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Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The rainbow trout (*Oncorhynchus mykiss*) exhibits high dietary amino acid requirements and an apparent inefficiency to use dietary carbohydrates. Using this species, we investigated the metabolic consequences of long-term high carbohydrates/low protein feeding. Fish were fed two experimental diets containing either 20 % carbohydrates/50 % proteins (C20P50), or high levels of carbohydrates at the expense of proteins (35 % carbohydrates/35 % proteins – C35P35). The expression of genes related to hepatic and muscle glycolysis (glucokinase (*GK*), pyruvate kinase and hexokinase) illustrates the poor utilisation of carbohydrates irrespective of their dietary levels. The increased postprandial GK activity and the absence of inhibition of the gluconeogenic enzyme glucose-6-phosphatase activity support the hypothesis of the existence of a futile cycle around glucose phosphorylation extending postprandial hyperglycaemia. After 9 weeks of feeding, the C35P35-fed trout displayed lower body weight and feed efficiency and reduced protein and fat gains than those fed C20P50. The reduced activation of eukaryotic translation initiation factor 4-E binding protein 1 (4E-BP1) in the muscle in this C35P35 group suggests a reduction in protein synthesis, possibly contributing to the reduction in N gain. An increase in the dietary carbohydrate:protein ratio decreased the expression of genes involved in amino acid catabolism (serine dehydratase and branched-chain α-keto acid dehydrogenase E1α and E1β), and increased that of carnitine palmitoyltransferase 1, suggesting a higher reliance on lipids as energy source in fish fed high-carbohydrate and low-protein diets. This probably also contributes to the lower fat gain. Together, these results show that different metabolic pathways are affected by a high-carbohydrate/low-protein diet in rainbow trout.

Key words: Metabolism: Rainbow trout: Gene expression: Signalling: Enzyme activities

Teleosts such as rainbow trout (*Oncorhynchus mykiss*) are recognised for their high protein requirement and their inefficiency in using dietary carbohydrates[1]. Oral or intravenous administration of glucose and a carbohydrate-rich diet result in prolonged postprandial hyperglycaemia in various fish species including rainbow trout despite efficient insulin secretion[2–6]. Even though most of the enzymes involved in carbohydrate metabolism have been detected in fish[7], the regulation of carbohydrate metabolism differs from that of mammals in several ways. Rainbow trout exhibit lower capacity for the phosphorylation of glucose by hexokinases in their muscles than mammalian species[1,8], and a carbohydrate-rich diet does not affect the activity or gene expression of hepatic key enzymes of gluconeogenesis such as glucose-6-phosphatase (G6Pase), fructose-1,6-biphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK)[9–13]. Moreover, increasing the proportion of carbohydrate in the rainbow trout diet at the expense of proteins impairs the activation of hepatic and muscle insulin signalling pathways after a single meal[12].

At the cellular level, the regulation of metabolism often depends on the cross-talk between nutritional and hormonal signals. A well-known cross-talk is that committed by insulin and amino acids. Besides their role as precursors for the synthesis of proteins and other N-containing compounds, amino acids are also involved in the regulation of major metabolic pathways[14] and are thus considered as signalling molecules. Amino acids regulate protein synthesis by activating the mammalian target of rapamycin (TOR)/p70 S6 kinase transduction pathway, together with insulin[15,16]. In trout, the combination

Abbreviations: 4E, BP1, eukaryotic translation initiation factor 4E binding protein 1; Akt, protein kinase B; Atg12l, autophagy-related 12-like; Atg4b, autophagy-related 4b; BCKDE1α, branched-chain α-keto acid dehydrogenase E1α; BCKDE1β, branched–chain α-keto acid dehydrogenase E1β; BCKDE2, branched-chain α-keto acid dehydrogenase E2 subunits; C20P50, 20 % carbohydrates/50 % proteins; C35P35, 35 % carbohydrates/35 % proteins; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; FBPase, fructose-1,6-biphosphatase; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GK, glucokinase; HK, hexokinase; LC3B, light chain 3B; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; SREBP1, sterol regulatory element-binding protein 1; TOR, target of rapamycin.

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of amino acids and insulin is also necessary to fully activate the TOR signalling pathway[17]. Recent studies have indicated that amino acids also play an important role in controlling gene expression in mammals and trout[17–19]. The mammalian TOR complex 1 has recently been identified as a critical element of the regulation of lipogenic gene expression[20,21]. In trout, amino acids regulate the expression of several genes related to glucose and lipid metabolism through pathways that are either TOR-dependent or not[17]. There is increasing evidence that amino acids interfere with insulin function, affecting glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis[22].

One particular feature of the rainbow trout diet is that total protein content may exceed 45 % of the DM. This consistently high dietary amino acid intake may thus have significant effects, particularly on insulin-regulated gene expression. In this regard, we demonstrated in a previous study that decreasing the protein:carbohydrate ratio of the trout diet strongly affected the insulin/amino acid signalling pathway and the expression pattern of several genes related to metabolism after a single meal[12].

The aim of the present study was to investigate the metabolic consequences of a long-term high carbohydrate/low protein intake. For this purpose, trout were fed diets containing either equivalent amounts of proteins and carbohydrates (35 % each) or decreased proportions of carbohydrates at the expense of proteins (20 % carbohydrates/50 % proteins). Postprandial activation of the protein kinase B (Akt)/TOR intracellular signalling pathway as well as the expression of several key genes related to hepatic and muscle metabolism were studied.

Experimental methods

Diets

For the present experiment, two diets were formulated with the carbohydrate:protein ratio as the main factor. The diets were named C35P35 (35 % carbohydrates/35 % proteins) and C20P50 (20 % carbohydrates/50 % proteins) according to the theoretical relative proportions of carbohydrate and crude protein levels. The formulation and ingredient composition of the diets are shown in Table 1. Casein and dextrin were used as protein and carbohydrate sources, respectively, and fish oil was used as the lipid source. The diets were formulated to meet the indispensable amino acid requirement profile of rainbow trout according to the National Research Council[23]. Diet C35P35 supplied quantitatively less total protein than diet C20P50. The diets were manufactured in our feed processing facilities (INRA). The ingredient mixtures of each diet were extruded, dried, sieved and stored in plastic bags. The analysed nutrient compositions of the diets are detailed in Table 1.

Table 1. Composition of the diets

Ingredients (%)	C35P35	C20P50
Casein*	31·0	45·9
Casein hydrolysate*	5·5	6·0
L-Arg*	0·7	1·1
Dextrin*	35·0	20·0
Soya lecithin†	2·0	2·0
Fish oil‡	13·0	13·0
$CaHPO_4.2H_2O$ (18 % P)*	1·3	0·5
Attractant mix§	1·5	1·5
Mineral premix‖	5·0	5·0
Vitamin premix¶	5·0	5·0
Analytical composition (%)		
DM (%)	87·5	80·9
Crude protein (% DM)	37·1	52·0
Lipids (% DM)	14·5	15·6
Gross energy (kJ/g DM)	21·7	22·7
NFE (Cbh)**	41·4	25·4

C35P35, 35 % carbohydrates/35 % proteins; C20P50, 20 % carbohydrates/50 % proteins; NFE (Cbh), nitrogen-free extract (carbohydrate).
* Sigma-Aldrich, St Louis, MO, USA.
† Louis François, St Maur des Fossés, France.
‡ North sea fish oil; Sopropèche, Boulogne-sur-Mer, France.
§ Glucosamine 0·5 g; taurine 0·3 g; betaine 0·3 g; glycine 0·2 g; alanine 0·2 g/100 g feed.
‖ 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 % Mn), 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 (Unité de Préparation des Aliments Expérimentaux, Jouy-en-Josas, INRA, France).
¶ Vitamin mixture (IU or mg/kg diet): DL-α-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; B_{12}, 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 (Unité de Préparation des Aliments Expérimentaux, Jouy-en-Josas, INRA, France).
** NFE = 100 − (crude protein + crude fat + ash).

Feeding trial and sampling

The experiments were carried out in accordance with the clear boundaries of European Union legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2001-464, 29 May 2001). The investigators carrying out the experiment had a 'level 1' or 'level 2' certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, 14 April 2002). The experiment was conducted at INRA St Pée-sur-Nivelle, certified for animal services under the permit number A64.495.1 by the French veterinary services, which is the competent authority. Juvenile rainbow trout were reared in our experimental facilities (INRA) at 18°C under natural photoperiod conditions and fed a commercial diet (T-3P classic; Trouw) until weighing 66 g. Trout were distributed into six circular tanks (500 litres, three tanks per diet) in groups of 100 fish each. Triplicate groups of fish were randomly allocated to one of the two semi-purified diets (INRA) and fed for 9 weeks. Each diet was distributed by hand to visual satiation twice per d for 6 out of 7 d and feed consumption was recorded every week. To follow growth and feed utilisation, fish were counted and group weighed every 3 weeks, and on the last day of the experiment, the final body weight was measured. At the end of the feeding trial, fish were left unfed for 48 h in order to obtain the basal levels of plasma metabolites. This fasting period also contributed to the homogeneous metabolic response of all the fish within the same group. Fish were refed

a single meal of their allocated diet to visual satiation and sampled (n 6) 2, 8 and 24 h after the meal. Gut content of the sampled animals was checked to ensure that the sampled fish had effectively consumed the diet. Blood was removed from the caudal vein and centrifuged (3·000 ***g***, 5 min), and the plasma recovered was immediately frozen and kept at −20°C. The liver and a sample of dorso-lateral white muscle from each fish were dissected, weighed and immediately frozen in liquid N_2 and kept at −80°C.

Chemical composition of the diets

Feed and freeze-dried whole fish were analysed for moisture (105°C for 24 h), ash (combustion in a muffle furnace at 550°C for 12 h), lipid (Soxtherm) and protein (N × 6·25, Kjeldahl Nitrogen analyser 2000; Fison Instruments) contents. Feed gross energy was determined using an adiabatic bomb calorimeter (IKA-Werke C5000; IKA® werke GmbH & Co. KG). Based on comparative carcass analyses, gain and retention values were computed.

Plasma metabolites

Plasma glucose, TAG and NEFA were undertaken with Glucose RTU (BioMerieux), PAP 150 (Biomérieux) and NEFA C (Wako Chemicals GmbH) kits, respectively, according to the recommendations of each manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction according to Moore[24] with glycine as the standard.

Western blot analysis

Protein extraction and Western blotting (20 μg protein for the liver and muscle) were undertaken as described previously[17,25] using anti-phospho Akt (Ser473), anti-Akt, anti-phospho-S6 (Ser235/Ser236), anti-S6, anti-phospho eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Thr37/Thr46) and anti-4E-BP1 (Ozyme) antibodies. These antibodies have been shown to successfully cross-react with protein from rainbow trout[17,25].

Gene expression analysis

Gene expression levels were determined by real-time quantitative RT-PCR as described previously[26]. We examined the expression of glucokinase (*GK*) and liver-type pyruvate kinase (hepatic *PK*) for hepatic glycolysis, hexokinase (*HK* and muscle-type pyruvate kinase (muscle *PK*) for muscle glycolysis, glucose-6-phosphatase isoforms 1 and 2 (*G6Pase1* and *G6Pase2*), fructose-1,6-biphosphatase (*FBPase*) and phosphoenolpyruvate carboxykinase (*PEPCK*) for hepatic gluconeogenesis, serine dehydratase (*SDH*) and branched-chain− α-keto acid dehydrogenase E1α, E1β and E2 subunits (*BCKDE1α*, *BCKDE1β* and *BCKDE2*) for hepatic amino acid catabolism, microtubule-associated light chain 3B (*LC3B*), γ-aminobutyric acid type A receptor-associated protein-like 1 (*GAB1*), autophagy-related 4b (*Atg4b*) and autophagy-related 12-like (*Atg12l*), involved in the autophagic/lysosomal proteolytic system and E3-ubiquitin ligases atrogin1/fbx32 and MuRF1 and α-type proteasome C2 and C3 subunits of the ubiquitin–proteasome-dependent proteolytic system for proteolysis. We also analysed fatty acid synthase (*FAS*), glucose-6-phosphate dehydrogenase (*G6PDH*) and the transcription factor sterol regulatory element-binding protein 1 (*SREBP1*) for lipogenesis and carnitine palmitoyltransferase 1 isoforms a and b (*CPT1a* and *CPT1b*) for hepatic fatty acid oxidation. Primers were designed to overlap an intron if possible (Primer3 v. 0.4.0; flodo.wi.mit.edu/) using known sequences in trout nucleotide databases (http://www.sigenae.org/) as described previously[12]. The quantification of the target gene transcript was done using *EF1* gene expression as reference as described previously[12,17,26]. No changes in *EF1* gene expression were observed in our studies (data not shown).

Enzyme activities

The protocols for determinations of hepatic GK, PEPCK and G6Pase activities were as described previously[8,27]. Enzyme activities (expressed per mg protein) were monitored by changes in the concentrations of reduced β-NAD and involved the use of a coupling enzyme added in excess. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as the standard. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol of substrate under the specified conditions (37°C).

Statistical analysis

Results are expressed as means with their standard errors. Statistical analyses of growth and nutrient utilisation parameters were performed by one-way ANOVA (Statview Software program, version 5; SAS Institute) to detect significant differences. Plasma parameters (glucose, TAG, NEFA and free amino acids), signalling data and gene expression data were analysed by two-way ANOVA. The Newman–Keuls multiple-range test was used to compare means in case of a significant effect ($P<0·05$).

Results

Growth performance, feed intake, and nutrient efficiency

Data on growth performance and feed utilisation are reported in Table 2. After the 9-week feeding trial, trout fed the C35P35 diet tended to have lower final body weight and growth rate than trout fed the C20P50 diet despite having a significantly higher feed intake over the trial period; however, the difference in final body weight and growth rate did not reach statistical significance ($P=0·6$ and $P=0·5$ for body weight and growth rate, respectively). Feed efficiency was thus better in fish fed the C20P50 diet than those fed the C35P35 diet. Increasing the proportion of carbohydrates at the expense of proteins significantly reduced daily N gain but had no effect on protein retention (percentage of intake). Fat gain and retention were significantly reduced in fish fed the C35P35 diet despite higher fat intake. Increasing the carbohydrate level led in fact to significantly reduced energy retention.

Table 2. Growth performance and nutrient utilisation†
(Mean values with their standard errors, *n* 3 in all groups)

	C35P35		C20P50		
	Mean	SEM	Mean	SEM	*P**
Initial body weight (g)	66·6	0·3	66·4	0·4	0·69
Final body weight (g)	152·4	1·6	172·3	7·7	0·06
Daily growth coefficient (%/d)‡	2·0	0·02	2·37	0·13	0·05
Feed intake (g DM/kg per d)§	14·1	0·1	11·5	0·03	<0·0001
N intake (mg/kg per d)	835·8	2·0	963·3	9·6	0·0002
Fat intake (g/kg per d)	2·0	0·03	1·8	0·01	0·0007
Feed efficiency‖	0·9	0·02	1·2	0·05	0·004
N gain (mg/kg per d)¶	333·2	2·4	388·9	12·7	0·01
Fat gain (g/kg per d)¶	1·0	0·1	1·3	0·1	0·02
Protein retention (% protein intake)**	39·9	0·7	40·4	1·4	0·77
Fat retention (% fat intake)**	50·4	2·4	70·8	3·7	0·01
Energy retention (% gross energy intake)	28·9	1·5	40·0	2·9	0·004

C35P35, 35 % carbohydrates/35 % proteins; C20P50, 20 % carbohydrates/50 % proteins.
* Mean values were significantly different between the diets ($P<0·05$).
† Weight is expressed as mean weight of fish within each tank (*n* 3 tanks). The other traits are expressed as mean of three tanks containing forty fish each.
‡ Daily growth coefficient = 100 × (mean final body weight$^{(1/3)}$ − mean initial body weight$^{(1/3)}$)/d.
§ Daily feed intake was calculated as the total amount of ingested food (g DM) divided by the mean biomass over the trial ((initial biomass + final biomass)/2, expressed as kg wet weight) and the number of days.
‖ Feed efficiency was estimated as the gain in total biomass (final biomass − initial biomass (kg wet weight)) divided by the amount of ingested DM (kg DM).
¶ N and fat gain were calculated as (final carcass nutrient content − initial carcass nutrient content) divided by the mean biomass over the trial ((initial biomass − final biomass)/2 (kg wet weight)) and the number of days, where nutrient refers to N and fat.
** Protein and fat retention were calculated as (100 × (final body weight × final carcass nutrient content) − (initial body weight × initial carcass nutrient content))/nutrient intake, where nutrient refers to protein and lipid.

Plasma metabolite levels

Data on plasma glucose, TAG, NEFA and free amino acids measured on 48 h-fasted fish (T0) and 2, 8 and 24 h after refeeding are presented in Fig. 1. Refeeding induced a significant rise in plasma glucose level with the highest level reaching 8 h after refeeding. In both groups, plasma glucose levels remained elevated 24 h after refeeding and were not significantly different from 8 h-refed fish. Plasma glucose levels were similar in both groups in fasted and 2 h-refed fish. Then, fish fed the C35P35 diet exhibited significantly higher plasma glucose levels 8 and 24 h after refeeding. Plasma TAG levels remained stable over the refeeding period and were not significantly different between the diets. NEFA levels significantly decreased 2 h after refeeding and then increased between 2 and 24 h after refeeding without reaching the level of NEFA measured in 48 h-fasted (T0) fish. No significant difference was recorded between the C35P35 and C20P50 diets. Total free amino acid levels were significantly affected by the diet with higher levels measured in fish fed the C20P50 diet at each sampling time. Plasma free amino acid levels changed significantly over the refeeding period. The levels measured 2 and 24 h after the meal were significantly lower than in fasted fish.

Hepatic intracellular signalling pathway

Using Western blot analysis, we investigated the effects of refeeding on hepatic phosphorylation of Akt and S6 proteins in fish fed the C35P35 and C20P50 diets by comparing the fasted fish and the 2- or 8 h-refed fish. As indicated in Fig. 2, refeeding did not enhance hepatic phosphorylation of Akt at least 2 and 8 h after refeeding irrespective of the diet. The ratio between hepatic phospho-S6 and total S6 significantly increased between the fasted and 2 h-refed fish in both diets. However, this increase was greater in C20P50-refed fish than in C35P35-refed fish as indicated by the statistical interaction between diet and sampling time at a significance level of $P=0·08$. When the fasted fish were compared with the 8 h-refed fish, the positive effect of refeeding on phosphorylated S6 was significant in both groups.

Muscle intracellular signalling pathway

In the muscle (Fig. 3), refeeding significantly enhanced the phosphorylation of Akt at both sampling times without any significant effect of the diets. Statistical analysis of the ratio between the phosphorylated and the total forms of 4E-BP1 indicated that the phosphorylation of 4E-BP1 increased 2 h after refeeding in both groups but reached a significantly higher level in fish fed the C20P50 diet than in fish fed the C35P35 diet as indicated by the positive interaction between diet and refeeding ($P<0·05$). When the fasted fish were compared with the 8 h-refed fish, a significant increase in 4E-BP1 phosphorylation was observed irrespective of the diet.

Hepatic gene expression

The effects of diet composition and refeeding on the expression of hepatic glucose metabolism-related genes were analysed by comparing the fasted, 8- and 24 h-refed fish (Fig. 4). Refeeding led to a strong increase in the expression of *GK* mRNA over the refeeding period.

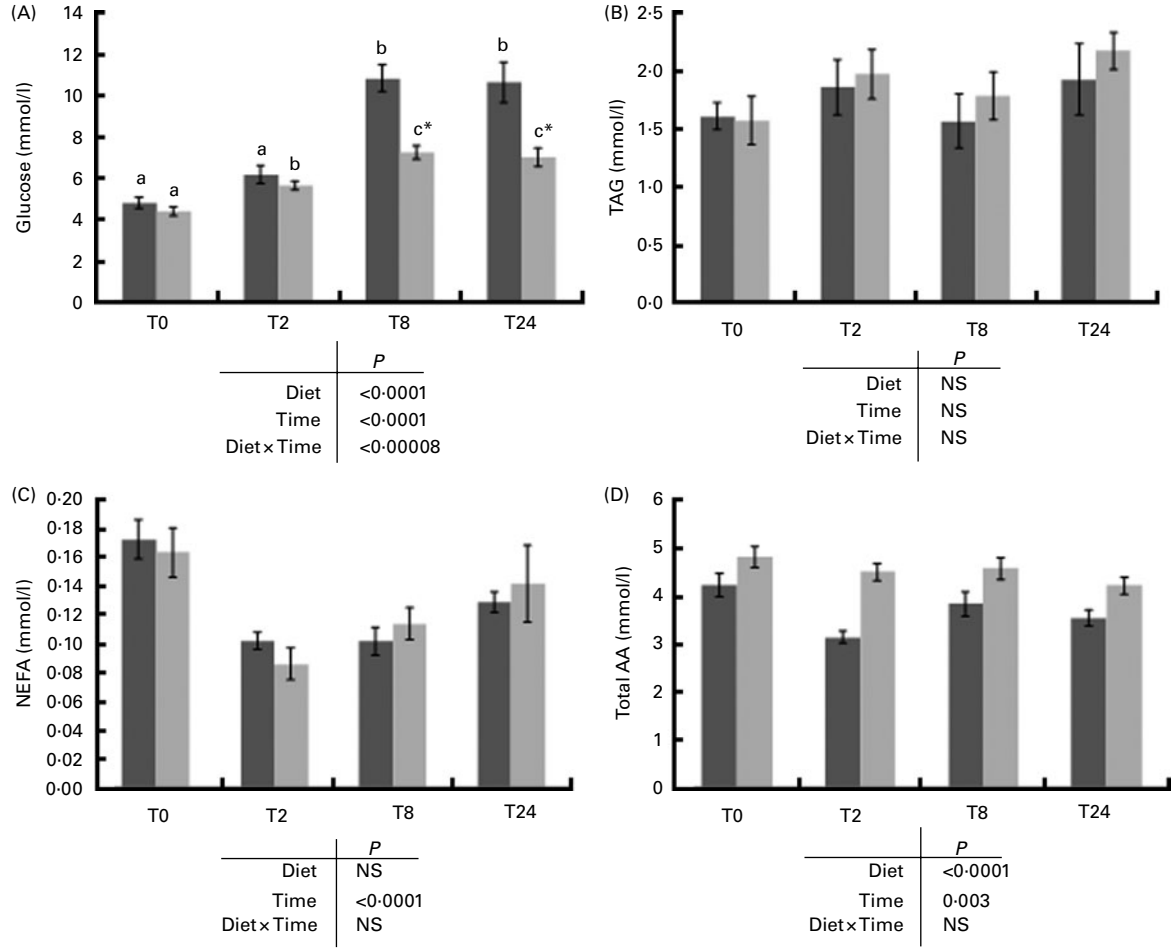

Fig. 1. Plasma levels of (A) glucose, (B) TAG, (C) NEFA and (D) total free amino acid (AA) in trout 2 (T2), 8 (T8) and 24 (T24) h after refeeding the 35 % carbohydrate/35 % protein (C35P35, ■) and 20 % carbohydrate/50 % protein (C20P50, ■) diets (fasted fish, T0). Values are means, with their standard errors represented by vertical bars (n 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. * Mean value was significantly different from that of the C35P35-fed group ($P<0·05$). ^{a,b,c} Mean values for a diet with unlike letters were significantly different ($P<0·05$). For glucose, there were significant effects for diet ($P<0·0001$) and time ($P<0·0001$) and the diet × time interaction was significant ($P=0·00008$). There was a significant effect of time ($P<0·0001$) for NEFA. For total AA, there were significant diet ($P<0·0001$) and time ($P=0·003$) effects.

The level of *GK* mRNA was also significantly higher in fish fed the C20P50 diet than in those fed the low-protein/high-carbohydrate diet. The expression of *PK* mRNA remained unchanged between the fasted and refed fish irrespective of the sampling time and diet. Among gluconeogenic genes, the levels of *PEPCK* mRNA declined from 8 h after refeeding and stabilised between 8 and 24 h after refeeding. An overall significantly lower expression of *PEPCK* was observed in the liver of fish fed the C35P35 diet compared with fish fed the C20P50 diet. Similar observations were made for *FBPase* gene expression except for the decrease in the *FBPase* mRNA level that was only significant between the fasted and 24 h-refed fish. Statistically significant interactions were observed between the diets and refeeding for each isoform of G6Pase. Refeeding significantly reduced *G6Pase1* mRNA levels 8 h after refeeding in both diets but the expression of *G6Pase1* remained at a low level between 8 and 24 h after refeeding in C20P50-refed fish, whereas in fish fed the C35P35 diet, the expression of this gene returned to the level found in the fasted fish. The comparison of fasted fish revealed that the expression of *G6Pase1* was higher in the liver of C20P50-fed fish than in their C35P35-fed counterparts. Refeeding inhibited the expression of the second isoform of G6Pase only in fish fed the C20P50 diet. As observed for *G6Pase1*, the level of *G6Pase2* mRNA was higher in fasted fish fed the C20P50 diet than in C35P35-fed fasted fish.

As indicated in Fig. 5, the expression of *SDH* enhanced 24 h after refeeding with both diets but the overall expression of *SDH* was greater in fish fed the C20P50 diet. *BCKDE1α* and *BCKDE1β* subunits exhibited a similar pattern of expression. They were significantly more expressed in fish fed the C20P50 diet than in those fed the C35P35 diet but were not regulated by refeeding. *BCKDE2* gene expression increased 8 h after refeeding and remained elevated 24 h after refeeding. The expression of *BCKDE2* was not affected by the composition of the diet. Regarding lipid metabolism, the expression of the lipogenic transcription factor *SREBP1* significantly increased 8 h after refeeding and then declined to reach values found in fasted fish. The level of expression of this transcription factor was not affected by the composition of the diet. A positive

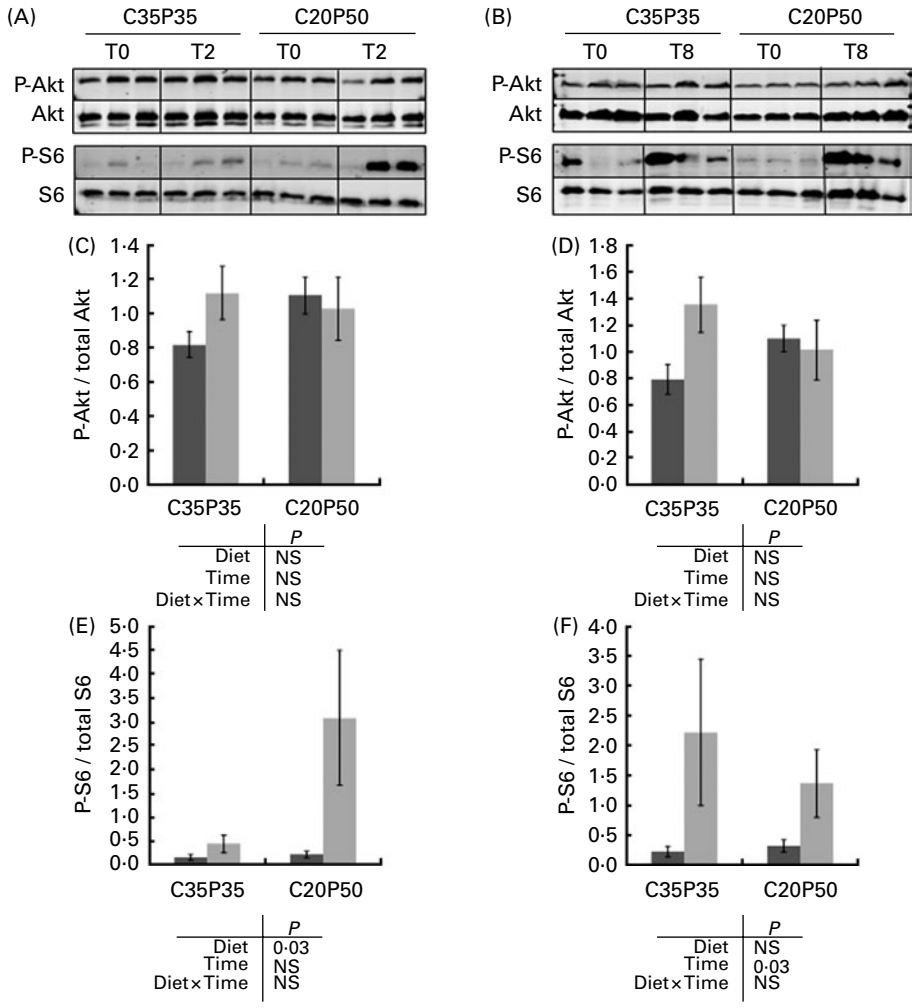

Fig. 2. Western blot analysis of hepatic protein kinase B (Akt) and S6 protein phosphorylation in fasted (T0, ■) and 35 % carbohydrate/35 % protein (C35P35) and 20 % carbohydrate/50 % protein (C20P50) diet-refed (■) rainbow trout. Comparisons between fasted and refed fish were performed (A, C, E) 2 h (T2) and (B, D, F) 8 h (T8) after refeeding. Equal amounts of 20 μg of total protein per lane were loaded on the gel. A representative blot is shown. (C–F) The ratio between the phosphorylated (P) protein and the total amount of the targeted protein. Values are means, with their standard errors represented by vertical bars (*n* 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. For the ratio P-S6:total S6, there were significant effects for (E) diet and (F) time (both $P=0{\cdot}03$).

effect of refeeding was also observed for the *G6PDH* mRNA level that significantly increased 24 h after refeeding in both groups. As indicated by the interaction between diet and time ($P=0{\cdot}03$), the abundance of *FAS* mRNA tended to be differentially regulated between the two dietary groups. Whereas refeeding tended to stimulate the expression of *FAS* in fish fed the C35P35 diet ($P=0{\cdot}07$), the level of *FAS* mRNA remained unchanged after refeeding in fish fed the C20P50 diet. The expression of isoforms a and b of *CPT1* significantly decreased 8 h after refeeding and remained at a low level at least 24 h after refeeding. A higher expression of *CPT1* was observed in fish fed the C35P35 diet; however, the difference reached statistical significance only for *CPT1a*.

Muscle gene expression

The effects of diet composition and refeeding on the expression of muscle metabolism-related genes were analysed by comparing the fasted, 8- and 24 h-refed fish (Fig. 6). The expression of *HK* and *PK* glycolytic genes was not subjected to refeeding regulation but diet composition affected significantly the expression of these two genes with a higher expression of *HK* and, conversely, a lower *PK* expression in the muscle of fish fed the C20P50 diet. The expression of C2 and C3 subunits of the ubiquitin–proteasome-dependent proteolytic system was neither affected by refeeding nor by diet composition, whereas the expression of fbx32 and MuRF1 ubiquitin ligases was significantly reduced from 8 h after refeeding. No difference in *fbx32* and *MuRF1* mRNA levels was observed between the two diets. Genes involved in the autophagic/lysosomal proteolytic system presented different kinds of regulation. Refeeding and diet composition did not affect *GAB1* mRNA levels. The *Atg4B* and *LC3B* genes exhibited a similar regulation pattern of expression. Whereas refeeding had no effect on the expression of *Atg4B* and *LC3B* in the muscle of fish fed the C20P50 diet, it significantly reduced mRNA levels of *Atg4B* and *LC3B* in fish fed the C35P35 diet from 8 h after refeeding. The levels of expression

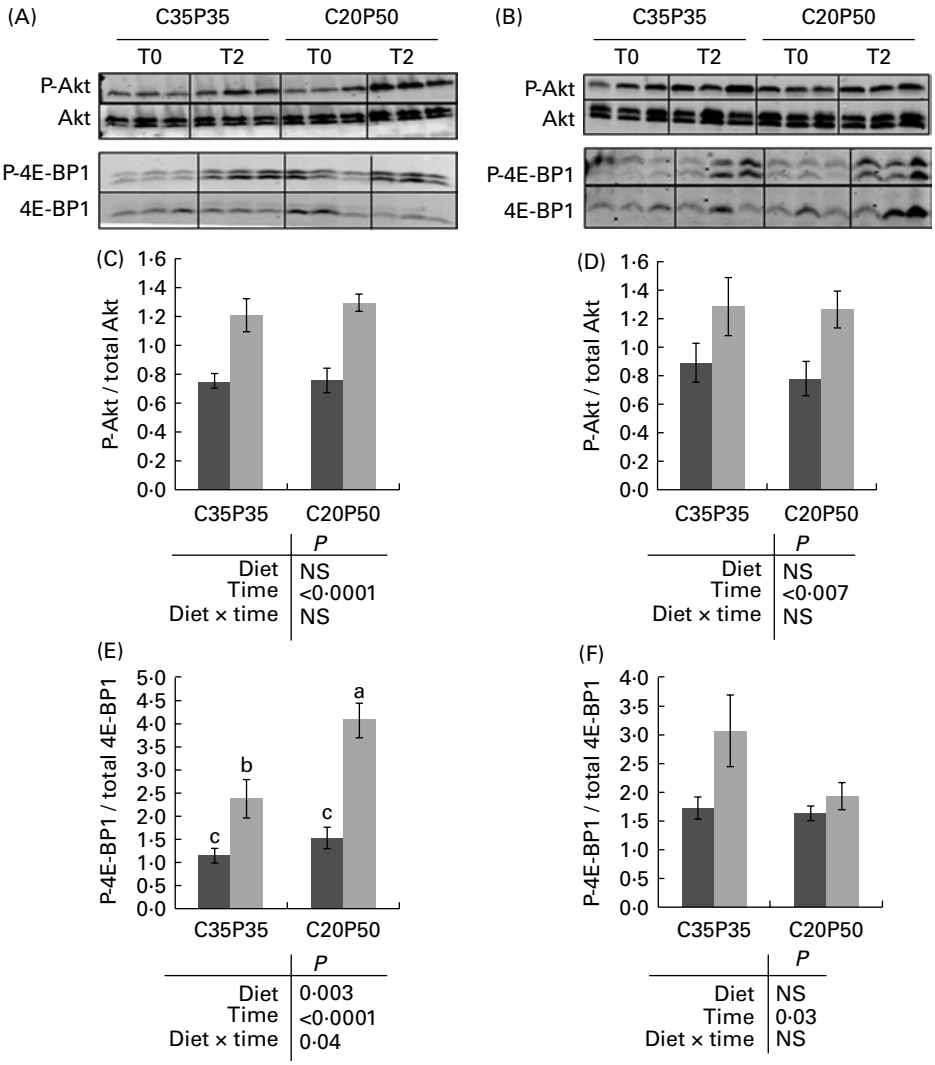

Fig. 3. Western blot analysis of muscle protein kinase B (Akt) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) protein phosphorylation in fasted (T0, ■) and 35 % carbohydrate/35 % protein (C35P35) and 20 % carbohydrate/50 % protein (C20P50) diet-refed (■) rainbow trout. Comparisons between fasted and refed fish were performed (A, C, E) 2 h (T2) and (B, D, F) 8 h (T8) after refeeding. Equal amounts of 20 μg of total protein per lane were loaded on the gel. A representative blot is shown. (C–F) The ratio between the phosphorylated (P) protein and the total amount of the targeted protein. Values are means, with their standard errors represented by vertical bars (n 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. [a,b,c]Mean values with unlike letters were significantly different ($P<0·05$). For the ratio P-Akt:total Akt, there were significant time effects ((C) $P<0·0001$ and (D) $P=0·007$). For the ratio P-4E-BP1:total 4E-BP1, there were significant effects for diet ((E) $P=0·003$) and time ((E) $P<0·0001$ and (F) $P=0·03$) and the diet × time interaction was significant ((E) $P=0·04$).

reached 8 h after refeeding was maintained 24 h after refeeding. Refeeding also decreased mRNA levels of *Atg12L* in both diets. However, the reduction was significant from 8 h after refeeding in fish fed the C35P35 diet against 24 h after refeeding in fish fed the C20P50 diet.

Enzyme activities

The effects of diet composition and refeeding on the enzyme activities of GK, G6Pase and PEPCK were analysed by comparing the fasted, 8- and 24 h-refed fish (Fig. 7). GK activities were similar between the two diets and significantly increased 24 h after refeeding. G6Pase activity remained stable over the sampling period and similar between the two diets. An increase in PEPCK activities was observed between the fasted and 8 h-refed fish. This increase was no longer observed 24 h after refeeding and was independent of the diet composition.

Discussion

The aim of the present study was to investigate the metabolic consequences of a high carbohydrate/low protein feeding and examine whether the rainbow trout was able to adapt to such a diet. Starch is the most common energy source for the majority of domestic terrestrial animals, but utilisation of native starch in fish is low and varies between fish species[1]. Omnivorous and herbivorous fish such as common carp, tilapia, channel catfish and Indian major carp species can easily be fed carbohydrate-rich diets without negative effects on growth and with good protein-sparing effect[1]. On the other

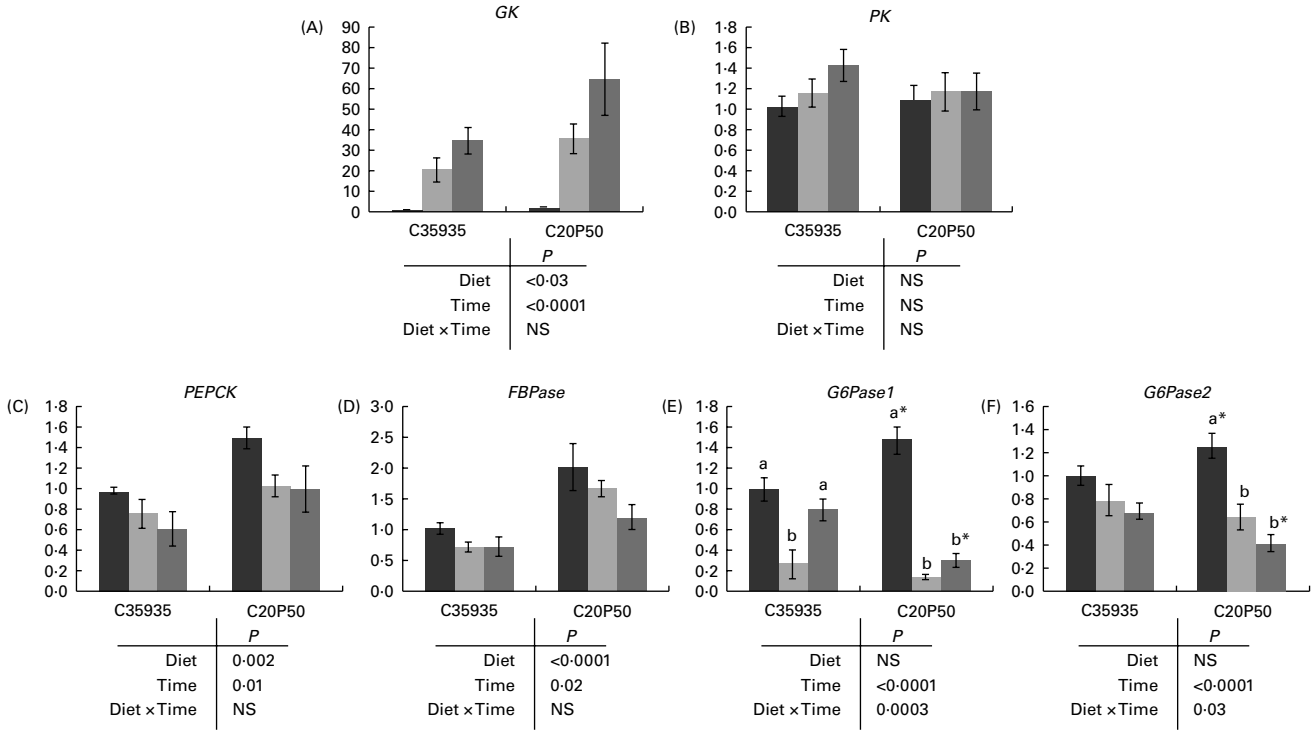

Fig. 4. Gene expression of selected enzymes related to glucose metabolism in the liver of fasted and 35 % carbohydrate/35 % protein (C35P35) and 20 % carbohydrate/50 % protein (C20P50) diet-refed rainbow trout. (A) Glucokinase (*GK*), (B) pyruvate kinase (*PK*), (C) phosphoenolpyruvate carboxykinase (*PEPCK*), (D) fructose-1,6-biphosphatase (*FBPase*), (E) glucose-6-phosphatase 1 (*G6Pase1*) and (F) glucose-6-phosphatase 2 (*G6Pase2*) mRNA levels were evaluated using real-time RT-PCR in fasted fish (■) and 8 h (■) and 24 h (■) after refeeding. Expression values are normalised with *EF1α*-expressed transcripts. Values are means, with their standard errors represented by vertical bars (n 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. * Mean value was significantly different from that of the C35P35-fed group ($P<0·05$). [a,b] Mean values for a diet with unlike letters were significantly different ($P<0·05$). For *GK*, there were significant effects for diet ($P<0·03$) and time ($P<0·0001$). There were significant effects of diet ($P=0·002$) and time ($P=0·01$) for *PEPCK*. For *FBPase*, there were significant effects of diet ($P<0·0001$) and time ($P=0·02$). There were significant effects of time ($P<0·0001$) and diet × time interaction ($P=0·0003$) for *G6Pase1*. For *G6Pase2*, there were significant effects for time ($P<0·0001$) and diet × time interaction ($P=0·03$).

hand, the growth of carnivorous fish including salmonids (Atlantic salmon and rainbow trout) and European sea bass is reduced when dietary levels of carbohydrates exceed 20 %[1,28]. We confirm in the present study that increasing the dietary carbohydrate level from 20 to 35 % while decreasing the protein content from 50 to 35 % reduced the growth of rainbow trout, although not significantly ($P=0·06$), after only 9 weeks of trial. This occurred despite an increased feed intake by this group to compensate for the low dietary protein level. The increase in the dietary carbohydrate:protein ratio also led to a reduction of N and fat gain. Whereas N gain reduction may be attributed to the reduction of protein intake since protein retention and efficiency were not modified, it seemed that fat gain reduction resulted from metabolic regulation decreasing fat retention such as oxidation of dietary fatty acids as energy supply. The high-carbohydrate/low-protein diet did not lead to any improved energy retention.

Protein kinase B/target of rapamycin signalling pathway

The decrease in feed efficiency suggests that the regulation of intermediary metabolism might have been modified by the reduction of the dietary protein:carbohydrate ratio. We reported previously that macronutrient composition of the diets affects the insulin/amino acid signalling pathway in rainbow trout liver and skeletal muscle after a single meal or, more precisely, the reduction of protein content in favour of carbohydrates impairs the activation of the Akt/TOR signalling pathway[12]. In the present study, we failed to demonstrate any such effect of dietary macronutrient composition on the phosphorylation of Akt. Refeeding had no effect on the phosphorylation of Akt in the liver, while muscle Akt phosphorylation was induced with both diets, further demonstrating that tissues may exhibit a differential regulation of phosphorylation events as already shown in mammalian models[29]. Nevertheless, the impaired postprandial phosphorylation of Akt in the liver is in agreement with previous studies showing that Akt activation is impaired after a single meal containing a high and low proportion of carbohydrate and protein, respectively[12]. Surprisingly, the phosphorylation of the Akt/TOR signalling downstream protein S6 was fully induced in the liver after refeeding irrespective of any changes in the diet. The hepatic activation of S6 without those of Akt remains unclear. Akt is known to be directly dependent on insulin for its activation[17], whereas targets of the TOR signalling pathway such as the ribosomal protein S6 require the association of insulin and amino acids. Low insulin secretion may be ruled out since previously published data indicate that increasing carbohydrates in the trout diet results in equivalent or even enhanced insulin secretion[3,30]. Further research is needed to evaluate the potential implication of the

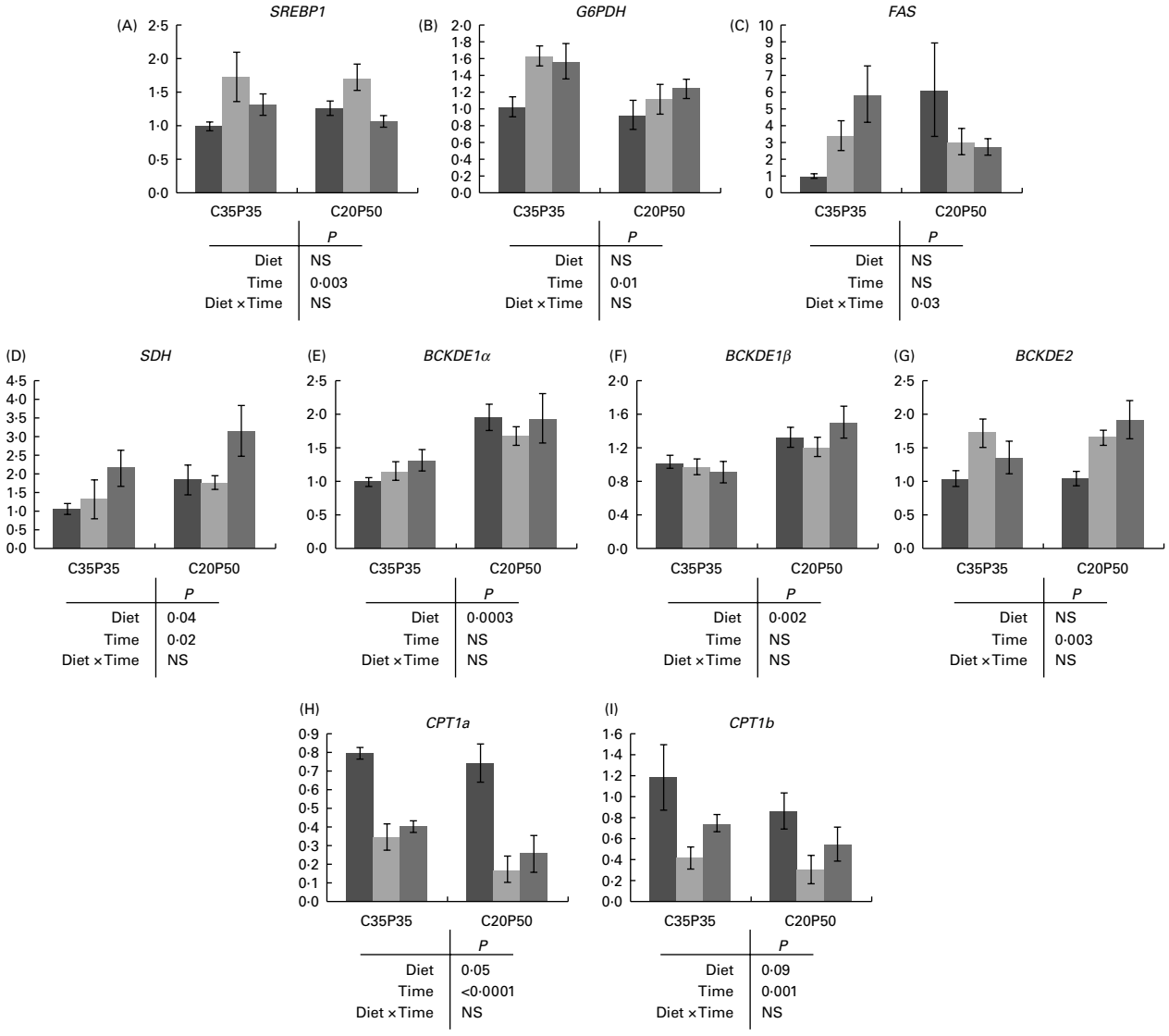

Fig. 5. Gene expression of selected enzymes related to lipid and energy metabolism in the liver of fasted and 35 % carbohydrate/35 % protein (C35P35) and 20 % carbohydrate/50 % protein (C20P50) diet-refed rainbow trout. (A) sterol regulatory element-binding protein 1 (*SREBP1*), (B) glucose-6-phosphate dehydrogenase (*G6PDH*), (C) fatty acid synthase (*FAS*), (D) serine dehydratase (*SDH*), branched-chain α-keto acid dehydrogenase (E) E1α (*BCKDE1α*), (F) E1β (*BCKDE1β*) and (G) E2 (*BCKDE2*) subunits and carnitine palmitoyltransferase 1 isoforms (H) a (*CPT1a*) and (I) b (*CPT1b*), mRNA levels were evaluated using real-time RT-PCR in fasted fish (■) and 8 h (■) and 24 h (■) after refeeding. Expression values are normalised with *EF1α*-expressed transcripts. Values are means, with their standard errors represented by vertical bars (n 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. * Mean value was significantly different from that of the C35P35-fed group ($P<0·05$). There were significant diet effects ($P=0·04$, $P=0·0003$, $P=0·002$, $P=0·05$ and $P=0·09$) for *SDH*, *BCKDE1α*, *BCKDE1β*, *CPT1a* and *CPT1b*, respectively. For *SREBP1*, *G6PDH*, *SDH*, *BCKDE2*, *CPT1a* and *CPT1b*, there were significant time effects ($P=0·003$, $P=0·01$, $P=0·02$, $P=0·003$, $P<0·0001$ and $P=0·001$, respectively). A significant effect was observed for the diet × time interaction ($P=0·03$) for *FAS*.

TOR/S6K1 negative feedback loop to IRS1[31] in the absence of postprandial hepatic activation of Akt in trout fed carbohydrate-rich diets. In the muscle, the phosphorylation of the Akt/TOR signalling downstream protein 4E-BP1 was also induced by refeeding, although this induction was significantly lower in C35P35-fed fish than in C20P50-fed fish 2 h after refeeding. This transient effect of increasing dietary carbohydrate:protein ratio on this key factor of muscle protein synthesis could account for the observed reduction in daily N gain. Further studies are warranted to monitor the consequences of long-term high carbohydrate/low protein feeding on the rate of muscle protein synthesis and degradation.

Muscle proteolytic pathways

Protein accretion reflects dynamic changes between synthesis and degradation. The latter depends on the activity of the main proteolytic routes that are the ubiquitin-proteasomal and the autophagy-lysosomal pathways. As already observed in this species[12,32,33], genes encoding proteasome subunits (C2 and C3) were not regulated by nutritional status. In contrast, genes encoding E3-ubiquitin ligases, atrogin1/MAFbx and MuRF1, were inhibited by feeding. However, the dietary carbohydrate:protein ratio had no impact on the expression of these genes. In mammals, the regulation of atrogin1/

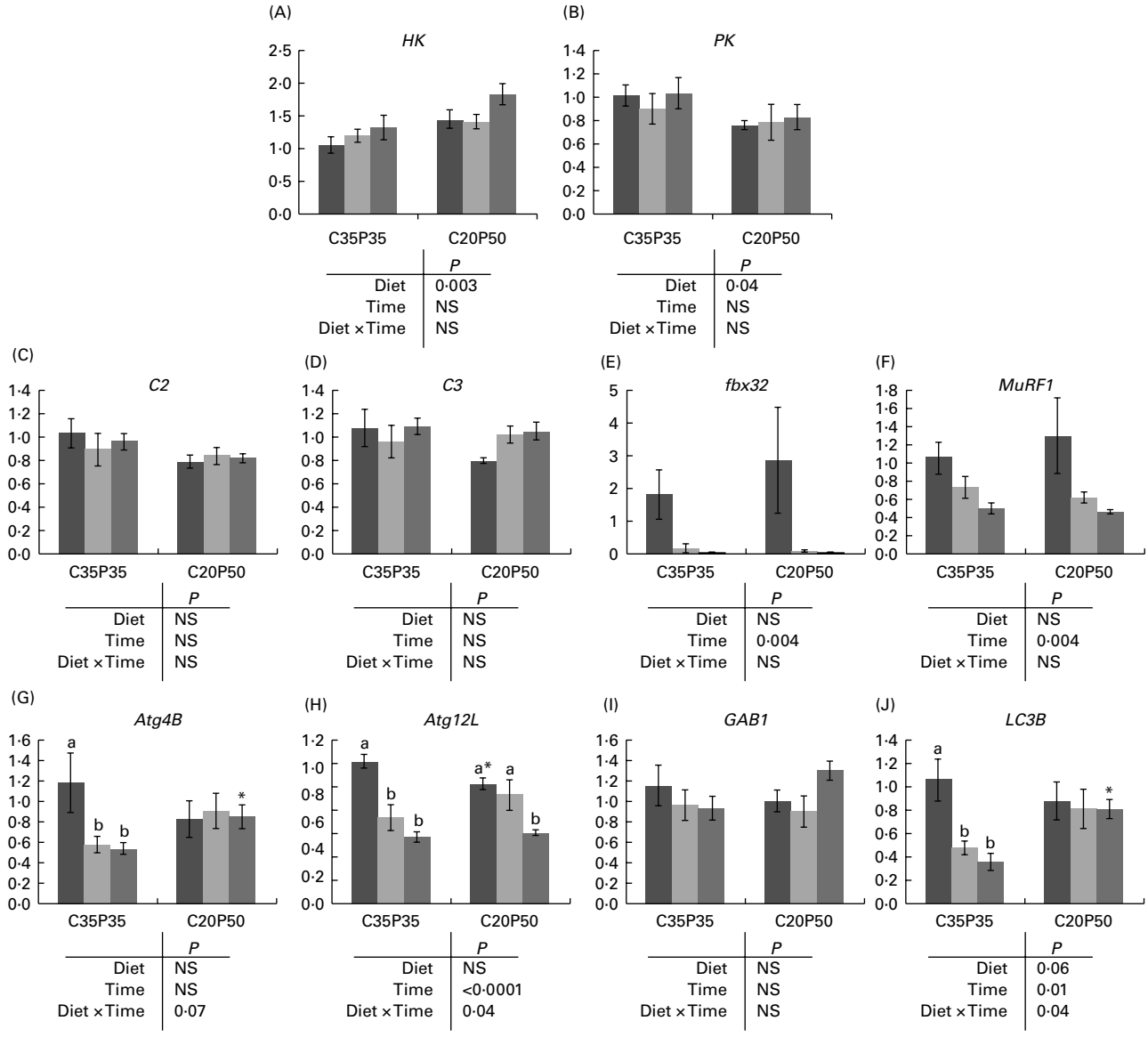

Fig. 6. Gene expression of selected enzymes in the muscle of fasted and 35 % carbohydrate/35 % protein (C35P35) and 20 % carbohydrate/50 % protein (C20P50) diet-refed rainbow trout. (A) Hexokinase (*HK*), (B) pyruvate kinase (*PK*), (C) *C2*, (D) *C3*, (E) *fbx32*, (F) *Murf*, (G) autophagy-related 4b (*Atg4b*), (H) autophagy-related 12-like (*Atg12l*), (I) γ-aminobutyric acid type A receptor-associated protein-like 1 (*GABA1*) and (J) microtubute-associated light chain 3B (*LC3B*) mRNA levels were evaluated using real-time RT-PCR in fasted fish (■) and 8 h (■) and 24 h (■) after refeeding. Expression values are normalised with *EF1α*-expressed transcripts. Values are means, with their standard errors represented by vertical bars (*n* 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. * Mean value was significantly different from that of the C35P35-fed group ($P<0·05$). [a,b] Mean values for a diet with unlike letters were significantly different ($P<0·05$). There were significant diet effects ($P=0·003$, $P=0·04$ and $P=0·06$) for *HK*, *PK* and *LC3B*, respectively. For *fbx32*, *Murf*, *Atg12l* and *LC3B*, there were significant time effects ($P=0·004$, $P=0·004$, $P<0·0001$ and $P=0·01$, respectively). There were significant effects observed for the diet × time interaction ($P=0·07$, $P=0·04$ and $P=0·04$) for *Atg4b*, *Atg12l* and *LC3B*, respectively.

MAFbx and MuRF1 has been shown to be associated with the activity of the FoxO1/3 (Forkhead box protein O1/3) transcription factors, the downstream targets of the phosphatidy Linositol 3-kinase (PI3K)/Akt signalling pathway[34–36]. In this regard, the correlation between the phosphorylation of Akt and the pattern of expression of the two E3-ubiquitine ligase-coding genes in the present study strongly suggests a strong conservation of the mechanisms involved in the regulation of the ubiquitin-proteasome-dependent proteolytic pathway between higher and lower vertebrates and confirms previous observation[37].

In contrast, no relationship was observed between the activation of Akt and the expression of autophagy-related genes. In mammals, the expression of these genes is recognised to be also dependent on the PI3K/Akt signalling pathway via the activation of the transcription factors FoxO[38,39]. However, recent *in vitro* and *in vivo* studies in trout have indicated that insulin growth factor 1 and insulin induces the activation of Akt and FoxO but has low or no effect on autophagy-related transcript levels, suggesting a minor role for this signalling axis on the autophagic/lysosomal pathway in this species[12,40,41]. The results presented here are consistent

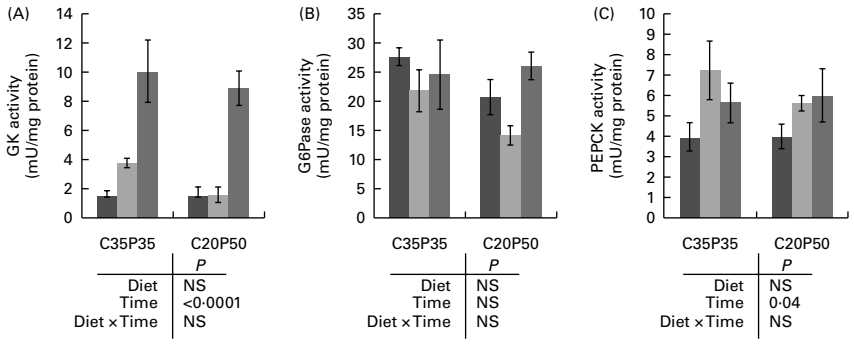

Fig. 7. Activities of (A) glucokinase (GK), (B) glucose-6-phosphatase (G6Pase) and (C) phosphoenolpyruvate carboxykinase (PEPCK) enzymes in the liver of fasted and 35 % carbohydrate/35 % protein (C35P35) and 20 % carbohydrate/50 % protein (C20P50) diet-refed rainbow trout. Activities were measured in fasted fish (■) and 8 h (■) and 24 h (■) after refeeding. Values are means, with their standard errors represented by vertical bars (n 6). Data were analysed using two-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. For GK and PEPCK activity, there were significant time effects ($P<0·0001$ and $P=0·04$, respectively).

with these data and indicate that other factors may account for the different expression patterns of the monitored autophagy-related genes between the two groups of fish. In this regard, new findings have provided direct evidence on the role of several other factors in mediating the induction of the expression of autophagy-related genes in muscle wasting[42]. Collectively, the present results and those previously published highlight the complexity of mechanisms involved in the control of the expression of autophagy-related genes.

Glucose metabolism

One hypothesis to explain the low dietary carbohydrate utilisation in trout is the atypical regulation of hepatic glucose metabolism in fish fed high levels of carbohydrates. A lack of induction of GK activity can be definitively excluded to explain the low dietary carbohydrate utilisation in fish. In fact, we observed that feeding trout with carbohydrates during 9 weeks led to the postprandial induction of this enzyme at both molecular and activity levels as already shown in trout after a single meal[12]. These results confirm that carbohydrates are necessary for GK induction in fish species[12,26,43,44]. Surprisingly, the induction of *GK* mRNA expression was less important in fish fed the diet containing the highest proportion of carbohydrates. It is possible that exposure to high plasma glucose levels generated by long-term feeding with a high-carbohydrate/low-protein diet may lead to the repression of *GK* expression. However, this difference in *GK* gene expression did not affect the level of GK activity reached 24 h after refeeding. The expression of the other studied hepatic and muscle glycolytic enzymes (hepatic PK, muscle HK and PK) was not modified between the fasted and refed fish irrespective of the dietary treatment, possibly resulting in a poor utilisation of dietary carbohydrates in these fish fed diets with carbohydrate levels at or above 20 % of the DM.

The second hepatic metabolic pathway that may be proposed to explain the low dietary utilisation in fish is the endogenous production of glucose. In contrast to mammals or some teleosts such as the gilthead seabream[45,46], no inhibition of gluconeogenesis was found in trout regardless of whether they were fed with or without carbohydrates[10,11,47]. It has been proposed that the absence of the regulation of postprandial gluconeogenesis could be related to the presence of a high level of gluconeogenic amino acids in the diets. Therefore, using a pair-feeding approach, Panserat *et al.*[48] demonstrated that at the same level of carbohydrate intake, a low dietary protein intake was associated with a significant decrease in hepatic gluconeogenic enzyme expression. We also suggested in a previous work that a futile cycle based on the phosphorylation and immediate dephosphorylation of glucose might contribute to the typical persistent postprandial hyperglycaemia in rainbow trout fed high levels of carbohydrates[12]. Indeed, based on fasting followed by a single meal, we previously found a concomitant increase in *GK* gene expression and an absence of the inhibition of gluconeogenic gene expression in fish fed high levels of carbohydrates[12]. In order to test the ability of trout to adapt their glucose metabolism to diets containing reduced and increased proportions of proteins and carbohydrates, respectively, we analysed here the effects of refeeding on gluconeogenic enzymes. We found that after 9 weeks of adaptation to the diets, the expression of gluconeogenic enzymes (PEPCK, FBPase, G6Pase1 and G6Pase2) were all down-regulated after refeeding, whereas they remained highly expressed after a single meal[12]. These results suggest that a long-term adaptation of the fish to carbohydrate-rich diets may restore the postprandial inhibition of gluconeogenic gene expression. It also appears that the dietary protein:carbohydrate ratio influenced the level of expression of gluconeogenic genes since all of them exhibited higher levels of expression in fish fed the lowest proportion of carbohydrate and a better response to refeeding. This higher expression of gluconeogenic genes in fish fed a diet with the highest protein:carbohydrate ratio is consistent in comparison with the positive role of amino acid on gluconeogenic gene expression[17,48]. Surprisingly, these molecular adaptations were without any effect on the enzyme activities, further strengthening the hypothesis of the existence of the futile cycle around the phosphorylation of glucose (due to the increased postprandial GK activity and the absence of the inhibition of G6Pase activity) that contributed to the persistence of high levels of plasma glucose in fish fed high levels of carbohydrates. Since a 9-week feeding

adaptation period was able to restore the postprandial inhibition of gluconeogenic gene expression without any modification of enzyme activities, we can wonder whether a longer feeding adaptation period may affect the activities of gluconeogenic enzymes. Nevertheless, we cannot rule out the possibility that enzyme activities were not measured at adequate timing. Further experiments are needed to explore this hypothesis.

Lipid metabolism

Increasing the dietary carbohydrate proportion from 20 to 35% while decreasing the protein content from 50 to 35% led to decreased fat gain and fat retention despite higher fat intake. These results clearly suggest that amino acids supplied through the high-protein diet led to higher lipogenesis and/or lower lipid catabolism (not measured here) than the diet containing less protein and more carbohydrate. However, our transcriptional data did not clearly corroborate an enhanced lipogenesis in fish fed the high-protein diet. Actually, expressions of the lipogenic transcription factor *SREBP1* and the enzyme of the pentose phosphate pathway G6PDH that produces reducing equivalents (NADPH) used as a cofactor for lipogenic enzymes were similarly increased after refeeding in both groups. Furthermore, the expression of *FAS* was up-regulated after refeeding in fish fed the high-carbohydrate/low-protein diet while remaining unchanged in fish fed the high-protein diet. It would have been interesting to analyse the activities of lipogenic enzymes since discrepancies between mRNA levels and enzyme activities have previously been demonstrated for lipogenic enzymes in fish[49,50].

The expression of *CPT1*, at least the isoform a, was higher in fish fed the low-protein diet ($P=0{\cdot}054$), suggesting that under situations of protein deficiency, energy production in fish highly relies on lipid catabolism. The measurement of CPT1 activity as well as fatty acid oxidation would be of great interest to confirm this hypothesis. The utilisation of lipids as an energy source probably led to the low fat deposition and fat gain in fish fed the diet containing the highest proportion of carbohydrates.

Amino acid catabolism

We observe that the expression of enzymes involved in amino acid catabolism is influenced by the composition of the diet. The expression of *SDH* and enzymes involved in branched-chain amino acid catabolism was higher in fish fed with the highest proportion of proteins. Therefore, unlike domestic cats, another example of carnivorous species that appears to have a limited ability to adjust protein oxidation to low dietary intakes of protein[51,52], it seemed that trout was able to adapt their hepatic catabolic capacity to the variation of protein intake.

In conclusion, the present study demonstrates that increasing the carbohydrate:protein ratio of the trout diet over a 9-week-long period affects protein synthesis and leads to the modification of expression of genes related to metabolism. However, gluconeogenesis was not inhibited after refeeding, despite the restoration of the postprandial inhibition of gluconeogenic gene expression, suggesting that the 'futile cycle' between GK and G6Pase still contributes to the persistence of hyperglycaemia. The present study also clearly indicates the ability of fish to adapt their amino acid catabolism to the dietary protein and carbohydrate levels. Further studies should explore the role of each macronutrient in energy production in fish fed high levels of carbohydrates.

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