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Dietary carbohydrate to protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal.

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ABSTRACT

Most teleost fish are known to require high levels of dietary proteins. Such high protein intake could have significant effects, particularly on insulin-regulated gene expression. We therefore analyzed the effects of an increase in the ratio of dietary carbohydrates/proteins on the refeeding activation of the Akt-TOR signaling pathways in rainbow trout and the effects on the expression of several genes related to hepatic and muscle metabolism and known to be regulated by insulin, amino acids and/or glucose. Fish were fed once one of three experimental diets containing high (H), medium (M) or low (L) protein (P) or carbohydrate (C) levels after 48h of feed deprivation. Activation of the Akt/TOR signaling pathway by refeeding was severely impaired by decreasing the proteins/carbohydrates ratio. Similarly, post-prandial regulation of several genes related to glucose (Glut4, glucose-6-phosphatase isoform 1), lipid (fatty acid synthase, ATP-citrate lyase, sterol responsive element binding protein, carnitine palmitoyltransferase 1 and 3-hydroxyacyl-CoA dehydrogenase) and amino acid metabolism (serine dehydratase and branched chain α-keto acid dehydrogenase E2 subunit) only occurred when fish were fed the high protein diet. On the other hand, diet composition had a low impact on the expression of genes related to muscle protein degradation. Interestingly, glucokinase was the only gene of those monitored whose expression was significantly up-regulated by increased carbohydrate intake. In conclusion, this study demonstrated that macro-nutrient composition of the diet strongly affected the insulin/amino acids signaling pathway and expression pattern of genes related to metabolism.

INTRODUCTION

Among teleosts, the rainbow trout is recognized for their inefficiency in using digestible carbohydrates (53) and are thus considered to be glucose intolerant. Oral administration of glucose or a carbohydrate-rich meal leads to prolonged postprandial hyperglycaemia despite normal insulin secretion. At the metabolic level, most of the key enzymes involved in carbohydrate metabolism have been described in fish (2). Induction of some of them such as glucokinase (GK) in liver and 6-phosphofructokinase (6PFK) and pyruvate kinase (PK) in liver and muscle by dietary carbohydrates is similar to that described in mammalian systems (5, 29, 33). However, findings concerning other metabolic mediators of glucose metabolism suggest the existence of atypical regulation after carbohydrate intake by trout, i.e. a lower capacity for glucose phosphorylation by hexokinases in the muscle of fish than in mammalian species (13, 53). Moreover, a carbohydrate-rich diet does not affect the activity or gene expression of key enzymes of gluconeogenesis such as glucose-6-phosphatase (G6Pase), fructose-1,6-biphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK) in rainbow trout (28, 30, 31, 51). Such impaired post-prandial down-regulation of gluconeogenesis in rainbow trout fed diets rich in carbohydrates is similar to the insulin resistance observed in human patients with type II diabetes. Moreover, trout also exhibit a low lipogenic response when fed a carbohydrate-rich diet (34). Despite the postprandial induction of insulin secretion (3) and the expected effects of insulin on the regulation of metabolism-related gene expression in the rainbow trout liver (35), the reasons for the particular regulation of metabolism-related genes in fish fed an increased proportion of carbohydrates remain unclear.

The mechanisms involved in the regulation of metabolism often depend on the crosstalk between nutritional and hormonal signals. This has been widely investigated for insulin and amino acids in recent years. Amino acids are not only considered to be precursors for the synthesis of proteins and other N-containing compounds, but are also involved in the regulation of major metabolic pathways (22) and are now considered as signaling molecules. Amino acids regulate protein synthesis by activating the mammalian target of rapamycin (mTOR) / p70 S6 kinase (S6K1) transduction pathway, together with insulin. The mechanism leading to the activation of TOR by insulin has already been established. Insulin acts through a tyrosine kinase membrane receptor that once activated by insulin binding, recruits and phosphorylates intracellular substrates known as IRS (insulin receptor substrates). Phosphorylated IRS then transmit the signal through several molecules, including successively phosphatidylinositol 3 Kinase (PI3K) and Akt (also known as protein kinase B), a critical node of the insulin signaling pathway leading to (at least) the regulation of glucose transport, glycogen synthesis, mRNA translation and gene expression (50). Indeed, Akt regulates intermediary metabolism-related gene expression via phosphorylation and nuclear exclusion of Forkhead-box Class O1 (FoxO1) transcription factor and also controls protein synthesis by the activation of TOR. Increasing evidence has emerged in recent years to show that amino acids may interfere with insulin function. For example, excessive levels of amino acids have detrimental effects on glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis (52), which may be due to the over-activation of TOR and S6K1. Recent findings have also indicated that activation of TOR contributes to the regulation of fatty acid biosynthesis by blocking nuclear accumulation of the mature form of the sterol responsive element binding protein (SREBP1) and subsequent expression of SREBP1 target genes such as fatty acid synthase (FAS) and ATP citrate lyase (ACLY) (38). This was

97 recently confirmed in rainbow trout hepatocytes where insulin and amino acids regulate
98 lipogenic as well as SREBP1 gene expression through a TOR-dependent pathway (14).
99
100 One particular feature of the rainbow trout diet is that total protein content may exceed 45%
101 of the dry matter. This consistently high dietary amino acid intake may thus have significant
102 effects, particularly on insulin-regulated gene expression. This could, for example, explain the
103 absence of post-prandial down-regulation of the expression of insulin target genes such as
104 G6Pase and PEPCK and the restoration of their inhibition by reducing dietary protein levels
105 (12, 32). The aim of the study presented here was therefore to analyze the effects of an
106 increase in the ratio of dietary carbohydrates/proteins of a single meal on the post-prandial
107 activation of the Akt-TOR intracellular signaling pathways as well as on the expression of
108 several key genes related to hepatic and muscle metabolism known to be regulated by insulin,
109 amino acids and/or glucose in higher vertebrates.
110

MATERIALS AND METHODS

Fish and diets

The experiments were carried out in accordance with French legislation governing the ethical treatment of animals and the investigators were certified by the French Government to carry out animal experiments. Three triplicate groups of juvenile rainbow trout (15 fish per tank; weights ranging from 50 to 60 g) were reared in our own experimental facilities (INRA, Donzacq, France) at 18°C and fed a commercial diet. Fish were left unfed for 48h in order to obtain the basal levels of plasma metabolites that are reached later in fish than in mammals due to slower intestinal transit and longer gastric emptying time at low temperatures compared to endothermic animals. Fish were then fed once ad libitum with one of the three semi-purified diets of high (H), medium (M) or low (L) levels of protein (P) or carbohydrates (C) (HPLC, MPMC and LPHC, respectively) (Table 1). The amount of feed distributed per tank was measured to ensure that feed intake was similar between diets (around 2% of their body weight). The diets were marginal, adequate and in excess of rainbow trout protein requirements (18, 37 and 65%, respectively) (25). Casein (normal or hydrolysate) and dextrin were used as protein and carbohydrate sources, respectively. Although, the diets differ slightly in terms of their energy content, they all fulfill the recommended requirements for the studied species (25). Three fish were sampled from each tank before feeding as well as 2, 8 and 24h after the meal. Trout were sacrificed by a sharp blow to the head. Gut content of the sampled animals was systematically checked to assert that the fish sampled 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-ventral white muscle from each fish were dissected, weighed and immediately frozen in liquid nitrogen and kept at –80°C.

Chemical composition of the diets

The chemical composition of the diets was analyzed using the following procedures: dry matter was determined after drying at 105°C for 24h, protein content (N×6.25) was determined by the Kjeldahl method after acid digestion (4), fat by petroleum ether extraction (Soxtherm) and gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany).

Plasma metabolites

Plasma glucose levels were determined using a commercial kit (Biomérieux, Marcy l'Etoile, France) adapted to a microplate format. Total plasma free amino acid levels were determined by the ninhydrin reaction according to Moore (24) with glycine as standard.

Western blot analysis

Protein extraction and western blotting (20 μg of protein for liver and muscle) were developed as previously described (14, 44) using anti-phospho Akt (Ser473), anti-Akt, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho S6K1 (Thr389), anti-phospho S6 (Ser235/Ser236), anti-S6, anti-phospho 4E-BP1 (Thr37/Thr46), anti-4E-BP1 (Ozyme, Saint Quentin Yvelines, France) and anti-S6K1 (Santa Cruz, Tebu, Le Perray-en-Yvelines, France)

159 antibodies. These antibodies have been shown to successfully cross-react with rainbow trout
160 (14, 44).

161 ***Gene expression analysis***

162

163 Gene expression levels were determined by real-time quantitative RT-PCR as previously
164 described (35). We examined expression of G6Pase1, G6Pase2, FBPase and PEPCK for
165 gluconeogenesis, glucose transporter 4 (Glut4), hexokinase (HK), GK, 6PFK and PK for
166 glucose transport and glycolysis, ACLY, FAS, glucose-6-phosphate dehydrogenase (G6PDH)
167 and the transcription factor SREBP1 for lipogenesis, carnitine palmitoyltransferase 1 isoforms
168 A and B (CPT1A and CPT1B), acetyl-CoA carboxylase (ACC), and 3-hydroxyacyl-CoA
169 dehydrogenase (HOAD) for fatty acid oxidation, serine dehydratase (SD) and branched chain
170 α-keto acid dehydrogenase E2 subunit (BCKD E2) and branched chain α-keto acid
171 dehydrogenase kinase (BCKD kinase) which are involved in the irreversible step of oxidation
172 of branched chain amino acids for amino acid catabolism and microtubule-associated light
173 chain 3B (LC3B), gamma-aminobutyric acid type A (GABA(A)) receptor-associated protein-
174 like 1 (Gabarapl1), autophagy-related 4b (atg4b) and autophagy-related 12-like (atg12l),
175 involved in the autophagic/lysosomal proteolytic system and E3-ubiquitin ligases
176 atrogin1/MAFbx and MuRF and alpha-type proteasome C2 and C3 subunits of the Ubiquitin-
177 Proteasome-Dependent Proteolytic system for proteolysis. Primers were designed to overlap
178 an intron if possible (Primer3 software) using known sequences in trout nucleotide databases
179 (http://www.sigenae.org/) as previously described (14, 35, 37, 45, 46). Primers targeting
180 genes encoding proteins from the proteasomal proteolytic system and the branched chain
181 amino acid oxidative system are detailed in Table 2. Primers targeting all other genes are
182 presented in previous publications of the same authors (14, 35, 37, 45, 46). Quantification of
183 the target gene transcript was done using usual EF1α gene expression as reference as

184 previously described (35). No changes in EF1α gene expression were observed in our studies
185 (data not shown).
186
187 ***Statistical analysis***
188
189 Results are expressed as means ± SEM. Statistical analyses were performed by one-way
190 ANOVA (Statview Software program, version 5; SAS Institute, Cary, NC) to detect
191 significant differences. The Newman-Keuls multiple-range test was used to compare means in
192 the case of a significant effect ($P < 0.05$).
193

RESULTS

Plasma glucose and amino acid levels.

Postprandial plasma glucose and free amino acid levels are presented in Figure 1. Compared to fasted trout (F) plasma glucose levels were significantly enhanced in trout fed high (LPHC) or medium (MPMC) levels of carbohydrates 8 and 24h after feeding. The highest plasma glucose levels were measured 24h after feeding in fish fed the diet containing the highest proportion of carbohydrates (Figure 1A). In contrast, no significant increase in plasma glucose level was observed in fish fed the HPLC diet. Compared to fasted fish, total plasma free amino acid (FAA) levels increased significantly in fish 2h after the meal, irrespective of the diet (Figure 1B). Total plasma FAA levels in fish fed the LPHC diet then decreased to reach levels not significantly different from those of the fasted group 8h after feeding and then to levels statistically lower than fasted levels 24h after refeeding. A similar pattern of changes was observed in fish fed the MPMC diet except that total plasma FAA levels remained significantly elevated 8h after refeeding. The highest total plasma FAA levels were measured 8h after feeding in fish fed the HPLC diet. In these fish, plasma levels of total free amino acids remained significantly higher than levels measured in fasted fish 24h after feeding.

Activation of hepatic Akt/TOR signaling pathway.

We investigated the effects of feeding the three experimental diets on the activation of hepatic Akt/TOR signaling pathways by comparing levels in fasted fish and 2h and 8h after the meal, respectively. Figure 2 presents the results of Western blot analyses of phosphorylated (p-) and total forms of Akt, TOR and S6 obtained in fasted and 2h refed fish. The ratio between the phosphorylated and total forms of the protein was calculated for each protein. Analysis of Akt

and S6 data indicated that the phosphorylation of Akt and S6 was significantly enhanced 2h after refeeding only in fish fed the HPLC diet. Analysis of TOR data showed no significant differences in the ratio of phosphorylated to total protein between the three dietary groups and unfed trout. Eight hours after refeeding, Akt/TOR signalling was no more activated whatever the diet (data not shown).

Messenger RNA levels of target genes in liver of fasted and 24h refed fish.

Compared to fasted fish, GK mRNA levels increased dramatically in fish fed the LPHC and MPMC diets. In contrast, no increase was observed in fish fed the HPLC diet. Expression of PK mRNA remained unchanged between fasted and refed fish whatever the diet. Of the four gluconeogenic genes explored (G6Pase1, G6Pase2, PEPCK and FBPase), the two isoforms of G6Pase were the only genes subjected to refeeding regulation. Expression of the first isoform of G6Pase was dramatically down-regulated in fish refed the HPLC diet, whereas expression of this isoform remained elevated in the livers of fish refed the LPHC and MPMC diets. mRNA levels of the second isoform of G6Pase were significantly decreased in refed fish whatever the diet. On investigating the regulation of expression of lipid metabolism-related genes, we found that mRNA expression of genes encoding FAS and ACLY was significantly upregulated 24h after refeeding the HPLC diet. Levels of G6PDH mRNA were not significantly affected by nutritional status, and there was a significant increase in mRNA levels of SREBP1 in HPLC refed fish only. We found that, whereas the LPHC diet had no effect on the post-prandial regulation of expression of CPT1A and B, feeding fish the HPLC diet resulted in a significant reduction in CPT1 A and B mRNA levels. When fish were fed the MPMC diet, fish exhibited intermediate levels of CPT1A and B mRNA since CPT1A and B gene expression was not significantly different from that measured in either HPLC or LPHC diets. We also investigated mRNA expression of SD as a potential source of pyruvate

for further gluconeogenesis or lipogenesis and recorded higher levels of expression in fish refed the LPHC and the MPMC diets, and even higher expression in fish fed the HPLC diet. Finally, we investigated mRNA expression of two genes involved in the catabolism of branched chain amino acids, namely BCKD E2 and BCKD kinase, and found higher levels of expression of the former in fish fed the HPLC diet compared to fasted fish.

Akt/TOR signaling pathway in muscle.

We next investigated the effects of feeding the three experimental diets on the Akt/TOR signaling pathways in skeletal muscle by comparing fasted and refed fish. Figure 4 represents the Western blot images of phosphorylated (p-) and total forms of Akt, S6K1, S6 and 4E-BP1 in fasted and 8h-refed fish. The ratio between the phosphorylated and total forms of the protein was calculated for each protein. Overall, the results indicated that the ratio between phosphorylated and total protein of all proteins studied reached significantly higher levels in fish fed the HPLC diet compared to fasted trout. In contrast, no significant increase was observed in fish fed the LPHC diet. Analysis of MPMC data showed intermediate results, with a significant increase in Akt, S6 and 4E-BP1 phosphorylation. The phosphorylation of the same molecules was also analysed in 2h refed fish. In agreement with previous findings in the same species showing that activation of S6K1 and 4E-BP1 occurs only from 5h following a meal in this tissue (44), no difference in S6K1, S6 and 4E-BP1 phosphorylation was observed between fasted and 2h-refed fish (data not shown).

Messenger RNA levels of target genes in skeletal muscle of fasted and 24h refed fish.

Messenger RNA levels of LC3B, Gabarapl1, atg4b, atg12l, atrogin1, MuRF, C2, C3, Glut4, HK, 6PFK, PK, ACC, CPT1A, CPT1B, HOAD, BCKD E2 and BCKD kinase were measured as indices of gene expression in fasted fish as well as 24h after refeeding the three

269 experimental diets (Figure 5). Except for the atg4b gene, mRNA levels of the autophagosomal
270 genes analysed in this study (LC3B, atg12l and Gabarapl1, panel A) were subjected to
271 significant reduction by refeeding. Although the three diets induced a reduction in LC3B and
272 atg12l gene expression, the Gabarapl1 mRNA level was only reduced in the skeletal muscle
273 of fish refed the HPLC diet. On the other hand, except for E3-ubiquitin ligases
274 atrogin1/MAFbx and MuRF, whose expression decreased in refed fish whatever the diet,
275 mRNA expression of the other proteasomal genes remained the same in fasted and refed fish
276 (panel B). Of the four glycolytic genes explored (panel C), Glut4 and PK were the only genes
277 subjected to regulation by refeeding, expression of the former increasing significantly in fish
278 fed the HPLC diet and that of the second decreasing regardless of the diet. On investigating
279 the regulation of expression of lipid metabolism-related genes (panel D), we found that
280 mRNA expression of the gene encoding ACC was not affected by nutritional status.
281 Messenger RNA expression of both isoforms of CPT1 were decreased by refeeding, with a
282 more marked effect in fish fed the HPLC diet and an intermediate effect when fish were refed
283 the MPMC diets. Messenger RNA levels of HOAD were also subjected to down regulation in
284 refed fish, but only with the HPLC diet. Finally, we investigated mRNA expression of two
285 genes involved in the catabolism of branched chain amino acids (panel E) and recorded higher
286 levels of BCKD E2 expression in fish fed the HPLC diet compared to fasted fish. The mRNA
287 levels of this gene tended to increase in fish fed the MPMC diet, but the increase did not reach
288 statistical significance.
289

DISCUSSION

Replacement of fish meal with plant raw materials in salmonid feeds represents a sustainable alternative for the stability and further expansion of aquaculture. Such substitution is often problematic since feedstuffs of plant origin are naturally rich in carbohydrates and many teleosts have metabolic limitations in glucose utilization even when fed highly digestible carbohydrate sources (23, 53). The aim of the present study was therefore to provide new information on the metabolic consequences of a single meal with increased ratio of dietary carbohydrates/proteins in the rainbow trout. For this purpose, we designed three experimental diets containing high, medium or low levels of proteins and carbohydrates, respectively, and focused on the refeeding regulation of the Akt/TOR insulin/nutrient signalling pathway as a major regulator of metabolism, as well as on the expression of several metabolism-related target genes in the liver and skeletal muscle of rainbow trout. Because of formulation constraints, the diets had slightly different gross energy contents, nevertheless much above the recommended energy levels for rainbow trout (25). Besides, we made sure that all groups had similar feed intakes during the single meal provided. As such, the observed differences in the studied metabolic targets are believed to reflect mainly dietary differences in macro-nutrient composition.

Akt/TOR signaling pathway

We reported previously that feeding even a single meal leads to the regulation of the Akt/TOR-signaling pathway in the liver and skeletal muscle of rainbow trout (15, 44), as in mammals (50, 54). In the present study, we demonstrated that macro-nutrient composition of the diet may affect the Akt/TOR response to feeding. We observed in rainbow trout that the

Akt/TOR signaling pathway in the liver and muscle was very poorly or even not activated by refeeding, 2 and 8h after the meal, respectively, when the proteins/carbohydrates ratio was severely decreased. In mammals, the Akt-TOR signaling pathway is activated by at least insulin and amino acids (11). Using primary cell culture of rainbow trout hepatocytes, we previously demonstrated that unlike Akt that is mainly activated by insulin, TOR and its downstream effectors need the combination of insulin and amino acids to be fully activated (14). Therefore, the reduction of protein proportion in MPMC ans LPHC diets perhaps contribute to attenuate the refeeding activation of S6 and 4EBP1. However, the reasons of the lower phosphorylation of Akt in fish fed diets with increased proportion of carbohydrates and the absence of TOR phosphorylation whatever the diet remain unclear. Low insulin secretion might explain the lack of significant activation of Akt in the high carbohydrate-refed fish, but unfortunately we were not able to measure plasma insulin levels in the present study. Nevertheless, previously published findings indicating that increasing carbohydrates in the trout diet resulted in equivalent or even enhanced rather than decreased post-prandial insulin secretion in rainbow trout (1, 3) tend to contradict the hypothesis of decreased insulin secretion (23). However, other arguments may favour the hypothesis of decreased insulin secretion. Decreased insulin secretion has been observed in rats and pigs fed very low-protein diets, and for the former this was due to a decrease in pancreatic islet volume and insulin storage (6, 27). However, it is also possible that the too small increase in total plasma FAA levels in fish fed the LPHC diet was also involved in the lack of Akt/TOR response to refeeding, since phosphorylation of Akt, S6K1 and 4E-BP1 in mammals has been found to be positively correlated with intravenous infusion levels of insulin and amino acids (26, 49).

Protein metabolism

As already observed in this species, genes encoding proteasome subunits (C2 and C3) in protein metabolism were not regulated by nutritional status (19, 40). In contrast, genes encoding E3-ubiquitin ligases atrogin1/MAFbx and MuRF1 and genes involved in the Autophagic/Lysosomal pathway were on the whole inhibited by feeding. However, diet composition had a low impact on the expression of these genes. In mammals, transcription factor FoxO3, a downstream target of Akt, has been found to be associated with expression of atrogin1/MAFbx and MuRF1 and is considered to be the link between Akt and both lysosomal and proteasomal pathways in skeletal muscle *in vivo* and in C2C12 myotubes (18, 42, 55). The post-prandial inhibition of expression of these E3-ubiquitin ligase genes in fish fed the LPHC diet that displayed low Akt phosphorylation is somewhat surprising and highlights the potential involvement of Akt-independent pathways (e.g., mediated by glucocorticoids) in the control of the genes studied (43). Overall, the results presented here show that genes related to muscle protein degradation are inhibited by both high proteins and high carbohydrates diets, whereas the factors controlling the proteins synthesis (S6K1, S6 and 4EBP1) are increased only in response to feeding the high proteins diet. These results are likely due to the existence of factors affecting specifically each system and highlight the complexity of mechanisms involved in the control of protein turnover. Whether these results are followed by similar diet composition effect on muscle proteolysis and protein synthesis rates is worth investigating.

Glucose metabolism

Once absorbed, glucose is normally taken up by cells such as hepatocytes and skeletal muscle cells and then directed to different metabolic pathways. In mammals, skeletal muscle is the main tissue contributing to the removal of glucose from the blood to maintain glucose

362 homeostasis (56). Glucose uptake in skeletal muscle is primarily operated by the glucose
363 transporter GLUT4 (39) and is under insulin regulation since insulin activates the
364 translocation of GLUT4 from intracellular vesicles to the plasma membrane and stimulates
365 the expression of GLUT4 (21). As previously reported in Atlantic cod (7) and rainbow trout
366 (36), GLUT4 gene expression was stimulated in rainbow trout muscle by refeeding in the
367 present study. However, this increase was only significant when fish were fed the protein
368 rich-diet, whereas in mammals, carbohydrates enhance the post-prandial stimulation of
369 GLUT4 in the skeletal muscle (41). This result suggests that glucose uptake might have been
370 stimulated following feeding in the present study but paradoxically only in those fish fed large
371 amounts of protein. However, the absence of stimulation of expression of glycolytic genes,
372 even the significant inhibition of PK gene expression, tends to refute this hypothesis and
373 suggests that there is very limited use of glucose by the muscle. This is also in agreement with
374 the well-known low rate of glucose phosphorylation by hexokinases in trout muscle compared
375 to mammalian species (10, 13, 53). This was furthermore confirmed by the stable level of
376 muscle glycogen levels in fasted and refed trout measured in the present study (data not
377 shown).

378 The regulation of glucose metabolism in the liver was also surprising. Unlike HK in muscle,
379 the expression of GK, the low affinity hepatic hexokinase, was upregulated by refeeding but
380 only when the diet contained medium or high levels of carbohydrates. We have previously
381 demonstrated in vitro using primary cell culture of rainbow trout hepatocytes that glucose
382 enhanced accumulation of GK mRNA (35). We confirmed in this in vivo study that, unlike
383 mammals in which insulin is a major regulator of GK gene expression (8), GK gene
384 expression is probably mainly controlled by carbohydrates in rainbow trout. Despite strong
385 induction of GK gene expression, expression of the downstream glycolytic genes such as PK
386 was not regulated by refeeding as previously observed (46). When looking at genes related to

gluconeogenesis, G6Pase, the last enzyme of gluconeogenesis, was the only gene inhibited by refeeding. According to the ancestral whole genome duplication that has occurred in the teleost lineage following its divergence from mammals, multiple copies of genes may be found in fish that may have evolved separately and acquired different patterns of expression, as previously reported for pro-opiomelanocortin (9, 17). We demonstrate here that the macro-nutrient composition of the diet affects the regulation of expression of the two isoforms of G6Pase differently, the first isoform being inhibited only in trout refed the HPLC diet and the second isoform being down regulated following refeeding, irrespective of diet composition. On the other hand, hepatic expression of PEPCK and FBPase was not subjected to regulation after a meal in any group. When we considered the glycolytic and the gluconeogenic pathways together, we observed that the opposite enzymes, GK and G6Pase (especially the first isoform), were the only enzymes exhibiting refeeding regulation of their respective mRNA levels. Furthermore, they presented contrasting regulation regarding the relative proportions of carbohydrates and proteins. Whereas refeeding increased GK gene expression only in fish fed carbohydrates, G6Pase (isoform 1) gene expression was inhibited only in fish fed the high protein / low carbohydrate diet. These results suggest that in trout refed once with a high level of carbohydrates, glucose taken up by the liver is phosphorylated then immediately dephosphorylated by G6Pase, whose expression of the first isoform is not inhibited by refeeding. This might possibly contribute to the prolonged post-prandial hyperglycaemia generally observed in trout. This hypothesis should to be carefully taken since the present study was restricted to cellular signaling analysis and mRNA quantification and should be completed by the analysis of the enzymatic activities or at least the evaluation of protein quantity by western blot analysis.

Lipid metabolism

411 Another metabolic pathway affected by the macronutrient composition of the diet was
412 lipogenesis. Previous reports have demonstrated that lipogenic gene expression is regulated
413 by refeeding in trout (15, 46). This post-prandial stimulation was confirmed for FAS, ACLY
414 and the transcription factor SREBP1, but only when fish were refed a high protein diet. It is
415 known that insulin regulation of FAS transcription in mammals is mediated by the PI3-
416 kinase/Akt signaling pathway (48). In mammals, SREBP1 is essential for the transcriptional
417 control of genes encoding enzymes of lipid biosynthesis such as FAS and ACLY and is
418 considered to be the link between TOR and control of lipogenic gene expression (38). The
419 link between lipogenic gene expression and the TOR pathway has also recently been
420 established in trout in which enhanced activation of the TOR signaling pathway leads to the
421 up-regulation of SREBP1 and lipogenic gene expression in the liver (46). This was then
422 confirmed in rainbow trout hepatocytes where insulin and amino acids regulate lipogenic as
423 well as SREBP1 gene expression through a TOR-dependent pathway (14). Since carbohydrate
424 rich-diets exhibited poor ability to activate the Akt/TOR signaling pathway, it is thus
425 compatible that there is no post-prandial induction of lipogenic gene expression. The greater
426 up-regulation of SD gene expression in fish fed the high protein diet is also in agreement with
427 the regulation of lipogenic gene expression. In mammals, SD is involved in the regulation of
428 liver gluconeogenesis from serine in different dietary, hormonal and developmental states by
429 providing pyruvate (47), but in the case of rainbow trout pyruvate originating from SD
430 activity might be directed to lipid synthesis and thus contribute to enhanced lipogenesis (46).

431

432 *Energy metabolism*

433 It is clear that regulation of CPT1 genes in mammals is under the control of dietary and
434 hormonal factors in tissues highly involved in fatty acid β-oxidation (16, 20). As expected,

refeeding decreased the mRNA levels of genes encoding CPT1 A and B both in the liver and muscle. This decrease was obviously dependent on the macronutrient composition of the diet since inhibition of CPT1 gene expression was observed only when fish were fed a high proportion of protein. Introduction of carbohydrate into the diet abolished the post-prandial down-regulation of CPT1 mRNA level. These results suggest that refeeding triggered a switch from the use of fatty acids as fuel to the use of amino acids in fish fed the largest amount of protein. Measurement of CPT1 activity as well as fatty acid oxidation would be of great interest to confirm this hypothesis. The similar expression of HOAD and the opposite expression of BCKD E2 (involved in the β-oxidation pathway and branched-chain amino acid degradation process, respectively) strengthen this hypothesis. However, these results were obtained after a single meal. It would be highly interesting to investigate if trout fed a low protein to carbohydrate diets during a longer period will improve the ability of trout to use carbohydrates as energy source.

Conclusion

In conclusion, this study demonstrated that activation of the insulin/amino acids signaling pathway and the regulation of the expression of a number of genes related to metabolism were severely impaired by decreasing the dietary proteins/carbohydrates ratio. The potential occurrence of the “futile cycle” between GK and G6Pase1, as well as the inability of fish to use dietary carbohydrates as fuel, probably contribute to the prolonged post-prandial hyperglycemic phenotype of the trout. This study clearly confirmed that this animal is adapted to use amino acids not only as an energy source but probably also as a substrate for lipogenesis. The findings obtained here were from fish fed a single meal and it would be

458 interesting to analyze the metabolic consequences of long term high carbohydrate/low protein
459 feeding and investigate how the rainbow trout is able to adapt to such a diet.

460

461 **Perspectives and significance**

462

463 The understanding of the role of nutrients as signaling molecules remains relatively limited,
464 although significant progresses have been achieved during the past few years, in particular
465 with regard to amino acid control of physiological functions. In this regard, carnivorous
466 rainbow trout (*Oncorhynchus mykiss*) is an interesting model to gain better understanding on
467 the role of nutrients and their interactions at physiological levels because of its very unusual
468 features to i) use (high level of) amino acids as the preferred energy source over carbohydrate
469 and ii) display a prolonged hyperglycemia after a glucose load or intake of carbohydrate
470 meals. The molecular basis of gene regulation by the different macronutrient intake is an
471 important field of research for an optimization of dietary nutrient supplies in various
472 physiological and physiopathological situations. Beyond gaining a basic understanding of
473 nutrient control of biological mechanisms, the characterization of how these processes
474 contribute to normal development and/or metabolic disorders has important implications for
475 optimizing the growth and development of livestock species, as well as for public health and
476 clinical medicine.

477

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482

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485 Low utilisation of dietary carbohydrates in carnivorous rainbow trout: role of amino acids,
486 glucose and insulin interactions?).

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663 **FIGURE LEGENDS**

664

665 **Fig. 1. Plasma levels of glucose and total free amino acids (AA) in trout 2, 8 and 24h**
666 **after refeeding HPLC, MPMC and LPHC diets (F = fasted fish).** Data are expressed as
667 means ± SE (n=9). § indicates significant difference compared to fasted fish. Letters indicate
668 significant difference between diets at a given hour after refeeding. ($P < 0.05$).

669

670 **Fig. 2. Western blot analysis of Akt, TOR and S6 protein phosphorylation in livers of**
671 **fasted (F) and HPLC, MPMC and LPHC diet 2h-refed rainbow trout.** Twenty
672 micrograms of total protein per lane were loaded on the gel. A representative blot is shown.
673 Graphs represent the ratio between the phosphorylated protein and the total amount of the
674 targeted protein. Results are means ± SE ($n = 6$) and were analyzed using one way ANOVA
675 followed by the Student-Newman-Keuls test for multiple comparisons. Mean values for a
676 selected group not sharing a common letter differ ($P < 0.05$).

677

678 **Fig. 3. Gene expression of selected enzymes in the livers of fasted (F) and HPLC, MPMC**
679 **and LPHC diet 24h-refed rainbow trout.** GK, PK, G6Pase1, G6Pase2, PEPCK, FBPase,
680 FAS, ACLY, G6PDH, SREBP1, CPT1a, CPT1b, SDH, BCKD E2 and BCKD kinase mRNA
681 levels were evaluated using real-time RT-PCR. Expression values are normalized with EF1α-
682 expressed transcripts. Results are means + SE ($n = 6$) and were analyzed using one way
683 ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Mean values
684 for a selected group not sharing a common letter differ ($P < 0.05$).

685

686 **Fig. 4. Western blot analysis of Akt, S6K1, S6 and 4E-BP1 protein phosphorylation in**
687 **muscle of fasted (F) and HPLC, MPMC and LPHC diet 8h-refed rainbow trout.** Twenty

688 micrograms of total protein per lane were loaded on the gel. A representative blot is shown.
689 Graphs represent the ratio between the phosphorylated protein and the total amount of the
690 targeted protein. Results are means ± SE (n = 6) and were analyzed using one way ANOVA
691 followed by the Student-Newman-Keuls test for multiple comparisons. Mean values for a
692 selected group not sharing a common letter differ ($P < 0.05$).
693
694 **Fig. 5. Gene expression of selected enzymes in muscle of fasted (F) and HPLC, MPMC**
695 **and LPHC diet 24h-refed rainbow trout.** LC3B, Gabarapl1, atg4b, atg12l,
696 atrogin1/MAFbx, MuRF, C2, C3, Glut4, HK, 6PFK, PK, ACC, CPT1a, CPT1b, HOAD,
697 BCKD E2 and BCKD kinase mRNA levels were evaluated using real-time RT-PCR.
698 Expression values are normalized with EF1α-expressed transcripts. Results are means + SE (n
699 = 6) and were analyzed using one way ANOVA followed by the Student-Newman-Keuls test
700 for multiple comparisons. Mean values for a selected group not sharing a common letter differ
701 ($P < 0.05$).

Table 1. Composition of diets.

Ingredients (%)	HPLC	MPMC	LPHC
Casein[1]	0.572	0.310	0.154
Casein hydrolysat[2]	0.100	0.055	0.021
L-arginine[3]	0.013	0.007	0.004
Dextrine[4]	0.050	0.350	0.537
Soy Lecithine[5]	0.020	0.020	0.020
Fish Oil[6]	0.130	0.130	0.130
CaHPO4.2H20 (18%P)[7]	0.000	0.013	0.019
Attractant mix[8]	0.015	0.015	0.015
Min. premix[9]	0.050	0.050	0.050
Vit. Premix[10]	0.0500	0.050	0.050
Analytical composition (%)			
Dry Matter (DM) (%)	90.96	92.61	93.09
Proteins (%DM)	65.15	36.69	17.62
Lipids (%DM)	17.05	15.72	15.59
Energy (kJ/g DM)	24.16	21.75	20.58
NFE (Cbh)[11]	6.38	32.52	48.84

1: Casein (Sigma-Aldrich, USA)
2: Casein hydrolysat (Sigma-Aldrich, USA)
3: L-arginine (Sigma-Aldrich, USA)
4: Dextrine (Sigma-Aldrich, USA)
5: Soy Lecithine (Louis François, St Maur des Fossés, France)
6: Feedoil (North sea fish oil, Sopropèche, Boulogne-sur-Mer, France)
7: CaHPO4.2H20 (18%P) (Sigma-Aldrich, USA)
8: Glucosamine 0,5g; Taurine 0,3g; Betaine 0,3g; Glycine 0,2g; Alanine 0,2g /100 g feed
9: Mineral mixture (g or mg/kg diet) : calcium carbonate (40%Ca), 2.15g; magnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2g;potassium iodide (75% I), 0.4mg; zinc sulphate (36% Zn), 0.4g; copper sulphate (25% Cu), 0.3g; manganese sulphate (33% Mib), 0.3g; dibasic calcium phosphate (20%Ca, 18%P), 5g; cobalt sulphate, 2mg; sodium selenite (30% Se), 3 mg; KCl, 0.9g; NaCl, 0.4g (UPAE (unité de préparation des aliments expérimentaux, Jouy, Inra, France))
10: 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, 15mg; B12, 0.05mg; 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))
11: Nitrogen-free extract (Carbohydrate) : 100 – (crude protein + crude fat + crude fiber + moisture + ash)

Table 2. Sequences of the primer pairs used for real-time quantitative RT-PCR

Gene	5'- 3' forward primer	5'- 3' reverse primer
Ubiquitin-proteasome pathway		
Atrogin-1	TGCGATCAAATGGATTCAAA	GATTGCATCATTTCCCCACT
MuRF	TTCCACAAAGCAAGACAGCA	CCGTGGTTCCTCCTGGTCGAT
C2	GCTGGCTGCTCACCAGAAGA	GGATTTGGGTTTTGCTTCCAAT
C3	TGGGAATCAAAGCCTCAAATGG	TGGGGATGGGCTCCTGGTA
Branched chain amino acids oxidation		
BCKD E2	CCCAAGATGAAGCCCACACC	CCAGGGAAGCAGCCTTGATG
BCKD kinase	TGGACGACCACAAGGACGTG	AGACGGGAGGTGAGGGTGGT

GenBank accession no. or sigenae accession no.: Atrogin1, CX026010; MuRF (Muscle ring finger), CA342294; C2 (alpha-type proteasome C2 subunit), CA347547; C3 (alpha-type proteasome C3 subunit), CA385654; BCKD E2 (branched chain α-keto acid dehydrogenase E2 subunit), AB050595; BCKD kinase (branched chain α-keto acid dehydrogenase kinase), BX076477.s.om.10.

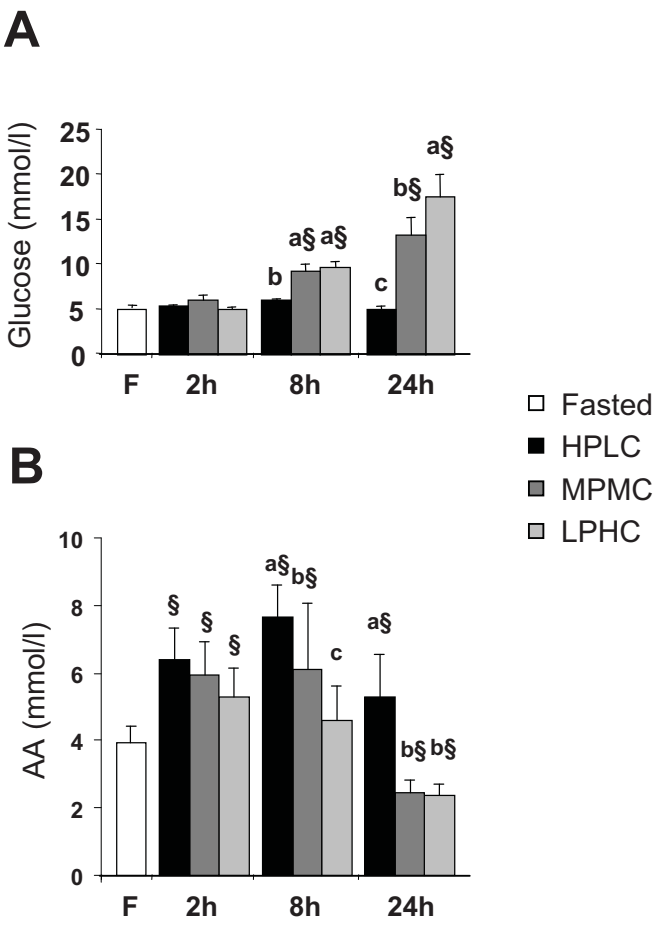
A
Glucose (mmol/l)
25
20
15
10
5
0
F
2h
8h
24h
b
a§
a§
c
b§
a§
B
AA (mmol/l)
10
8
6
4
2
0
§
§
§
a§
b§
c
a§
b§
b§
F
2h
8h
24h
Fasted
HPLC
MPMC
LPHC

Fig. 1

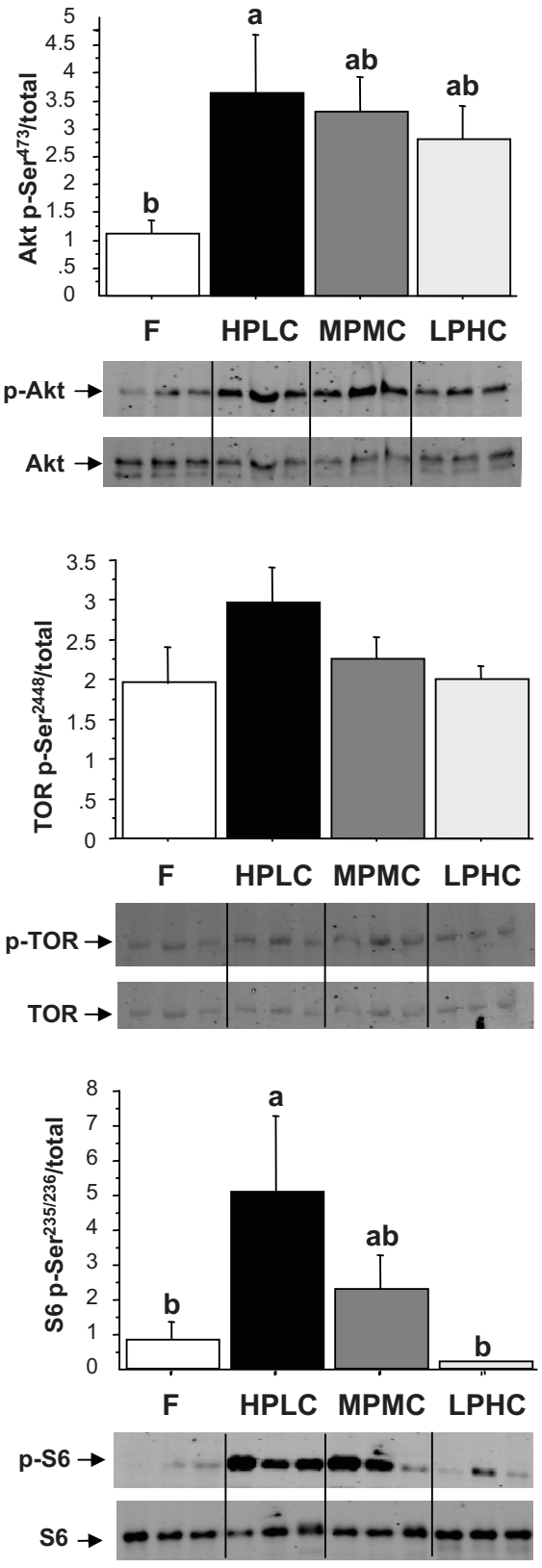
Akt p-Ser473/total
5
4.5
4
3.5
3
2.5
2
1.5
1
.5
0
a
ab
ab
b
F
HPLC
MPMC
LPHC
p-Akt
Akt
TOR p-Ser2448/total
3.5
3
2.5
2
1.5
1
.5
0
F
HPLC
MPMC
LPHC
p-TOR
TOR
S6 p-Ser235/236/total
8
7
6
5
4
3
2
1
0
a
ab
b
b
F
HPLC
MPMC
LPHC
p-S6
S6

Fig. 2

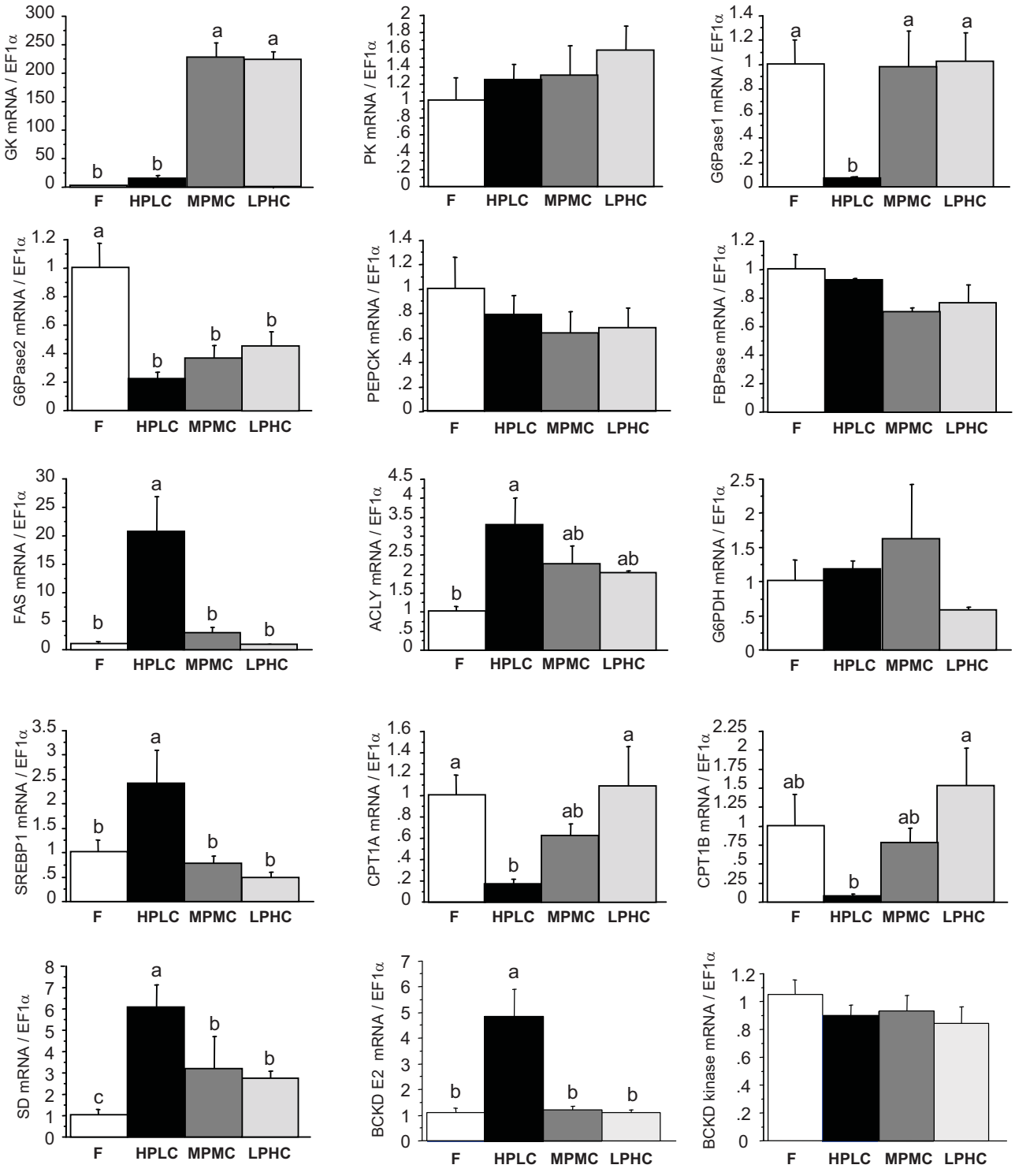
GK mRNA / EF1α
PK mRNA / EF1α
G6Pase1 mRNA / EF1α
G6Pase2 mRNA / EF1α
PEPCK mRNA / EF1α
FBPase mRNA / EF1α
FAS mRNA / EF1α
ACLY mRNA / EF1α
G6PDH mRNA / EF1α
SREBP1 mRNA / EF1α
CPT1A mRNA / EF1α
CPT1B mRNA / EF1α
SD mRNA / EF1α
BCKD E2 mRNA / EF1α
BCKD kinase mRNA / EF1α
F HPLC MPMC LPHC

Fig. 3

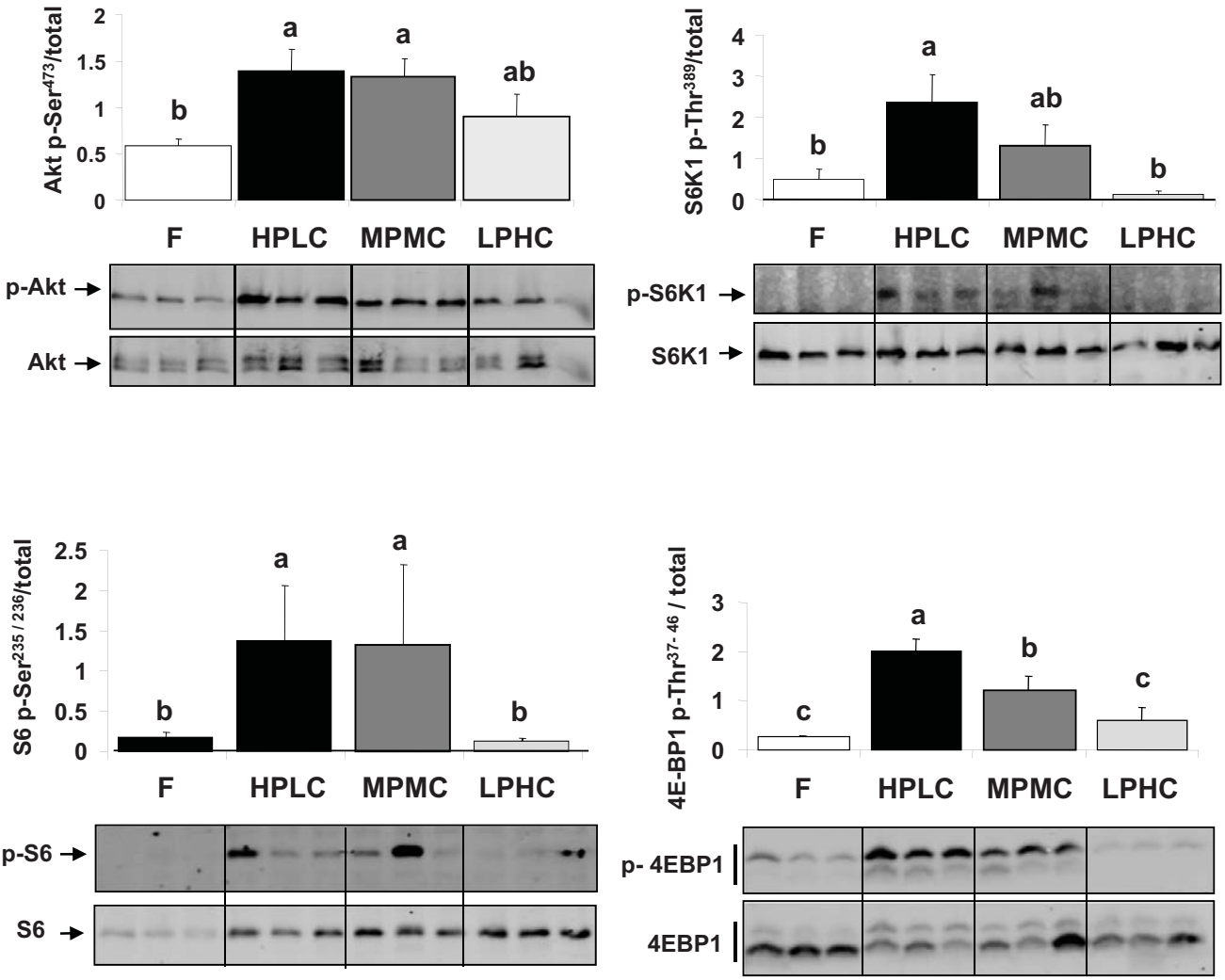
Akt p-Ser473/total
2
1.5
1
0.5
0
a
a
ab
b
F
HPLC
MPMC
LPHC
p-Akt
Akt
S6K1 p-Thr389/total
4
3
2
1
0
a
ab
b
b
F
HPLC
MPMC
LPHC
p-S6K1
S6K1
S6 p-Ser235 / 236/total
2.5
2
1.5
1
0.5
0
a
a
b
b
F
HPLC
MPMC
LPHC
p-S6
S6
4E-BP1 p-Thr37- 46 / total
3
2
1
0
a
b
c
c
F
HPLC
MPMC
LPHC
p- 4EBP1
4EBP1

Fig. 4

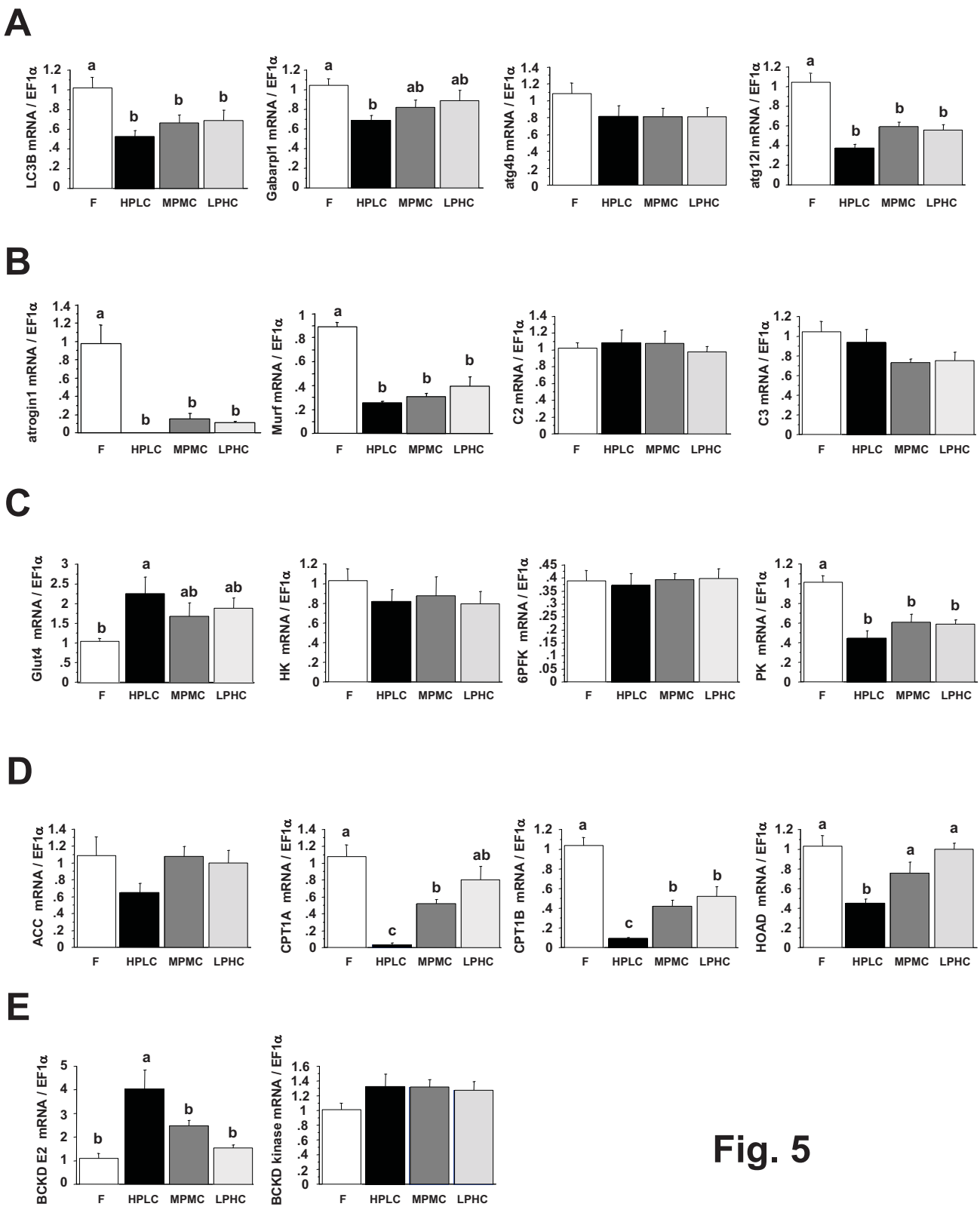
A
LC3B mRNA / EF1α
Gabarpl1 mRNA / EF1α
atg4b mRNA / EF1α
atg12l mRNA / EF1α
B
atrogin1 mRNA / EF1α
Murf mRNA / EF1α
C2 mRNA / EF1α
C3 mRNA / EF1α
C
Glut4 mRNA / EF1α
HK mRNA / EF1α
6PFK mRNA / EF1α
PK mRNA / EF1α
D
ACC mRNA / EF1α
CPT1A mRNA / EF1α
CPT1B mRNA / EF1α
HOAD mRNA / EF1α
E
BCKD E2 mRNA / EF1α
BCKD kinase mRNA / EF1α
F
HPLC
MPMC
LPHC

Fig. 5