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RESEARCH ARTICLE

Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways

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ABSTRACT

Using rainbow trout fed with low-fat or high-fat diets, we aimed to determine whether the response of food intake, mRNA abundance of hypothalamic neuropeptides involved in the metabolic regulation of food intake and fatty acid sensing systems in the hypothalamus and liver are similar to results previously observed when levels of specific fatty acids were raised by injection. Moreover, we also aimed to determine if the phosphorylation state of intracellular energy sensor 5′-AMP-activated protein kinase (AMPK), and proteins involved in cellular signaling such as protein kinase B (Akt) and target of rapamycin (mTOR) display changes that could be related to fatty acid sensing and the control of food intake. The increased levels of fatty acids in the hypothalamus and liver of rainbow trout fed with a high-fat diet only partially activated fatty acid sensing systems and did not elicit changes in food intake, suggesting that the fatty acid sensing response in fish is more dependent on the presence of specific fatty acids, such as oleate or octanoate, rather than to the global increase in fatty acids. We also obtained, for the first time in fish, evidence for the presence and function of energy sensors such as AMPK and proteins involved in cellular signaling, like mTOR and Akt, in the hypothalamus. These proteins in the hypothalamus and liver were generally activated in fish fed the high-fat versus low-fat diet, suggesting that cellular signaling pathways are activated in response to the increased availability of fatty acids.

KEY WORDS: Lipid-enriched diet, Rainbow trout, Fatty acid sensing, AMPK, mTOR, Akt

INTRODUCTION

In previous studies, we have characterized the presence and function of fatty acid sensing systems in the hypothalamus, liver and Brockmann bodies (BB, main accumulation of pancreatic endocrine tissue in this species) of the teleost fish model rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) (Librán-Pérez et al., 2012, 2013a,b,c, 2014a,b, 2015a,b). These systems respond to changes not only in long-chain fatty acids (LCFAs), such as oleate but, unlike mammals, also in medium-chain fatty acids (MCFAs) such as octanoate, and correlate with the control of food intake (hypothalamus), hormone release (BB) or metabolic homeostasis (liver). They are based on: (1) fatty acid metabolism through inhibition of carnitine palmitoyltransferase 1 (CPT-1) to import fatty-acid–CoA into the mitochondria for oxidation; (2) binding to fatty acid translocase (FAT/CD36) and further modulation of transcription factors, such as peroxisome proliferator-activated receptor type α (PPARα) and sterol regulatory element-binding protein type 1c (SREBP1c); and (3) mitochondrial production of reactive oxygen species (ROS) by electron leakage, resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity (Soengas, 2014). The activation of these systems is associated with the inhibition of the orexigenic factors agouti-related protein (AgRP) and neuropeptide Y (NPY) and the enhancement of the anorexigenic factors pro-opio melanocortin (POMC) and cocaine and amphetamine-related transcript (CART), ultimately leading to decreased food intake (Librán-Pérez et al., 2012, 2014a). Since a reduced food intake has been observed after feeding fish, such as sea bass (Boujard et al., 2004) or rainbow trout (Gélineau et al., 2001), with lipid-enriched diets, changes in fatty acid sensing systems are expected in fish fed with diets containing different lipid levels.

Evidence obtained in recent years demonstrated that the integrative energy and nutrient sensor 5′-AMP-activated protein kinase (AMPK) is activated by phosphorylation when cellular fuel availability is low, resulting in enhanced catabolism and breakdown of energy stores (Hardie and Ashford, 2014). In fish, there is evidence in rainbow trout for the presence and functioning of AMPK in the liver (Craig and Moon, 2011, 2013; Polakof et al., 2011a; Fuentes et al., 2013) and muscle (Craig and Moon, 2013; Magnoni et al., 2014); however, to date, there is no information in any fish tissue regarding the response of AMPK to changes in the levels of nutrients such as fatty acids, as demonstrated in mammals (Hardie and Ashford, 2014).

Furthermore, proteins involved in cellular signaling, such as target of rapamycin (mTOR) and protein kinase B (Akt), are also suggested to be involved in the nutritional regulation of carbohydrate and lipid metabolism in fish. Thus, in rainbow trout liver, activation of mTOR contributes to the regulation of fatty acid biosynthesis (Skiba-Cassy et al., 2009), and the increase in Akt phosphorylation is essential for the antilipolytic action of insulin (Polakof et al., 2011b). However, as for AMPK, there are no available studies in fish assessing the response of these proteins to changes in circulating levels of fatty acids, as demonstrated in mammals (Berthoud and Morrison, 2008; Benoit et al., 2009; de Morentin et al., 2011).

Therefore, the aim of this study in rainbow trout fed with a low-fat or high-fat diet was: (1) to determine whether the response of food intake, mRNA abundance of hypothalamic neuropeptides involved in the metabolic regulation of food intake, and fatty acid sensing systems in hypothalamus and liver is similar to that previously observed when levels of specific fatty acid were raised by injection;

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and (2) to determine if the phosphorylation state of intracellular energy sensors (AMPK) and proteins involved in cellular signaling (Akt and mTOR) display changes in the hypothalamus and liver in response to different dietary lipid levels that could be linked to variations in parameters related to fatty acid sensing and the control of food intake.

RESULTS

No mortality was observed throughout the 4 week feeding trial. Body weight, growth rate and feed intake values are shown in Table 1. Final fish body weight, relative weight gain and specific growth rate were significantly (P<0.05) higher in the group fed with the high-fat diet. The value of feed efficiency was higher in the group fed with the high-fat diet. There were no significant differences between diets in the feed intake values, either expressed on an absolute (g per fish) or a relative (per unit body weight) basis, nor in cumulative feed intake (Fig. 1).

Considering the composition of the diets (Tables 2,3) as well as food intake, fish fed the low-fat diet ingested a total of 0.09±0.01 g of lipid per fish day^{-1} and fish fed the high-fat diet ingested 0.29±0.01 g of lipid per fish day^{-1} (Table 1). Considering the fatty acid composition of the diets (Table 3), intake of specific fatty acids (in mg fatty acids per fish day^{-1}) was also markedly different when comparing both diets (Table 4). This was especially relevant for C14:0 (myristate), C16:0 (palmitate), C16:1 (palmitoleate), C18:1 (oleate), C18:2 n-6 (linoleate), C18:3 n-3 (α-linolenate), C20:5 n-3 (eicosapentanoate), and C22:6 n-3 (docosahexanoate).

Metabolite levels in plasma and tissues 6 h after feeding are shown in Fig. 2. Free fatty acid levels in plasma (Fig. 2A), hypothalamus (Fig. 2B), and liver (Fig. 2C) increased in the group of fish fed with high-fat diet compared with the group fed with low-fat diet. Triglyceride levels increased in plasma of fish fed with the high-fat diet (Fig. 2D) but there were no significant differences in hypothalamus (Fig. 2E) and liver (Fig. 2F). No significant changes were noted for glucose levels in plasma (data not shown).

Fig. 3 represents phosphorylated and total forms of Akt, AMPK and mTOR in the hypothalamus. The ratios for Akt (Fig. 3A) increased 6 h after the meal in fish fed with the high-fat diet compared with the low-fat fish. The ratios of AMPK (Fig. 3B) in fish fed the high-fat diet decreased 3 h after the meal and increased 6 h after the meal compared with fish fed the low-fat diet whereas in fish fed the high-fat diet the ratio observed 3 h after the meal was lower than that observed after 1 or 6 h. Finally, the value of mTOR (Fig. 3C) increased 3 h after the meal in the fish fed the high-fat diet compared with fish fed the low-fat diet. No differences with time were noted for Akt (Fig. 3A) and mTOR (Fig. 3C), whereas AMPK values in fish fed with the high-fat diet were lower 3 h after the meal compared with those observed after 1 and 6 h (Fig. 3B).

The ratios of phosphorylated versus total forms of Akt, AMPK and mTOR in the liver are shown in Fig. 4. The ratio for Akt was higher 6 h after the meal in the fish fed with the high-fat diet (Fig. 4A). No

Table 1. Mass and food intake measurements for rainbow trout fed with low-fat or high-fat diets for 4 weeks

	Diet	
	Low fat	High fat
Initial M_b (g)	34.51±0.52	34.09±0.42
Final M_b (g)	86.29±3.21	95.29±3.42*
Weight gain (%)	51.79±2.89	61.20±3.50*
Specific growth rate (%M_b day^{-1})	3.26±0.10	3.54±0.14*
Food intake (g per fish day^{-1})	1.60±0.10	1.64±0.06
Food intake (%M_b day^{-1})	2.65±0.11	2.54±0.13
Food intake (g kg^{-1}M_b)	16.42±0.78	16.26±0.73
Feed efficiency	1.11±0.04	1.29±0.09*
Total lipid intake (g per fish day^{-1})	0.09±0.01	0.29±0.01*

Data are means±s.e.m. of five different tanks (each containing 20 fish) per diet.

*Significantly different (P<0.05) from fish fed with the LF diet at the same time.

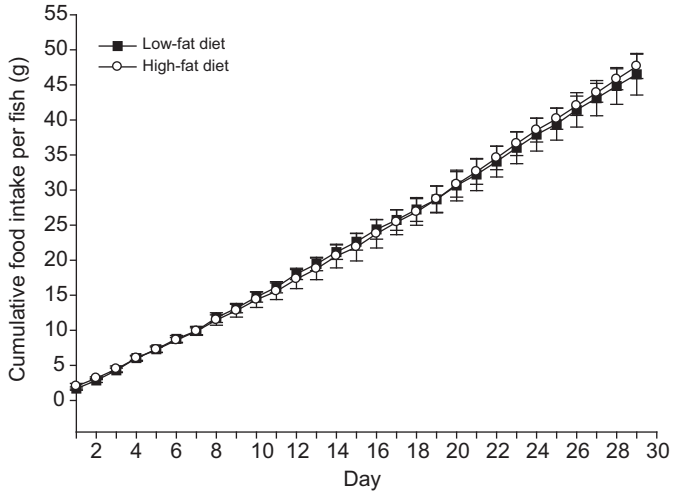

Fig. 1. Cumulative food intake in rainbow trout fed with low-fat or high-fat diets for 4 weeks. Data are means±s.d. of 5 different tanks (each containing 20 fish). No significant difference between diets (P≥0.05) was noted.

Table 2. Ingredients and proximate composition of low-fat and high-fat diets used to feed rainbow trout for 4 weeks

	Diet	
	Low-fat	High-fat
Ingredients (% diet)		
LT Fishmeal[1]	45.0	45.0
CPSP G[1]	5.0	5.0
Wheat gluten[2]	5.0	5.0
Corn gluten meal[3]	5.0	5.0
Gelatinised corn starch[2]	12.0	12.0
Whole wheat[2]	10.0	10.0
Oil Blend[4]	1.6	16
Cellulose[5]	14.4	0.0
Mineral and vitamin premix[6]	2.0	2.0
Analyzed proximate composition (% DM)		
Dry matter (DM, % diet)	96.2	95.8
Crude protein	46.3	46.6
Crude lipid	5.8	19.0
Ash	9.8	10.0
Starch	18.0	18.4
Gross energy (GE, kJ g^{-1} DM)[7]	20.0	23.2
Calculated digestible energy (DE) content (kJ g^{-1} DM)[8]		
DE from protein	9.9	9.9
DE from carbohydrates	2.6	2.7
DE from fat	2.1	7.0
Total DE	14.6	19.6

[1]LT Fishmeal and Soluble fish protein concentrate (CPSP G), Sopropêche 56100 Lorient, France; [2]Roquette 62080 Lestrem, France; [3]Inzo, France; [4]Fish oil/Rapeseed oil (ratio 6/10); [5]Rettenmeier et Söhne 73494 Rosenberg, Germany; [6]INRA UPAE 78200 Jouy en Josas, France; [7]GE value of low-fat diet includes the caloric value of cellulose; [8]Calculated using apparent digestibility coefficients of 90%, 93% and 82% and caloric values (kJ g^{-1}) of 23.7, 39.6 and 17.7 for protein, fat and carbohydrates, respectively.

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Table 3. Fatty acid composition of low-fat and high-fat diets used to feed rainbow trout for 4 weeks

	Diet	
Fatty acid	Low-fat	High-fat
Saturated		
C14:0	4.00	3.99
C15:0	0.23	0.32
C16:0	12.25	14.83
C17:0	0.18	0.18
C18:0	2.31	2.39
C20:0	0.33	0.25
C22:0	0.15	0.10
Monounsaturated		
C16:1	4.19	4.71
C17:1	0.07	0.07
C18:1	39.38	30.42
C20:1	1.61	3.89
C22:1	0.97	3.54
Polyunsaturated		
C16:2 n-4	0.74	0.47
C16:3 n-4	0.73	0.61
C16:4 n-1	1.13	0.69
C18:2 n-6	13.70	12.37
C18:3 n-3	4.89	3.18
C18:3 n-6	0.11	0.11
C18:4 n-3	0.88	1.09
C20:2 n-6	0.09	0.17
C20:3 n-3	0.04	0.13
C20:3 n-6	0.04	0.02
C20:4 n-3	0.23	0.34
C20:4 n-6	0.39	0.47
C20:5 n-3	6.28	6.06
C21:5 n-3	0.23	0.24
C22:2 n-6	0.04	0.05
C22:5 n-3	0.48	0.58
C22:6 n-3	2.61	5.30

Values are g per 100 g of total fatty acid.

Table 4. Daily fatty acid intake of fish fed with low-fat or high-fat diets for 4 weeks

	Diet	
Fatty acid	Low fat	High fat
Saturated		
C14:0	3.58±0.10	11.95±0.16*
C15:0	0.29±0.008	0.69±0.009*
C16:0	13.32±0.39	36.61±0.50*
C17:0	0.16±0.005	0.54±0.007*
C18:0	2.15±0.06	6.90±0.09*
C20:0	0.22±0.007	0.99±0.01*
C22:0	0.09±0.003	0.45±0.006*
Monounsaturated		
C16:1	4.23±0.12	12.52±0.17*
C17:1	0.06±0.002	0.21±0.003*
C18:1	27.32±0.79	117.68±1.62*
C20:1	3.49±0.10	4.78±0.07*
C22:1	3.18±0.09	2.90±0.04*
Polyunsaturated		
C16:2 n-4	0.42±0.01	2.21±0.03*
C16:3 n-4	0.55±0.02	2.18±0.03*
C16:4 n-1	0.62±0.02	3.38±0.05*
C18:2 n-6	11.11±0.32	40.94±0.60*
C18:3 n-3	2.86±0.08	14.61±0.20*
C18:3 n-6	0.10±0.003	0.33±0.005*
C18:4 n-3	0.97±0.03	2.63±0.04*
C20:2 n-6	0.15±0.004	0.27±0.004*
C20:3 n-3	0.12±0.003	0.12±0.002
C20:3 n-6	0.02±0.001	0.12±0.002*
C20:4 n-3	0.31±0.009	0.69±0.009*
C20:4 n-6	0.42±0.01	1.17±0.016*
C20:5 n-3	5.44±0.16	18.74±0.26*
C21:5 n-3	0.22±0.006	0.69±0.009*
C22:2 n-6	0.04±0.001	0.12±0.002*
C22:5 n-3	0.52±0.01	1.43±0.02*
C22:6 n-3	4.76±0.14	7.77±0.11*

Data are means±s.e.m. of five different tanks (each containing 20 fish) per diet.
*Significantly different (P<0.05) from fish fed with the low-fat diet at the same time.

significant changes were noted between groups for the P-AMPK/AMPK ratio (Fig. 4B). The P-mTOR/mTOR ratio increased 3 and 6 h after the meal in fish fed with the high-fat diet compared with fish fed with the low-fat diet (Fig. 4C) whereas values in fish fed the low-fat diet were higher 1 h after the meal than after 3 or 6 h.

Changes in mRNA abundance of transcripts assessed in hypothalamus 6 h after the last meal, are shown in Fig. 5. Values of FAT/CD36, CPT1c, liver X receptor α (LXRα), PPARα, SREBP1c, CART, NPY and POMC-A1 were higher in the group fed with the high-fat diet than in the group fed with the low-fat diet. No significant changes were noted for mRNA abundance of acetyl-CoA carboxylase (ACC), ATP-citrate lyase (ACLY), fatty acid synthetase (FAS), hydroxyacil-CoA dehydrogenase (HOAD), mitochondrial uncoupling protein 2a (UCP2a), inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like) or AgRP.

Changes in mRNA abundance of transcripts assessed in the liver are shown in Fig. 6. ACLY mRNA abundance in the group fed with the high-fat diet was lower than in the group fed with the low-fat diet whereas ACC, CPT1a, HOAD, UCP2a and PPARα mRNA levels were higher in the group fed with the high-fat diet. No significant changes were noted for mRNA levels of the other proteins assessed.

DISCUSSION

We previously demonstrated the activation of fatty acid sensing systems in rainbow trout after experimental increases in the levels of oleate or octanoate (Soengas, 2014). There is, however, no evidence for the response of these systems when fish are fed with diets containing different lipid contents. We therefore fed rainbow trout for 4 weeks with two experimental diets differing in lipid content (6 vs 19% of diet dry matter). Levels of circulating fatty acids and triglycerides were markedly increased in plasma of rainbow trout fed with the high-fat diet. Moreover, those differences were also reflected in the free fatty acid levels of the two tissues assessed, i.e. hypothalamus and liver, thus validating the experimental design, and supporting the assessment of changes in fatty acid sensing systems in both tissues. The observed changes in fatty acid levels are similar to those observed in other studies with rainbow trout fed comparable diets (Figueiredo-Silva et al., 2012b).

Effects on fatty acid sensing systems in the hypothalamus and liver

In the hypothalamus, the fatty acid sensing system based on fatty acid metabolism was apparently not activated in fish fed the high-fat diet since no significant changes were noted in the mRNA abundance of ACC, ACLY and FAS, whereas the increase in the mRNA abundance of CPT1c was contrary to that expected (Librán-Pérez et al., 2012, 2013b, 2014a). In contrast, the fatty acid sensing system related to binding to FAT/CD36 and further modulation of transcription factors was activated in fish fed the high-fat versus low-fat diet, as seen from increased mRNA abundance of FAT/

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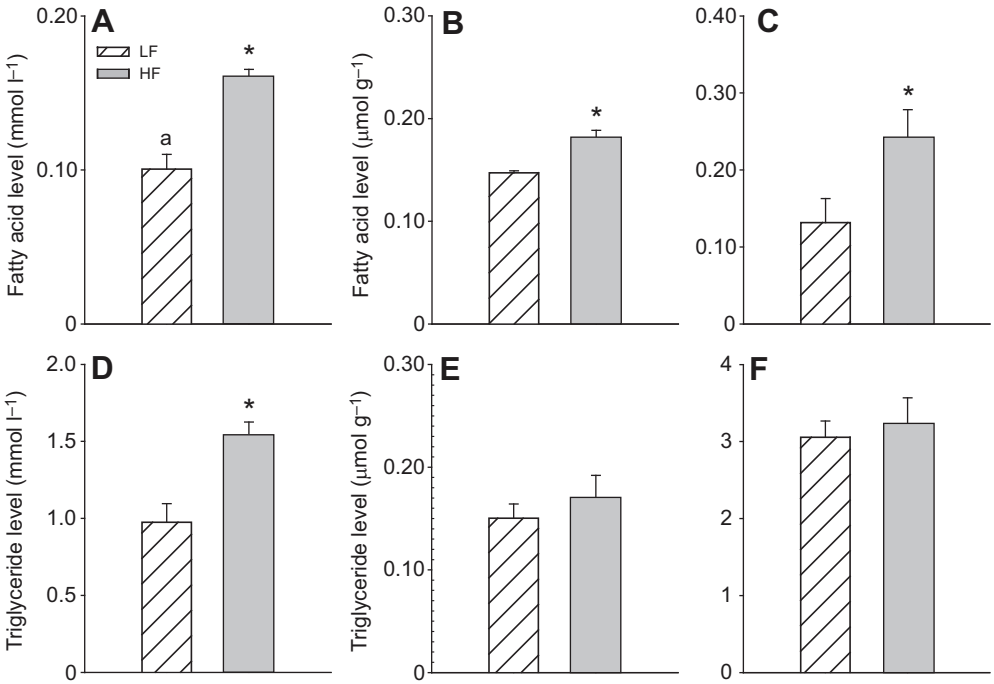

Fig. 2. Levels of non-esterified fatty acids and triglycerides in rainbow trout fed on low-fat or high-fat diets. Fatty acid and triglyceride levels were measured in plasma (A,D), hypothalamus (B,E) and liver (C,F), respectively, 6 h after the last meal in fish fed on low-fat (LF) or high-fat (HF) diets. Each value is the mean+s.e.m. of *N*=9 fish per diet. **P*<0.05 compared with fish fed with the low-fat diet.

CD36, LXRα, PPARα and SREBP1c. The fatty acid sensing system associated with mitochondrial production of ROS and further inhibition of K_{ATP} was not modified by feeding diets with different lipid content since no significant changes were noted for mRNA abundance of HOAD, UCP2a and Kir6.x-like. These data differ from results obtained previously in the same species after raising levels of specific fatty acids, such as oleate or octanoate (Librán-Pérez et al., 2012, 2013b, 2014a), although in those studies short-term (hours) effects were assessed instead of the long-term (4 weeks) period of the present study. However, the specific single fatty acid injections activated the systems related to fatty acid metabolism, binding to FAT/CD36, and mitochondrial activity, whereas the rise in fatty acid levels induced in the present study by feeding diets with different amount of lipids only activated one of the fatty acid sensing systems – that related to fatty acid binding to FAT/CD36. It therefore appears that oleate and octanoate induce changes in the fatty acid sensing systems related to fatty acid metabolism and mitochondrial activity, which cannot be mimicked by the unspecific increased supply of various fatty acids together, as in the present study. In this way, it is interesting to compare the present results with those obtained in trout hypothalamus following an unspecific decrease in circulating fatty acid levels induced by pharmacological treatment with SDZ WAG 994 (Librán-Pérez et al., 2014b). In that study, the fatty acid sensing systems related to fatty acid metabolism and mitochondrial activity also responded partially to the decrease in circulating levels of fatty acids. Therefore, the fatty acid sensing systems are apparently designed to respond to changes in the level of specific fatty acids such as oleate and octanoate in fish, but not so clearly to changes in the levels of various fatty acids together, such as those induced by

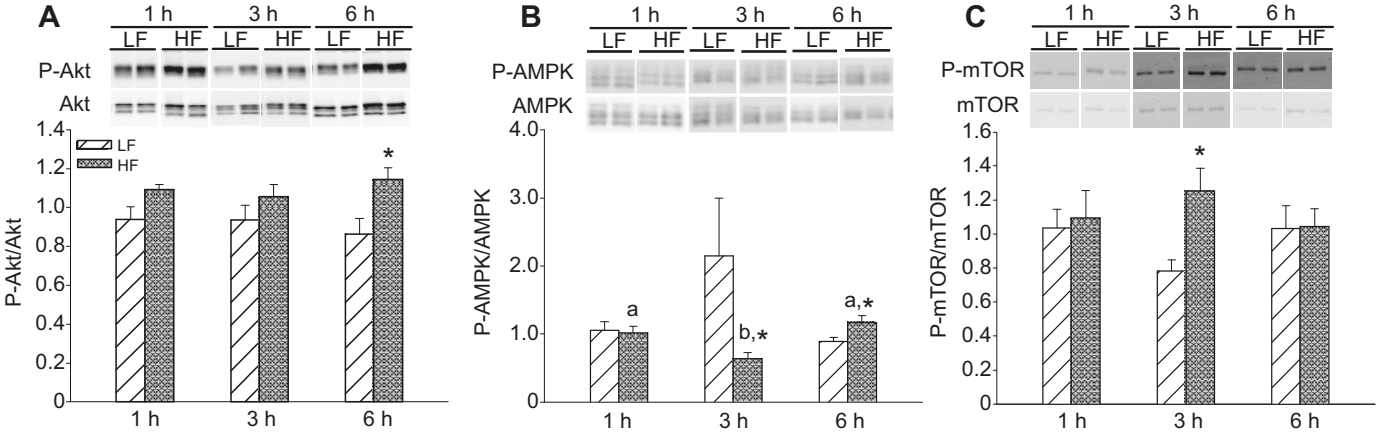

Fig. 3. Western blot analysis of Akt, AMPK and mTOR phosphorylation in the hypothalamus of rainbow trout fed with low-fat or high-fat diets. Akt (A), AMPK (B) and mTOR (C) was measured 1 h, 3 h and 6 h after the last meal. 20 μg of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and two representative blots per time and diet are shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Each value is the mean+s.e.m. of *N*=6 fish per diet and per time point. **P*<0.05 compared with fish fed with the low-fat diet. Different letters indicate significant differences (*P*<0.05) from different times in fish fed the same diet. There was no significant interaction between factors. LF, low-fat diet; HF, high-fat diet.

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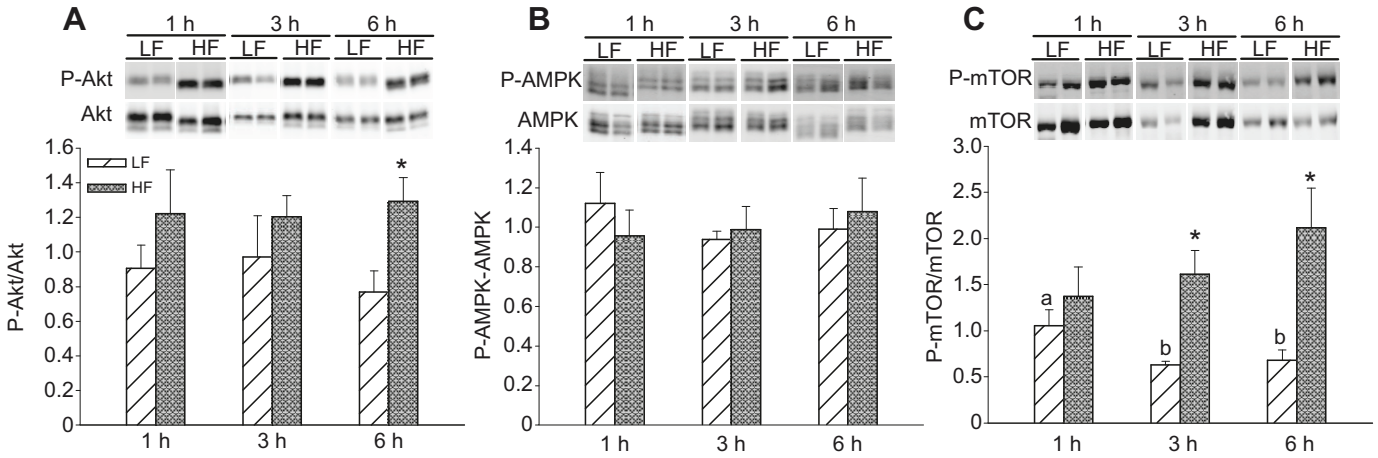

Fig. 4. Western blot analysis of Akt, AMPK and mTOR phosphorylation in the liver of rainbow trout fed with low-fat or high-fat diets. Akt (A), AMPK (B) and mTOR (C) was measured 1 h, 3 h and 6 h after the last meal. 20 μg of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and two representative blots per time and diet are shown here. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Each value is the mean+s.e.m. of *N*=6 fish per treatment and per time. **P*<0.05 compared with fish fed with the low-fat diet. Different letters indicate significant differences (*P*<0.05) from different times in fish fed the same diet. There was no significant interaction between factors. LF, low-fat diet; HF, high-fat diet.

the experimental diets used in the study where clear increases in the levels of ingested fatty acids were observed, not only in the case of oleate but also in palmitate, oleate, α-linolenate, eicosapentanoate and docosahexanoate among others. Similar specificities in the response of fatty acid sensing systems in the hypothalamus have been documented before in rat where oleate (López et al., 2007), but not other fatty acids such as octanoate (Obici et al., 2002) or palmitate (Benoit et al., 2009), was able to stimulate these systems.

In the liver of rainbow trout, we had previously suggested that the fatty acid sensing capacity appears to be an efferent response elicited by previous hypothalamic sensing followed by vagal and/or sympathetic outflow (Librán-Pérez et al., 2013a,b, 2015a). The parameters related to hepatic fatty acid-sensing in the present study displayed a partial response to changes in dietary lipid level, as seen for the fatty acid-sensing system based on fatty acid metabolism, where the mRNA abundance of only ACLY decreased, as expected in fish fed the high-fat diet, whereas the abundance of FAS mRNA was unchanged. In the fatty acid sensing system based on FAT/CD36, no changes were noted in the mRNA abundance of FAT/CD36, LXRα, and SREBP1c and only PPARα mRNA was enhanced in fish fed the high-fat diet, similar to other findings in the liver of rainbow trout (Martinez-Rubio et al., 2013) and Atlantic salmon (Kennedy et al., 2006) fed a lipid-enriched diet. Finally, the fatty acid sensing system based on mitochondrial activity was also partially activated in liver of fish fed with the high-fat diet since increased abundance of HOAD and UCP2a mRNA was noted, although no changes were noted in the mRNA of the components of the K_{ATP} channel, namely Kir6.x-like. In general, the response noted in the liver is more important than in the hypothalamus, and comparable to that already observed in this species when subjected to a treatment with fish oil (Librán-Pérez et al., 2013a) whose composition (especially rich in long-chain PUFAs such as eicosapentanoate and docosahexanoate) would be comparable to that of the high-fat diet used in the present study.

Effects on food intake

In line with previous studies in trout (Geurden et al., 2006; Figueiredo-Silva et al., 2012a,b), the high-fat compared with low-fat diet improved growth and food efficiency. Feeding the high-fat diet, however, did not decrease the amount of food intake (either considering the absolute amount per fish day or the relative amount corrected for differences in body weight). Other comparable studies carried out with rainbow trout similarly observed no decreased intake due to the higher dietary lipid content (Geurden et al., 2006; Saravanan et al., 2012; Figueiredo-Silva et al., 2012a,b), whereas trout fed with a fish-oil-enriched diet for 15 weeks displayed a significant decrease in food intake (Gélineau et al., 2001). The different response may relate to the difference in feeding duration (4 weeks in the present study) or to the fatty acid amount and composition of the lipids used for preparing the high-fat diet (a mixture of fish oil and rapeseed oil in this study), which together with the amount of lipids ingested, result in fish fed the high-fat diet having an increased intake of several fatty acids, especially myristeate, palmitate, pamitoleate, oleate, linoleate, α-linolenate, eicosapentanoate and docosahexanoate. Regarding the changes in mRNA abundance of hypothalamic neuropeptides involved in the regulation of food intake, we observed an increase in the values of the anorexigenic peptides POMC and CART in fish fed with the high-fat diet, whereas the expression of the orexigenic peptide NPY increased and no changes were noted in the orexigenic peptide AgRP. Trout subjected to increased levels of specific fatty acids such as oleate or octanoate also showed an increased anorexigenic potential as reflected in these hypothalamic neuropeptides, which was related to the inhibition of food intake (Librán-Pérez et al., 2012, 2014a). However, in our study, the global increase in the anorexigenic potential (considering changes in the four neuropeptides assessed) did not correlate with changes in food intake, which could be hypothesized to relate to the activation of only some of the different fatty acid sensing systems involved in the modulation of neuropeptide expression in fish fed with the high-fat diet. However, this situation is not so different from that known in mammals. In rat, the inhibition of food intake induced by treatment with oleate is not observed when animals were treated with other fatty acids like octanoate or palmitate (López et al., 2007) or when animals were fed a high-fat diet supplying a mixture of various fatty acids at once (Benoit et al., 2009), as is the case in the present study since the oil blend used despite being rich in oleate, also contains other fatty acids and resulted in a high intake of palmitate, among others. Also, for instance, in mammals palmitate, but not oleate, decreases activation of PI3K induced by insulin (Benoit et al.,

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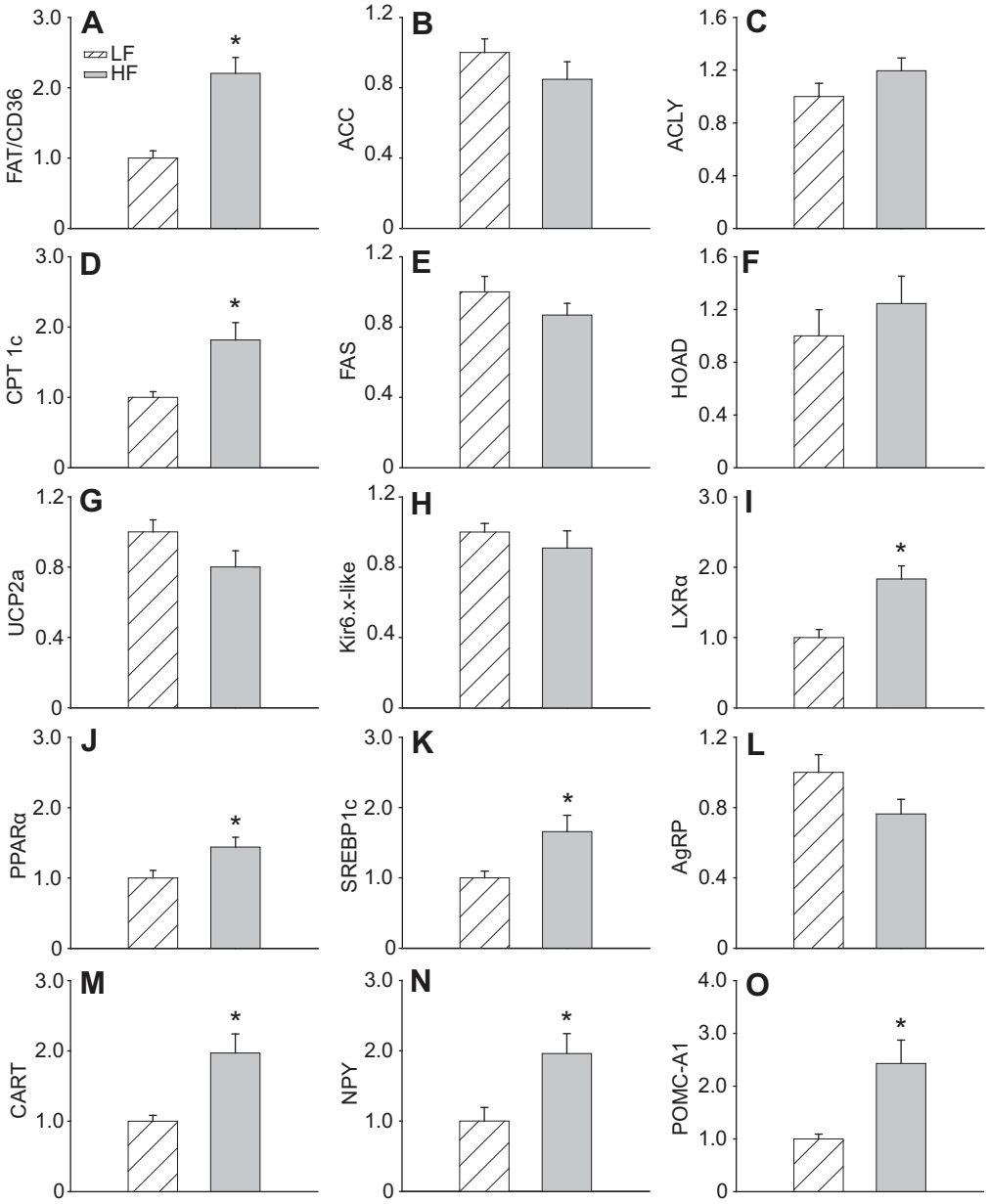

Fig. 5. Relative mRNA abundance of a range of metabolites in the hypothalamus of rainbow trout fed with low-fat or high-fat diets. Measurements were taken 6 h after the last meal. Data represent means+s.e.m. of 6 measurements. The results are normalized to β-actin expression and referenced to fish fed on the low-fat diet. **P*<0.05 compared with fish fed with the low-fat diet. LF, low-fat diet; HF, high-fat diet.

2009), suggesting that this fatty acid, among others, could be responsible for the differential response between oleate alone and the present high-fat diet. A similar behaviour in rainbow trout induced by fatty acids other than oleate or octanoate present in the high-fat diet, such as palmitate, could also help to explain the observed results in food intake. But considering the enhanced growth elicited by feeding the high-fat diet, the positive energy balance in these fish might also contribute to the observed changes in the expression of hypothalamic neuropeptides.

Effects on integrative energy and nutrient sensors and cellular signaling pathways

This is the first study in the fish literature, as far as we are aware, in which the expression and phosphorylation state of AMPK has been assessed in hypothalamus, whereas several others studied its presence in the liver and muscle of rainbow trout (Craig and Moon, 2013; Magnoni et al., 2014). In fish fed with the high-fat diet, an apparent decrease in the activation of AMPK was noted 3 h after a meal based on the decrease in the phosphorylation status in hypothalamus, although an apparent increase was also noted after 6 h. Considering that AMPK activation leads to the inhibition of energy-consuming biosynthetic pathways (Florant and Healy, 2012), such activation by feeding enhanced levels of dietary lipid are expected to decrease the lipogenic potential and increase fatty acid oxidation (de Morentin et al., 2011). There are no comparable studies available in the fish brain, although there is evidence from other tissues, such as liver or muscle. In the liver of rainbow trout fed with a diet rich in carbohydrates contradictory results were obtained since increased mRNA abundance of AMPKα1 was observed in one study (Craig and Moon, 2013) but a decreased P-AMPK/AMPK ratio was observed in another (Kamalam et al., 2012). In rainbow trout muscle, the activation of AMPK activity by swimming coincides with increased mRNA abundance of CPT1 (Magnoni et al., 2014). In mammals, the increase in AMPK phosphorylation state in hypothalamus inhibits ACC activity, resulting in decreased FAS and enhanced CPT1 activity (Chari et al., 2010). In our study, the decreases noted in abundance of ACC and FAS mRNA in the hypothalamus were not significant, but a

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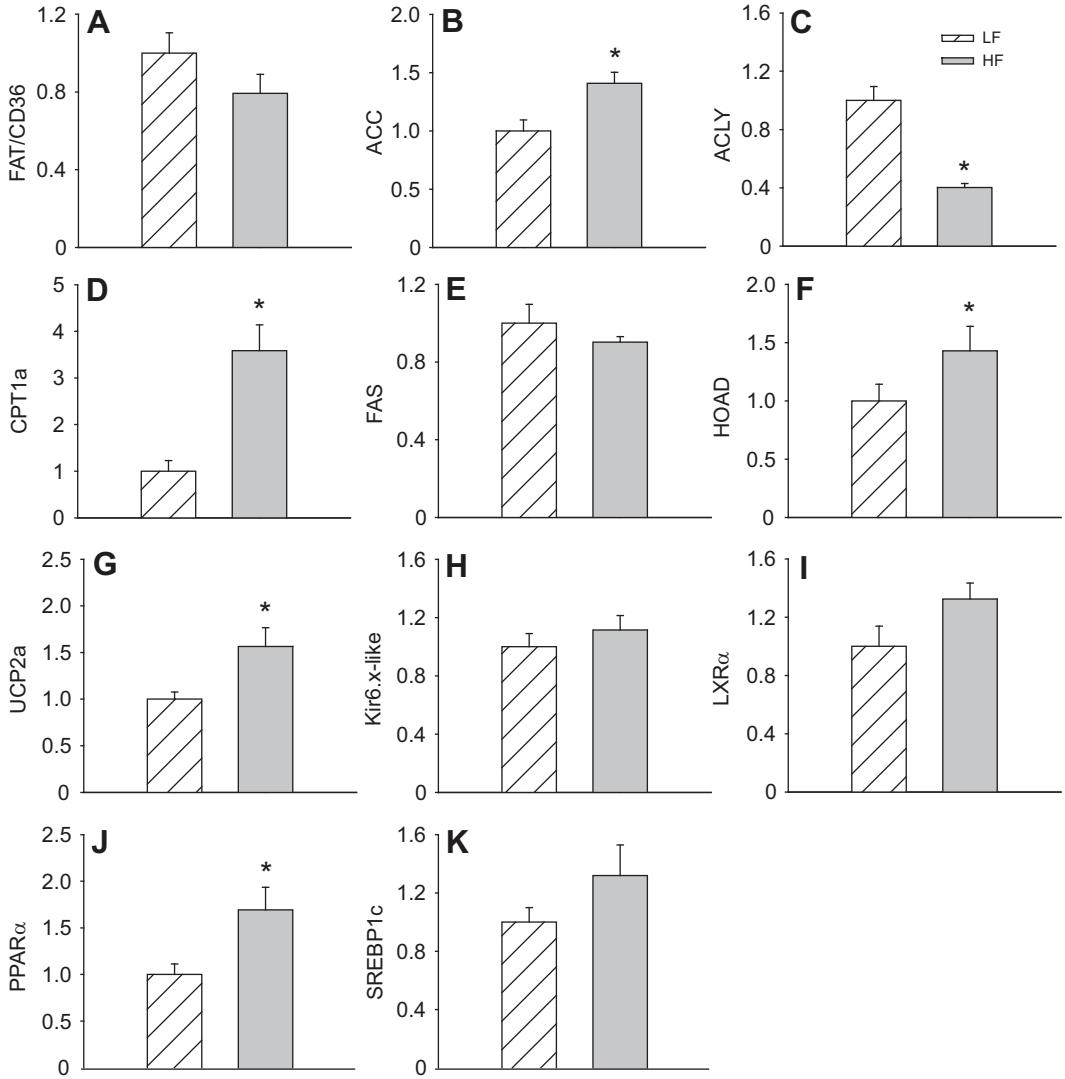

Fig. 6. Relative mRNA abundance of a range of metabolites in the liver of rainbow trout fed with low-fat or high-fat diets. Measurements were taken 6 h after the last meal. Data represent means+s.e.m. of 6 measurements. The results are normalized to β-actin expression and referenced to fish fed on the low-fat diet. *P<0.05 compared with fish fed with the low-fat diet. LF, low-fat diet; HF, high-fat diet.

clear increase was noted in CPT1c mRNA abundance, whereas in the liver, mRNA abundance of ACC and CPT1a increased and no changes were noted in FAS. In contrast, AMPK is known to exert a negative control over mTOR signaling in mammals (de Morentin et al., 2011). In the present study, the higher mTOR phosphorylation in the hypothalamus of fish fed the high-fat diet after 3 h coincided with reduced phosphorylation of AMPK. In general, AMPK is responding to the increased availability of fatty acid resulting from feeding fish a lipid-enriched diet. However, the changes observed in the mRNA abundance of parameters related to its signaling do not agree with those expected from mammalian literature (Chari et al., 2010), suggesting a differential response in fish.

The cellular signaling pathways associated with mTOR and Akt in the mammalian hypothalamus and liver are activated in response to increased levels of circulating fatty acids (Berthoud and Morrison, 2008; de Morentin et al., 2011). Also in our study, the phosphorylation state of mTOR and Akt tended to be higher in fish fed with the high-fat compared with the low-fat diet in both the hypothalamus and liver, and the increase was significant 6 h after the last meal in most cases (except for mTOR in the hypothalamus). In fish, there are no studies describing the presence and functioning of these cellular signaling pathways in the hypothalamus, although they have been characterized in the liver and muscle of rainbow trout under varying conditions of nutrient availability, showing the lack of changes in muscle Akt phosphorylation state after changes in dietary fat level (Figueiredo-Silva et al., 2012b) or the lack of changes in liver and muscle Akt and mTOR upon changes in the dietary protein level (Seiliez et al., 2011). Moreover, in re-fed rainbow trout, a situation of increased nutrient levels, increased phosphorylation of mTOR and Akt was noted in muscle (Seiliez et al., 2008) and liver (Lansard et al., 2009; Skiba-Cassy et al., 2009; Mennigen et al., 2012). Not surprisingly, these cellular signaling pathways are also activated in response to the treatment with anabolic hormones, which can be compared with a situation of abundance of nutrients. Thus, insulin treatment enhanced phosphorylation state of Akt in rainbow trout adipocytes (Bouraoui et al., 2010), adipose tissue (Polakof et al., 2011b), liver and muscle (Polakof et al., 2010b), and mTOR in adipocytes (Bou et al., 2014), whereas IGF-1 treatment enhanced the P-Akt/Akt ratio in muscle of rainbow trout (Codina et al., 2008).

Our data thus confirm that the enhanced availability of nutrients (lipid) induces the activation of the cellular signaling pathways

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related to mTOR and Akt, indicative of the anabolic state experienced by fish. Several parameters involved in fatty acid sensing and metabolism are related in mammals to these intracellular signaling pathways, such as the Akt-induced expression of SREBP1, which enhances expression of its target genes encoding proteins such as FAS and ACLY. In the present study, we observed in hypothalamus a simultaneous enhancement of Akt phosphorylation and abundance of SREBP1c mRNA, although without significant changes in the abundance of ACLY and FAS mRNA, whereas in liver, a decrease was noted in ACLY, indicative of a reduced lipogenic capacity. In line with this, in the study carried out by Figueiredo-Silva et al. (2012b), rainbow trout fed with a high-fat diet also displayed decreased lipogenic potential (FAS and G6PDH activities) in the liver. Therefore, the changes observed in metabolic parameters related to fatty acid sensing do not directly reflect those of the analyzed cellular signaling pathways, suggesting the existence of more complex interactions between them.

In summary, the fatty acid sensing systems characterized in rainbow trout whose activation in response to increased levels of oleate or octanoate has been found to result in decreased food intake (Librán-Pérez et al., 2012, 2014a) did not respond in the same way when fish were fed for 4 weeks with a lipid-enriched diet. The increased levels of fatty acid in hypothalamus and liver of rainbow trout fed the high-fat diet only partially activated fatty acid sensing systems with no changes in food intake, suggesting that fatty acid sensing response in fish to increased levels of fatty acid is more dependent on the presence of specific fatty acids such as oleate or octanoate, rather than to the global increase in fatty acid. However, we also obtained, for the first time in fish, evidence for the presence and functioning of energy sensors such as AMPK and proteins involved in cellular signaling, such as mTOR and Akt, in the hypothalamus. These proteins in hypothalamus and liver were generally activated in fish fed the high-fat versus low-fat diet, suggesting the activation of the cellular signaling pathways in response to the increased availability of fatty acids. This response was, however, not always accompanied by expected changes in the abundance of mRNA encoding parameters that are normally related, suggesting a complex interaction of fatty acid sensing and related mechanisms, including the control of food intake, which deserves further study.

MATERIALS AND METHODS

Experimental diets

Two diets based on fish meal (Table 2) were formulated to be isonitrogenous, but to contain two different levels of crude lipid. The low-fat diet contained 1.6% oil blend, whereas the high-fat diet contained 16% oil blend (50:50, fish oil:rapeseed oil). The difference in lipid level was compensated for by adding non-digestible cellulose to the low-fat diet in order to have only differences in digestible energy coming from lipids in both diets (Table 3). The two diets were manufactured using a twin screw extruder (Clextral, France) at the experimental feed unit (Donzacq, France) of the French National Institute of Agronomy Research (INRA, France). The diet ingredients and proximate composition are provided in Table 2, whereas fatty acid composition is shown in Table 3.

Fish and experimental conditions

The experiment was conducted in the INRA experimental facility of St Pée-sur Nivelle with rainbow trout obtained from the INRA experimental fish farm of Donzacq (Landes, France). The trout were acclimatised two weeks prior to the start of the experiment to the laboratory conditions: 12:12-h light-dark photoperiod and dechlorinated tap water at 17°C. Fish (34.4±0.47 g initial body weight) were randomly distributed into ten experimental tanks (20 fish per tank).

After acclimation, each of the two experimental groups were fed by hand (twice per day at 08:00 h and 14:00 h) to visual satiation in five replicate groups of 20 fish each for 4 weeks. The fish in each tank were weighed at the start and end of the trial in order to calculate the initial and final body mass. Food intake was assessed every day. Thus, the food uneaten remaining at the bottom (conical tanks) and feed waste were withdrawn, dried and weighed. The amount of food consumed by all fish in each tank was calculated as the difference from the feed offered. Results are shown as the means±s.e.m. of the data obtained in five different tanks (containing 20 fish each) per diet. Weight gain (%)=100%×(final body mass−initial body mass)/initial body mass; daily food intake (FI, %BW day^{-1})=100%×dry feed intake/[(initial tank biomass+final tank biomass)/2×days of trial duration]; feed efficiency (FE)=mass increase/dry feed intake. The experiment was conducted in strict accordance with EU legal frameworks related to the protection of animals used for scientific purposes (directive 2010/63/EU) and guidelines of French legislation governing the ethical treatment of animals (decree No. 2001-464; May 29, 2001). It was approved by the ethics committee of INRA (INRA 2002-36; April 14, 2002). The INRA experimental station is certified for animal services under the permit number A64.495.1 by the French veterinary services, which is the competent authority.

Sampling procedures

After 4 weeks of feeding the experimental diets, we evaluated postprandial changes in several parameters in fish. We carried out two experimental sets using different tanks per set, time and diet. In a first set, 6 fish per diet were sampled from 3 different tanks per diet 1, 3 and 6 h after the meal to assess changes in the levels of proteins involved in cellular signaling. In a second set, 15 fish per diet were sampled from 2 different tanks per diet 6 h after the meal to assess changes in mRNA abundance (6 fish per diet) and metabolite levels (9 fish per diet). We used 6 h in this second set because changes in gene expression are expected at the same time or later than those of cell signaling.

On each sampling, fish were anesthetized in tanks with 2-phenoxyethanol (Sigma, 0.2% v/v) weighed and killed by decapitation, and hypothalamus and liver were taken, immediately frozen in liquid nitrogen and stored at −80°C. Blood was collected by caudal puncture with ammonium heparinized syringes, and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 0.6 mol l^{-1} perchloric acid) and neutralized (using 1 mol l^{-1} potassium bicarbonate) before freezing in liquid nitrogen and storage at −80°C until further assay.

Diet analysis

The chemical composition of the diets was analyzed by the following methods: dry matter after drying at 105°C for 24 h, ash by combustion at 600°C for 4 h in a muffle furnace, crude protein (Nx6.25) by Kjeldahl method in acid-digested samples, crude lipid by petroleum ether extraction using Soxhlet method (Soxtherm), gross energy content in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany) and starch content by enzymatic method (InVivo labs, France). Fatty acid composition was determined in the total lipid extract as described in Kamalam et al. (2013).

Western blot analysis

Expression of selected phosphorylated and unphosphorylated proteins was analyzed in the liver and hypothalamus of fish (N=6) sampled 1 h, 3 h and 6 h after a meal. Frozen samples (200 mg) were homogenized in 1 ml (hypothalamus) or 2 ml (liver) of buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} sodium fluoride, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% NP40-IGEPAL and 1.02 mg ml^{-1} protease inhibitor cocktail (Roche, Basel, Switzerland), using an Ultraturrax homogenizer. Tubes were kept on ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 1000 ***g*** for 15 min at 4°C and supernatants were again centrifuged at 20,000 ***g*** for 30 min. The resulting supernatants were recovered and stored at −80°C. The concentration of protein in each sample was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with bovine serum albumin as standard. Liver and hypothalamus protein lysates (10 µg of protein for Akt; 20 µg for

AMPK and mTOR) were subjected to SDS-PAGE and western blotting using appropriate antibodies. Anti-phospho Akt (Ser473), anti-carboxyl terminal Akt, anti-phospho AMPK (Thr172), anti-AMPK, anti-phospho-mTOR (Ser2448), anti-mTOR antibodies were used (Cell Signaling Technology, Saint Quentin Yvelings, France). All these antibodies have been shown to crossreact successfully with rainbow trout proteins of interest (Skiba-Cassy et al., 2009; Kamalam et al., 2012). After washing, membranes were incubated with an IRDye infrared secondary antibody (Li-COR Biosciences, Lincoln, Nebraska, USA) and spots were quantified by Odyssey Infrared Imaging System software (Version 3.0, Li-COR Biosciences).

Assessment of metabolite levels

Levels of FFAs, triglyceride and glucose in plasma were determined enzymatically using commercial kits adapted to a microplate format (Wako Chemicals, Neuss, Germany, for fatty acids and Biomérieux, Grenoble, France, for triglyceride and glucose). Samples used to assess metabolite levels in tissues were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 mol l^{-1} perchloric acid and neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged (10,000 ***g***), and the supernatant used to assay tissue metabolites. Tissue FFA and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples.

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies, Grand Island, NY, USA) and treated with RQ1-DNAse (Promega, Madison, WI, USA). Two µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Promega) and random hexaprimers (Promega). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ (Bio-Rad). Analyses were performed on 1 µl cDNA using the MAXIMA SYBR Green qPCR Mastermix (Thermo Scientific, Waltham, MA, USA), in a total PCR reaction volume of 15 µl, containing 50–500 nmol l^{-1} of each primer. Sequences of the forward and reverse primers used for expression of each gene are shown in supplementary material Table S1. CPT1 isoforms for each tissues (liver, hypothalamus) were chosen based on previous studies (Librán-Pérez et al., 2012, 2013a,b,c, 2014a,b, 2015a,b).

Relative quantification of the target gene transcript was done using β-actin gene expression as reference, which was stably expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 90 s using hot-start iTaq DNA polymerase activation; 35 steps of PCR were performed, each consisting of heating at 95°C for 20 s for denaturing, and at specific annealing and extension temperatures. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C s^{-1} from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl (2001) method. This mathematical algorithm computes an expression ratio based on q-PCR efficiency and the crossing point deviation of the unknown sample versus a control group:

$$R = \frac{(E_{\text{target gene}})^{\Delta CT_{\text{target gene}}(\text{control}-\text{sample})}}{(E_{\beta-\text{actin}})^{\Delta CT_{\beta-\text{actin}}(\text{control}-\text{sample})}}, \quad (1)$$

where E is PCR efficiency determined using a standard curve of cDNA serial dilutions (cDNA dilutions from 1/32 up to 1/512) and ΔCT is the crossing point deviation of an unknown sample versus a control.

Statistics

Comparisons between low-fat and high-fat in proteins involved in cell signaling were carried out with a two-way ANOVA in which diet and time (1, 3 and 6 h) were the main factors. When a significant difference was observed within a factor, *post hoc* comparisons were carried out using the Student's t (diet) or Student-Newman-Keuls (time) tests. Comparisons between low-fat and high-fat diets in levels of metabolites and mRNA abundance were carried out with a Student's t-test. When necessary, data were log transformed to fulfil the conditions of the analysis of variance. Differences were considered statistically significant at $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.L.-P., I.G., G.C., S.P., and J.L.S. conception and design of research; M.L.-P., K.D., I.G., and G.C. performed experiments; M.L.-P., K.D., I.G., and G.C. analyzed data; M.L.-P., I.G., G.C., S.P., and J.L.S. interpreted results of experiments; M.L.-P., I.G., G.C., S.P., and J.L.S. prepared figures; M.L.-P., I.G., G.C., S.P., and J.L.S. edited and revised manuscript; M.L.-P., I.G., G.C., S.P., and J.L.S. drafted manuscript. J.L.S. approved final version of manuscript.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.123802/-/DC1

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