i.10_3.1.om.4	CN7B_SCHPO COP9/signalosome complex subunit 7B	0·004
tcal0001a.o.19	15029915.1.om.4	CCR4_RAT C-X-C chemokine receptor type 4	0·004
tcbk0058c.g.14	15060181.1.om.4	CWFF_SCHPO Cell cycle control protein cwf15	0·004
tcba0006c.l.19	tcay0018b.i.17_5.1.om.4	TFR1_CRIGR Transferrin receptor protein 1	0·004
tcbk0044c.l.01	15025792.1.om.4	RLA2_BRAFL 60S acidic ribosomal protein P2	0·005
tcab0002c.e.12	tcab0002c.e.12_5.1.om.4	YFW7_SCHPO Hypothetical protein PB2B4.07 in chromosome I	0·005
tcbk0051c.f.12	tcbk0005c.o.13_5.1.om.4	RS16_HUMAN 40S ribosomal protein S16	0·005
1RT119C21_A_B11	tcad0001a.m.03_3.1.om.4	CIA1_HUMAN WD-repeat containing protein Ciao 1	0·005
tcaa0002c.f.02	tcaa0002c.f.02_5.1.om.4	VATB_CHICK Vacuolar ATP synthase subunit B (*EC* 3.6.3.14)	0·005
tcay0012b.b.15	tcay0030b.e.18_3.1.om.4	CWFO_SCHPO Cell cycle control protein cwf24	0·005
tcam0002b.e.05	tcad0009a.e.24_3.1.om.4	SPRC_RAT SPARC precursor	0·005
tcad0008a.p.16	tcad0003a.i.20_5.1.om.4	IUNH_CRIFA Inosine-uridine preferring nucleoside hydrolase (*EC* 3.2.2.1)	0·005
tcbk0018c.n.07	tcay0017b.j.17_3.1.om.4	ACPM_MOUSE Acyl carrier protein	0·005
tcba0009c.f.23	tcba0009c.f.23_5.1.om.4	ABC1_HUMAN ATP-binding cassette	0·006
tcay0013b.g.06	15068153.1.om.4	SGTB_HUMAN Small glutamine-rich tetratricopeptide repeat-containing protein B	0·006
tcay0031b.j.13	tcay0018b.l.03_3.1.om.4	TPM4_HORSE Tropomyosin alpha 4 chain	0·006
tcay0008b.h.15	15073662.1.om.4	ARGI_HUMAN Arginase 1 (*EC* 3.5.3.1)	0·006
tcbk0053c.i.13	tcba0028c.p.14_5.1.om.4	INPP_MOUSE Inositol polyphosphate 1-phosphatase (*EC* 3.1.3.57)	0·006
tcad0002a.k.18	tcad0002a.k.18_5.1.om.4	RGS5_MOUSE Regulator of G-protein signaling 5 (RGS5)	0·006
tcbk0002c.l.10	tcay0025b.o.12_3.1.om.4	PSB7_RAT Proteasome subunit beta type 7 precursor (*EC* 3.4.25.1)	0·006
tcbk0036c.m.09	tcay0016b.p.23_3.1.om.4	M2OM_MOUSE Mitochondrial 2-oxoglutarate/malate carrier protein (OGCP)	0·006
tcbk0018c.b.15	15019342.1.om.4	SHP_MOUSE Orphan nuclear receptor SHP	0·007
tcay0028b.g.04	tcay0028b.g.04_3.1.om.4	IF2A_RAT Eukaryotic translation initiation factor 2 subunit 1 (eIF-2-alpha)	0·007
tcay0003b.g.15	15073721.1.om.4	QOR_HUMAN Quinone oxidoreductase (*EC* 1.6.5.5)	0·007
tcay0020b.j.18	tcay0020b.j.18_3.1.om.4	ICMT_XENLA Protein-S isoprenylcysteine O-methyltransferase (*EC* 2.1.1.100)	0·007
tcbk0024c.f.12	17239246.2.om.4	RL28_MOUSE 60S ribosomal protein L28	0·008
tcac0001c.i.22	tcac0001c.i.22_3.1.om.4	UNKNOWN	0·008
tcac0006c.n.11	15064666.1.om.4	DIM1_HUMAN Spliceosomal U5 snRNP-specific 15 kDa protein	0·008
tcbk0005c.c.21	tcbk0005c.c.21_5.1.om.4	CT20_HUMAN Protein C20orf20	0·008
tcbk0033c.o.02	tcbk0033c.o.02_5.1.om.4	PRTP_HUMAN Lysosomal protective protein precursor (*EC* 3.4.16.5) (Cathepsin A)	0·008
1RT102K01_A_F01	15017476.1.om.4	UNKNOWN	0·008
tcav0003c.h.07	tcav0003c.h.07_3.1.om.4	ARH7_HUMAN Rho guanine nucleotide exchange factor 7	0·008
tcba0001c.m.17	tcba0001c.m.17_5.1.om.4	KBTA_RAT Kelch repeat and BTB domain containing protein 10	0·009
tcac0006c.p.12	AF115536.1.om.4	TAP1_HUMAN Antigen peptide transporter 1 (APT1)	0·009
tcbk0022c.f.06	tcay0028b.c.20_3.1.om.4	P44_PANTR Non-A non-B hepatitis-associated microtubular aggregates protein (Antigen p44)	0·009
tcad0006a.e.03	tcad0002a.o.04_3.1.om.4	ARLL_HUMAN ADP-ribosylation factor-like protein 4L	0·009
tcba0002c.p.17	17244760.2.om.4	COXO_MOUSE Cytochrome c oxidase polypeptide VIIc	0·009
tcay0001b.i.04	tcay0016b.j.19_3.1.om.4	AR34_HUMAN ARP2/3 complex 34 kDa subunit (P34-ARC)	0·009
tcad0005a.c.22	tcad0005a.c.22_5.1.om.4	AATM_RAT Aspartate aminotransferase	0·009
tcbk0050c.m.05	tcay0018b.g.16_3.1.om.4	ASAH_HUMAN Acid ceramidase precursor (*EC* 3.5.1.23)	0·01
tcad0006a.d.14	tcad0006a.d.14_3.1.om.4	CB45_RAT 45 kDa calcium-binding protein precursor	0·01
tcbk0057c.p.24	tcay0027b.m.18_3.1.om.4	TAM2_HUMAN Translocation associated membrane protein 2	0·01
tcac0003c.k.15	15014990.1.om.4	PSA1_HUMAN Proteasome subunit alpha type 1 (*EC* 3.4.25.1)	0·01
tcay0039b.h.02	tcay0033b.m.11_5.1.om.4	RL2_GEOSL 50S ribosomal protein L2	0·01

* Where $P < 0{\cdot}01$ by *t* test (TIGR Multiple Experiment Viewer software; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA). The sixty genes are classified following the *P* values.
† http://public-contigbrowser.sigenae.org:9090/index.html

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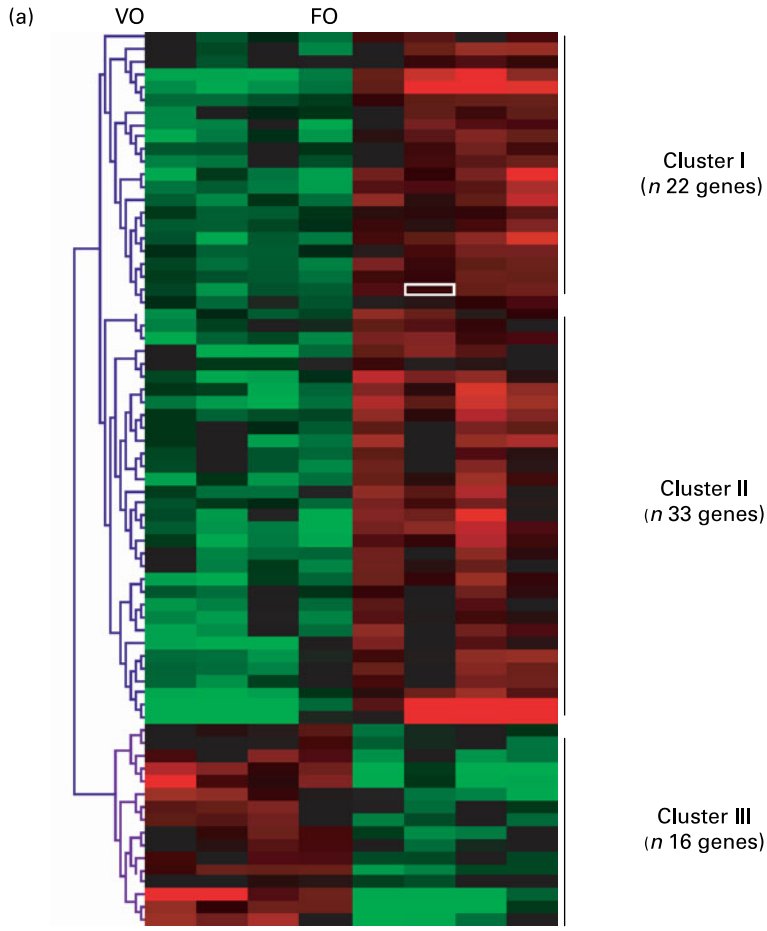

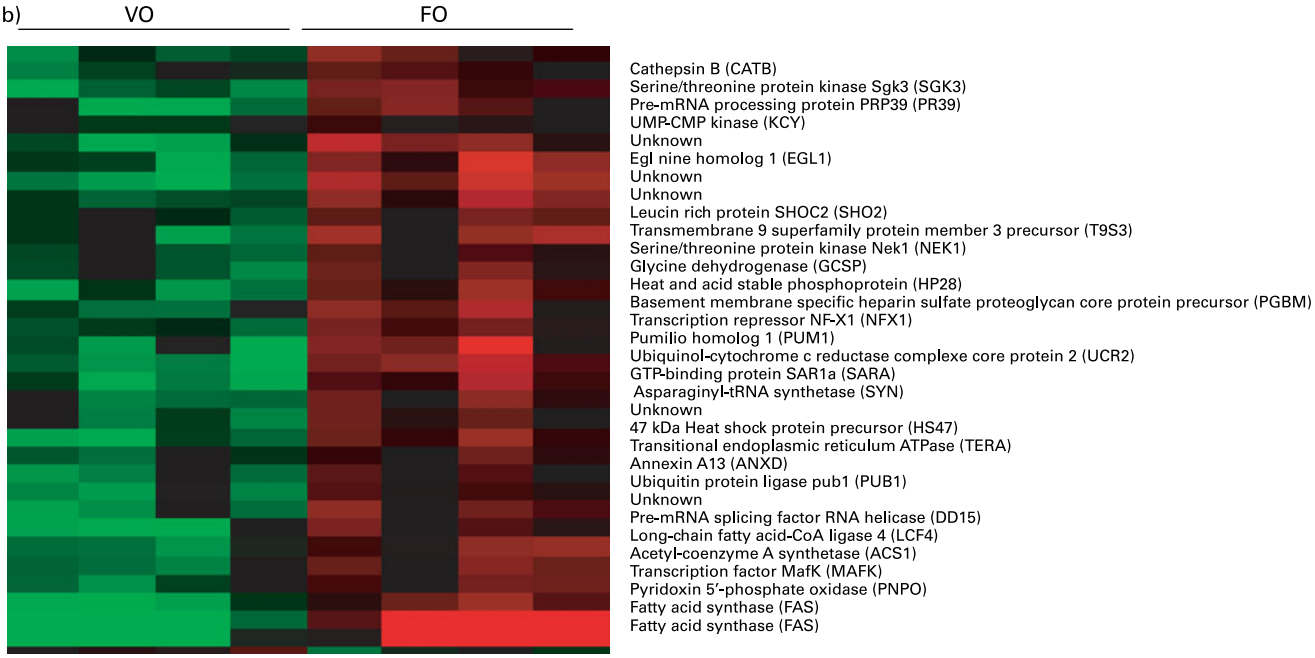

Fig. 1. (a) Hierarchical classification of differentially expressed genes between fish fed with or without fish oil (FO) ($P<0{\cdot}01$, *t* test, TIGR Multiple Experiment Viewer software; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA). Seventy-one hepatic genes were differentially expressed between the two dietary groups: sixteen were over-expressed (in red) and fifty-five were under-expressed (in green) in fish fed with vegetable oils (VO). (b) Detailed description of cluster II.

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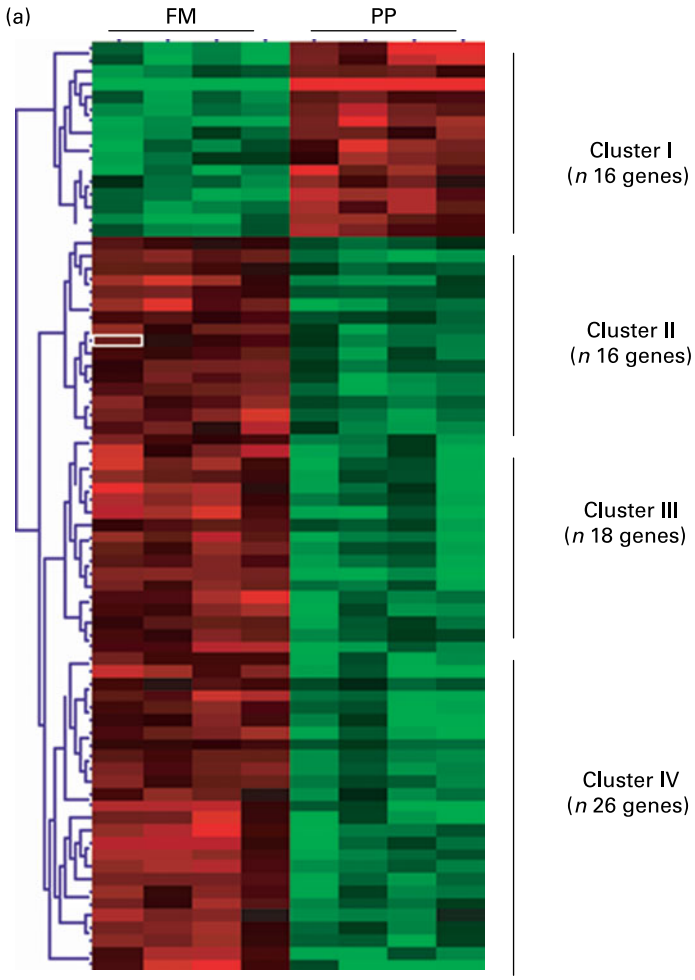

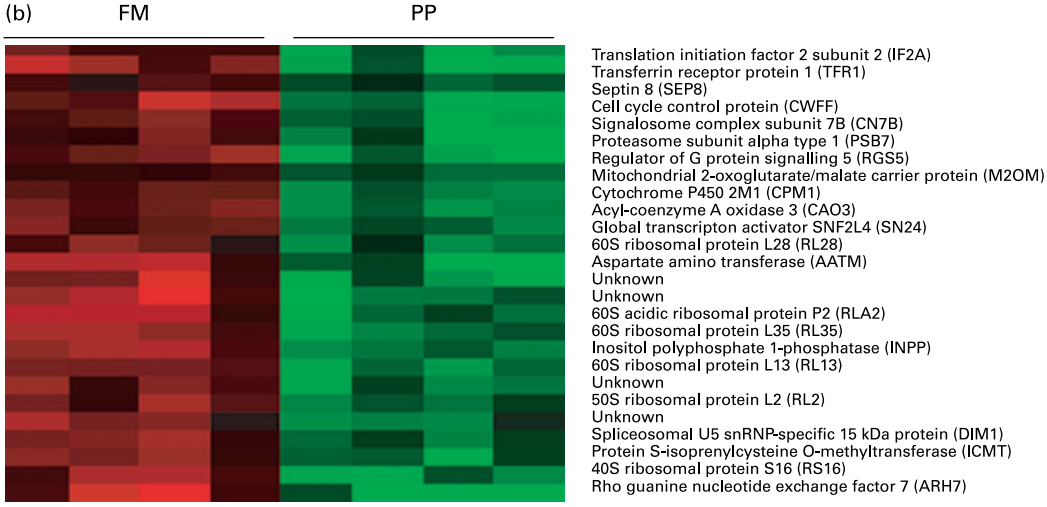

Fig. 2. (a) Hierarchical classification of differentially expressed genes between fish fed with or without fishmeal (FM) ($P<0{\cdot}01$, *t* test, TIGR Multiple Experiment Viewer software; The Institute for Genomic Research, J. Craig Venter Institute, Rockville, MD, USA). Seventy-five hepatic genes were differentially expressed between the two dietary groups: fifteen were over-expressed (in red) and sixty were under-expressed (in green) in fish fed with plant proteins (PP). (b) Detailed description of cluster IV.

Table 11. Major functional groups of hepatic transcripts exhibiting lower abundance in fish fed with vegetable oils after gene ontology analysis (GoMiner)*

Biological process	Function	best_swissprot_hit_accession number
Lipid metabolism	Fatty acid biosynthesis	FAS_CHICK
	Fatty acid biosynthesis	FAS_ANSAN
	Fatty acid metabolism	LCF4_RAT
	Sphingolipid metabolism	LCB1_HUMAN
	Fatty acid, steroid and xenobiotic oxidation	CP13_ONCMY
	Lipid biosynthesis (isoprenoid biosynthesis)	ACS1_RHIME
Protein catabolism	Protein metabolism	EGL1_HUMAN
	Protein repair	YCD1_HUMAN
	Proteolysis	CATD_CLUHA
	Proteolysis	UCR2_HUMAN
	Proteolysis	CATB_CHICK
	Proteolysis (ubiquitin mediated)	PUB1_SCHPO
	Protein catabolism	PRSX_HUMAN
	Amino acid metabolism (glycine, serine and threonine metabolism)	GCSP_MOUSE
Protein modification	Protein amino acid phosphorylation; mitosis	NEK1_HUMAN
	Protein amino acid phosphorylation	SGK3_HUMAN
	Protein biosynthesis	SYQ_HUMAN
	Protein folding	TCPB_HUMAN
Transcription regulation	Aminoacyl-tRNA biosynthesis	SYN_HUMAN
	mRNA splicing	SMD2_HUMAN
	mRNA splicing	PR39_YEAST
	mRNA splicing	DD15_MOUSE

* GoMiner software (Genomics and Bioinformatics Group, National Institutes of Health, Bethesda, MD, USA; http://discover.nci.nih.gov/gominer/).

cytochrome P450s described here can be proposed as molecular markers of dietary fish oil replacement by vegetable oils even though measures of enzymic activities will be necessary to confirm this status.

Gene profiling after fishmeal replacement by plant proteins: focus on specific genes

As the trout fed with plant proteins had significantly lower growth than the fish fed fishmeal, it was worth searching potential molecular actors to explain this phenotype, such as ribosomal proteins and glutamine synthetase.

A relatively high number of genes (*n* 8) coding for ribosomal proteins were detected in microarrays to be down-regulated in these fish. Moreover, we could classify them in the same cluster (IV) and considered that this set to be related to the lower growth of fish (for example, lower protein synthesis). Unfortunately, it was not possible to confirm these data by quantitative RT-PCR. We have no explanation to understand the discrepancy between the microarray and quantitative RT-PCR data. It is possible that these genes are false positive even though the data of microarrays seem unequivocal in humans (eight ribosomal genes with differential expression). An another explanation may be found in the specificity of the ribosomal genes: (i) there are more than seventy-nine ribosomal genes and, in trout, these are potentially in higher number due to pseudotetraploidy of the salmonids linked to a recent duplication of the genome[42,43] and (ii) all these genes are not under the same control of their expression[44]. Thus, it is possible that we have not analysed the most appropriate ribosomal protein genes by quantitative RT-PCR. Overall, we prefer to take these data about ribosomal proteins with caution even though the growth rate and feed efficiency (which are major endpoints of the present nutritional experiments linked to the proteosynthesis potential) were unambiguously lower in the fish fed plant proteins than the fish fed fishmeal ($P<0{\cdot}01$).

We found that the glutamine synthetase mRNA levels were lower in the fish fed with plant proteins. Indeed, the lower glutamine synthetase gene expression (8-fold) observed in microarrays seems to be due to the lower levels of the two isoforms of the glutamine synthetase, i.e. GS01 (7-fold) and GS03 (6·9-fold), two genes highly correlated with rising levels of ammonia in rainbow trout[45]. Glutamine synthetase catalyses the transformation of glutamic acid into glutamine, leading to the elimination of ammonia[46]. Rainbow trout do have an active ammonia detoxification system, and glutamine synthetase activity increases after a meal naturally rich in proteins[47] not only in the brain but also in the liver[45]. In the present study, the fishmeal replacement by plant proteins was associated with lower hepatic glutamine synthetase gene expression, thus potentially lowering the capacity of ammonia detoxification, possibly explaining the lower growth of these fish. However, the link between fishmeal replacement by plant proteins (naturally rich in glutamate) and lower glutamine synthetase gene expression is not clear and needs further study, especially at the level of enzyme activities.

Comparison with others nutrigenomic studies in fish

Very few studies have used nutrigenomics as a tool for the analysis of dietary fatty acids–gene interactions in aquaculture nutrition. Jordal *et al.* [48] found regulation of several individual genes (for example, Δ6-desaturase, peroxysome proliferator-activated receptor, mitochondrial genes) after replacement of fish oil by 75 % rapeseed oil in Atlantic salmon. We do not find any common differentially expressed genes between the two studies. Indeed, we do not observe any change in Δ6-desaturase gene expression following the microarray analysis (data not shown) in contrast to Jordal *et al.* [48], suggesting that the effects on gene profiling by vegetable oil replacement

Table 12. Major functional groups of hepatic transcripts exhibiting lower abundance in fish fed with vegetable proteins after gene ontology analysis (GoMiner)*

Biological process	Function	best_swissprot_hit_accession number
Lipid metabolism	Fatty acid biosynthesis; oxidative phosphorylation	ACPM_MOUSE
	Fatty acid metabolism (sphingolipid metabolism)	ASAH_HUMAN
	Fatty acid oxidation	CAO3_HUMAN
	Cholesterol metabolism	ABC1_HUMAN
Amino acid metabolism	Amino acid catabolism	AATM_RAT
	Amino acid metabolism	GLNA_SQUAC
	Amino acid metabolism	MTL2_MOUSE
	Amino acid metabolism	ARGI_HUMAN
Protein biosynthesis	Ribosome assembly	RLA2_BRAFL
	Ribosome assembly	RL27_HUMAN
	Ribosome assembly	CB45_RAT
	Ribosome assembly	RL28_MOUSE
	Ribosome assembly	RL35_HUMAN
	Ribosome assembly	RL13_BRARE
	Ribosome assembly	RL2_GEOSL
	Ribosome assembly	RS16_HUMAN
	Protein biosynthesis	PAB5_ARATH
	Translation initiation	IF2A_RAT
Protein transport	Intracellular protein transport	TAM2_HUMAN
	Intracellular protein transport	PRTP_HUMAN
	Intracellular protein transport	ICMT_XENLA
Transcription regulation	mRNA splicing	DIM1_HUMAN
	mRNA splicing	CWFO_SCHPO
	mRNA splicing	CWFF_SCHPO
	mRNA splicing	U520_HUMAN
	mRNA splicing	SFR2_MOUSE
	Transcription regulation	CT20_HUMAN
	Transcription regulation	SN24_HUMAN
Generation of metabolite precursors and energy	ATP biosynthesis	VATB_CHICK
	Oxidative phosphorylation (electron transport)	CPM1_ONCMY
	Oxidative phosphorylation (electron transport)	UCR1_MOUSE
	Oxidative phosphorylation, electron transport	COXO_MOUSE
	Protein catabolism (proteasome)	PSB7_RAT
	Protein catabolism (proteasome)	PSA1_HUMAN
	Purine salvage	IUNH_CRIFA
	Repair iron-sulfur proteins	NIFU_RICPR
Signal transduction	Signal transduction	KAP2_BOVIN
	Signal transduction	INPP_MOUSE
	Signal transduction	RGS5_MOUSE
	Signal transduction	ARLL_HUMAN

* GoMiner software (Genomics and Bioinformatics Group, National Institutes of Health, Bethesda, MD, USA; http://discover.nci.nih.gov/gominer/).

in fish may possibly vary with vegetable oil source and/or with fish species.

Concerning the analysis of dietary protein–gene interaction at the integrative level, an earlier study analysed the effect of dietary plant-protein substitution on hepatic metabolism using a proteomic approach[14]. The majority of the up-regulated proteins affected by the plant-protein diets were involved in energy production (NADPH, electron transferring flavoproteins). This is in contrast to data from the present study, in which lower expression levels of genes involved in energy

Table 13. Selected genes analysed by real-time PCR: effect of dietary fish oil (FO) replacement by vegetable oils (VO)*

Gene	Fold-regulation (in VO fish)†	*P*	CV (%) FO	CV (%) VO
Fatty acid synthase	−10·1	<0·02	2·5	18·4
Ubiquinol-cytochrome c reductase	−3·1	0·001	1·1	1·3
Cathepsin B	−2·3	<0·05	1·7	1·1
CYP1A (3A/A1)	−2·7	<0·02	4·2	2·5
CYP3A (A27)	+2·7	<0·005	2·9	2·3

* Statistical differences in gene expression were evaluated between group means (six samples per group) by randomisation tests using the Relative Expression Software tool (REST©)[23]. The transcript level of target genes was normalised with EF1α-expressed transcripts.

† Positive and negative regulation means that the target gene is expressed at a higher or lower level, respectively.

Table 14. Selected genes analysed by real-time PCR: effect of dietary fishmeal (FM) replacement by plant proteins (PP)*

Gene	Fold-regulation (in PP fish)†	*P*	CV (%) FM	CV (%) PP
Glutamine synthetase	−8·0	0·001	0·5	0·2
Glutamine synthetase GS03	−7·1	0·007	1·22	9·06
Glutamine synthetase GS02	+2·1	0·26	11·73	13·03
Glutamine synthetase GS01	−6·9	<0·02	3·85	5·14
Ribosomal protein L35	+1·4	0·42	1·9	4·4
Ribosomal protein L27	−1·1	0·48	1·0	1·0
Ribosomal protein S6	−1·4	0·08	0·2	0·9
Ribosomal protein S7	+1·4	0·08	1·0	1·6

* Statistical differences in gene expression were evaluated between group means (six samples per group) by randomisation tests using the Relative Expression Software tool (REST©)[23]. The transcript level of target genes was normalised with EF1α-expressed transcripts.
† Positive and negative regulation means that the target gene is expressed at a higher or lower level, respectively.

metabolism were found. However, we should recognise that comparison between transcriptomic and proteomic data is difficult. A recent study by Salem *et al.*[49] on the hepatic gene expression profiles between fasted and fed rainbow trout showed an inhibition of protein synthesis gene expression (ribosomal protein) in fasted fish; these data can be related to ours on trout fed with plant-protein diets having reduced growth.

Conclusion

Our data based on a transcriptomic approach and confirmed by quantitative RT-PCR enable us to identify modifications of hepatic gene expression after intake of a plant-based diet by rainbow trout. This non-exhaustive list of genes could be useful and used in the future as powerful tools to more closely monitor the effects of the evolution of feeds used for farmed fish[50].

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F. P. and D. E. were responsible for generating the trout microarrays. C. K. was co-responsible for generating statistical analysis of the microarray data. E. P.-J. generated the quantitative RT-PCR data. N. R., G. C., F. M. and S. K. conducted the nutritional experiments in fish. S. P. was responsible for project development, drafted the manuscript and is the corresponding author.

There are no conflicts of interest.

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