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Reduced lipid intake leads to changes in digestive enzymes in the intestine but has minor effects on key enzymes of hepatic intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*)

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*For sustainable aquaculture, the removal of marine resource ingredients in fish diets is an important objective. While most studies focus on the replacement of fish oil by vegetable oil, little is known on the nutritional effects of presence (which corresponds to the control diet) or absence of dietary fish oil. We studied fatty acid composition of brush-border membranes and digestive enzyme activities of the intestine and measured the expression and activities of several enzymes involved in the hepatic intermediary metabolism of rainbow trout (*Oncorhynchus mykiss*) fed for 7 weeks with or without fish oil. The diets were pair-fed to ensure that fish fed either diet had comparable carbohydrate and protein intakes. Absence of fish oil significantly reduced growth rate, protein efficiency and plasma lipid components. Activities of intestinal digestive enzymes were significantly decreased in the anterior intestine in fish fed without fish oil. In liver, dietary fish oil removal did not affect the transcript levels or activities of the main enzymes involved in lipogenesis (fatty acid synthase) and fatty acid β-oxidation (3-hydroxyacyl-CoA dehydrogenase), glycolysis or amino acid oxidation. It lowered the expression of the genes coding for gluconeogenic enzymes (glucose-6-phosphatase and phosphoenolpyruvate carboxykinase), but their enzyme activities were not affected. The activities, but not gene expression of lipogenic enzymes, involved in NADPH and malonyl-CoA formation were also modified after fish oil removal as reflected by higher activities of isocitrate dehydrogenase/glucose-6-phosphate dehydrogenase and acetyl-CoA carboxylase enzymes. Overall, our results indicate that the intestinal digestive capacity was strongly modified by dietary fish oil removal, while hepatic intermediary metabolism was only marginally affected, in fed rainbow trout.*

Keywords: aquaculture, digestion, fish oil, metabolism, rainbow trout

Introduction

Compound diets used for intensive fish farming still contain high levels of marine feedstuffs. This impairs the sustainability of fish production, while aquaculture should be a solution to the decrease in marine natural resources (Naylor *et al.*, 2000; New and Wijkstroem, 2002). Diets for salmonids, for instance, include large amounts of fish oil, because the addition of dietary lipids has a sparing action on proteins (Sargent *et al.*, 2002). Given that fish oil is a limited natural resource (New and Wijkstroem, 2002), much effort is being devoted towards research on alternatives to fish oil in the diets of farmed fish.

Increasing dietary fish oil has a nitrogenous sparing effect and improves the quality of fish flesh for human consumption (Sargent *et al.*, 2002). Flesh content of n-3 polyunsaturated fatty acids (PUFAs) reflects the dietary content of these fatty acids. Attention must be paid to conserving in fish flesh high levels of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), known to play an essential role in protection against cardiovascular risk, diabetes type 2 and neurodegenerative

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disease in humans (Bourre, 2005; Ismail, 2005, Nettleton and Katz, 2005). The implications of replacing dietary fish oil by a mix of vegetable oils have been widely studied in fish in recent years. It does not have marked effects on growth but liver fatty acid metabolism as well as muscle n-3 PUFA contents are affected in Atlantic salmon (*Salmo salar*) (Torstensen *et al*., 2000; Bell *et al*., 2001 and 2002; Stubhaug *et al*., 2005). However, using a fish oil 'finishing diet' at the end of the experiment can partially restore the EPA and DHA levels in fish flesh (Bell *et al*., 2003 and 2004; Torstensen *et al*., 2005). This illustrates once again that the quality of the lipid depots is driven by the fatty acid composition of the diet. This effect may be directed through the modification of the intermediary metabolism. Indeed, it is well known that fatty acids such as EPA and DHA regulate hepatic gene expressions by controlling the activities or abundances of key transcription factors involved in the regulation of hepatic metabolic enzymes (Jump *et al*., 2005; Sampath and Ntambi, 2005). In studies dealing with the substitution of fish oil by other lipid sources, the effects of removing fish oil is confounded with the effects of adding a source with a different fatty acid profile on overall metabolism (Bell *et al*., 2001 and 2002; Jordal *et al*., 2005; Stubhaug *et al*., 2005). From a quantitative point of view, we ourselves have found that an increase in supply of dietary fish oil modified the activities and expressions of key enzymes of the glucose metabolism and led to less-efficient carbohydrate metabolism in rainbow trout (*Oncorhynchus mykiss)* (Panserat *et al*., 2002). Thus, it appears that the interaction between fish oil and intermediary metabolism must be better understood to provide the basis necessary for the successful replacement of fish oil in fish diets.

Thus, in the present study, we analysed the effects of the removal of dietary fish oil (residual fatty acids being supplied by fish meal) on two tissues: liver, as the centre of intermediary metabolism, and intestine, as the first organ in contact with nutrients. In order to keep constant the protein and carbohydrate ingestion, the animals were pair-fed, a quite original strategy in fish. We measured several enzyme activities and gene expression for candidate proteins involved in hepatic intermediary metabolism. In order to assess the effects parallel to digestive capacity, we measured the evolution of the fatty acid composition of the brush-border membranes (BBMs) and the activity of selected enzymes.

Material and methods

Fish and diets

A 7-week growth study was undertaken with duplicate groups of juvenile rainbow trout (100 fish per tank; initial body weight 139 g) reared in our experimental fish farm (INRA, Donzacq, France) at 18°C under natural photoperiod in spring. Two diets were formulated and manufactured in our experimental unit. Diet HL contained 15.5% fish oil (Table 1) and diet LL had no fish oil but had a higher level of fish meal (Table 1). Based on the analytical composition of the diet, the ration for each group was determined to ensure the pair-feeding of the animals (Table 2). Fish were hand-fed twice a day at 1.50% and 1.27% of body weight per day for groups HL and LL, respectively. Fish were group weighed by tanks each week, to adjust the rations.

Table 1 *Composition of the experimental diets*

	Diets	
	HL	LL
Ingredients (%)[†]		
Fish meal 70	46.5	55
CPSP G	20	24
Fish oil	15.5	0
Binder	1	1
Gelatinised starch	15	18
Mineral pre-mix	1	1
Vitamin pre-mix	1	1
Analytical composition		
Dry matter (DM; %)	90	84
Crude protein (CP; % DM)	50	61
Crude lipid (% DM)	25	10
Gross energy (kJ/g DM)	24	21
Starch[‡] (% DM)	14	17
Fatty acid composition (% fatty acids)		
Σ Saturated	30.6	36.4
Σ Monounsaturated	38.4	38.0
Σ Polyunsaturated fatty acids n-6	4.6	4.5
Σ Polyunsaturated fatty acids n-3	20.6	14.4

Abbreviations are: HL: fish fed with fish oil; LL: fish fed without fish oil.
[†]Fish meal (Sopropèche, Boulogne-sur-Mer, France); CPSP: soluble concentrate of fish protein 70 (Sopropèche, Boulogne-sur-Mer, France); Fish oil: Feedoil (North Sea fish oil, Sopropèche, Boulogne-sur-Mer, France); Binder: Alginate GF 150 (Louis François exploitation, Saint-Maur, France); Gelatinised starch Amidaine A 200 (Amylum Aquitaine, Bordeaux, France); Mineral mixture (g or mg/kg diet): calcium carbonate (40% Ca), 2.15 g; magnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25% Cu), 0.3 g; manganese sulphate (33% Mib), 0.3 g; dibasic calcium phosphate (20% Ca, 18% P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g (UPAE (unité de préparation des aliments expérimentaux, Jouy, INRA, France)); Vitamin mixture (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15 000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (UPAE (unité de préparation des aliments expérimentaux, Jouy, INRA, France)).
[‡]Starch deduction = 100 × (energy − (39.6 × %lipid + 23.2 × %protein))/17.2.

Tissue and blood sampling, and brush-border membranes isolation

At the end of the growth trial, 12 fishes from each group were randomly sampled 8 h after the meal and sacrificed by a sharp blow on the head. This delay was chosen because we have previously shown that it corresponds to the post-prandial peak of nutrient absorption at this water temperature (Panserat *et al*., 2002). Gut contents of the sampled animals were systematically checked to make sure

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Table 2 *Calculated feed intake after use of pair-feeding protocol for fish weighting 100 g*

	Diets	
	HL	LL
Ration size (g/day per 100 g fish)	1.5	1.27
Feed intake† (g/day per 100 g fish)		
Protein	0.75	0.77
Lipid	0.37	0.13
Carbohydrate	0.21	0.22
Fatty acid intake‡ (g/day per 100 g fish)		
Σ Saturated	0.11	0.05
Σ Monounsaturated	0.14	0.05
Σ Polyunsaturated fatty acids n-6	0.02	0.006
Σ Polyunsaturated fatty acids n-3	0.08	0.02

Abbreviations are: HL: fish fed with fish oil; LL: fish fed without fish oil.
†Feed intake: [analytical composition of diets in nutrient (%) × daily ration size (g)]/100 g fish.
‡Fatty acid intake: [analytical composition for each fatty acids (in % of fatty acids) × analytical composition of diets in lipid (%)/100 × daily ration size (g)]/100 g fish.

that the fish sampled had consumed the diet. The liver was immediately dissected and weighed and the intestine was open and washed for remaining digesta in sterile ice-cold saline solution (NaCl 9‰). Tissues were weighed and immediately frozen in liquid nitrogen and kept at −80°C pending assays. Blood was removed from the caudal vein and centrifuged (3000 × **g**, 5 min), and the recovered plasma was immediately frozen and kept at −20°C pending the analyses of glucose, free fatty acids and triacylglycerols.

A fraction of the liver samples was reserved for the analysis of gene expression; the remaining part was used for enzyme activity measurements. The BBMs were purified from the intestine (avoiding any contamination by perivisceral fat and pancreatic tissues from the caeca) prior to the enzyme assays. To this end, two samples of intestines were pooled, so that we constituted six replicates per tank. Intestines were homogenised in Tris–Mannitol buffer. Of this homogenate, 10% was kept for lipase assays and the rest was used for purifying the BBMs of the enterocytes using the method of Crane *et al*. (1979).

Chemical composition

Chemical composition of the diets. The two experimental diets were analysed using the following procedures: dry matter after drying at 105°C for 24 h, starch by the gluco-amylase glucose oxydase method (Thivend *et al*., 1972) and gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany). Protein content (N × 6.25) was determined by the Kjeldahl method after acid digestion. Total liver lipids were determined by the method of Folch *et al*. (1957), after extraction by dichloromethane rather than chloroform. Fatty acid composition of diets was determined in the total lipid extract. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride (BF_3) in methanol according to Shantha and Ackman (1990) and were separated using a Varian 3400 gas chromatograph. FAME were identified by comparison with known standard mixtures (Sigma, St Louis, MO, USA) and quantified.

Fatty acid composition of the brush-border membranes. Of the purified BBM, 80% was used for lipid extraction. This was performed using the micro-method described by Jones *et al*. (1992). FAME prepared from the lipidic extract of the BBM fraction were separated by gas chromatography, in an AutoSystem PerkinElmer (PerkinElmer, Waltham, MA, USA) with a flame ionisation detector, BPX 70 capillary column (25 m × 0.22 mm i.d. × 0.25 μm film thickness), split–splitless injector, with helium as the carrier gas. The injector and detector temperatures were, respectively, 220°C and 260°C. Initial temperature of the oven was 50°C, increasing to 180°C by increments of 15°C/min, maintained for 5 min, and finally increased to 220°C by increments of 3°C/min.

Plasma metabolites

Plasma glucose concentration was determined using the glucose oxidase method in a Beckman glucose analyser (Beckman II; Beckman Instruments Inc., Brea, CA, USA). Plasma triacylglycerol levels were measured by colorimetric enzymatic assay using hepatic lipase (EC 3.1.1.3), glycerokinase (EC 2.7.1.30), glycerol-3-phosphate oxidase (EC 1.1.3.21) and peroxidase (EC 1.1.11) as enzymes (PAP 150 kit; Biomérieux, Marcy-l'étoile, France). Plasma fatty acid levels were measured by colorimetric enzymatic assay using acyl-CoA synthetase, acyl-CoA oxydase and peroxydase as enzymes (Wako Nefa C kit; Wako Chemicals GmbH, Neuss, Germany).

Enzyme activities

Enzyme activities were performed on frozen-thawed tissues. Enzyme activities were expressed per mg of protein. Protein concentration was determined according to Bradford (1976) using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as standard.

Measurement of activities of digestive enzymes in intestine

We analysed the lipase-like enzymes, a non-specific lipase that can hydrolyse carboxyl ester bonds of acyl-glycerols and other minor dietary fats, including cholesterol esters. Its activity likely reflects the effective lipolytic capacity of the intestinal tract. The other enzymes were indicators of BBM functionality: aminopeptidase N (EC 3.4.11.2); γ-glutamyl-transpeptidase (EC 2.3.2.2); alkaline phosphatase (EC 3.1.3.1) and maltase (EC 3.2.1.20). Enzymatic activities were assayed on 20% of the purified BBM fraction except for lipase-like activities, which were assayed directly with the homogenates following the method described by Iijima *et al*. (1998). Aminopeptidase N, γ-glutamyl-transpeptidase, maltase and alkaline phosphatase were assayed in purified BBM fractions, according to Maroux *et al*. (1973), Meister

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et al. (1981), Dahlqvist (1970) and Bessey *et al*. (1946), respectively.

Measurement of activities for hepatic metabolic enzymes
Among the enzymes involved in lipogenesis and fatty acid metabolism, we selected fatty acid synthase (FAS, EC.2.3.1.48), acetyl-CoA carboxylase (ACC, EC6.4.1.2), 3-hydroxyacyl-CoA dehydrogenase (HOAD, EC.1.1.1.35) known to be highly regulated by nutrients (Sul and Wang, 1998; Reddy and Hashimoto, 2001; Tong, 2005). We measured also activity and expression of glucose-6-phosphate dehydrogenase (G6PD, EC.1.1.1.49) and NADP-isocitrate dehydrogenase (ICDH-NADP, EC.1.1.1.42) because these NADPH-productive enzymes are linked to lipid metabolism (Salati and Amir-Ahmady 2001, Koh *et al*., 2004). Measures of lipogenic FAS and G6PD activities were carried out as described by Richard *et al*. (2006). ACC activity was assayed using an isotopic method as previously described for rainbow trout (Rollin *et al*., 2003), where the activities of the two ACC isoforms, ACCα and ACCβ, were probably measured together. HOAD enzyme activities were determined following the method by Kobayashi *et al*. (1996). ICDH-NADP enzyme activities have been obtained by measuring NADP reduction at 340 nm after addition of 89.7 mmol/l TEA-HCl at pH 7.4, 41 mmol NaCl, 0.34 mmol/l NADP, 0.43 mmol/l $MnSO_4$, 4.17 mmol/l DL-isocitrate in samples after sonication and centrifugation at 12 000 $\times$ **g** (liver samples have been homogenised in a cold ice buffer: 20 mmol.l Tris–HCl pH 7.4, 250 mmol/l manitol, 2 mmol/l EDTA, 100 mmol/l NaF, 10 mmol/l mercaptoethanol, 0.5 mmol/l PMSF). The glycolysis pathway was analysed through assaying glucokinase (GK, EC 2.7.1.2), 6-phosphofructokinase (6-PF1K, EC) and pyruvate kinase (PK, EC.2.7.1.40). We also measured the activities of glucose-6-phosphatase (G6Pase, EC.3.1.3.9), fructose-1.6-biphosphatase (FBPase, EC.3.1.3.1.1) and phosphoenolpyruvate carboxykinase (PEPCK, EC.4.1.132), key regulators of the gluconeogenic pathway (Pilkis and Granner, 1992; Van Schaftingen and Gerin, 2002). Glycolytic (GK and PK) and gluconeogenic (G6Pase, FBPase and PEPCK) enzyme activities were measured as previously described (Kirchner *et al*., 2003b). Measures of 6-PFK1 activities have been performed following Meton *et al*. (1999) with aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase as co-enzymes. Protein metabolism is also particular in fish because it is orientated towards the oxidation of dietary amino acids for energetic purposes (Cowey and Walton, 1989). We measured as described by Mambrini *et al*. (1998) the expression of the key enzyme of this pathway, glutamate dehydrogenase (GDH, EC.1.4.1.3), which has a higher activity in fish compared with mammals (Dabrowski and Guderley, 2002).

Gene expression analysis by real-time quantitative RT-PCR
Total RNAs were extracted from rainbow trout liver or from the anterior intestinal segment using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). A total of 1 μg of total RNA was reverse transcribed to cDNA with the Superscript™ III RNAse H Reverse Transcriptase kit (Invitrogen) using oligo dT primers. Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ™ (BIO-RAD, Hercules, CA, USA). Analyses were performed on 10 μl of the RT reaction mixture using the iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, CA, USA). The total volume of the PCR reaction was 25 μl, containing 200 nmol/l of each primer. Primers were designed so that they are overlapping an intron if possible (Primer3 software; Whitehead Institute for Biomedical Research/MIT Center, Cambridge, MA, USA) using known sequences in nucleotide databases (Table 3). Database (Genbank, http://www.genome.ad.jp/htbin/www_bfind?dna today; National Institute of Agronomic Research (INRA) – Sigenae http://ensembl-sigenae.jouy.inra.fr/; Tigr Gene Index; http://compbio.dfci.harvard.edu/tgi/) accession numbers for rainbow trout genes were: gb|AF135403|-Genbank (*gk*), gb|AF120150|-Genbank (*g6pase*), gb|AF333188|-Genbank (*fbpase*), gb|AF246149|-Genbank (*m-peck*), tcaa0001c.m.06_5.1.om.4-Sigenae (*fas*), tcbk0010c.b.21_5.1.om.4-Sigenae (*acc*: highly similar to ACC irrespective of the isoform: α or β), tcad0001a.i.15_3.1.om.4-Sigenae (*hoad* short chain), TC72180-Tigr (*g6pd*), 15072560.1.om.4-Sigenae (*icdh-nadp*) and gb|AJ419571|-Genbank (*gdh* isoform 2). We measured the level of *acc* transcripts without discerning *acc*α and *acc*β because the genes coding for the two isoform cDNAs are still not characterised at the molecular level in all the rainbow trout genomic resources we screened (Tigr, Unigene, Sigenae EST databases). GDH is encoded by a multigene family; because the expression of each gene is tissue specific, we chose to focus on the major transcript in the liver, *gdh2*, and used the primers specific for this transcript that are already designed (M. Mambrini, personal communication). The q-PCR amplified products were checked for specificity by sequencing.

Relative quantification of the target gene transcript was done using *ef1*α gene expression as reference (Olsvik *et al*., 2005). Thermal cycling was initiated with the incubation at 95°C for 90 s for hotstart iTaq™ DNA polymerase activation. In all, 35 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, except for *gdh2*, for which the temperature was set at 60°C. Following the final cycle of the PCR, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55°C to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNAs were run for each reaction as negative controls.

Data analysis
Data are presented as means $\pm$ standard deviation. Except for q-PCR data analysis, we analysed the different diets with an unpaired two-tailed Student's *t*-test (Statistical Packages for the Social Sciences, 1999). Relative quantification

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Table 3 *Sequence of the primer pairs used for real-time quantitative PCR determinations of the trasncript level for several rainbow trout genes*

Genes	Name (position†)	5′–3′ Forward primer	Name (position*)	5′–3′ Reverse primer
Glucokinase (gk)	GK1 (E2/E3)	tgaaggatcagaggtgggtgat	GK2 (E4)	gaaggtgaaacccagaggaagc
6-Phosphofructokinase (6-pfk1)‡	6PFK1 (E1)	(a) ggtggagatgcacaaggaa	6PFK2 (E1/2)	(a) cttgatgttgtcccctccat
	6PFK3 (E4)	(b) ggcatcaccaacctgtgygt	6PFK4 (E4)	(b) gtcrttrtcgatggagcccac
Pyruvate kinase (pk)	PK1 (E10)	ccatcgtcgcggtaacaaga	PK (E10/E11)	gcccctggccttttcctatgt
Glucose-6-phosphatase (g6pase)	G6PAse1 (E4)	tgcccacttcccacacca	G6PASE2 (E5)	agcccacagcaaaggagag
Fructose biphosphatase (fbpase)	FBPASE1 (E4/E5)	gctggacccttccatcgg	FBPASE2 (E6)	cgacataacgcccaccatagg
Mitochondrial phosphoenolpyruvate carboxykinase (m-pepck)	PECK1 (5′UTR)	gttggtgctaaagggcacac	PEPCK2 (E1)	cccgtcttctgataagtccaa
Fatty acid synthase (fas)	FAS1 (E36)	gagacctagtggaggctgtc	FAS2 (E37)	tcttgttgatggtgagctgt
Acetyl-coA-carboxylase (acc)	ACC1 (E49)	tgagggcgttttcactatcc	ACC2 (E50/E51)	ctcgatctccctctccact
3-Hydroxyacyl-CoA dehydrogenase (hoad)	HOAD1 (E3/E4)	ggacaaagtggcaccagcac	HOAD2 (E4)	gggacggggttgaagaagtg
Glucose-6-phosphate dehydrogenase (g6pd)	G6PD1 (E2/E3)	cactacctgggcaaggagat	G6PD2 (E3)	ttgctcatcatcttggcgta
NADP-isocitrate dehydrogenase (icdh-NAPP)	ICDH1 (E7)	gacagcaccaacagggcaa	ICDH2 (E8)	aagccagcctcgatggtctc
Glutamate dehydrogenase type 2 (gdh2)	GDH2-1 (E1)	atcaagccctgcaaccacgtcct	GDH2-2 E2)	tcttcactgtaacggatccccccttt
Elongation factor 1 alpha (ef1α)	EF1TS (E7/E8)	tcctcttggtcgtttcgctg	EF1TAS (3′UTR)	acccgagggacatcctgtg

†Positioned following the corresponding (heterologous) zebrafish or mammalian gene sequences: exon n (En), exon boundaries (En/En+1), or untranslated region (UTR) (http://www.ensembl.org/).
‡Primer sequences were chosen either on (a) specific trout sequence highly similar to liver 6PFK1 isoform (5′ UTR) (tcbk0069c.k.05_5.1.om.4 (Agenae EST name)) or (b) on conserved nucleotide sequences between liver 6-PFK1 in different animal species: *Canis familiaris* (Ensembl Gene ID ENSCAFG00000010846); *Homo sapiens* (Genbank: BC006422); *Rattus norvegicus* (genbank: BC061791) (using degenerated primers chose where Y = C/T; R = G/A).

of the target gene transcript with *ef1α* reference gene transcript was made following the Pfaffl method with the Relative Expression Software tool (REST©) (Pfaffl *et al.*, 2001 and 2002). This mathematical algorithm computes an expression ratio, based on q-PCR efficiency and the crossing point deviation of the unknown sample *v.* a control group: $R = [(E_{\text{target gene}})^{\Delta CT\ \text{target gene}\ (\text{mean control} - \text{mean unknown sample})}]/[(E_{EF1\alpha})^{\Delta CT\ EF1\alpha\ (\text{mean control} - \text{mean unknown sample})}]$ where E is the PCR efficiency determined by a standard curve using the serial dilution of cDNA (cDNA dilutions from 1/16 up to 1/512) (E has been accepted when >90%); ΔCT the crossing point deviation of an unknown sample *v.* a control. Statistical differences in gene expression between control and sample were evaluated in groups means by randomisation tests (Pfaffl *et al.*, 2002) using REST© software. In all, 2000 random allocations were performed. For all the statistical tests, significant differences were considered at $P < 0.05$.

Results

During the growth study, mortality was low in all tanks and unrelated to experimental treatments and there were no external pathological signs. Fish consumed their ration entirely, so with pair-feeding, it was mainly the intake of lipids (such as PUFA n-3 fatty acid) that was different between the HL and LL fish (Table 2). Growth performance of juvenile rainbow trout fed with the HL diet was significantly higher than that of fish fed LL (Table 4; $P < 0.01$, Student's *t*-test). The best protein efficiency ratio was observed in fish fed HL (Table 4; $P < 0.05$, Student's *t*-test). The liver weight and the hepatosomatic index were significantly higher in HL- than in LL-fed fish ($P < 0.05$, Student's *t*-test). We observed that LL-fed fish had much lower mass of perivisceral fat tissue than HL-fed fish (visual observation; data not shown). Glycaemia was not affected by the nature of the dietary treatment (Table 4). Plasma free fatty acid and triglyceride levels were higher in trout fed with HL than in those fed with the LL diet (Table 4; $P < 0.01$, Student's *t*-test).

Reduction in the intake of lipids induced changes in intestinal digestive capacities (Table 5). Alkaline phosphatase, aminopeptidase N and γ-glutamyl peptidase-specific activities were significantly lower in LL fish than in HL fish ($P < 0.05$, Student's *t*-test). The most important decrease in activity associated with fish oil removal was observed for lipase-like enzymes ($P < 0.05$, Student's *t*-test). We did not notice significant effects on the fatty acid composition of the BBM (Table 5).

The removal of dietary fish oil did not affect the activity and expression of hepatic FAS and HOAD (Tables 6 and 7). ACC activity was three times higher in fish fed the LL diet compared with those fed the HL diet (Table 6, $P < 0.01$, Student's *t*-test). In contrast, *acc* gene expression coding, irrespective of α or β isoform, was not significantly different between HL and LL fish (Table 7). The activity of the cytosolic enzymes producing NADPH (G6PD and ICDH-NADP) were 1.5 times higher in fish fed the LL diet compared with fish fed the HL diet (Table 6, $P < 0.05$, Student's *t*-test). By contrast, the expression of the genes coding for these enzymes was not affected by the dietary treatment (Table 7). Glycolytic enzyme activities and gene expressions

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Table 4 *Effects of dietary lipid levels on growth performance, feed efficiency, liver and plasmatic parameters in juvenile rainbow trout over 7 weeks*

	Diets		
	HL	LL	*P*-value[†]
Zootechnical parameters ($n = 2$ tanks with 100 fish per tank)[‡]			
Initial body weight (IBW) (g)	139.50 ± 0.50	138.50 ± 2.00	ns
Final body weight (FBW) (g)	236.03 ± 3.28	200.11 ± 2.86	$P = 0.01$
Daily growth index	1.91 ± 0.07	1.30 ± 0.01	$P < 0.05$
Feed efficiency	0.92 ± 0.05	0.77 ± 0.02	ns
Protein efficiency ratio	1.86 ± 0.10	1.26 ± 0.03	$P < 0.05$
Liver parameters ($n = 12$ per diet)			
Final liver weight (g)	4.19 ± 0.81	3.30 ± 1.05	$P < 0.05$
Hepato-somatic index[§] (%)	1.99 ± 0.62	1.63 ± 0.28	$P < 0.05$
Plasma parameters[¶] ($n = 12$ per diet)			
Glucose (g/l)	1.03 ± 0.22	0.95 ± 0.16	ns
Free fatty acids (mEq/l)	0.14 ± 0.05	0.07 ± 0.02	$P < 0.001$
Triacylglycerols (g/l)	9.77 ± 5.99	4.44 ± 1.68	$P < 0.01$

Abbreviations are: HL: fish fed with fish oil; LL: fish fed without fish oil.
Data are expressed as means ± standard deviation.
[†]*P*-values are shown when the differences are significant ($P < 0.05$, Student's *t*-test); ns: not significant (Student's *t*-test, $P > 0.05$).
[‡]Daily growth index: $100 \times (FBW^{1/3} - IBW^{1/3})$/duration (49 days). Feed efficiency: wet weight gain (g)/dry feed intake (g). Protein efficiency ratio: Wet weight gain (g)/crude protein intake (g).
[§]Hepatosomatic index: (liver weight/body weight) × 100.
[¶]Plasma parameters measured 8 h after feeding.

Table 5 *Intestinal biochemical parameters measured 8 h after feeding between juvenile rainbow trout fed with a diet contained 15% fish oils (HL) or not (LL). Enzymatic activities of digestive enzymes were expressed as mU/mg protein. Data are expressed as means ± standard deviation (*n = *6 pools of two samples per diet)*

	Diets		
	HL	LL	*P*-value[†]
Enzymatic activities			
Intestinal homogenate			
Lipase-like	6.1 ± 1.39	2.8 ± 0.39	<0.01
Brush border membranes			
Digestive enzymes			
Alkaline phosphatase	2.3 ± 0.34	1.7 ± 0.06	<0.01
Aminopeptidase	1.8 ± 0.16	1.5 ± 0.16	<0.01
γ-glutamyl transpeptidase	244 ± 17.4	167 ± 40.3	<0.01
Maltase	222 ± 20.5	198 ± 28.4	ns
Fatty acid composition of brush border membranes (% fatty acids)			
Σ Saturated	31.6 ± 0.9	31.3 ± 0.7	ns
Σ Monosaturated	14.3 ± 1.2	17.0 ± 1.7	ns
Σ Polyunsaturated fatty acids n-6	2.1 ± 0.1	1.9 ± 0.1	ns
Σ Polyunsaturated fatty acids n-3	35.3 ± 1.8	33.2 ± 3.4	ns
Including linolenic acid	0.5 ± 0.1	0.4 ± 0.1	ns
Including EPA	5.0 ± 0.6	3.6 ± 03	ns
Including DHA	28.5 ± 1.5	28.2 ± 3.3	ns

Abbreviations are: HL: fish fed with fish oil; LL: fish fed without fish oil; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.
[†]*P*-values are shown when the differences are significant ($P < 0.05$, Student's *t*-test); ns: not significant ($P > 0.05$).

were not affected by the feeding regimes (Tables 6 and 7). In contrast, gluconeogenic enzymes were affected by the dietary treatment at both molecular and enzymatic levels: (i) transcripts of the first and last gluconeogenic enzymes (*g6pase* and *mpepck*) were significantly expressed at lower level in fish fed the LL diet compared with fish fed the HL

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Table 6 *Enzymatic activities of proteins involved in hepatic intermediary metabolism 8 h after feeding. Specific enzymatic activities have been measured (mU/mg protein)*

	Diets		
	HL	LL	*P*-value[†]
Lipid metabolism			
Fatty acide synthase	0.21 ± 0.07	0.26 ± 0.04	ns
3-hydroxyacyl-CoA dehydrogenase	122 ± 23	118 ± 26	ns
Acetyl-CoA-carboxylase[‡]	0.44 ± 0.06	1.40 ± 0.10	$P < 0.01$
Glucose metabolism			
Glycolysis			
Glucokinase	29.6 ± 9.0	31.3 ± 12.8	ns
6-phosphofructokinase	44.6 ± 12.3	51.3 ± 10.8	ns
Pyruvate kinase	164 ± 64	137 ± 80	ns
Gluconeogenesis			
Glucose-6-phosphatase	26.1 ± 1.2	24.7 ± 5.8	ns
Fructose biphosphatase	19.1 ± 4.3	26.8 ± 3.8	$P < 0.05$
Phosphoenolpyruvate carboxykinase[§]	33.0 ± 4.3	29.6 ± 3.8	ns
Amino acid metabolism			
Glutamate dehydrogenase	153 ± 16	169 ± 32	ns
NADPH production			
Glucose-6-phosphate dehydrogenase	232 ± 41	342 ± 66	$P < 0.01$
NADP-isocitrate dehydrogenase	108 ± 14	150 ± 41	$P < 0.05$

Abbreviations are: HL: fish fed with fish oil; LL: fish fed without fish oil.
Data are expressed as means ± standard deviation ($n = 12$) except for fatty acid synthase, Glucose-6-phosphate dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, Acetyl-CoA-carboxylase and NADP-isocitrate dehydrogenase ($n = 6$).
[†]*P*-values are shown when the differences are significant ($P < 0.05$, Student's *t*-test); ns: not significant ($P > 0.05$, Student's *t*-test).
[‡]Total acetyl-coA-carboxylase (ACC) activity: ACCα + ACCβ.
[§]Total phosphoenolpyruvate carboxykinase (PEPCK) activity: mitochondrial PEPCK + cytosolic PEPCK. In trout liver, 90% of PEPCK activity is mitochondrial (Kirchner *et al.*, 2003a).

diet (Table 7, $P < 0.01$, Rest test) but without any modification of enzymatic activities (Table 6); and (ii) the FBPase enzyme had higher activity in fish fed the LL diet compared with fish on HL (Table 6; *t*-test, $P < 0.05$) but *fbpase* gene expression was not affected by the dietary treatment (Table 7). Finally, the key enzyme involved in liver amino acid oxidation, the GDH enzyme, presented similar levels of molecular expression and enzymatic activity between the two dietary conditions (Tables 6 and 7).

Discussion

The main objective of our study was to evaluate the effects of dietary fish oil removal on the integrity of the digestive capacity and on liver intermediary metabolism in rainbow trout. We used a pair-feeding protocol in order to analyse the effect of dietary fish oil removal alone and not, as usually done, its replacement by vegetable oils (Bell *et al.*, 2001 and 2002; Stubhaug *et al.*, 2005). This strategy was rendered possible because the diet deficient in fish oil was based on fish meal containing on average 8% fat. This amount is sufficient to provide to rainbow trout the amount of essential fatty acids corresponding to the requirements (National Research Council, 1993). With the pair-feeding technique, we succeeded in modifying the lipid intake of fish while maintaining the protein and carbohydrate supplies. This is shown by the plasma level of glucose, which was not affected by the dietary treatment, when plasma levels of free fatty acids and triglycerides were lower for fish fed the diet without added fish oil. The removal of fish oil led to an overall decrease in growth performance, feed and protein efficiencies as expected due to the lower level of total energy intake.

Fish oil removal in the diet had several effects at the digestive tract level: lipolytic activity was lowered by 2.2-fold; activities of BBM digestive enzymes not linked to lipid metabolism were also greatly decreased (up to 40%) in the LL group. This reveals that the enterocyte membrane has a lower digestive capacity if fish are fed diets with low lipids. This result was not the consequence of a modification in fatty acid composition of the enterocyte BBM and therefore of membrane fluidity, because this composition remained unchanged after 7 weeks of fish oil removal in the diet. Our results contrast with those obtained with European sea bass (*Dicentrarchus labrax*), where the activities of the digestive enzymes of the BBM were reduced and the fatty acid composition of the BBM was significantly modified

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Table 7 *Real-time quantitative RT-PCR analyses of the transcript level of several genes expressed in the liver of trout juveniles fed a diet with no fish oil (LL) in comparison to a diet containing 15.5% fish oil (HL). Analyses were performed on samples taken 8 h after feeding. Statistical differences in gene expression level between LL and HL samples were evaluated in groups means by randomisation tests (Pfaffl* et al., *2002) using REST© software. A negative value of target genes was normalised with elongation factor 1 alpha (*ef1α*) expressed transcripts. For each diet, organs from 12 individuals were analysed independently*

Genes	*n*-fold variation, LL *v.* HL	*P*-value†
Lipid metabolism		
Fatty acid synthase	+1.80	ns
3-hydroxyacyl-CoA dehydrogenase	+1.08	ns
Acetyl-CoA-carboxylase	−1.48	ns
Glucose metabolism		
Glycolysis		
Glucokinase	+1.04	ns
6-phosphofructokinase	nd‡	
Pyruvate kinase	+1.62	ns
Gluconeogenesis		
Glucose-6-phosphatase	−2.51	$P = 0.01$
Fructose biphosphatase	+1.42	ns
Mitochondrial phosphoenolpyruvate carboxykinase	−1.54	$P < 0.05$
Amino acid metabolism		
Glutamate dehydrogenase type 2	+1.16	ns
NADPH production		
Glucose-6-phosphate dehydrogenase	+1.12	ns
NADP-isocitrate dehydrogenase	+1.07	ns

Abbreviations are: HL: fish fed with fish oil; LL: fish fed without fish oil.
†ns: not significant (randomisation tests REST©, $P > 0.05$).
‡nd: non-detectable. Irrespective of the primers used in our assays (see Table 2), no successful amplification by RT-PCR for this gene has been obtained using hepatic total RNAs; the success was obtained only with total RNAs from muscle.

when the dietary lipid level was increased from 21% to 30% (Cahu *et al*., 2000). The fact that, in the present study, fatty acid composition of the enterocyte membranes was not affected by the dietary lipid level may be due to: (1) the lower lipid level of the control diet (HL diet) compared with the one employed in the study of Cahu *et al*. (2000), (2) the rigorous control of the nutrient intake in the present study and (3) a difference between species in the lipid metabolism, e.g. higher capacity to synthesise and metabolise fatty acid in the trout intestine. In general, PUFA synthesis in enterocytes could be increased in fish fed diets in which dietary fish oil was replaced with a vegetable oil blend (Tocher *et al*., 2003 and 2004) strengthened by the similar levels of EPA and DHA in BBMs observed in this study. Studies on lipid metabolism (β-oxidation, biosynthesis) in the intestine are in progress. Overall, our data indicate that the digestive capacity was highly modulated in response to dietary fish oil removal.

It is well known that hepatic fatty acid metabolism is sensitive to regulation after feeding different quantities of dietary lipids in animals (Bergen and Mersmann, 2005). Moreover, previous studies have shown a close relationship between dietary lipids and hepatic lipid profiles in salmonids (Bell *et al*., 2001 and 2002; Tocher *et al*., 2001). In spite of this, no dietary effects at the molecular and enzymatic levels were observed in the present study for two key enzymes catalysing both the mitochondrial fatty acid β-oxidation (HOAD) and fatty acid biosynthesis (FAS). Concerning the absence of FAS regulation, our results are different from that found in mammals (Sampath and Ntambi, 2005) but in accordance with data obtained on rainbow trout fed diets with distinct (highly unsaturated) fatty acid profiles (Richard *et al*., 2006; Tapia-Salazar *et al*., 2006) or different levels of lipids (Henderson and Sargent, 1981; Gelineau *et al*., 2001): therefore, our data confirm the low control by dietary lipid intake of hepatic FAS enzyme in rainbow trout. The absence of HOAD regulation by lipid intake suggests an absence of regulation of the mitochondrial fatty acid β-oxidation but other key enzymes such as the carnitine palmitoyltransferase I, which mediates the transport of fatty acids across the outer mitochondrial membrane have to be further studied. In contrast to FAS and HOAD enzymes, ACC, G6PD and ICDH-NADPH enzymatic activities were significantly higher in fish fed the diet deprived of fish oil suggesting higher production of malonyl-CoA and NADPH in LL fish as has been described in mammals (Sampath and Ntambi, 2005) and rainbow trout (Rollin *et al*., 2003). Globally, the increase of G6PD, ICDH-NADP and ACC activities did not seem to be dependent on the mRNA levels. Thus, only three of the five hepatic enzymes of lipid metabolism studied here responded to the fish oil removal and this regulation was not linked to concomitant differential gene expression in the present nutritional challenge.

Due to the intricate nature of glucose, amino acid and lipid metabolism in liver, we analysed also the enzymes involved in glucose and amino acid metabolism. Moreover, there are particularities of the protein and carbohydrate nutrition, i.e. protein metabolism in fish is mainly orientated towards the oxidation of dietary amino acids for energetic purposes (Cowey and Walton, 1989) and dietary glucose is poorly used at the metabolic level (Hemre *et al*., 2002). While there were no effects at the molecular and enzymatic levels for glycolytic enzymes (GK, 6PFK1 and PK) and for the key enzyme of amino acid oxidation (GDH), the expression of genes coding for key enzymes of the gluconeogenic pathway, *g6pase* and *pepck* were significantly modified. These genes are expressed at a high level in liver of carnivorous fish and are poorly controlled by dietary carbohydrates and fasted-fed status in rainbow trout (Panserat *et al*., 2000, 2001a and b). Their expressions were significantly reduced by the removal of fish oil in the present study. Our results confirm our previous finding in rainbow trout (Panserat *et al*., 2002) and are in agreement with data obtained in mammals (Lam *et al*., 2003; Collins

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et al., 2006). Our data suggest that the usual level of dietary fish oil in fish nutrition may be associated with the stimulation of the gluconeogenesis pathway, which at least partly explains the low metabolic dietary glucose utilisation in this carnivorous fish species (Hemre *et al.*, 2002). Intriguingly, we found higher FBPase activities (another gluconeogenic key enzyme) in fish fed the LL diet in contrast to the lower gluconeogenic *g6pase* and *pepck* gene expression observed in the same fish group. At present, we have no explanation for the apparent discordant regulation by dietary fish oil of the three gluconeogenic key enzymes in our study.

We succeeded to disconnect lipid supply to carbohydrate and protein supply with pair-feeding. However, our results are linked to three major intricate factors: the lowering of the overall energy (e.g. lipid) supply, the proper effect of fatty acid reduction and the restriction of intake of fish fed the LL diet. Because dietary and, in particular, lipid restriction extends the life spans, studies of the biological mechanism behind dietary restriction are of interest in many species (Han and Hickey, 2005). In rat liver, dietary restriction results in up-regulation of gluconeogenic *g6pase* and *pepck* gene expression and a reduction of glycolytic (*gk*, *pk*), *acc*α and *icdh-nadp* gene expressions (Dhabbi *et al.*, 1999; Spinder, 2001). These variations of gene expression were not observed in the trout fed diet LL, suggesting that our data were more related to the dietary fish oil removal *per se* than a lowering of energy intake. Moreover, we cannot determine which fatty acids were linked to the observed data because every fatty acid intake was more or less modified. Further studies using oral administration of fish oil/fatty acids (capsules filled with fish oil/fatty acids) or cell cultures will be necessary to delimitate the specific role of fish oil and/or fatty acids in rainbow trout.

Finally, it seems that dietary fish oil removal was well tolerated in rainbow trout and had no major effects on liver metabolism. Intestine was more sensitive to reduction in fish oil level. Further studies are now in progress to investigate globally the effects of fish oil in rainbow trout liver and intestine using integrative approaches.

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