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

Marta Librán-Pérez[1], Inge Geurden[2], Karine Dias[2], Genevieve Corraze[2], Stephane Panserat[2], and José L. Soengas[1]

[1]Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Universidade de Vigo, E-36310 Vigo, Spain.
[2]INRA, UR 1067 Nutrition Metabolism Aquaculture, Aquapôle, CD918, F-64310 St-Pée-sur-Nivelle, France.

Corresponding author:

    Prof. José L Soengas

    Laboratorio de Fisioloxía Animal

    Facultade de Bioloxía

    Edificio de Ciencias Experimentais

    Universidade de Vigo

    E-36310 Vigo

    Spain

    Tel. +34-986 812 564

    Fax +34-986 812 556

    E-mail jsoengas@uvigo.es

SUMMARY

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

INTRODUCTION

In previous studies, we have characterized in 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) the presence and function of fatty acid (FA)-sensing systems (Librán-Pérez et al., 2012; 2013a,b,c; 2014a,b; 2015a,b). These systems respond to changes not only in a long-chain FA (LCFA) such as oleate but, unlike mammals, also in medium-chain FA (MCFA) like octanoate, and correlates with the control of food intake (hypothalamus), hormone release (BB) or metabolic homeostasis (liver). They are based on i) FA metabolism through inhibition of carnitine palmitoyltransferase 1 (CPT-1) to import FA-CoA into the mitochondria for oxidation; ii) binding to FA translocase (FAT/CD36), and further modulation of transcription factors like peroxisome proliferator-activated receptor type α (PPARα), and sterol regulatory element-binding protein type 1c (SREBP1c); and, iii) 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-Perez et al., 2012; 2014a). Since a reduced food intake has been observed after feeding fish like sea bass (Boujard et al., 2004) or rainbow trout (Gélineau et al., 2001) with lipid-enriched diets, changes in FA sensing systems are expected in fish fed with diets containing different lipid levels that have not been assessed yet.

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 liver (Craig and Moon, 2011, 2013; Polakof et al., 2011a; Fuentes et al., 2013) and muscle (Craig and Moon, 2013; Magnoni et al., 2014) but to date there is no information in any fish tissue regarding the response of AMPK to changes in the levels of nutrients like fatty acids, as demonstrated in mammals (Hardie and Ashford, 2014).

Furthermore, proteins involved in cellular signaling like target of rapamycin (mTOR) and protein kinase B (Akt) have been 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 FA 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, there are no available studies in fish assessing the response of these proteins to changes in levels of circulating nutrients like FA, 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 low fat or high-fat diets was 1) to determine if the response of food intake, mRNA abundance of hypothalamic neuropeptides involved in the metabolic regulation of food intake, and FA sensing systems in hypothalamus and liver is similar to that previously observed when levels of specific FA were raised by injection; and 2) to determine if the phosphorylation state of intracellular energy sensors (AMPK), and proteins involved in cellular signalling (Akt and mTOR) display changes in hypothalamus and liver in response to different dietary lipid levels that could be linked to variations in parameters related to FA 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 HF diet. The value of feed efficiency was higher in the group fed with the HF 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.

Changes in cumulative feed intake are shown in Fig. 1 and there were no significant differences between diets.

Considering composition of the diets as well as food intake, fish fed the LF diet ingested 0.09 ± 0.01g of lipid/fish/day and fish fed the HF diet ingested 0.29 ± 0.01g of lipid/fish/day. Considering FA composition of the diets (Table 3), FA intake of specific FA (in mg FA/fish/day) 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 hours after feeding are shown in Fig. 2. Free FA levels in plasma (Fig. 2A), hypothalamus (Fig. 2B), and liver (Fig. 2C) increased in the group of fish fed with HF diet compared with the group fed with LF diet. Triglyceride levels increased in plasma of fish fed with the HF diet (Fig. 2D) but there were no significant differences in hypothalamus (Fig. 2E) and liver (Fig. 2F). Finally, 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 6h after the meal in fish fed with the HF diet compared with fish fed LF. The ratios of AMPK (Fig. 3B) in fish fed the HF diet decreased 3h after the meal and increased 6h after the meal compared with fish fed the LF diet whereas in fish fed the HF diet the ratio observed 3h after the meal was lower than that observed after 1 or 6h. Finally, the value of mTOR (Fig. 3C) increased 3h after the meal in the fish fed the HF diet compared with fish fed the LF diet. No differences with time were noted for Akt (Fig. 3A) and mTOR (Fig. 3C) whereas for AMPK values in fish fed HF diet were lower 3h after the meal compared with those observed after 1 and 6h (Fig. 3B).

The ratios of phosphorylated vs. total forms of Akt, AMPK, and mTOR in liver are shown in Fig. 4. The ratio for Akt was higher 6h after the meal in the fish fed with the HF diet (Fig. 4A). No significant changes were noted between groups for the ratio P-AMPK/AMPK (Fig. 4B). The ratio P-mTOR/mTOR increased 3 and 6h after the meal in fish fed with the HF diet compared with fish fed the LF diet (Fig. 4C) whereas values in fish fed the LF diet were higher 1h after the meal than after 3 or 6h.

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

Changes in mRNA abundance of transcripts assessed in liver are shown in Fig. 6. ACLY mRNA abundance (Fig. 6C) in the group fed with the HF diet was lower than the group fed with the LF diet whereas ACC (Fig. 6B), CPT1a (Fig. 6D), HOAD (Fig. 6F),

UCP2a (Fig. 6G), and PPARα (Fig. 6J) mRNA levels were higher in the group fed with HF diet than in the group fed with LF. No significant changes were noted for levels of FAT/CD36 (Fig. 6A), FAS (Fig. 6E), Kir6.x-like (Fig. 6H), LXRα (Fig. 6I), and SREBP1c (Fig. 6K).

DISCUSSION

We previously demonstrated the activation of FA-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 of different lipid content. 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 FA and triglycerides were markedly increased in plasma of rainbow trout fed with the HF diet. Moreover, those differences were also reflected in the free FA levels of the two tissues assessed, i.e. hypothalamus and liver, thus validating the experimental design, and supporting the assessment of changes in FA-sensing systems in both tissues. The observed changes in FA levels are similar to those observed in other studies with rainbow trout fed comparable diets (Figueiredo-Silva et al., 2012b).

Effects on FA-sensing systems in hypothalamus and liver

In hypothalamus, the FA-sensing system based on FA metabolism was apparently not activated in fish fed the HF 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 FA-sensing system related to binding to FAT/CD36 and further modulation of transcription factors was activated in fish fed the HF vs LF diet, as seen from increased mRNA abundance of FAT/CD36, LXRα, PPARα and SREBP1c. Finally, the FA-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 FA such as oleate or octanoate (Librán-Pérez et al., 2012, 2013b, 2014a) though 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 FA injections activated the systems

related to FA metabolism, binding to FAT/CD36, and mitochondrial activity, whereas the rise in FA levels induced in the present study by feeding diets with different amount of lipids only activated one of the FA-sensing systems, i.e. that related to FA binding to FAT/CD36. It hence appears that oleate and octanoate induced changes in the FA sensing systems related to FA metabolism and mitochondrial activity, which cannot be mimicked by the unspecific increased supply of various FAs 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 FA levels induced by pharmacological treatment with SDZ WAG 994 (Librán-Pérez et al., 2014b). In that study, the FA-sensing systems related to FA metabolism and mitochondrial activity also responded partially to the decrease in circulating levels of FA. Therefore, the FA-sensing systems are apparently designed to respond to changes in the level of specific FAs such as oleate and octanoate in fish, but not so clearly to changes in the levels of various FAs together such as those induced by the experimental diets used in the study where clear increases in the levels of ingested FA were observed not only in the case of oleate but also in palmitate, oleate, α-linolenate, eicosapentanoate, and docosahexanoate among others. In this way, specificities in the response of FA-sensing systems in hypothalamus have been documented before in rat where oleate was able to stimulate these systems (López et al., 2007) but not other FA such as octanoate (Obici et al., 2002) or palmitate (Benoit et al., 2009).

In liver of rainbow trout, we had previously suggested that the FA 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 FA-sensing in the present study displayed a partial response to changes in dietary lipid level, as seen for the FA-sensing system based on FA metabolism where only mRNA abundance of ACLY decreased, as expected in fish fed the HF diet whereas FAS mRNA abundance was unchanged. In the FA-sensing system based on FAT/CD36 no changes were noted in the mRNA abundance of FAT/CD36, LXRα, and SREBP1c and only mRNA abundance of PPARα was enhanced in fish fed the HF diet, similar to other findings in liver of rainbow trout (Martinez-Rubio et al., 2013) and Atlantic salmon (Kennedy et al., 2006) fed a lipid-enriched diet. Finally, the FA-sensing system based on mitochondrial activity was also partially activated in liver of fish fed with the HF diet since increased mRNA abundance of HOAD and UCP2a was noted though no changes were noted in the mRNA abundance of the components of the K_{ATP} channel namely

Kir6.x-like. In general, the response noted in liver is more important than in 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 PUFA such as eicosapentanoate and docosahexanoate) would be comparable to that of HF 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 HF diet compared to LF diet improved growth and food efficiency. Feeding the HF 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 (Gèlineau 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 HF 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 HF diet in an increased intake of several FAs, especially for 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 in fish fed with the HF diet an increase in the values of the anorexigenic peptides POMC and CART 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 FA like 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 FA-sensing systems involved in the modulation of neuropeptide expression in fish fed with the HF 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 FA like

octanoate or palmitate (López et al., 2007) or when animals were fed a high fat diet supplying a mixture of various FA at once (Benoit et al., 2009) as it is the case in the present study since the oil blend used despite rich in oleate also contains other FA 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.,2009) suggesting that this FA, among others, could be responsible of the differential response between oleate alone and the present HF diet. A similar behaviour in rainbow trout induced by FA other than oleate or octanoate present in the HF diet, such as palmitate, could also help to explain the observed results in food intake. On the other hand, considering the enhanced growth elicited by feeding the HF 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 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 liver and muscle of rainbow trout (Craig and Moon, 2013; Magnoni et al., 2014). In fish fed with the HF diet an apparent decrease in the activation of AMPK was noted 3h after meal based on the decrease in the phosphorylation status in hypothalamus though an apparent increase was also noted after 6h. 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 FA oxidation (de Morentin et al., 2011). There are no comparable studies available in fish brain, though there is evidence obtained in other tissues like liver or muscle. In 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 decreased P-AMPK/AMPK ratio was observed in another one (Kamalam et al., 2012). In rainbow trout muscle, the activation of AMPK activity by swimming coincides in time 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 activities (Chari et al., 2010). In our study, the decreases noted in ACC and FAS mRNA abundance in hypothalamus were not significant but a clear increase was noted in CPT1c mRNA

abundance whereas in liver mRNA abundance of ACC and CPT1a increased and no changes were noted in FAS. On the other hand, AMPK is known to exert a negative control over the mTOR signaling in mammals (de Morentin et al., 2011). In the present study, the higher mTOR phosphorylation in hypothalamus of fish fed the HF diet after 3h coincided with a lower phosphorylation of AMPK at the same time. In general, AMPK is responding to the increased availability of FA resulting from feeding fish a lipid-enriched diet. However, the changes observed in the mRNA abundance of parameters related to its signaling are not 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 mammalian hypothalamus and liver are activated in response to increased levels of circulating FA (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 HF compared to the LF diet in both hypothalamus and liver, and the increase was significant 6h after the last meal in most cases (except for mTOR in hypothalamus). In fish, there are no studies in literature describing the presence and functioning of these cellular signaling pathways in hypothalamus, though they have been characterized in liver and muscle of rainbow trout under varying conditions of nutrient availability, showing the lack of changes in muscle Akt phosphorylation state by changes in dietary fat level (Figueiredo-Silva et al 2012b) or the lack of changes in liver and muscle Akt and mTOR by changes in dietary protein level (Seiliez et al. 2011). Moreover, in refed 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 to 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 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 related to mTOR and Akt indicative of the anabolic state experienced by fish. Several parameters involved in FA 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 such as FAS and ACLY. In the present study, we observed in hypothalamus a simultaneous enhancement of Akt phosphorylation and mRNA abundance of SREBP1c though without significant changes in mRNA abundance of ACLY and FAS, whereas in liver a decrease was noted in ACLY indicative of a reduced lipogenic capacity. In line, in the study carried out by Figueiredo-Silva et al. (2012b) rainbow trout fed with a high fat diet also displayed in liver decreased lipogenic potential (FAS and G6PDH activities). Therefore, the changes observed in metabolic parameters related to FA-sensing do not directly reflect those of the analysed cellular signaling pathways, suggesting the existence of more complex interactions between them.

In summary, the FA-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 an lipid-enriched diet. The increased levels of FA in hypothalamus and liver of rainbow trout fed the HF diet only partially activated FA-sensing systems with no changes in food intake, suggesting that FA-sensing response in fish to increased levels of FA is more dependent on the presence of specific FA such as oleate or octanoate rather than to the global increase in FA. On the other hand, we also obtained, for the first time in fish, evidence for the presence and functioning in hypothalamus of energy sensors like AMPK and proteins involved in cellular signaling like mTOR and Akt. These proteins in hypothalamus and liver were generally activated in fish fed the HF vs LF diet suggesting the activation of the cellular signaling pathways to the increased availability of FA. This response was however not always accompanied by expected changes in mRNA abundance of parameters that are normally related to them suggesting a complex interaction of these systems with FA-sensing and mechanisms related, including the control of food intake, which deserves further study.

MATERIALS AND METHODS

Experimental diets

Two fish meal based diets (Table 2) were formulated to be isonitrogenous, but to contain two different levels of crude lipid. Low-fat (LF) diet contained 1.6 % oil blend, whereas high-fat (HF) diet contained 16% oil blend (fish oil/ rapeseed oil, 50/50). The difference in lipid level was compensated for by adding non-digestible cellulose in diet LF in order to have only differences in DE coming from lipids between both diets (Table 2), 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 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 diets was fed by hand (twice per day at 08:00 and 14:00) to visual satiation to five replicate groups of fish 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 weight. 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 mean ± SEM of the data obtained in 5 different tanks (containing 20 fish each) per diet. Weight gain (%) = 100% ∗ (final body weight − initial body weight) /initial body weight; daily food intake (FI, %BW per d) = 100% ∗ dry feed intake / ((initial tank biomass + final tank biomass) / 2 × days of trial duration); feed efficiency (FE) = weight 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 the French

legislation governing the ethical treatment of animals (Decree No. 2001-464, May 29th, 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 signalling. In a second set, 15 fish per diet were sampled from 2 different tanks per diet 6h after the meal to assess changes in mRNA abundance (6 fish per diet) and metabolite levels (9 fish per diet). We used 6h in this second set because changes in gene expression are expected at the same time or later than those of cell signalling.

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

Diet analysis

The chemical composition of the diets was analysed 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 on the total lipid extract as described in Kamalam et al. (2013).

Western blot analysis

Expression of selected phosphorylated and not phosphorylated proteins was analyzed in the liver and hypothalamus of fish (n = 6) sampled 1 h, 3h and 6h after the meal. Frozen samples (200 mg) were homogenized in 1 mL (hypothalamus) or 2 mL (liver) of buffer containing 150 mM NaCl, 10 mM Tris, 1 mM EGTA, 1 mM EDTA (pH 7.4), 100 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP40-IGEPAL, and 1.02 mg/ml protease inhibitor cocktail (Roche, Basel, Switzerland), using an Ultraturrax homegenizer. Tubes were kept in ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 1000g for 15 min at 4ºC and supernatants were again centrifuged at 20,000g for 30 min. The resulting supernatants were recovered and stored at -80ºC. 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 cross-react 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 FFA, triglyceride, and glucose in plasma were determined enzymatically using commercial kits adapted to a microplate format (Wako Chemicals, Neuss, Germany, for FA, 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 M perchloric acid, and neutralized (using 1 M 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^{TM} (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 nM of each primer. Sequences of the forward and reverse primers used for each gene expression are shown in Table 5. 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 stable expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 90s using hot-start iTaq DNA polymerase activation; 35 steps of PCR were performed, each one consisting of heating at 95°C for 20s 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 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 method (2001). 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: $R = [(E_{\text{target gene}})^{\Delta CT_{\text{Target gene}} \text{(mean control- mean unknown sample)}}] / [(E_{\beta actin})^{\Delta CT_{\beta actin} \text{(mean control- mean unknown sample)}}]$ 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 LF and HF in proteins involved in cell signaling were carried out with a two-way ANOVA in which diet (LF and HF) and time (1, 3, and 6h) were the main factors. When a significant difference was observed within a factor, post-hoc comparisons were carried out using the Student t (diet) or Student-Newman-Keuls (time) tests. Comparisons between LF and HF in levels of metabolites and mRNA abundance were carried out with a Student t test. When necessary data were Log transformed to fulfil the conditions of the analysis of variance. Differences were considered statistically significant at $P<0.05$.

Competing interest
No competing interest declared

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Tables

Table 1. Initial body weight (IBW), final body weight (FBW), weight gain, specific growth rate (SGR), feed intakes and feed efficiency (FE) of rainbow trout fed with low fat (LF) and high fat (HF) diets for 4 weeks. Data are mean ± S.E.M of 5 different tanks (containing 20 fish each) per diet.

	Diets	
	LF	HF
IBW (g)	34.51 ± 0.52	34.09 ± 0.42
FBW (g)	86.29 ± 3.21	95.29 ± 3.42 *
Weight gain (%)	51.79 ± 2.89	61.20 ± 3.50 *
SGR (%BW per d)	3.26 ± 0.10	3.54 ± 0.14 *
FI (g per fish per d)	1.60 ± 0.10	1.64 ± 0.06
FI (%BW per d)	2.65 ± 0.11	2.54 ± 0.13
FI (g/kg met BW)	16.42 ± 0.78	16.26 ± 0.73
FE	1.11 ± 0.04	1.29 ± 0.09 *

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

Table 2. Ingredients and proximate composition of low fat (LF) and high fat (HF) diets used to feed rainbow trout for 14 days

	Diet	
	LF	HF
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
Analysed 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 DM)[7]	20.0	23.2
Calculated digestible energy (DE) content (kJ/g 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 diet LF 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.

Table 3. Fatty acid composition (g/100 of total FA) of low fat (LF) and high fat (HF) diets used to feed rainbow trout for 4 weeks

	Diet	
Fatty acid	LF	HF
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

Table 4. Daily fatty acid intake (mg/fish/day) of fish fed with low fat (LF) and high fat (HF) diets for 4 weeks. Data are mean ± S.E.M of 5 different tanks (containing 20 fish each) per diet.

	Diet	
Fatty acid	LF	HF
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*

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

Table 5. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Data base	Accession Number
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	GenBank	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	Sigenae	tcbk0010c.b.21_5.1.om.4
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	GenBank	CA349411.1
AgRP	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	GenBank	CR376289
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	GenBank	NM_001124627
CPT-1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	GenBank	AF327058
CPT-1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	GenBank	AJ619768
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	Sigenae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	DFCI	AY606034.1
HOAD	GGACAAAGTGGCACCAGCAC	GGGACGGGGTTGAAGAAGTG	Sigenae	tcad0001a.i.15 3.1.om
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	Sigenae	CA346261.1.s.om.8:1:773:1
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	GenBank	FJ470291
NPY	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	GenBank	NM_001124266
POMC-A1	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	Tigr	TC86162
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	GenBank	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank	CA048941.1
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	GenBank	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; AgRP, Agouti related peptide; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; HOAD, hydroxyacil-CoA dehydrogenase; Kir6.x-like, inward rectifier K⁺ channel pore type 6.x-like; LXRα, liver X receptor α; NPY, neuropeptide Y; POMC-A1, pro-opio melanocortin A1; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; UCP2a, mitochondrial uncoupling protein 2a. mRNA abundance were determined as described in the same species (Panserat et al., 2000; Ducasse-Cabanot et al., 2007; Kolditz et al., 2008; Lansard et al., 2009; Cruz-García et al., 2009; Conde-Sieira et al., 2010; Polakof et al., 2008,2010a,2011b; Figueiredo-Silva et al., 2012c; Sánchez-Gurmaches et al., 2012; MacDonald et al., 2014).
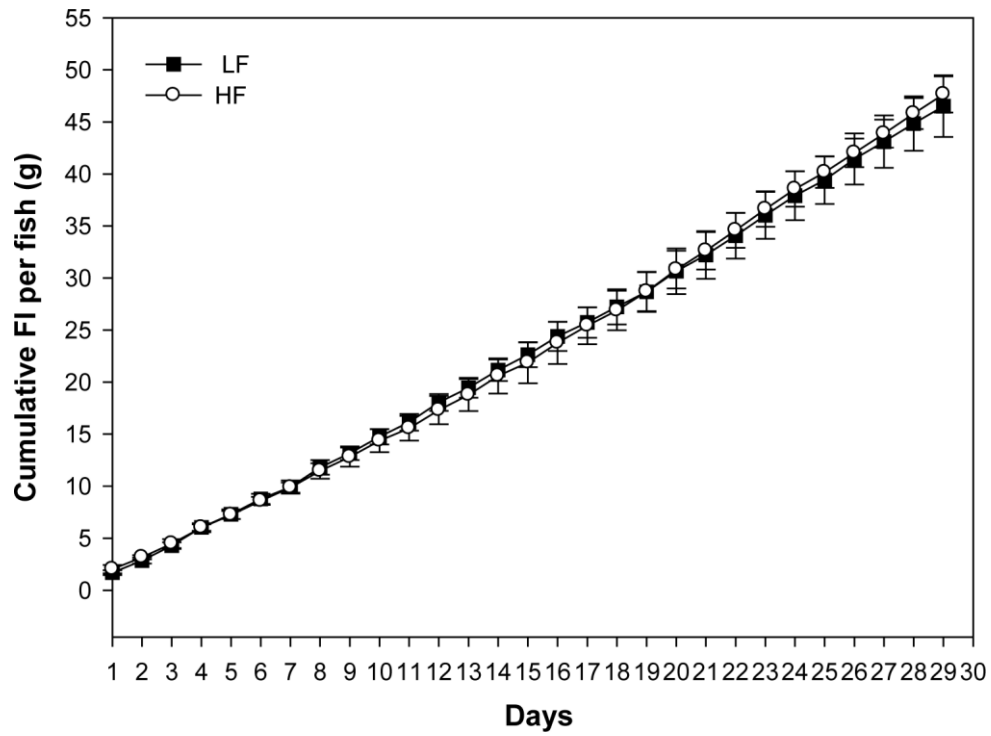
Figures

Fig. 1. Cumulative food intake (FI) in rainbow trout fed during 4 weeks with low fat (LF) or high fat (HF) diets. Data are means ±SD of 5 different tanks (containing 20 fish each) per tank. No significant difference between diets (P≥0.05) was noted.
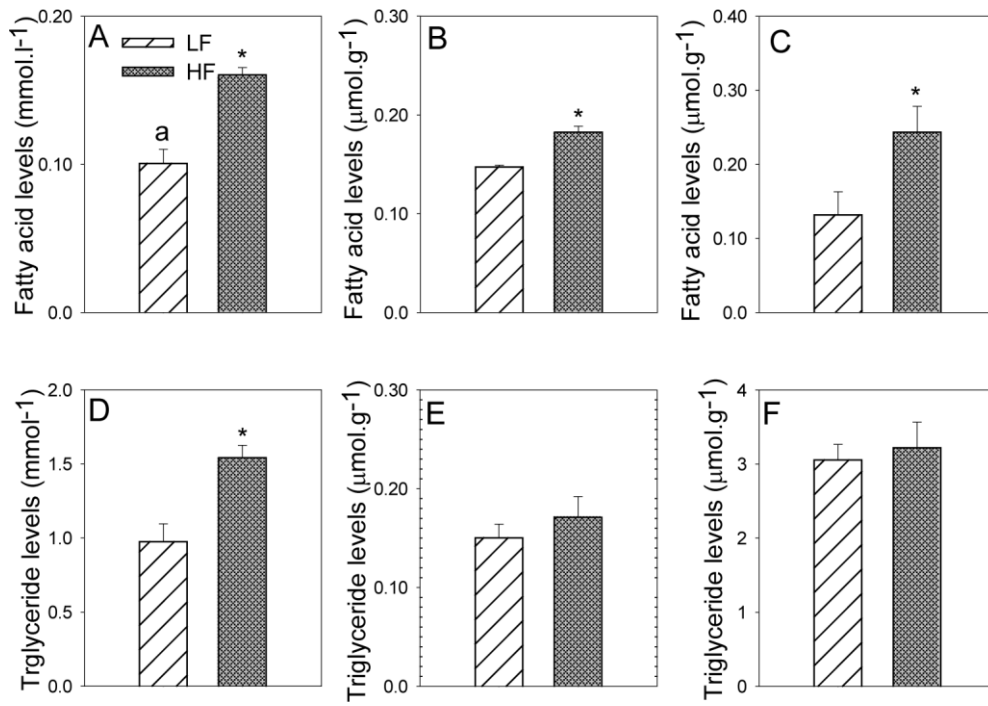
Fig. 2. Levels of non-esterified fatty acids and triglycerides in plasma (A), hypothalamus (B), and liver (C), and levels of triglyceride in plasma (D), hypothalamus (E), and liver (F) of rainbow trout 6h after the last meal with low fat (LF) or high fat (HF) diets. Each value is the mean + SEM of n = 9 fish per diet. *, significantly different (P<0.05) from fish fed with the LF diet.
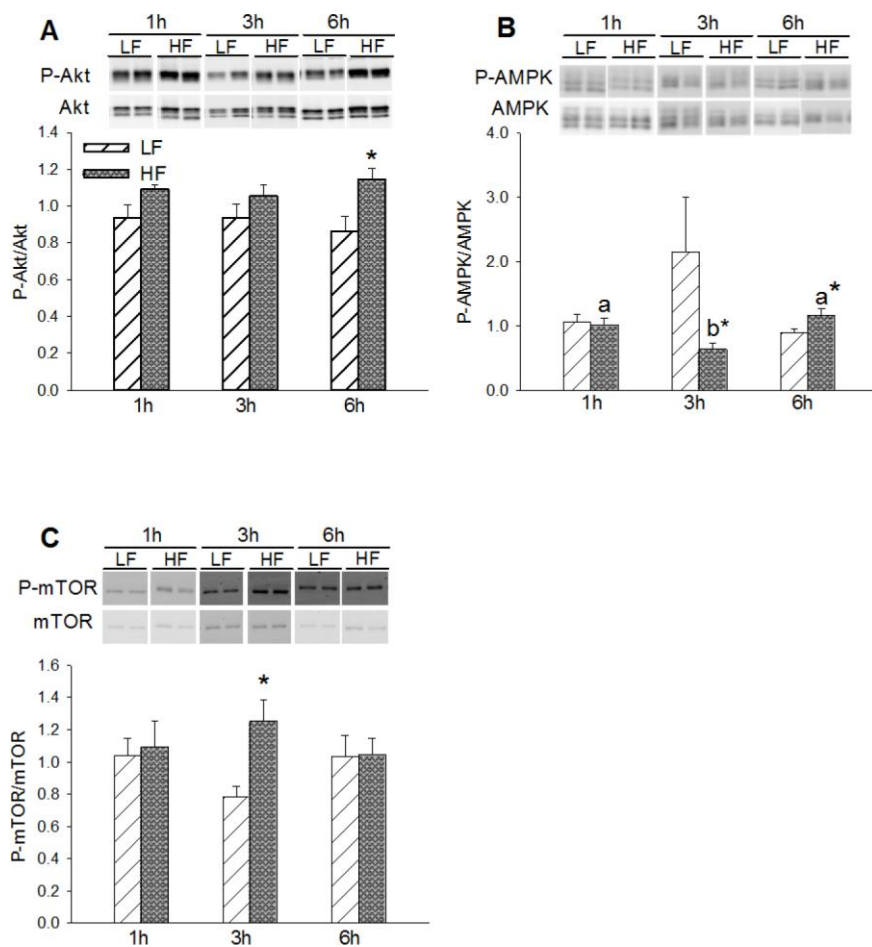
Fig. 3. Western blot analysis of Akt (A), AMPK (B), and mTOR (C) phosphorylation in hypothalamus of rainbow trout 1h, 3h and 6h after the last meal with low fat (LF) or high fat (HF) diets. 20 µg of total protein per lane were loaded on the gel respectively. Western blots were performed on 6 individual 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 + SEM of n = 6 fish per diet and per time. *, significantly different ($P<0.05$) from fish fed with the LF diet at the same time. Different letters indicate significant differences ($P<0.05$) from different times in fish fed the same diet. There was no significant interaction between both factors.
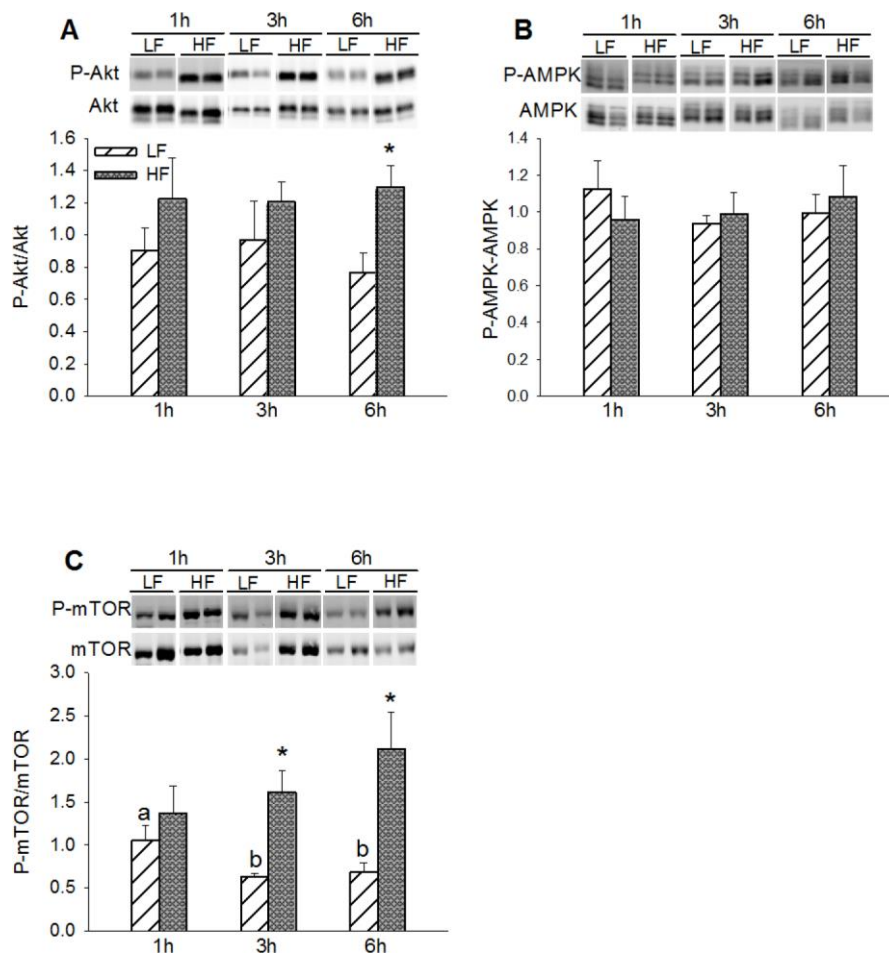
Fig. 4. Western blot analysis of Akt (A), AMPK (B), and mTOR (C) phosphorylation in liver of rainbow trout 1h, 3h and 6h after the last meal with low fat (LF) or high fat (HF) diets. 20 μg of total protein per lane were loaded on the gel respectively. Western blots were performed on 6 individual samples 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 + SEM of n = 6 fish per treatment and per time. *, significantly different (P<0.05) from fish fed with the LF diet at the same time. Different letters indicate significant differences (P<0.05) from different times in fish fed the same diet. There was no significant interaction between both factors.
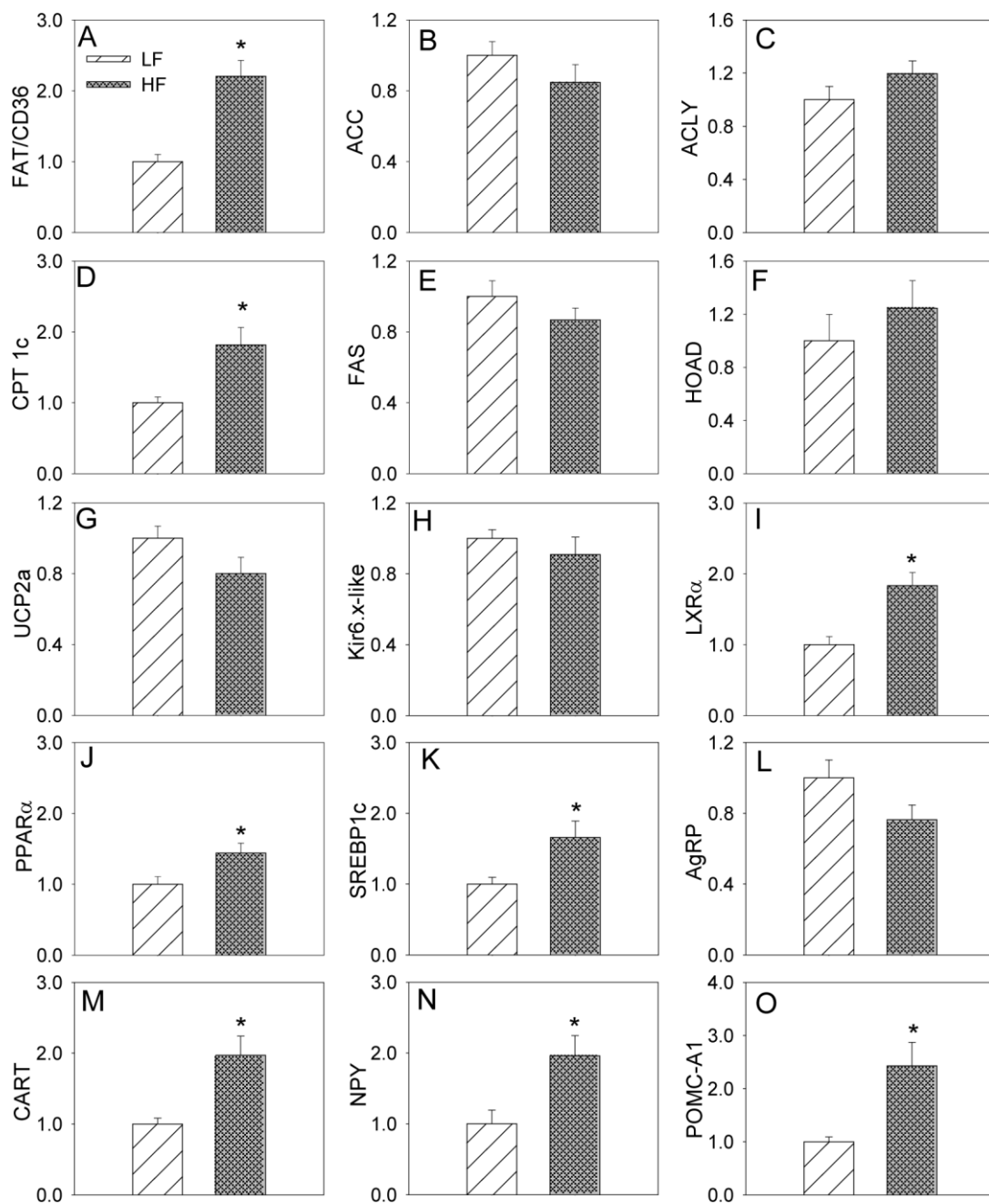
Fig. 5. Relative mRNA abundance of FAT/CD36 (A), ACC (B), ACLY (C), CPT 1c (D), FAS (E), HOAD (F), UCP2a (G), Kir6.x-like (H), LXRα (I), PPARα (J), SREBP1c (K), AgRP (L), CART (M), NPY (N) and POMC-A1 (O) in hypothalamus of rainbow trout 6 h after the last meal with low fat (LF) or high fat (HF) diets. Data represent mean + SEM of 6 measurements. The results are referred to LF group and are normalized by β-actin expression. *, significantly different (P<0.05) from fish fed with the LF diet
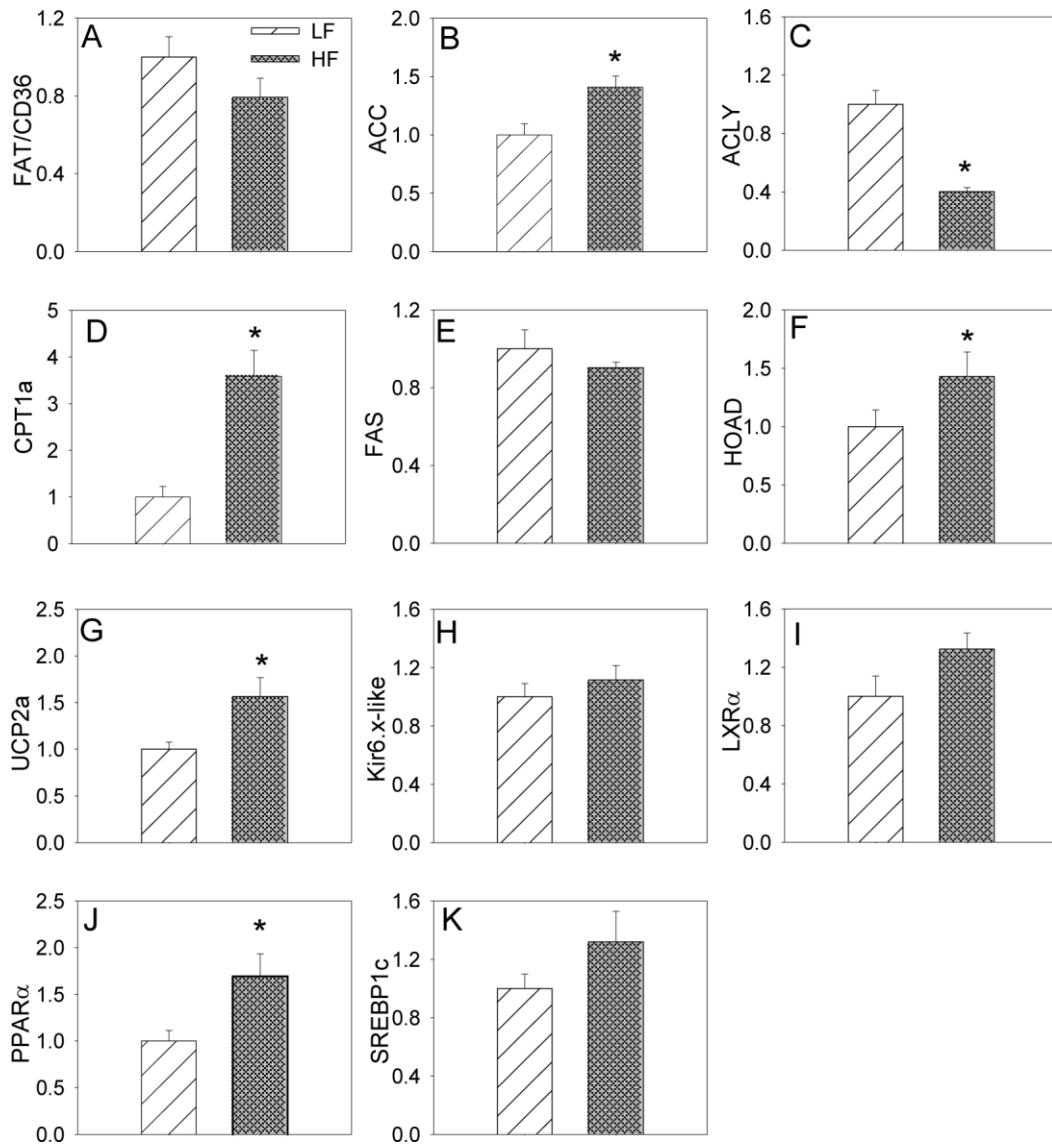
Fig. 6. Relative mRNA abundance of FAT/CD36 (A), ACC (B), ACLY (C), CPT1a (D), FAS (E), HOAD (F), UCP2a (G), Kir6.x-like (H), LXRα (I), PPARα (J), and SREBP1c (K) in liver of rainbow trout 6 h after the last meal o with low fat (LF) or high fat (HF) diets. Data represent mean + SEM of 6 measurements. The results are referred to LF group and are normalized by β-actin expression. *, significantly different (P<0.05) from fish fed with the LF diet.

Table S1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR)

	Forward primer	Reverse primer	Database	Accession number
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	GenBank	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	Sigenae	tcbk0010c.b.21_5.1.om.4
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	GenBank	CA349411.1
AgRP	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	GenBank	CR376289
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	GenBank	NM_001124627
CPT-1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	GenBank	AF327058
CPT-1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	GenBank	AJ619768
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	Sigenae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	DFCI	AY606034.1
HOAD	GGACAAAGTGGCACCAGCAC	GGGACGGGGTTGAAGAAGTG	Sigenae	tcad0001a.i.15 3.1.om
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	Sigenae	CA346261.1.s.om.8:1:773:1
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	GenBank	FJ470291
NPY	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	GenBank	NM_001124266
POMC-A1	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	Tigr	TC86162
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	GenBank	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank	CA048941.1
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	GenBank	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; AgRP, Agouti related peptide; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; HOAD, hydroxyacil-CoA dehydrogenase; Kir6.x-like, inward rectifier K^+ channel pore type 6.x-like; LXRα, liver X receptor α; NPY, neuropeptide Y; POMC-A1, pro-opio melanocortin A1; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; UCP2a, mitochondrial uncoupling protein 2a. mRNA abundance were determined as described in the same species (Panserat et al., 2000; Ducasse-Cabanot et al., 2007; Kolditz et al., 2008; Lansard et al., 2009; Cruz-Garcia et al., 2009; Conde-Sieira et al., 2010; Polakof et al., 2008, 2010a, 2011b; Figueiredo-Silva et al., 2012c; Sánchez-Gurmaches et al., 2012; MacDonald et al., 2014).