Universidade Vigo

Facultade de Bioloxía

PROGRAMA DE DOCTORADO EN ACUICULTURA

Mecanismos centrales y periféricos sensores de ácidos grasos en la trucha arco iris como modelo de pez teleósteo. Implicación en el control de la ingesta de alimento

Tesis Doctoral para optar al

Título de Doctorado por la
Universidad de Vigo con Mención Internacional

Marta Librán Pérez

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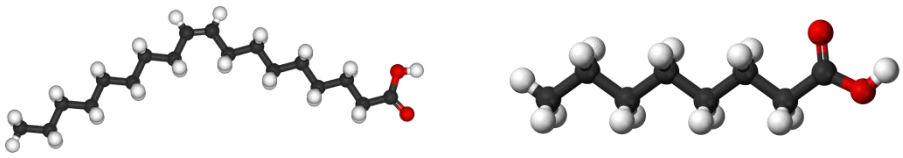

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2015

JOSÉ LUIS SOENGAS FERNÁNDEZ Catedrático de Fisiología en el Departamento de Biología Funcional y Ciencias de la Salud de la Universidad de Vigo,

CERTIFICA:

Que la presente memoria titulada "Mecanismos centrales y periféricos sensores de ácidos grasos en la trucha arco iris como modelo de pez teleósteo. Implicación en el control de la ingesta de alimento" presentada por Dña. Marta Librán Pérez para optar al Título de Doctorado por la Universidad de Vigo con Mención Internacional, ha sido realizada bajo su dirección, y que, hallándose concluida, autoriza su presentación para que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste y surta los efectos oportunos, firmo el presente en Vigo, a 19 de marzo de 2015.

Dr. D. José Luis Soengas Fernández.

Esta tesis doctoral ha sido realizada gracias a la concesión de una beca-contrato de Formación de Personal Investigador (FPI) del Ministerio de Ciencia e Innovación (BES-2011-043394) a Marta Librán Pérez.

El trabajo experimental de esta tesis doctoral ha sido subvencionado por los siguientes proyectos de investigación:

1. Influencia de señales intrínsecas (sensores metabólicos y sistema circadiano) y del estrés sobre el control de la alimentación en peces teleósteos. Aplicación en piscicultura.
   Ministerio de Ciencia e Innovación-FEDER (AGL2010-22247-C03-03/ACU).

2. Integración de señales metabólicas y neuroendocrinas en la regulación de la ingesta y su interacción con el estrés en peces.
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3. Consolidación y estructuración de unidades de investigación competitivas del SUG (Grupo con potencial de crecimiento).
   Xunta de Galicia (CN 2012/004)

Esta tesis ha sido realizada y consecuentemente será defendida de forma adecuada para la obtención del título de Doctorado por la Universidad de Vigo con Mención Internacional.

Este trabajo ha sido evaluado por dos evaluadores internacionales independientes del centro donde se ha realizado la tesis doctoral:

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β-HSD: 3β-hidroxiesteroide deshidrogenasa
5HT: serotonina
6PGDH: 6-fosfogluconato deshidrogenasa (*EC* 1.1.1.44)
11-βH: 11β-hidroxiesteroide deshidrogenasa
ACC: Acetil-CoA carboxilasa (*EC* 6.4.1.2)
ACLY: ATP-citrato liasa (*EC* 4.1.3.8)
ACTH: Hormona adrenocorticotropa
ADN/DNA: Ácido desoxirribonucleico
ADNc/DNAc: Ácido desoxirribonucleico complementario
ADP: Adenosina 5'-difosfato
AgRP: Péptido relacionado con agouti
Akt: Proteína quinasa B
AMPK: Proteína quinasa activada por AMP (EC 2.7.1.109)
ANOVA: Análisis de varianza
ARC: Núcleo arcuato
ARN/RNA: Ácido ribonucleico
ARNm/mRNA: Ácido ribonucleico mensajero
ATP: Adenosina 5´-trifosfato
BB: Cuerpos de Brockmann
BBS: Bombesina
BSA: Ácido bicinchonínico
C75: 4-metileno-2-octil-5-oxotetrahidrofurano-3-ácido carboxílico
CART: Tránscrito relacionado con la cocaína y la anfetamina
CCK: Colecistoquinina
cDNA: Ácido desoxirribonucleico complementario
CPT-1: Carnitina palmitoil transferasa 1 (*EC* 2.3.1.21)
CRF: Factor liberador de corticotropina
CRFBP: Proteína de unión al factor liberador de corticotropina
CS: Citrato sintetasa (*EC* 4.1.3.7)
DA: Dopamina
DHA: ácido docohexanoico
DMH: Núcleo dorsomedial
DMSO: Dimetilsulfóxido
DTNB: 5,5´-ditiobis-2-nitrobenzoico
DTT: 1,4-dithiothreitol
EDTA: Ácido etilendiamino tetraacético
EEM: Error estándar de la media
EF1α: Factor de elongacion 1α
FA: Ácido graso
FABPs: Proteína específica con alta afinidad por los ácidos grasos.
FATP: Proteína trasnportadora de ácidos grasos.
FAS: Ácido graso sintetasa (*EC* 2.3.1.85)
FAT/CD36: Proteína translocadora de ácidos grasos (cluster de diferenciación 36)
FBPasa: Fructosa-1,6-bifosfatasa (*EC* 3.1.3.11)
FBW: Peso final
FFA: ácidos grasos libres
FE: Eficiencia de alimentación
FI: Ingesta de alimento
G6Pasa: Glucosa 6-fosfatasa (*EC* 3.1.3.9)
GE: Neurona estimulada por glucosa
GH: Hormona del crecimiento
GI: Neurona inhibida por glucosa
GK: Glucoquinasa (*EC* 2.7.1.2)
GLP-1: Péptido análogo al glucagón tipo1
GPasa: Glucógeno fosforilasa (*EC* 2.4.1.1)
GRP: Péptido liberador de gastrina
GSasa: Glucógeno sintetasa (*EC* 2.4.1.11)
HF: alto en grasas
HK: Hexoquinasa (*EC* 2.7.1.1)
HOAD: Hidroxiacil-CoA deshidrogenasa (*EC* 1.1.1.35)
HPA: Eje hipotálamo-hipófisis-adrenal
HPB: Hidroxipropil-β-ciclodextrin
HPI: Eje hipotálamo-hipófisis-interrenal
HUFA: ácidos grasos saturados

IBW: Peso inicial
ICV: Intracerebroventricular
IP: Intraperitoneal
K^+_{ATP}: Canal de potasio dependiente de ATP
Kir6.x-Like: Proteína formadora del poro tipo 6x-like del canal de K^+ dependiente de ATP
LCFA: ácido graso de cadena larga
LF: Bajo en grasas
LH/LHA: Área hipotalámica lateral
LPL: lipoprotein lipasa
LXRα: Receptor hepático X tipo α
MCD: Malonil CoA deshidrogenasa (*EC* 4.1.1.9)
MCFA: ácido graso de cadena media
MCH: Hormona concentradora de melanina
MCR: Receptor de melanocortinas
MS-222: Éster etílico del ácido 3-aminobenzoico
MSH: Hormona estimuladora de melanocitos
mTOR: Diana de rapamicina en células de mamífero
NA: Noradrenalina
NADH: Forma reducida de la nicotinamida adenina dinucleótido
NLT: *Nucleus lateralis tuberis*
NPO: *Nucleus preopticus*
NPY: Neuropéptido Y
OCT: Octanoato/ácido octanoico
OL: oleato/ácido oleico
OX: Orexinas
PCA: Ácido perclórico
PK: Piruvato quinasa (*EC* 2.7.1.40)
PKC- δ: Proteína quinasa C tipo δ
POMC: Pro-opio melanocortina
PPARα: Receptor tipo α activado por la proliferación de peroxisomas
PUFA: ácidos grasos poliinsaturados
PVN: Núcleo paraventricular
P450scc: Citocromo P450 de escisión de la cadena lateral
q-PCR: PCR cuantitativa
ROS: Especies reactivas de oxígeno
RT-PCR: Retrotranscripción-reacción en cadena de la polimerasa
SCFA: ácido graso de cadena corta
SDZ: SDZ WAG 994
SGR: Tasa de crecimiento específico
SNC/CNS: Sistema nervioso central
SNK: Student-Newman-Keuls
SREBP1c: Proteína tipo 1c que se liga a elementos reguladores de esteroles
StAR: Proteína reguladora de la esteroidogénesis aguda
SUR-like: Receptor de sulfonilurea-like
TAG: Triacilgliceroles
TGI: Tracto gastrointestinal
TOFA: Ácido *5-(*tetradeciloxi*)*-2-furoico
UCP2a: Proteína desacoplante mitocondrial tipo 2a
VLDL: Lípidos de muy baja densidad
VMH: Núcleo ventromedial hipotalámico
VMN: Núcleo ventromedial
β-NADH: Nicotinamida adenin dinucleótido, forma reducida
β-$NADP^+$: Nicotinamida adenin dinucleótido fosfato
β-NADPH: Nicotinamida adenin dinucleótido fosfato, forma reducida

1. INTRODUCCIÓN GENERAL

1.1 METABOLISMO LIPÍDICO EN PECES

Los lípidos constituyen un grupo diverso de compuestos, cuya característica común y definitoria es su insolubilidad en agua. Sus funciones biológicas son diversas. En muchos organismos las grasas y los aceites son las formas principales de almacenamiento energético, mientras que los fosfolípidos y los esteroles constituyen la mitad de la masa de las membranas biológicas. Otros lípidos, aún estando presentes en cantidades relativamente pequeñas, juegan papeles cruciales como cofactores enzimáticos, transportadores electrónicos, agentes emulsionantes, hormonas y mensajeros intracelulares (Tocher, 2003).

Debido a su alto contenido calórico, los lípidos constituyen una eficiente reserva energética. En peces, junto con las proteínas, los lípidos se metabolizan rápida y eficazmente con el fin de proporcionar la energía necesaria para cubrir las necesidades del animal (Cowey y Walton, 1989). Su papel más importante es el almacenamiento y provisión de energía metabólica en forma de ATP a través de la β-oxidación de los ácidos grasos (Sargent *et al.*, 1989; Froyland *et al.,* 2000).

Los lípidos en los animales, incluidos los peces, se pueden dividir en dos grandes grupos: lípidos polares, compuestos principalmente por los fosfolípidos, y lípidos neutrales, compuestos principalmente por triacilgliceroles (TAG) (triglicéridos)(Figura 1.1).

Los **ácidos grasos** (FA) son los componentes característicos de muchos lípidos y están formados por una cadena larga hidrocarbonada de tipo lineal, y con un número par de átomos de carbono. Tienen en un extremo de la cadena un grupo carboxilo (-COOH) y se designan en base a la longitud de sus cadenas, al grado de saturación y a la posición de sus enlaces de etileno. Se dividen en dos grupos, los ácidos grasos saturados, que solo poseen enlaces simples entre sus átomos de carbono y los ácidos grasos insaturados (PUFA), que poseen uno o varios enlaces dobles entre sus átomos de carbono. Son precursores de varios metabolitos importantes (eicosanoides). Además, los lípidos de peces son ricos en ácidos grasos de cadena larga altamente insaturados (HUFA), que juega un papel importante en la nutrición animal, incluyendo la nutrición en peces y humanos, reflejando su particular papel en procesos fisiológicos críticos (Tocher, 2003).

Los **TAG** son ésteres de glicerol con tres moléculas de ácidos grasos, siendo la principal forma de almacenamiento y de transporte de los ácidos grasos.

Los **fosfoglicéridos,** pertenecientes al grupo de los fosfolípidos, están compuestos por ácido fosfatídico, una molécula compleja compuesta por glicerol, en el que se han esterificado dos ácidos grasos (uno saturado y otro insaturado) y un grupo fosfato. A su vez, al grupo fosfato se une un alcohol o un aminoalcohol.

Los **esfingolípidos** son un grupo de lípidos polares que contienen un aminoalcohol complejo de cadena larga (esfingosina). El centro de cada esfingolípido es una ceramida derivado de una amida de ácido graso de la molécula de alcohol. Son componentes importantes de las membranas.

El **colesterol**, es el lípido simple más importante en todos los animales, incluidos los peces. Es el componente más común de los esteroles que son derivados complejos de los triterpenos y pueden existir sin esterificar como componentes esenciales de las membranas celulares o almacenados como lípidos neutrales en su forma esterificada a ácidos grasos.

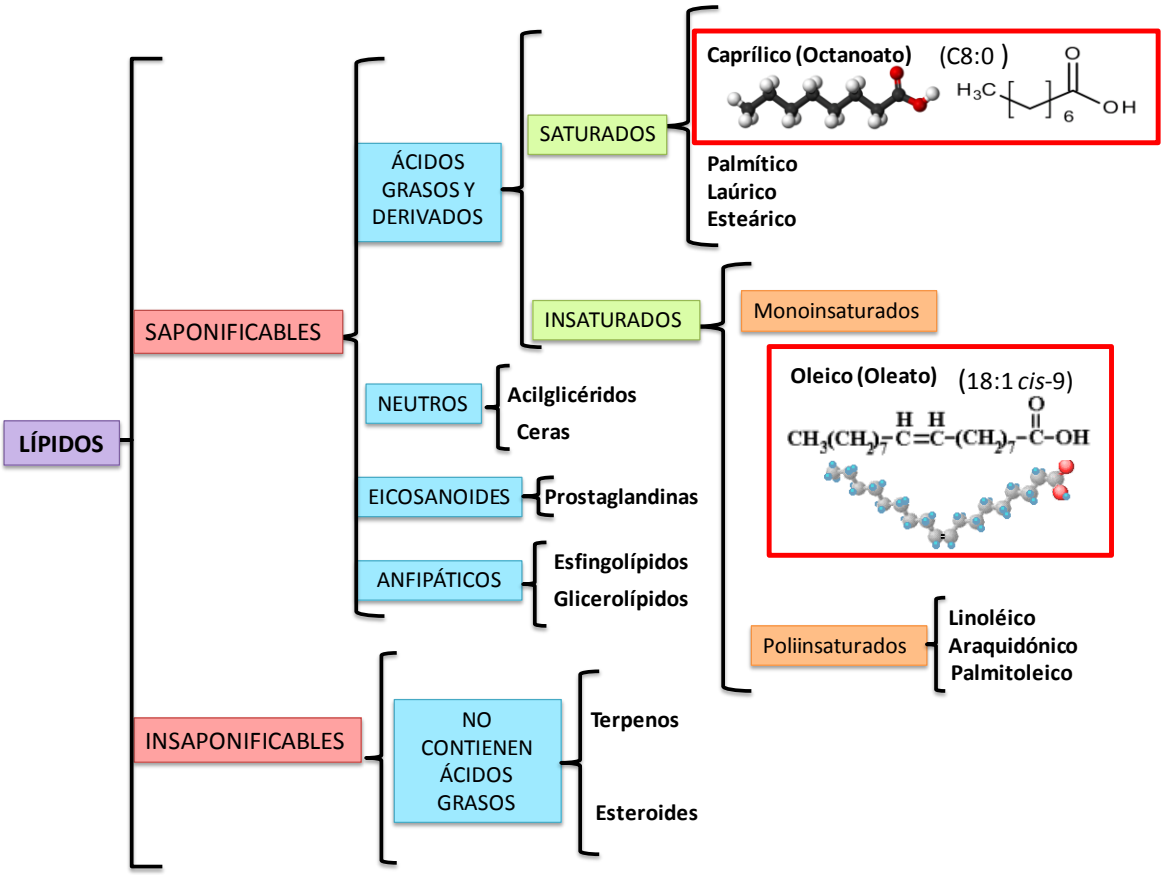

Fig. 1.1. Clasificación general de lípidos.

La principal fuente de lípidos en los peces es la dieta , que incluye principalmente TAG junto con los fosfolípidos y las ceras. Son importantes por varias razones. En primer lugar, por aportar componentes que son necesarios para el crecimiento como son los constituyentes de las membranas de las células (constituidas principalmente por fosfolípidos y colesterol). En segundo lugar, porque aportan energía para el mantenimiento de las funciones del cuerpo, lo cual permitirá que parte de las proteínas de la dieta se ahorren para el crecimiento. Tercero, por ser la única fuente de unos compuestos esenciales (que el pez no puede sintetizar y ha de incorporarlos con la dieta) como son los ácidos grasos esenciales. Y en cuarto lugar, por ser el vehículo de otros componentes necesarios para la función normal de los seres vivos, como son las vitaminas liposolubles (A,D,E y K). La oxidación de los TAG, las ceras y los fosfolípidos, constituye una fracción considerable del metabolismo energético de la mayoría de los tejidos (Weber y Haman, 1996).

En general, los procesos de digestión, absorción y transporte de lípidos en peces son similares a los de mamíferos (Sargent *et al.*, 1989). En cuanto a la **digestión**, los lípidos procedentes de la dieta, principalmente los TAG, se hidrolizan en el lumen intestinal por la acción de las enzimas lipasas pancreáticas (Figura 1.2). El páncreas enxocrino, es la mayor fuente de enzimas digestivas lipasas tanto en peces como en mamíferos (Kapoor *et al.,* 1975; Fange y Grove, 1979) aunque en varios estudios llevados a cabo en peces, se ha observado actividad de las lipasas en el estómago, sugiriendo que este tejido también puede ser una fuente de lipasas, y que el origen bacteriano de algunas actividades lipolíticas en el tracto digestivo de peces no puede ser descartado (Olsen y Ringoe, 1997). En intestino de mamíferos, la hidrólisis de los TAG la llevan a cabo las triacilglicerol lipasas, existiendo dos tipos de sistemas, el complejo pancreático lipasa-colipasa y el menos específico de sales biliares que activan las lipasas cuya presencia ha sido demostrada en peces teleósteos (Olsen y Ringoe, 1997). Este sistema ha sido bien caracterizado en bacalao (*Gadus morhua*), cuya actividad lipolítica en los fluídos intestinales se estimula por las sales biliares y la subsiguiente activación de la lipasa de los ciegos pilóricos del pez (Gjellesvik *et al.,* 1989; Gjellesvik, 1991a,b). Sin embargo, son escasas las evidencias de la existencia del complejo pancreático lipasa-colipasa en peces. En trucha arco iris (*Oncorhynchus mykiss*), se ha descrito una enzima similar a la lipasa pancreática (Leger *et al.*, 1977,1979). La digestibilidad de los lípidos en general es elevada (alrededor del 90%) y este valor va a depender tanto del origen o naturaleza de los lípidos como del punto de fusión que presentan. Este punto de fusión aumenta de manera directa con el grado de saturación de sus ácidos grasos. El principal producto de hidrólisis resultante tras la digestión de lípidos en peces son los ácidos grasos libres (FFA).

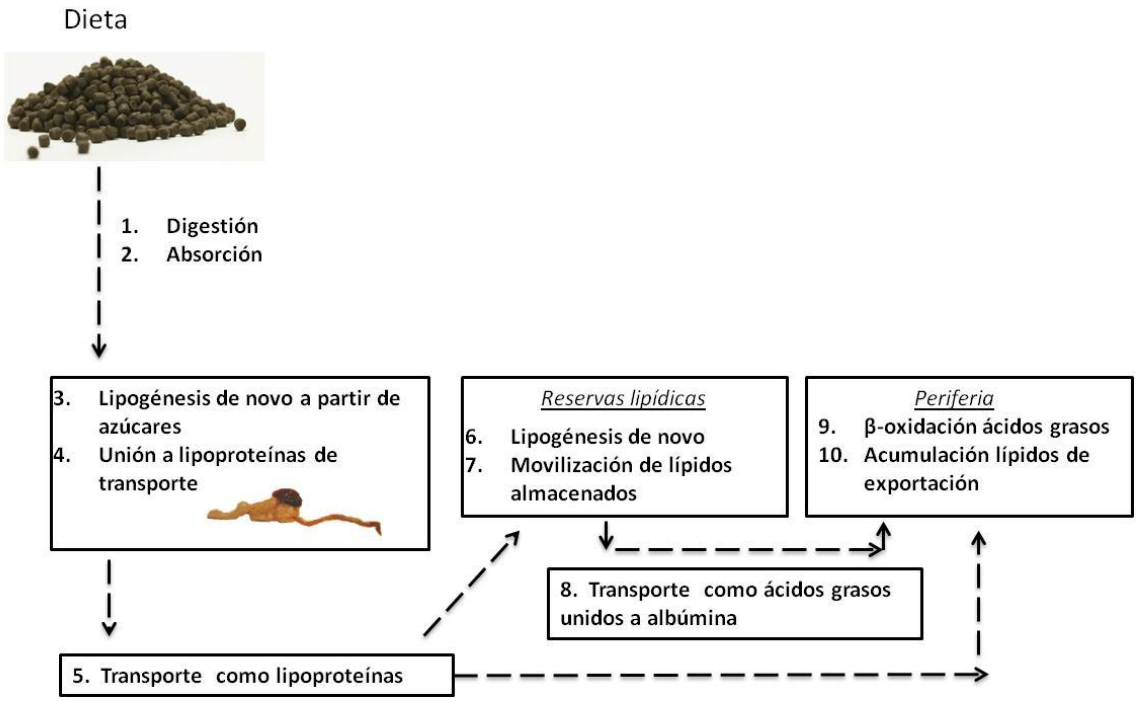

Fig. 1.2. Representación esquemática de la incorporación de lípidos de la dieta (modificada de Castelló-Orvay F., 1993)

La **absorción** de los productos de la digestión lipídica no ha sido muy estudiada en peces, pero los procesos físicos básicos, incluyendo la secreción biliar para la emulsificación y el transporte de los productos hidrolizados, son en general similares a los de mamíferos. La absorción de lípidos en peces ocurre predominantemente en la parte proximal del intestino coincidiendo con la mayor actividad lipolítica. Sin embargo, los lípidos se pueden absorber en menor cantidad a lo largo de toda la longitud del intestino. En las células de la mucosa intestinal, el principal destino de los FFA absorbidos es la reesterificación con glicerol, para resintetizarse en TAG y fosfoglicéridos (Sargent *et al.*, 1989). Los esteroles y las ceras se pueden regenerar en parte, aunque el colesterol libre se transporta fácilmente desde las células de la mucosa, y la gran mayoría de los alcoholes grasos se oxidan al correspondiente FA en el epitelio celular (Bauermeister y Sargent, 1979). La mayoría de los fosfoglicéridos se digieren y se absorben para a continuación reesterificarse antes de ser exportados desde las células intestinales.

En cuanto al **transporte** extracelular, los lípidos transportados en sangre surgen de tres fuentes: lípidos de la dieta, que son absorbidos por la mucosa intestinal y luego trasportados a la sangre como quilomicrones, lípidos endógenos que son procesados en el hígado y transportados a la sangre principalmente como lípidos de muy baja densidad VLDL (Goldstein *et al.* , 1983) y finalmente lípidos movilizados en forma de FFA (Figura 1.3).

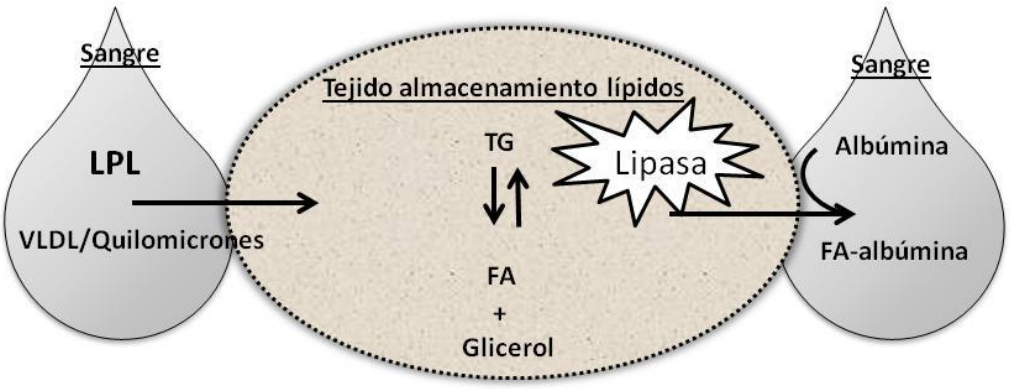

Fig. 1.3. Modelo de tejido de almacenamiento de lípidos y funcionamiento de la enzima lipasa (Modificado de Sheridan, 1994).

Los FA se transportan en la sangre ligados a la albúmina (Sheridan, 1988). La albúmina, por lo tanto, va a ser un factor limitante en la movilización de los lípidos. El siguiente paso, es la captación celular. La absorción de FA por las células involucra proteínas de membrana con una alta afinidad por los ácidos grasos. Entre estas proteínas de transporte de ácidos grasos nos encontramos con la proteína transportadora de ácidos grasos (FATP) y la ácido graso translocasa (FAT/CD36) que transportan los ácidos grasos al interior de la célula para ser metabolizados mediante la β-oxidación (Le Foll *et al.,* 2009).

El transporte intracelular de los FFA en mamíferos está facilitado por proteínas específicas con una alta afinidad por los ácidos grasos (FABPs). Las FABPs son específicas de cada tejido y han sido caracterizadas en hígado, intestino, tejido adiposo, cerebro y corazón de mamíferos (Veerkamp y Maatman, 1995) y en algunos tejidos de peces (Bass *et al.,* 1991; Baba *et al.,* 1999; Cordoba *et al.,* 1999). También se ha caracterizado ADNc de FABPs en corazón de trucha y en intestino, hígado y cerebro de pez cebra (*Danio rerio)* (Ando *et al*., 1998; Andre *et al*., 2000; Denovan-Wright *et al.,* 2000a,b).

En cuanto al **almacenamiento**, los lípidos se depositan fundamentalmente en el tejido adiposo, en el hígado y en el músculo (Sheridan y Kao, 1988) y la capacidad de almacenaje varía según la especie (Jobling *et al.*, 1991), la estación y el estadío de desarrollo del pez (Henderson y Tocher, 1987; Sheridan, 1988), aumentando en animales alimentados con dietas ricas en lípidos, con la edad y tamaño del animal (Company *et al.,* 1999; Corraze, 2001) .

La **lipogénesis** es el término usado para describir las reacciones biosintéticas para la formación de nuevos lípidos endógenos y así ser transformados en ácidos grasos y glicerol para cubrir las necesidades energéticas. La última fuente de carbón para la biosíntesis de nuevos lípidos es el acetil-CoA formado en la mitocondria debido a la descarboxilación oxidativa de piruvato o la degradación oxidativa de algún aminoácido. El paso clave en la lipogénesis está catalizado por el complejo citosólico multienzimático

ácido graso sintetasa (FAS) que está caracterizado en peces (Sargent *et al.,* 1989). El principal órgano lipogénico en peces es el hígado, aunque se sabe que en trucha arco iris el tejido adiposo tiene también capacidad lipogénica (Henderson y Sargent, 1981). La tasa de lipogénesis está regulada por el número de ingestas y por factores nutricionales, especialmente en peces de agua dulce. Los cambios en el contenido lipídico de la dieta en los vertebrados superiores inducen modificaciones sobre la lipogénesis hepática (Salati y Amir-Ahmady, 2001), el tranporte de lípidos en plasma (Fernández y West, 2005) y su captación en los tejidos (Raclot *et al.,* 1997). En peces, un incremento en el contenido de lípidos en la dieta y un descenso en el contenido de carbohidratos deprimen la actividad de varias enzimas lipogénicas en el salmón plateado (*Oncorhynchus kisutch*) (Lin *et al.,* 1977a,b), bagre del canal (*Ictalurus punctatus*) (Likimani y Wilson, 1982) y lubina (*Dicentrarchus labrax*) (Dias *et al.,*1988).

Los TAG que almacenan los peces se obtienen a partir de FFA por la acción de la lipoproteín lipasa (LPL) (Arnault *et al.*, 1996; Oku *et al;* 2002), cuya regulación es nutricional, de modo que su actividad y expresión disminuye durante el ayuno y se activa con la alimentación (Liang *et al.*, 2002; Albalat et *al.,* 2006). La **lipólisis** mediante la cual los TAG sufren una hidrólisis en FFA y glicerol mediada por la TAG-lipasa (Sheridan y Allen, 1984) depende también del estatus nutricional de animal, incrementándose la tasa lipolítica y el contenido plasmático en animales ayunados (Albalat *et al.,* 2005) y disminuyendo cuando se realimenta a los peces (Pottinger *et al.,* 2003). Este proceso metabólico puede verse alterado debido a la composición de la dieta, por ejemplo dietas ricas en proteínas vegetales incrementan los procesos lipolíticos y hacen caer los niveles de lípidos corporales totales (Albalat *et al.,* 2005).

El proceso de **β-oxidación** de los FA y la formación de los **cuerpos cetónicos** está establecida en peces. La oxidación de los FA en peces es una fuente importante de energía no sólo en hígado sino también en varios tejidos como en corazón, músculo rojo y músculo blanco (Froyland *et al*., 1998, 2000). Hay evidencias de que tanto los FA saturados como los insaturados se catabolizan en la β-oxidación mitocondrial en peces (Sargent *et al*., 1989). En teleósteos, tanto en hígado como en cerebro, se han caracterizado algunas actividades enzimáticas implicadas en el metabolismo de los cuerpos cetónicos, ácido acetoacético (acetoacetato) y el ácido betahidroxibutírico (β-hidroxibutirato), lo que sugiere que existe una implicación de éste en el metabolismo energético de dichos animales, especialmente durante el ayuno (Leblanc y Ballantyne, 2000; Soengas y Aldegunde, 2002). El proceso de β-oxidación de los FA no sólo ocurre a nivel mitocondrial, si no que los peroxisomas son otras localizaciones posibles de la β-oxidación. El proceso es similar en ambas con la salvedad de que en el peroxisoma no se genera energía en forma de ATP. En el músculo rojo de de salmón Atlántico (*Salmo salar*) se han observado altos niveles de β-oxidación peroxisomal (Froyland *et al*., 2000).

En mamíferos, el cerebro capta ácidos grasos y los metaboliza, como por ejemplo el octanoato (Hamprecht y Dringen, 1995). En peces, sólo algunos estudios en teleósteos, han abordado el papel de los PUFA en los tejidos neuronales. Pero se sabe que cerebros de muchas especies de teleósteos son particularmente ricos en n-3 PUFA, sobre todo en ácido docohexanoico (DHA) (Tocher y Mackinlay, 1990; Mourente y Tocher, 1992; Tocher *et al.,* 1996).

1.2 INGESTA Y REGULACIÓN DE ALIMENTO EN PECES

El concepto de nutrición animal abarca diferentes procesos como la ingesta de alimento, la digestión, la absorción y el metabolismo de nutrientes y finalmente la excreción de los productos de desecho. La cantidad de comida ingerida y la eficiencia por la cual ese alimento se convierte en último lugar en crecimiento son procesos que dependen de múltiples factores que afectan a la fisiología del animal (Conde-Sieira, 2012a,b).

La ingesta de alimento en vertebrados, entre ellos los peces, es un fenómeno complejo que implica diversos factores endocrinos centrales y periféricos. Las acciones de estos factores están moduladas por variables intrínsecas y extrínsecas, incluyendo entre otras a las reservas energéticas y el uso de energía metabólica (Tabla.1.1).

Tabla 1.1. Factores que afectan a la ingesta de alimento en peces (modificado de Volkoff *et al.,* 2009).

FACTORES QUE AFECTAN A LA INGESTA DE ALIMENTO EN PECES			
Estado del pez	**Ambiente (condiciones físico-químicas)**	**Características de la dieta (Presentación /Formulación)**	**Factores hormonales**
-Estado de salud -Fase de desarrollo -Fase de reproducción -Especie -Estrés	-Fotoperiodo -Intensidad de la luz -Depredadores -Influencia humana -Temperatura -Oxígeno -Turbidez -Contaminación -Iones/pH	-Frecuencia -Abundancia -Formulación -Presentación -Composición -Anteriores experiencias -Sistema de administración -Atractantes/Estimulantes alimenticios	-Ritmos biológicos -Neuropéptidos -Estado reproductivo

En peces, los lípidos son nutrientes principales para numerosos procesos fisiológicos (Sheridan, 1994; Tocher *et al.,* 2003; Polakof *et al.,* 2010a) por lo que es razonable que los niveles de lípidos estén relacionados con el control de ingesta. En este sentido se ha observado que en peces alimentados con una dieta con alto contenido en grasas se produce un descenso en la ingesta de alimento (Shearer *et al*., 1997; Rasmussen *et al*., 2000; Gélineau *et al*., 2001; Johansen *et al*., 2002; Luo *et al*., 2014). Esto es debido a que los peces, al igual que el resto de animales, se alimentan para satisfacer sus requerimientos energéticos y nutricionales inmediatos y para acumular cantidades suficientes de energía y nutrientes en los depósitos corporales para poder mantener así su homeostasis energética. Para explicar este fenómeno se propuso hace 60 años el modelo de modulación lipostática de la ingesta (Kennedy, 1953) mediante el cual existirían señales inhibitorias generadas en proporción a los contenidos grasos corporales que actuarían en el cerebro para reducir la ingesta de alimento. Más tarde se propuso (Gibbs *et al.,* 1973) que durante las comidas se generarían señales (denominadas "factores de saciedad") que incluirían péptidos secretados desde el tracto gastrointestinal (TGI), que aportarían información al sistema nervioso central a través de diversas vías de modo que se inhibiría la ingesta y se finalizaría el consumo de alimento (Schwartz *et al.,* 2000). Este modelo se enmarcaría en un modelo más amplio de modulación metabólica de la ingesta que ha sido desarrollado en mamíferos. En este modelo, la regulación metabólica de la ingesta en mamíferos incluye bucles de regulación negativa y positiva a través de mecanismos que actúan a diferentes tiempos y en diferentes lugares (Forbes, 1992, 1998; Langhans y Scharrer, 1992; Langhans, 1999). En general, el bucle positivo determina el inicio y continuación de la ingesta y es el resultado de la relación entre las propiedades sensitivas del alimento, la experiencia previa en relación a la disponibilidad de nutrientes, y el estatus fisiológico del animal. El bucle negativo incluye fases gastrointestinales y metabólicas o fases pre y post-absortivas (Langhans, 1999).

Por lo tanto, la regulación de la ingesta en vertebrados puede actuar a diferentes escalas de tiempo para determinar el tamaño de una comida, ajustar el balance de los nutrientes esenciales o mantener el equilibrio energético de todo el organismo. En mamíferos se conoce la existencia de factores que regulan estos procesos, tanto a corto como a largo plazo, creándose así un circuito de información modulado continuamente por la entrada de señales procedentes de los órganos de los sentidos y sensores internos (Carter *et al.,* 2001). Este circuito de regulación determina el comportamiento alimenticio y la ingesta. El proceso está coordinado por el sistema nervioso central mediante diversas rutas en las que el hipotálamo y el cerebro posterior son los principales centros de regulación.

En peces todavía es difícil establecer qué factores actúan a corto o largo plazo, aunque la información obtenida hasta el momento parece indicar que los mecanismos

básicos que llevan a cabo la regulación de la ingesta son similares a los de mamíferos a pesar de las claras diferencias entre ambos (Volkoff *et al.,* 2009).

1.2.1 Regulación a corto plazo

El inicio del proceso de ingesta y la duración de la misma, cuando un animal dispone de alimento, responde al resultado de la integración por parte del cerebro de señales procedentes tanto de estímulos externos (principalmente visuales y olfativos) como de estímulos internos (hormonas, metabolitos o factores nerviosos). El cerebro responde a toda esta información produciendo factores que o bien estimulan (factores orexigénicos) o inhiben (anorexigénicos) la ingesta de alimento (Volkoff *et al.* 2005). Estas señales efectoras del sistema nervioso central (SNC) serían las responsables de decidir no solo cuándo se inicia y termina una comida, sino que a medio plazo también determinarían el tipo de nutriente ingerido.

El sistema de control de apetito puede verse como una red de señales conceptualizada en tres dominios (Blundell y King, 1996). El primer nivel se caracteriza por eventos psicológicos (percepción del hambre, apetito) y relacionados con el comportamiento. El segundo nivel se corresponde con eventos fisiológicos y metabólicos y el tercer nivel se corresponde con las interacciones metabólicas y neurotransmisoras que ocurren en el cerebro. El apetito refleja el funcionamiento coordinado de los eventos y procesos que tienen lugar en los tres niveles (Fig.1.4).

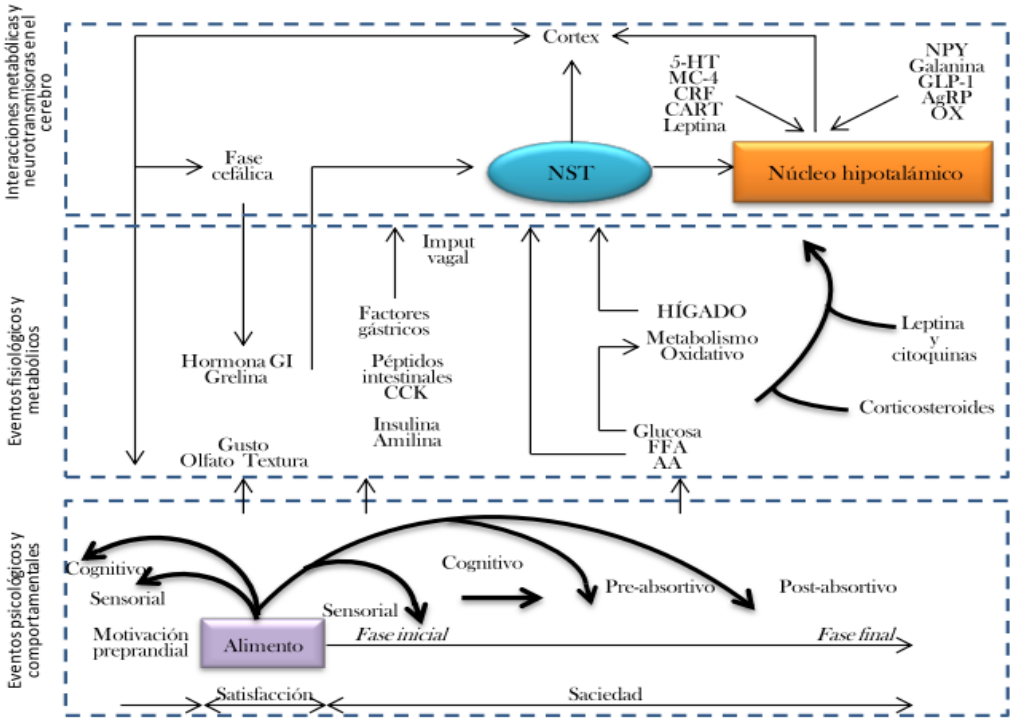

Fig.1.4. Representación de la red psicobiológica que regula el apetito conceptualizada en tres dominios distintos y coordinados (Modificado de Harrold *et al*., 2012).

1.2.2 Regulación a largo plazo

La regulación de la ingesta también va a responder a procesos de almacenamiento de energía y al estado energético del organismo. Los tejidos que actúan como almacén de energía envían señales "tónicas" que informan sobre el nivel de reservas energéticas induciendo al consumo de alimentos cuando las reservas energéticas son bajas.

Se sabe que en mamíferos existe una regulación a largo plazo del contenido lipídico corporal (teoría lipostática) que está mediado por la leptina y la insulina (Fig.1.5) que van a informar del contenido lipídico al organismo (Langhans *et al.,* 1999).

Se ha comprobado experimentalmente que las inyecciones centrales y periféricas de **leptina** reducen la ingesta de alimento y el peso corporal en mamíferos (Seeley *et al.,* 1996). Además, también se ha visto que en situación de ayuno se reducen los niveles de esta hormona en plasma, estómago y tejido adiposo y tras una sobrealimentación se eleva su concentración (Ahima y Osei. 2004). En peces, se ha visto que la leptina produce un efecto anoréctico similar al encontrado en mamíferos, tras su tratamiento intraperitoneal (IP) y/o intracerebroventricular (ICV) en carpín *Carassius auratus* (Volkoff *et al.*, 2003; De Pedro *et al.,* 2006) y en trucha arco iris (Murashita *et al.,* 2008; Kling *el al.,* 2009; Aguilar *et al.,* 2010).

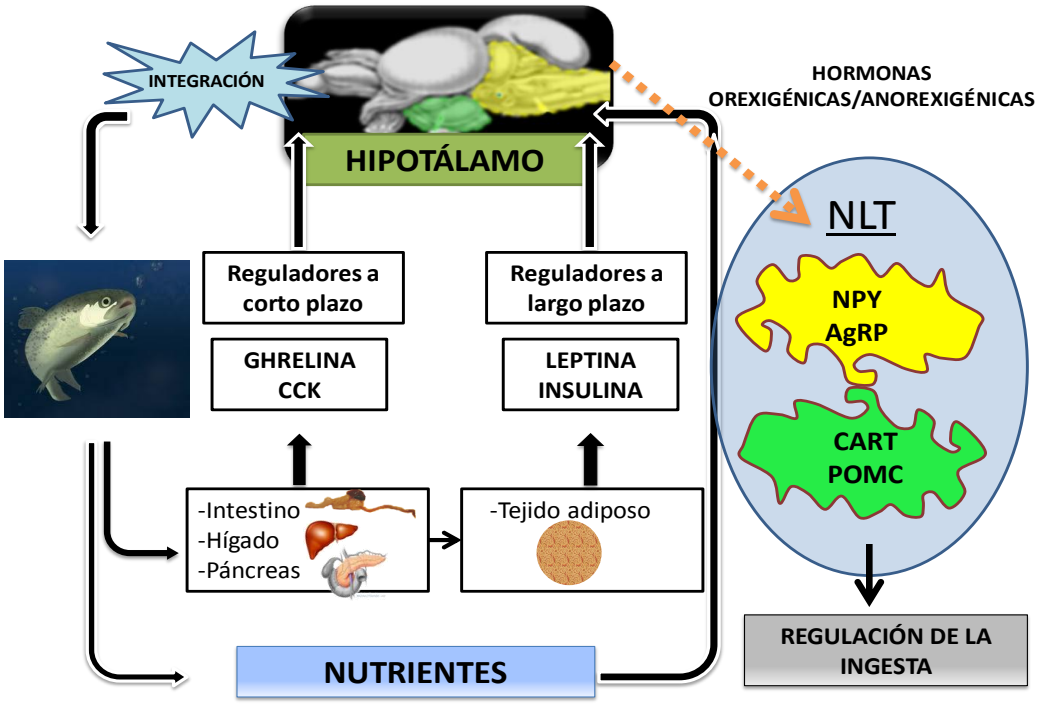

Fig. 1.5. Representación esquemática de la regulación de la ingesta de alimento a corto y largo plazo (Modificado de Konturek *et al.,* 2005).

En carpín se ha visto que la leptina puede inhibir los efectos potenciadores de la ingesta que ejercen factores orexigénicos como el neuropéptido Y (NPY) y la Orexina-A (OX-A) y potenciar las acciones de factores anorexigénicos como el tránscrito regulado por cocaína y anfetamina (CART) y la colecistoquinina (CCK) (Volkoff *et al.,* 2003). En cultivos *in vitro* de hipotálamo de trucha arco iris tratados con leptina, se produjo un descenso dosis-dependiente en los niveles de ARNm de NPY (Aguilar *et al.*, 2010, 2011). Todo esto sugiere que, al igual que en mamíferos, la leptina interactúa con otras señales del apetito y en este caso con vías hipotalámicas para producir un descenso en la ingesta de alimento. En perca, la leptina además estimula el metabolismo lipídico (Londraville y Duvall, 2002) y en carpín además de estimular el metabolismo lipídico, también produce cambios en el metabolismo glucídico (De Pedro *et al.,* 2006).

Otra hormona que regula la ingesta de alimento, el balance energético y la adiposidad corporal a largo plazo junto con la leptina es la **insulina**. En mamíferos se sabe que atraviesa la barrera hematoencefálica y una vez en el SNC se une a receptores situados en las mismas regiones hipotalámicas que los receptores de leptina. Sin embargo, aunque en la ingesta y metabolismo su efecto es similar, la insulina se secreta de forma aguda tras las comidas (Schwartz *et al*., 2000). También interactúa con otras señales del apetito como NPY, las melanocortinas o la CCK, y al igual que la leptina, induce un descenso en el consumo de alimento y, en peces, estimula la síntesis de glucógeno, la lipogénesis y la síntesis de proteínas (Nelson y Sheridan, 2006). En este sentido, los niveles de insulina en peces son más bajos después del ayuno que tras la alimentación (Navarro *et al.,* 2006; Monserrat *et al.,* 2007) y su administración inhibe la ingesta en trucha arco iris (Soengas y Aldegunde, 2004; Polakof *et al.,* 2008a).

1.3 FACTORES ENDOCRINOS CENTRALES Y PERIFÉRICOS REGULADORES DE LA INGESTA

El hipotálamo es el tejido que integra todas las señales que participan en el control de la alimentación procedentes de tejidos periféricos como el tracto gastrointestinal, el hígado, el tejido adiposo y el páncreas (Valassi *et al.,* 2008; Volkoff *et al.,* 2009). La ingesta de alimento está bajo el control de un sistema central constituido por los niveles de neuropéptidos y monoaminas combinado con un sistema periférico de saciedad que involucraría varios péptidos y hormonas gastrointestinales. Además, se sabe que gran parte de los péptidos que participan en el control de la ingesta tienen un efecto inhibidor de la misma, mientras que solo unos pocos la estimulan (De Pedro y Björnsson, 2001). (Tabla 1.2).

Tabla 1.2. Principales péptidos, hormonas y monoaminas involucrados en la regulación de la ingesta en peces tanto a nivel central como periférico.

FACTORES ENDOCRINOS CENTRALES Y PERIFÉRICOS REGULADORES DE LA INGESTA			
ESTIMULADORES DEL APETITO		**INHIBIDORES DEL APETITO**	
Centrales	**Periféricos**	**Centrales**	**Periféricos**
NPY	Ghrelina	CRF	Leptina
AgRP		CART	Insulina
Galanina		Melanocortinas	CCK/gastrina
Orexinas		Monoaminas	BBS/GRP
MCH			Melatonina
Apelina			Amilina

1.3.1 Sistema de regulación central

En peces no hay muchos trabajos sobre el control neuronal de la alimentación, casi todos están basados en estimulaciones eléctricas, lesiones en determinadas áreas cerebrales o el corte de vías neuronales. Estos estudios parecen indicar que el hipotálamo está involucrado en el control de la ingesta de alimento al igual que en mamíferos (Demski, 1973; Lin *et al.,* 2000). Estudios llevados a cabo en peces teleósteos y elasmobranquios demuestran que la estimulación eléctrica de los lóbulos inferiores del hipotálamo (Demski, 1973; Roberts y Savage, 1978) provoca una respuesta en la ingesta, concretamente en telencéfalo ventral, núcleos gustativos secundarios y lóbulos ópticos (Peter, 1979) induciendo una estimulación del comportamiento alimenticio (Demski y Knigge, 1971), mientras que la lesión del tracto olfatorio provoca un descenso de la ingesta de alimento (Demski y Knigge, 1971; Stacey y Kyle, 1983). Esto demostraría que, al igual que en mamíferos, existe un centro dual para la regulación de la alimentación en el hipotálamo, funcionando el hipotálamo lateral (LH) como centro de alimentación y el núcleo ventromedial (VMN) como centro de saciedad (Anand y Brobeck, 1951). El hipotálamo en peces también tiene un papel fundamental en el control integrado del centro de alimentación, en el cual se producen señales orexigénicas y anorexigénicas mediante la síntesis de diferentes péptidos y monoaminas en función de la información que reciben de la periferia.

1.3.1.1. *Factores estimuladores del apetito (orexigénicos) involucrados en la regulación central*

Los péptidos reguladores de la ingesta que se han identificado en peces mediante procesos de aislamiento, secuenciación y clonación son homólogos a los de mamíferos. Existen diversos estudios de los efectos de estos péptidos en la ingesta y el metabolismo en peces que han sido evaluados mediante administración por vía periférica (IP), por vía central (ICV), oral, mediante implantes (Silverstein *et al.,* 2001; Volkoff *et al.,* 2003; Johansson *et al,* 2005; Bernier, 2006), o indirectamente mediante la cuantificación de los niveles hormonales en sangre, o niveles de expresión del ARNm o proteína en peces sometidos a diferentes condiciones como ayuno o estrés.

El **neuropéptido Y** está presente en todos los peces estudiados (Kehoe y Volkoff, 2007; Liang *et al.,* 2007). En mamíferos, está presente en el núcleo arcuato y paraventricular del hipotálamo, que son áreas claves en la regulación de la ingesta (Halford *et al.,* 2004), siendo uno de los agentes orexigénicos más potente y que además interactúa con otras señales orexigénicas y anorexigénicas del hipotálamo. En peces, las neuronas productoras de NPY están distribuidas en el SNC en dipnoos, elasmobranquios y teleósteos (Sundstrom *et al.,* 2005; Kehoe y Volkoff, 2007). Varios estudios vinculan al NPY con la regulación de la alimentación en teleósteos. Inyecciones ICV de NPY en carpín, salmón plateado, o bagre del canal, provocan un incremento dosis-dependiente de la ingesta (López-Patiño *et al.,* 1999; Silverstein y Plisetskaya, 2000; De Pedro *et al.,* 2000; Narnaware *et al.,* 2000). Además tanto en mamíferos como en peces, la acción de NPY puede deberse al menos en parte a la modulación de otros reguladores del apetito como el factor liberador de corticotropina (CRF) y cortisol (Bernier *et al.,* 2004) , CART (Volkoff y Peter, 2000), leptina (Volkoff *et al.,* 2003), hormona concentradora de melanina (MCH) (Matsuda *et al.,* 2007), orexina y galanina (Volkoff y Peter, 2000), hormona del crecimiento (GH) (Mazumdar *et al.,* 2007) y ghrelina (Miura *et al.,* 2006).

La **proteína relacionada con agouti (AgRP)** es otro péptido clave en la regulación de la ingesta en mamíferos. En peces, se ha identificado AgRP en carpín (Cerdá-Reverter y Peter, 2003a), en pez cebra (Song *et al.,* 2003) y en pez globo (*Takifugu rubripes)* (Klovins *et al.,* 2004). En carpín y de pez cebra se ha visto que el ayuno regula los niveles de ARNm de AgRP en el hipotálamo (Cerdá-Reverter y Peter, 2003; Song *et al.,* 2003), por lo que AgRP podría tener un papel en la regulación de la ingesta de alimento en peces (Volkoff *et al.,* 2005).

La **galanina** se ha descrito en varias especies de peces, tanto en tejidos centrales (Adrio *et al.,* 2005; Volkoff *et al.,* 2005) como periféricos (Johnsson *et al.,* 2001; Unniappan *et al.,* 2002). Se comporta como un agente estimulador de la ingesta tras la administración a nivel central en el carpín dorado y la tenca, mientras que la administración por vía periférica no produce efectos en la alimentación de estas especies

(De Pedro *et al.,* 1995; Volkoff y Peter, 2001a). Además interactúa de forma sinérgica con otros estimuladores del apetito como la OX-A y NPY (Volkoff y Peter, 2001a).

Las **orexinas (OXs)**, son dos péptidos: OX-A y orexina B (OX-B). En mamíferos hay estudios que sugieren que el sistema de las orexinas puede constituir un mecanismo de iniciación y finalización de las comidas para modular la homeostasis de la glucosa (Harrold *et al.,* 2012). En peces, la administración central de OXs estimula el apetito en carpín (Nakamachi *et al.,* 2006). Además en carpín y pez cebra (*Danio rerio)* durante el ayuno se incrementan los niveles de pre-pro-orexinas en el cerebro (Novak *et al.,* 2005; Nakamachi *et al*., 2006). También los niveles de expresión de ARNm de pre-pro-orexina se incrementaron en peces alimentados con pequeñas cantidades de comida (Xu y Volkoff, 2007) y en pez cebra y en carpín se comprobó una regulación de los estados de vigilia de estos peces mediante la inyección de orexinas por lo que están involucradas indirectamente en su ingesta y metabolismo (Volkoff *et al.,* 2009).

MCH está presente en el hipotálamo lateral y caudal, así como en la hipófisis de los peces (Huesa *et al.,* 2005). No está muy claro su efecto en la ingesta de alimento ya que en carpín tras su inyección no hubo cambios importantes en su alimentación (Cerdá-Reverter y Peter, 2003), sin embargo, en platija (*Platichthys flesus*) en condiciones de ayuno se produjo un aumento de la expresión hipotalámica de MCH (Takahashi *et al.,* 2004).

La **apelina** es un péptido cuya función en la ingesta de alimento no está muy clara. La administración a nivel central de apelina-13 (que es uno de sus metabolitos activos) en rata produce una inhibición de la ingesta (Sunter *et al.,* 2003; Clarke *et al.,* 2009). Lo mismo ocurre si se inyecta durante la noche a nivel central otro de sus metabolitos activos, la apelina-12, sin embargo, si se administra durante el día hay una estimulación de la ingesta (O´Shea *et al.,* 2003). En peces, la apelina se produce en varios tejidos incluyendo cerebro y tejido adiposo. Existen estudios en carpín mediante los cuales se ha observado un incremento de la ingesta tras la administración ICV e IP de apelina siendo además los niveles de ARNm en hipotálamo y telencéfalo de ésta más altos en peces ayunados que en peces alimentados (Volkoff y Wyatt, 2009). También se ha observado una estimulación de la ingesta de alimento en pez de cueva (*Astyanax mexicanus)* tras la administración a nivel periférico de apelina (Penney y Volkoff, 2014).

1.3.1.2. *Factores inhibidores del apetito (anorexigénicos) involucrados en la regulación central*

El **CRF** en mamíferos tiene un efecto anorexigénico muy potente (Heinrichs, 1999). Es la hormona hipotalámica primaria que estimula la liberación de la hormona adrenocorticotropina (ACTH) hipofisaria, que a su vez, estimula la secreción de cortisol por el tejido interrenal (Flik *et al.,* 2006). Como función secundaria, el CRF se encarga de la regulación de la ingesta y el balance energético (Richard *et al.,* 2002; Bernier, 2006). Aunque inyecciones a nivel periférico de CRF en carpín no producen cambios en la ingesta de alimento (De Pedro *et al.,* 1993) sí que lo hacen inyecciones ICV suprimiendo la ingesta tanto en carpín como en tenca (De Pedro *et al.,* 1995; Bernier y Peter 2001a). También se ha observado que CRF produce cambios en la expresión de neuropéptidos como NPY y CART en hipotálamo de trucha arco iris a través de la alteración del patrón de respuesta ante cambios en los niveles de glucosa por lo que la inhibición de la ingesta en condiciones de estrés puede estar mediada por la acción de CRF en las áreas glucosensoras (Conde-Sieira, 2011).

CART es un neuropéptido anoréctico que ha sido descrito en carpín (Volkoff y Peter, 2001a) y bacalao (Kehoe y Volkoff, 2007). En carpín la inyección ICV de CART produce una disminución de la ingesta de alimento. El ayuno produce un descenso en los niveles de ARNm de CRF en varias regiones cerebrales de carpín, bagre de canal y en bacalao (Volkoff y Peter 2001b; Kehoe y Volkoff, 2007; Kobayashi *et al.,* 2008). CART actúa de forma sinérgica con la leptina, mientras antagoniza la estimulación de la ingesta por parte de NPY y de la OX-A (Volkoff y Peter, 2000).

Las **melanocortinas** son un grupo de hormonas hipofisarias que derivan de la pro-opiomelanocortina (POMC) (Metz *et al.,* 2006). Entre ellas se encuentran la ACTH y la hormona estimuladora de los melanocitos (MSH). En mamíferos se sabe que las melanocortinas están involucradas en el control de la ingesta y de la homeostásis energética (Cone, 2006) y que interactúan con otros sistemas. NPY inhibe la expresión de POMC (Rahmouni *et al.,* 2001) y la leptina hace que se incremente su expresión en el núcleo arcuato de mamíferos (Gorissen *et al.,* 2006). En peces, la administración ICV de agonistas o antagonistas del receptor de melanocortinas 4 (MC4R) en carpines ayunados durante 24 horas resulta en una inhibición o estimulación respectivamente de la ingesta (Cerdá-Reverter *et al.,* 2003b; Kobayashi *et al.,* 2008).

Los **neurotransmisores monoaminérgicos** como la noradrelina (NA), la dopamina (DA) y la serotonina (5-HT) interactúan con los neuropéptidos y hormonas involucrados en el control de ingesta de alimento. La administración de NA tanto en mamíferos como en peces estimula la ingesta de alimento a través de receptores α2-adrenérgicos, mientras que la estimulación de los receptores α1 en mamíferos y en peces y los β2 y β3 solo en mamíferos, produce una disminución de la ingesta (De Pedro *et al.,* 1995, 1998a;

Ramos *et al*., 2005). En cuanto a la DA, tratamientos con agonistas de receptores D1 y D2 produjeron un descenso de la ingesta en peces (De Pedro *et al.,* 1998a). En trucha arco iris, la administración de fenfluramina, un fármaco que induce niveles extracelulares elevados de 5HT, produce una inhibición de la ingesta (Ruibal *et al.,* 2002). En carpín la acción central de la serotonina está mediada por el CRF (De Pedro *et al*., 1998b).

1.3.2 Sistema de regulación periférico

Al sistema de regulación central de la alimentación llegan señales de saciedad procedentes del tracto gastrointestinal, que en presencia de comida estimula la liberación de péptidos reguladores que están involucrados en el control de la motilidad y secreción intestinal, causando el cese de la ingesta de alimento. Cuando el contenido de nutrientes en el intestino es elevado, éste proporciona señales al estómago reduciéndose así la tasa de llenado gástrico y prolongando la distensión gástrica postprandial (Morley, 1987).

1.3.2.1. *Factores estimuladores del apetito (orexigénicos) involucrados en la regulación periférica*

La **ghrelina** es un péptido secretado principalmente en el estómago pero también en el cerebro. Es la única hormona gastrointestinal con propiedades orexigénicas confirmadas (Olszewski *et al.,* 2008). Tanto en mamíferos como en peces hay una mayor expresión de ARNm de ghrelina en el estómago que en el cerebro (Kaiya *et al.,* 2008). En mamíferos, la administración periférica de ghrelina induce sensación de hambre y potencia la ingesta de alimento (Wren *et al*., 2001) y su administración central la aumenta tanto como el NPY (Tschop *et al.,* 2000). En peces, en carpín, tilapia y trucha arco iris la administración de ghrelina IP e ICV produce un incremento en la ingesta (Unniappan *et al.*, 2002, 2004; Riley *et al.,* 2005; Matsuda *et al.,* 2006; Miura *et al.*, 2006, 2007). Además, en carpín se ha observado que la ghrelina interactúa con otros factores reguladores de la ingesta de alimento como el NPY y las orexinas lo que sugiere que el efecto de esta hormona está mediado por una vía dependiente del NPY y las OXs (Miura *et al*., 2006, 2007).

1.3.2.2. *Factores inhibidores del apetito (anorexigénicos) involucrados en la regulación periférica*

La **CCK y la gastrina** son péptidos gastrointestinales que poseen una secuencia tertrapeptídica C-terminal. La CCK en mamíferos está presente en el cerebro y en el tracto gastrointestinal, mientras que la gastrina solo se produce en las células gástricas endocrinas. La CCK disminuye el vaciado gástrico, estimula las secreciones gástricas y reduce la ingesta de alimento actuando de forma periférica por vía vagal aunque su unión a receptores del cerebro también provoca saciedad (Raybould, 2007). En peces, la CCK inhibe la ingesta en carpín y bagre de canal tras la administración a nivel central y periférico y el tratamiento con sus antagonistas produce un aumento en la ingesta de alimento en trucha arco iris (Gelineau y Boujard, 2001). Diversos péptidos relacionados con la CCK se liberan cuando hay comida en el intestino, inhibiéndose así el vaciado intestinal e incrementándose la motilidad intestinal (Forgan y Foster, 2007). La liberación de CCK está afectada por la composición de la dieta en trucha arco iris (Jonsson *et al.,* 2006). Todavía se desconoce el papel de la gastrina en la regulación de la ingesta en peces, pero se sabe que influye en la motilidad intestinal (Forgen y Foster, 2007).

La **bombesina (BBS)** y el **péptido liberador de gastrina (GRP)** en mamíferos están distribuidos en el TGI y SNC (McCoy y Avery, 1990). Cuando se administran IP o ICV péptidos relacionados con BBS, se produce una potente inhibición de la ingesta (Lin *et al.,* 2000). En peces, estos péptidos están distribuidos en cerebro, TGI y sistema cardiovascular (Xu y Volkoff, 2009). Existen estudios que muestran que tras la administración i.p. o i.c.v. de BBS se produce una inhibición de la ingesta en carpa (*Cyprinus carpio)* (Himick y Peter, 1994). La expresión del ARNm de GRP en intestino de bacalao es más alta en peces sobrealimentados que en los que recibieron poca cantidad de alimento (Xu y Volkoff, 2009).

La **melatonina** es una hormona que se sintetiza principalmente en la glándula pineal en respuesta a una ritmicidad circadiana (Falcón *et al.*, 2010). Tanto en mamíferos como en peces, la melatonina se sintetiza también en el TGI dependiendo de la presencia de alimento en el intestino (Bubenik y Pang, 1997). La administración IP de melatonina produce una inhibición de la ingesta de alimento en carpin (Pinillos *et al.,* 2001; De Pedro *et al.,* 2008) y lo mismo ocurre con su administración oral en la tenca *(Tinca tinca*) (López-Olmeda *et al.,* 2006) y la lubina (López-Olmeda *et al.,* 2009). Sin embargo, este efecto anorexigénico no se observa cuando se administra i.c.v. (Pinillos *et al.,* 2001) por lo que parte de sus acciones podrían ser periféricas. Tanto en regiones centrales como periféricas la hormona produce cambios en los niveles de parámetros relacionados con el metabolismo de la glucosa (Soengas *et al.,* 1998; De Pedro *et al.,* 2008). En hipotálamo de trucha arco iris, el tratamiento *in vitro* con melatonina aumenta la expresión de péptidos orexigénicos (NPY) y reduce la de anorexigénicos (CART, CRF, POMC), lo que discrepa con

el papel anorexigénico descrito para la melatonina en peces teleósteos, por lo que su efecto anoréctico podría darse a través de la interacción con tejidos periféricos y formaría parte de una compleja red de regulación del apetito (Conde-Sieira *et al.,* 2012a).

La **amilina** es un péptido segregado en mamíferos en las células β-pancreáticas a la vez que secreta la insulina después de la comida. Para ello, se une a receptores específicos en el SNC produciéndose una disminución en la ingesta de alimento (Lutz, 2006). En carpín, tras la administración de amilina vía IP y vía ICV se produce una inhibición de la ingesta (Thavanathan y Volkoff, 2006).

1.3.3 Integración a nivel hipotalámico de las señales implicadas en la ingesta

Como se ha descrito antes, diversos tejidos periféricos como el tracto gastrointestinal, el tejido adiposo, el hígado y el páncreas participan en el control de la alimentación, aunque el hipotálamo es el encargado de integrar todas estas señales junto con otras regiones como puede ser el tronco cerebral (Valassi *et al.,* 2008; Volkoff *et al.,* 2009).

El hipotálamo es un área especializada del cerebro que anatómicamente está definida por diversos grupos o núcleos neuronales. Entre otras funciones, participa en la regulación de la ingesta de alimento y dicha función está presente en todos los vertebrados incluidos los peces (Volkoff *et al.,* 2009).

En el hipotálamo se pueden diferenciar varias regiones interconectadas entre sí mediante vías neuronales y que son las encargadas de regular el apetito y la ingesta. En la regulación de la ingesta el sistema nervioso central no actúa solo, sino que interactúa con el TGI (López *et al.,* 2007). El TGI degrada y transforma la comida ingerida mediante procesos mecánicos y químicos, lo que estimula la síntesis por parte de células endocrinas del TGI de hormonas gastrointestinales que informan sobre la situación nutritiva y metabólica en la que se encuentra el individuo (López *et al.,* 2007).

Todo este proceso está acompañado de una serie de señales nerviosas originadas en sensores gastrointestinales y enviadas periféricamente hasta el sistema nervioso central, donde se integran a nivel del hipotálamo para el control de la ingesta (Solomon y Martínez, 2006). El núcleo arcuato (ARC) del hipotálamo cuyo equivalente en peces es el NLT, que se encuentra en la base del tercer ventrículo, juega un papel importante en la regulación de la ingesta, puesto que presenta diversas neuronas con receptores específicos para una gran variedad de señales (López *et al*., 2007). El ARC recibe múltiples señales endocrinas periféricas, siendo la ghrelina, CCK, leptina e insulina las más importantes al aportar información sobre el estado nutricional y metabólico del individuo (López *et al*., 2007) (Figura 1.6). Desde aquí se proyectan fibras nerviosas que conectan con las demás regiones, liberando neuropéptidos orexigénicos (NPY, AgRP) o

anorexigénicos (CART, POMC) que producen señales hacia otras neuronas de orden superior situadas en el núcleo paraventricular (Pvn), núcleo dorsomedial (Dmh), el área lateral (Lha) y el hipotálamo ventromedial (Vmh), donde se expresan otros neuropéptidos involucrados en el gasto energético y a su vez conectados mediante vías neuronales implicadas en el control de ingesta y apetito (Gao y Lane., 2003).

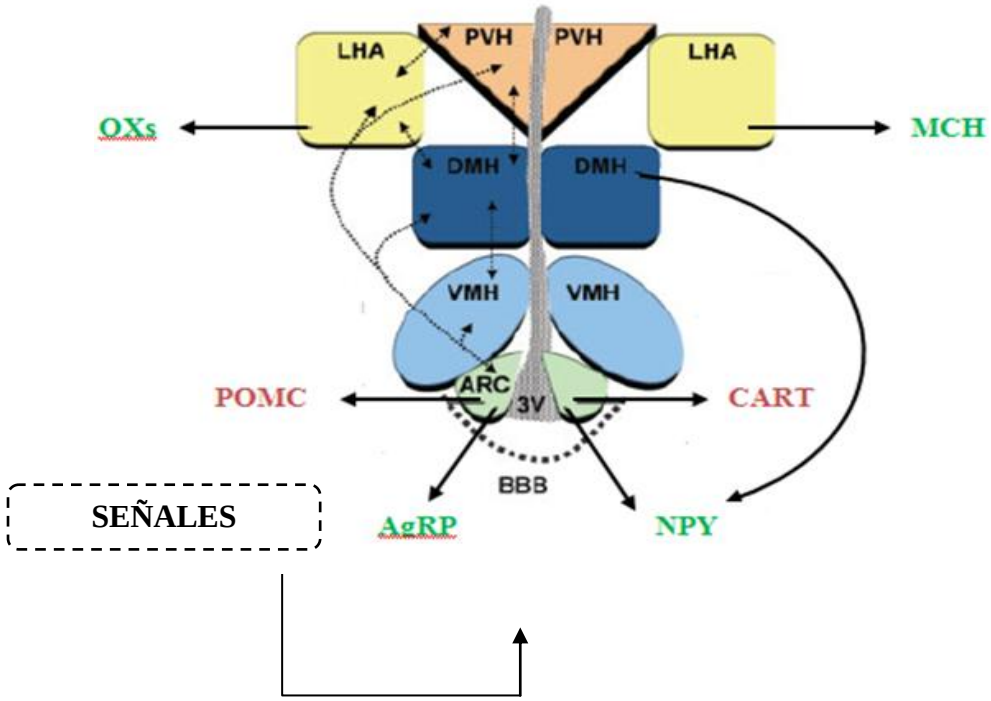

Figura 1.6. Esquema de los diferentes núcleos que conforman el hipotálamo y las conexiones existentes entre ellos (flechas). Los neuropéptidos orexigénicos se muestran en color verde y los anorexigénicos en color rojo (Modificado de López *et al.,* 2007).

El hipotálamo integra esas señales periféricas produciendo neuropéptidos que pueden estimular o inhibir la sensación de hambre (orexigénicos o anorexigénicos respectivamente), siendo el Lha (región orexigénica), coloquialmente conocida como el "centro del hambre", la encargada de la regulación de la ingesta (Solomon y Martínez, 2006).

Aunque la información sobre la regulación de la ingesta en otros organismos diferentes de mamíferos es escasa, en la figura 1.7 se representa de forma esquemática la regulación integral de los factores que afectan a la ingesta de alimento en peces, cuyos mecanismos parecen ser básicamente similares (Gorissen *et al.,* 2006), aunque existen algunas diferencias relativas a los núcleos hipotalámicos implicados.

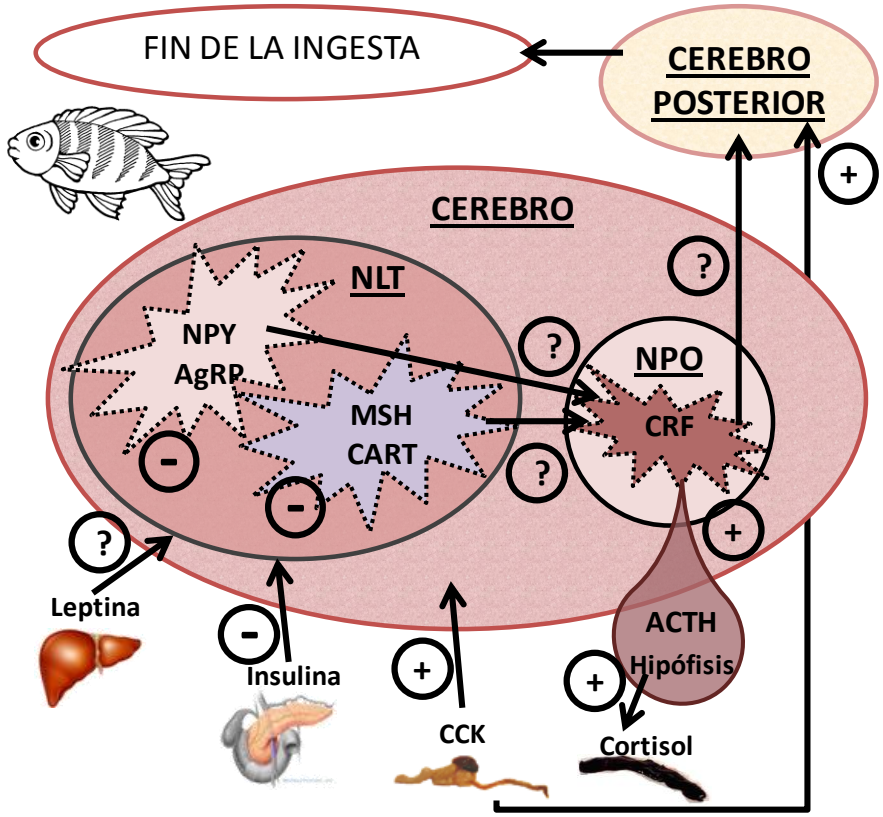

Figura 1.7. Esquema de la regulación de la ingesta de alimento en peces mediante señales periféricas y centrales, orexigénicas (+) o anorexigénicas (-). (?) indica interacciones todavía desconocidas en peces. El NLT en peces es el equivalente al ARC en mamíferos, mientras que el NPO corresponde al PVN. (Modificada de Gorissen *et al*., 2006).

1.3.4 Regulación metabólica de la ingesta de alimento

Aunque existen numerosas señales periféricas que pueden estar implicadas en el comportamiento alimenticio, es importante reconocer que el balance energético y la ingesta a corto y largo plazo están regulados a través de mecanismos muy diferentes que interactúan entre sí por medio de vías neuronales o humorales. Algunas señales como los nutrientes (carbohidratos, lípidos y aminoácidos) y las hormonas gastrointestinales, actúan como determinantes de la saciedad para regular la ingesta de alimento (De la Higuera, 2001; Carter *et al.,* 2001). Por una parte, tras la ingesta, los nutrientes procedentes de la dieta que se encuentran en el estómago y en la zona proximal del intestino activan quimiorreceptores que producen señales aferentes que viajan por las fibras del nervio vago provocando la inhibición de la ingesta. Además, los nutrientes que llegan por la vena portal también pueden desencadenar señales vagales aferentes desde el hígado. De este modo, los niveles de glucosa, lípidos y aminoácidos pueden modular la ingesta de alimento limitando la cantidad de comida ingerida en una toma (Fig. 1.8.A).

En mamíferos se ha descrito la existencia de mecanismos sensores que informan de la disponibilidad de nutrientes como la glucosa y ácidos grasos, que le permiten al cerebro modular la adquisición y el gasto de energía y las funciones metabólicas periféricas como un medio para controlar la homeostasis energética general (Blouet y Schwartz, 2010). El principal centro de convergencia e integración de las múltiples señales nutricionales parece ser el hipotálamo. La detección hipotalámica de nutrientes, de forma directa o indirecta mediante información aferente, activa neurocircuitos involucrados en la regulación de la ingesta de alimento, la homeostasis de la glucosa, el metabolismo del tejido adiposo y el gasto energético (Morton *et al.,* 2006). A su vez, las neuronas del complejo dorsal-vagal integran la información que les llega desde el cerebro anterior relacionada con los nutrientes y la adiposidad con las señales intestinales de saciedad para así regular el comportamiento alimenticio (Morton *et al*., 2006). Además, las proyecciones hipotalámicas que terminan en el complejo dorsal-vagal también provocan un flujo de información eferente a varios efectores del balance energético como el hígado, el páncreas y el tejido adiposo (Fig.1.8.B).

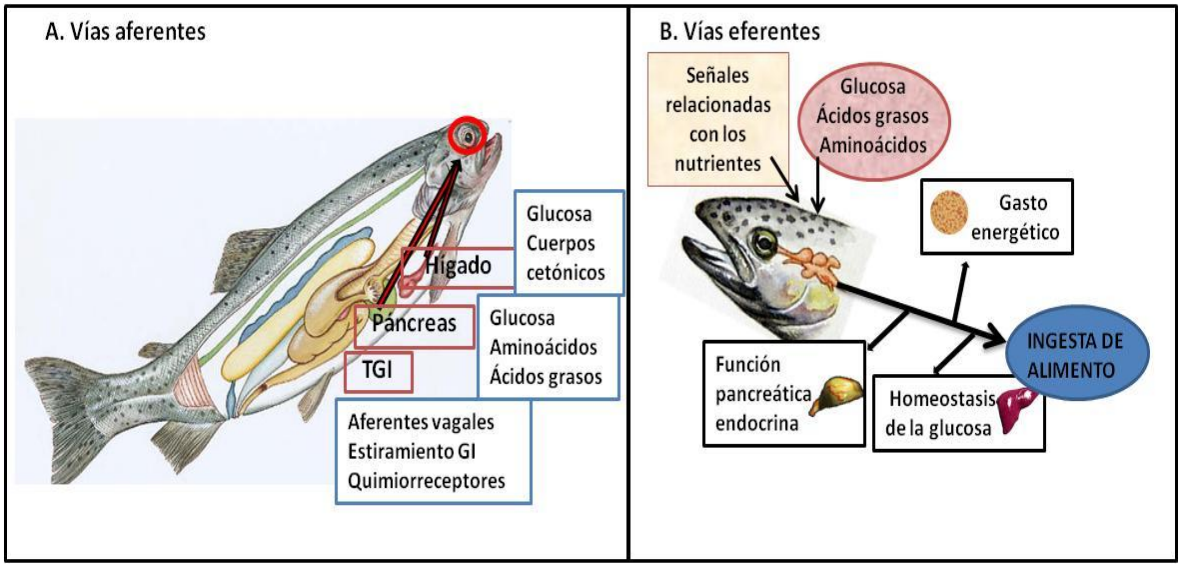

Figura 1.8. A: Señales periféricas aferentes (hormonales y nutricionales) que regulan la ingesta a corto plazo (Adaptada de Havel, 2001). **B**: Efectores metabólicos y comportamentales del balance energético regulados por la detección de nutrientes en el hipotálamo (Adaptada de Blouet y Schwartz, 2010).

Entre los mecanismos mediante los cuales se podría producir la regulación de la ingesta a este nivel, destacan los circuitos de neuronas del sistema de las melanocortinas ya que existen datos que revelan que estas neuronas pueden detectar cambios en la disponibilidad de nutrientes (Song *et al.,* 2001; Yh *et al*., 2009). Estos circuitos incluyen neuronas que expresan péptidos orexigénicos (NPY y AgRP) y otras anorexigénicas que

expresan POMC y CART y que se encuentran en las proximidades del 3er ventrículo y la eminencia media, áreas hipotalámicas que presentan una relativa porosidad en la barrera hematoencefálica, quedando accesibles para los nutrientes y las hormonas circulantes (Blouet y Schwartz, 2010). Así, las neuronas anorexigénicas POMC/CART se despolarizan, mientras que las orexigénicas NPY/AgRP se hiperpolarizan en respuesta al incremento en el nivel de nutrientes (Fioramonti *et al.,* 2007).

Las respuestas de la ingesta basadas en cambios en los niveles circulantes de determinados metabolitos parecen actuar también a medio plazo. Existen experimentos con alimentos de contenido extremo en proteínas y aminoácidos en los que los peces parecen detectar el desequilibrio en la dieta y regulan su ingesta en función del mismo (Rodehutscord *et al*., 1995). Incluso cuando se les ofrece a los peces diferentes alimentos a elegir, como ocurre en otros animales, tienden a ajustar la ingesta de los diferentes componentes para conseguir equilibrar su contenido final (Cuenca *et al.*, 1993). Así, aunque es difícil probar que los animales seleccionan la dieta para cubrir sus necesidades nutricionales cuando se les da a elegir entre varios tipos de alimentos (Forbes, 1988), parece claro que en muchas situaciones los peces presentan evidencias de cierto comportamiento de selección de nutrientes (De la Higuera, 2001). Cuando los animales se alimentan con dietas deficientes en algún nutriente específico, puede ocurrir un incremento en la ingesta para alcanzar el nivel de requerimiento absoluto, si la deficiencia es leve, o una inhibición de la respuesta cuando la deficiencia es grave, ya que una reducción de la ingesta a tiempo puede prevenir la aparición de desórdenes metabólicos (De la Higuera, 2001).

En trucha y anguila (*Anguilla anguilla*) alimentadas con dietas isocalóricas pero basadas en proteínas de origen vegetal la ingesta disminuye, posiblemente debido a la deficiencia en ciertos aminoácidos, ya que la ingesta se recupera tras añadir esos aminoácidos a la dieta (García-Gallego *et al.,* 1998; De la Higuera *et al.,* 1999), por lo que el nivel y fuente de proteína en la dieta afecta a la ingesta de forma inversamente proporcional al contenido de energía, digestibilidad y metabolización (Morales *et al.,* 1994). Con respecto a los lípidos en la dieta, se ha encontrado una relación negativa con la ingesta de alimento, como lo demuestran estudios con varias especies de teleósteos (Rasmussen *et al.*, 2000). Los carbohidratos se utilizan con menor eficiencia y el nivel máximo permisible en la dieta difiere entre las distintas especies de peces (Wilson, 1994). Teniendo en cuenta su baja proporción relativa en la dieta, los carbohidratos deberían interactuar débilmente con la regulación de la ingesta de alimento en peces. Sin embargo, varios estudios describen una disminución en la ingesta de alimento tras alimentar con dietas ricas en carbohidratos a especies de peces como la lubina (Peres *et al.,* 2002), trucha arco iris (Kaushik *et al.,* 1989; Polakof *et al*., 2008c), salmón atlántico (Hemre *et al*., 1995) y pez gato (*Clarias batrachus)* (Erfanullah, 1998). Ello avala la existencia de un mecanismo regulador de los niveles de glucosa sobre la ingesta de alimento.

Aparte del contenido nutritivo de la dieta, la energía que ésta contiene es un factor fundamental que interviene en la ingesta de los peces. Los resultados procedentes de estudios que abordaron este control de la alimentación a partir de la energía indican que en varias especies de peces (trucha, lubina y carpín dorado) se incrementa la ingesta cuando la dieta incorpora elementos inertes que disminuyen su contenido energético (Rozin y Mayer, 1961; Grove *et al*., 1978; Dias *et al.,* 1998). Se ha descrito que salmónidos alimentados con dietas isoproteicas de diferente contenido energético, consumían más cantidad de la dieta menos energética pero siempre alcanzando sus requerimientos mínimos (Boujard y Médale, 1994). La ingesta de energía con el fin de alcanzar ese estatus energético determinado, puede estar influenciada también por el contenido de reservas energéticas del pez (Liebelt *et al.,* 1965).

En cuanto a los mecanismos sensores de nutrientes, se conocen bastante bien en mamíferos los implicados en proporcionar información sobre cambios en los niveles circulantes de glucosa (glucosensores) y ácidos grasos (sensores de ácidos grasos). En peces teleósteos también existen evidencias de la presencia de sistemas sensores a nivel central y periférico. En trucha arco iris el sistema glucosensor está relativamente bien caracterizado, así como sus implicaciones en el control de la ingesta de alimento (Polakof *et al.,* 2011, 2012). Sin embargo, en peces no existen hasta la fecha estudios sobre la existencia y funcionamiento de sistemas sensores tanto de ácidos grasos como de aminoácidos.

El sistema glucosensor en mamíferos (Levin *et al.,* 1999) y en peces (Polakof *et al.*, 2011b) tiene implicaciones directas sobre el control de ingesta, siendo la glucosa una señal reguladora importante que controla la secreción de hormonas y activa neuronas en el sistema nervioso central y periférico (Levin *et al.,* 1999). Debido a que existen tejidos como el sistema nervioso central que reciben su energía metabólica casi exclusivamente de la glucosa (Marty *et al.,*2007; Polakof *et al.,* 2011b), es necesario que sus niveles no disminuyan significativamente por lo que necesitan de sistemas ubicados a nivel central que informen sobre las variaciones en los niveles circulantes de glucosa (Marty *et al.,* 2007; Polakof *et al.,* 2011b). Además, existen sistemas glucosensores en otros órganos y tejidos que se encargan de monitorizar continuamente los niveles de glucosa en sangre. Estos sensores responden a los cambios en los niveles de glucosa generando la producción de hormonas o activando el sistema nervioso autónomo, para así regular los niveles de ingesta y el gasto energético (Marty *et al*., 2007; Polakof *et al.*, 2011b).

En mamíferos se conoce relativamente bien el funcionamiento de los mecanismos glucosensores periféricos en las células β-pancreáticas, que relacionan las variaciones en los niveles circulantes de glucosa con la secreción de insulina (Schwartz *et al.,* 2000). En este mecanismo juega un papel fundamental la enzima glucokinasa (GK) que incrementa su actividad de forma directamente proporcional al aumento de los niveles de glucosa en plasma (Lynch *et al.,* 2000). También se ha demostrado la existencia de glucosensores

basados en la GK en el hígado, el cual responde a los cambios en la concentración de glucosa de forma compleja pudiendo alternar entre su utilización (mediante la glucólisis y síntesis de glucógeno) o su producción (mediante la glucogenolisis y la gluconeogénesis), jugando un papel muy importante en la homeostasis glucídica del organismo (Lynch *et al.,* 2000).

El cerebro depende directamente de la concentración de glucosa para satisfacer su demanda de energía, lo que sugiere que es muy importante la existencia de mecanismos glucosensores en el sistema nervioso central, como el hipotálamo, el cual presenta células específicas que se pueden estimular o inhibir en presencia de glucosa (Dunn-Meynell *et al.,* 2002). Esas regiones cerebrales implicadas en el control de la homeostasis y de la ingesta de alimento son capaces de recibir señales de los niveles de nutrientes y hormonas en sangre, así como de los sistemas sensoriales central periférico usando esta información para generar las respuestas fisiológicas apropiadas (Polakof *et al.,* 2011b).

Los mecanismos glucosensores conocidos hasta la fecha se pueden agrupar en cuatro categorías: 1) Mecanismo metabólico, dependiente de la enzima glucokinasa, el transportador GLUT2 y el canal de potasio dependiente de ATP (K^+_{ATP}); 2) Receptores gustativos que dependen de T1R2+T1R3 y α-gustducina; 3) La expresión del transportador de glucosa SGLT-1 y 4) La expresión del receptor LXR. Se han encontrado evidencias de la existencia de estos mecanismos en distintos tejidos de mamíferos como distintas áreas cerebrales, el hígado o el TGI (Schwartz *et al*., 2000; Lynch *et al.,* 2000). En peces, se ha demostrado la presencia de mecanismos glucosensores dependientes de GK en áreas centrales (hipotálamo y cerebro posterior) y periféricas (hígado y cuerpos de Brockmann) (Polakof *et al.,* 2011b), así como su implicación y participación en distintos procesos, incluyendo el control de la ingesta. Asimismo, se han obtenido evidencias preliminares sobre la posible presencia de los otros mecanismos glucosensores (Soengas, 2014).

Además de la glucosa, se ha demostrado en mamíferos la existencia de varios mecanismos sensores de ácidos grasos, fundamentalmente a nivel de hipotálamo. El principal mecanismo es el ligado al metabolismo de ácidos grasos, de modo que el incremento de los niveles circulantes de ácidos grasos en plasma induce un aumento de los niveles de malonil CoA en hipotálamo (López *et al.,* 2005, 2007). Asimismo, se han demostrado mecanismos sensores alternativos como el transporte de ácidos grasos a través del transportador de ácidos grasos FAT/CD36 (Le Foll *et al.,* 2009), la activación de nuevas isoformas de PKC (Blouet *et al.,* 2010) y la producción mitocondrial de especies reactivas de oxígeno (ROS) por intercambio de electrones que acaban inhibiendo la actividad del canal de potasio dependiente de ATP (K^+_{ATP}) (Blouet y Schwartz, 2010). Los cambios en la actividad de estos sistemas se han relacionado con la regulación de la ingesta a través de cambios en la expresión de factores orexigénicos y anorexigénicos como NPY, AgRP, POMC y CART (López *et al.,* 2005, 2007).

En peces, hasta la fecha, no existen estudios que demuestren la existencia de alguno de estos mecanismos sensores de lípidos.

1.4 SISTEMAS SENSORES DE ÁCIDOS GRASOS Y SU IMPLICACIÓN EN EL CONTROL DE LA INGESTA

En mamíferos, se ha demostrado la existencia de neuronas especializadas en hipotálamo, y probablemente en cerebro posterior que detectan cambios en los niveles circulantes de ácidos grasos de cadena larga (LCFA), pero no de cadena corta (SCFA) o cadena media (MCFA) contribuyendo al control nervioso de la homeostasis energética (Gao *et al.,* 2003; Migrenne *et al*., 2007). Como se ha descrito en el apartado anterior, existen varios mecanismos sensores de ácidos grasos fundamentalmente a nivel hipotalámico (López *et al.,* 2005, 2007). La figura 1.9 representa de forma esquemática el funcionamiento de los diferentes mecanismos sensores de ácidos grasos en mamíferos.

1.4.1 Mecanismos sensores de ácidos grasos en mamíferos

El principal mecanismo mediante el cual los LCFAs son detectados en el cerebro es de naturaleza metabólica. Un incremento en los niveles de FA en plasma induce un aumento de los niveles de malonil CoA en hipotálamo, lo que desencadena la inhibición de la enzima carnitina palmitoiltransferasa 1 (CPT-1), encargada de transferir los ácidos grasos al interior de la mitocondria para su oxidación (López *et al.,* 2005, 2007).

En el mecanismo dependiente de FAT/CD36, un incremento en los niveles de FA resulta en un mayor transporte mediante el transportador FAT/CD36 y la subsiguiente modulación de factores de transcripción como el receptor activado de proliferación de los peroxisomas de tipo α (PPARα) y la proteína tipo 1c reguladora del elemento de unión de esteroles (SREBP1c) (Le Foll *et al.,* 2009).

En el tercer mecanismo conocido, el incremento en la concentración de ácidos grasos induce una translocación y subsiguiente activación de la protein quinasa C-δ (PKC-δ) resultando en una inhibición de PI3K proporcional al incremento en los niveles de ácidos grasos (Benoit *et al.,* 2009; Blouet y Schwartz, 2010).

Finalmente, el incremento en los niveles circulantes de ácidos grasos estimula la producción mitocondrial de especies reactivas de oxígeno por intercambio de electrones que acaban inhibiendo la actividad del canal de potasio dependiente de ATP (K^+_{ATP}) (Blouet y Schwartz, 2010).

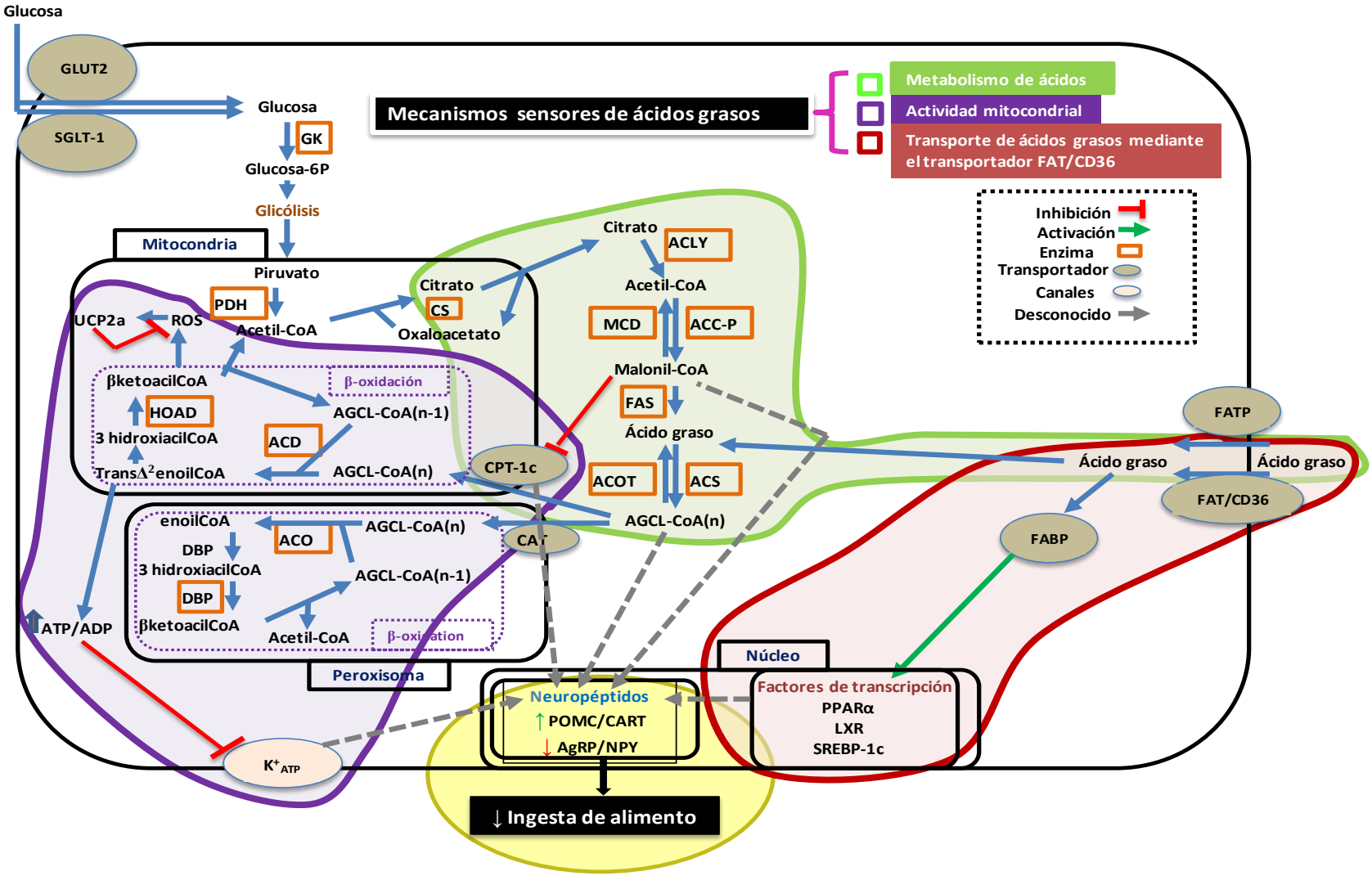

Figura 1.9. Esquema de los mecanismos sensores de ácidos grasos en mamíferos y su funcionamiento.

Como resultado de la acción de estos mecanismos se produce una regulación de la ingesta a través de cambios en la expresión de factores orexigénicos y anorexigénicos (López *et al.,* 2005, 2007). Así pues, un incremento en los niveles de LCFA en mamíferos desencadena una inhibición en la expresión de AgRP y de NPY, y un aumento en la expresión de POMC y CART lo que acaba resultando en la reducción de la ingesta de alimento (López *et al.,* 2005).

La relación entre el metabolismo de los ácidos grasos y el control de la ingesta de alimento se ha demostrado mediante el efecto anoréctico de inhibidores de FAS, como el 4-metileno-2-octil-5-oxotetrahidrofurano-3-ácido carboxílico (C75), cuyos efectos se revierten por la presencia de inhibidores de la acetil-CoA carboxilasa (ACC) como el *ácido* 5-(*tetradeciloxi*)-*2-furoico* (TOFA) (Loftus *et al.,* 2000; Gao y Lane, 2003; Hu *et al.,* 2011).

El descenso en los niveles circulantes de ácidos grasos produce una respuesta sensora a nivel hipotalámico que desencadena una respuesta a nivel periférico para restaurar los niveles circulantes de ácidos grasos. Los estudios realizados al respecto son escasos pero sugieren la activación de diferentes hormonas de naturaleza lipolítica entre las que destacan los glucocorticoides producidos en el tejido adrenal como resultado de la activación del eje hipotalámico-hipofisario-adrenal (HPA) (Oh *et al.,* 2012, 2014).

Además, se sabe que la detección a nivel central de glucosa y FA están relacionadas a través de una vía vagal y simpática para la regulación homeostática de la glucosa afectando a la liberación de insulina en páncreas y la producción endógena de glucosa en hígado (Blouet y Schwartz, 2010).

1.4.2 Sistema sensor de ácidos grasos en peces

En los peces teleósteos, el metabolismo energético es diferente al de los mamíferos, ya que estos últimos utilizan la glucosa como su mayor fuente de energía para desarrollar sus actividades fisiológicas y la mayoría de los peces son relativamente intolerantes a ella, dependiendo en mayor medida del metabolismo de aminoácidos y lípidos (ver reviews: Polakof *et al.,* 2011b, 2012). Los peces utilizan los lípidos como una fuente importante de energía para llevar a cabo procesos fisiológicos y reproductivos (Sheridan, 1994; Tocher *et al*., 2003; Polakof *et al*., 2010a).

El hígado en peces juega un papel central en el metabolismo de los ácidos grasos, y las interacciones entre el metabolismo de la glucosa y de los lípidos están bien caracterizadas en este tejido (Panserat *et al.,* 2002; Figuereido-Silva *et al.,* 2012b). Además, las actividades y expresión de enzimas lipogénicas hepáticas como la ácido graso

sintetasa FAS y sus auxiliares para ésta como la glucosa-6-fosfato–deshidrogenasa (G6PDH) y la enzima málica (ME) no resultan modificadas por el reemplazamiento total o parcial de lípidos animales por vegetales en la dieta del salmón atlántico (Tortensen *et al*., 2004), rodaballo (*Scopthalmus máxima)* (Raclot *et al*., 1997) y trucha arco iris (Richard *et al.,* 2006). Tampoco el metabolismo intermediario hepático se ve modificado por la retirada de los lípidos de la dieta (Ducasse-Cabanot *et al.,* 2007), si bien manipulaciones de la misma han demostrado que la lipogénesis en los peces está regulada del mismo modo que en mamíferos: un incremento en el contenido de lípidos en la dieta y un descenso concominante en el contenido de carbohidratos deprimen la actividad de varias enzimas lipogénicas en el salmón plateado, (Lin *et al.,* 1977a,b), bagre del canal (Likimani y Wilson, 1982) y lubina (Dias *et al.,* 1998). Sin embargo, la lipogénesis parece estar regulada con menos rigor en los peces que en los mamíferos, dado que para deprimir este proceso en los primeros se necesita una cantidad triple de lípidos en la dieta (Henderson y Sargent, 1981) que en los segundos (Chillard, 1993).

Algunas hormonas como la insulina están fuertemente ligadas al metabolismo de los lípidos en peces (Caruso y Sheridan, 2011). Tras un incremento en los niveles circulantes de ácidos grasos se produce un aumento en la liberación de insulina (Barma *et al.*,2006). En hígado de trucha arco iris el tratamiento con esta hormona incrementa la expresión de ARNm de FAS e inhibe la expresión de CPT1, enzimas implicadas en la síntesis de lípidos y la oxidación respectivamente (Plagnes-Juan *et al.,* 2008).

Debido a que los lípidos son una fuente importante de energía para los peces, no es sorprendente que estén relacionados con el control de la ingesta de alimento. En este sentido, se ha demostrado en varias especies de peces que una alimentación basada en dietas con altos contenidos en lípidos provoca una reducción de la ingesta de alimento (Shearer *et al.,* 1997; Rasmussen *et al.,* 2000; Gélineau *et al.,* 2001; Johansen *et al.,* 2002) lo que sugiere que existe un sistema sensor de lípidos que regula la ingesta de alimento en peces. Sin embargo, el mecanismo a través del cual los lípidos inhiben la ingesta de alimento y las vías implicadas en una posible regulación de la homeostasis energética no ha sido estudiado. Además, tampoco existen estudios que evalúen cual es el efecto en particular de los FA sobre la ingesta de alimento y el metabolismo de FA en tejidos periféricos como el hígado y el páncreas endocrino.

Hasta la fecha, en peces solo se han evaluado los efectos de un aporte metabólico lipídico en tejidos periféricos, como hígado y músculo (Boujard *et al*., 1993; Shearer *et al.,* 1997; Boujard *et al*., 2004; Torstensen *et al.*, 2009; Sánchez-Gurmaches *et al.*, 2010; Figuereido-Silva *et al.,* 2012a,b, 2013; Martinez-Rubio *et al.,* 2013) pero nunca han sido evaluados en cerebro.

Asimismo, se ha caracterizado hasta la fecha en peces la presencia a nivel central y periférico de componentes de mecanismos sensores de ácidos grasos que poseen los mamíferos. Así, se ha demostrado la presencia de los factores de transcripción SREBP-1c y PPARα que juegan un papel muy importante en la homeostásis lipídica y de los ácidos grasos (Chinetti *et al.,* 2000; Horton *et al*., 2002; Ibabe *et al.*, 2002; Minghetti *et al*., 2011; Cunha *et al.*, 2013). También LXR que es otro factor de transcripción existente en mamíferos, ha sido caracterizado en salmónidos (Cruz-Garcia *et al.*, 2009) y su función en la regulación de transcripción ha sido descrita en miocitos y adipocitos de trucha arco iris (Cruz-Garcia *et al.,* 2011). Asimismo, la enzima CPT-1, encargada de transferir los ácidos grasos al interior de la mitocondria está presente en hígado y músculo cardiaco y esquelético de peces y su expresión génica se ha descrito en trucha arco iris (Gutieres *et al.,* 2003). También se sabe que componentes de mecanismos sensores del metabolismo de los FA están presentes en hígado (Kolditz *et al.,* 2008; Plagnes-Juan *et al.,* 2008; Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Polakof *et al.,* 2010a) y páncreas (Polakof *et al.,* 2012a) de la misma especie. Sin embargo, hasta la fecha no se ha caracterizado la participación de estos componentes en mecanismos sensores funcionales y su posible respuesta frente a cambios en los niveles circulantes de FA.

Teniendo en cuenta que en peces al cambiar el contenido lipídico de la dieta se produce una regulación de la ingesta de alimento, que las áreas donde se localizan los sistemas glucosensores que responden a una serie de hormonas y metabolitos incluidos los ácidos grasos (Levin *et al.,* 2004) son las mismas que en mamíferos y que componentes de los mecanismos sensores de lípidos existentes en mamíferos han sido descritos también en peces, podemos hipotetizar que existan mecanismos de detección de lípidos en esas mismas áreas.

2. OBJETIVOS

OBJETIVOS

La regulación de la ingesta de alimento es un proceso complejo que involucra numerosas interacciones entre el cerebro y señales periféricas. En peces, esta regulación parece verse afectada tras una alimentación basada en dietas con altos contenidos en lípidos. En mamíferos, se ha demostrado la existencia de neuronas especializadas en hipotálamo, y probablemente en cerebro posterior, que detectan cambios en los niveles circulantes de ácidos grasos de cadena larga (LCFA), pero no de cadena corta (SCFA) o cadena media (MCFA). Además en los últimos años, en trucha arco iris, se ha caracterizado la existencia de mecanismos glucosensores centrales (hipotálamo y cerebro posterior) y periféricos (hígado y cuerpos de Brockmann), implicados en el control de la ingesta de alimento y liberación de hormonas. Teniendo en cuenta que en mamíferos las áreas donde se localizan los sistemas glucosensores son las mismas que responden a una serie de hormonas y metabolitos incluidos los ácidos grasos, es posible que existan mecanismos de detección de lípidos en esas mismas áreas, que no han sido evaluados hasta la fecha en peces.

En base a lo mencionado anteriormente, en la presente Tesis doctoral se ha determinado como objetivo general la caracterización, usando como modelo de pez teleósteo la trucha arco iris, de sistemas sensores de ácidos grasos en tejidos claves para el control de la homeostasis de los lípidos y la ingesta de alimento como son el hipotálamo, cerebro posterior, hígado y cuerpos de Brockmann (BB). Partiendo de este objetivo general, se han planteado una serie de objetivos específicos:

1. Evaluar la presencia de componentes de mecanismos sensores de ácidos grasos en cerebro y BB, y su respuesta tanto a nivel metabólico como en la regulación de la ingesta ante un incremento de los niveles circulantes de ácidos grasos mediante la administración intraperitoneal de un ácido graso de cadena larga como el oleato y otro de cadena media como el octanoato. Con este objetivo se ha desarrollado el trabajo experimental Nº1: **"A metabolic fatty acid sensing mechanism is present in hypothalamus and Brockmann bodies of rainbow trout".**

2. Evaluar la respuesta del metabolismo hepático de la glucosa y de los lípidos tras el tratamiento oral con una mezcla de aceites de pescado o tras la administración intraperitoneal con ácidos grasos como oleato y octanoato. Este objetivo ha sido desarrollado en el trabajo experimental Nº2: **"Effects of a mixture or single fatty acids treatment on the response of hepatic lipid and glucose metabolism. Possible presence of fatty acid-sensing systems"**.

3. Evaluar *in vitro* (en ausencia de influencias externas) si el hipotálamo y los BB responden o no a cambios en la concentración de FA de forma similar a lo previamente observado *in vivo* en el trabajo experimental Nº1. Asimismo, se estimará el efecto en hipotálamo del oleato y octanoato del tratamiento *in vitro* en presencia de inhibidores relativos a componentes del sistema sensor de FA. Para dilucidarlo se ha desarrollado el trabajo experimental Nº3: **"*In vitro* evidences of direct fatty acid-sensing capacity in hypothalamus and Brockmann bodies of rainbow trout"**.

4. Determinar si el hígado responde tras su incubación *in vitro* con diferentes concentraciones de oleato y octanoato de forma similar a la previamente observada *in vivo* en el trabajo experimental Nº2. Este objetivo se aborda en el trabajo experimental Nº4: **"Response of hepatic fatty acid-sensing systems in rainbow trout to *in vitro* oleate or octanoate treatment"**.

5. Evaluar los efectos del tratamiento intracerebroventricular con oleato y octanoato en la regulación de la ingesta de alimento y en parámetros relativos al sistema sensor de ácidos grasos en hipotálamo. Así se determinará la posible existencia de un mecanismo directo de detección de FA en dicho tejido. Los resultados obtenidos se presentan en el trabajo experimental Nº5: **"Effects of oleate or octanoate intracerebroventricular treatment on food intake and hypothalamic fatty acid systems in rainbow trout".**

6. Evaluar los efectos del tratamiento intracerebroventricular con oleato y octanoato en parámetros relativos al sistema sensor de FA en tejidos periféricos como hígado y BB. Así, se comprobará si la respuesta es similar a la previamente observada tras el tratamiento intraperitoneal en los trabajos Nº1 y Nº2 y tras el tratamiento *in vitro* en los trabajos Nº3 y Nº4 o si la respuesta es diferente, posiblemente debido a la interacción con otros sistemas endocrinos. Este objetivo se ha abordado en el trabajo experimental Nº6: **"Fatty acid sensing systems are affected by central oleate or octanoate administration in liver and Brockmann bodies of rainbow trout".**

7. Determinar si el descenso en los niveles de ácidos grasos circulantes inducido farmacológicamente produce una inhibición en los sistemas sensores de ácidos grasos a nivel hipotalámico y ello se refleja en una activación de la ingesta. Si la respuesta que se observa es producto de dicha caída, debería ser contrarrestada si además del tratamiento con el fármaco se trata a los animales con un preparado (intralipid) que produce un incremento en los niveles de ácidos grasos circulantes. Asimismo, se evaluará la posible participación del eje hipotálamo-

hipófisis-interrenal (HPI). Los resultados obtenidos se presentan en el trabajo experimental Nº7: **"Hypothalamic response to decreased levels of fatty acid in rainbow trout. Possible involvement of the hypothalamus-pituitary-interrenal axis".**

8. Evaluar la repuesta contra-reguladora periférica (en hígado y BB) ante la reducción inducida farmacológicamente de los niveles circulantes de FA. Asimismo, se evaluará la posible participación del eje HPI. Este ha sido el propósito del trabajo experimental Nº8: **"Brockmann bodies and liver metabolic response to decreased circulating fatty acid levels in rainbow trout; involvement of the hypothalamus-pituitary-interrenal (HPI) axis".**

9. Determinar si la respuesta de los sistemas sensores de FA en hipotálamo, BB e hígado y de la ingesta de alimento frente al incremento en los niveles oleato y octanoato se modifica en presencia de una hormona como es la insulina. Este objetivo ha sido considerado para la realización del trabajo Nº9: **"Insulin modulation of fatty acid sensing in rainbow trout".**

10. Determinar si la respuesta de los sistemas sensores de ácidos grasos y el mecanismo de regulación de la ingesta responden a cambios en los niveles de ácidos grasos circulantes inducidos mediante la alimentación con dietas con un contenido diferente en FA. Además se pretende determinar si el estado de fosforilación de sensores energéticos intracelulares (AMPK), y proteínas implicadas en la señalización intracelular (Akt, mTOR) muestran cambios en hipotálamo e hígado que puedan estar relacionados con los cambios en el mecanismo sensor de FA y en el control de la ingesta de alimento. Este objetivo se ha llevado a cabo en el trabajo experimental Nº10: **"Effects on fatty acid sensing, food intake regulation and cellular signaling pathways in hypothalamus and liver of rainbow trout fed with a lipid-enriched diet."**

3. TRABAJOS EXPERIMENTALES

3.1 TRABAJO EXPERIMENTAL Nº1

A metabolic fatty acid sensing mechanism is present in hypothalamus and Brockmann bodies of rainbow trout

INTRODUCTION

In mammals, detection of substrates such as glucose and fatty acids (FA) allows the brain to modulate energy intake and expenditure and peripheral metabolic functions as a means of controlling overall energy homeostasis. Evidence in mammals demonstrate that specialized neurons within the hypothalamus (and possibly brainstem) are able to detect changes in plasma levels of long-chain fatty acid (LCFA), but not short- (SCFA) or medium-chain FA (MCFA), thus contributing to nervous control of energy homeostasis (Migrenne *et al.,* 2007). The most accepted mechanism through which LCFAs are sensed in brain is of metabolic nature. Thus, increased FA levels in plasma induced enhanced malonyl-CoA levels and subsequent inhibition of carnitine palmitoyltransferase 1 (CPT-1) to import FA-CoA into the mitochondria for oxidation (López *et al.,* 2005, 2007). However, FA metabolism only accounts for part of fatty acid sensing in hypothalamus, since alternative mechanisms have been suggested to be present, such as 1) transport through cluster of differentiation 36 (fatty acid transporter, FAT/CD36) (Le Foll *et al.,* 2009), 2) FA-induced activation of novel protein kinase C (PKC) isoforms (Blouet and Schwartz, 2010), 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 (Blouet and Schwartz, 2010). Changes in the activity of those systems in mammalian hypothalamus have been associated, through not completely understood mechanisms (López *et al.,* 2005, 2007), with the regulation of food intake through changes in the expression of orexigenic and anorexigenic factors. Thus, enhanced levels of LCFA in mammals resulted in an inhibition of agouti-related protein (AgRP) and neuropeptide Y (NPY) expression, and an enhancement of pro-opio melanocortin (POMC) and cocaine and amphetamine related transcript (CART) expression with a concomitant reduction in food intake (López *et al.,* 2005). The relationship between metabolic fatty acid sensing and control of food intake has been also demonstrated by the anorectic effect of fatty acid synthase (FAS) inhibitors such as 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) whose effects are reverted by the presence of acetyl-CoA carboxylase (ACC) inhibitors like 5-(tetradecyloxy)-2-furoic acid (TOFA) (Loftus *et al.,* 2000; Gao and Lane, 2003; Hu *et al*., 2011). In addition to feeding, central glucose and FA detection has been related, through vagal and sympathetic outflow, to the regulation of glucose homeostasis by affecting insulin release in pancreas and endogenous glucose production in liver (Blouet and Schwartz, 2010).

There are no studies available in vertebrates other than mammals, as far as we are aware, regarding the presence of fatty acid sensing mechanisms and their possible relationship with the control of food intake. Fish energy metabolism is rather different than that of mammals since most fish are relatively intolerant to glucose and therefore they rely more on amino acid and lipid metabolism (Polakof *et al.,* 2011b). Fish use lipids

as a major energy source supporting numerous physiological, developmental, and reproductive events (Sheridan, 1994; Tocher *et al.,* 2003; Polakof *et al*., 2010a). Fish liver plays a central role in FA metabolism, and interactions between glucose and lipid metabolism are well characterized in this tissue (Panserat *et al*., 2002; Figueiredo-Silva *et al.,* 2012b). Furthermore, a reduced food intake has been observed in several fish species fed with high fat diets or containing high fat stores (Shearer *et al.,* 1997; Rasmussen *et al.,* 2000; Gélineau *et al.,*2001; Johansen *et al.,* 2002) suggesting that lipid sensor mechanisms regulating food intake may be present in fish. However, there are no studies in which the effect of particular FA on food intake were evaluated. The mechanism through which lipids inhibit food intake in fish has been never evaluated so far, and the pathways involved in a possible regulation of energy homeostasis also remain unresolved. Actually, evidence of some of the actors involved in lipid-sensing are scarce and limited to the rainbow trout, including the presence of FAS, ATP-citrate lyase (ACLY) and CPT-1 in Brockmann bodies (Polakof *et al.,* 2012a) and FAS in the hypothalamus (Polakof *et al.,* 2008b) at the molecular level.

In rainbow trout we previously demonstrated (see Polakof *et al.,* 2011b) for a review) the existence of glucosensors either at central (hypothalamus and hindbrain) or peripheral (Brockmann bodies) locations involved in the control of food intake and hormone release. Considering that in mammals glucosensor areas are full metabolic sensors responding to a host of metabolites and hormones (Levin *et al.*, 2004) we hypothesize the presence of lipid sensing mechanisms in the same areas. Therefore, using as a teleost fish model the rainbow trout we aimed to evaluate the presence of putative components of fatty acid sensing mechanisms (as described in mammalian models) and their response to enhanced levels of circulating FA. Accordingly, we evaluated in putative metabolic sensor areas changes in parameters related to: 1) fatty acid metabolism such as fatty acid, triglyceride and malonyl-CoA levels, ACLY, hydroxyacil-CoA dehydrogenase (HOAD) and FAS activities, and mRNA abundance of ACLY, FAS, CPT1, citrate synthase (CS), and acetyl-CoA oxidase (ACO), 2) transport of FA, such as mRNA abundance of FAT and fatty acid transport protein 1 (FATP1), 3) nuclear receptors and transcription factors involved in lipid metabolism, such as mRNA abundance of peroxisome proliferator-activated receptor type α (PPARα), liver X receptor (LXR), and sterol regulatory element-binding protein type 1c (SREBP1c), 4) components of the K_{ATP} channel, such as mRNA abundance of inward rectifier K^+ channel pore type 6.-like (Kir6.x-like) and sulfonylurea receptor-like (SUR-like), and 5) neuropeptides related to the metabolic control of food intake, such as mRNA abundance of NPY, POMC, and CART after IP administration of increasing doses of an LCFA like oleate or an MCFA like octanoate.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish weight was 101 ± 3 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 1201/2005) for the use of animals in research.

Experimental design

Following 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks. Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. Fish were lightly anaesthetized with MS-222 (50 $mg \cdot l^{-1}$) buffered to pH 7.4 with sodium bicarbonate, and weighed. Then, 12 fish per group received intraperitoneally (IP) 10 $ml \cdot kg^{-1}$ injection of saline solution alone (control) or containing oleate (60 or 300 $\mu g \cdot kg^{-1}$) or octanoate (60 or 300 $\mu g \cdot kg^{-1}$). To safely deliver FA they were solubilized in 45% hydroxypropyl-β-cyclodextrin (HBP) to a final concentration of 17 mM (Morgan *et al*., 2004). The HPB-fatty acid solution was diluted in saline to the appropriate concentration used for each injection. HPB alone at a similar concentration as in the fatty acid studies was used in all vehicle control studies (no effects of the vehicle alone were noticed for any of the parameters assessed, data not shown). Blood, hypothalamus, hindbrain and Brockmann bodies samples were taken 6h after treatment. Concentrations of FA were selected based on studies carried out previously in mammals (Lam *et al.,* 2005; Pocai *et al*., 2006; Caspi *et al*., 2007) whereas 6h was chosen since in previous studies in the same species that was the time period necessary to achieve changes in the sensing mechanisms for another closely related nutrient such as glucose (Polakof *et al.,* 2007a,b, 2008a,b,c). In each group, 8 fish were used to assess enzyme activities and metabolite levels whereas the remaining 4 fish were used for the assessment of mRNA levels by qRT-PCR. In each sampling fish were anesthesized as above and blood was taken from the caudal vein with a heparinised syringe. Fish were then sacrificed by decapitation and hypothalamus, hindbrain and Brockmann bodies were taken and stored as previously described (Polakof *et al.,* 2007a).

A second set of fish was used to evaluate changes in food intake after IP administration of FA. Fish were randomly assigned to experimental groups in different tanks and fasted for 24 h before injection. Then, 8 fish per group received IP 10 ml·kg^{-1} injection of saline solution alone (control) or containing oleate (60 or 300 µg·kg^{-1}) or octanoate (60 or 300 µg·kg^{-1}) previously solubilised in HBP as described above. Food intake was registered for 3 days before treatment (to define basal line data) and then 6 and 24 h after IP treatment. On each time, food was supplied in batches of approx. 10g every 2 min until satiation. After feeding, 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 previously described as the difference from the feed offered (De Pedro *et al.,* 1998; Polakof *et al.,* 2008a,b). Food intake values registered after treatment are referred to those of basal values, and results are shown as the mean ± SEM of the data obtained in three different tanks per treatment.

A third set of fish was used to evaluate changes in food intake after IP administration of a FAS inhibitor (C75) or a ACC inhibitor (TOFA). Fish were randomly assigned to experimental groups in different tanks and fasted for 24 h before injection. Then, 8 fish per group received IP 10 ml·kg^{-1} injection of saline solution alone (control) or containing C75 (5 mg·kg^{-1}) or TOFA (5 mg·kg^{-1}) or TOFA/C75 (5 mg·kg^{-1} each). TOFA and C75 were previously dissolved in DMSO. Food intake was registered as described above.

Assessment of metabolite levels and enzyme activities

Plasma FA, total lipids, triglyceride, and glucose levels were determined enzymatically using commercial kits (Wako Chemicals for FA, Spinreact for total lipids and triglyceride, and Biomérieux for glucose) adapted to a microplate format.

Samples used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vol of ice-cooled 6% PCA, 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 FA, total lipids, and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples. Tissue malonyl-CoA levels were determined by ELISA (Cusabio Biotech). Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols ice-cold-buffer consisting of 50 mmol·l^{-1} Tris (pH 7.6), 5 mmol·l^{-1} EDTA, 2 mmol·l^{-1} 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of supernatant (15 µl) at a pre-established protein concentration, omitting the substrate in control wells (final

volume 265-295 µl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma, USA) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ACLY (*EC* 4.1.3.8), FAS (*EC* 2.3.1.85), and HOAD (*EC* 1.1.1.35) activities were determined as described previously (Alvarez *et al.,* 2000; Kolditz *et al.,* 2008; Polakof *et al*., 2011b).

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues using Trizol reagent (Invitrogen) and treated with RQ1-DNAse (Promega). Two µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and random hexaprimers (Invitrogen). 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 of cDNA using the MAXIMA SYBR$^{®}$ Green qPCR Mastermix (Fermentas), in a total PCR reaction volume of 15 µl, containing 50-500 nM of each primer. mRNA abundance of fatty acid synthetase (FAS), inward rectifier K^+ channel pore type 6.-like (Kir6.x-like), sulfonylurea receptor-like (SUR-like), neuropeptide Y (NPY), pro-opio melanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) were determined as previously described (Polakof *et al.,* 2008b; Conde-Sieira *et al.,* 2010). mRNA abundance of ATP-citrate lyase (ACLY), acetyl-CoA oxidase (ACO), cluster of differentiation 36 (FAT/CD36), carnitine palmitoyl transferase type 1 (CPT1), citrate synthetase (CS), fatty acid transport protein 1 (FATP1), liver X receptor (LXR), peroxisome proliferator-activated receptor type α (PPARα), and sterol regulatory element-binding protein type 1c (SREBP1c) were determined as previously described by other authors in the same species (Kolditz *et al.,* 2008; Cruz-García *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Sánchez-Gurmaches, 2010; Polakof *et al.,* 2010a,b, 2011). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1.

Relative quantification of the target gene transcripts 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 15 min using hot-start iTaq^{TM} DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15s for denaturing, and at specific annealing for 30s and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analysed in triplicate. All the replicates of each sample were located in the same plate for each gene to allow comparisons. We included in all the

plates the standard curve (by triplicate), and controls for NTC and RT negative control (by duplicate). Only efficiency values between 85-100% were accepted (the R^2 for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl method (Pfaffl, 2001).

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward Primer	Reverse Primer	Annealing Temperature (ºC)	Accession Number
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	NM_ 001124235.1
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	CA349411.1
ACO	GCGCCAAGTACTTCCTCAAC	TCACAAACTCCTGTGTGCTG	55	BX085367
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	NM_001124627
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62	AY606034.1 (DFCI)
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55	AF327058
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	55	AF606076
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59	AJ619768
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	59	AJ620356
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	55	TC89195 (Tigr)
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59	tcab0001c.e.06 5.1.s.om.8
FATP1	AGGAGAGAACGTCTCCACCA	CGCATCACAGTCAAATGTCC	60	CA373015 (DFCI)
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60	CA346261.1.s.om.8:1:773:1
LXR	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	62	FJ470291
NPY	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58	NM_001124266
POMC	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	TC86162 (Tigr)
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	CA048941.1
SUR-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	62	tcce0019d.e.20_3.1.s.om.8

ACLY, ATP-citrate lyase; ACO, Acetyl-CoA oxidase; CART, cocaine- and amphetamine-related transcript ; CD36, cluster of differentiation 36 (fatty acid transporter); CPT1, carnitine plamitoyl transferase type 1; CS, citrate synthetase; FAS, fatty acid synthetase; FATP1, fatty acid transport protein 1; Kir6.x-like, inward rectifier K^+ channel pore type 6.-like; LXR, liver X receptor; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; SUR-like, sulfonylurea receptor-like.

Statistics

Comparisons among groups were carried out using Student t test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$. When necessary data were log transformed to fulfill the conditions of the analysis of variance.

RESULTS

Changes in food intake are shown in Fig. 1. The administration of oleate or octanoate induced dose-dependent decreases in food intake, which were also time-dependent (Fig. 1A). Food intake decreased after C75 administration, and this effect was cunteracted by the presence of TOFA (Fig. 1B).

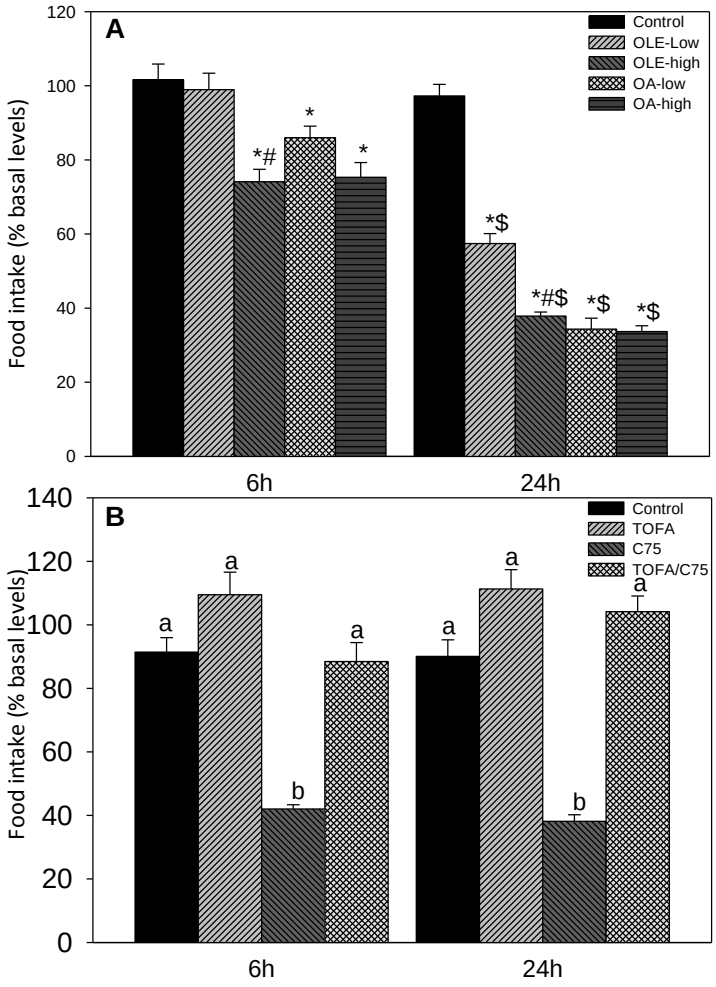

Fig. 1. A. Effect of intraperitoneal treatment with saline solution alone (control) or containing 60 or 300 μg·kg^{-1} of oleate or octanoate on food intake of rainbow trout. *, significantly different (P<0.05) from control fish at the same time. #, significantly different (P<0.05) from low dose of fatty acid of the same treatment and time. $, significantly different (P<0.05) from 6h of the same treatment. B. Effect of intraperitoneal treatment with saline solution alone (control) or containing C75 (5 mg·kg^{-1}) or TOFA (5 mg·kg^{-1}) or TOFA/C75 (5 mg·kg^{-1} each) on food intake in rainbow trout. Different letters indicate significant differences (*P*<0.05) among treatments at the same time. Food intake is displayed as mean + S.E.M. of the percentage of food ingested with respect to basal levels (calculated as the average of food intake the three days previous to experiment). The results are shown as mean + S.E.M. of the results obtained in three different tanks in which 8 fish were used per group in each tank.

Changes in parameters assessed in plasma are shown in Fig. 2. Treatment with oleate or octanoate increased levels of FA (Fig. 2A), triglycerides (Fig. 2B), and total lipids (Fig. 2C) without affecting glucose (Fig. 2D).

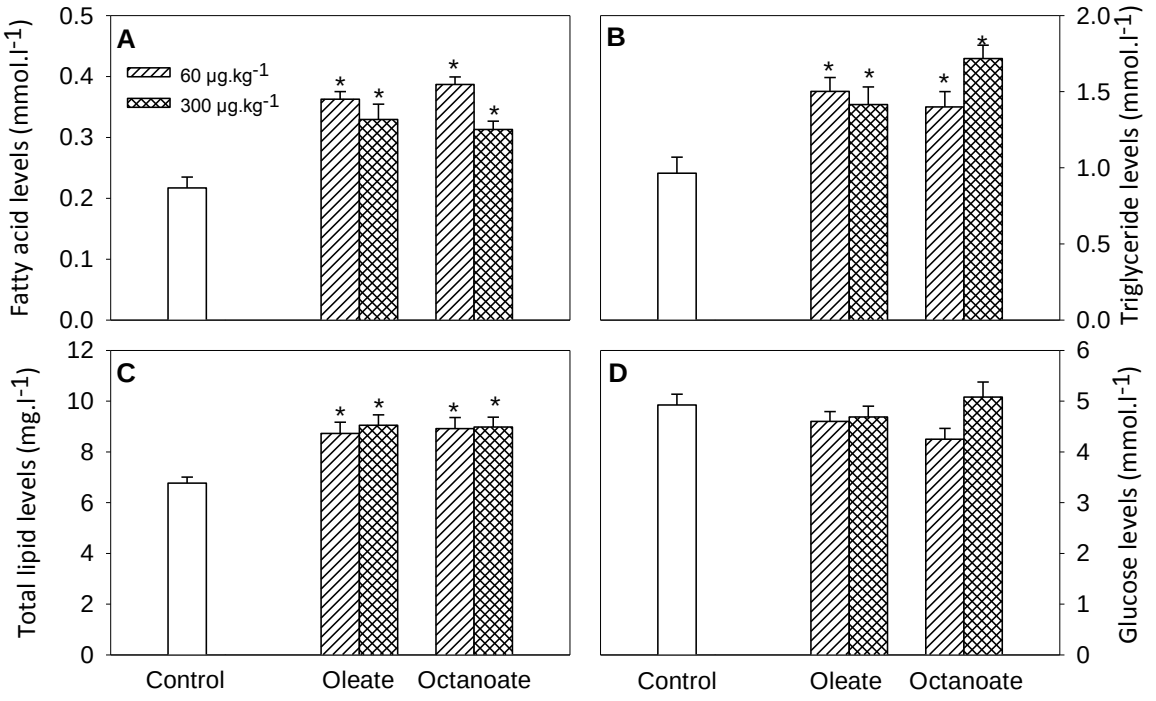

Fig. 2. Levels of FA, triglycerides, total lipids, and glucose in plasma of rainbow trout intraperitoneally injected with saline solution alone (control) or containing 60 or 300 µg·kg^{-1} of oleate or octanoate. Fish were sampled 6h after the injection. Each value is the mean + SEM of n=8 fish per treatment. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose at the same treatment.

Metabolite levels and enzyme activities evaluated in hypothalamus are shown in Fig. 3. IP treatment with increased doses of FA was therefore effective in inducing an increase not only in circulating FA but also in hypothalamic levels of total lipids. These results validate the experimental design, and are in agreement with preliminary experiments in which we evaluated a wide range of fatty acid doses. Thus, treatment with oleate or octanoate increased levels of FA (Fig. 3A, higher at the low doses used), total lipids (Fig 3C, not with the high dose of octanoate), and malonyl-CoA (Fig. 3D) and the activities of ACLY (Fig. 3E, not with the low dose of oleate) and FAS (Fig. 3F, high dose of oleate and low dose of octanoate) whereas a decrease was noticed for triglyceride levels (Fig. 3B, not with the high dose of octanoate). No significant changes were noticed for HOAD activity (Fig. 3G).

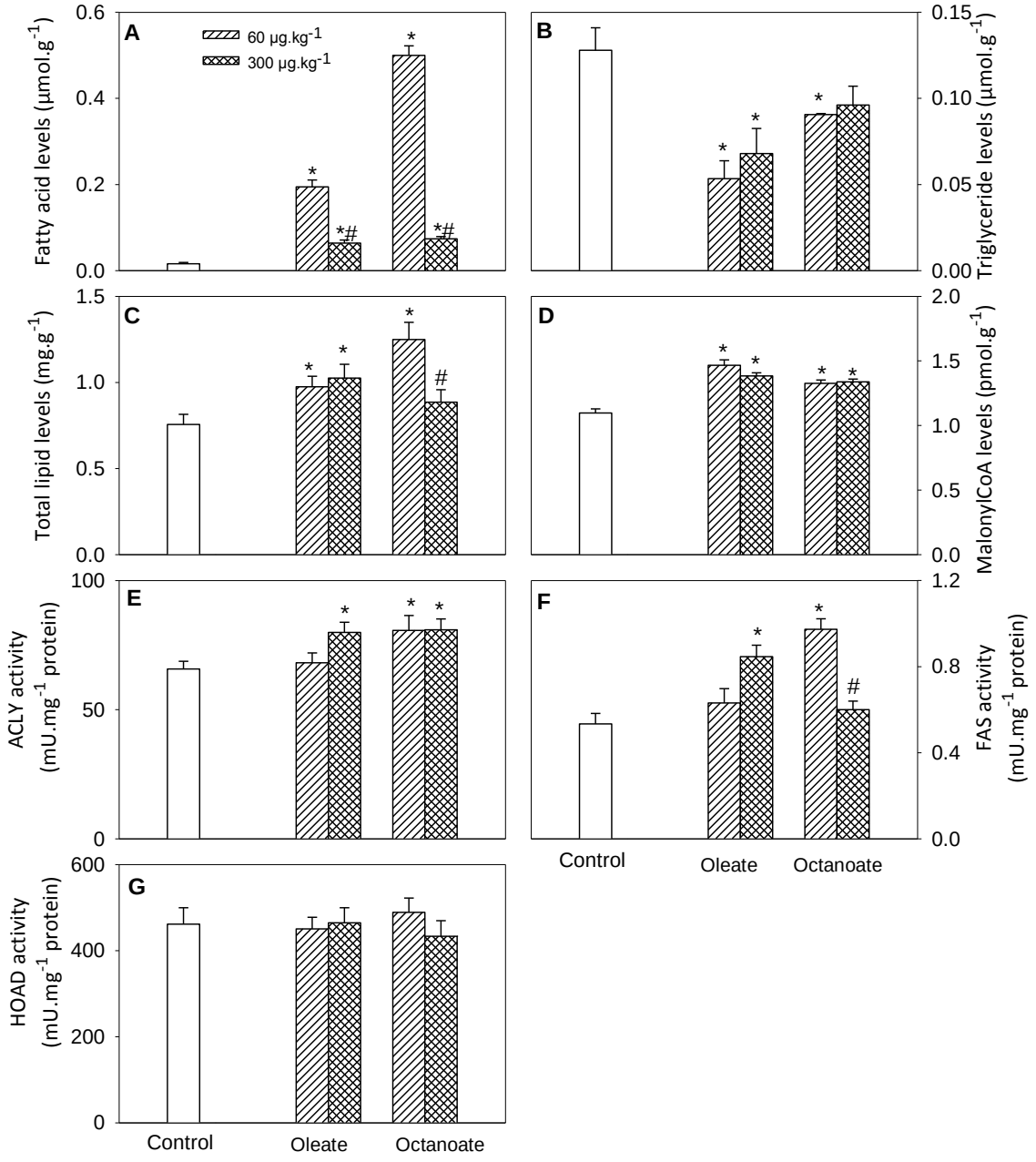

Fig. 3. Levels of FA, triglycerides, total lipids, and malonyl-CoA, and activities of ATP-citrate lyase (ACLY), fatty acid synthetase (FAS), and hydroxyacyl-CoA dehydrogenase (HOAD) in hypothalamus of rainbow trout intraperitoneally injected with saline solution alone (control) or containing 60 or 300 µg·kg^{-1} of oleate or octanoate. Further details as in legend of Fig. 2. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose at the same treatment.

Changes in mRNA abundance assessed in hypothalamus are shown in Table 2. The treatment with oleate or octanoate induced down-regulation of mRNA levels of ACLY, CS and Kir6.x-like (dose-dependent effects for both FA in the 3 genes), ACO (only high dose

for oleate, dose-dependent effects for octanoate), CART (only at the low dose for oleate and the high dose for octanoate), LXR (only at high doses), and SUR-like whereas an up-regulation was noticed for PPARα (dose-response effect of oleate). Moreover, oleate treatment up-regulated mRNA abundance of FAT/CD36, POMC, and SREBP1c and down-regulated mRNA abundance of CPT1c and NPY (only low dose). The high dose of octanoate decreased mRNA levels of FATP1. No significant changes were noticed for mRNA levels of CPT1d.

Table 2. mRNA levels in hypothalamus of rainbow trout after intraperitoneal administration of saline alone (control) or containing 60 or 300 μg·kg^{-1} of oleate or octanoate. Data represent mean of 4 measurements.

	Oleate		Octanoate	
	60 μg·kg^{-1}	300 μg·kg^{-1}	60 μg·kg^{-1}	300 μg·kg^{-1}
Fatty acid transport				
CD36	+2.39*	+2.06*	+1.28	+1.04
FATP1	+1.24	+1.17	-1.06	-1.82*
Fatty acid metabolism				
ACLY	-1.58*	-27.0*#	-16.9*	-27.8*#
ACO	-1.24	-26.7*#	-2.44*	-154.1*#
CPT1c	-1.41*	-1.78*	-1.01	-1.32
CPT1d	-1.15	-1.17	+1.12	+1.18
CS	-1.63*	-4.21*#	-2.67*	-9.65*#
FAS	-1.48*	-5.01*#	-2.16*	-1.99*
K_{ATP} channel				
Kir6.x-like	-1.98*	-3.26*#	-2.35*	-5.04*#
SUR-like	-2.26*	-2.49*	-2.12*	-3.41*
Transcription factors				
LXR	-1.27	-2.10*#	-1.33	-3.30*#
PPARα	+1.87*	+5.89*#	+6.61*	+8.55*
SREBP1c	+1.54*	+1.72*	+1.14	+1.03
Neuropeptides				
CART	-1.53*	+1.24#	+1.12	-1.75*#
NPY	-2.14*	-1.09#	+1.35	+1.14
POMC	+1.85*	+2.02*	+1.16	+1.01

Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by β-actin mRNA levels, mRNA levels-no variation). *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose of the same treatment.

Metabolite levels and enzyme activities evaluated in hindbrain are shown in Fig. 4. Treatment with oleate or octanoate increased levels of triglycerides (Fig. 4B, not with low dose of oleate, dose-dependent effects of octanoate), and total lipids (Fig. 4C, dose-dependent changes for octanoate), and ACLY activity (Fig. 4E, only high dose of octanoate, dose-dependent changes for oleate). Moreover, the high dose of octanoate elevated fatty acid levels (Fig. 4A) whereas the low dose of

oleate elevated HOAD activity (Fig. 4F). No significant changes were noticed for malonyl CoA levels (Fig. 4D).

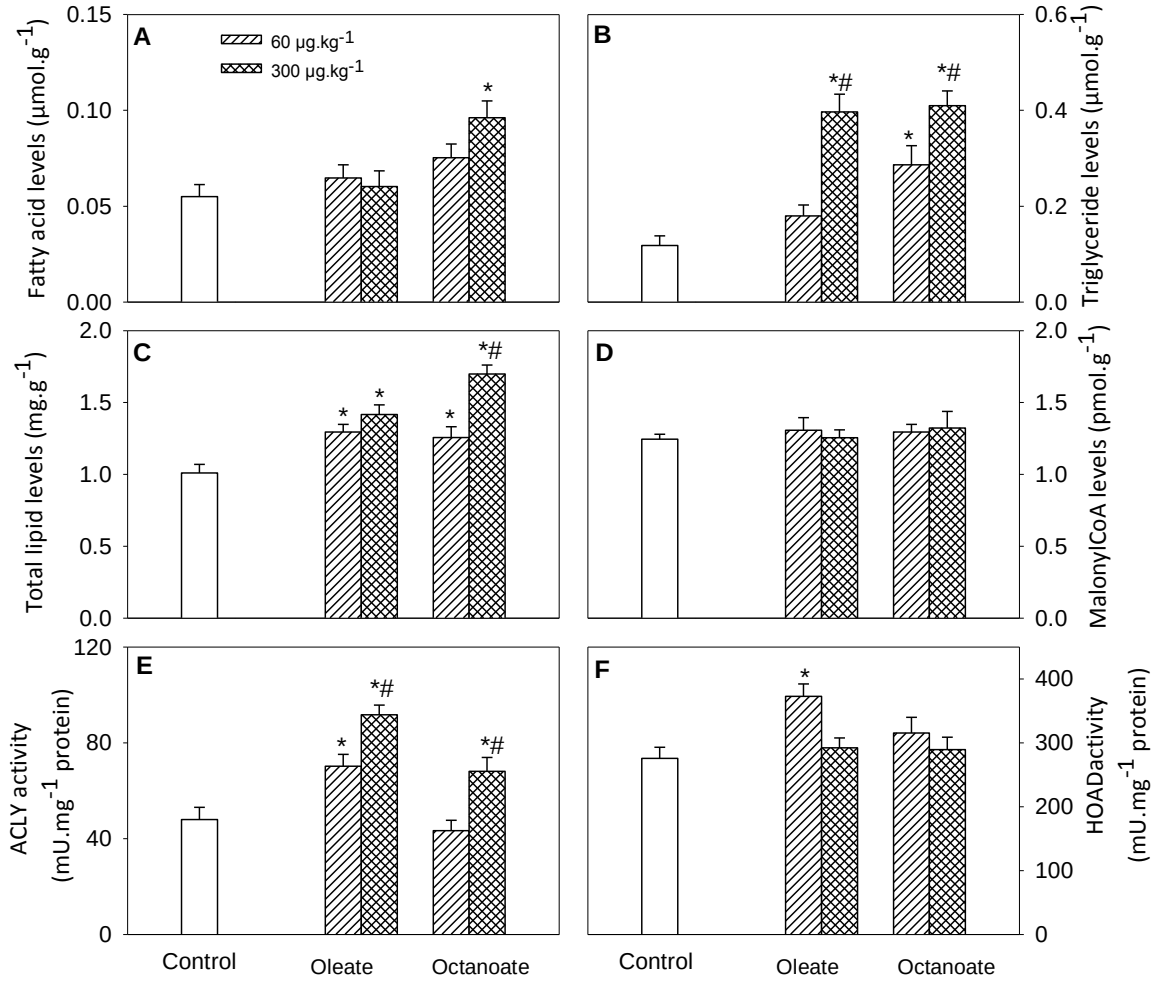

Fig. 4. Levels of FA, triglycerides, total lipids, and malonyl-CoA, and activities of ATP-citrate lyase (ACLY), and hydroxyacyl-CoA dehydrogenase (HOAD) in hindbrain of rainbow trout intraperitoneally injected with saline solution alone (control) or containing 60 or 300 µg·kg^{-1} of oleate or octanoate. Further details as in legend of Fig. 2. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose at the same treatment.

Changes in mRNA abundance assessed in hindbrain are shown in Table 3. The treatment with oleate or octanoate up-regulated mRNA levels of CPT1c (only low dose for oleate), FATP1, and NPY (only high dose of octanoate), and down-regulated mRNA levels of CPT1d and FAS (only at high doses for both FA). Furthermore the treatment with the low dose of oleate up-regulated mRNA levels of ACLY, and CS whereas the treatment with the high dose of octanoate down-regulated PPARα mRNA levels. No significant changes

were noticed for mRNA abundance of ACO, CART, Kir6.x-like, LXR, POMC, SREBP1c, and SUR-like.

Table 3. mRNA levels in hindbrain of rainbow trout after intraperitoneal administration of saline alone (control) or containing 60 or 300 μg·kg^{-1} of oleate or octanoate.

	Oleate		Octanoate	
	60 μg·kg^{-1}	300 μg·kg^{-1}	60 μg·kg^{-1}	300 μg·kg^{-1}
Fatty acid transport				
CD36	+1.64*	+1.31	+1.03	+1.04
FATP1	+3.98*	+1.75*#	+3.04*	+2.54*
Fatty acid metabolism				
ACLY	+2.31*	+1.01#	+1.32	+1.23
ACO	-1.01	+1.29	-1.14	+1.07
CPT1c	+3.57*	+1.26#	+3.32*	+4.49*
CPT1d	-1.71*	-2.28*	-1.58*	-1.77*
CS	+2.39*	+1.23#	+1.26	+1.35
FAS	-1.32	-1.76*	+1.18	-3.53*
K_{ATP} channel				
Kir6.x-like	+1.22	+1.02	-1.16	-1.32
SUR-like	+1.35	+1.46	+1.31	+1.05
Transcription factors				
LXR	+1.36	-1.13	+1.05	-1.01
PPARα	+1.02	-1.37	-1.12	-1.63*#
SREBP1c	+1.33	+1.15	+1.19	+1.28
Neuropeptides				
CART	+1.13	+1.37	+1.25	-1.06
NPY	+5.02*	+3.19*	+1.13	+1.97*#
POMC	+1.23	+1.32	-1.02	-1.15

Data represent mean of 4 measurements. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by β-actin mRNA levels, mRNA levels-no variation). *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose of the same treatment.

Metabolite levels and enzyme activities assessed in Brockmann bodies are shown in Fig. 5. Treatment with oleate or octanoate increased levels of FA (Fig. 5A) and triglycerides (Fig. 5B, only for the low dose for octanoate) and decreased ACLY activity (Fig. 5D). Treament with the low dose of octanoate increased FAS activity (Fig. 5E) whereas treatment with the low dose of oleate decreased HOAD activity (Fig. 5F). No significant changes were noticed for total lipid levels (Fig. 5C).

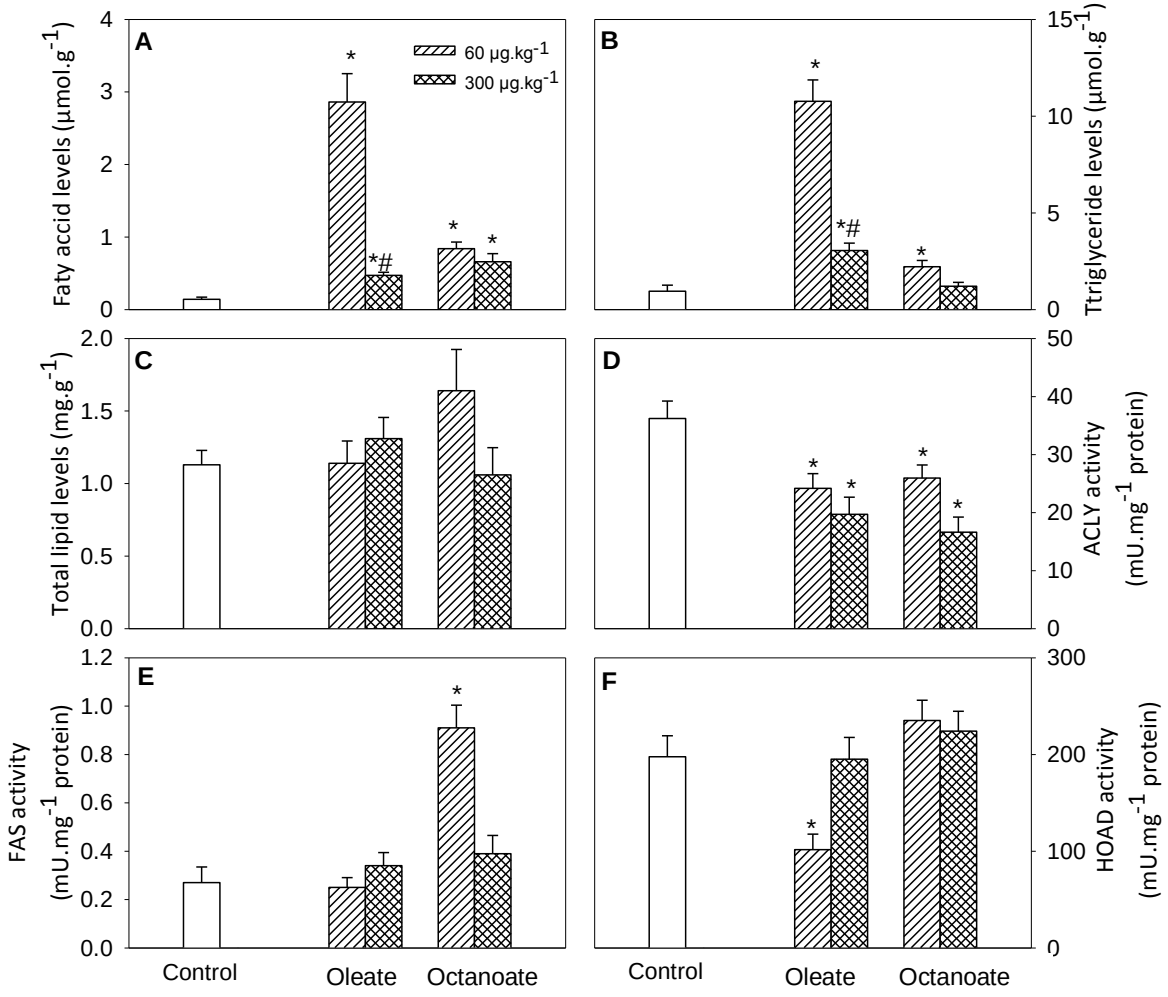

Fig. 5. Levels of FA, triglycerides, and total lipids, and activities of ATP-citrate lyase (ACLY), fatty acid synthetase (FAS), and hydroxyacyl-CoA dehydrogenase (HOAD) in Brockmann bodies of rainbow trout intraperitoneally injected with saline solution alone (control) or containing 60 or 300 µg·kg^{-1} of oleate or octanoate. Further details as in legend of Fig. 2. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose at the same treatment.

Changes in mRNA abundance in Brockmann bodies are shown in table 4. The treatment with oleate or octanoate significantly down-regulated mRNA levels of CPT1b (dose-response effect for octanoate), FAS (dose-response effect for both acids) and LXR (only at high doses) whereas an up-regulation was noticed for PPARα (dose-response for oleate, no effect for the low dose of octanoate) and SREBP1c (only high doses). Moreover, the treatment with oleate up-regulated mRNA levels of ACLY (low dose) and FAT/CD36 whereas the treatment with the high dose of octanoate down-regulated SUR-like mRNA levels. No changes were noticed for mRNA levels of CPT1a, CS and Kir6.x-like.

Table 4. mRNA levels in Brockmann bodies of rainbow trout after intraperitoneal administration of saline alone (control) or containing 60 or 300 μg·kg^{-1} of oleate or octanoate.

	Oleate		Octanoate	
	60 μg·kg^{-1}	300 μg·kg^{-1}	60 μg·kg^{-1}	300 μg·kg^{-1}
Fatty acid transport				
CD36	+2.51*	+2.19*	+1.37	+1.45
FATP1	+2.88*	+2.36*	+1.51	+1.93*
Fatty acid metabolism				
ACLY	+3.73*	+1.49#	+1.47	+1.41
CPT1a	+1.26	+1.35	+1.18	-1.22
CPT1b	-1.54*	-2.12*	-1.87*	-3.02*#
CS	+1.39	+1.24	+1.46	+1.35
FAS	-1.53*	-22.1*#	-6.99*	-23.6*#
K_{ATP} channel				
Kir6.x-like	+1.19	-1.09	-1.26	+1.43
SUR-like	+1.41	+1.40	-1.25	-3.61*#
Transcription factors				
LXR	-1.11	-1.54*	-1.19	-1.40*
PPARα	+1.55*	+2.32*#	+1.35	+1.52*
SREBP1c	+1.04	+6.91*#	+1.08	+1.73*#

Data represent mean of 4 measurements. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by β-actin mRNA levels, mRNA levels-no variation). *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose of the same treatment.

DISCUSSION

High FA circulating levels reduce food intake but do not alter plasma glucose levels

Besides their role as energy source, lipids have been shown to act as important signalling molecules in mammals (Migrenne *et al.,* 2007). In fish, high circulating lipids levels have been traditionally associated to reduced food intake (Shearer *et al.,* 1997; Rasmussen *et al.,* 2000; Gélineau *et al.,* 2001; Johansen *et al.,* 2002; Nordrum *et al.,* 2003; Williams *et al.,* 2006), while more recently a deleterious effect on glucose homeostasis was described in rainbow trout fed in a high fat diet (Figueiredo-Silva *et al.,* 2012b). However, these effects had been never evaluated before using individual FA. In the present study, we evaluated, for the first time in fish, if a treatment with an individual FA, such as oleate (a LCFA) or octanoate (a MCFA), was able to alter food intake and glucose homeostasis in rainbow trout. The decrease in food intake noticed under our experimental conditions, clearly indicates that FA are involved in the lipid-induced

inhibition of food intake, which allows us to suggest the presence of a central FA sensing system involved in the control of food intake. In contrast, no changes were noticed in glycaemia, suggesting that the negative effects of high circulating FA levels could be developed only in fasted fish during chronic exposures or as the result of the interaction with other nutrients or endocrine systems.

Fatty acid sensing through changes in fatty acid metabolism: impact on food intake and hypothalamic neuropeptides

In mammals circulating LCFA act as signals of nutrient surplus in the hypothalamus (López *et al*., 2007; Migrenne *et al.,* 2011). In the present study, the increase in circulating FA are reflected in hypothalamus by increased levels of FA (higher at low doses) and total lipids, and decreased levels of triglycerides. These changes support an increased entry of FA into rainbow trout hypothalamus that once within the tissue may elicit a response in metabolism consistent with a FA sensing mechanism. Therefore, we evaluated changes in several parameters of FA metabolism, which in mammals are related to the main mechanism involved in FA sensing, especially in the hypothalamus. One of those mechanisms is that in which increased LCFA levels in plasma enhanced malonyl-CoA levels in hypothalamus that inhibit the ability of CPT-1 to import FA-CoA into mitochondria for oxidation. Moreover, in mammalian hypothalamus FAS is known to be pharmacologically inhibited under conditions of enhanced FA levels (López *et al*., 2005, 2007), and enhanced malonyl-CoA levels can lead to an inhibition of food intake through effects in the expression of orexigenic and anorexigenic neuropeptides. In fish to date there were no similar studies. In rainbow trout hypothalamus the first important issue was to characterize the presence of those components in the brain regions assessed. We were successful in general, and therefore we then evaluated changes in those parameters when rainbow trout were treated with a LCFA like oleate and with a MCFA like octanoate. In mammalian hypothalamus the FA sensing mechanism related to FA metabolism is activated by LCFA but not by MCFA or SCFA (López *et al*., 2007). The situation in rainbow trout seems to be rather different since in general treatment with octanoate (MCFA) induced similar effects than those induced by oleate (LCFA). This different behaviour between fish and mammals could be related to the findings that body lipids in teleosts contain considerable amounts of MCFA (Davis *et al.,* 1999; Trushenski, 2009) and that in rainbow trout there is no preferential oxidation of MCFA compared with LCFA (Figueiredo-Silva *et al.,* 2012) in contrast with the mammalian situation (Ooyama *et al.,* 2009). Changes observed in parameters assessed in trout hypothalamus after treatment with both FA agree in general with the mammalian model (Hu *et al.,* 2011) such as for the increase in malonyl-CoA levels, or for the decrease in mRNA abundance of ACLY (an increase was noticed in activity), ACO, and CS. It is interesting that mRNA levels of FAS (but not activity) also decreased in rainbow trout

hypothalamus when fish were treated with either oleate or octanoate since such inhibition had been reported in mammals only after pharmacological (but not physiological) treatments. The main differences between the results obtained for oleate and octanoate were those related to the decrease observed in mRNA abundance of CPT1c after oleate (but not octanoate) treatment. This difference can be attributed to the fact that in mammals octanoate does not require CPT1 for entry in the mitochondria (Wolfgang and Lane, 2007), and therefore a similar mechanism could be present in trout.

Given the apparently successful characterization of the main lipid-sensing markers in the hypothalamus of rainbow trout, we assessed if this system could be involved in the changes observed in food intake. Thus, the treatment with C75 (FAS inhibitor) induced a decrease in food intake that was reverted by the presence of TOFA (ACC inhibitor) since fish co-treated with C75 and TOFA did not display changes in food intake compared with controls. This response is similar to that observed in mammals (Loftus *et al.,* 2000; Gao and Lane 2003; Hu *et al.,* 2011) supporting that several of the known mechanisms of FA metabolism involved in the FA sensing system related to the central control of food intake in mammals may be also present in fish.

Thus, changes in FA metabolism are known to inhibit food intake in mammals through reduced expression of NPY/AgRP and increased expression of POMC/CART (López *et al.,* 2005, 2007). In rainbow trout hypothalamus treatment with oleate decreased NPY and increased POMC mRNA abundance. Both changes are in the direction of inhibiting food intake and are therefore in agreement with the reduced food intake observed in the present study when rainbow trout was treated with FA. In contrast to the metabolic changes described above in which oleate induced similar responses than those elicited by octanoate, no major changes were noticed in the expression of neuropeptides like NPY, POMC or CART after octanoate treatment, which is also in contrast with the above findings that both octanoate and oleate induced a decrease in food intake. In mammals, it has been hypothesized that changes in CPT1c (the CNS specific isoform of CPT1) would be mediating the expression of peptides in mammalian hypothalamus (Chari *et al.,* 2010). If no changes occur in CPT1c after octanoate treatment in rainbow trout hypothalamus this could be related to the lack of effect of that fatty acid in neuropeptide expression. In this way, in mammals, the anorexigenic effect of FA could depend on the rate of mitochondrial activity since LCFA produce a higher inhibition than MCFA or SCFA (Benani *et al.,* 2007). In general, we may suggest that rainbow trout hypothalamus posses a FA sensing system based on FA metabolism which was related to the inhibition of food intake. The system seems to be fully activated by a LCFA like oleate but only partially activated by a MCFA like octanoate since in this later case no changes were noticed in the mRNA abundance of orexigenic and anorexigenic peptides. A recent paper by Figueiredo-Silva *et al*. (Figueiredo-Silva *et al.,* 2012a) also suggest that triglycerides formed by MCFA are not dependent on CPT1c for oxidation when compared with triglycerides formed by LCFA. In any case, the partial activation of a putative FA sensing system induced by

octanoate is different than the mammalian model. There are no other comparable studies carried out in fish hypothalamus to date since the available studies so far evaluated the metabolic effects of dietary lipid in peripheral tissues, such as the liver or muscle (Kennedy *et al.,* 2006; Kolditz *et al*., 2008; Plagnes-Juan *et al.,* 2008; Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Figueiredo-Silva *et al.,* 2012b).

Fatty acid sensing in hindbrain and Brockmann bodies

In hindbrain of rainbow trout the treatment with FA did not induce large changes in FA levels, which is in clear contrast with the results obtained in hypothalamus suggesting a different behaviour of hindbrain in dealing with FA arriving from blood stream. Compared with the results obtained in parameters related to fatty acid metabolism in hypothalamus, we observed in hindbrain absence of response in some cases (levels of FA and malonyl-CoA, and mRNA levels of ACO, CS, and POMC) or converse changes in others (triglyceride and total lipid levels and mRNA levels of ACLY, CPT1c, NPY), whereas only for mRNA levels of FAS changes in hindbrain were similar to those observed in hypothalamus. We may therefore suggest that, in contrast to the mammalian situation where hindbrain has been suggested to be involved in fatty acid sensing through specific changes in FA metabolism (Migrenne *et al.,* 2007), this seems not to be the case in rainbow trout.

In mammals the ability to sense FA affects insulin secretion in the endocrine pancreas (Obici *et al.,* 2002; Migrenne *et al.,* 2006). In fish, insulin release in pancreatic endocrine tissue is influenced not only by circulating glucose but also by lipid levels, which is not surprising considering the lipogenic role of insulin in fish (Caruso and Sheridan, 2011). Therefore, we hypothesize that a system detecting changes in circulating lipid levels may be present in the main accumulation of endocrine pancreatic tissue in fish, the Brockmann bodies, and therefore we evaluated the response of the same components than those addressed in the hypothalamus. The results obtained in BB were in some cases similar to those observed in hypothalamus, like the decreased mRNA levels of FAS and CPT1c after treatment with oleate or octanoate, which suggests that FA metabolism may be involved in FA sensing capacity in BB. Further studies assessing insulin levels will confirm if this mechanism is related to hormone secretion.

Other possible pathways of fatty acid sensing

FA metabolism only accounts for part of FA sensing in mammalian hypothalamus since alternative mechanisms have been suggested to be present such as binding to the FA transporter FAT/CD36 (Nordrum *et al.,* 2003), which would act as a receptor rather a transporter (Migrenne *et al.,* 2011). The participation of FAT/CD36 on this mechanism

was evaluated in rainbow trout by assessing changes in its mRNA abundance, which was enhanced in hypothalamus by the oleate administration without being affected by octanoate treatment. The specific action of oleate in FAT/CD36 is in agreement with the lack of changes noticed in mRNA abundance of the other carrier presumably involved in fatty acid uptake such as FATP1. In mammals the activation of the FAT/CD36 pathway has been related to the activation of several transcription factors, including PPARα and SREBP1c (Le Foll *et al.,* 2009). In rainbow trout hypothalamus the treatment with oleate enhanced not only FAT/CD36 mRNA abundance, but also PPARα and SREBP1c gene expression. Moreover, PPARα has been reported in mammalian hypothalamus to act as a fatty acid sensor binding FA to activate gene transcription (Wolfrum, 2007; Miyauchi *et al.,* 2010), a possibility that has also to be taken into account in the present study. In fish, as in mammals (Wolfrum, 2007), PPARα attenuates the expression of LXR (Cho *et al.,* 2012). Therefore, a decrease in LXR mRNA levels could be expected when PPARα mRNA levels increased, as observed in the present study in hypothalamus of fish treated with FA. Therefore, it seems that raised FA levels, especially oleate, are also sensed in trout hypothalamus through changes in the expression of FAT/CD36 carrier and related transcription factors. In hindbrain no changes were noticed in the same parameters whereas in BB the pathway seems also to be present since enhanced mRNA levels of FAT/CD36, PPARα, and SREBP1c were noticed after treatment with oleate or octanoate. In a way similar to that observed in hypothalamus LXR mRNA levels decreased in BB of fish treated with FA suggesting again a inhibitory relationship with PPARα as demonstrated in flounder liver (Cho *et al.,* 2012).

In mammalian hypothalamus increased FA levels also result in enhanced mitochondrial production of ROS by electron leakage, finally resulting in an inhibition of the K_{ATP} channel activity (Blouet and Schwartz, 2010). In our study, we evaluated changes in the mRNA abundance of the components of the K_{ATP} channel (Kir6.x-like and SUR-like) (Polakof *et al.,* 2008b) observing a clear inhibition by treatment with both FA. This is in agreement with the response observed in mammals, suggesting that this channel is sensitive to circulating FA and may participate in fatty acid sensing. In contrast, in hindbrain and in BB the pathway related to mitochondrial activity seems not to be working since no changes comparable to those observed in hypothalamus were observed in the mRNA abundance of the components of the K_{ATP} channel (Kir6.x-like and SUR-like).

Conclusions and perspectives

In the present study, we describe for the first time in fish, altered food intake in response to the administration of single FA. Moreover, we have found that several metabolic and molecular components of the main lipid sensing system described in mammals are sensitive to the high circulating FA levels induced in our experimental

design. We may then suggest that the increase in circulating LCFA or MCFA levels in rainbow trout are sensed in the hypothalamus through a complex mechanism in which FA metabolism, binding to FAT/CD36 and mitochondrial activity are apparently involved. The mechanisms are quite similar to those suggested in mammals except for the apparent capacity of rainbow trout hypothalamus to detect increases not only in LCFA but also in MCFA, which could be related to a higher importance of MCFA in teleosts. Changes in those hypothalamic pathways could be related to the control of food intake since food intake was inhibited when the FA metabolism was perturbed (using FAS or ACC inhibitors) and, only after oleate treatment, changes in mRNA levels of specific neuropeptides such as NPY and POMC were also noticed in hypothalamus. This response seems to be exclusive of the hypothalamus, since the other centre controlling food intake (hindbrain) was unaffected by treatments. Since FA treatment was given IP further intracerebroventricular (ICV) experiments are necessary to establish that the effects of FA are related to nutrient sensing and not to peripheral hormonal effects.

The results obtained in BB suggest that at least two of the components of a putative fatty acid sensing system (based on fatty acid metabolism and binding to FAT/CD36) could be present in BB of rainbow trout responding with changes similar to those observed in hypothalamus. However, since no changes were observed in the mRNA levels of the components of the K^{+}_{ATP} channel the fatty acid detection in BB does not seem to be easily related to insulin secretion as suggested in mammals (Cho *et al.,* 2012). Furthermore, the possibility that in a way similar to mammals the response of BB to raised fatty acid levels in plasma be a consequence of hypothalamic fatty acid sensing through vagal and sympathetic outflow (Blouet and Schwartz, 2010) cannot be ruled out and must be addressed in future studies using ICV treatments with FA.

In conclusion, lipids had been considered to date in fish only as an important source of energy with an inhibitory relationship with food intake that had not been evaluated before. The present study provides for the first time in fish evidence for a specific role for FA (MCFA and LCFA) as metabolic signals in hypothalamus and Brockmann bodies where the detection of those FA can be associated with the control of food intake and hormone release. Further studies are necessary to characterize the underlying mechanisms as well as the global impact of lipid sensing in energy metabolism in fish.

3.2 TRABAJO EXPERIMENTAL Nº2

Effects of a mixture or single fatty acids treatment on the response of hepatic lipid and glucose metabolism. Possible presence of fatty acid-sensing systems

INTRODUCTION

Liver plays a central role in fatty acid (FA) metabolism in fish, and interactions between glucose and FA metabolism have been partially characterized in this tissue. An increase in dietary lipid level (either from vegetable oil, fish oil or a mixture of both) was shown to induce hyperglycemia, reduce glycolytic and lipogenic potential, and increase gluconeogenic potential in the liver of rainbow trout (Gélineau *et al.,* 2001; Panserat *et al.,* 2002; Figueiredo-Silva *et al.,* 2012a,b) .Increased glycaemia after the consumption of high fat diets was also seen in other studies with salmonids (Hemre and Sandnes, 1999; Mazur *et al.,* 1992), grouper (*Epinephelus coioides*) (Cheng *et al.,* 2006) and sunshine bass (*Morone chrysops×M. saxatilis*) (Hutchins *et al.,* 1998), and sometimes associated with hyperinsulinemia (Shearer *et al*., 1997). Differences in hepatic lipogenesis have also been suggested to help in explaining differences in glucose use among fish species (Figueiredo-Silva *et al.,* 2013) and rainbow trout lines divergently selected for muscle fat content (Skiba-Cassy *et al.,* 2009; Kamalam *et al.,* 2012). Because this seems to be particular true when a high lipid level is combined with a high carbohydrate in the diet, reduction of dietary lipid level may thus be expected to have a protective role in improving the glycaemic control and increasing insulin sensitivity in carnivorous fish fed high carbohydrate diets (Figueiredo-Silva *et al.,* 2012b, Polakof *et al.,* 2012).

The impact of high FA supply on hepatic metabolism and in particular on the control of glycaemia of fish seems, however, to be time-related. While the consumption of a high fat diet for 14 days resulted in prolonged hyperglycaemia and reduced plasma glucose clearance in response to an exogenous glucose or insulin challenge, a single high fat meal reduced glycaemia compared with a low fat meal in rainbow trout (Figueiredo-Silva *et al.,* 2012b). It seems, therefore, that metabolism of fish as of mammals (Paolisso *et al.,* 1995) respond differently to short- or long-term exposure to FA. However, while considerable information is available on the metabolic response of fish to long-term exposure to high FA level, information on the acute response to FA and in particular on the mechanisms by which FA exert their effects is practically inexistent.

We previously reported in rainbow trout (Librán-Pérez *et al.,* 2012) that acute administration of oleate (C18:1), a long-chain fatty acid (LCFA), and octanoate (C8:0), a medium chain FA (MCFA), activate putative components of a FA sensing system (based in FA metabolism, binding to FAT/CD36 and mitochondrial activity) in hypothalamus (possibly related to the control of food intake) and Brockmann bodies (possibly related to insulin release). Considering that several of these putative components of FA sensing mechanisms are also present in the liver of rainbow rout (Kolditz *et al.,* 2008; Plagnes-Juan *et al.,* 2008; Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Polakof *et al.,* 2010a), one

may expect for a similar FA sensing system operating in liver in response to FA administration.

The present study focused, therefore, on the acute response of hepatic lipid and glucose metabolism to a mixture (Experiment 1: fish oil (FO), for being a common source of FA in fish diets) or single FA (oleate or octanoate FA, for their presence and absence in FO, respectively). Evidence for the presence of a metabolic FA sensing system in liver was also investigated. For that, two experiments were conducted to investigate the impact of an acute FO (experiment 1) or single FA (experiment 2) administration on glucose metabolism (glucose, glycogen and lactate levels, GK, PK, FBPase, and G6Pase activities, and mRNA abundance of GK, PK, and FBPase), FA metabolism (circulating FA, triglyceride and total lipid levels, ACLY, MCD, HOAD and FAS activities, and mRNA abundance of ACC, ACLY, ACO, FAS, CPT1, CS, and HOAD), FA transport (mRNA abundance of FAT/CD36 and FATP1), and nuclear receptors and transcription factors involved in FA metabolism (PPARα and SREBP1c).

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from local fish farms (INRA experimental fish farm of Donzacq, Landes, France in experiment 1, and Soutorredondo, Spain in experiment 2). In experiment 1, fish with an initial body weight of 100 ± 10 g were kept in 70 L litre tanks (24 fish per tank) in a recirculating rearing system (INRA, St-Pée-sur-Nivelle, France) at a water temperature of 17°C and controlled photoperiod (12L:12D). The fish were weighed and sorted prior to stocking in order to constitute homogenous groups of fish with similar (100 ± 10 g) individual body weight. In experiment 2, fish with an initial body weight of 101 ± 3 g were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish were fed a commercial trout diet during the pre-experimental period (48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed).

The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), the Spanish Government (RD 1201/2005), and the French Government (Decret nº 2001-464 of May 29, 2001) and were approved by the Ethics Committee of INRA (according to INRA 2002-36 of April 14, 2002) and Universidade de Vigo for the use of animals in research.

Experimental designs

Experiment 1: Oral administration of FO

Oral FO administration was performed in fasted fish to ensure the complete emptying of the digestive tract. Trout were slightly anaesthetized (0.05% phenoxyethanol) and received, through a cannula coupled to a syringe, 10 mL·Kg^{-1} of tank water (control) or FO (Sopropêche, 56100 Lorient, France) whose composition is described in Table 1. Immediately after oral administration of tank water or FO, fish received an intraperitoneal (IP) injection of glucose (250 mg·kg^{-1}). The lack of oil in the water tank and its presence in the gastrointestinal tract was systematically checked in order to assure that the whole volume administrated was digested. Blood and liver samples were taken 6h after treatment and frozen at -80ºC. In each group, 6 fish were used to assess enzyme activities and metabolite levels whereas the remaining 6 fish were used for the assessment of mRNA levels by qRT-PCR.

Table 1. Fatty acid composition (g/100 of total FA) of fish oil orally administered to rainbow trout.

Fatty acid	(g/100 of total FA)
Saturated	
C12:0	0.1
C14:0	7.5
C15:0	0.5
C16:0	16.8
C17:0	0.3
C20:0	0.2
Monounsaturated	
C14:1	0.1
C15:1	0.1
C16:1	9.0
C17:1	0.2
C18:1	14.2
C20:1	1.0
C22:1	0.9
Polyunsaturated	
C14:PUFA	0.2
C16:2 n-4	1.3
C16:3 n-4	1.9
C16:4 n-1	2.3
C18:2 n-6	1.6
C18:3 n-3	0.9
C18:3 n-6	0.3
C18:4 n-3	2.3
C20:2 n-6	0.2
C20:3 n-3	0.1
C20:3 n-6	0.1
C20:4 n-3	0.8
C20:5 n-3	16.5
C20:4 n-6	0.9
C21:5 n-3	0.7
C22:2 n-6	0.1
C22:5 n-3	2.1
C22:6 n-3	8.6

Experiment 2: Intraperitoneal administration of FA

Following 1 month acclimation period, fish were fasted before treatment to ensure the complete emptying of the digestive tract. Fish were slightly anaesthetized with MS-222 (50 mg·l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed and randomly assigned to 100 litre experimental tanks. Then, 12 fish per group received IP 10 ml·kg^{-1} injection of saline solution alone (control) or containing oleate (60 or 300 µg·kg^{-1}) or octanoate (60 or 300 µg·kg^{-1}). To safely deliver FA (Morgan *et al.,* 2004) they were solubilized in 45% hydroxypropyl-β-cyclodextrin (HBP) to a final concentration of 17 mM. Concentrations of FA were selected based on studies carried out previously in mammals (Lam *et al*., 2005; Pocai *et al*., 2006; Caspi *et al*., 2007). The HPB-FA solution was diluted in saline for each injection. HPB alone at a similar concentration as in the FA studies was used in all vehicle control studies (no effects of the vehicle alone were noticed for any of the parameters assessed, data not shown). Blood and liver samples were taken 6h after treatment, shown as the time period necessary to achieve changes in the sensing mechanisms for other nutrients such as glucose (Polakof *et al.,* 2007a,b; 2008a,b,c). In each group, 8 fish were used to assess enzyme activities and metabolite levels whereas the remaining 4 fish were used for the assessment of mRNA levels by qRT-PCR.

Assessment of metabolite levels and enzyme activities

Blood glucose levels were determined with one drop of blood using the Accu-Check™ glucometer (Roche). Plasma free FA (non-esterified fatty acids, NEFA C from Wako Chemicals GmbH, Germany) and triglycerides (TAG, Biomerieux, France) levels were determined using colorimetric commercial kit adapted to microplates. Liver samples used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vol of ice-cooled 6% perchloric acid (PCA), and neutralized (using 1 mol·l^{-1} potassium bicarbonate). The homogenate was centrifuged, and the supernatant used to assay tissue metabolites. Liver FA, total lipids, and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples. Total lipid and lactate levels were assessed enzymatically using commercial kits (Spinreact) adapted to microplates. Tissue glycogen levels were assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux).

Samples for enzyme assays were homogenized by ultrasonic disruption with 9 vols ice-cold-buffer consisting of 50 mmol·l^{-1} Tris (pH 7.6), 5 mmol·l^{-1} EDTA, 2 mmol·l^{-1} 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged and the supernatant used immediately for determination of enzyme activity. Enzyme activity was determined using a microplate reader INFINITE 200 Pro (Tecan, Grödig, Austria) and microplates. Enzyme activity (units IU), defined as µmoles of substrate

converted to product per min, at assay temperature (37°C) was expressed per mg of soluble protein (specific activity). Protein was assayed in triplicate according to the bicinchoninic acid method with bovine serum albumin (Sigma, USA) as standard. Fructose 1,6-bisphosphatase (FBPase, *EC* 3.1.3.11), glucose 6-phosphatase (G6Pase, *EC* 3.1.3.9), glucose 6-phosphate dehydrogenase (G6PDH, *EC* 1.1.1.49), glycogen phosphorylase (GPase, *EC* 2.4.1.1), glucokinase (GK, *EC* 2.7.1.2), and pyruvate kinase (PK, *EC* 2.7.1.40) activities were estimated as described previously (Polakof *et al.,* 2007a,b, 2008a,b). ATP-citrate lyase (ACLY, *EC* 4.1.3.8), fatty acid synthase (FAS, *EC* 2.3.1.85), hydroxyacyl-CoA dehydrogenase (HOAD, *EC* 1.1.1.35), and malonyl-CoA decarboxylase (MCD, *EC* 4.1.1.9) activities were determined as described by Alvarez *et al.* (2000), Polakof *et al.* (2011a), Kolditz *et al.* (2008), and Zhou *et al*. (2004), respectively.

Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues using Trizol reagent (Life Technologies) and treated with RQ1-DNAse (Promega). Two µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexaprimers (Life Technologies). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ™ (BIO-RAD, Berkeley, CA, USA). Analyses were performed on 1 µl of the diluted cDNA using the MAXIMA SYBR® Green qPCR Mastermix (Fermentas), in a total PCR reaction volume of 15 µl, containing 50-500 nM of each primer. Fatty acid synthetase (FAS), and glucokinase (GK) qPCRs were performed using primers and protocol as described previously (Polakof *et al*., 2008c). Acetyl-CoA decarboxylase (ACC), ATP-citrate lyase (ACLY), acetyl-CoA oxidase (ACO), fatty acid transporter cluster of differentiation 36 (FAT/CD36), carnitine palmitoyl transferase type 1 (CPT1), citrate synthetase (CS), fatty acid transporter type 1 (FATP1), peroxisome proliferator-activated receptor type α (PPARα), and sterol regulatory element-binding protein type 1c (SREBP1c) qPCRs were performed using primers and protocol as described previously (Ducasse-Cabanot *et al.,* 2007; Kolditz *et al.,* 2008; Lansard *et al.,* 2009; Polakof *et al.,* 2010b, 2011a; Sánchez-Gurmaches *et al.,* 2012). mRNA abundance of MCD was determined using specific primers developed for rainbow trout based on available sequences (BX869708.s.om.10, Sigenae database, INRA). Sequences of the forward and reverse primers, and annealing temperature used for each gene expression are shown in Table 2.

Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq™ DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15s for denaturing, and at specific annealing for 30s and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 95°C) to ensure that only

one fragment was amplified. Each sample was analysed in triplicate. All the replicates of each sample were located in the same plate for each gene to allow comparisons. We included in all the plates the standard curve (by triplicate), and blanks for DNA, PCR and retrotranscription (by duplicate). Only efficiency values between 85-100% were accepted (the R^2 for all the genes assessed was always higher than 0.985). mRNA levels of the target genes were normalized with the housekeeping gene α-elongation factor 1 (EF1α) and expression levels calculated following the Pfaffl method (2001).

Table 2. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Annealing temperature (°C)
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	59
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60
ACO	GCGCCAAGTACTTCCTCAAC	TCACAAACTCCTGTGTGCTG	55
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	55
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	55
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59
FATP1	AGGAGAGAACGTCTCCACCA	CGCATCACAGTCAAATGTCC	60
FBPase	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	59
G6Pase	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	55
G6PDH	CTCATGGTCCTCAGGTTTG	AGAGAGCATCTGGAGCAAGT	59
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	60
PK	CCATCGTCGCGGTAACAAGA	ACATAGGAAAGGCCAGGGGC	59
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; ACO, acetyl-CoA oxidase; CPT1, carnitine plamitoyl transferase type 1; CS, citrate synthetase; EF1α, elongation factor 1α; FAS, fatty acid synthetase; FBPase, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase; G6PDH, glucose 6-phosphate dehydrogenase; GK, glucokinase; MCD, malonyl-CoA dehydrogenase; PK, pyruvate kinase; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c.

Statistics

Comparisons among groups were carried out using Student t test and one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$. When necessary data were log transformed to fulfill the conditions of the analysis of variance.

RESULTS

Experiment 1

The acute administration of FO in concert with a glucose load resulted in lower blood glucose (~50%) and higher triglyceride (about 3-fold) plasma levels at 6 h when compared to the control (Table 3). On the one hand, acute administration of FO did not result in any change of FAS, G6PDH, G6Pase or GK activities. One the other hand, the acute administration of FO in concert with a glucose load resulted in higher G6PDH (1.6-fold), FAS (1.5), CS (2.4-fold) but reduced ACC (2.3-fold) and GK (12.5-fold) transcripts levels (Table 4). Concerning proteins involved in the oxidation of FA and its regulation, mRNAs of both CPTs were down-regulated while the transcription factor PPARα was up-regulated (2.5-fold). No effect was found on the transcription levels of ACLY, MCD, G6Pase or SREBP1c.

Table 3. Plasma glucose (mM), triglycerides (TAG, mM), free fatty acids (FFA, meq L^{-1}), and glycogen levels (µmol glycosyl units·g^{-1} wt), and enzyme activities (mU·mg^{-1} protein or µU·mg^{-1} protein for FAS) in liver of rainbow trout receiving oral administration of fish oil (10 mL·kg^{-1}, one time) for 6h.

		Treatment	
	Parameters	Water	Fish oil
Plasma	Glucose	10.71 ± 0.80	5.68 ± 0.75*
	TAG	1.24 ± 0.17	3.66 ± 0.32*
	FFA	0.18 ± 0.01	0.21 ± 0.01
Liver	Glycogen	94.1 ± 14.1	92.4 ± 9.54
	FAS activity	9.17 ± 3.97	5.34 ± 1.78
	G6PDH activity	151.9 ± 14.5	217.9 ± 46.6
	G6Pase activity	5.44 ± 0.46	4.26 ± 0.57
	GK activity	2.78 ± 0.64	2.30 ± 0.19

Fish were subjected to a glucose tolerance test (glucose solution 250 mg·kg^{-1}, IP) and killed 6h after injection. Fish were fasted before treatments, and tank water was used as control for fish oil treatment. Each value is the mean ± SEM of n=6 fish per treatment. *, significantly different ($P<0.05$) from water group.

Table 4. mRNA levels in liver of rainbow trout receiving oral administration of fish oil (FO, 10 mL·kg^{-1}, one time).

Parameters	Treatment
ACC	-2.3*
ACLY	-1.4
MCD	-1.4
G6PDH	+1.6*
FAS	+1.5*
SREBP1c	-1.1
CPT1a	-1.6*
CPT1b	-5.3*
PPARα	+2.5*
CS	+2.4*
G6Pase	+1.0
GK	-12.5*

Fish were subjected to a glucose tolerance test (glucose solution 250 mg·kg^{-1}, ip) and killed 6h after the injection. Fish were fasted before the treatments, and tank water was used as control for fish oil treatment. Each value is the mean of n=6 fish per treatment Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by EF1α mRNA levels, mRNA levels-no variation). *, significantly different (P<0.05) from control fish.

Experiment 2

Changes in liver metabolites in response to the administration of single FA are shown in Fig. 1. Treatment with oleate or octanoate decreased hepatic FFA levels (Fig. 1A) in a dose-dependent manner, being higher at the lower administration level. Triglycerides (Fig. 1B) and total lipid content (Fig. 1C) were also reduced in a dose-dependent manner but only by oleate or octanoate, respectively. Higher hepatic glucose levels (Fig. 1D) were found after the administration of oleate or octanoate, being higher at the higher administration level. Hepatic glycogen levels were enhanced only by the administration of oleate (Fig 1E). Finally, octanoate treatment has significantly increased lactate levels, in particular at high dose administration (Fig. 1F).

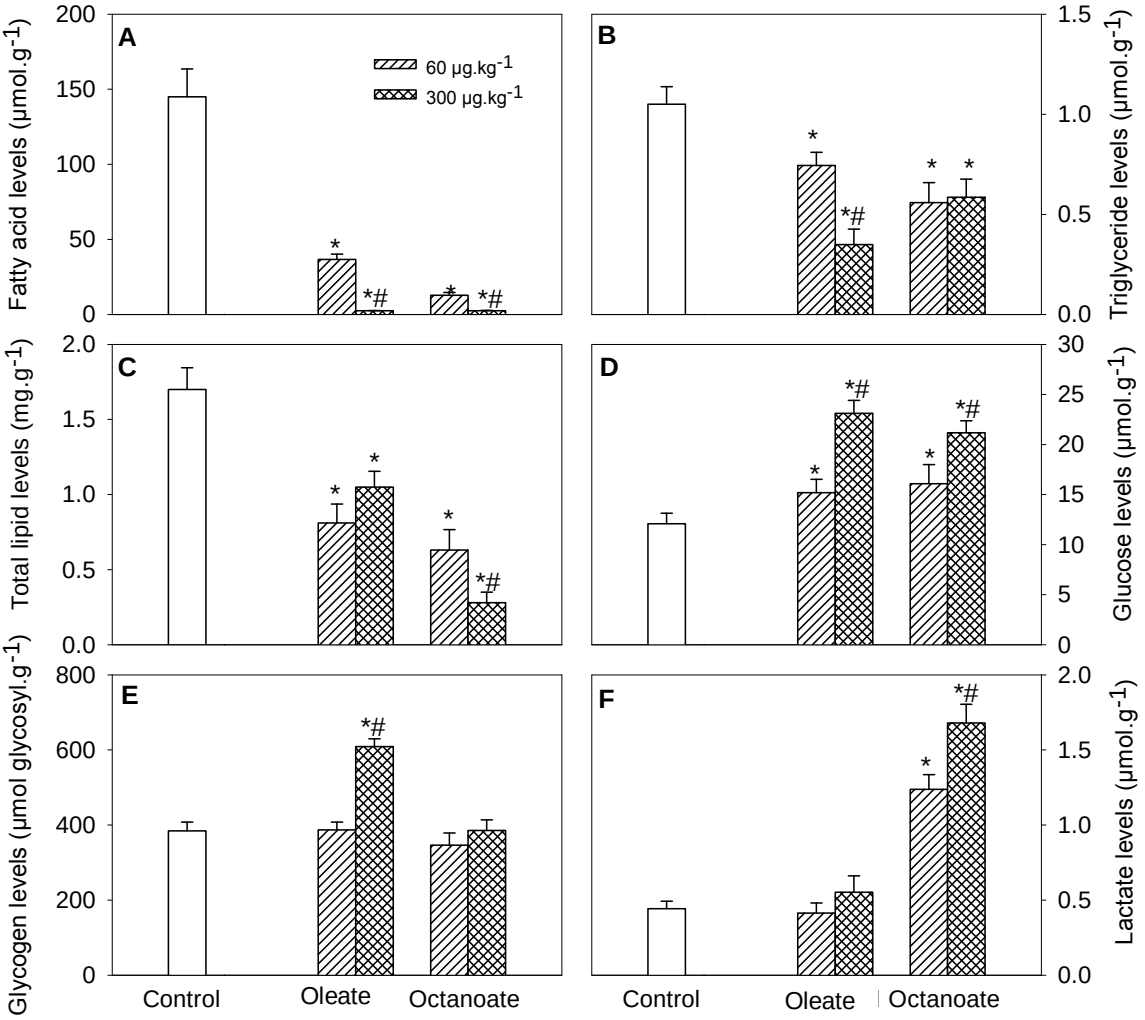

Fig. 1. Levels of fatty acids, triglycerides, total lipids, glucose, glycogen, and lactate in liver of rainbow trout intraperitoneally injected with saline solution alone (control) or containing 60 or 300 µg·kg^{-1} of oleate or octanoate. Fish were sampled 6h after the injection. Each value is the mean + SEM of n=8 fish per treatment. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose at the same treatment.

Enzyme activities in liver are shown in Fig. 2. Treatment with oleate or octanoate has clearly decreased activities of FAS (Fig. 2B; > 30-fold) and PK (Fig. 2G, >2-fold), and GPase activity at high doses (Fig. 2I; about 2-fold). An effect of oleate and octanoate was also found on the activities of ACLY (Fig. 2A), G6PDH (Fig. 2E), MCD activity (Fig. 2D), and FBPase (Fig. 2H). However, while oleate increased the activities of ACLY and G6PDH at low dose and FBPase at both doses (but higher at low dose), octanoate decreased activities of ACLY and FBPase at both doses and activity of G6PDH at high dose. MCD responded in a similar way to oleate and octanoate, being increased at low administration. GK activity

has only responded to low administration of oleate (increased about 2-fold) and no effect of single FA administration was noticed for HOAD activity (Fig. 2C).

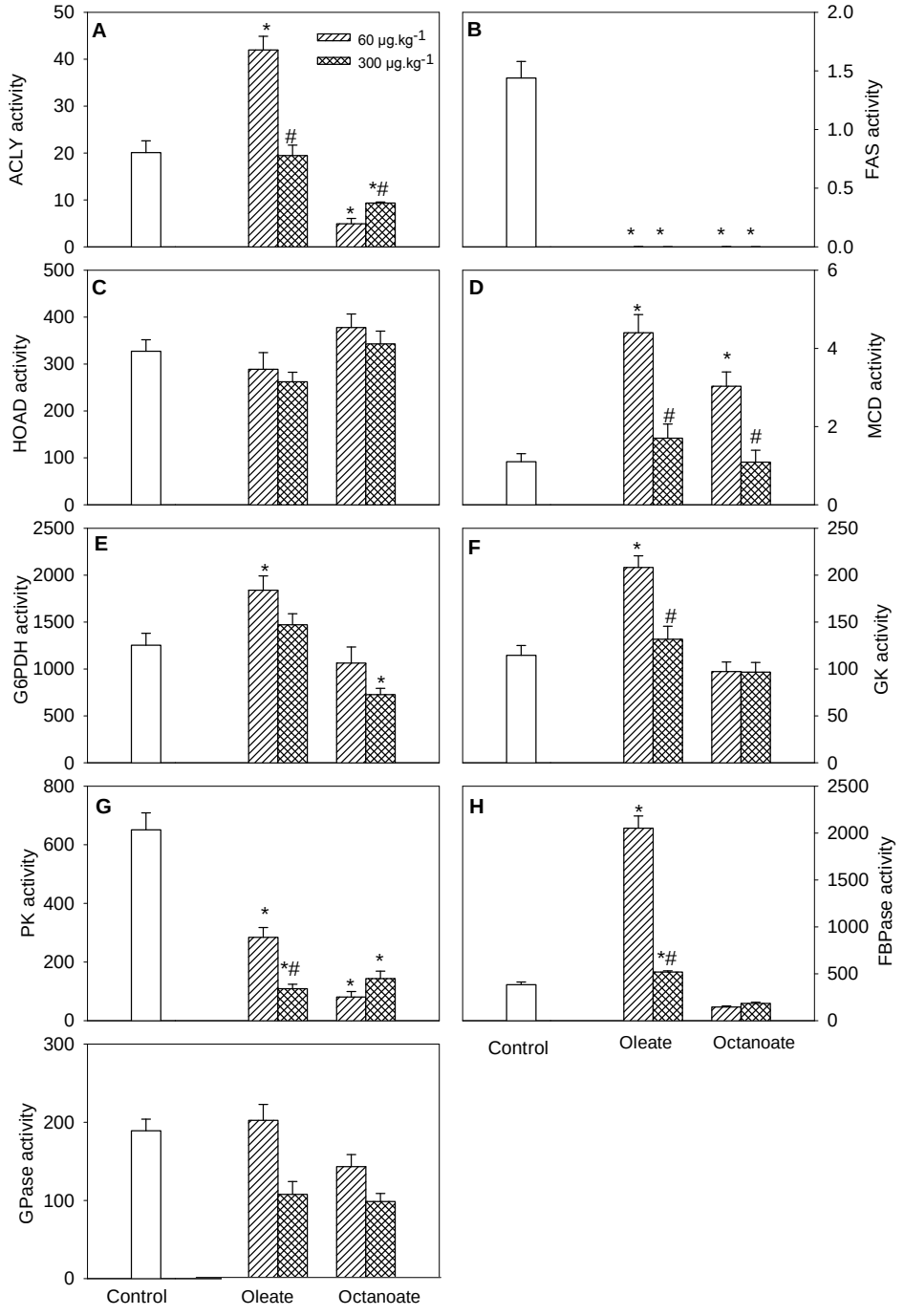

Fig. 2. Activities of ATP-citrate lyase (ACLY), fatty acid synthetase (FAS), hydroxyacyl-CoA dehydrogenase (HOAD), malonyl-CoA decarboxylase (MCD), glucose 6-phosphate dehydrogenase (G6PDH), glucokinase (GK), pyruvate kinase (PK), fructose 1,6-bisphosphatase (FBPase), and glycogen phosphorylase (GPase) in liver of rainbow trout intraperitoneally injected with saline solution alone (control) or containing 60 or 300 µg·kg^{-1} of oleate or octanoate. Fish were sampled 6h after the injection. Each value is the mean + SEM of n=8 fish per treatment. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from low dose at the same treatment.

Changes in mRNA abundance in liver are shown in Table 5. The treatment with oleate or octanoate significantly down-regulated mRNA levels of CPT1a (dose-response for octanoate), CPT1b (dose-response for both fatty acids), CS (not for low dose of oleate), FAS, and GK (dose response for oleate, no effect for the low dose of octanoate) and up-regulated mRNA levels of G6PDH (no effect for the low dose of oleate). Moreover, the treatment with oleate up-regulated mRNA levels of MCD and down-regulated those of FBPase (only low dose). Finally the treatment with octanoate down–regulated mRNA levels of SREBP1c and up-regulated mRNA levels of PPARα (only high dose). No changes were noticed for mRNA abundance of ACC, ACLY, ACO, FAT/CD36, FATP1, and PK.

Table 5. mRNA levels in liver of rainbow trout after Intraperitoneal (IP) administration of saline alone (control) or containing 60 or 300 $\mu g \cdot kg^{-1}$ of oleate or octanoate.

Parameters	Oleate60	Oleate300	Octanoate60	Octanoate300
FAT/CD36	-1.1	+1.2	-1.0	-1.1
FATP1	+1.1	+1.1	+1.2	+1.2
ACC	+1.1	+1.2	+1.3	+1.1
ACLY	+1.4	+1.4	+1.5	+1.4
MCD	+2.3*	+2.0*	+1.1	-1.3
G6PDH	+1.2	+1.8*#	+2.0*	+1.6*
FAS	-5.0*	-5.2*	-3.2*	-3.2*
SREBP1c	+1.2	-1.2	-1.6*	-2.0*
ACO	-1.0	+1.3	-1.2	+1.1
CPT1a	-3.0*	-3.2*	-5.8*	-19.1*#
CPT1b	-3.6*	-6.2*#	-1.7*	-5.0*#
PPARα	-1.1	+1.3	-1.0	+1.5*
CS	-1.1	-2.3*#	-1.9*	-1.6*
GK	-8.0*	-68.6*#	+1.6	-29.8*#
PK	+1.3	-1.0	+1.1	-1.4
FBPase	+1.3	-1.7*#	-1.3	-1.2

Data represent mean of 4 measurements. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by EF1α mRNA levels, mRNA levels-no variation). *, significantly different ($P<0.05$) from control fish. #, significantly different ($P<0.05$) from low dose at the same treatment.

DISCUSSION

Recent findings suggest that interaction between FA and glucose at the metabolic level may result in the development of impaired glucose tolerance and a reduced sensitivity to exogenous insulin in rainbow trout (Figueiredo-Silva *et al.,* 2012b). Impact of FA on hepatic lipid and glucose metabolism seems however to be time-related, and a single high fat meal or acute FA administration shown to induce a better glucose tolerance in fish (Figueiredo-Silva *et al.,* 2012b) as in mammals (Paolisso *et al.,* 1995). Moreover, FA have been shown to play a central role in signalling nutrient abundance at the central level and thus on overall energy homeostasis in both mammals (Pocai *et al.,* 2006; López *et al.,* 2007) and fish (Librán-Pérez *et al.,* 2012). The present study provides relevant information on the possible components by which FA exert their effects on hepatic lipid and glucose metabolism and contribute for a better control of glycaemia in rainbow trout.

A single oral FO administration improves glycaemia directing glucose towards lipogenesis

We evaluated whether oral FO administration modified the response of trout to a glucose tolerance test (GTT) in terms of circulating metabolites and hepatic metabolism. The FO treatment effectively increased plasma triglycerides, validating the experimental design. Interestingly, acute administration of FO reduced (~50%) circulating glucose levels relative to that seen in the control group (oral water). Results agree with recent findings in rainbow trout showing that a single high fat/high carbohydrate meal reduced glycaemia compared with a low fat/high carbohydrate meal (Figueiredo-Silva *et al.,* 2012b), and seem to confirm the idea that an acute administration of FA has a positive impact on the control of glycaemia in fish.

The basis of the FO-induced changes in glycaemia remains elusive. In humans an acute administration of FA has been reported to stimulate insulin secretion (Paolisso *et al.,* 1995). Although circulating insulin levels were not analysed here, changes observed in several insulin-sensitive parameters (at both biochemical and molecular level) suggest that these may be responsible for the fast drop in glycaemia following the administration of FO in concert with glucose load.

The most relevant change noticed in parameters related to glucose metabolism was the drastic decrease in liver GK mRNA levels following FO administration. This agrees with observations in rats where mixture FA administration also reduced GK mRNA levels (Jump *et al.,* 1994). In rainbow trout, hepatic GK activity and expression are generally induced by high glucose circulating levels in the same (Panserat *et al.,* 2000, 2002; Polakof

et al 2010b; Figueiredo-Silva *et al.,* 2012b, 2013) and other fish species (Caseras *et al.,* 2000; Enes *et al.,* 2006) but information on the regulation of GK by FA is still inexistent in fish. Nevertheless, it has been evidenced that hepatic glycolytic potential itself does not fully explain the poor control of glycaemia in fish (Panserat *et al.,* 2000; Figueiredo-Silva *et al.,* 2012b, 2013). Moreover, unlike in mammals (Iynedjian, 2009), GK gene expression in rainbow trout is highly repressed by insulin (about 200-fold) (Lansard *et al.,* 2010, Polakof *et al.,* 2010b). Given the inability of glucose to stimulate GK gene expression during the GTT in FO-treated fish, we hypothesize that an interaction between FA and glucose resulted in elevated insulin levels as documented in humans (Paolisso *et al.,* 1995) and thereby reduced plasma glucose levels. However, some caution must be taken since insulin levels were not assessed here, no PFK-1 or PK activities were assessed, and no changes were noticed in GK activity, suggesting an effect only at molecular level. Also at molecular level, the upregulation of both FAS and G6PDH transcripts following FO administration comply with the idea that the increases in lipogenic potential affected the use of glucose, as suggested in other studies in fish (Panserat *et al.,* 2002, 2009; Polakof *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Figueiredo-Silva *et al.,* 2012b, 2013; Kamalam *et al.,* 2012).

Under the present short-term conditions, the increased lipogenic potential in liver of FO-treated fish was accompanied by decreased FA oxidation potential, illustrated by reduced mRNA levels of CPT1a and CPT1b following FO administration though caution must be taken since no protein measurements are shown. This contrasts with data from studies with long-term high dietary fat supply, which generally increases FA oxidation and decreases FAS, as seen in rainbow trout (Kolditz *et al.,* 2008) or Atlantic salmon (Kennedy *et al.,* 2006), probably as a result of complex nutrient interactions controlling long-term energy homeostasis.

PPARα may function as a transcription factor for the expression of FA oxidation-related genes in fish as seen mammals (Viana Abranches *et al.,* 2011). In the flounder, PPARα stimulation enhances the expression of FA oxidation-related genes such as CPT1a, MCD and ACO (Cho *et al.,* 2012). Strikingly, our results do not indicate such a relationship in trout, since when PPARα was up-regulated the FA oxidation capacity was reduced. Accordingly, the apparently enhanced potential for FA oxidation observed in the present study can be also associated with increased mRNA levels of this transcription factor, as occurs in the liver of Atlantic salmon fed a diet rich in FO (Kennedy *et al.,* 2006).

Similar glycogen levels in the liver of trout given the FO or water (control) also agrees with the idea that excess of circulating glucose is being directed towards lipogenesis rather than stored as glycogen. However, no changes were noticed in mRNA abundance of SREBP1c, which is unexpected since this transcription factor seems to be involved in the regulation of lipid biosynthesis, at least under normal nutritional and physiological conditions (Skiba-Cassy *et al.,* 2009).

Hepatic sensing of FA induce changes in glucose and lipid metabolism

In a second experiment trout received IP single doses of oleate, a representative FA in the FO FA mixture. Oleate is present in vegetable oils (olive oil, rapeseed oil) and like other monounsaturated FA is involved in preventing/promoting insulin resistance (Ryan *et al*., 2000). Effects of oleate were further compared with the effects of octanoate, absent from FO. We recently documented that both FA activate a FA sensing system in the hypothalamus and Brockmann bodies of rainbow trout (Librán-Pérez *et al.,* 2012). Several of these putative components of a FA sensing system are also present in the liver of rainbow rout (Kolditz *et al.,* 2008; Plagnes-Juan *et al.,* 2008; Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Polakof *et al.,* 2010a).

Plasma levels of FA, triglyceride and total lipids increased after an acute IP injection of oleate or octanoate (see data in Librán-Pérez *et al.,* 2012) thus supporting the validity of the experimental design. In contrast, no changes were noticed in glycemia after treatment with any of the FA, though, it should be remembered that no GTT was carried out in the FA experiment. The present results show a reduced glycolytic potential (PK activity) in the presence of both oleate or octanoate whereas an increased gluconeogenic potential (FBPase activity) occurred only in the presence of oleate. Also GK mRNA levels decreased, comparable or even stronger than in the first experiment with FO as well as with other studies in which rainbow trout were fed a high fat diet (Figueiredo-Silva *et al.,* 2012b). This repressive effect on GK mRNA was seen with both FA (though GK activity was clearly enhanced with oleate treatment). In a previous study, both FA also induced similar responses in glucose metabolism in other tissues of rainbow trout such as hypothalamus and Brockmann bodies (Librán-Pérez *et al.,* 2012). This similar effect of MCFA and LCFA in rainbow trout (in contrast with mammals (López *et al.,* 2007)) may be associated with the fact that fish are able to store considerable amounts of MCFA (Davis *et al.,* 1999; Trushenski, 2009) and in rainbow trout there is no preferential oxidation of MCFA (lauric acid) compared with LCFA (Figueiredo-Silva *et al.,* 2012a).

The increase in liver glucose and glycogen (only after oleate treatment) levels may suggest that FA stimulate glucose metabolism for accumulation instead of redirecting to TCA cycle, which is also consistent with the reduced expression of oxidative enzymes, like CS. Changes observed in glycogen levels and in GPase activity would partially support such contention, and are in agreement with increased glycogen levels reported in the same species when fed a high fat diet (Figueiredo-Silva *et al.,* 2012b).

The lipogenic potential was clearly inhibited in liver by treatment with both of the FA as demonstrated by decreased activities of ACLY and FAS and decreased FAS mRNA. However, G6PDH activity in the presence of oleate and G6PDH mRNA levels in the presence of any of the FA used did not show similar decreases than those observed for other lipogenic parameters, actually showing an increase. Considering that SREBP1c in

mammals targets genes expressing lipogenic enzymes such as FAS and ACLY (Kim *et al.,* 2007), a down-regulation of SREBP1c by octanoate treatment (though not with oleate) is in agreement with the decreased lipogenic potential observed in liver.

The acute FA treatment also modified the cytoplasmatic catabolic FA pathway, which was enhanced by the presence of oleate or octanoate, as illustrated by increased MCD activity and mRNA levels. In Atlantic salmon catabolism of FA in liver was principally due to peroxisomal, rather than mitochondrial β-oxidation, and therefore CPT1 may not have a prominent role as a regulatory element of FA oxidation (Kennedy *et al.,* 2006). In the present study, the potential for oxidation, either in the peroxisomes (ACO) or in the mitochondria (CPT1) was not enhanced and in fact actually decreased in mitochondria after treatment with both FA (supported by decreased mRNA levels of CPT1a, CPT1b and CS). This is in accordance with the changes observed in CPTs mRNA levels following acute FO administration.

Since the acute FA treatments did not change the FA transport potential (FAT/CD36 or FATP1 mRNA), the decreased level of hepatic lipids probably results from changes in the pathways within the hepatocyte. In this way, the decreased lipid levels agree with the reduced lipogenic potential but not with the reduced FA use. The explanation for this paradoxical concomitant down-regulation of both lipogenesis and beta-oxidation potentials may be related to changes expected in malonyl-CoA. The highly reduced lipogenic potential (strong repression of the FAS gene and the almost undetectable FAS activity) together with the increase in MCD activity (about 2.5-fold) may lead to an accumulation of malonyl-CoA, known to inhibit CPTs in rainbow trout liver (Gutières *et al.,* 2003) though no changes were noticed in ACC mRNA levels.

In a first approach in which fish were challenged with a glucose and lipid tolerance test, we observed that when both nutrients were administered at the same time, an increased potential for lipogenesis occurred concomitantly with a lower level of glycemia. Since metabolic response to single FA administration of oleate and octanoate was similar, an interaction between FA and glucose rather than FA alone is probably in the origin of results reported here (experiment 1) and in our previous study (Figueiredo-Silva *et al.,* 2012b). Although administration of the individual FA did not enhance lipogenesis nor did it reduce plasma glucose levels as seen with FO, results provide clear evidence that both FA (with minor differences in their effects) even provided at a low dose play a key role in the regulation of several putative components of a FA sensing system present in rainbow trout liver. Further studies are necessary to further characterize the FA-sensing capabilities of rainbow trout liver, as well as to verify whether the observed effects on metabolism are a pure lipid effect or attributable to specific FA.

3.3 TRABAJO EXPERIMENTAL Nº3

In vitro evidences of direct fatty acid-sensing capacity in hypothalamus and Brockmann bodies of rainbow trout

INTRODUCTION

In mammals specialized neurons within the hypothalamus are able to detect changes in plasma levels of long-chain fatty acid (LCFA), but not short-chain (SCFA) or medium-chain (MCFA) FA, thus contributing to nervous control of energy homeostasis (Le Foll *et al.,* 2009). This capacity has been suggested to be achieved through 4 different mechanisms (López *et al.,* 2005, 2007; Le Foll *et al.,* 2009; Blouet and Schwartz, 2010) such as i) FA metabolism through inhibition of carnitine palmitoyltransferase 1 (CPT-1) to import FA-CoA into the mitochondria for oxidation; ii) transport through fatty acid translocase (FAT/CD36); iii) FA-induced activation of novel protein kinase C (PKC) isoforms; and iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity. Changes in the activity of those systems in mammalian hypothalamus in response to enhanced LCFA levels have been associated, through not completely understood mechanisms (López *et al.,* 2005, 2007), with the reduction in food intake through 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). In addition to feeding, central glucose and FA detection has been related, through vagal and sympathetic outflow, to the regulation of glucose homeostasis by affecting insulin release in pancreas and endogenous glucose production in liver (Blouet and Schwartz, 2010) though FA also directly regulate insulin release from pancreatic β-cells (Migrenne *et al.,* 2006).

Fish energy metabolism is rather different than that of mammals since most fish are relatively intolerant to glucose, and they rely more on amino acid and lipid metabolism (Tocher, 2003; Polakof *et al.,* 2011b, 2012b). Furthermore, a reduced food intake has been observed in several fish species fed with lipid-enriched diets or containing high fat stores (Shearer *et al.,* 1997; Rasmussen *et al.,* 2000; Gélineau *et al.,* 2001; Johansen *et al.,* 2002; Figueiredo-Silva *et al.,* 2012b) suggesting that lipid sensor mechanisms regulating food intake may be present in fish. In a previous study in rainbow trout *Oncorhynchus mykiss* (Librán-Pérez *et al.,* 2012) we observed that intraperitoneal acute administration of oleate (LCFA) or octanoate (MCFA) elicited an inhibition in food intake and induced in hypothalamus a response compatible with fatty acid sensing in which fatty acid metabolism, binding to FAT/CD36 and mitochondrial activity were apparently involved, which is similar to that suggested in mammals except for the apparent capacity of rainbow trout to detect changes in MCFA levels. Changes in those hypothalamic pathways can be related to the control of food intake, since food intake was inhibited when FA metabolism was perturbed (using a fatty acid synthetase (FAS) inhibitor) and changes in mRNA levels of specific neuropeptides such as NPY and POMC were also noticed (Librán-Pérez *et al.,* 2012). The results obtained in Brockmann bodies

(BB, main accumulation of pancreatic endocrine cells in this species) also suggested the presence of components of putative fatty acid sensing systems based on fatty acid metabolism and binding to FAT/CD36 (Librán-Pérez *et al.,* 2012), which could be related to insulin release since hyperinsulinemia has been demonstrated in fish after increasing circulating FA levels (Barma *et al.,* 2006). However, we cannot discard that i) those effects of FA on hypothalamus and BB could be attributed to an indirect effect mediated by changes in other endocrine systems, ii) changes were dependent on fatty acid concentration, and iii) the response of BB may be a consequence of vagal and sympathetic outflow from the hypothalamus in a way similar to that described in mammals (Obici *et al.,* 2002) or to a direct action similar to that observed for the stimulation of insulin release by FA (Migrenne *et al.,* 2006). Furthermore, additional information is required regarding the precise mechanisms through which those systems are informing of changes in FA levels.

Therefore, we aimed to evaluate *in vitro* (in the absence of external influences) whether or not hypothalamus and BB respond to changes in FA concentration in a way similar to that previously observed *in vivo*. Accordingly, in a first set of experiments, we evaluated in hypothalamus and BB exposed to increased oleate or octanoate concentrations changes in parameters related to: i) FA metabolism, ii) FA transport iii) nuclear receptors and transcription factors involved in lipid metabolism, iv) a ROS effector, v) components of the K_{ATP} channel, and vi) only in hypothalamus, neuropeptides related to the metabolic control of food intake. In a second set of experiments, we evaluated in hypothalamus *in vitro* the response of those parameters to oleate or octanoate in the presence of selected inhibitors related to fatty acid sensing components.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish weight was 103 ± 4 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed).

Ethics statement

The experiments described were carried out in strict accordance with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 1201/2005) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Experiment 1: *In vitro* incubation of hypothalamus and BB at increased concentrations of oleate or octanoate

Freshly obtained tissues were incubated as previously described (Polakof *et al.,* 2007b). Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. Every morning of an experiment, fish were dipnetted from the tank, anaesthesized with MS-222 (50 $mg \cdot l^{-1}$) buffered to pH 7.4 with sodium bicarbonate, euthanized by decapitation, and weighed. The hypothalamus and BB were removed and dissected as described previously (Polakof *et al.,* 2007b). Tissues were rinsed with modified Hanks´medium (128 mM NaCl; 3.63 mM KCl, 2.81 mM $NaHCO_3$, 0.85 mM $CaCl_2$, 0.55 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.23 mM Na_2HPO_4, 7.5 mM HEPES, 50 $U \cdot ml^{-1}$ penicillin, and 50 $\mu g \cdot ml^{-1}$ streptomycin sulphate, pH 7.4; referred to a basal medium), sliced on chilled Petri dishes, and placed in a chilled Petri dish containing 100 ml of modified Hanks´medium.g^{-1} tissue that was gassed with 0.5% CO_2/99.5% O_2. To ensure adequate mass, tissues were combined from different fish resulting in pools of 3-4 hypothalami and 3-4 BB. Tissues were incubated in 48-well culture plates at 15ºC for 1 h with 250 µl of modified Hanks´medium per well containing 25 mg of tissue that was gassed with a 0.5% CO_2/99.5% O_2 mixture. Control wells contained modified Hanks´medium with 2 mM D-glucose. Treated wells contained medium at the same glucose concentration and increased concentrations (1, 10 or 100 µM) of oleate or octanoate. After 1h incubation, tissues were quickly removed, rinsed, frozen in liquid nitrogen, and stored at -80°C until assayed. FA concentrations were selected based on FA levels assessed in rainbow trout *in vivo* in hypothalamus and BB (Librán-Pérez *et al.,* 2012) and plasma (Boujard *et al.,* 1993; Polakof *et al.,* 2011a; Figueiredo-Silva *et al.,* 2012b), as well as by the concentrations used in comparable *in vitro* studies in rainbow trout (Sánchez-Gurmaches *et al.,* 2010).

On each experiment, one set of 7 tissue pools per tissue was assessed for enzyme activities, another set of 7 tissue pools was used for the assay of tissue metabolites, and another set of 7 tissue pools was used for the assay of mRNA levels. The number of independent experiments (one set of 7 tissue pools each) carried out was five (N=5).

Experiment 2: *In vitro* incubation of hypothalamus with oleate or octanoate alone or in the presence of inhibitors of different pathways of fatty acid signalling and metabolism

Freshly obtained hypothalamus were obtained and incubated as described in experiment 1. Control wells contained medium with 2 mM D-glucose. Treated wells contained medium at the same glucose concentration and 100 µM oleate or 100 µM octanoate alone or in the presence of selected inhibitors of parameters related to fatty acid sensing capacity in mammals. These included: 40 $\mu g.ml^{-1}$ 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75, FAS (fatty acid synthase) inibitor), 10 µM R(+)-2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylic acid (etomoxir, CPT1 inhibitor), 1 µM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, ROS scavenger), 20 µM methyl (1*R*,2*R*,6*S*)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate (genipin, UCP2 (mitochondrial uncoupling protein 2) inhibitor), 500 µM 7-chloro-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide, sulfonylurea receptor (SUR-1) antagonist), 5 µM C, *N*-(((2*E*,4*E*,7*E*)-undeca-2,4,7-trienylidene)amino)nitrous amide (triacsin C, acyl-CoA synthetase (ACS) inhibitor), 50 nM sulfo-N-succinimidyl oleate (SSO, FAT/CD36 inhibitor), and 40 $\mu g.ml^{-}$15-(tetradecyloxy)-2-furoic acid (TOFA, acetyl CoA carboxylase (ACC) inhibitor). Reagents were previously dissolved in modified Hanks´ medium (etomoxir), ethanol (trolox), or DMSO (c75, TOFA, diazoxide, triacsin C, SSO, and genipin); no effects were observed due to the vehicle alone (data not shown). The concentrations of the different agents used were those previously used in fish (Polakof *et al.,* 2007b; Prieto *et al.,* 2009; Sánchez-Gurmaches *et al.,* 2010)and mammals (Landree *et al.,* 2004; Lam *et al.,* 2005; Le Foll *et al.,* 2009). After 1h incubation, tissues were quickly removed, rinsed, frozen in liquid nitrogen, and stored at -80°C until assayed.

On each experiment, one set of 19 tissue pools per tissue (1 control, 1 oleate alone, 1 octanoate alone, 8 oleate plus different agents, 8 octanoate plus different agents) was assessed for enzyme activities, another set of 19 tissue pools was used for the assay of tissue metabolites, and another set of 19 tissue pools was used for the assay of mRNA levels. The number of independent experiments (one set of 19 tissue pools each) carried out was five (N=5).

Assessment of metabolite levels and enzyme activities

Samples used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vol of ice-cooled 0.6M perchloric acid, and neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged (10,000 g), and the supernatant used to assay tissue metabolites. FA, total lipids, and triglyceride levels were determined enzymatically using commercial kits (Wako Chemicals for FA, and Spinreact for total lipids and triglyceride) adapted to a microplate format.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA complex (DTNB CoA complex) at 412 nm. The reactions were started by the addition of supernatant (15 µl) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ATP-citrate lyase (ACLY, *EC* 4.1.3.8), FAS (*EC* 2.3.1.85), and hydroxyacil-CoA dehydrogenase (HOAD, *EC* 1.1.1.35) activities were determined as described previously (Alvarez *et al.,* 2000; Kolditz *et al.,* 2008; Polakof *et al*., 2010b).CPT (*EC* 2.3.1.21) activity was assessed in a tris-HCl buffer (75 mM, pH 8.0) containing 1.5 mM EDTA, 0.25 mM DTNB, 0.035 mM palmitoyl CoA and 0.7 (hypothalamus) or 2 (BB) mM L-carnitine (omitted for controls).

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies) and treated with RQ1-DNAse (Promega). Two µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexaprimers (Life Technologies). 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 (Fermentas), in a total PCR reaction volume of 25 µl, containing 50-500 nM of each primer. mRNA abundance of ACC, ACLY, CART, FAT/CD36, CPT1, citrate synthase (CS), FAS, inward rectifier K^{+} channel pore type 6.-like (Kir6.x-like), liver X receptor α (LXRα), malonyl CoA dehydrogenase (MCD), NPY, POMC, peroxisome proliferator-activated receptor type α (PPARα), sterol regulatory element-binding protein type 1c (SREBP1c), sulfonylurea receptor-like (SUR-like), and UCP2a were determined as previously described in the same species (Ducasse-Cabanot *et al.,* 2007; Kolditz *et al.,* 2008; Cruz-Garcia *et al.,* 2009; Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Polakof *et al.,* 2010a, 2011a; Figueiredo-Silva *et al.,* 2012c; Librán-Pérez *et al.,* 2013a). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1. Relative quantification of the

target gene transcripts 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 15 min using hot-start iTaq^{TM} DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15s for denaturing, annealing at specific temperatures (Table 1) for 30s, and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analyzed in triplicate. All the replicates of each sample were located in the same plate for each gene to allow comparisons. We included in all the plates the standard curve (by triplicate), and controls for NTC and RT negative control (by duplicate). Only efficiency values between 85-100% were accepted (the R^2 for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl method (Pfaffl, 2001) .

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Annealing Tª(°C)	Accession Number (GenBank or others)
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	59	tcbk0010c.b.21_5.1.om.4- Sigenae
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	CA349411.1
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	NM_001124627
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55	AF327058
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	55	AF606076
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59	AJ619768
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	59	AJ620356
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	55	TC89195 Tigr
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59	tcab0001c.e.065.1.s.om.8 .Sigenae
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62	AY606034.1 DFCI
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60	CA346261.1.s.om.8:1:773:1Sigenae
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	62	FJ470291
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	60	BX869708.s.om.10 Sigenae
NPY	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58	NM_001124266
POMC	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	TC86162 Tigr
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	CA048941.1
Sur-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	62	tcce0019d.e.20_3.1.s.om.8.Sigenae
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; CART, cocaine- and amphetamine-related transcript; CPT1, carnitine palmitoyl transferase type 1; CS, citrate synthetase; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; Kir6.x-like, inward rectifier K^+ channel pore type 6.-like; LXRα, liver X receptor α; MCD, malonyl CoA dehydrogenase; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; SUR-like, sulfonylurea receptor-like; UCP2a, mitochondrial uncoupling protein 2a

Statistics

Comparisons among groups were carried out using Student t test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$. When necessary data were log transformed to fulfill the conditions of the analysis of variance.

RESULTS

Experiment 1: In vitro incubation of hypothalamus and BB at increased concentrations of oleate or octanoate

Changes in metabolite levels and enzyme activities assessed in hypothalamus are shown in Fig. 1. Treatment with oleate or octanoate elicited increased levels of FA (Fig. 1A, dose-response), triglycerides (Fig. 1B, dose-response) and total lipids (Fig. 1C), and activities of ACLY (Fig. 1D, dose-response) and FAS (Fig. 1E), and decreased CPT activity (Fig. 1G, dose-response). No significant differences were noticed for HOAD activity (Fig. 1F).

Changes in mRNA abundance in hypothalamus are shown in Table 2. A down-regulation after oleate or octanoate treatment was observed for mRNA levels of FAT/CD36 (dose-response for oleate), ACC, ACLY (dose-response), CS, FAS (dose-response), UCP2a, Kir6.x-like (dose-response for octanoate), SUR-like (dose-response for oleate), LXRα (dose-response), and only after oleate treatment for NPY (dose-response). An up-regulation after oleate or octanoate treatment was observed for CPT1c, MCD (dose-response), PPARα (dose-response), CART (dose-response for oleate), and POMC (dose-response for octanoate), and only after octanoate treatment for CPT1d and SREBP1c.

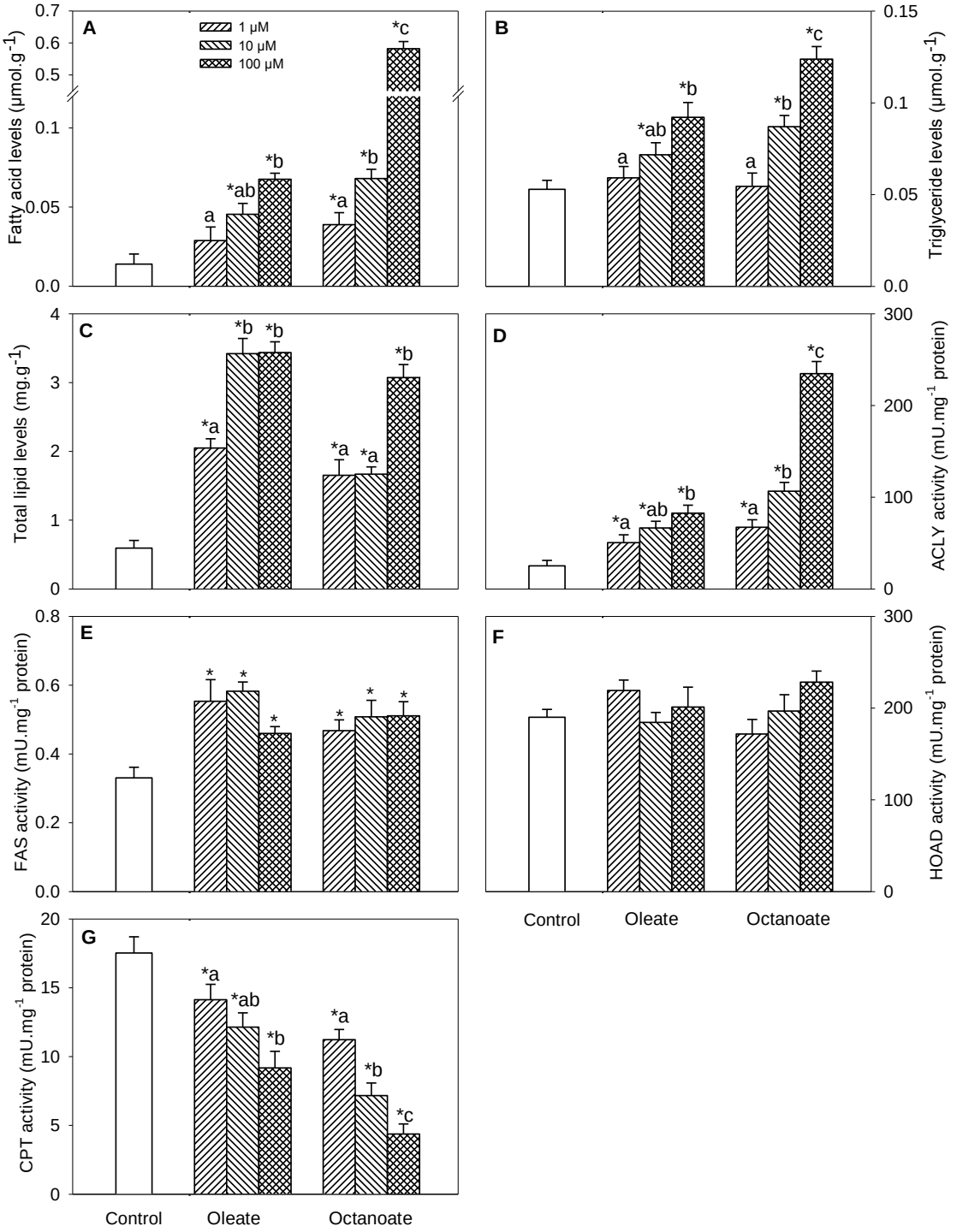

Fig. 1. Levels of metabolites and enzyme activities in hypothalamus incubated with oleate or octanoate. Levels of fatty acid (A), triglyceride (B) and total lipid (C), and activities of ACLY (D), FAS (E), HOAD (F), and CPT (G) in hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate. Each value is the mean + S.E.M. of 5 independent experiments carried out with pools of hypothalami from 3-4 different fish. *, significantly different (P<0.05) from control fish. Different letters indicate significant differences (P<0.05) among fatty acid concentration within each fatty acid treatment.

Table 2. mRNA levels in hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate.

	Oleate (µM)			Octanoate(µM)		
	1	**10**	**100**	**1**	**10**	**100**
Fatty acid transport						
FAT/CD36	-1.78*a	-2.21*ab	-2.94*b	-2.56*a	-3.12*b	-3.03*ab
Fatty acid metabolism						
ACC	-1.13a	-1.85*b	-1.57*b	-1.33*a	-1.37*a	-1.69*b
ACLY	-1.05a	-1.41*ab	-1.79*b	-1.13	-1.48*ab	-2.03*b
CPT1c	-1.03a	+1.06a	+1.48*b	+1.59*	+1.74*	+1.43*
CPT1d	-1.06	-1.02	+1.01	+5.01*	+4.98*	+4.82*
CS	-1.81*	-1.98*	-2.12*	-1.54*	-1.81*	-1.56*
FAS	+1.04a	-1.22ab	-1.43*b	-1.13a	-1.18a	-1.46*b
MCD	+1.02a	+1.71*b	+2.61*b	+1.25a	+1.87*b	+2.03*b
Mitochondrial uncoupling						
UCP2a	+1.00a	-2.12*b	-2.22*b	-1.85*	-1.97*	-2.42*
K_{ATP} channel						
Kir6.x-like	-1.33*	-1.42*	-1.66*	-1.47*a	-1.82*ab	-2.50*b
SUR-like	-1.16a	-1.62*b	-1.80*b	+1.07a	-1.48*b	-1.39*b
Transcription factors						
LXRα	-1.35*a	-1.49*a	-1.87*b	-1.23a	-1.37*a	-1.64*b
PPARα	+1.04a	+1.32*b	+1.41*b	+1.01a	+1.41*b	+1.50*b
SREBP1c	+1.17	+1.01	+1.02	+1.58*	+1.44*	+1.56*
Food intake control						
CART	+1.31a	+1.44*ab	+1.78*b	+1.19	+1.39*	+1.57*
NPY	-1.14a	-1.39*a	-1.98*b	-1.08	+1.07	+1.12
POMC	+1.55*	+1.43*	+1.49*	+1.51*a	+1.73*ab	+2.32*b

Each value is the mean of 5 independent experiments carried out with pools of hypothalami from 3-4 different fish. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalized by β-actin mRNA levels, mRNA levels-no variation). *, significantly different (P<0.05) from control fish. Different letters indicate significant differences (P<0.05) among concentration within each fatty acid treatment.

Changes in metabolite levels and enzyme activities assessed in BB are shown in Fig. 2. Treatment with oleate or octanoate elicited increased levels of FA (Fig. 2A, dose-response for octanoate), triglycerides (Fig. 2B, dose-response) and total lipids (Fig. 2C, dose-response), and activities of ACLY (Fig. 2D, dose-response), FAS (Fig. 2E, dose-response for octanoate), and CPT (Fig. 2G), and only after oleate treatment for HOAD activity (Fig. 2F).

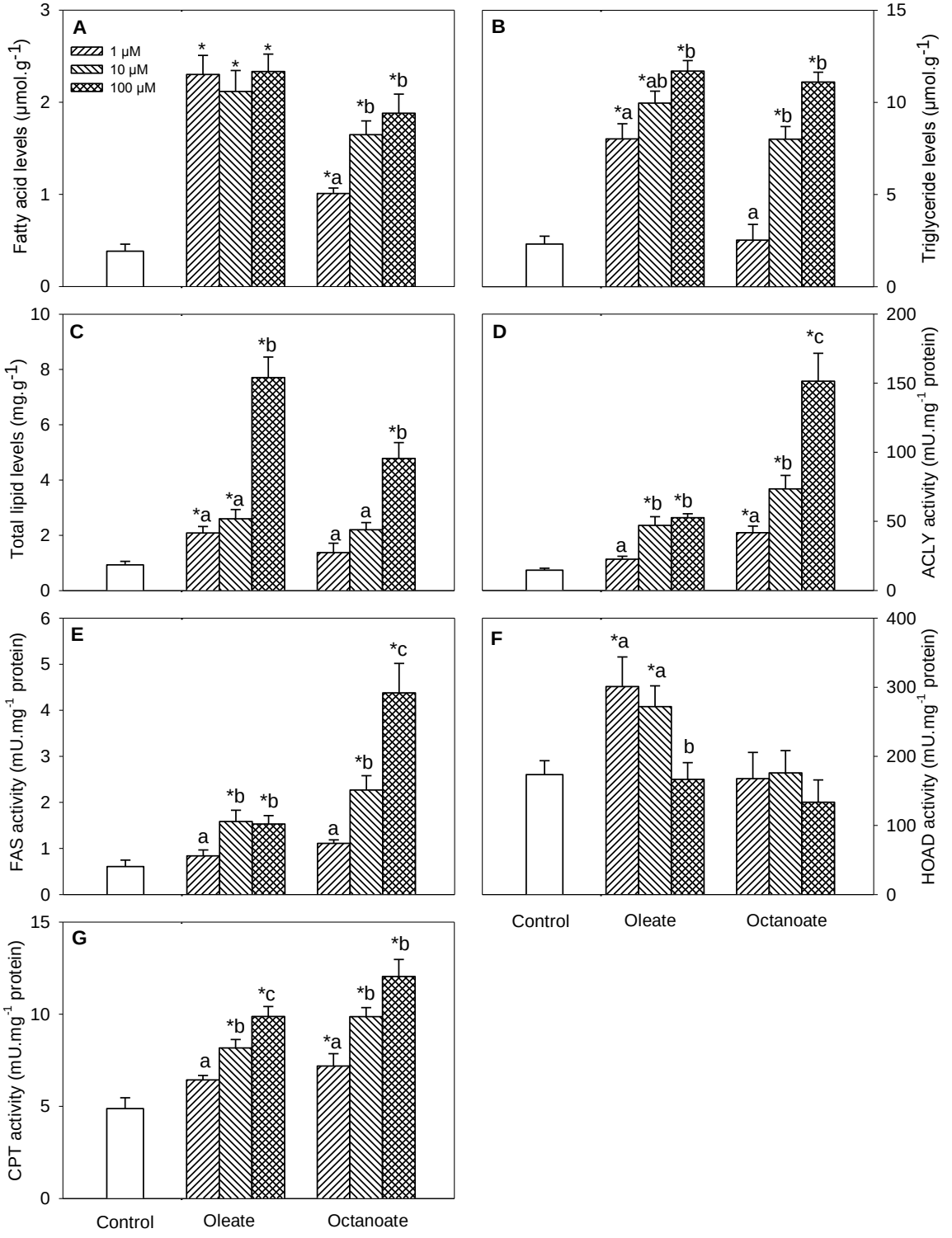

Fig. 2. **Levels of metabolites and enzyme activities in Brockmann bodies incubated with oleate or octanoate.** Levels of fatty acid (A), triglyceride (B) and total lipid (C), and activities of ACLY (D), FAS (E), HOAD (F), and CPT (G) in Brockmann bodies of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate. Each value is the mean + S.E.M. of 5 independent experiments carried out with pools of Brockmann bodies from 3-4 different fish. *, significantly different ($P<0.05$) from control fish. Different letters indicate significant differences ($P<0.05$) among fatty acid concentration within each fatty acid treatment.

Changes in mRNA abundance in BB are shown in Table 3. A down-regulation after oleate or octanoate treatment was observed for mRNA levels of ACC (dose-response for octanoate), ACLY (dose-response for oleate), Kir6.x-like (dose-response for oleate), SUR-like (dose-response), PPARα and SREBP1c, and only after octanoate treatment for CPT1a. An up-regulation after oleate or octanoate treatment was observed for CPT1b, CS, FAS (dose-response for oleate), only after oleate treatment for CPT1a, and only after octanoate treatment for LXRα (dose-response). mRNA levels of FAT/CD36 after treatment with any of the fatty acids and mRNA levels of MCD after oleate treatment displayed changes at only one fatty acid concentration.

Table 3. mRNA levels in Brockmann bodies of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate.

	Oleate (µM)			**Octanoate(µM)**		
	1	**10**	**100**	**1**	**10**	**100**
Fatty acid transport						
FAT/CD36	+1.20a	+1.98*b	-1.17a	-1.49*	-1.33	-1.07
Fatty acid metabolism						
ACC	-1.47*a	-1.45*a	-2.70*b	-1.65*a	-2.08*ab	-2.57*b
ACLY	-1.24a	-1.38*a	-1.87*b	-1.53*	-1.59*	-1.41*
CPT1a	+1.78*a	+4.68*b	+2.08*a	-3.45*a	-1.60*b	-2.32*b
CPT1b	+1.07a	+1.61*b	+1.79*b	+1.61*	+2.32*	+1.85*
CS	+1.39*	+1.42*	+1.63*	+2.11*	+2.27*	+2.18*
FAS	+1.16a	+2.75*b	+3.29*b	+1.06a	+1.58*b	+1.52*b
MCD	-1.09a	-1.12a	-2.16*b	+1.08	+1.09	+1.26
K_{ATP} channel						
Kir6.x-like	-1.04a	-1.39*b	-1.55*b	-1.11a	-2.23*b	-2.03*b
SUR-like	-1.03a	-1.51*b	-1.79*b	-1.16a	-1.56*b	-2.56*c
Transcription factors						
LXRα	-1.11	-1.08	+1.10	+1.86*a	+2.06*a	+2.59*b
PPARα	-1.87*ab	-1.59*a	-2.47*b	-1.79*	-1.52*	-1.83*
SREBP1c	-4.18*	-4.99*	-4.70*	-3.97*	-4.43*	-3.53*

Each value is the mean of 5 independent experiments carried out with pools of Brockmann bodies from 3-4 different fish. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalized by β-actin mRNA levels, mRNA levels-no variation). *, significantly different (P<0.05) from control fish. Different letters indicate significant differences (P<0.05) among concentration within each fatty acid treatment.

Experiment 2: In vitro incubation of hypothalamus with oleate or octanoate alone or in the presence of inhibitors of different pathways of fatty acid signalling and metabolism

In all cases, the effects of oleate or octanoate alone compared with controls were similar than those assessed in the first experiment. Considering that we evaluated treatment with oleate or octanoate alone or in the presence of 8 different inhibitors, representing with standard figures all the results obtained in this experiment would mean the use of an unmanageable amount of multiple figures. Therefore, in favour of simplicity, the effects of inhibitors are summarized in table 4 describing in which cases the presence of the inhibitor significantly ($P<0.05$) counteracted the effects produced by the treatment with 100 µM oleate or 100 µM octanoate alone. HOAD activity, for which no significant differences were found in experiment 1 was not assessed in this experiment.

C75 in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only octanoate) and total lipids, activities of ACLY, CPT, and FAS, and mRNA levels of FAT/CD36 (only oleate), ACC, CS (only octanoate), FAS (only oleate), MCD, LXRα (only octanoate), and NPY (only oleate).

Etomoxir in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only octanoate) and total lipids (only octanoate), activities of ACLY, CPT, and FAS, and mRNA levels of ACC (only octanoate), CPT1c, CS, FAS (only oleate), MCD, LXRα (only oleate), NPY (only octanoate), and POMC (only octanoate).

Trolox in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides and total lipids (only for octanoate in both cases), FAS activity (only octanoate), and mRNA levels of UCP2a, Kir6.x-like (only oleate), SUR-like, LXRα, PPARα (only octanoate), SREBP1c, NPY (only oleate), and POMC.

Genipin in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only octanoate) and total lipids (only octanoate), FAS activity (only octanoate), and mRNA levels of FAS (only octanoate), UCP2a, Kir6.x-like, SUR-like, LXRα (only octanoate), PPARα (only oleate), SREBP1c, NPY (only oleate), and POMC.

Diazoxide in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only oleate), FAS activity (only oleate), and mRNA levels of FAT/CD36, ACLY, MCD (only oleate in the three cases), UCP2a, Kir6.x-like, SUR-like, LXRα, PPARα, SREBP1c, NPY, and POMC (only oleate in the last four cases).

Triacsin C in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only oleate) and total lipids (only octanoate), CPT activity, and mRNA levels of FAT/CD36 (only oleate), ACLY, CPT1c, CPT1d (only octanoate), CS, FAS, MCD, PPARα (only oleate), CART (only octanoate), NPY (only oleate), and POMC (only octanoate).

SSO in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only oleate), and mRNA levels of FAT/CD36, Kir6.x-like (only oleate), LXRα, PPARα, SREBP1c, NPY (only oleate), and POMC (only oleate). TOFA in the presence of oleate or octanoate counteracted the effects of FA alone for levels of triglycerides (only oleate), activities of ACLY, CPT (only oleate), and FAS (only octanoate), and mRNA levels of FAT/CD36 (only oleate), ACC, CPT1c (only oleate), CS, FAS (only oleate), MCD (only oleate), Kir6.x-like (only octanoate), NPY, and POMC.

Table 4. Response of metabolite levels, enzyme activities, and mRNA abundance of several parameters related to fatty acid sensing in hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 100 µM oleate (Ol) or 100 µM octanoate (Oc) alone (controls) or 100 µM oleate or 100 µM octanoate and selected inhibitors related to fatty acid sensing capacity in mammals. These included: 40 µg.ml^{-1} C75 (FAS inhibitor), 50 µM etomoxir (CPT1 inhibitor), 1 µM trolox (ROS scavenger), 20 µM genipin (UCP2 inhibitor), 500 µM diazoxide (sulfonyl urea receptor 1 antagonist), 5 µM triacsin C (ACS inhibitor), 50 nM SSO (FAT/CD36 inhibitor), and 40 µg.ml^{-1} TOFA (ACC inhibitor).

	Inhibitors																	
	C75		Etomoxir		Trolox		Genipin		Diazoxide		Triacsin C		SSO		TOFA	
Param.	**Ol**	**Oc**	**Ol**	**Oc**	**Ol**	**Oc**	**Ol**	**Oc**	**Ol**	**Oc**	**Ol**	**Oc**	**Ol**	**Oc**	**Ol**	**Oc**
Metabolite levels																
Fatty acid																
Triglycer.		+		+		+		+	+		+		+		+	
T.lipid	+	+		+		+	+	+				+				
Enzyme activities																
ACLY	+	+	+	+											+	+
CPT	+	+	+	+							+	+			+	
FAS	+	+	+	+		+		+	+							+
mRNA abundance																
FAT/ CD36	+								+		+		+	+	+	
ACC	+	+		+											+	+
ACLY									+		+	+				
CPT1c			+	+							+	+			+	
CPT1d												+				
CS		+	+	+							+	+			+	+
FAS	+		+					+			+	+			+	
MCD	+	+	+	+					+		+	+			+	
UCP2a					+	+	+	+	+	+			+			
Kir6.xlike					+		+	+	+	+						+
SUR-like					+	+	+	+	+	+						
LXRα		+	+		+	+		+	+	+			+	+		
PPARα						+	+		+		+		+	+		
SREBP1c					+	+	+	+	+				+	+		
CART												+				
NPY	+			+	+		+		+		+		+		+	+
POMC				+	+	+	+	+	+			+	+		+	+

Only those parameters for which significant effects of oleate or octanoate treatment alone were noticed (Fig. 1 and Table 2) were evaluated for inhibitor action. Values represent the mean of 5 independent experiments carried out with pools of hypothalami from 3-4 different fish. +, inhibitor significantly ($P<0.05$) counteracted the effect of oleate or octanoate alone. C75, 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid. Etomoxir, R(+)-2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylic acid). Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Genipin, methyl (1*R*,2*R*,6*S*)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate. Diazoxide, 7-chloro-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide. Triacsin C, *N*-(((2*E*,4*E*,7*E*)-undeca-2,4,7-trienylidene)amino)nitrous amide). SSO, sulfo-N-succinimidyl oleate. TOFA, 5-(tetradecyloxy)-2-furoic acid

DISCUSSION

The effects of oleate or octanoate on parameters related to lipid sensing in hypothalamus in vitro are similar to those previously observed in vivo

The treatment with oleate or octanoate is reflected in the hypothalamus by increased levels of FA, triglycerides and total lipids. These changes support an increased entry of FA that may elicit responses consistent with FA sensing mechanisms. Accordingly, the treatment with both FA elicited in most parameters dose-dependent changes, which were similar for both FA, and also in general similar to those observed previously *in vivo* (Librán-Pérez *et al.,* 2012) for levels of FA and total lipids, activities of ACLY, FAS, and HOAD, and mRNA levels of ACLY, FAS, NPY, PPARα, LXRα, CS, Kir6.x-like, and SUR-like, and only after treatment with oleate for mRNA levels of CPT1d and POMC. Only few parameters displayed different responses than those previously observed *in vivo* after treatment with both FA, such as for levels of triglycerides and mRNA levels of CART, SREBP1c, and CPT1c. The finding of similar results *in vitro* than those observed *in vivo* allow us to suggest that the action of FA on putative fatty acid sensing mechanisms in hypothalamus of rainbow trout is direct and not mediated by other endocrine systems or nutrients.

In the fatty acid sensing system characterized in mammals based on FA metabolism (López *et al.,* 2005, 2007), increased LCFA levels inhibit the ability of hypothalamic CPT1 to import FA-CoA into mitochondria for oxidation, which can lead to inhibition of food intake through effects on the expression of orexigenic and anorexigenic neuropeptides. This system was apparently working in rainbow trout hypothalamus *in vitro* since the most important parameters involved in such mechanism displayed changes compatible with those of the mammalian model, except for the fact that in mammals the system does not respond to MCFA like octanoate (López *et al.,* 2007) as it does in rainbow trout. These changes include the decreased mRNA levels of ACLY and CS (Hu *et al*., 2011) whereas the decreased mRNA levels of FAS are also in agreement with the pharmacological inhibition observed in mammals (López *et al.,* 2005, 2007). mRNA levels of CPT1c as well as activities of FAS and ACLY did not respond in the same way than in the mammalian model. The changes in FAS and ACLY activities were similar than those previously observed *in vivo* whereas the increase noticed in mRNA levels of CPT1c was unexpected compared with previous *in vivo* results (Librán-Pérez *et al.,* 2012). However, CPT activity (not assessed in our previous *in vivo* study) did show a clear inhibition after treatment with both FA.

The mechanism for FA sensing through FAT/CD36 (Le Foll *et al.,* 2009) is also apparently working in hypothalamus of rainbow trout *in vitro* since increased mRNA levels of PPARα and SREBP1c (the later only for octanoate) and decreased mRNA levels of

LXRα were noted, which are compatible with those observed in the mammalian model (Wolfrum, 2007; Miyauchi *et al.,* 2010) though in mammals no effects of octanoate are present. The increase observed in mRNA levels of PPARα in parallel with increases in mRNA levels of related genes such as CPT1 and MCD is also in agreement with that observed in liver of rainbow trout (Morash and McClelland, 2011) and flounder (Cho *et al.,* 2012). However, mRNA levels of FAT/CD36 displayed a clear decrease in response to increased FA levels which was different to that previously observed *in vivo* (Librán-Pérez *et al.,* 2012).

Another mechanism for lipid sensing in mammals is through mitochondrial production of ROS by electron leakage resulting in an inhibition of K^+_{ATP} (Blouet and Schwartz, 2010). Hypothalamus of rainbow trout exposed to increased levels of oleate or octanoate displayed a clear inhibition of mRNA levels of the components of the K^+_{ATP} channel, which is in agreement with that expected for oleate but not octanoate in the mammalian model (Benani *et al.,* 2007). We also observed a clear down regulation of UCP2a mRNA levels in hypothalamus exposed to oleate or octanoate in agreement with the known effects of increased ROS levels as described in mammalian models (Benani *et al.,* 2007). It is interesting to mention that activated UCP2 may reduce mitochondrial ROS levels in zebrafish brain (Tseng *et al.,* 2011).

The activation of FA sensing mechanisms in mammalian hypothalamus has been related to the control of food intake through not well understood mechanisms involving the control of the expression of anorexigenic (POMC and CART) and orexigenic (AGRP and NPY) factors (López *et al.,* 2005, 2007). In our previous study (Librán-Pérez *et al.,* 2012), we observed that the inhibition of food intake in rainbow trout elicited by *in vivo* treatment with oleate (but not octanoate) acid coincided with decreased mRNA levels of NPY and increased levels of POMC. Moreover, Figueiredo-Silva *et al.* (Figueiredo-Silva *et al*., 2012c) also observed changes in mRNA levels of NPY and CART in hypothalamus of rainbow trout fed a lipid-enriched diet. In the present study, we observed that the presence of oleate or octanoate stimulated mRNA levels of anorexigenic peptides (POMC and CART) whereas mRNA levels of the orexigenic NPY decreased after oleate treatment. The differences with the results obtained *in vivo* could indicate that the enhanced FA levels are inducing changes in the expression *in vitro*, i.e. the mechanisms are present in the tissue and respond to FA, but under *in vivo* conditions other systems are interacting with the expression of those neuropeptides and therefore the response is masked. In any case, changes in mRNA levels of neuropeptides are in agreement with the reduction of food intake addressed *in vivo* for oleate (Librán-Pérez *et al.,* 2012) lending further support for the physiological role of these lipid sensing systems in rainbow trout. In mammals changes in CPT1c appear to modulate the expression of neuropeptides in hypothalamus (Chari *et al.,* 2010). In our previous *in vivo* study there were no effects of octanoate treatment on mRNA levels of CPT1c and neuropeptides whereas in the present study both parameters were affected by octanoate treatment. Therefore, the FA metabolic

sensing system was apparently fully activated by LCFA and MCFA *in vitro* though *in vivo* the responses of neuropeptides were not apparent after MCFA treatment. Therefore, the response *in vivo*, at least for the octanoate, displays an interaction with other systems/factors.

The effects of oleate or octanoate on parameters related to lipid sensing in Brockmann bodies in vitro are similar to those observed in hypothalamus but different than those previously observed in vivo in Brockmann bodies

In mammals lipid metabolism in the β-cell is critical for the normal regulation of insulin secretion (MacDonald *et al.,* 2008) and FA directly act to regulate insulin release from pancreatic β-cells [5]. In fish, the studies available described that lipid metabolism is indeed important in BB (Polakof *et al.,* 2008b, 2012a) where insulin release is also influenced by circulating FA levels (Barma *et al.,* 2006; Caruso and Sheridan, 2011).

In the present study, the treatment with oleate or octanoate elicited increased levels of FA, triglycerides and total lipids in a way similar to that observed in hypothalamus but different than that described in mammals where pancreatic cells do not respond to MCFA such as octanoate (MacDonald *et al.,* 2008). Changes observed in BB in metabolite levels, enzyme activities and mRNA levels were in general similar to those observed in hypothalamus thus supporting direct FA sensing in this tissue. However, in contrast to that observed in hypothalamus, changes observed in most parameters assessed *in vitro* in BB were in general different (in certain cases even converse) than those observed previously *in vivo* in the same tissue (Librán-Pérez *et al.,* 2012) such as for levels of total lipids, activities of ACLY, FAS (oleate), and HOAD (oleate), and mRNA levels of ACLY, FAS, SREBP1c, PPARα, LXRα, FAT/CD36 (oleate), CS, Kir6.x-like, SUR-like, CPT1a, and CPT1b. Only few parameters displayed similar responses than those previously observed *in vivo* after treatment with oleate or octanoate (Librán-Pérez *et al.,* 2012), such as for levels of FA and triglycerides, and only after octanoate treatment for FAS and HOAD activities, and mRNA levels of FAT/CD36. Those responses can be generalized to the three putative FA sensing systems evaluated (FA metabolism, transport through FAT/CD36, and mitochondrial activity).

The finding of such a different response *in vitro* than that observed *in vivo* allow us to suggest that the action of FA on putative fatty acid sensing mechanisms in BB of rainbow trout observed *in vivo* is influenced by other endocrine systems like insulin (Barma *et al.,* 2006; Caruso and Sheridan, 2011).However, we may also hypothesize that the *in vivo* response of BB could be a consequence of vagal and sympathetic outflow from the hypothalamus in a way similar to that described in mammals (Le Foll *et al.,* 2009).

The use of specific inhibitors provide further evidence for the specific mechanisms of fatty acid sensing in rainbow trout hypothalamus

To obtain additional information regarding the mechanisms through which putative FA-sensing systems might directly inform of changes in FA levels, we incubated hypothalamus *in vitro* with oleate or octanoate alone or in the presence of specific inhibitors and antagonists of the FA-sensing mechanisms present in mammalian hypothalamus. As for the use of those inhibitors in fish, we previously demonstrated in rainbow trout the effects of C75 and TOFA on food intake (Librán-Pérez *et al.,* 2012), and the inhibitory effects of diazoxide in mRNA levels of components of the K^{+}_{ATP} channel (Polakof *et al.,* 2007b) whereas other studies validated the use of etomoxir (Sánchez-Gurmaches *et al.,* 2010), trolox (Prieto *et al.,* 2009), SSO (Zhou *et al.,* 2010), and triacsin C (Grove and Sidell, 2004). However, there are no references available, as far as we are aware, for the use of genipin in fish. Considering the high number of multiple comparisons carried out it is possible that some of the significant differences observed might be false positives. This fact precluded us for discussing specific actions of all antagonists tested in the response of all parameters assessed. However, several general trends are apparent from the results obtained, and these can be summarized as follows: i) All the antagonists in the presence of oleate or octanoate counteracted the action of the FA alone for several of the parameters assessed, and for many of the antagonists the action was comparable for oleate or octanoate with the exception of trolox, which was more effective counteracting the effects of octanoate, and diazoxide, which was more effective counteracting the effects of oleate. ii) The inhibitors related to FA metabolism such as C75, etomoxir, triacsin C and TOFA generally counteracted the effects of oleate or octanoate alone in parameters related to that mechanism (levels of metabolites, enzyme activities and mRNA levels of ACC, ACLY, CPT1c, CPT1d, CS, and FAS). Moreover, those inhibitors counteracted few of the effects of oleate or octanoate alone on parameters related to other FA sensing mechanisms such as those involved in FAT/CD36 transport or mitochondrial activity (mRNA levels of FAT/CD36, UCP2a, Kir6.x-like, SUR-like, LXRα, PPARα or SREBP1c). iii) The inhibitors related to transport through FAT/CD36 and mitochondrial activity such as trolox, genipin, SSO, and diazoxide generally counteracted the effects of oleate or octanoate alone in related parameters (mRNA levels of FAT/CD36, UCP2a, Kir6.x-like, SUR-like, LXRα, PPARα or SREBP1c). In contrast, few effects of those inhibitors were noted in parameters related to FA metabolism.

There are almost no effects of inhibitors counteracting the effects of oleate or octanoate in the expression of CART whereas the effects of oleate or octanoate on expression of NPY and POMC were mainly counteracted by the presence of inhibitors of FA sensing mechanisms acting through FAT/CD36 and mitochondrial activity like trolox, genipin, SSO, and diazoxide. It is interesting to mention that counteractive effects were noted for oleate or octanoate for POMC whereas for NPY counteractive effects were mainly noted for oleate.

These results give therefore further support for the presence of FA sensing mechanisms in rainbow trout hypothalamus similar to those already described in mammals (López *et al.,* 2005, 2007; Le Foll *et al.,* 2009; Blouet and Schwartz, 2010) and possibly related to the control of food intake but, in contrast to mammals, with the ability of responding either to MCFA like octanoate or LCFA like oleate. The similar responses elicited by octanoate and oleate could be related to the fact that fish are able to store considerable amounts of MCFA (Davis *et al.,* 1999; Trushenski, 2009) and in rainbow trout there is no preferential oxidation of MCFA compared with LCFA (Figueiredo-Silva *et al.,* 2012c). However, since the later study was carried out after dietary replacement of fish oil (rich in LCFA) by coconut oil (rich in MCFA) the possibility of differences existing in the transport and oxidation of LCFA and MCFA cannot be discarded.

In conclusion, we provide information supporting that components of different FA sensing systems are present in rainbow trout hypothalamus displaying dose-dependent changes in response to increased levels of octanoate (MCFA) or oleate (LCFA). Changes in those parameters are also reflected in the expression of anorexigenic and orexigenic peptides related to the control of food intake. The responses observed *in vitro* in hypothalamus are comparable to those observed in a previous study *in vivo* (Librán-Pérez *et al.,* 2012) allowing us to suggest that the increase of circulating LCFA or MCFA levels in rainbow trout is directly sensed in hypothalamus. Further support was obtained by incubating hypothalamus with oleate or octanoate in the presence of specific inhibitors, whose presence counteracted in many cases the effects of the FA alone. These results give further support to the capacity of rainbow trout hypothalamus to sense changes either in MCFA or LCFA levels through mechanisms related to FA metabolism, binding to FAT/CD36 and mitochondrial activity comparable to those addressed in mammals but with differences in their responses, especially the capacity of responding to octanoate in rainbow trout.

The incubation of BB *in vitro* in the presence of increased concentrations of oleate or octanoate resulted in changes that in general were comparable to those observed in hypothalamus thus supporting the existence of direct FA sensing capacity in this tissue. However, these *in vitro* changes were not coincident with those previously observed *in vivo* in this species (Librán-Pérez *et al.,* 2012). Therefore, we may hypothesize that the FA sensing capacity of BB previously characterized *in vivo* is influenced by other neuroendocrine systems. Therefore, further studies, using intracerebroventricular treatments with oleate and octanoate are necessary to elucidate if the activation of those sensing systems in BB is a consequence of hypothalamic FA sensing through vagal and sympathetic outflow and/or can be attributed to the interaction with other endocrine systems.

3.4 TRABAJO EXPERIMENTAL Nº4

Response of hepatic fatty acid-sensing systems in rainbow trout to *in vitro* oleate or octanoate treatment

INTRODUCTION

The hepatic metabolism of rainbow trout (Figueiredo-Silva *et al.,* 2012b) as of mammals (Paolisso *et al.,* 1995) seems to respond differently to short- or long-term exposure to fatty acids (FA). However, while considerable information is available on the metabolic response of fish to long-term exposure to high FA level (Gélineau *et al.,* 2001; Panserat *et al.,* 2002; Skiba-Cassy *et al*., 2009; Figueiredo-Silva *et al.,* 2012a,b, 2013; Kamalam *et al.,* 2012), information on the acute response to FA and in particular on the mechanisms by which FA exert their effects is practically inexistent. We previously reported in rainbow trout (Librán-Pérez *et al.,* 2012, 2013a) that acute administration of oleate, a long-chain FA (LCFA), or octanoate, a medium chain FA (MCFA), activate putative components of FA sensing systems. Those systems would be based on FA metabolism, binding to cluster of differentiation 36 (CD36, FA transporter) and mitochondrial activity in hypothalamus (possibly related to the control of food intake), Brockmann bodies (possibly related to insulin release), and liver. In liver, increased glucose and glycogen accumulation, decreased lipogenic potential, and enhanced cytoplasmatic FA catabolism (without affecting peroxisomal and mitochondrial FA oxidation capacity and FA transport) were noted after intraperitoneal treatment of rainbow trout with oleate or octanoate (Librán-Pérez *et al.,* 2013a). However, we cannot discard that i) those effects of FA could be attributed to an indirect effect mediated by changes in other endocrine systems, ii) changes were dependent on fatty acid concentration, and iii) the response of liver may be a consequence of vagal and sympathetic outflow from the hypothalamus in a way similar to that described in mammals (Obici *et al*., 2002).

Therefore, we aimed to evaluate *in vitro* (in the absence of extrahepatic regulatory mechanisms) whether liver responds to changes in FA concentration in a way similar to that previously observed *in vivo* (Librán-Pérez *et al.,* 2013a). Accordingly, we evaluated in liver slices exposed to increased oleate or octanoate concentrations changes in parameters related to: i) FA metabolism, such as levels of FA, triglycerides, and total lipids, activities of ATP-citrate lyase (ACLY), carnitine plamitoyl transferase (CPT), fatty acid synthetase (FAS), glucose 6-phosphate dehydrogenase (G6PDH), hydroxyacil-CoA dehydrogenase (HOAD), and malonyl CoA dehydrogenase (MCD), and mRNA abundance of acetyl-CoA carboxylase (ACC), ACLY, CPT1, FAS, and MCD; ii) nuclear receptors and transcription factors involved in lipid metabolism, such as mRNA abundance of peroxisome proliferator-activated receptor type α (PPAR*α*), and sterol regulatory element-binding protein type 1c (SREBP1c); iii) a ROS effector, such as mRNA abundance of mitochondrial uncoupling protein 2 (UCP2); and iv) parameters related to glucose metabolism, such as levels of glucose, glycogen, and lactate, activities of fructose 1,6-bisphosphatase (FBPase), glucokinase (GK), glucose 6-phosphatase (G6Pase), glycogen phosphorylase (GPase), and pyruvate kinase (PK), and mRNA abundance of GK.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish mass was 103 ± 4 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 1201/2005) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Freshly obtained tissues were incubated as previously described (Polakof *et al.,* 2007b). Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. Every morning of an experiment, fish were dipnetted from the tank, anaesthesized with MS-222 (50 $mg \cdot L^{-1}$) buffered to pH 7.4 with sodium bicarbonate, euthanized by decapitation, and weighed. The liver was removed and rinsed with modified Hanks´medium (128 mM NaCl; 3.63 mM KCl, 2.81 mM $NaHCO_3$, 0.85 mM $CaCl_2$, 0.55 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.23 mM Na_2HPO_4, 7.5 mM HEPES, 50 $U \cdot mL^{-1}$ penicillin, and 50 $\mu g \cdot mL^{-1}$ streptomycin sulphate, pH 7.4; referred to a basal medium), sliced (approx. 300 µm) on chilled Petri dishes, and placed in a chilled Petri dish containing 100 ml of modified Hanks´medium.g^{-1} tissue that was gassed with 0.5% CO_2/99.5% O_2. Tissues were combined from different fish resulting in pools of 3-4 livers. Tissues were incubated in clear polystyrene 48-well culture plates at 15ºC for 1 h with 250 µL of modified Hanks´medium per well containing 25 mg of tissue that was gassed with a 0.5% CO_2/99.5% O_2 mixture. Control wells contained modified Hanks´medium with 2 mM D-glucose. Treated wells contained medium at the same glucose concentration and increased concentrations (1, 10 or 100 µM) of oleate or octanoate (previously dissolved in 5% ethanol). After 1h incubation, tissues were quickly removed, rinsed, snap-frozen in liquid nitrogen, and stored at -80°C until assayed. FA concentrations were selected based on FA levels assessed in rainbow trout *in vivo* in liver (Librán-Pérez *et al.,* 2013a) and plasma (Boujard *et al.,* 1993; Polakof *et al.,* 2011a; Figueiredo-Silva *et al.,* 2012a) as well as by the concentrations used in comparable *in vitro* studies in rainbow trout (Sánchez-Gurmaches *et al.,* 2010). Liver slices are easy to obtain, with a high mass that allows the measurement of several parameters. However, potassium leaking into the medium, clumping of tissue and overloading of wells may be present.

On each experiment, one set of 7 tissue pools was assessed for enzyme activities, another set of 7 tissue pools was used for the assay of tissue metabolites, and another set of 7 tissue pools was assayed for mRNA abundance. The number of independent experiments (one set of 7 tissue pools each) carried out was five (N=5).

Assessment of metabolite levels and enzyme activities

Samples used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 N perchloric acid, and neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged (10,000 g), and the supernatant assayed for tissue metabolites. FA, total lipids, triglyceride levels, lactate and glucose were determined enzymatically using commercial kits (Wako Chemicals for FA, Spinreact for total lipids, triglyceride, and lactate, and Biomérieux for glucose) adapted to a microplate format. Tissue glycogen levels were assessed using the method of Keppler and Decker (1974), and glucose levels obtained after glycogen breakdown were assessed as above for plasma samples.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme activities using a microplate reader INFINITE 200 Pro (Tecan) and microplates. Reaction rates of enzymes were determined by changes in absorbance of NAD(P)H at 340 nm or, in the case of CPT-1 activity, of 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA complex (DTNB CoA complex) at 412 nm. The reactions were started by the addition of supernatant (15 µL) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µL), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Fructose 1,6-bisphosphatase (FBPase, *EC* 3.1.3.11), glucose 6-phosphatase (G6Pase, *EC* 3.1.3.9), glucose 6-phosphate dehydrogenase (G6PDH, *EC* 1.1.1.49), glycogen phosphorylase (GPase, *EC* 2.4.1.1), glucokinase (GK, *EC* 2.7.1.2), and pyruvate kinase (PK, *EC* 2.7.1.40) activities were estimated as described previously (Polakof *et al.,* 2007a,b, 2008a,b,c). ATP-citrate lyase (ACLY, *EC* 4.1.3.8), fatty acid synthase (FAS, *EC* 2.3.1.85), hydroxyacyl-CoA dehydrogenase (HOAD, *EC* 1.1.1.35), malonyl CoA decarboxylase (MCD, *EC* 4.1.1.9), and CPT-1 (*EC* 2.3.1.21) activities were determined as described by Alvarez *et al.* (2000), Polakof *et al.* (2011a), Kolditz *et al.* (2008), Zhou *et al*. (2004), and Ditlecadet and Driedzic (2012), respectively.

Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies) and treated with RQ1-DNAse (Promega). Two µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexaprimers (Life Technologies). 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 (Fermentas), in a total PCR reaction volume of 25 µl, containing 50-500 nM of each primer. mRNA abundance of ACC, ACLY, CPT1, FAS, MCD, UCP2a, GK, PPARα, and SREBP1c were determined using primers and procedures previously described in the same species (Ducasse-Cabanot *et al.,* 2007; Kolditz *et al.,* 2008; Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Polakof *et al.,* 2010b, 2011a; Figueiredo-Silva *et al.,* 2012c; Librán-Pérez *et al.,* 2013a). Sequences of the forward and reverse primers of each gene are shown in Table 1. Relative quantification of the target gene transcripts was done using β-actin gene expression as reference, which was stably expressed in this experiment.

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Annealing Tª(°C)	Accession Number (GenBank or others)
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	59	tcbk0010c.b.21_5.1.om.4-(Sigenae)
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	CA349411.1
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55	AF327058
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	55	AF606076
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59	tcab0001c.e.06 5.1.s.om.8 (Sigenae)
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60	AF053331
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	60	BX869708.s.om.10 (Sigenae)
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	CA048941.1
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; CPT1, carnitine plamitoyl transferase type 1; FAS, fatty acid synthetase; GK, glucokinase; MCD, malonyl-CoA dehydrogenase; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; UCP2, mitochondrial uncoupling protein 2

Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq^{TM} DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15s for denaturing, and at specific annealing temperatures (Table 1) for 30s and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analyzed in triplicate. All the replicates of each sample were located in the same plate for each gene to allow comparisons. We included in all the plates the standard curve (by triplicate), and controls for NTC and RT negative control (by duplicate). Only efficiency values between 85-100% were accepted (the R^2 for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl method (2001)

Statistics

Comparisons among groups were carried out using Student t test (paired comparisons) or one-way ANOVA (multiple comparisons) followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$.

RESULTS

Changes in metabolite levels related to lipid metabolism are shown in Fig. 1. Oleate treatment increased levels of fatty acids (Fig. 1A, only at 100 µM) and total lipids (Fig. 1C, dose-response). Octanoate treatment increased levels of fatty acids (Fig. 1A, only at 100 µM) and total lipids (Fig. 1C, only at 100 µM).

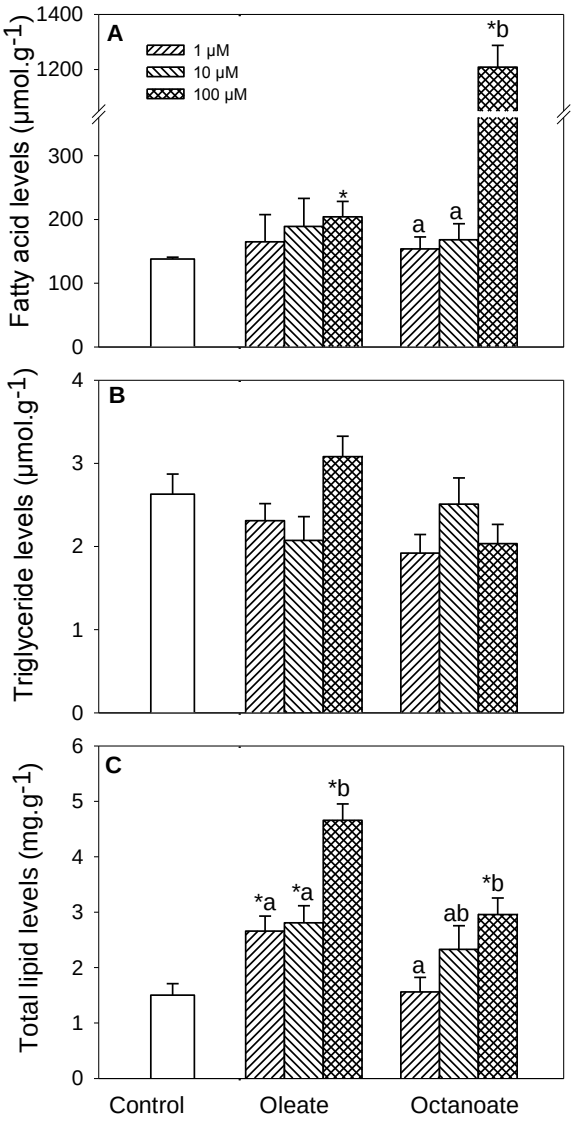

Fig. 1. Levels of fatty acid (A), triglyceride (B), and total lipid (C) in liver of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate. Each value is the mean + S.E.M. of 5 independent experiments carried out with pools of livers from 3-4 different fish. *, significantly different ($P<0.05$) from control fish. Different letters indicate significant differences ($P<0.05$) among concentrations within each fatty acid treatment.

In metabolite levels related to glucose metabolism oleate treatment increased levels of glucose (Fig. 2A), glycogen (Fig. 2B, only at 100 µM) and lactate (Fig. 2C, dose-response). Octanoate treatment increased levels of glucose (Fig. 2A, only at 10 µM) and lactate (Fig. 2C, 10 and 100 µM).

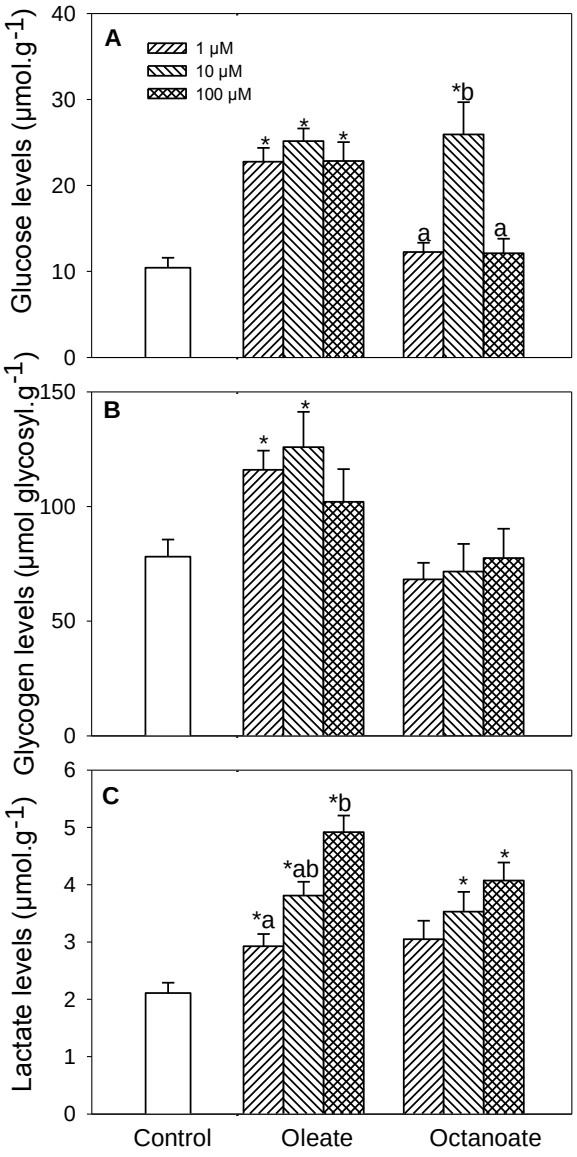

Fig. 2. Levels of glucose (A), glycogen (B), and lactate (C) in liver of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate. Each value is the mean + S.E.M. of 5 independent experiments carried out with pools of livers from 3-4 different fish. *, significantly different (P<0.05) from control fish. Different letters indicate significant differences (P<0.05) among concentrations within each fatty acid treatment.

Enzyme activities related to lipid metabolism were affected by treatment with oleate or octanoate that increased activities of ACLY (Fig. 3A, dose-response for oleate) and G6PDH (Fig. 3E, dose-response for oleate), and decreased activities of FAS (Fig. 3B, dose-response), MCD (Fig. 3C, not at 1 µM oleate), and HOAD (Fig. 3D, not at 1 µM). No changes were noticed for CPT activity (Fig. 3F).

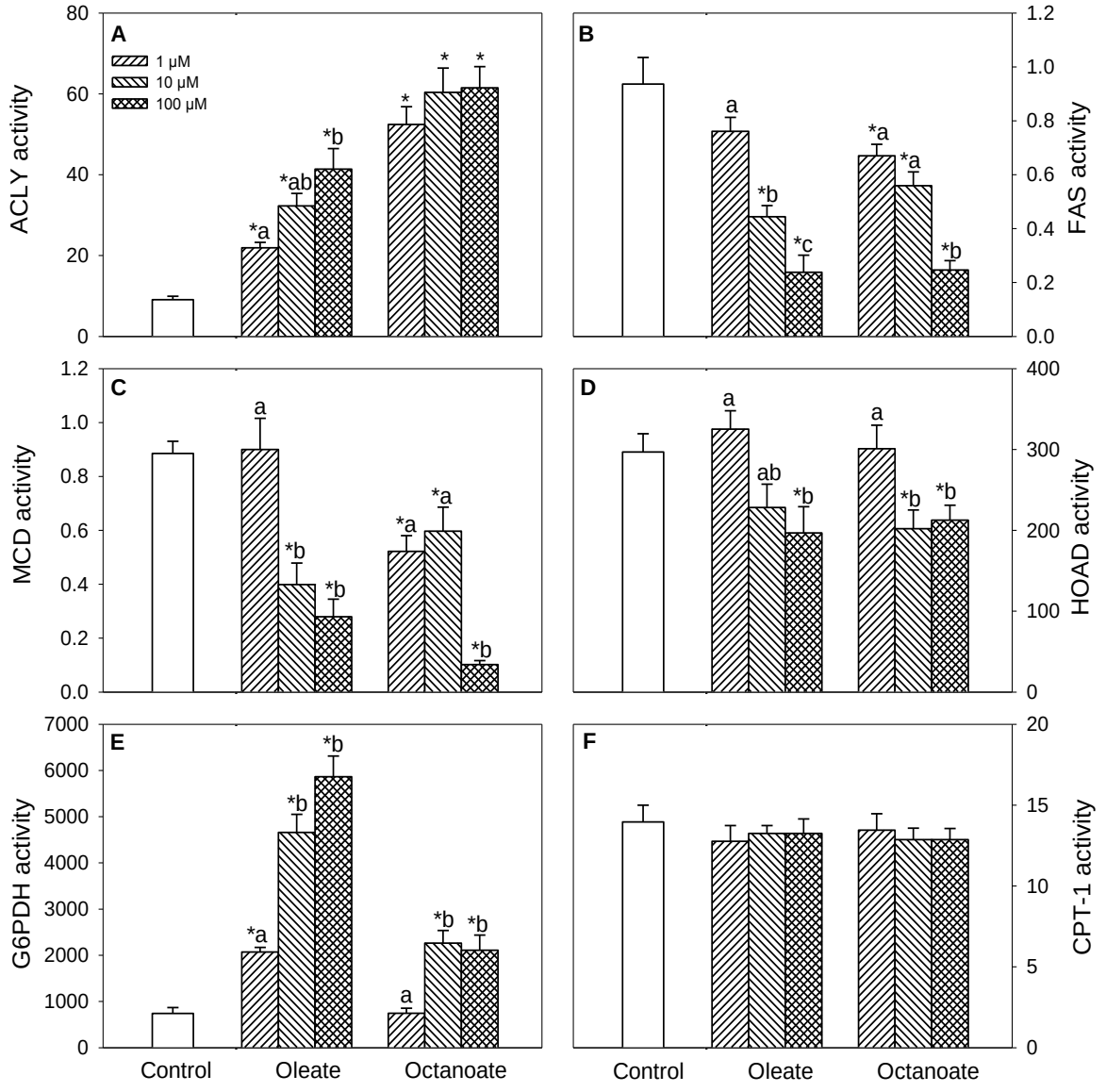

Fig. 3. Activities (mU.mg^{-1} protein) of ACLY (A), FAS (B), MCD (C), HOAD (D), G6PDH (E), and CPT (F) in liver of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate. Each value is the mean + S.E.M. of 5 independent experiments carried out with pools of livers from 3-4 different fish. *, significantly different (P<0.05) from control fish. Different letters indicate significant differences (P<0.05) among concentrations within each fatty acid treatment.

Treatment with oleate or octanoate affected activities of enzymes related to glucose metabolism such as the increase of GK (Fig. 4A), FBPase (Fig. 4B), and G6Pase (Fig. 4D, not at 1 µM oleate), and the decrease of GPase (Fig. 4E, only at 1 µM for octanoate). Oleate treatment increased PK activity (Fig. 4C, not at 1 µM).

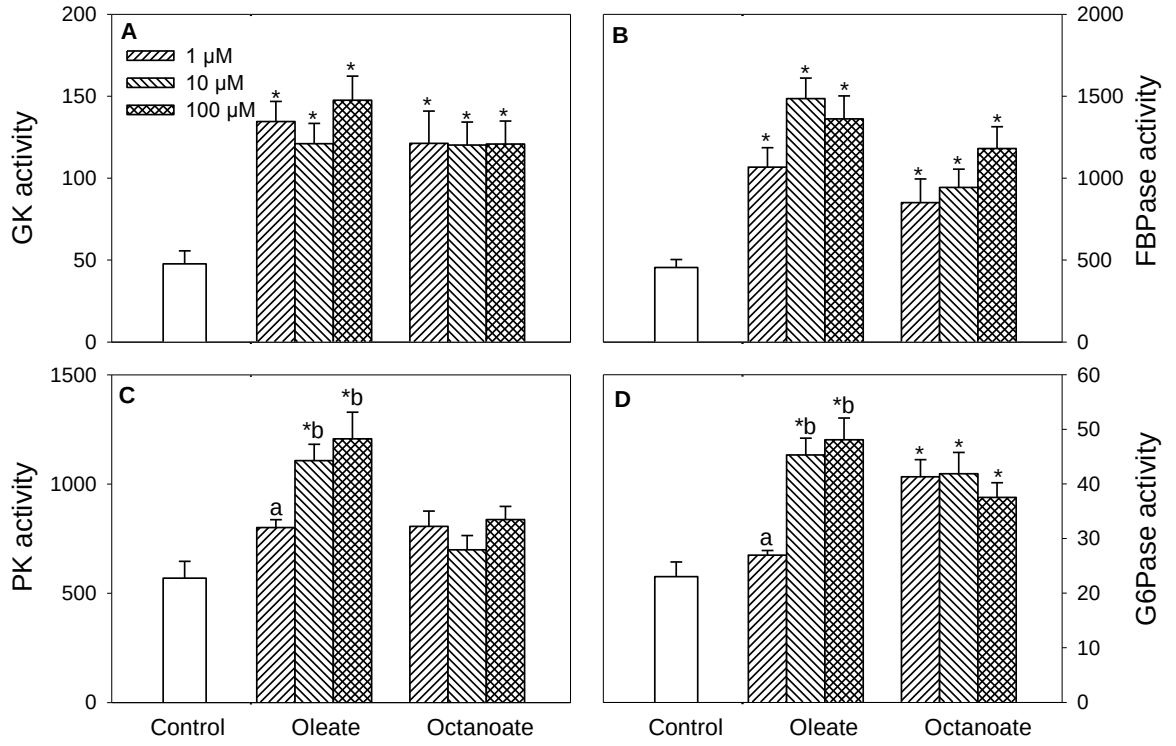

Fig. 4. Activities (mU.mg^{-1} protein) of GK (A), FBPase (B), PK (C), and G6Pase (D) in liver of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate. Each value is the mean + S.E.M. of 5 independent experiments carried out with pools of livers from 3-4 different fish. *, significantly different ($P<0.05$) from control fish. Different letters indicate significant differences ($P<0.05$) among concentrations within each fatty acid treatment.

Treatment with oleate or octanoate up-regulated in a dose-dependent way mRNA levels of ACC, FAS, MCD, GK, and PPARα, and down-regulated those of UCP2 (Table 2). Treatment with oleate up-regulated mRNA levels of ACLY (only 100 µM) and CPT1b (dose-response). Treatment with octanoate induced a dose-dependent increase in CPT1a mRNA levels (Table 2).

Table 2. mRNA levels in liver of rainbow trout incubated *in vitro* for 1 h at 15 °C in modified Hanks' medium containing 2 mM D-glucose alone (control) or containing 1, 10, or 100 µM oleate or octanoate.

	Oleate (µM)			**Octanoate (µM)**		
	1	10	100	1	10	100
Fatty acid metabolism						
ACC	+1.12a	+1.46*b	+1.47*b	+4.82*a	+6.69*b	+7.53*b
ACLY	+1.08a	+1.18a	+1.42*b	+1.08	+1.09	+1.27
CPT1a	+1.09	-1.14	-1.19	+1.40*a	+1.61*a	+2.37*b
CPT1b	+1.46*a	+1.59*a	+2.18*b	-1.26	-1.09	-1.28
FAS	+1.70*a	+1.79*a	+2.34*b	+1.86*a	+2.26*ab	+2.59*b
MCD	+1.68*a	+1.95*ab	+2.16*b	+37.4*a	+39.5*a	+62.2*b
Mitochondrial uncoupling						
UCP2a	+1.07a	-1.38*b	-2.87*c	+1.14a	-1.65*b	-4.81*c
Glucose metabolism						
GK	+2.18*a	+4.62*b	+5.16*b	+1.47*a	+2.96*b	+3.52*b
Transcription factors						
PPARα	+1.13a	+1.43*b	+1.49*b	+1.26a	+1.48*ab	+1.67*b
SREBP1c	+1.01	+1.05	+1.03	+1.02	-1.01	+1.03

Each value is the mean of 5 independent experiments carried out with pools of livers from 3-4 different fish. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalized by β-actin mRNA levels, which did not show changes among groups). *, significantly different ($P<0.05$) from control fish. Different letters indicate significant differences ($P<0.05$) among fatty acid concentration within each fatty acid treatment.

DISCUSSION

In a previous study (Librán-Pérez *et al.,* 2013a) we described the response of rainbow trout liver to *in vivo* intraperitoneal administration of oleate or octanoate resulting in reduced lipogenic potential, increased FA oxidation, and increased mitochondrial activity. As a whole those changes are a signal of FA abundance and are in agreement with the so-called FA-sensing systems (Pocai *et al.,* 2006; López *et al.,* 2007).

In the present study changes observed in most parameters assessed *in vitro* were in general different than those observed previously *in vivo* (Librán-Pérez *et al.,* 2013a). The differential response *in vitro* included enhanced lipogenic potential, reduced FA oxidation potential, and decreased mitochondrial activity thus affecting components of the FA-sensing systems evaluated but with a response almost converse than that observed *in vivo*.

The lipogenic potential, which was inhibited in liver by treatment with any of the FA *in vivo* (Librán-Pérez *et al.,* 2013a) was activated *in vitro* as supported by increased activities of ACLY and G6PDH and increased mRNA levels of FAS and ACC. However, the lack of changes noted in SREBP1c mRNA levels is not apparently in agreement since SREBP1c in mammals targets genes expressing lipogenic enzymes such as FAS and ACLY (Kim *et al.,* 2007). This difference could indicate that the activation of SREBP1c preceded in time that of the lipogenic enzymes whose transcription is regulating.

The cytoplasmatic catabolic FA pathway, which was enhanced by the presence of oleate or octanoate *in vivo* (Librán-Pérez *et al.,* 2013a) was inhibited *in vitro*, as illustrated by decreased MCD activity. The enhanced lipogenic potential is likely related to reduced FA use since decreased MCD activity may lead to a decrease in the accumulation of malonyl CoA, known to inhibit CPTs in rainbow trout liver (Gutières *et al.,* 2003). Accordingly, the potential for oxidation in the mitochondria was apparently decreased based on decreased HOAD activity though no changes were noted in CPT activity (contradictory changes were noted in mRNA levels of CPT1a and CPT1b). PPARα mRNA levels increased after treatment with oleate or octanoate, a response similar to that observed in Atlantic salmon fed a fish oil-enriched diet (Kennedy *et al.,* 2006) but in contrast with flounder liver where PPARα mRNA levels changed in parallel with those of FA oxidation genes (Cho *et al.,* 2012).

A byproduct of increased FA oxidation is increased ROS production that is neutralized by UCP2 in mammalian FA-sensing tissues (Andrews *et al.,* 2008). In the only reference available in fish regarding a role for UCP2 in liver no changes were noted in UCP2 mRNA levels in red sea bream under food deprivation conditions or after feeding a high lipid diet (Liang *et al.,* 2003) not reflecting a relationship between mitochondrial

activity and oxidation. In the present study the decreased mRNA UCP2 levels are compatible with the reduced potential observed for FA oxidation.

The increase in liver glucose and glycogen (only the later after oleate treatment) levels may suggest that FA stimulate glucose metabolism for accumulation instead of redirecting to TCA cycle. Changes observed in glycogen levels would partially support such contention, and are similar to increased glycogen levels reported in the same species when fed a high fat diet (Figueiredo-Silva *et al.,* 2012b). The present results show an increased gluconeogenic potential (FBPase activity) and capacity for glucose release (G6Pase activity) in the presence of FA. Also GK activity and mRNA levels increased after treatment with any of the FA; those changes are the converse than those previously observed *in vivo* (Librán-Pérez *et al.,* 2013a) but again similar to those observed in rainbow trout fed a high fat diet (Figueiredo-Silva *et al.,* 2012b). The excess of FA in the medium is probably inducing less use of glucose and therefore an accumulation of glucose and glycogen and subsequent changes in pathways such as glycolysis resulting in enhanced lactate production.

Since increased glucose and glycogen contents implies an available source of glucose in the medium we cannot discard that at least part of the changes observed could be attributed to a glucose/FA interaction. Such an interaction would be in agreement with the fact that similar responses were induced by oleate and octanoate. Furthermore, oleate and octanoate cannot be discarded to circulate at least in part complexed to albumin in trout plasma *in vivo*. No protein was present in the incubation medium in the present experiments. This could partially affect to the differential response observed when comparing *in vivo* and *in vitro* studies since oleate and octanoate could have been complexed to albumin under the *in vivo* conditions of our previous IP treatment (Librán-Pérez *et al.,* 2013a). Moreover, we may also suggest that the action of FA on hepatic lipid and glucose metabolism of rainbow trout observed *in vivo* (Librán-Pérez *et al.,* 2013a) is of indirect nature since in the absence of extrahepatic regulatory mechanisms the response changed dramatically.

The differential response could be attributed to an indirect action of FA through effects on any hormone related to fatty acid and glucose metabolism, such as insulin (Caruso and Sheridan, 2011). In this way, feeding fish with diets enriched in FA is known to increase insulin levels (Barma *et al.,* 2006), and insulin treatment in rainbow trout is known to enhance the potential of lipogenesis as well as to decrease the potential for FA oxidation in liver (Plagnes-Juan *et al.,* 2008; Lansard *et al.,* 2009; Polakof *et al.,* 2010a). Therefore, a rise in insulin levels would result in changes that in general agree with those previously reported *in vivo* (Librán-Pérez *et al.,* 2013a), which would not be observed in the present *in vitro* study due to the isolation of liver slices.

Alternatively, the *in vivo* response of liver could be a consequence of central FA detection. Thus, in mammals, fatty acid sensing in hypothalamus is related to the

regulation of glucose homeostasis by affecting pancreatic endocrine function and liver energy metabolism (Blouet and Schwartz, 2010). The downstream mechanism(s) by which FA modulate hepatic glucose fluxes are likely based on sympathetic and parasympathetic systems that provide direct innervations of the liver via the splanchnic nerve and vagus nerve, respectively (Morgan *et al.,* 2004; Migrenne *et al.,* 2006). We observed in rainbow trout hypothalamus the presence of putative fatty acid-sensing systems responding to increased levels of oleate or octanoate (Librán-Pérez *et al.,* 2012). Moreover, vagus and splanchnic nerves are present in rainbow trout though it is not clear whether or not branches of those nerves arrive to the liver (Burnstock, 1959; Seth and Axelsson, 2010). Therefore, the results observed in liver after *in vivo* treatment with oleate or octanoate (Librán-Pérez *et al.,* 2013a) could be the result of hypothalamic FA detection followed by hypothetical vagal and/or sympathetic outflow to the liver, which would not be observed in the present *in vitro* study because liver slices were not under hypothalamic influence. On the other hand, since in mammals insulin release from pancreas is also related to hypothalamic FA sensing (Obici *et al.,* 2002; Caspi *et al.,* 2007) a similar mechanism cannot be discarded to be also present in rainbow trout.

In conclusion, we provide information supporting that several components of different FA sensing systems display in rainbow trout liver slices *in vitro* dose-dependent changes in response to increased levels of octanoate or oleate similar for both FA. However, those changes were in general not coincident with those previously observed *in vivo* in the liver of this species (Librán-Pérez *et al.,* 2013a) allowing us to hypothesize, among other reasons, that the FA sensing capacity of liver observed *in vivo* would be of indirect nature. Further studies are necessary to elucidate such hypothesis including intracerebroventricular treatments to elucidate whether or not *in vivo* responses in liver are a consequence of hypothalamic FA sensing and/or can be attributed to the interaction with other endocrine systems.

3.5 TRABAJO EXPERIMENTAL Nº5

Effects of oleate or octanoate intracerebroventricular treatment on food intake and hypothalamic fatty acid systems in rainbow trout

INTRODUCTION

There is evidence that specialized neurons within mammalian hypothalamus detect changes in plasma levels of long-chain fatty acid (LCFA), but not short-chain (SCFA) or medium-chain (MCFA) FA through four different mechanisms (López *et al.,* 2005, 2007; Le Foll *et al.,* 2009; Blouet and Schwartz, 2010). These include: 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); iii) activation of protein kinase C (PKC) isoforms; and, iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity. Changes in these systems have been associated, through not completely understood mechanisms, 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) (López *et al.,* 2005, 2007). Altogether, these changes could be involved in the decreased food intake observed after rising circulating LCFA levels in mammals.

Most teleost fish are relatively intolerant to glucose thus relying more on amino acids and lipids to fuel metabolism (Tocher, 2003; Polakof *et al.,* 2011b, 2012b). A reduced food intake has been observed in fish fed with lipid-enriched diets or containing high fat stores (Shearer *et al.,* 1997; Silverstein *et al*., 1999; Rasmussen *et al.,* 2000; Gélineau *et al.,* 2001; Johansen *et al.,* 2002, 2003; Figueiredo-Silva *et al.,* 2012a) raising the question whether lipid sensing mechanisms regulating food intake may be present in fish (Froiland *et al.,* 2012). Accordingly, in a previous study in rainbow trout *Oncorhynchus mykiss* (Librán-Pérez *et al.,* 2012) we observed that intraperitoneal (IP) acute administration of oleate (LCFA) or octanoate (MCFA) elicited an inhibition in food intake, and induced in hypothalamus a response compatible with FA sensing in which FA metabolism, binding to FAT/CD36, and mitochondrial activity were apparently involved. Changes in these hypothalamic pathways can be also related to the control of food intake, since changes in mRNA levels of specific neuropeptides such as NPY and POMC were also noted (Librán-Pérez *et al.,* 2012). The main difference in the responses with those known in the mammalian model (López *et al.,* 2007) is the effect of octanoate. This could be related to the fact that body lipids in teleosts contain considerable amounts of MCFA (Davis *et al.,* 1999; Trushenski, 2009). Accordingly, MCFA have been used as a dietary lipid source in fish (Figueiredo-Silva *et al.,* 2012a), and in rainbow trout there is no preferential oxidation of MCFA compared with LCFA (Figueiredo-Silva *et al.,* 2012c). In a subsequent study (Davis *et al.,* 1999) we observed that rainbow trout hypothalamus *in*

vitro (in the absence of extrahypothalamic regulatory mechanisms) displayed responses in parameters related to FA-sensing similar to those previously observed after IP treatment suggesting that the increase of circulating LCFA or MCFA levels in rainbow trout is directly sensed in hypothalamus. However, we cannot reject that the effects of IP treatment with FA (Librán-Pérez *et al.,* 2012) could be attributed to an indirect effect mediated by changes elicited in levels of peripheral hormones. Therefore, in the present study, we evaluated in rainbow trout the effects of intracerebroventricular (ICV) treatment with oleate or octanoate on food intake, and in parameters related to putative FA sensing systems in hypothalamus. If FA are directly sensed in hypothalamus, central administration should elicit effects similar to those of intraperitoneal treatment, as we reported in rainbow trout (Librán-Pérez *et al.,* 2012), and as described in mammals (Obici *et al.,* 2002; Morgan *et al.,* 2004).

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish weight was 101 ± 2 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Following 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks. Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. On the day of experiment fish were lightly anaesthetized with MS-222 (50 $mg \cdot l^{-1}$) buffered to pH 7.4 with sodium bicarbonate, and weighed. ICV administration was performed as previously described (Polakof and Soengas, 2008). Briefly, fish were placed on a plexiglass board with Velcro© straps adjusted to hold them

in place. A 29½ gauge needle attached through a polyethylene cannula to a 10 µl Hamilton syringe was aligned with the 6th preorbital bone at the rear of the eye socket, and from this point the syringe was moved through the space in the frontal bone into the third ventricle. The plunger of the syringe was slowly depressed to dispense 1µl·100g^{-1} body mass of Hanks´ saline alone or containing 1 µmol oleate (O-1008, Sigma Chemical Co.) or octanoate (C-2875, Sigma Chemical Co.). To safely deliver FA they were solubilized in 45% hydroxypropyl-β-cyclodextrin (HBP) to a final concentration of 17 mM (Morgan *et al.,* 2004). The HPB-FA solution was diluted in saline to the appropriate concentration used for each injection. HPB alone at a similar concentration as in the FA studies was used in controls (no effects of the vehicle alone were noted for any of the parameters assessed, data not shown).

In a first set of experiments food intake was registered for 3 days before treatment (to define basal line data) and then 6 and 24 h after ICV treatment with saline-HPB alone (control, n=10 for each time point) or containing oleate (n=10 for each time point) or octanoate (n=10 for each time point). After feeding, 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 previously described as the difference from the feed offered (De Pedro *et al.,* 1998; Polakof *et al.,* 2008c). The experiment was repeated three times, and therefore results are shown as the mean ± SEM of the data obtained in three different tanks per treatment.

In a second set of experiments fish were ICV injected with saline-HPB alone (control, n=15 at 2h and n=15 at 6h) or containing oleate (n=15 at 2h and n=15 at 6h) or octanoate (n=15 at 2h and n=15 at 6h) with the same concentrations described above. After 2h or 6h, fish were lightly anaesthetized with MS-222 (50 mg·l^{-1}) buffered to pH 7.4 with sodium bicarbonate. 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. Fish were sacrificed by decapitation and hypothalamus were taken, snap-frozen in liquid nitrogen, and stored at -80 °C. At each time, 10 fish per group were used to assess enzyme activities and metabolite levels whereas the remaining 5 fish were used for the assessment of mRNA levels by qRT-PCR.

In both experiments, we were interested in comparing the differential effects of oleate or octanoate, and therefore we used similar molar concentrations. Due to the different molecular weight of both FA this results in a difference in the utilizable energy of both treatments, which we cannot discard to be responsible, at least in part, of the differences observed.

Assessment of metabolite levels and enzyme activities

Levels of FA, total lipids, triglyceride, glucose, and lactate in plasma were determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany, for FA; Spinreact, Barcelona, Spain for total lipid, triglyceride, and lactate; Biomérieux, Grenoble, France, for glucose) adapted to a microplate format. The determination of total lipid is based on the sulfo-phosho-vanillin method (Kaplan, 1984).

Samples used to assess hypothalamic metabolite levels 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 FA, total lipid, and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples. Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma, St Louis, Mo, USA). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT-1 activity, of 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA complex at 412 nm. The reactions were started by the addition of supernatant (15 µl) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ATP-citrate lyase (ACLY, *EC* 4.1.3.8), fatty acid synthase (FAS, *EC* 2.3.1.85), and CPT-1 (*EC* 2.3.1.21) activities were determined as described previously (Davis *et al.,* 1999; Trushenski, 2009; Librán-Pérez *et al.,* 2012, 2013).

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 (Life Technologies) and random hexaprimers (Life Technologies).

Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ™ (BIO-RAD, Hercules, CA, USA). Analyses were performed on 1 µl cDNA using the MAXIMA SYBR® Green qPCR Mastermix (Fermentas, Vilnius, Lithuania), in a total PCR reaction volume of 25 µl, containing 50-500 nM of each primer. mRNA abundance of acetyl-CoA carboxylase (ACC), ACLY, CART, FAT/CD36, CPT-1, citrate synthase (CS), FAS, inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), liver X receptor α (LXRα), malonyl CoA dehydrogenase (MCD), NPY, POMC, PPARα, SREBP1c, sulfonylurea receptor-like (SUR-like), and mitochondrial uncoupling protein 2a (UCP2a) were determined as described in the same species (Ducasse-Cabanot *et al.,* 2007; Kolditz *et al.,* 2008; Cruz-Garcia *et al.,* 2009; Lansard *et al.,* 2009; Conde-Sieira *et al.,* 2010; Polakof *et al.,* 2008a,b, 2010a, 2011a; Figueiredo-Silva *et al.,* 2012a; Librán-Pérez *et al.,* 2013). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1.

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Annealing Tª(°C)	Accession Number (GenBank or others)
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	59	tcbk0010c.b.21_5.1.om.4- Sigenae
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	CA349411.1
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	NM_001124627
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59	AJ619768
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	59	AJ620356
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	55	TC89195 Tigr
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59	tcab0001c.e.065.1.s.om.8 .Sigenae
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62	AY606034.1 DFCI
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60	CA346261.1.s.om.8:1:773:1Sigenae
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	62	FJ470291
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	60	BX869708.s.om.10 Sigenae
NPY	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58	NM_001124266
POMC	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	TC86162 Tigr
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	CA048941.1
Sur-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	62	tcce0019d.e.20_3.1.s.om.8.Sigenae
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; CS, citrate synthetase; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; Kir6.x-like, inward rectifier K^+ channel pore type 6.-like; LXRα, liver X receptor α; MCD, malonyl CoA dehydrogenase; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; SUR-like, sulfonylurea receptor-like; UCP2a, mitochondrial uncoupling protein 2a.

Relative quantification of the target gene transcript was done using β-actin gene expression as reference (Migrenne *et al.,* 2007) whose expression was stable in this experiment. Thermal cycling was initiated with incubation at 95°C for 90s using hot-start iTaq^{TM} DNA polymerase activation; 35 steps of qPCR 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 (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 (Plaffl, 2001) with the Relative Expression Software tool (REST$^{©}$). 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: $R = [(E_{\text{target gene}})^{\Delta CT_{\text{Target gene}}(\text{mean control- mean unknown sample})}] / [(E_{EF1\alpha})^{\Delta CT_{EF1\alpha}(\text{mean control- mean unknown sample})}]$ where E is PCR efficiency determined using a standard curve of cDNA serial dilutions (cDNA dilutions from 1/32 to 1/512), and ΔCT is the crossing point deviation of an unknown sample versus a control.

Statistics

Comparisons among groups were carried out with two-way ANOVA with time (2 and 6h) and treatment (control, oleate, and octanoate) as main factors. In those cases where a significant effect was noted for a factor, post-hoc comparisons were carried out by a Student-Newman-Keuls test. Differences were considered statistically significant at $P<0.05$.

RESULTS

Changes in food intake are shown in Fig. 1. Oleate or octanoate significantly decreased food intake after 6h and 24h of ICV treatment compared with controls, and the decrease was lower after 24h for both FA. Food intake was lower after octanoate treatment than after oleate treatment after 24h.

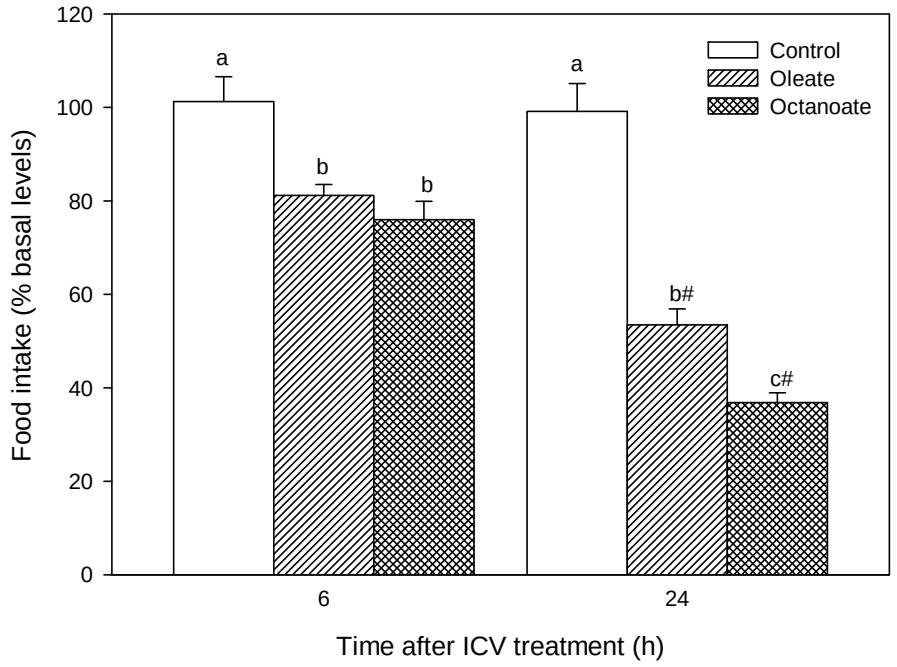

Fig. 1. Food intake in rainbow trout 6 and 24h after intracerebroventicular administration of 1 µl saline alone (control) or containing 1 µmol oleate or octanoate. Different letters indicate significant differences (P<0.05) among treatments within each time. #, significantly different (P<0.05) from 6h at the same treatment. The results are shown as mean + S.E.M. of the results obtained in three different tanks in which 10 fish were used per group in each tank.

Levels of plasma metabolites are shown in Fig. 2. There were no significant differences vs. controls in any parameter due to treatment with oleate or octanoate. Levels of FA (Fig. 2a), triglyceride (Fig. 2b), and total lipid (Fig. 2c) were lower after 6h than after 2h of octanoate treatment.

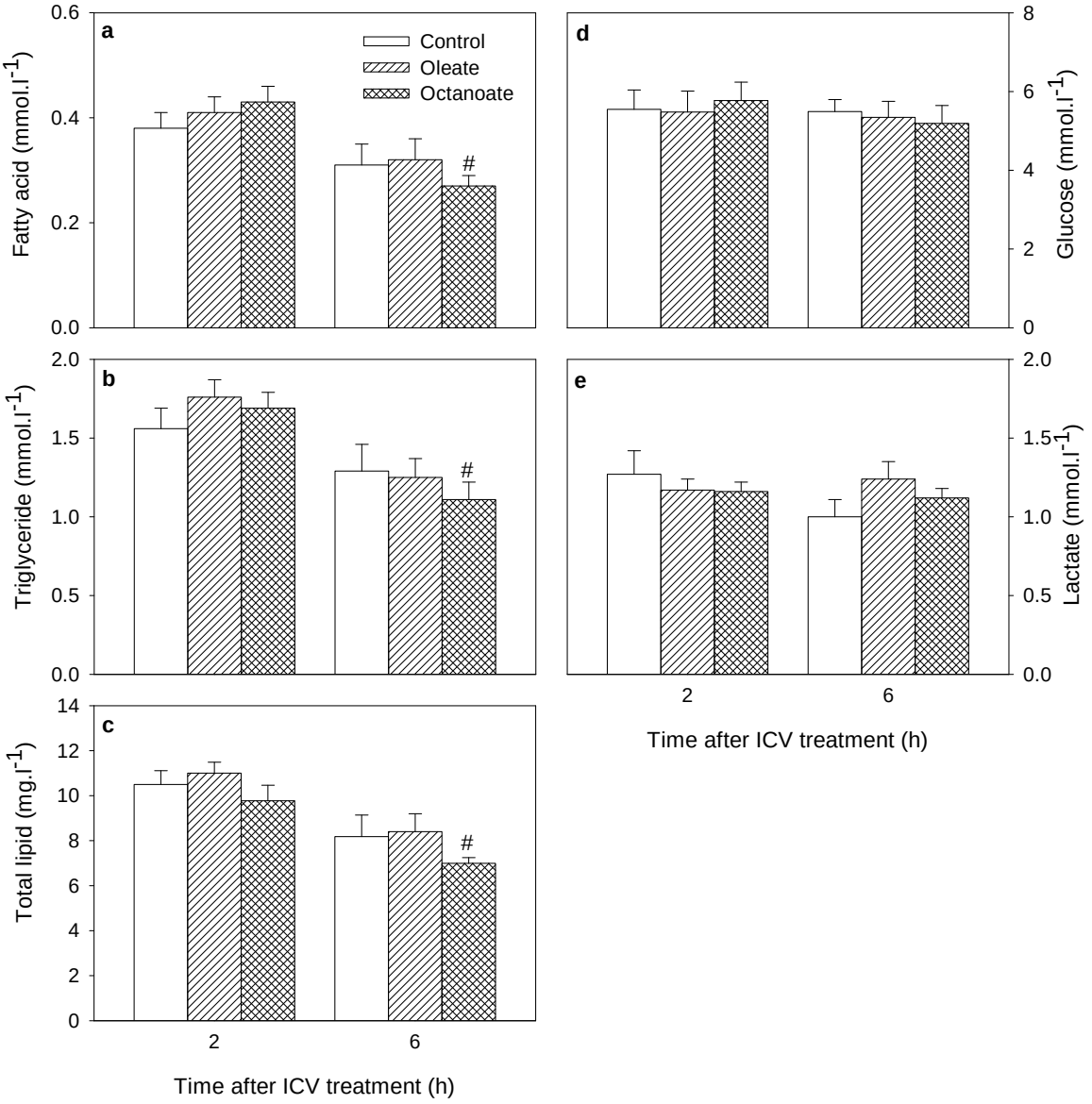

Fig. 2. Levels of FA (a), triglyceride (b), total lipid (c), glucose (d), and lactate (e) in plasma of rainbow trout after 2h or 6h of intracerebroventicular administration of 1 µl saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. #, significantly different (P<0.05) from 2h at the same treatment.

Hypothalamic metabolite levels are shown in Fig. 3. FA levels (Fig. 3a) increased compared with controls after treatment with oleate (2h) and octanoate (2 and 6h), and levels after octanoate treatment were higher at 6h than at 2h. Triglyceride levels (Fig. 3b) increased compared with controls after treatment with oleate (2h) and octanoate (6h); levels after 6h of octanoate treatment were higher than those observed after 2h treatment with the same FA and after 6h treatment with oleate. Compared with controls, total lipid levels (Fig. 3c) increased after 6h treatment with oleate or octanoate; the increase produced by oleate was higher than that elicited by octanoate, and also higher than levels observed after 2h treatment with oleate.

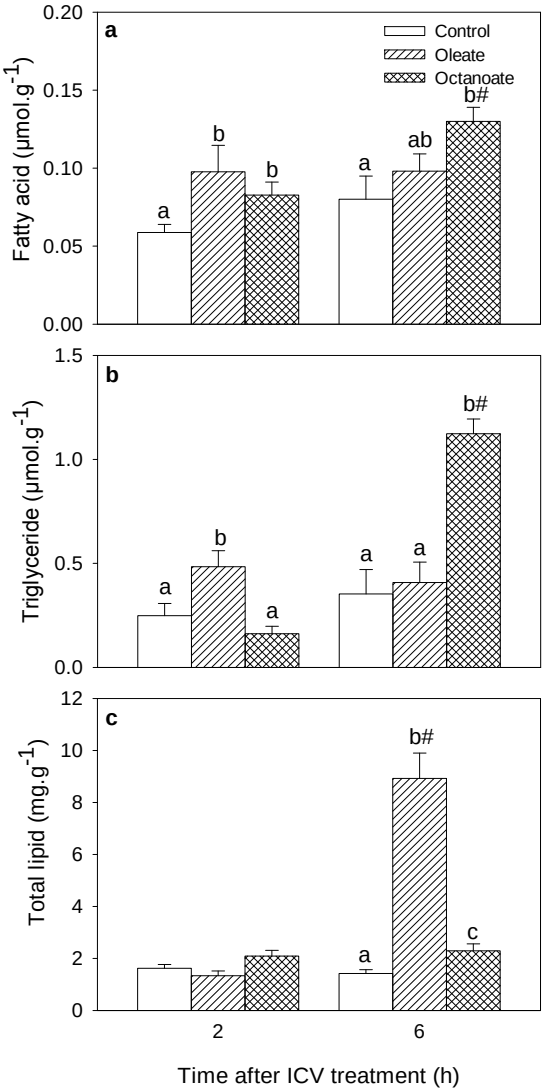

Fig. 3. Levels of FA (a), triglyceride (b), and total lipid (c) in hypothalamus of rainbow trout after 2h or 6h of intracerebroventicular administration of 1 µl saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences ($P<0.05$) among treatments within each time. #, significantly different ($P<0.05$) from 2h at the same treatment.

Enzyme activities in hypothalamus are shown in Fig. 4. Compared with controls FAS activity (Fig. 4a) decreased after 2h treatment with oleate or octanoate whereas activity increased after 6h treatment with octanoate; activities after 6h treatment with oleate or octanoate were higher than those observed after 2h. ACLY activity (Fig. 4b) after 2h treatment with octanoate was higher than that of controls, and also higher than that observed after 6h treatment with the same FA. CPT-1 activity (Fig. 4c) decreased after 2h and 6h treatment with oleate or octanoate, and the activity was lower after octanoate treatment.

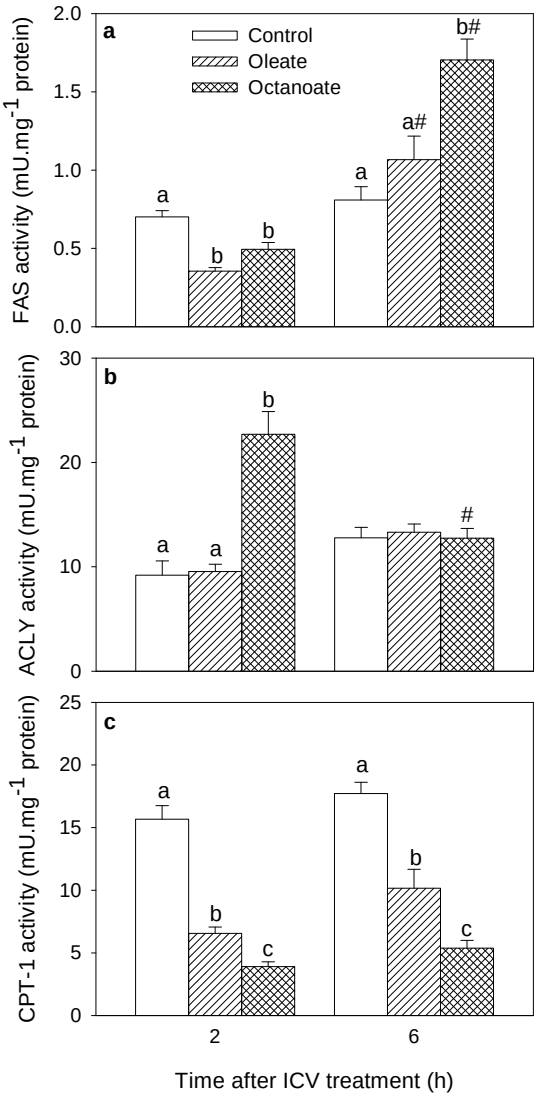

Fig. 4. Activities of FAS (a), ACLY (b), and CPT-1 (c) in hypothalamus of rainbow trout after 2h or 6h of intracerebroventicular administration of 1 µl saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences ($P<0.05$) among treatments within each time. #, significantly different ($P<0.05$) from 2h at the same treatment.

Changes in mRNA levels assessed in hypothalamus are shown in table 2. Oleate treatment compared with controls down-regulated mRNA levels of FAT/CD36 (2h and 6h), ACC (2h), ACLY (2h), CS (2h), FAS (2h), Kir6.x-like (2h and 6h), SUR-like (2h and 6h), LXRα (2h and 6h), and NPY (2h), and up-regulated those of CS (6h), MCD (2h), CART (2h), and POMC (2h and 6h). The treatment with octanoate compared with controls resulted in down-regulation of mRNA levels of ACC (2h), Kir6.x-like (2h), SUR-like (2h and 6h), and NPY (2h), and up-regulation of mRNA levels of CPT-1c (2h), CS (6h), MCD (2h), UCP2a (2h), and POMC (2h and 6h). Changes elicited after 2h treatment with octanoate were significantly different than those elicited at the same time by oleate for mRNA levels of ACLY, CPT-1c, FAS, UCP2a, and LXRα; no such differences were noted after 6h. Changes induced by 6h treatment with oleate were significantly different than those observed after 2h treatment with the same FA for mRNA levels of ACC, ACLY, FAS, CART, and NPY. Changes induced by 6h treatment with octanoate were significantly different than those observed after 2h treatment with the same FA for mRNA levels of ACLY, CPT-1c, UCP2a, and NPY.

Table 2. mRNA levels in hypothalamus of rainbow trout after 2 or 6h of intracerebroventicular administration of 1 µl saline alone (control) or containing 1 µmol oleate or octanoate.

	Oleate		**Octanoate**	
	2h	**6h**	**2h**	**6h**
Fatty acid transport				
FAT/CD36	-1.37*	-1.51*	+1.09	-1.24
Fatty acid metabolism				
ACC	-1.54*	+1.15#	-1.38*	+1.09
ACLY	-1.54*	+1.15#	-1.38*	+1.09
CPT-1c	-1.46*	+1.07#	+1.26†	-1.05#
CPT-1d	-1.04	+1.23	+1.63*†	+1.09#
CS	-1.05	-1.07	+1.23	+1.01
FAS	-1.41*	+1.43*	+1.13	+1.49*
MCD	-1.40*	+1.20#	+1.19†	-1.07
Mitochondrial uncoupling	+1.44*	+1.13	+1.80*	+1.21
UCP2a				
K_{ATP} channel	+1.27	+1.04	-1.36*	+1.23#
Kir6.x-like				
SUR-like	-1.43*	-1.34*	-1.36*	-1.18
Transcription factors	-1.35*	-1.54*	-1.34*	-1.69*
LXRα				
PPARα	-1.68*	-1.34*	+1.11†	+1.03
SREBP1c	-1.29	-1.16	+1.10	+1.31
Neuropeptides	+1.08	-1.05	+1.17	-1.20
CART				
NPY	+1.38*	-1.18#	+1.03	-1.09
POMC	-1.51*	+1.01#	-1.53*	-1.03#

Data represent mean of 5 measurements. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by β-actin mRNA levels, which did not show changes among groups). *, significantly different (P<0.05) from control fish at the same time and treatment. #, significantly different (P<0.05) from 2h at the same treatment. †, significantly different (P<0.05) from oleate treatment at the same time.

DISCUSSION

Minor changes in plasma metabolite levels of treated fish vs. controls indicates that no major metabolic changes occurred in the periphery after central treatment with FA thus validating our experimental design.

FA-sensing systems are partially activated by central administration of oleate or octanoate

The increase observed in FA levels in hypothalamus support the increased entry of FA after ICV injection. In mammals, when circulating FA levels increase, hypothalamic malonyl CoA levels increase in keeping with a decreased need for FA oxidation, and FA are preferentially esterified to produce diacylglycerol and triglycerides (Gutières *et al.,* 2003), which is in agreement with the increased triglyceride levels observed in hypothalamus in the present study. The FA-sensing mechanism based on FA metabolism relates FA abundance with decreased lipogenic potential resulting on CPT-1 inhibition (López *et al.,* 2007). Accordingly, in the present study, many parameters displayed changes comparable with those of the mammalian model after oleate treatment including decreased CPT-1 activity and mRNA levels of ACC, ACLY, and FAS (also activity after 2h). The apparent down-regulation of lipogenic potential and mitochondrial FA oxidation potential would be explained by malonyl CoA action. Thus, the apparent reduced lipogenic potential together with the increase observed in MCD mRNA levels may lead to an accumulation of malonyl CoA, which is known to inhibit CPT-1 activity in rainbow trout (Benani *et a*l., 2007; Morash *et al.,* 2008) in agreement with the decreased activity noted for CPT-1, as well as with the decreased ACC mRNA levels observed after oleate treatment in the present study. However, changes noted after treatment with octanoate were not so comparable since despite decreased CPT-1 activity and ACC mRNA levels were noted, no logical changes were observed in several of the remaining parameters evaluated such as FAS, CS or ACLY mRNA levels. Therefore, it seems that octanoate affects only some parameters related to FA metabolism whereas oleate is modulating most of them. This situation is different however than that reported in mammals where MCFA like octanoate did not affect any parameters related to FA sensing based on FA metabolism (Obici *et al.,* 2002). Moreover, ICV treatment elicited in many parameters changes similar to those observed previously after IP treatment (Librán *et al.,* 2012) either for oleate treatment, such as levels of FA (2h) and total lipid (6h), and mRNA levels of FAS (2h), ACLY (2h), CPT-1d (2 and 6h) and CS (2h) or for octanoate treatment, such as for levels of FA (2 and 6h) and total lipid (6h), activities of FAS (6h) and ACLY (2h), and mRNA levels of CPT-1d (6h). In general, coincident responses with those of IP treatment (Librán-Pérez *et al.,* 2012) were more

numerous than those not coincident allowing us to suggest that the FA sensing mechanism based on FA metabolism is directly responding to FA in rainbow trout hypothalamus. The time course of responses was different when comparing effects of oleate and octanoate. Thus, in general, most of the coincident responses with IP treatment for oleate occurred after 2h of treatment whereas for octanoate most of the coincidences occurred after 6h of treatment. This is suggesting a different time needed to elicit the FA sensing response depending on the nature of the FA, i.e. shorter for LCFA like oleate and longer for MCFA like octanoate.

ICV treatment with oleate or octanoate resulted in decreased mRNA levels of the components of the K^+_{ATP} channel Kir6.x-like and SUR-like, which are related to the FA sensing system mediated by mitochondrial activity (Blouet and Schwartz, 2010). The decreases were similar to those observed after IP (Librán-Pérez *et al.,* 2012) and *in vitro* (Librán-Pérez *et al.,* 2013b) treatments, and were similar for both FA evaluated. The similar effect of MCFA and LCFA in mitochondrial activity is different than that observed in mammals where LCFA produce a higher effect than MCFA (Andrews *et al.,* 2008). Also in mammals, CPT-1 activity can enhance LCFA entry into mitochondria and increase β-oxidation resulting in generation of ROS, which together with FA promote UCP2 transcription and activity (Torstensen *et al.,* 2009). UCP2 activity neutralizes ROS thereby allowing continuous CPT-1 promoted FA β-oxidation that enables continuous support of the energetic needs to maintain firing of NPY/AgRP cells (Torstensen *et al.,* 2009). Therefore, UCP2 expression is usually increased with enhanced mitochondrial activity, and this is usually associated with higher lipid levels, as demonstrated for instance in Atlantic salmon liver (Liang *et al.,* 2003). However, no changes were noted in UCP2a mRNA levels in the present study, in contrast to that previously observed *in vitro* (Davis *et al.,* 1999) but in agreement with the absence of changes reported in liver of red sea bream fed a high fat diet (Moullé *et al.,* 2012).

The FA sensing system associated with binding to FAT/CD36 and subsequent modulation of transcription factors and nuclear receptors did not display in general changes after ICV treatment with oleate or octanoate comparable to those observed after IP treatment (Librán-Pérez *et al.,* 2012). mRNA levels of FAT/CD36 were expected to increase after central oleate treatment in agreement with that previously observed after IP treatment in the same species (Librán-Pérez *et al.,* 2012) and in mammals (Kennedy *et al.,* 2006) but a decrease was noted after oleate treatment in the present study. Furthermore, mRNA levels of PPARα and SREBP1c did not change after ICV treatment with oleate in contrast with the increase observed after IP treatment (Librán-Pérez *et al.,* 2012) and with the increase reported in mRNA levels of PPARα in liver of Atlantic salmon fed a diet rich in fish oil (Wolfrum, 2007). Only mRNA levels of LXRα changed after oleate treatment in a way comparable to that addressed before (Librán-Pérez *et al.,* 2012) and compatible with the mammalian model (Richards *et al.,* 2004). In contrast, all these parameters changed in hypothalamus after oleate treatment *in vitro* (Librán-Pérez *et al.,*

2013b) in a way comparable to that observed after IP treatment (Librán-Pérez *et al.,* 2012). Therefore, the direct sensing based on binding to FAT/CD36 is presumably masked under the *in vivo* conditions of IP treatment (Librán-Pérez *et al.,* 2012) by other unknown factors. Moreover, octanoate treatment did not induce any changes in parameters related to FA sensing through FAT/CD36 in contrast with the results obtained *in vitro* (Librán-Pérez *et al.,* 2013b) but similar to those previously assessed after IP treatment (Librán-Pérez *et al.,* 2012). The lack of changes after octanoate treatment could be related to the finding that in other tissues of rainbow trout, such as red and white muscle, octanoate (MCFA) do not compete with palmitate (LCFA) for FAT/CD36 binding (Barma *et al.,* 2006).

Possible interaction of hypothalamic FA-sensing systems with peripheral hormones

Since IP treatment with oleate or octanoate elevated circulating FA levels in rainbow trout (Librán-Pérez *et al.,* 2012) the synthesis and release of peripheral hormones may be altered, which could be responsible in part for the changes observed after IP treatment. In this way, it is known that increased levels of circulating FA enhanced insulin release in fish (Caruso and Sheridan, 2011), which is involved in controlling lipid metabolism (Caruso *et al.,* 2008). Moreover, expression of insulin (Caruso *et al.,* 2010) and several insulin receptors subtypes (Sánchez-Gurmaches *et al.,* 2012) has been demonstrated in rainbow trout brain regions including hypothalamus. Thus, we cannot reject that an increase occurred in circulating insulin levels after IP treatment with FA (Librán-Pérez *et al.,* 2012), which would not be observed either *in vitro* (due to the isolation of the hypothalamus from external influences) (Librán-Pérez *et al.,* 2013b) or in the present ICV study since no changes were noted in the levels of plasma metabolites including FA.

Of the three FA-sensing systems assessed, any hypothetical endocrine regulation would be more important in those cases where no similar effects were noted when comparing results after IP and ICV treatments, such as the case of the mechanism associated with FA binding to FAT/CD36. Furthermore, this hypothetical hormone action would be important in those parameters where the results observed *in vitro* (Librán-Pérez *et al.,* 2013b) were similar to those observed after ICV treatment (this study) but different to those noted after IP treatment (Librán-Pérez *et al.,* 2012), such as the case of mRNA levels of FAT/CD36 and SREBP1c after oleate treatment. Accordingly, the rise in FAT/CD36 mRNA levels observed after IP oleate treatment (Librán-Pérez *et al.,* 2012) would be comparable to the enhancement of FAT/CD36 mRNA levels elicited in liver of the same species by insulin treatment (Sánchez-Gurmaches *et al.,* 2012). That situation would be also comparable to that observed in mammalian hypothalamus where FAT/CD36 mRNA

levels increased after feeding a high fat diet (Kennedy *et al.,* 2006), a situation known to elicit increased insulin levels in plasma.

A possible interaction of peripheral hormone levels with parameters related to other FA sensing systems cannot be rejected since insulin for instance is known to enhance FAS gene expression and to inhibit CPT-1 gene expression in liver of rainbow trout (Lansard *et al.,* 2009). However, results obtained after ICV treatment in parameters related to putative FA sensing systems based on FA metabolism and mitochondrial activity were in general similar to those obtained after *in vitro* (Librán-Pérez *et al.,* 2013b) and IP (Librán-Pérez *et al.,* 2012) treatments. Therefore, we believe that hormone interaction on FA sensing through those systems would be (if any) of minor importance.

Food intake is inhibited by central administration of oleate or octanoate and could be related to changes in neuropeptide expression

The inhibition of food intake observed after ICV treatment with any of the FA assessed was comparable to that previously observed after IP treatment in the same species (Librán-Pérez *et al.,* 2012). It is important to emphasize that the decrease was higher for octanoate than for oleate either after IP or ICV treatment. The decrease in food intake noted under our experimental conditions clearly supports that the lipid-induced inhibition of food intake in fish (Shearer *et al.,* 1997; Silverstein *et al*., 1999; Rasmussen *et al.,* 2000; Gélineau *et al.,* 2001; Johansen *et al.,* 2002, 2003; Figueiredo-Silva *et al.,* 2012a) is probably mediated by central FA sensing.

The expression of neuropeptides was in general in agreement with changes noted in food intake. Thus, mRNA abundance of the orexigenic factor NPY decreased 2h after ICV treatment whereas mRNA abundance of the anorexigenic factor POMC increased 2 and 6h after ICV treatment, and (only for oleate) an increase was noted 2h after ICV treatment in mRNA abundance of the anorexigenic factor CART. As a whole, changes in mRNA levels of those neuropeptides support increased anorexigenic potential in agreement with the decrease noted in food intake. These changes agree with those previously addressed by Figueiredo-Silva *et al*. (Figueiredo-Silva *et al.,* 2012c) in rainbow trout where decreased NPY mRNA levels and increased CART mRNA levels were observed in hypothalamus of fish fed a lipid-enriched diet whereas in Atlantic salmon increased NPY levels were also noted in the preoptic area of lean fish compared with fat fish (Silverstein *et al.,* 1999). Comparable decreases in NPY mRNA levels were also observed in mammalian hypothalamus after ICV treatment with oleate (Blouet and Schwartz, 2010). It is important to mention that those changes in mRNA levels of neuropeptides are also similar to those observed after IP (Librán-Pérez *et al.,* 2012) and *in vitro* (Librán-Pérez *et al.,* 2013b) treatments with oleate in hypothalamus of the same species whereas in the

case of octanoate changes were similar to those observed *in vitro* (Librán-Pérez *et al.,* 2013b) but not after IP treatment (Librán-Pérez *et al.,* 2012). It is important to consider that other neuropeptides known to be involved in the control of food intake such as AgRP are also known to respond to changes in nutrient levels in mammalian hypothalamus (López *et al.,* 2007), and therefore they could be also partially responsible for the response observed in food intake of rainbow trout, especially in the case of octanoate.

The precise connection between changes in FA sensing systems and the expression of anorexigenic and orexigenic factors is not known even in mammals (López *et al.,* 2007) though several possibilities have been suggested such as direct action of malonyl CoA or CPT1c in the signaling mechanisms regulating neuropeptide expression, indirect action through inhibition of CPT-1, or modulation of transcription factors. Any of them, among others, could be involved in the integrative response occurring in rainbow trout hypothalamus.

In conclusion, the results obtained in this study support direct FA sensing of rainbow trout hypothalamus to increased levels of oleate and octanoate through reduced potential of lipogenesis and FA oxidation, and decreased potential of K^{+}_{ATP}. The FA sensing through binding to FAT /CD36 and subsequent expression of transcription factors appears to be also direct but an interaction induced by changes in levels of peripheral hormones cannot be rejected. The activation of those systems would be integrated in hypothalamus resulting in altered production of anorexigenic and orexigenic factors explaining the reduced food intake observed after IP and ICV treatment with oleate or octanoate. Accordingly, decreased expression of NPY and increased expression of POMC and CART (the later only after oleate treatment) were observed in parallel with the activation of FA sensing systems and the decrease in food intake, allowing us to suggest the involvement of at least those peptides in the response of decreased food intake noted after oleate and octanoate treatment.

3.6 TRABAJO EXPERIMENTAL Nº6

Fatty acid sensing systems are affected by central oleate or octanoate administration in liver and Brockmann bodies of rainbow trout

INTRODUCTION

The capacity of detecting changes in circulating levels of long-chain fatty acid (LCFA) is present in mammalian hypothalamus through four different mechanisms (López *et al*. 2005, 2007; Le Foll *et al*. 2009; Blouet and Schwartz 2010). These include: 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, iii) activation of protein kinase C (PKC) isoforms; and iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity. The activation of those systems in hypothalamus has been related to food intake inhibition. In addition to feeding, central FA detection has been related, through vagal and sympathetic outflow, to homeostasis regulation by affecting insulin release in pancreas and endogenous glucose production in liver (Blouet and Schwartz 2010) though FA also directly regulate insulin release from pancreatic β-cells (Migrenne *et al*. 2006).

Most teleost fish are relatively intolerant to glucose thus relying more on amino acid and lipid metabolism for fuelling purposes (Tocher 2003; Polakof *et al.* 2011a, 2012c). Accordingly, a reduced food intake has been observed in fish fed with lipid-enriched diets or containing high fat stores (Shearer *et al*. 1997; Silverstein *et al*. 1999; Rasmussen *et al.* 2000; Gélineau *et al*. 2001; Johansen *et al*. 2002, 2003; Figueiredo-Silva *et al*. 2012a). Thus, several authors suggested that lipid sensing mechanisms regulating food intake may be present in fish (Frøiland *et al*. 2012). Accordingly, in previous studies in rainbow trout *Oncorhynchus mykiss* we observed that intraperitoneal (IP) (Librán-Pérez *et al*. 2012), *in vitro* (Librán-Pérez *et al*. 2013b) or intracerebroventricular (ICV) (Librán-Pérez *et al*. 2014a) administration of a LCFA like oleate or a medium-chain FA (MCFA) like octanoate induced changes in parameters related to FA sensing systems in hypothalamus comparable to those observed in mammals, with the main difference being the lack of response to octanoate in mammals. Those systems, based on FA metabolism, binding to FAT/CD36, and mitochondrial activity would be activated by increased levels of FA, and would be related to the control of food intake since decreased food intake and changes in the expression or orexigenic and anorexigenic peptides were also noted (Librán-Pérez *et al*. 2012, 2014a).

In peripheral tissues of rainbow trout like liver and Brockmann bodies (BB, main accumulation of pancreatic endocrine cells in this species) our previous studies also suggested the presence of components of putative FA sensing systems responding to increased levels of oleate or octanoate after IP treatment (Librán-Pérez *et al.* 2012, 2013a). However, the incubation of liver and BB *in vitro* in the presence of increased concentrations of oleate or octanoate (Librán-Pérez *et al.* 2013b, 2013c) resulted in

changes that in general were not coincident with those previously observed *in vivo* after IP treatment.

Vagus and splanchnic nerves are present in gastrointestinal tract of rainbow trout (Burnstock 1959; Seth and Axelsson, 2010). Therefore, we may hypothesize that the FA sensing capacity of BB and liver previously characterized *in vivo* after IP treatment (Librán-Pérez *et al*. 2012, 2013a) could be the consequence of hypothalamic FA sensing followed by vagal and/or sympathetic outflow in a way similar to that suggested in mammals (Obici *et al.* 2002; Blouet and Schwartz 2010). Alternatively, the differential response (when comparing IP vs. *in vitro* treatments) might be attributed to an indirect action of FA in IP treatment through effects on any hormone related to FA metabolism in fish, such as insulin (Barma *et al*. 2006; Caruso and Sheridan 2011). Therefore, in the present study, we evaluated in rainbow trout the effects of ICV treatment with oleate or octanoate in parameters related to putative FA sensing systems in BB and liver. If FA sensing in those tissues is related to previous FA sensing in hypothalamus then changes observed in parameters after ICV treatment should be similar to those previously observed after IP treatment. In contrast, if changes after ICV treatment are not similar to those observed after IP treatment then a hypothetical interaction with other endocrine systems would be more likely.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish weight was 103 ± 4 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Following 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks. Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. Fish were lightly anaesthetized with MS-222 (50 $mg \cdot L^{-1}$) buffered to pH 7.4 with sodium bicarbonate, and weighed. Intracerebroventricular (ICV) administration was performed as previously described (Polakof and Soengas 2008). Briefly, fish were placed on a plexiglass board with Velcro© straps adjusted to hold them in place. A 29½ gauge needle attached through a polyethylene cannula to a 10 µL Hamilton syringe was aligned with the 6th preorbital bone at the rear of the eye socket, and from this point the syringe was moved through the space in the frontal bone into the third ventricle. The plunger of the syringe was slowly depressed to dispense $1\mu L \cdot 100g^{-1}$ body mass of Hanks´ saline alone (control, n=15) or containing 1 µmol oleate (n=15) or octanoate (n=15). To safely deliver FA, they were solubilized in 45% hydroxypropyl-β-cyclodextrin (HBP) to a final concentration of 17 mM (Morgan *et al.,* 2004). The HPB-FA solution was diluted in saline to the appropriate concentration used for each injection. HPB alone at a similar concentration as in the fatty acid studies was used in all vehicle control studies (no effects of the vehicle alone were noticed for any of the parameters assessed, data not shown). After 6h, fish were anaesthetized with MS-222 (50 $mg \cdot L^{-1}$) buffered to pH 7.4 with sodium bicarbonate. Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 0.6 $mol \cdot L^{-1}$ perchloric acid) and neutralized (using 1 $mol \cdot L^{-1}$ potassium bicarbonate) before freezing on liquid nitrogen and storage at -80ºC until further assay. Fish were then sacrificed by decapitation, and liver and Brockmann bodies were taken as previously described (Polakof *et al*. 2007a,b), snap-frozen in liquid nitrogen and stored at -80 °C. In each group, 10 fish were used to assess enzyme activities and metabolite levels whereas the remaining 5 fish were used for the assessment of mRNA levels by qRT-PCR.

Assessment of metabolite levels and enzyme activities

Samples used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 $mol \cdot L^{-1}$ perchloric acid, and neutralized (using 1 $mol \cdot L^{-1}$ potassium bicarbonate). The homogenate was centrifuged (10,000 g), and the supernatant assayed for tissue metabolites. FA, total lipid, triglyceride, lactate, and glucose levels were determined enzymatically using commercial kits (Wako Chemicals for FA, Spinreact for total lipid, triglyceride, and lactate, and Biomérieux for glucose) adapted to a microplate format. Tissue glycogen levels were assessed using the method of Keppler

and Decker (1974), and glucose levels obtained after glycogen breakdown were assessed as above for plasma samples.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mmol·L^{-1} Tris (pH 7.6), 5 mmol·L^{-1} EDTA, 2 mmol·L^{-1} 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme activities using a microplate reader INFINITE 200 Pro (Tecan) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA complex (CPT-1 activity) at 412 nm. The reactions were started by the addition of supernatant (15 µL) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µL), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Fructose 1,6-bisphosphatase (FBPase, *EC* 3.1.3.11), glucose 6-phosphatase (G6Pase, *EC* 3.1.3.9), glucose 6-phosphate dehydrogenase (G6PDH, *EC* 1.1.1.49), glucokinase (GK, *EC* 2.7.1.2), and pyruvate kinase (PK, *EC* 2.7.1.40) activities were estimated as described previously (Polakof *et al.* 2007a,b, 2008a,b,c). ATP-citrate lyase (ACLY, *EC* 4.1.3.8), fatty acid synthase (FAS, *EC* 2.3.1.85), hydroxyacyl-CoA dehydrogenase (HOAD, *EC* 1.1.1.35), malonyl CoA decarboxylase (MCD, *EC* 4.1.1.9), and carnitine palmitoyltransferase-1 (CPT-1, *EC* 2.3.1.21) activities were determined as described by Alvarez *et al*. (2000), Polakof *et al*. (2011a), Kolditz *et al*. (2008), Zhou *et al*. (2004), and Ditlecadet and Driedzic (2013), respectively.

Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies) and treated with RQ1-DNAse (Promega). 2 µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexaprimers (Life Technologies). 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 (Fermentas), in a total PCR reaction volume of 25 µl, containing 50-500 nM of each primer. mRNA abundance of acetyl-CoA carboxylase (ACC), ACLY, FAT/CD36, CPT-1, citrate synthase (CS), FAS, FBPase, G6Pase, GK, inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), liver X receptor α (LXRα), MCD, peroxisome proliferator-activated receptor type α (PPARα), PK, sterol regulatory element-binding protein type 1c (SREBP1c), sulfonylurea receptor-like (SUR-like), and mitochondrial uncoupling protein 2a (UCP2a) were determined as

previously described in the same species (Panserat *et al*. 2000; Ducasse-Cabanot *et al*. 2007; Kolditz *et al*. 2008; Polakof *et al*. 2008c, 2010a, 2011a; Cruz-Garcia *et al*. 2009; Lansard *et al*. 2009; Figueiredo-Silva *et al*. 2012a; Librán-Pérez *et al.* 2013a). Sequences of the forward and reverse primers of each gene are shown in Table 1.

Table 1 Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Annealing Tª(°C)	Accession Number (GenBank or others)
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	59	tcbk0010c.b.21_5.1.om.4- Sigenae
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	CA349411.1
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55	AF327058
CPT1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	55	AF606076
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	55	TC89195 Tigr
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59	tcab0001c.e.065.1.s.om.8 .Sigenae
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62	AY606034.1 DFCI
FBPasa	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	59	AF333188
G6Pase	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	55	cay0019b.d.18_3.1.s.om.8.1-1693 Sigenae
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60	AF053331
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60	CA346261.1.s.om.8:1:773:1Sigenae
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	62	FJ470291
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	60	BX869708.s.om.10 Sigenae
PK	CCATCGTCGCGGTAACAAGA	ACATAGGAAAGGCCAGGGGC	59	AF246146
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	CA048941.1
Sur-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	62	tcce0019d.e.20_3.1.s.om.8.Sigenae
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; CPT-1, carnitine palmitoyl transferase type 1; CS, citrate synthetase; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; FBPase, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase; GK, glucokinase; Kir6.x-like, inward rectifier K^+ channel pore type 6.-like; LXRα, liver X receptor α; MCD, malonyl CoA dehydrogenase; PK, pyruvate kinase; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; SUR-like, sulfonylurea receptor-like; UCP2a, mitochondrial uncoupling protein 2a

Relative quantification of the target gene transcript was done using β-actin gene expression as reference (Olsvik *et al*. 2005), which was stable expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 90s using hot-start iTaq^{TM} 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 (Pfaffl 2001) with the Relative Expression Software tool (REST©). 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: $R = [(E_{\text{target gene}})^{\Delta CT_{\text{Target gene}}(\text{mean control- mean unknown sample})}] / [(E_{EF1\alpha})^{\Delta CT_{EF1\alpha}(\text{mean control- mean unknown sample})}]$ 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 among groups were carried out with one-way ANOVA followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$. When necessary data were log transformed to fulfill the conditions of the analysis of variance.

RESULTS

Metabolite levels and enzyme activities in Brockmann bodies are shown in Fig. 1. FA levels (Fig. 1A) increased after treatment with oleate or octanoate with the increase being higher with oleate. Triglyceride levels (Fig. 1B) decreased after treatment with octanoate compared with controls and fish treated with oleate. Total lipid levels (Fig. 1C) did not show significant changes. FAS activity (Fig. 1D) was lower in fish treated with octanoate than in fish treated with oleate. ACLY activity (Fig. 1E) increased in fish treated with oleate compared with controls and fish treated with octanoate. CPT-1 activity (Fig. 1F) increased in fish treated with oleate or octanoate compared with controls.

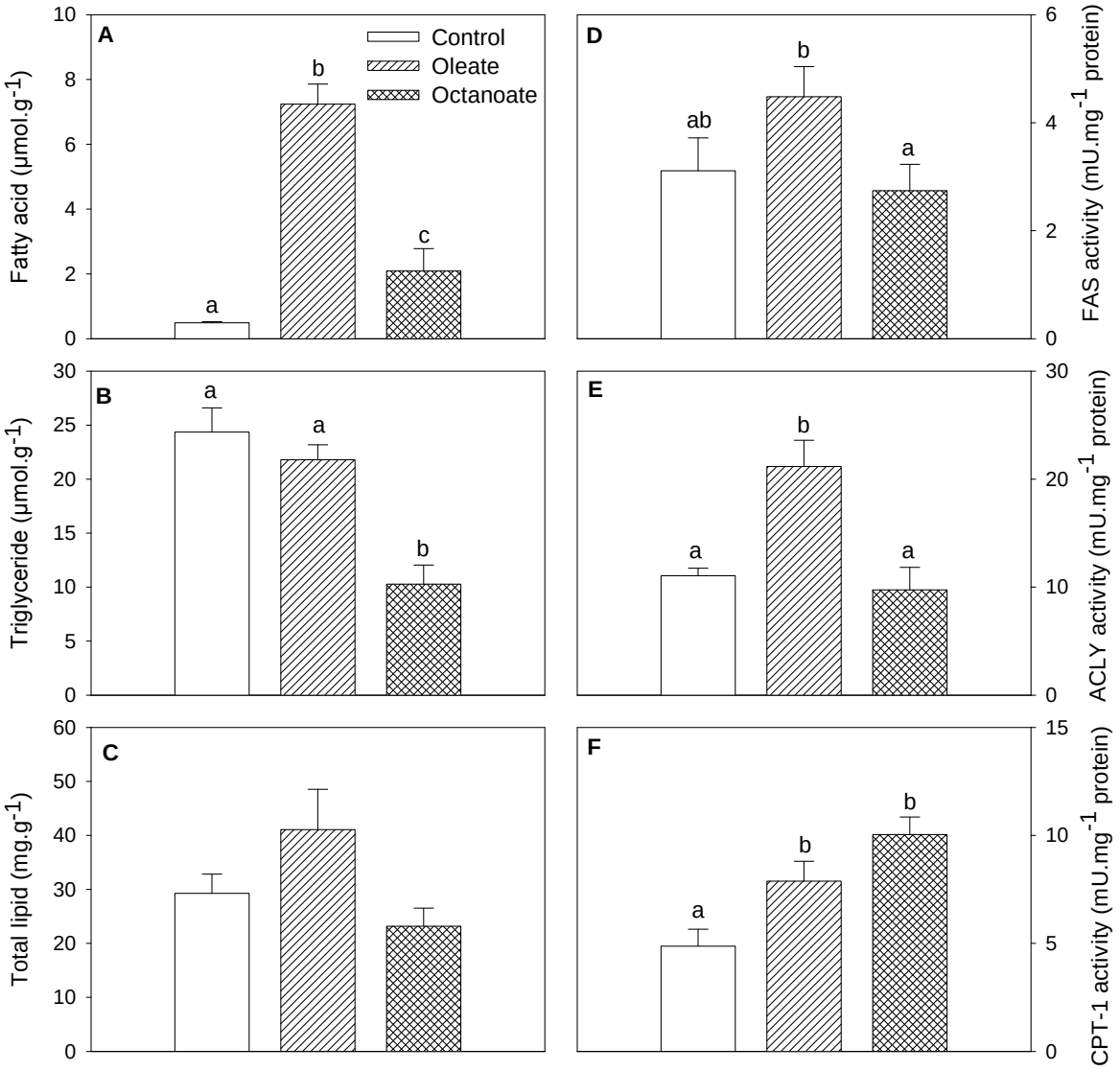

Figure 1 Levels of FA (A), triglyceride (B), and total lipid (C), and activities of FAS (D), ACLY (E), and CPT-1 (F) in Brockmann bodies of rainbow trout after 6h of intracerebroventicular administration of 1 µL saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences ($P<0.05$) among treatments.

Levels of metabolites assessed in liver are shown in Fig. 2. Levels of FA (Fig. 2A) and total lipid (Fig. 2C) decreased in fish treated with oleate or octanoate compared with controls. Glucose levels (Fig. 2D) increased in fish treated with oleate or octanoate compared with controls. Glycogen levels (Fig. 2E) increase in fish treated with oleate compared with controls. No significant changes were noticed for levels of triglyceride (Fig. 2B) or lactate (Fig. 2F).

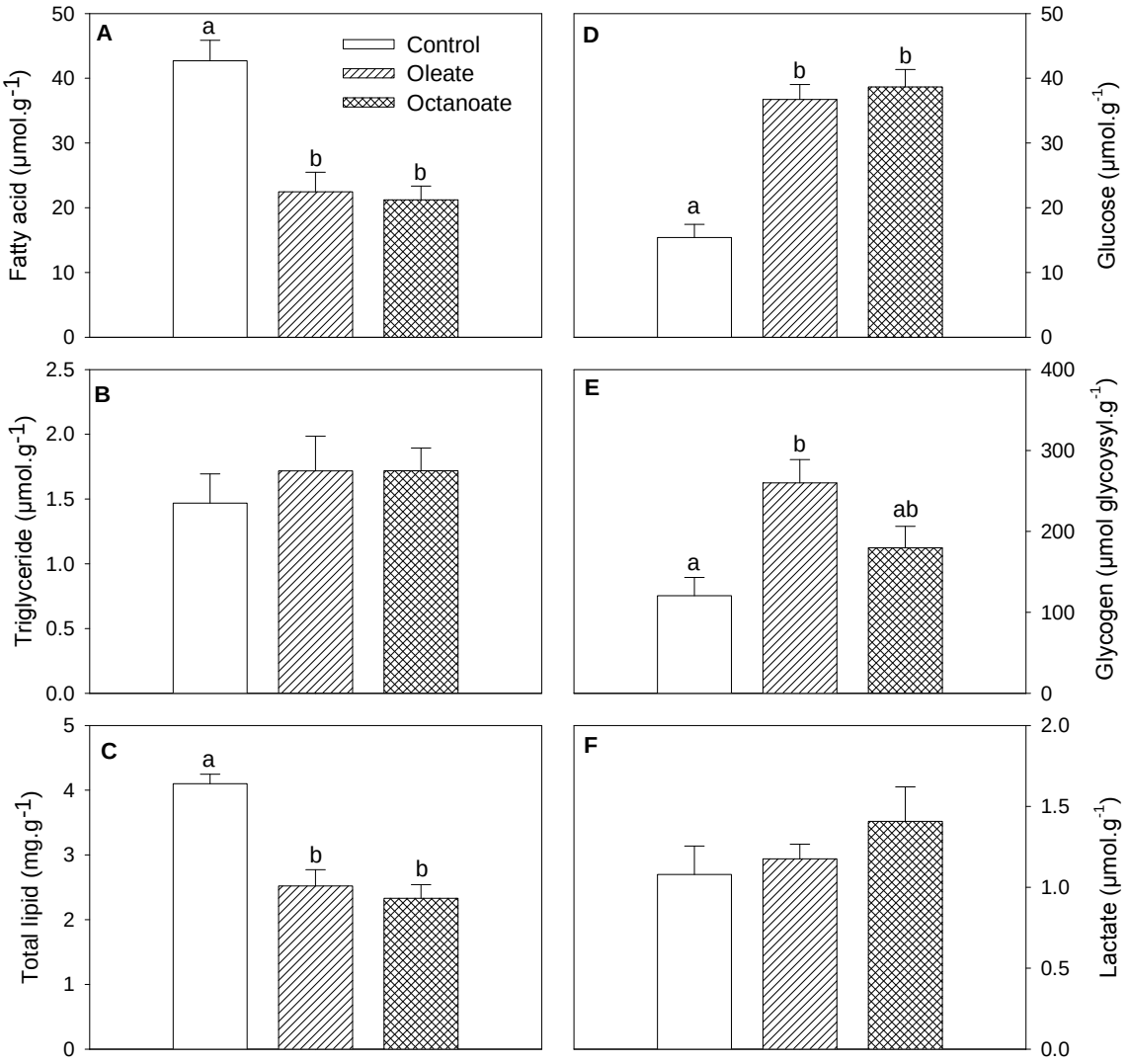

Figure 2 Levels of FA (A), triglyceride (B), total lipid (C), glucose (D), glycogen (E), and lactate (E) in liver of rainbow trout after 6h of intracerebroventicular administration of 1 µL saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

The activity of liver enzymes involved in lipid metabolism is shown in Fig. 3. FAS activity (Fig. 3A) decreased after treatment with oleate or octanoate compared with controls with the activity being lower after treatment with oleate. ACLY activity (Fig. 3B) decreased after treatment with octanoate compared with controls. MCD activity (Fig. 3C) increased after treatment with oleate compared with controls and fish treated with octanoate. No significant changes were noticed for HOAD activity (Fig. 3D). CPT-1 activity (Fig. 3E) decreased after treatment with oleate or octanoate compared with controls. Finally, G6PDH activity (Fig. 3F) decreased in fish treated with oleate compared with controls.

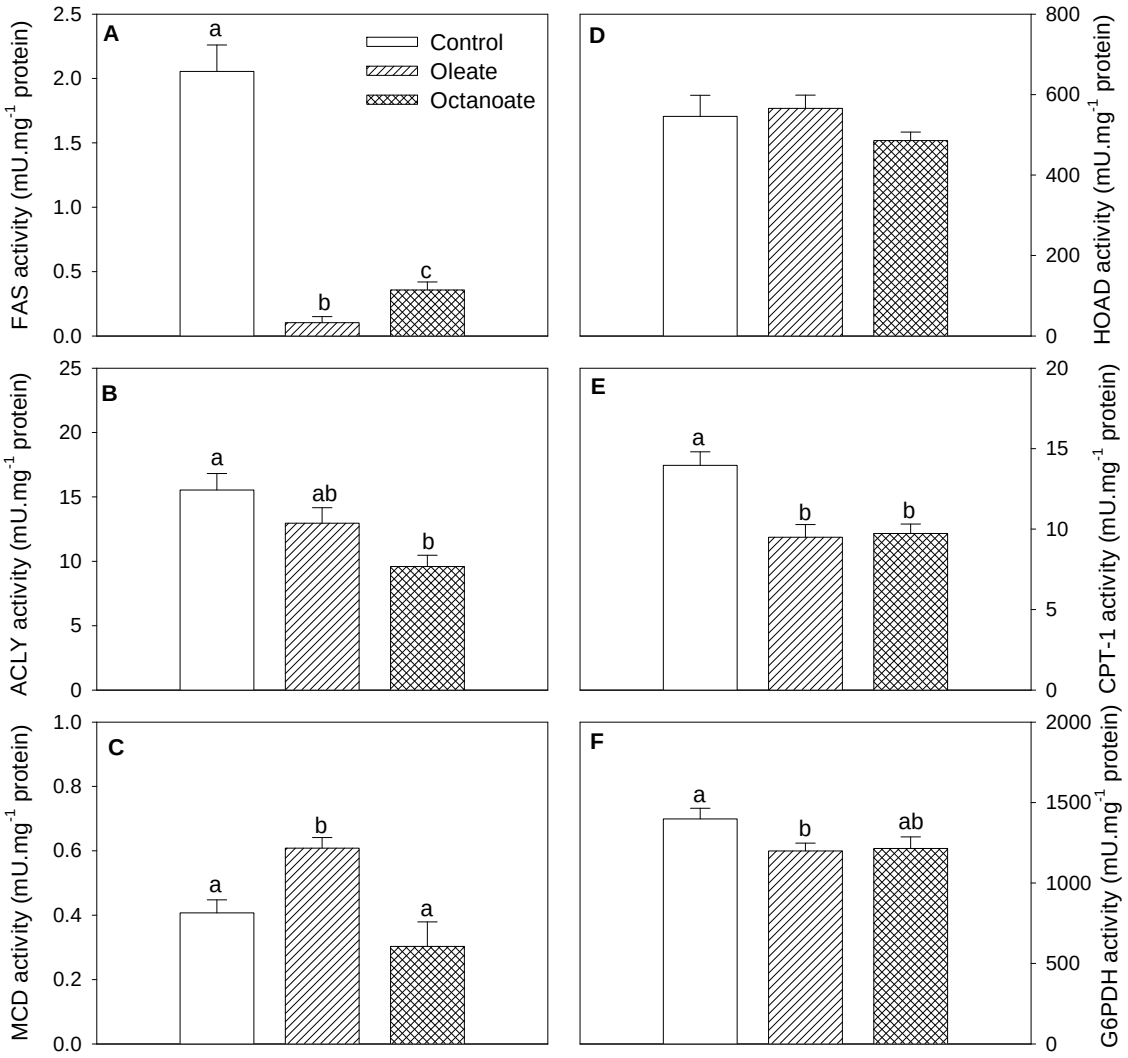

Figure 3 Activities of FAS (A), ACLY (B), MCD (C), HOAD (D), CPT-1 (E), and G6PDH (F) in liver of rainbow trout after 6h of intracerebroventicular administration of 1 µL saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

No significant differences were observed in the activity of liver enzymes involved in glucose metabolism (Fig. 4).

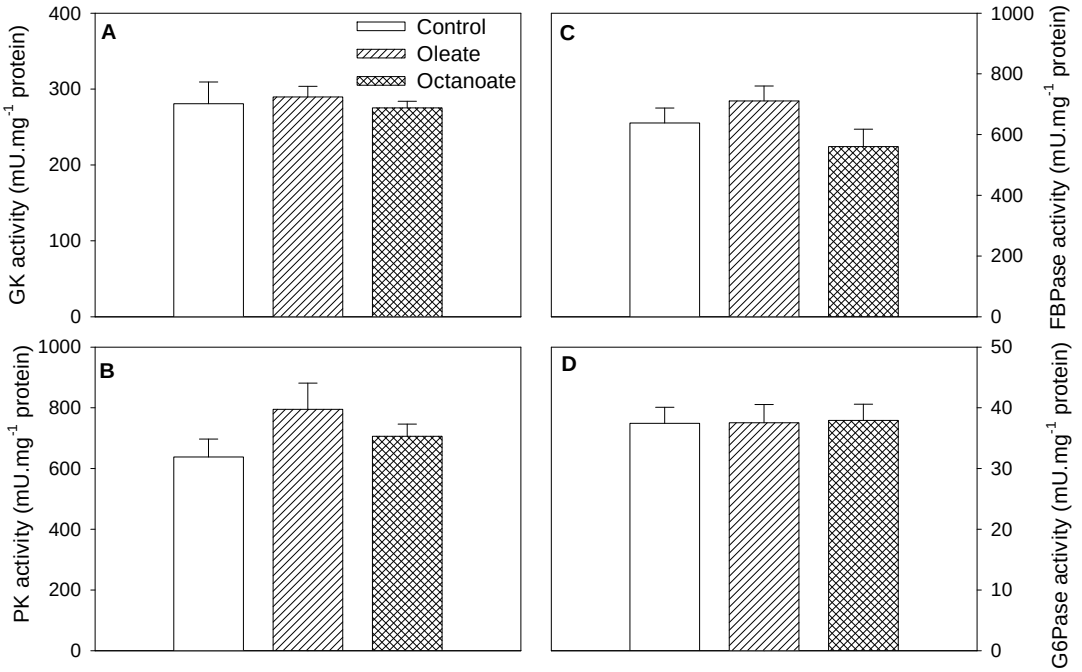

Figure 4 Activities of GK (A), PK (B), FBPase (C), and G6Pase (D) in liver of rainbow trout after 6h of intracerebroventicular administration of 1 µL saline alone (control) or containing 1 µmol oleate or octanoate. Each value is the mean + SEM of n = 10 fish per treatment.

Changes in mRNA levels assessed in Brockmann bodies are shown in table 2. Oleate treatment compared with controls down-regulated mRNA levels of ACLY and FAS, and up-regulated those of CPT-1a, CS, and SREBP1c. Octanoate treatment compared with controls down-regulated mRNA levels of FAT/CD36, ACLY, CPT-1b, FAS, and PPARα, and up-regulated those of CS and MCD. Changes elicited by oleate treatment were significantly different than those elicited by octanoate treatment for mRNA levels of FAT/CD36, ACLY, CPT-1a, CPT-1b, PPARα, and SREBP1c.

Table 2 mRNA levels in Brockmann bodies of rainbow trout after 6h of intracerebroventicular administration of 1 µL saline alone (control) or containing 1 µmol oleate or octanoate.

	Treatment	
Parameter	**Oleate**	**Octanoate**
Fatty acid transport		
FAT/CD36	-1.14	-1.95*#
Fatty acid metabolism		
ACC	+1.17	-1.23
ACLY	-2.58*	-1.49*#
CPT-1a	+2.07*	-1.24#
CPT1-b	+1.27	-2.02*#
CS	+1.62*	+1.69*
FAS	-1.56*	-1.71*
MCD	+1.18	+1.58*
Mitochondrial uncoupling		
UCP2a	-1.28	-1.27
K_{ATP} *channel*		
Kir6.x-like	-1.15	+1.26
SUR-like	-1.16	+1.12
Transcription factors		
LXRα	-1.23	+1.03
PPARα	+1.16	-2.36*#
SREBP1c	+1.50*	+1.05#

Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by β-actin mRNA levels, which did not show changes among groups), and represent the mean of 5 measurements. *, significantly different (P<0.05) from control fish. #, significantly different (P<0.05) from oleate treatment.

Changes in mRNA levels assessed in liver are shown in table 3. Oleate treatment compared with controls down-regulated mRNA levels of CPT-1b, PPARα, FBPase, and GK, and up-regulated those of CPT-1a, MCD, and UCP2a. Octanoate treatment compared with controls down-regulated mRNA levels of CPT-1b, PPARα, and GK, and up-regulated those of MCD, UCP2a, and SREBP1c. Changes elicited by oleate treatment were significantly different than those elicited by octanoate treatment for mRNA levels of CPT-1a, SREBP1c, FBPase, and GK.

Table 3 mRNA levels in liver of rainbow trout after 6h of intracerebroventicular administration of 1 µL saline alone (control) or containing 1 µmol oleate or octanoate

	Treatment	
Parameter	**Oleate**	**Octanoate**
Fatty acid metabolism		
ACC	+1.04	-1.19
ACLY	+1.32	+1.14
CPT-1a	+2.08*	+1.22#
CPT1-b	-1.53*	-2.06*
FAS	+1.17	+1.25
MCD	+1.64*	+1.79*
Mitochondrial uncoupling		
UCP2a	+1.70*	+1.64*
Transcription factors		
PPARα	-1.77*	-1.94*
SREBP1c	+1.28	+1.62*#
Glucose metabolism		
FBPasa	-1.49*	+1.16#
G6Pase	-1.11	-1.22
GK	-2.25*	-4.35*#
PK	-1.30	-1.22

Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalised by β-actin mRNA levels, which did not show changes among groups), and represent the mean of 5 measurements. *, significantly different ($P<0.05$) from control fish. #, significantly different ($P<0.05$) from oleate treatment.

DISCUSSION

The present study was designed to assess whether or not FA sensing previously characterized in liver and Brockmann bodies of rainbow trout (Librán-Pérez *et al.* 2012, 2013a) is an indirect response related to previous FA sensing in hypothalamus or be the result a hypothetical interaction with other endocrine systems. The results obtained pointed to a different behaviour of both tissues. Since the present study aims to compare with results obtained in previous studies carried out after IP or *in vitro* treatments it is important to emphasize that all these studies were carried out under equivalent conditions (size of the same origin, similar size, same stocking conditions, same experimental facilities, same diet, etc).

No changes in levels of plasma metabolites were noted after ICV treatment as described in Librán-Pérez *et al*. (2014a) where the results obtained in parameters measured in plasma and hypothalamus in the same experiment are shown. Therefore, we may suggest that changes in parameters assessed in peripheral tissues after ICV treatment with oleate or octanoate should be the result of central FA detection and transmission of such information, and not the result of changes induced by altered levels of plasma metabolites.

FA sensing capacity in BB is direct but probably influenced by circulating hormone levels

ICV treatment with oleate or octanoate elicited changes in several parameters of BB related to FA metabolism such as FA levels, activities of ACLY, CPT-1, and CS, and mRNA levels of FAS, ACLY, CPT-1a (oleate), FAT/CD36 (octanoate) and PPARα (octanoate). More important, few of those changes were comparable either to those described in the same tissue after IP treatment (Librán-Pérez *et al*. 2012) or to those described in mammals under FA sensing situations (MacDonald *et al*. 2008). Thus, only changes in levels of FA, and mRNA levels of FAS (octanoate), ACLY, CPT-1b (octanoate) and MCD (octanoate) would support a FA sensing response in that tissue.

When the results were compared with those previously observed after IP treatment (Librán-Pérez *et al*. 2012) few coincidences were noted, such as those of FAS (activity and mRNA levels) and mRNA levels of CPT-1b (octanoate) and SREBP1c (oleate). If FA sensing in BB would be related to previous FA sensing in hypothalamus then similar results than those observed after IP treatment should have been observed in the present ICV study. Furthermore, the lack of coincident results was general for the three FA sensing systems assessed, such as those mediated by FA metabolism, FA transport

through FAT/CD36, and mitochondrial activity. It is interesting that for the two later systems the coincidences vs. IP treatment were almost inexistent (only SREBP1c mRNA levels after oleate treatment). Therefore, these results support that the direct FA sensing capacity observed *in vitro* in BB is modified under the *in vivo* conditions of IP treatment by the interaction with the effects of peripheral endocrine systems induced by changes in circulating FA levels.

In this way, feeding fish with diets enriched in FA is known to increase insulin levels (Barma *et al*. 2006), and insulin treatment in rainbow trout is known to enhance the potential of lipogenesis as well as to decrease the potential for FA oxidation in several fish tissues (Plagnes-Juan *et al*. 2008; Lansard *et al.* 2009; Polakof *et al*, 2010a, 2011a; Caruso and Sheridan, 2011). Therefore, a rise in insulin levels would result in changes that in general agree with those previously reported *in vivo* after IP treatment (Librán-Pérez *et al*. 2012). There are no studies available regarding the effects of insulin treatment in parameters related to FA metabolism and transport in fish BB (Polakof *et al*. 2008c, 2012), but there are references available in other tissues describing changes in parameters such as FAS, CPT-1, and FAT/CD36 (Plagnes-Juan *et al*. 2008; Lansard *et al*. 2009; Cruz-García *et al*. 2011; Sánchez-Gurmaches *et al*. 2012).

Since in mammals insulin release from pancreas is also related to hypothalamic FA sensing (Obici *et al*. 2002; Caspi *et al.* 2007) a similar mechanism cannot be discarded to be also present in rainbow trout. In that case, the hypothetical rise in insulin levels in plasma after IP treatment with FA would be the result of the addition of two components, i.e. the enhancement of insulin release due to the increased action of FA in β-cells, and the increased insulin release in BB induced by previous hypothalamic FA sensing mediated by vagal and/or splanchnic outflow. Considering that the effects of ICV treatment in BB were completely different than those observed after IP treatment the first possibility seems more likely.

FA sensing capacity in liver is indirect and probably be the result of previous hypothalamic FA sensing

In contrast to the results obtained in BB, many of the parameters assessed in liver displayed responses to ICV treatment with oleate and octanoate in parameters related to FA metabolism similar than those previously addressed after IP treatment (Librán-Pérez *et al*. 2013a) and different than those observed after *in vitro* treatment (Librán-Pérez *et al*. 2013c). These include FA and total lipid levels, activities of FAS, ACLY (octanoate), MCD (oleate) and HOAD, and mRNA levels of ACC, ACLY, CPT-1b, and MCD (oleate). Moreover, many of the changes noted after ICV treatment in liver were clearly coincident with the

known model of FA sensing including inhibition of FAS, ACLY (octanoate), and CPT-1 as well as MCD activation.

In mammals, FA sensing in hypothalamus is related to the regulation of glucose homeostasis by affecting liver energy metabolism (Blouet and Schwartz 2010). The downstream mechanism(s) by which FA modulate hepatic glucose fluxes are likely based on sympathetic and parasympathetic systems that provide direct innervations of the liver via the splanchnic nerve and vagus nerve, respectively (Morgan *et al.* 2004; Migrenne *et al.* 2006). We observed in rainbow trout hypothalamus the presence of putative FA-sensing systems responding directly to increased levels of oleate or octanoate (Librán-Pérez *et al.* 2012, 2013b, 2014). Moreover, vagus and splanchnic nerves are present in rainbow trout gastrointestinal tract though it is not clear whether or not branches of those nerves arrive to the liver (Burnstock 1959; Seth and Axelsson, 2010). We may therefore suggest that the results obtained after IP treatment for FA-sensing parameters related to FA metabolism are the result of previous hypothalamic FA sensing.

In the FA sensing mechanism related to FAT/CD36, only changes in SREBP1c mRNA levels after octanoate treatment were comparable to those previously observed in the same tissue after IP treatment (Librán-Pérez *et al.* 2013a). Therefore, the putative FA sensing mechanism based on FAT/CD36 in liver is not responding to ICV treatment. Considering that responses to FA treatment were noted after IP treatment for parameters related to FAT/CD36 we may suggest that FA sensing observed in liver after IP treatment (Librán-Pérez *et al*. 2013a) via FAT/CD36 was not related to previous FA hypothalamic sensing and therefore could be related to changes in circulating levels of peripheral hormones induced by raised FA levels. In this way, a recent study by Sánchez-Gurmaches *et al.* (2012) reported that insulin treatment in rainbow trout enhanced levels of FAT/CD36 mRNA in liver, which would be in agreement with the hypothetical effect induced by raised levels of circulating insulin after IP treatment with oleate or octanoate (Librán-Pérez *et al*. 2013a).

As for the FA sensing mechanism related to mitochondrial activity only minor changes had been observed after IP treatment (Librán-Pérez *et al*. 2013a), and a similar response was noted in the present ICV study. Therefore, we may suggest that this FA sensing mechanism is not directly present in liver, and is not influenced by previous hypothalamic FA sensing and/or changes in peripheral hormone levels.

In mammals central FA detection in hypothalamus controls not only food intake but also peripheral energy expenditure, body fat content, and hepatic glucose production (Folmes and Lopaschuk 2007). Thus, ICV administration of oleate (but not octanoate) in mammals results in a marked decrease in hepatic glucose production via decreased glycogenolysis and glucose release (Obici *et al*. 2002; Morgan *et al.* 2004). This metabolic effect requires intact vagus nerve efferences and central activation of K^{+}_{ATP}. When we

consider changes observed in parameters related to glucose metabolism in liver we observed that ICV treatment with oleate or octanoate increased levels of glucose and glycogen (the later not in the case of octanoate) and decreased mRNA levels of GK and FBPase (oleate), which were similar to those previously observed after IP (Librán-Pérez *et al*. 2013a) but not after *in vitro* (Librán-Pérez *et al*. 2013c) treatments. Therefore, the present results would support that hypothalamic FA sensing induces in liver changes in glucose metabolism similar to those observed in mammals with the main difference of the lack of effects of octanoate in mammals (Obici *et al.* 2002). Changes in liver glucose metabolism therefore support outflow from the hypothalamus to the liver, thus indirectly supporting the results obtained in parameters related to FA metabolism.

In conclusion, central treatment of rainbow trout with oleate or octanoate induced changes in several parameters related to FA sensing in peripheral tissues of rainbow trout such as BB and liver. Changes observed in BB were completely different than those previously observed after IP treatment with the same FA and also different than those observed under *in vitro* conditions. Therefore, FA sensing in BB is apparently direct and under IP conditions of raised FA levels in plasma is not related to previous FA sensing in hypothalamus but can be influenced by changes in the levels of peripheral hormones like insulin. In contrast, results obtained after ICV treatment with FA in liver were comparable to those observed after IP treatment but different than those observed after *in vitro* treatment with the same FA. Therefore, FA sensing in liver is apparently indirect and be the consequence of previous hypothalamic FA sensing followed by vagal and/or sympathetic outflow. As a whole, this study confirms previous studies suggesting that liver and Brockmann bodies are able to sense increased circulating levels of oleate and octanoate though the nature of their FA sensing response is completely different being a direct response (probably related to hormone secretion) in Brockmann bodies whereas the response in liver appears to the an efferent response elicited by previous hypothalamic sensing. Further studies are needed to obtain more information regarding such interesting differential behavior.

3.7 TRABAJO EXPERIMENTAL Nº7

Hypothalamic response to decreased levels of fatty acid in rainbow trout. Possible involvement of the hypothalamus-pituitary-interrenal axis

INTRODUCTION

Specialized neurons within mammalian hypothalamus have been suggested to detect increases in plasma levels of long-chain fatty acid (LCFA), but not short-chain (SCFA) or medium-chain (MCFA) FA through several mechanisms (López *et al*., 2007; Benoir *et al*., 2009; Blouet and Schwartz 2010), such as 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); iii) activation of protein kinase C-θ; and iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity. Changes in these systems have been associated (Diéguez *et al.,* 2011) with the modulation of hypothalamic homeobox domain transcription factor (BSX), forkhead box 01 (Fox01), and phosphorylated cAMP response-element binding protein (pCREB). The action of these factors would result in 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 (López *et al*., 2007; Benoir *et al*., 2009; Blouet and Schwartz 2010; Diéguez *et al.,* 2011).

In fish, a reduced food intake has been observed after feeding fish with lipid-enriched diets or in fish containing high fat stores (Shearer *et al*., 1997; Silverstein *et al*., 1999; Rasmussen *et al.,* 2000; Gélineau *et al*., 2001; Johansen *et al*., 2002, 2003; Figueiredo-Silva *et al*., 2012a) raising the question whether lipid sensing mechanisms regulating food intake may be also present in fish (Froiland *et al.,* 2012; Soengas, 2014). Accordingly, we observed in rainbow trout *Oncorhynchus mykiss* that intraperitoneal (Librán-Pérez *et al.,* 2012) or intracerebroventricular (Librán-Pérez *et al.,* 2014a) administration of oleate (LCFA) or octanoate (MCFA) elicited an inhibition in food intake. Furthermore, the treatment induced a response in the hypothalamus compatible with FA sensing including reduced potential of lipogenesis and FA oxidation, decreased potential of K_{ATP}, and modulation of FAT/CD36 with subsequent changes in the expression of transcription factors (Librán-Pérez *et al.,* 2012, 2013b, 2014a). This response is comparable in general with that reported in mammals with the main difference of the capacity of fish to respond to increased levels of an MCFA like octanoate (Soengas, 2014). Changes in these hypothalamic pathways can be also related to the control of food intake, since changes in mRNA levels of neuropeptides such as NPY and POMC-A1 were also noted (Librán-Pérez *et al.,* 2012, 2013b, 2014a). In the hypothalamus of another fish species, the orange-spotted grouper (*Epinephelus coioides*), the involvement of FA

metabolism and mitochondrial activity in the orexigenic effects of NPY has been also suggested (Tang *et al.,* 2013).

The fall of blood glucose levels is sensed in central glucosensor areas eliciting counter-regulatory responses to restore glucose levels as demonstrated in mammals (Marty *et al.,* 2007) and fish (Polakof *et al*., 2011b, 2012b). This mechanism has been evidenced in rat for metabolites other than glucose, such as FA. Thus, the counter-regulatory response to decreased circulating levels of FA has been associated with the activation of the hypothalamus-pituitary-adrenal (HPA) axis, and, therefore, to enhanced circulating levels of glucocorticoids whose lipolytic action would restore plasma FA levels (Clément *et al.,* 2002; Oh *et al.,* 2012, 2014). In fish fed with diets containing low lipid levels, an increase in food intake has been described (Silverstein *et al*., 1999; Gélineau *et al*., 2001; Johansen *et al*., 2002, 2003; Schrama *et al*., 2012; Saravanan *et al.,* 2013) but to date there is no evidence available regarding the existence of counter-regulatory responses to decreased FA levels. We hypothesize that the decrease in circulating levels of FA in rainbow trout would result in the down-regulation of putative hypothalamic FA sensing systems (Soengas, 2014) with concomitant changes in the expression of orexigenic and anorexigenic factors ultimately leading to a stimulation of food intake. To assess this hypothesis, we lowered circulating FA levels in rainbow trout treating fish with SDZ WAG 994 (SDZ), a selective A1 adenosine receptor agonist that inhibits lipolysis (Jacobson and Gao, 2006). We also evaluated if the presence of intralipid (a lipid emulsion of phospholipid-stabilized soybean oil for intravenous administration, Sigma Chemical Co, Ref. I-141) was able to counteract changes induced by SDZ treatment. Furthermore, we also evaluated the possible involvement of the hypothalamus-pituitary-interrenal (HPI) axis (fish equivalent to mammalian HPA) in the counter-regulatory response. Thus, we modified an accessible part of the HPI axis such as cortisol levels by treating fish with SDZ in the presence of metyrapone, which decreases cortisol synthesis in fish (Bernier and Peter, 2001; Dindia *et al.,* 2013).

MATERIALS AND METHODS

Ethics statement

The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research. The Ethics Committee of the Universidade de Vigo approved the procedures.

Fish

Rainbow trout obtained from a local fish farm (A Estrada, Spain) were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish weight was 99 ± 3 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain).

Experimental design

Following acclimation, fish were fasted for 24h before treatment to ensure fish had basal levels of metabolic hormones including cortisol. On the day of experiment, a first set of fish were anaesthetized in tanks with 2-phenoxyethanol (Sigma, 0.2% v/v), and weighed. Then, 15 fish per group received intraperitoneally (IP) 10 $mL.Kg^{-1}$ injection of saline solution alone (control, C), or containing SDZ (SDZ; Tocris, 60 $\mu g.Kg^{-1}$), metyrapone (M; Sigma, 1 $mg.Kg^{-1}$), both SDZ and metyrapone (SDZ+M), or both SDZ and intralipid (SDZ+IL; Sigma I-141, 3 $mL.Kg^{-1}$). Blood, hypothalamus, and head kidney samples were taken 6h after treatment, which was chosen on the basis of previous studies in which such time period was necessary to achieve changes in the FA sensing mechanisms when levels of FA were increased (Librán-Pérez *et al.,* 2012, 2013b). Initial concentrations of SDZ were selected based on studies carried out previously in mammals (Cox et *al.,* 1997; Oh *et al.,* 2012), and then in preliminary studies (data not shown) we evaluated different SDZ doses. Since SDZ is known to reduce mean arterial pressure and heart rate at high doses (Cox *et al.,* 1997) we selected a dose (60 $\mu g.Kg^{-1}$) able to lower levels of circulating FA without inducing any other apparent alteration. The concentrations of metyrapone and intralipid were selected based on previous studies carried out in rainbow trout (Bernier and Peter, 2001; Milligan, 2003; Dindia *et al.,* 2013) and mammals (Oh *et al.,* 2012), respectively. In each group, 10 fish were used to assess enzyme activities and metabolite levels whereas the remaining 5 fish were used for the assessment of mRNA levels by qRT-PCR. In each sampling, fish were anesthesized as above, and blood was collected from the caudal vein with a heparinised syringe. Fish were then sacrificed by decapitation, and hypothalamus and head kidney (area containing interrenal cells, i.e. those involved in glucocorticoid synthesis in fish) were taken and stored as previously described (Polakof *et al.,* 2008a; Conde-Sieira *et al.,* 2013).

In a second set of fish, we evaluated changes in food intake after IP administration of SDZ or SDZ + intralipid. Fish were randomly assigned to experimental groups in different tanks and fasted for 24 h before injection. Then, 8 fish per group were anesthesized and IP injected as above. Food intake was assessed 3 days before treatment (to define baseline data), and then 6 and 24 h after treatment. After feeding, 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 previously described as the difference from the feed offered (De Pedro *et al.,* 1998; Polakof *et al.,* 2008c) . Results are shown as the mean ± SEM of the data obtained in three different tanks per treatment.

Assessment of metabolite levels and enzyme activities

Levels of FA, total lipid, triglyceride, glucose, and lactate in plasma were determined enzymatically using commercial kits (Wako for FA; Spinreact for total lipid, triglyceride, and lactate; Biomérieux for glucose) adapted to a microplate format. Plasma cortisol levels were assessed by ELISA using a commercially available kit (Cayman).

Samples used to assess hypothalamic metabolite levels 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 FA, total lipid, and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT-1 activity, of 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA (DTNB) complex at 412 nm. The reactions were started by the addition of supernatant (15 µl) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed per protein level, which was assayed according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Enzyme activities were assessed at maximum rates determined by preliminary tests to determine optimal substrate concentrations. ATP-citrate lyase (ACLY, *EC* 4.1.3.8) activity was assessed in a tris-HCl buffer (50 mM, pH 7.8) containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM citrate, 10 mM β-mercaptoethanol, 5 mM ATP, 0.3 mM NADH, 7 $U.ml^{-1}$ malate dehydrogenase, and 50 µM Coenzyme A (omitted for controls). Fatty acid synthase (FAS, *EC* 2.3.1.85) activity was assessed in a phosphate buffer (100 mM, pH 7.6) containing 0.1 mM NADPH, 25 µM Acetyl-CoA, and 30 µM Malonyl-CoA (omitted for controls). Hydroxyacil-CoA dehydrogenase (HOAD, *EC* 1.1.1.35) activity was assessed in a imidazole buffer (50 mM, pH 7.6) containing 0.15 mM NADH and 3.5 mM Acetoacetyl-CoA (omitted for controls). CPT-1 (*EC* 2.3.1.21) activity was assessed in a tris-HCl buffer

(75 mM, pH 8.0) containing 1.5 mM EDTA, 0.25 mM DTNB, 35 µM palmitoyl CoA, and 0.7 mM L-carnitine (omitted for controls).

mRNA abundance analysis by quantitative RT-PCR

Total RNA extracted from tissues using Trizol reagent (Life Technologies) was treated with RQ1-DNAse (Promega). 4 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 Fisher Scientific), in a total PCR reaction volume of 25 µl, containing 50-500 nM of each primer. mRNA abundance of transcripts 3β-hydroxysteroid dehydrogenase (3βHSD), 11β-hydroxylase (11βH), acetyl-CoA carboxylase (ACC), ACLY, CART, corticotrophin releasing factor (CRF), corticotrophin releasing factor binding protein (CRFBP), FAT/CD36, CPT-1, citrate synthetase (CS), FAS, inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), liver X receptor α (LXRα), malonyl CoA dehydrogenase (MCD), NPY, cytochrome P450 cholesterol side chain cleavage (P450scc), POMC-A1, PPARα, SREBP1c, steroidogenic acute regulatory protein (StAR), sulfonylurea receptor-like (SUR-like), and mitochondrial uncoupling protein 2a (UCP2) was determined as previously described in the same species (Geslin, 2004; Ducasse-Cabanot *et al*., 2007; Geurden *et al.,* 2007; Kolditz *et al*., 2008; Cruz-Garcia *et al*., 2009; Lansard *et al*., 2009; Polakof *et al*., 2010a, 2011a; Conde-Sieira *et al.,* 2010; Figueiredo-Silva *et al*., 2012a; Librán-Pérez *et al.,* 2013a). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1. Relative quantification of the target gene transcripts was done using elongation factor 1α (EF-1α) gene expression as reference, which was stably expressed in this experiment.

Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq^{TM} DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15s for denaturing, annealing at specific temperatures for 30s, and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient from 55 to 95°C) to ensure amplification of only one fragment. Each sample was assessed in triplicate. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Only efficiency values between 85-100% were accepted (the R^2 for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the EF-1α reference gene transcript was made following the Pfaffl method (Pfaffl, 2001).

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Data Base	Accession Number
3β-HSD	TCACAGGGTCAACGTCAAAG	CCTCCTTCTTGGTCTTGCTG	GenBank	S72665.1
11βH	ATTTGCCCTGTACGAGTTGG	GGATGATGATGTCTCTGACTG	GenBank	AF179894
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	Sigenae	tcbk0010c.b.21_5.1.om.4
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	GenBank	CA349411.1
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	GenBank	NM_001124627
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	GenBank	AJ619768
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	GenBank	AJ620356
CRF	ACAACGACTCAACTGAAGATCTCG	AGGAAATTGAGCTTCATGTCAGG	GenBank	AF296672
CRFBP	GGAGGAGACTTCATCAAGGTGTT	CTTCTCTCCCTTCATCACCCAG	GenBank	AY363677
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	Tigr	TC89195
EF-1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	GenBank	AF498320
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	Sigenae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	DFCI	AY606034.1
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	Sigenae	CA346261.1.s.om.8:1:773:1
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	GenBank	FJ470291
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	Sigenae	BX869708.s.om.10
NPY	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	GenBank	NM_001124266
P450scc	ATGCGTCAGGACACTAACAC	CAGCGGTATCATCTTCAGCA	GenBank	S57305.1
POMC-A1	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	Tigr	TC86162
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	GenBank	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank	CA048941.1
StAR	CTCCTACAGACATATGAGGAAC	GCCTCCTCTCCCTGCTTCAC	GenBank	AB047032
Sur-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	Sigenae	tcce0019d.e.20_3.1.s.om.8
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	GenBank	DQ295324

3βHSD, 3β-hydroxysteroid dehydrogenase; 11βH, 11β-hydroxylase; ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; CRF, corticotrophin releasing factor; CRFBP, corticotrophin releasing factor binding protein; CS, citrate synthetase; EF-1α, elongation factor 1α; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; Kir6.x-like, inward rectifier K^+ channel pore type 6.x-like; LXRα, liver X receptor α; MCD, malonyl CoA dehydrogenase; NPY, neuropeptide Y; P450scc, cytochrome P450 cholesterol side chain cleavage; POMC-A1, pro-opio melanocortin A1; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; StAR, steroidogenic acute regulatory protein; SUR-like, sulfonylurea receptor-like; UCP2a, mitochondrial uncoupling protein 2a.

Statistics

Comparisons among groups were carried out with two-way ANOVA with treatment and time as main factors for food intake data whereas the remaining parameters were compared with one-way ANOVA. Post-hoc comparisons were carried out with a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$.

RESULTS

Food intake increased after 6 and 24h of treatment with SDZ, and the presence of intralipid counteracted the increase (Fig. 1).

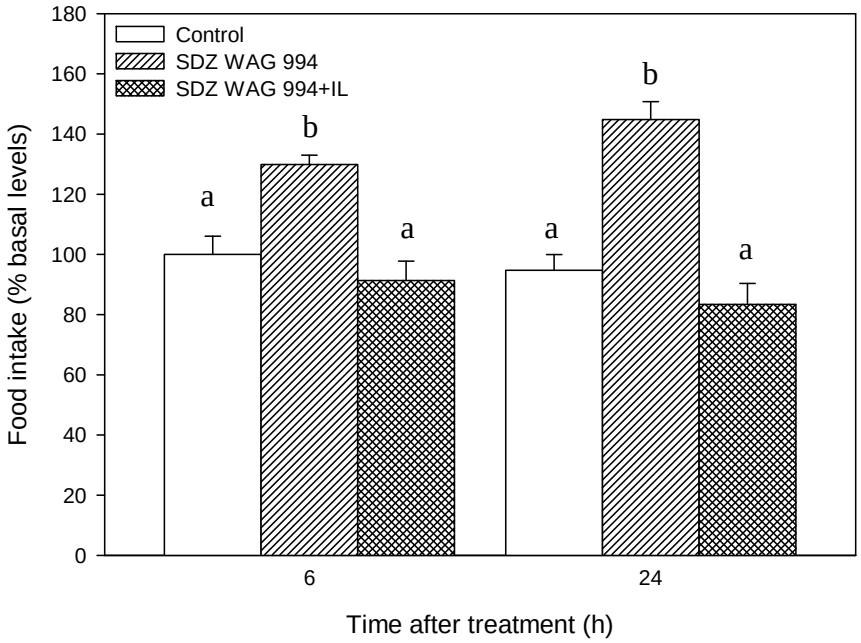

Fig. 1. Changes in food intake after intraperitoneal treatment. Food intake of rainbow trout after 6h or 24h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control) or containing SDZ WAG 994 alone (60 µg.Kg^{-1}) or containing SDZ WAG 994 (60 µg.Kg^{-1}) together with intralipid (3 mL.Kg^{-1}) solution. Different letters indicate significant differences (P<0.05) among treatments at the same time. Food intake is shown as mean + S.E.M. of the percentage of food ingested with respect to basal levels (calculated as the average of food intake the three days previous to experiment). The results are shown as mean + S.E.M. of the results obtained in three different tanks in which 8 fish were used per group in each tank. Different letters indicate significant differences (P<0.05) among treatments at each time.

Levels of metabolites assessed in plasma are shown in Fig. 2. FA (Fig. 2A) and triglyceride (Fig. 2B) levels decreased after SDZ treatment compared with all other groups. Total lipid levels (Fig. 2C) decreased after treatment with SDZ or SDZ +IL compared with the remaining groups, and the decrease was weaker in the SDZ +IL group. Glucose levels increased after treatment with SDZ +M compared with control and SDZ groups (Fig. 2D). Lactate levels increased after SDZ +IL treatment compared with SDZ +M (Fig. 2E). Cortisol levels were higher in the SDZ group than in all other groups (Fig. 2F).

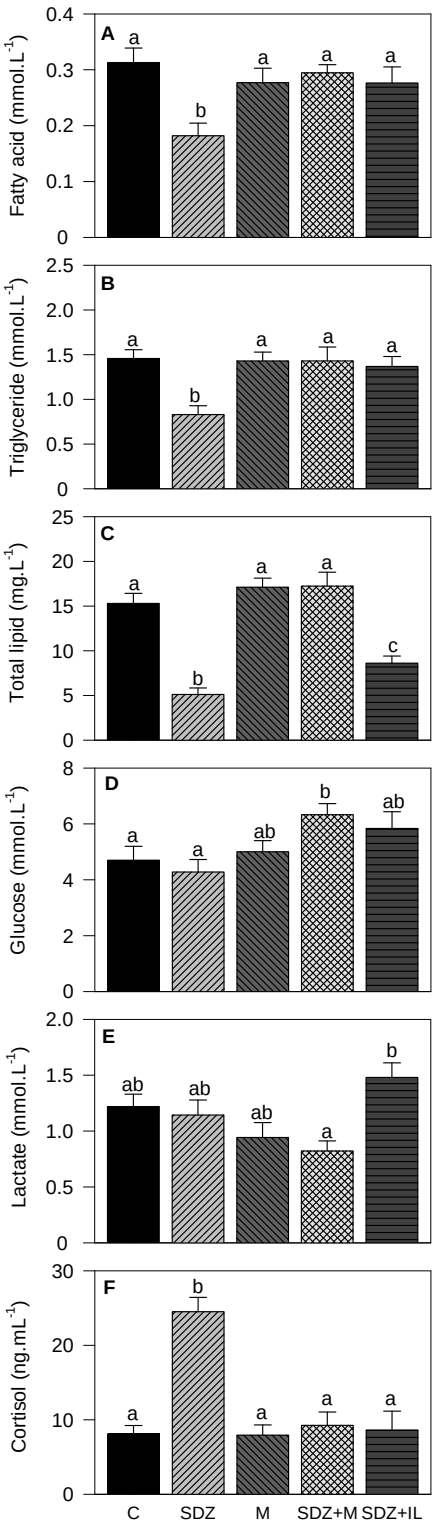

Fig. 2. Changes in the levels of plasma metabolites after intraperitoneal treatment. Levels of fatty acid (A), triglyceride (B), total lipid (C), glucose (D), lactate (E), and cortisol (F) in plasma of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), both SDZ WAG 994 and metyrapone (SDZ+M), or both SDZ WAG 994 and intralipid (3 mL.Kg^{-1}) solution (SDZ+IL). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

In hypothalamus (Fig. 3) SDZ treatment decreased levels of FA (vs. all other groups) and triglycerides (vs. control, SDZ +M and SDZ +IL groups) whereas no significant changes were noted for total lipid levels.

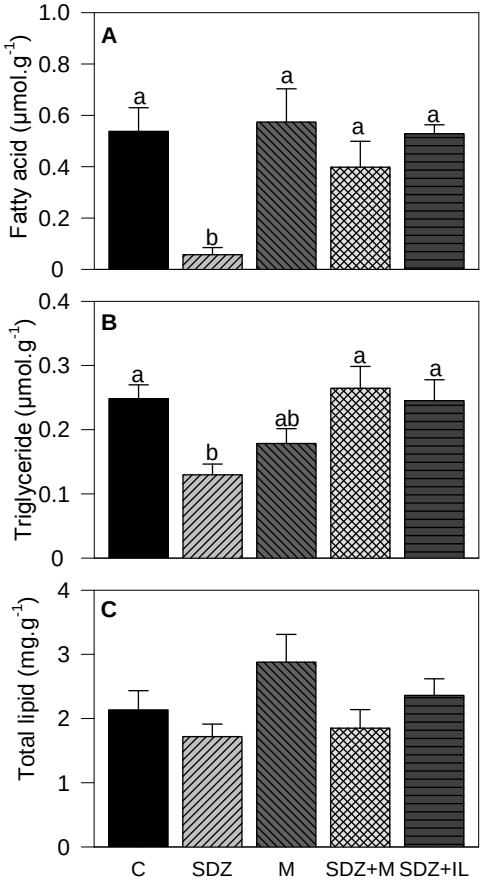

Fig. 3. **Changes in the levels of metabolites in hypothalamus after intraperitoneal treatment.** Levels of fatty acid (A), triglyceride (B), and total lipid (C) in hypothalamus of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), both SDZ WAG 994 and metyrapone (SDZ+M), or both SDZ WAG 994 and intralipid (3 mL.Kg^{-1}) solution (SDZ+IL). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

Enzyme activities assessed in hypothalamus are shown in Fig. 4. FAS activity was lower in the SDZ and M groups compared with the remaining groups (Fig. 4B). HOAD activity was lower in the SDZ group than in control, SDZ +M, and SDZ +IL groups (Fig. 4D). We did not observe significant differences for CPT-1 (Fig. 4A) and ACLY (Fig. 4C) activities.

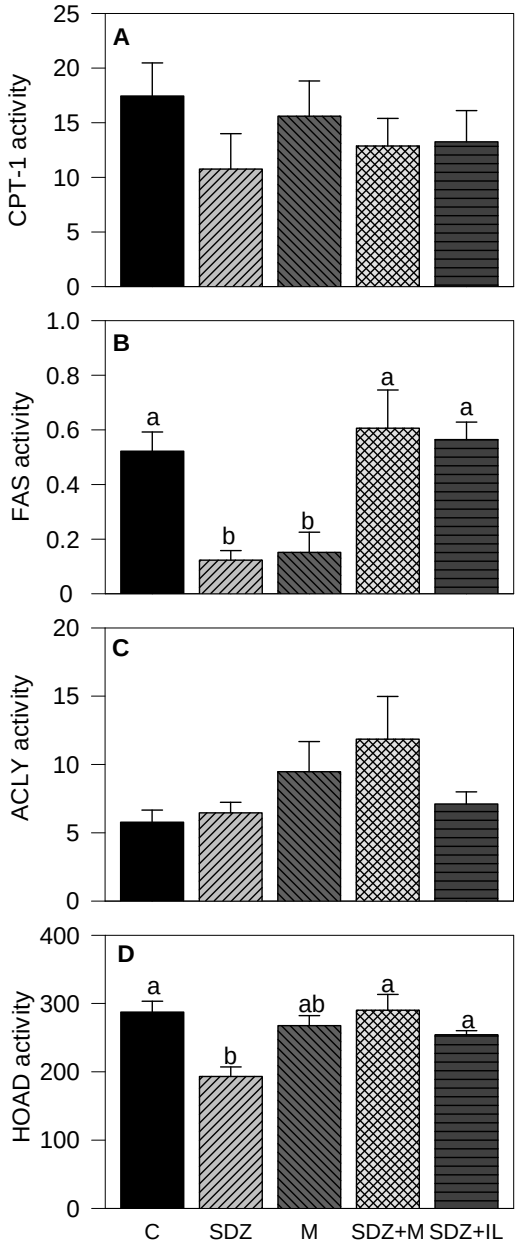

Fig. 4. **Changes in the activity of enzymes in hypothalamus after intraperitoneal treatment.** Activities (mU.mg^{-1} protein) of CPT-1 (A), FAS (B), ACLY (C), and HOAD (D) in hypothalamus of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), both SDZ WAG 994 and metyrapone (SDZ+M), or both SDZ WAG 994 and intralipid (3 mL.Kg^{-1}) solution (SDZ+IL). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

RNA abundance of transcripts related to fatty acid sensing in hypothalamus is shown in Table 2. FAT/CD36 level was lower in the SDZ group compared with control, M, and SDZ +IL groups. ACC level increased in the M group compared with control, SDZ +M, and SDZ +IL groups. ACLY level increased after M treatment compared with control, SDZ, and SDZ +IL treatments. CPT-1c level decreased in the SDZ group compared with control and SDZ +IL groups. Levels of CPT-1d in the SDZ group were lower than in control, M, and SDZ +IL groups. FAS level increased in the group treated with SDZ compared with all other groups. MCD values were lower in the SDZ group than in control and SDZ +IL groups. UCP2a values were higher in the SDZ group than in control, SDZ +M, and SDZ +IL groups whereas those of SDZ +IL group were lower than in all other groups. The level of SUR-like was lower in the M and SDZ +M groups than in all other groups. PPARα level increased after treatment with M or SDZ +M compared with all other groups. CART level decreased after treatment with SDZ or SDZ +M compared with control and SDZ +IL groups. POMC-A1 level decreased after treatment with SDZ or SDZ +M compared with control and SDZ +IL groups with the decrease being more important for SDZ +M; the value of SDZ +IL was higher than in all other groups. Finally, no significant changes were apparent for CS, Kir6.x-like, LXRα, and NPY.

Table 2. Relative mRNA abundance of transcripts related to fatty acid sensing in hypothalamus of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), both SDZ WAG 994 and metyrapone (SDZ+M), or both SDZ WAG 994 and intralipid (3 mL.Kg^{-1}) solution (SDZ+IL).

	C	SDZ	M	SDZ+M	SDZ+IL
Fatty acid transport					
FAT/CD36	1 ± 0.05a	0.63 ± 0.04b	1.13 ± 0.09a	0.89 ± 0.12ab	1.23 ± 0.08a
Fatty acid metabolism					
ACC	1 ± 0.16a	1.15 ± 0.12ab	1.80 ± 0.25b	0.99 ± 0.08a	1.06 ± 0.16a
ACLY	1 ± 0.11a	1.07 ± 0.11a	1.70 ± 0.14b	1.30 ± 0.20ab	0.99 ± 0.14a
CPT-1c	1 ± 0.08a	0.62 ± 0.05b	0.90 ± 0.09ab	0.69 ± 0.10ab	1.15 ± 0.11a
CPT-1d	1 ± 0.06ab	0.57 ± 0.04c	0.96 ± 0.12ab	0.64 ± 0.08bc	1.24 ± 0.11a
CS	1 ± 0.16	0.88 ± 0.12	1.46 ± 0.20	0.93 ± 0.16	1.15 ± 0.12
FAS	1 ± 0.08a	1.58 ± 0.09b	1.17 ± 0.12a	1.16 ± 0.09a	1.14 ± 0.13a
MCD	1 ± 0.08a	0.57 ± 0.06b	0.75 ± 0.13ab	0.72 ± 0.05ab	1.43 ± 0.23a
Mitochondrial uncoupling					
UCP2a	1 ± 0.12a	1.51 ± 0.10b	1.34 ± 0.14a	0.82 ± 0.14a	0.47 ± 0.07c
K_{ATP} channel					
Kir6.x-like	1 ± 0.13	0.98 ± 0.08	0.92 ± 0.11	0.86 ± 0.09	1.40 ± 0.21
SUR-like	1 ± 0.12a	0.81 ± 0.07a	0.58 ± 0.07b	0.49 ± 0.03b	1.03 ± 0.10a
Transcription factors					
LXRα	1 ± 0.13	0.73 ± 0.06	1.22 ± 0.16	0.86 ± 0.09	1.03 ± 0.12
PPARα	1 ± 0.15a	1.02 ± 0.13a	2.39 ± 0.18b	1.88 ± 0.14b	1.26 ± 0.16a
SREBP1c	1 ± 0.11	0.96 ± 0.11	1.31 ± 0.15	0.90 ± 0.09	1.32 ± 0.12
Neuropeptides					
CART	1 ± 0.11a	0.53 ± 0.13b	0.75 ± 0.05ab	0.57 ± 0.08b	1.13 ± 0.11a
NPY	1 ± 0.07	1.09 ± 0.09	1.28 ± 0.15	1.06 ± 0.09	1.03 ± 0.12
POMC-A1	1 ± 0.06a	0.54 ± 0.05b	0.75 ± 0.08ab	0.37 ± 0.06b	2.50 ± 0.22c

Data represent mean of 5 measurements. Data is expressed with respect to the control group (expression results were normalized by EF-1α mRNA levels, which did not show changes among groups). Different letters indicate significant differences (P<0.05) among treatments.

RNA abundance of transcripts related to HPI axis in hypothalamus and head kidney is shown in Table 3. In hypothalamus, CRF value was higher in the SDZ group compared with all other groups whereas value of SDZ +IL group was higher than in M and SDZ +M groups. CRFBP levels increased after treatment with SDZ or M compared with control, SDZ +M, and SDZ +IL groups. 3βHSD level increased after treatment with SDZ compared with all other groups; values in the SDZ +M group were lower than in all other groups.

11βH level increased after treatment with SDZ or M compared with all other groups with the increase being higher for M group, and decreased after treatment with SDZ +M compared with all other groups. P450scc level increased in the group treated with SDZ compared with the remaining groups; the level in the M and SDZ +M groups was lower than in all other groups with the decrease being more important for SDZ +M. Finally, StAR value increased after treatment with SDZ compared with all other groups.

Table 3. Relative mRNA abundance of transcripts related to hypothalamus-pituitary-interrenal axis in hypothalamus and head kidney of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), both SDZ WAG 994 and metyrapone (SDZ+M), or both SDZ WAG 994 and intralipid (3 mL.Kg^{-1}) solution (SDZ+IL).

	C	SDZ	M	SDZ+M	SDZ+IL
Hypothalamus					
CRF	1 ± 0.11a	1.74 ± 0.10b	0.69 ± 0.09a	0.63 ± 0.07a	1.19 ± 0.11a
CRFBP	1 ± 0.09a	1.49 ± 0.07b	1.88 ± 0.17b	1.35 ± 0.10a	1.01 ± 0.12a
Head Kidney					
3βHSD	1 ± 0.17a	1.77 ± 0.12b	1.30 ± 0.15a	0.32 ± 0.03c	0.86 ± 0.14a
11βH	1 ± 0.13a	1.91 ± 0.14b	3.17 ± 0.39c	0.31 ± 0.09d	1.28 ± 0.20a
P450scc	1 ± 0.15a	1.68 ± 0.07b	0.52 ± 0.11c	0.08 ± 0.01d	1.08 ± 0.15a
StAR	1 ± 0.18a	1.92 ± 0.13	1.23 ± 0.10a	0.77 ± 0.09a	1.26 ± 0.11a

Data represent mean of 5 measurements. Data is expressed with respect to the control group (expression results were normalized by EF-1α mRNA levels, which did not show changes among groups). Different letters indicate significant differences (P<0.05) among treatments.

DISCUSSION

Lowering circulating FA levels in rainbow trout decreased anorexigenic potential in hypothalamus and increased food intake

Treatment with SDZ was effective in reducing not only circulating FA levels but also those of triglyceride and total lipid, which validates the experimental design. These changes are similar to those reported in rat after similar treatment (Kashiwagi. 1995; Cox *et al.,* 1997; Oh *et al.,* 2012). There are no comparable references available in fish, though in zebrafish bezafibrate treatment was also able to decrease plasma triglyceride and cholesterol levels (Velasco-Santamaría *et al.,* 2012) . Moreover, the presence of intralipid was able to counteract the action of SDZ in rainbow trout resulting in levels of FA and triglyceride similar to those of controls, again in agreement with that reported in rat (Oh *et al.,* 2012).

Food intake increased in fish treated with SDZ. This allows us to suggest, for the first time in fish, that hypothalamus sense the decreased levels of FA resulting in an orexigenic response stimulating food intake. We cannot exclude the possibility that SDZ could have a direct effect on food intake independent of that elicited by the inhibition of lipolysis. However, the finding that the presence of intralipid counteracted the increased food intake suggest that the effect of reduced FA levels is more likely. This increased food intake is in agreement with that reported in different fish species, including rainbow trout, after feeding diets with low lipid content (Silverstein *et al.,* 1999; Gélineau *et al.,* 2001; Johansen *et al.,* 2002, 2003; Schrama *et al.,* 2012; Saravanan *et al.,* 2013). Furthermore, the food intake response is the opposite of that observed when the same species was treated with specific FA such as oleate or octanoate (Librán-Pérez *et al.,* 2012). Changes elicited in the levels of circulating lipids relate to changes observed in hypothalamus since SDZ treatment decreased levels of FA and triglyceride, and levels recovered those of controls after additional treatment with intralipid. Therefore, changes in circulating metabolites in plasma are translated into changes in metabolite levels in hypothalamus thus supporting the FA sensing capacity of that tissue not only to increased levels of FA as previously described (Librán-Pérez *et al.,* 2012, 2013b, 2014a) but also to decreased levels of circulating FA as herein reported. Accordingly, we evaluated changes in parameters related to putative FA sensing systems in hypothalamus in order to relate them to changes observed in food intake.

SDZ treatment affected the FA sensing system related to FA metabolism since it decreased the activity of FAS and HOAD as well as mRNA abundance of CPT1c, CPT1d, and MCD and increased mRNA abundance of FAS. Furthermore, the presence of intralipid counteracted these changes. We have previously observed that this system responds to increased levels of FA with decreased lipogenic potential and decreased capacity of FA

oxidation (Librán-Pérez *et al.,* 2012, 2013b, 2014a). Therefore, we expected that changes observed in parameters related to FA metabolism would be different than those observed after increasing levels of specific FA such as oleate or octanoate (Librán-Pérez *et al.,* 2012). Accordingly, we observed opposite changes for FAS activity and mRNA abundance as well as for mRNA abundance of CPT1c and MCD. However, other parameters that displayed changes in situations of increased circulating levels of oleate or octanoate (Librán-Pérez *et al.,* 2012, 2013b, 2014a) such as CPT-1 and ACLY activities and mRNA abundance of ACC, ACLY, and CS did not display any change in the present study. The responses observed suggest that the FA sensing system related to FA metabolism only respond partially to decreased circulating levels of FA. There are no similar studies available in fish hypothalamus (Soengas, 2014) whereas in peripheral tissues like liver lipogenic potential is up-regulated when fish were fed with diets with low lipid content (Martínez-Rubio *et al.,* 2013).

The putative FA sensing system related to FA transport through FAT/CD36 and subsequent modulation of transcription factors partially responded to decreased circulating levels of FA since several parameters did not show changes compared with controls (mRNA abundance of PPARα, SREBP1c, and LXRα). Only mRNA abundance of FAT/CD36 decreased after SDZ treatment. This change is the opposite of that previously observed when FA levels increased (Librán-Pérez *et al.,* 2012) suggesting a decreased potential for binding capacity of FAT/CD36 in parallel with the decreased circulating FA levels.

As for the putative FA sensing system related to K_{ATP} channel and mitochondrial activity, Kir6.x-like and SUR-like mRNA abundance displayed no changes when circulating FA levels decreased. However, levels of UCP2a mRNA were higher in the group treated with SDZ than in controls, a change opposite of that described when FA levels increased (Librán-Pérez *et al.,* 2012, 2014a).

Despite the relatively few changes observed in the FA sensing systems evaluated, mRNA abundance of anorexigenic peptides (POMC-A1 and CART) clearly decreased in hypothalamus of fish treated with SDZ. This response is the opposite of that observed under conditions of increased circulating FA levels where the abundance of these transcripts increased (Soengas, 2014). Moreover, the increase observed in the mRNA abundance of POMC-A1 in the SDZ+IL group could be associated with the returning of food intake in that group to values similar of those in control group. Furthermore, we did not observe significant changes in NPY mRNA abundance when FA levels decreased, in contrast with the decrease observed when FA levels increased (Librán-Pérez *et al.,* 2012, 2014a). These changes suggest a decreased anorexigenic potential in hypothalamus of SDZ-treated fish, which is in agreement with changes noted in food intake. However, CRF mRNA levels increased under the same conditions what considering the anorexigenic

nature of this peptide suggest a more complex relationship between changes in mRNA levels of neuropeptides and food intake.

Therefore, it seems that the decrease in circulating FA levels induced by SDZ treatment resulted in decreased levels of FA in hypothalamus but only few of the parameters involved in putative FA sensing systems addressed in hypothalamus changed accordingly. However, since the expression of several neuropeptides and, more important, food intake changed under those conditions, we can suggest, for the first time in non-mammalian vertebrates, that central capacity for sensing decreased FA levels exists in fish. However, this modulation of FA sensors is not exactly the opposite of that observed when FA levels increase (Librán-Pérez *et al.,* 2012, 2013b, 2014a). The difference in the response of these systems to increased or decreased FA levels could be due to different factors. Any of these factors could be: i) the involvement of another putative FA sensing mechanism, such as that mediated by PKC-θ (Benoit *et al.,* 2009), or ii) the fact that we evaluated the decrease of FA in general whereas in the other studies carried out in rainbow trout (Librán-Pérez *et al.,* 2012, 2013b, 2014a) we evaluated the increase of specific FA, such as oleate and octanoate. In fact, in a recent study in rat, the counter-regulatory response to decreased FA levels depends on the type of FA (Oh *et al.,* 2014).

The activation of the HPI axis is probably involved in the FA counter-regulatory response to decreased levels of FA

In mammals, different hormones of lipolytic action such as growth hormone (Kreitschmann-Adermahr *et al.,* 2010), epinephrine (Watt *et al.,* 2004), glucagon (Quabbe *et al.,* 1983), or glucocorticoids (Clément *et al.,* 2002; Oh *et al.,* 2012, 2014) were associated with the counter-regulatory response to decreased levels of FA. In the present study, we have evaluated the possibility that cortisol (main glucocorticoid in fish) could be involved in the counter-regulatory response observed in rainbow trout.

SDZ treatment induced an increase in circulating levels of cortisol, which was not observed in the SDZ+IL group. This allows us to suggest that the activation of the HPI axis is involved in the response to decreased levels of FA. This response is comparable to that observed in rat where the presence of intralipid also counteracted the decrease in circulating FA levels induced by SDZ treatment (Oh *et al.,* 2012). We have also monitored in head kidney the mRNA abundance of proteins involved in cortisol synthesis such as 11βH, 3βHSD, P450scc and StAR. Abundance of these transcripts increased after treatment with SDZ, and values returned to normality when fish were co-treated with SDZ and intralipid. In rainbow trout up-regulation of these transcripts corresponds with enhanced cortisol levels in plasma (Aluru and Vijayan, 2006; Alderman *et al.,* 2012). Therefore, changes observed in these transcripts agree well with those of cortisol levels.

Furthermore, we have also monitored hypothalamic CRF and CRFBP mRNA abundance that in the same species are normally changing in parallel with the levels of cortisol in plasma under short-term periods (Jeffrey *et al*., 2014). SDZ treatment increased levels of both transcripts and, once more, the presence of intralipid counteracted such elevation. Interestingly, an inverse relationship between plasma glucose and FA was observed in plasma in agreement with that seen in many fish species under stress situations in which the HPI axis is activated (Wendelaar, 1997). Altogether, these results suggest that decreased circulating levels of FA activate the HPI axis. However, we cannot discard a direct action of SDZ on cortisol synthesis.

Further support of the involvement of the HPI axis comes from results obtained in the groups treated with metyrapone. Metyrapone is an inhibitor of 11βH, and its treatment resulted in decreased circulating levels of cortisol in previously stressed fish (Bernier and Peter, 2001; Milligan, 2003; Dindia *et al.,* 2013) or no changes if fish were not previously stressed (Leach and Taylor, 1980; Tripathi and Verma, 2003), as observed in the present study. A glucocorticoid feedback response (i.e., increased CRF mRNA levels in response to decreased cortisol levels) has been demonstrated in rainbow trout when plasma cortisol levels changed after metyrapone treatment (Bernier and Peter, 2001; Doyou *et al.,* 2006). Thus, the lack of changes in cortisol levels in the M group is also reflected by the absence of changes in hypothalamic CRF mRNA levels. Changes observed in hypothalamus of metyrapone-treated fish (up-regulation of ACC, ACLY and PPARα mRNA abundance) generally fit with those previously observed in catfish brain (Tripathi and Verma, 2003) where increased lipogenic capacity occurred after metyrapone treatment. However, since metyrapone treatment alone induced changes in several parameters assessed in hypothalamus and head kidney, we cannot discard that several of the effects could be attributable to an interactions between SDZ and metyrapone.

Based on the comparison between SDZ and SDZ+M groups, metyrapone treatment was effective in counteracting the increased cortisol levels elicited by SDZ treatment. This effect of metyrapone is in agreement with that previously observed in the same species under different stressful conditions (Bernierand Peter, 2001; Milligan, 2003; Dindia *et al.,* 2013). Furthermore, the treatment with both metyrapone and SDZ induced in plasma changes in parameters in a way that values were comparable to those of the control group and different than those of the SDZ group, such as for plasma FA, triglyceride or total lipid levels. This return to normality is also evident in parameters assessed in hypothalamus where FA and triglyceride levels that decreased in the SDZ group turned back to normal values in the group treated with SDZ and metyrapone. In parameters related to the HPI axis, an effective blockade of its functioning was apparent as demonstrated by the strong decrease observed in transcript abundance of the four proteins related to cortisol metabolism assessed in head kidney in the SDZ + metyrapone group. These changes are associated with those observed in the levels of cortisol in plasma, which were similar to those of the control group and lower than in the SDZ-

treated group. Further support comes from changes observed in CRF and CRFBP mRNA abundance in the hypothalamus where a significant inhibition of the response observed with the SDZ treatment was evident. Altogether, these results suggest us that the HPI axis is involved in the response induced by decreased circulating FA levels produced by SDZ treatment.

The remaining parameters assessed in hypothalamus related to FA sensing displayed in general in the group treated with SDZ and metyrapone values similar to those of controls and different than those elicited by SDZ treatment alone. Thus, metyrapone treatment effectively counteracted changes elicited by SDZ treatment in a way similar to those elicited by the presence of intralipid. Therefore, we suggest that the changes observed in FA sensing mechanisms involved in the counter-regulatory orexigenic response in hypothalamus could relate to the activation of the HPI axis. Since increased CRF production in hypothalamus activates this axis, we could speculate that CRF is modulating the activity of hypothalamic neurons that integrate metabolic information resulting in altered production of anorexigenic and orexigenic neuropeptides and finally in the food intake response, in a way comparable to that previously suggested for glucosensing in the same species (Conde-Sieira *et al.,* 2011). However, blocking the HPI axis with metyrapone did not alter the decreased anorectic potential deduced by the down regulation observed in POMC-A1 and CART transcripts by SDZ treatment in a way similar to the lack of effect of metyrapone treatment on mRNA abundance of NPY in hypothalamus of stressed rainbow trout (Doyou *et al.,* 2006). Moreover, stress situations and factors related to the HPI axis such as CRF have been demonstrated to be clearly anorexigenic in several fish species including rainbow trout (Bernier and Craig, 2005; Bernier *et al.,* 2008) and a clear increase in mRNA levels of CRF was also observed in hypothalamus of SDZ-treated fish. Therefore, the modulation of food intake control by HPI activation is complex and other factors besides those herein assessed, are likely involved.

In summary, we have obtained evidence, for the first time in fish (and in a non-mammalian vertebrate), about the existence of a counter-regulatory response to a fall in circulating FA levels. The response is apparently associated with food intake control and the activation of HPI axis. Thus, the decrease in circulating levels of FA in rainbow trout induces an increase in food intake that is associated with the decrease of the anorexigenic potential in hypothalamus and with changes in several parameters related to putative FA-sensing mechanisms in hypothalamus. The treatment with intralipid counteracted these changes. The decrease in FA levels apparently induces a counter-regulatory response in rainbow trout in which the activation of the HPI axis is likely involved. This activation probably not related to the control of food intake through FA sensor systems (Soengas, 2014) but to the modulation of lipolysis in peripheral tissues to restore FA levels in plasma. This counter-regulatory response initiated in the hypothalamus, probably through changes in CRF, and this activation would arrive to

peripheral tissues such as liver where metabolic changes would occur accordingly. However, we cannot exclude the possibility that i) the fall in plasma FA was sensed outside the brain, and the information be transmitted via an afferent neural pathway or a humoral factor, or ii) that other lipolytic hormones in fish such as GH, glucagon or catecholamines (Harmon *et al.,* 1993; O´Connor *et al.,* 1993; Fabbri *et al*., 1998; Albalat *et al.,* 2005; Sangiao-Alvarellos *et al.,* 2006) could be involved in the FA counter-regulatory response. All these possibilities clearly deserve further studies.

3.8 TRABAJO EXPERIMENTAL Nº8

Brockmann bodies and liver metabolic response to decreased circulating fatty acid levels in rainbow trout; involvement of the hypothalamus-pituitary-interrenal (HPI) axis

INTRODUCTION

In vertebrates circulating levels of fatty acids (FA) fall during the post-absorptive phase and are restored after the meal. The treatment with SDZ WAG 994 (SDZ), a selective A1 adenosine receptor agonist that inhibits lipolysis (Jacobson and Gao, 2006) has been used in mammals to experimentally induce decreased levels of FA, which are sensed in central areas through FA-sensing mechanisms (Le Foll *et al.* 2009; Blouet and Schwartz 2010). The detection elicits counter-regulatory responses to restore FA levels, which have been associated with the activation of the hypothalamus-pituitary-adrenal (HPA) axis (Oh *et al.* 2012, 2014). Thus, the enhanced lypolysis (especially in liver) induced by raised levels of circulating glucocorticoids would restore plasma FA levels (Oh *et al.,* 2012, 2014).

In fish, we previously demonstrated the presence of putative FA-sensing systems responding to increases in the levels of FA like oleate or octanoate in central (hypothalamus) and peripheral (liver and Brockmann bodies; BB, main accumulation of pancreatic endocrine cells in this species) areas of rainbow trout (Soengas 2014).

In peripheral tissues of rainbow trout like liver and BB our previous studies suggested that these tissues are able to sense increased circulating levels of oleate and octanoate. However, the nature of their FA sensing response appear to be different. In BB, the response, probably related to hormone secretion, appears to be direct (not related to previous FA sensing in the hypothalamus), but modulated by the interaction with the effects of peripheral endocrine systems (Librán-Pérez *et al.* 2012, 2013a, 2013b, 2013c). In liver, 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, 2013c, 2015a).

In this species, we also observed that SDZ treatment decreased levels of circulating FA together with increased food intake (Librán-Pérez *et al.* 2014b). The increase in food intake is associated in hypothalamus with the decrease of the anorexigenic potential and with changes in several parameters related to putative FA-sensing mechanisms (Librán-Pérez *et al.* 2014b). The hypothalamus-pituitary-interrenal axis (HPI, equivalent to mammalian HPA) seems to be activated in a counter-regulatory response to reduced FA levels (LIbrán-Pérez *et al.* 2014b). We hypothesize that this activation would enhance lipolysis (targeting to restore FA levels) in peripheral tissues rather than participate in the regulation of food intake. In order to assess this hypothesis, we evaluated the peripheral counter-regulatory response to a pharmacologically-induced reduction in FA circulating levels in rainbow trout. Therefore, we present results obtained in liver and BB of rainbow trout after SDZ treatment including fatty acid, triglyceride, and

total lipid levels as well as parameters related to putative FA-sensing systems based on 1) FA metabolism, such as activity and mRNA abundance of fatty acid synthase (FAS), malonyl CoA decarboxylase (MCD, only in liver), and carnitine palmitoyltransferase-1 (CPT-1), and mRNA abundance of acetyl-CoA carboxylase (ACC), ACLY, CPT-1a, CPT-1b, and MCD; 2) binding to FA translocase (FAT/CD36) and further modulation of transcription factors such as mRNA abundance of peroxisome proliferator-activated receptor type α (PPARα), and sterol regulatory element-binding protein type 1c (SREBP1c), and FAT/CD36 in liver, and liver X receptor α (LXRα) in BB; and, 3) mitochondrial production of reactive oxygen species (ROS), such as mRNA abundance of mitochondrial uncoupling protein 2a (UCP2a), and hydroxyacyl-CoA dehydrogenase (HOAD) activity in liver, and mRNA abundance of inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), and sulfonylurea receptor-like (SUR-like) in BB. In additional groups, we also evaluated the possible involvement of the HPI axis by treating fish with SDZ in the presence of metyrapone (M), an inhibitor of HPI axis in fish (Dindia *et al*. 2013). Accordingly, we also evaluated parameters related to the response of liver to increased cortisol levels in plasma, such as glucose, glycogen, and lactate levels, mRNA abundance of glucocorticoid receptor 1 (GR1) and 2 (GR2) and parameters related to glucose metabolism, such as activity and mRNA abundance of fructose 1,6 bisphosphatase (FBPase), glucose 6-phoshatase (G6Pase), glucose 6-phosphate dehydrogenase (G6PDH), glucokinase (GK), and pyruvate kinase (PK), and activities of glycogen phosphorylase (GPase), and glycogen synthase (GSase).

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) obtained from a local fish farm (A Estrada, Spain) were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 15 °C. Fish weight was 99 ± 3 g. Fish were fed once daily to satiety with commercial fish pellets (Dibaq-Diproteg SA, Spain). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research.

Experimental design

Following 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks. Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. On the day of experiment fish were anaesthetized in tanks with 2-phenoxyethanol (Sigma, 0.2% v/v), and weighed. Then, 15 fish per group received intraperitoneally (IP) 10 $mL.Kg^{-1}$ injection of saline solution alone (control, C), or containing SDZ (Tocris Bioscience, Abingdon, UK, 60 $\mu g.Kg^{-1}$), metyrapone (M; Sigma, 1 $mg.Kg^{-1}$), or both SDZ and metyrapone (SDZ+M). Blood, liver and BB samples were taken 6h after treatment, which was chosen since in previous studies in the same species that was the time period necessary to achieve changes in the FA sensing mechanisms when levels of FA were increased (Librán-Pérez *et al.* 2012, 2013b, 2014a). Initial concentrations of SDZ were selected based on studies carried out previously in mammals (Cox *et al.* 1997; Oh *et al*. 2012), and then in preliminary studies (data not shown) we evaluated different SDZ doses. Since SDZ is known to reduce mean arterial pressure and heart rate at high doses (Cox *et al.* 1997) we selected a dose (60 $\mu g.Kg^{-1}$) able to lower levels of circulating FA without inducing any other alteration. The dose of metyrapone was selected based on previous studies carried out in rainbow trout (Bernier and Peter 2001; Milligan 2003; Dindia *et al.* 2013). After 6h, fish were anesthetized as above. Fish were sacrificed by decapitation, and liver and BB were taken as previously described (Polakof *et al.* 2007a,b), snap-frozen, and stored at -80 °C. In each group, 10 fish were used to assess enzyme activities and metabolite levels whereas the remaining 5 fish were used for the assessment of mRNA levels by qRT-PCR.

Assessment of metabolite levels and enzyme activities

Samples used to assess metabolite levels 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 assayed for tissue metabolites. FA, total lipid, triglyceride, lactate, and glucose levels were determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany, for FA; Spinreact, Barcelona, Spain for total lipid, triglyceride, and lactate, and Biomérieux, Grenoble, France, for glucose) adapted to a microplate format. Tissue glycogen levels were assessed using the method of Keppler and Decker (1974), and glucose levels obtained after glycogen breakdown were assessed as above for plasma samples.

Samples for enzyme activities were homogenized by ultrasonic disruption in 10 vol ice-cold phosphorylation-dephosphorylation stopping buffer containing: 50 mM

imidazole-HCl (pH 7.6), 15 mM 2-mercaptoethanol, 100 mM KF, 5 mM EDTA, 5 mM EGTA, and a protease inhibitor cocktail (Sigma, P-2714). The homogenate was centrifuged (10,000 g) and the supernatant used for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA complex (CPT-1 activity) at 412 nm. The reactions were started by the addition of supernatant (15 µL) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µL), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein, which was assayed according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. FBPase (*EC* 3.1.3.11), G6Pase (*EC* 3.1.3.9), G6PDH (*EC* 1.1.1.49), GK (*EC* 2.7.1.2), GPase (*EC* 2.4.1.1.), GSase (*EC* 2.4.1.11), and PK (*EC* 2.7.1.40) activities were estimated as described previously (Polakof *et al.* 2007a,b; 2008a,b,c; Conde-Sieira *et al.* 2013). ACLY (*EC* 4.1.3.8), FAS (*EC* 2.3.1.85), HOAD (*EC* 1.1.1.35), MCD (*EC* 4.1.1.9), and CPT-1 (*EC* 2.3.1.21) activities were determined as described by Alvarez *et al*. (2000), Polakof *et al.* (2011a), Kolditz *et al.* (2008), Zhou *et al.* (2011), and Ditlecadet and Driedzic (2013), respectively

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. mRNA abundance of ACC, ACLY, CPT-1, FAS, FBPase, G6Pase, G6PDH, GK, GR-1, GR-2, Kir6.x-like, LXRα,, MCD, PK, PPARα, SREBP1c, SUR-like, and UCP2a were determined as previously described in the same species (Panserat *et al.* 2000; Ducasse-Cabanot *et al.* 2007; Kolditz *et al.* 2008; Polakof *et al*. 2008c, 2010a, 2011a; Cruz-Garcia *et al.* 2009; Lansard *et al.* 2009; Figueiredo-Silva *et al.* 2012a; Librán-Pérez *et al.* 2013a). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1. Relative quantification of the target gene transcripts was done using elongation factor 1α (EF-1α) gene expression as housekeeping, which was stably expressed in this experiment.

Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq DNA polymerase activation; 40 steps of PCR were performed, each one consisting of

heating at 95°C for 15s for denaturing, annealing at specific temperatures for 30s, and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (temperature gradient at 0.5°C/s from 55 to 95°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 EF-1α as reference was made following the Pfaffl (2001) method.

Table 1 Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

	Forward primer	Reverse primer	Data Base	Accession Number
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	Sigenae	tcbk0010c.b.21_5.1.om.4
ACLY	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	GenBank	CA349411.1
CPT-1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	GenBank	AF327058
CPT1-1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	GenBank	AF606076
CS	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	Tigr	TC89195
EF-1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	GenBank	AF498320
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	Sigenae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	DFCI	AY606034.1
FBPase	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	GenBank	AF333188
G6Pase	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	Sigenae	cay0019b.d.18_3.1.s.om.8.1-1693
G6PDH	CTCATGGTCCTCAGGTTTG	AGAGAGCATCTGGAGCAAGT	GenBank	CA351434
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	GenBank	AF053331
GR-1	ACTGGTTCCCACAATGCTGT	ATCGGTTCAGGGTCGTCAT	GenBank	NM001124730.1
GR-2	GCTCCTGGCTCTTTCTGATG	TTTGCTCACACTGGTCGTTC	GenBank	AY495372.1
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	Sigenae	CA346261.1.s.om.8:1:773:1
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	GenBank	FJ470291
MCD	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	Sigenae	BX869708.s.om.10
PK	CCATCGTCGCGGTAACAAGA	ACATAGGAAAGGCCAGGGGC	GenBank	AF246146
PPARα	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	GenBank	AY494835
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank	CA048941.1
SUR-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	Sigenae	tcce0019d.e.20_3.1.s.om.8
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	GenBank	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; CPT-1, carnitine palmitoyl transferase type 1; CS, citrate synthetase; EF-1α, elongation factor 1α; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; FBPase, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase; G6PDH, glucose 6-phosphate dehydrogenase; GK, glucokinase, GR-1, glucocorticoid receptor type 1; GR-2, glucocorticoid receptor type 2; Kir6.x-like, inward rectifier K^+ channel pore type 6.x-like; LXRα, liver X receptor α; MCD, malonyl CoA dehydrogenase; PK, pyruvate kinase; PPARα, peroxisome proliferator-activated receptor type α; SREBP1c, sterol regulatory element-binding protein type 1c; SUR-like, sulfonylurea receptor-like; UCP2a, mitochondrial uncoupling protein 2a.

Statistics

Comparisons among groups were carried out using one-way ANOVA followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$. When necessary data were log transformed to fulfill the conditions of the analysis of variance.

RESULTS

Fig. 1 shows levels of metabolites in liver. FA levels decreased in groups treated with SDZ and SDZ+M compared with control and M groups (Fig. 1A). No significant changes occurred in triglyceride (Fig. 1B) levels. Total lipid levels decreased in groups SDZ and SDZ+M compared with control group (Fig. 1C). Glucose levels decreased in group treated with SDZ compared with control group (Fig. 1D). Glycogen levels (Fig. 1E) decreased in fish treated with SDZ compared with control and M groups. Finally, no significant changes occurred in lactate levels (Fig. 1F).

Enzyme activities related to lipid metabolism in liver are shown in Fig. 2. CPT-1 activity decreased in fish treated with SDZ+M compared with control and M groups (Fig. 2A). FAS activity (Fig. 2B) decreased in the SDZ group compared with control, M, and SDZ+M groups. ACLY activity (Fig 2C) was lower in the group treated with M compared with control and SDZ groups. No significant changes were noted for HOAD activity (Fig. 2D). MCD activity (Fig. 2E) increased in the group treated with M compared with control and SDZ groups. Finally, no significant changes were noted for G6PDH (Fig. 2F) activity.

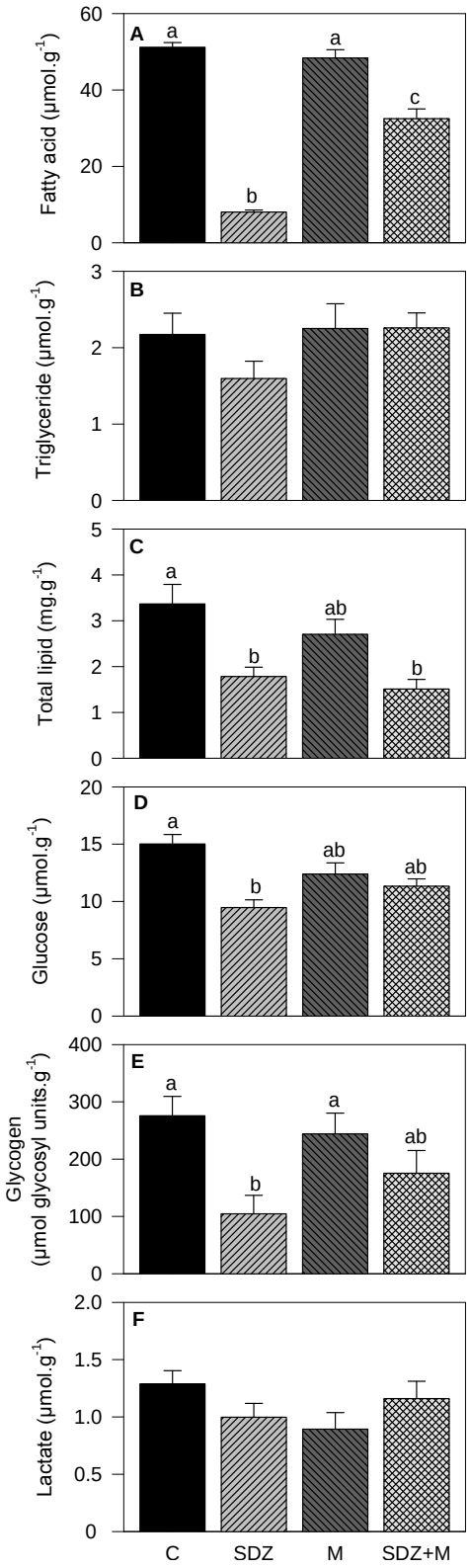

Fig. 1 Levels of fatty acid (A), triglyceride (B), total lipid (C), glucose (D), glycogen (E), and lactate (F) in liver of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among

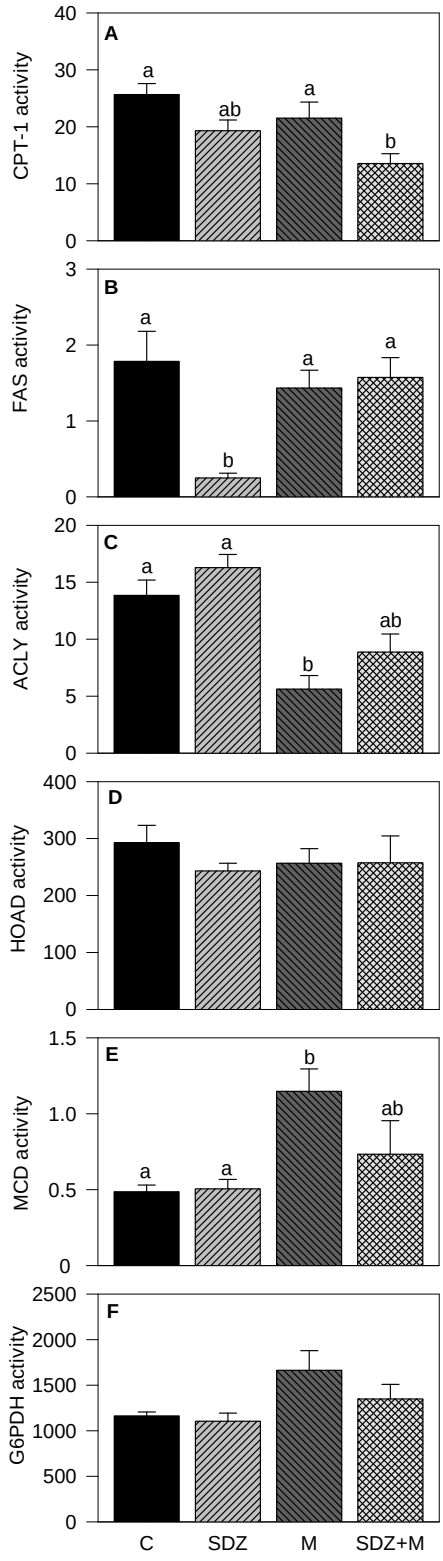

Fig. 2 Activities (mU.mg^{-1} protein) of CPT-1 (A), FAS (B), ACLY (C), HOAD (D), MCD (E), and G6PDH (F) in liver of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

Fig. 3 displays enzyme activities related to glucose metabolism in liver. No significant changes were noted for GK (Fig. 3A). PK activity (Fig. 3B) decreased in fish treated with SDZ compared with control, M, and SDZ+M groups. FBPase (Fig. 3C) and G6Pase (Fig. 3D) activities increased in fish treated with SDZ compared with all other groups (except vs. SDZ+M group in G6Pase activity). Finally, no significant changes were noted for GPase (Fig. 3E), and GSase (Fig. 3F) activities.

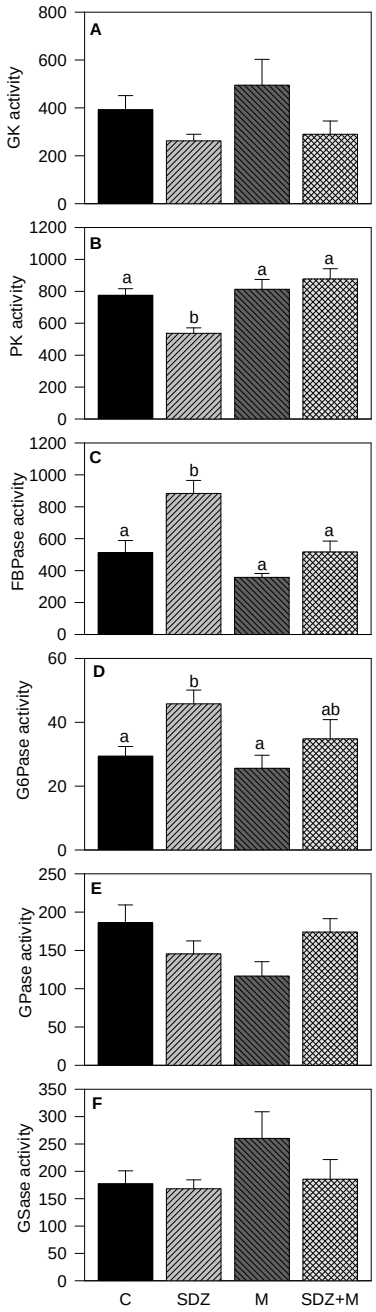

Fig. 3 Activities (mU.mg^{-1} protein) of GK (A), PK (B), FBPase (C), G6Pase (D), GPase (E), and GSase (F) in liver of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

Fig. 4 shows levels of metabolites assessed in BB. FA levels in the SDZ group were lower than in all other groups (Fig. 4A). Triglyceride levels (Fig. 4B) decreased in the SDZ group compared with control and M groups. Total lipid levels in the SDZ group were lower than in control and M groups whereas levels in the M group were higher than in all other groups (Fig. 4C).

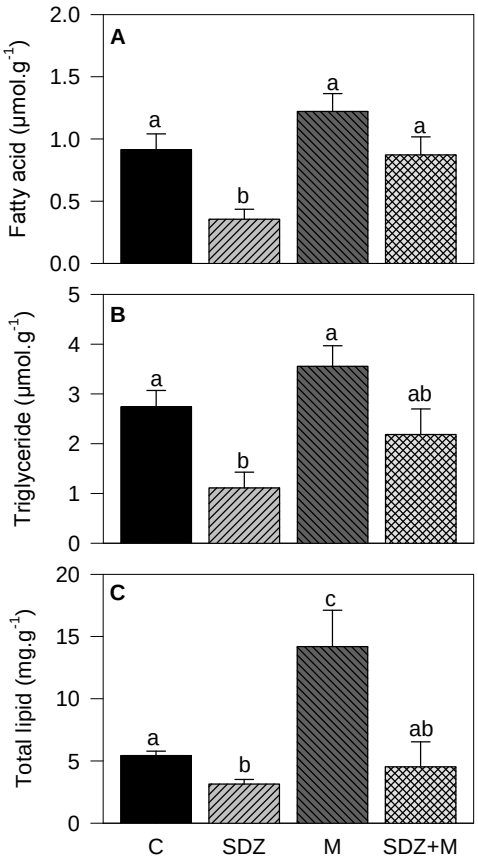

Fig. 4 Levels of fatty acid (A), triglyceride (B), and total lipid (C) in Brockmann bodies of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

Enzyme activities in BB are shown in Fig. 5. CPT-1 activity increased after treatment with SDZ compared with control and M treatments (Fig. 5A). FAS activity (Fig. 5B) increased after treatment with SDZ compared with all other groups.

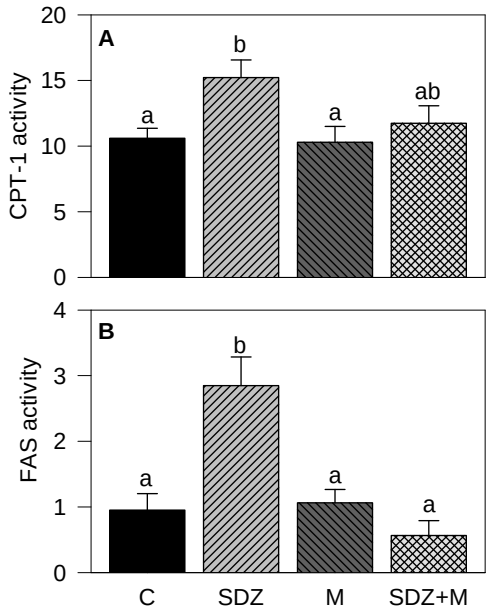

Fig. 5 Activities (mU.mg^{-1} protein) of CPT-1 (A) and FAS (B) in Brockmann bodies of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control, C) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M). The results are shown as mean + S.E.M. of 10 fish per treatment. Different letters indicate significant differences (P<0.05) among treatments.

Table 2 displays mRNA abundance of transcripts assessed in liver. ACC level decreased in the SDZ group compared with all other groups. ACLY level was lower in the SDZ group compared with the M group. CPT-1a level increased after SDZ treatment compared with control and M groups with the increase being more important for SDZ treatment. CPT-1b level was lower in the SDZ+M than in any other group. FAS level increased after treatment with SDZ or SDZ+M compared with the remaining groups with the increase being more important in the SDZ+M group. MCD level was higher after treatment with SDZ compared with control and M groups. UCP2a level decreased after treatment with SDZ compared with all other groups whereas level in the SDZ+M group was lower than that of M group. PPARα level increased in the SDZ group compared with all other groups. GK level decreased compared with control after treatment with SDZ, M, and SDZ+M with the decrease being more important for SDZ and SDZ+M. PK and FBPase values were lower in the SDZ group compared with control and M groups. The value of G6PDH was lower in the SDZ group than in all other groups. Finally, no significant changes were noted for levels of SREBP1c, G6Pase, GR-1, and GR-2.

Table 2 Relative mRNA abundance of transcripts in liver of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M).

	SDZ	M	SDZ+M
Fatty acid metabolism			
ACC	-1.98*a	+1.15b	-1.10b
ACLY	-1.39a	+1.52b	+1.30ab
CPT-1a	+2.48*a	-1.49b	+1.32c
CPT-1b	+1.13a	-1.10a	-1.89*b
FAS	+1.53*a	+1.11a	+2.36*b
MCD	+1.65*a	+1.04b	+1.51a
Mitochondrial uncoupling			
UCP2a	-2.74*a	+1.33b	-1.46c
Transcription factors			
PPARα	+2.27*a	+1.43b	+1.55b
SREBP1c	-1.37	+1.22	-1.36
Glucose metabolism			
GK	-3.05*a	-1.71*b	-3.93*a
PK	-1.79*a	-1.12b	-1.59ab
FBPase	-1.79*a	+1.01b	-1.49ab
G6Pase	+1.13	+1.02	+1.11
G6PDH	-1.59*a	-1.13b	+1.11b
Glucocorticoid receptors			
GR-1	-1.21	+1.06	-1.31
GR-2	-1.41	-1.42	-1.32

Data represent mean of 5 measurements. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalized by EF-1α mRNA levels, which did not show changes among groups). *, significantly different ($P<0.05$) from control fish. Different letters indicate significant differences ($P<0.05$) among treatments

Table 3 shows mRNA abundance of transcripts in BB. ACC value decreased after treatment with SDZ compared with control group. ACLY level decreased after treatment with SDZ compared with control and SDZ+M groups. CPT-1a abundance decreased after treatment with SDZ and SDZ+M compared with all other groups with the decrease being more important for SDZ+M. The level of CPT-1b decreased in SDZ group compared with the remaining groups. CS value was higher in the M group than in any other group. FAS value was higher after treatment with SDZ or SDZ+M compared with control and M groups. UCP2a level increased after treatment with SDZ compared with control and SDZ+M groups. SREBP1c levels were higher in the SDZ+M group than in control and SDZ groups. Finally, no significant differences were found for levels of FAT/CD36, MCD, Kir6.x-like, SUR-like, LXRα, and PPARα.

Table 3 Relative mRNA abundance of transcripts in Brockmann bodies of rainbow trout after 6h of intraperitoneal administration of 10 mL.Kg^{-1} of saline solution alone (control) or containing SDZ WAG 994 (SDZ, 60 µg.Kg^{-1}), metyrapone (M, 1 mg.Kg^{-1}), or both SDZ WAG 994 and metyrapone (SDZ+M).

	SDZ	**M**	**SDZ+M**
Fatty acid transport			
FAT/CD36	-1.17	+1.34	+1.09
Fatty acid metabolism			
ACC	-1.59*a	+1.42b	+1.24b
ACLY	-1.64*a	-1.07ab	+1.37b
CPT-1a	-2.61*a	-1.59b	-7.06*c
CPT-1b	-2.07*a	-1.50b	-1.42b
CS	+1.08a	+2.56*b	+1.14a
FAS	+2.21*a	+1.12b	+1.79*a
MCD	-1.02	-1.07	+1.37
Mitochondrial uncoupling			
UCP2a	+1.66*a	+1.37a	-1.44b
K_{ATP} channel			
Kir6.x-like	-1.02	+1.47	+1.42
SUR-like	+1.14	+1.21	+1.39
Transcription factors			
LXRα	-1.36	-1.15	-1.42
PPARα	-1.06	+1.49	+1.19
SREBP1c	-1.06a	+1.46ab	+1.82*b

Data represent mean of 5 measurements. Data is expressed as fold-induction (+, increase; -, decrease) with respect to the control group (expression results were normalized by EF-1α mRNA levels, which did not show changes among groups). *, significantly different ($P<0.05$) from control fish. Different letters indicate significant differences ($P<0.05$) among treatments

DISCUSSION

Changes in the levels of plasma metabolites after SDZ treatment were described in a preceding manuscript (Librán-Pérez *et al.* 2014b) showing a decrease in circulating levels of FA, triglyceride and total lipid, and an increase in cortisol levels. The additional presence of metyrapone counteracted these changes. In this manuscript, we show the effects observed in liver and BB in the same experiment.

Putative FA-sensing systems in liver and BB respond differentially to decreased levels of circulating FA

The decrease in circulating levels of FA elicited by SDZ treatment reflected in comparable changes in FA levels in liver. However, this response cannot be extended to parameters involved in putative FA-sensing systems, such as those involved in FA metabolism, binding to FAT/CD36 and subsequent modulation of transcription factors like PPARα or SREBP1c, and K_{ATP} capacity (Soengas 2014). We expected changes in these parameters related to FA sensing converse to those previously observed when levels of FA were increased in the same tissues and species (Librán-Pérez *et al.* 2013a, 2013c, 2015a). This, however, happened in very few of them including mRNA abundance of FAS or UCP2a. The increase in mRNA levels of FAS (but not activity) is in agreement with a similar increase found in the same species when fed a diet with a low lipid content (Martínez-Rubio *et al.* 2013). Overall, we may suggest that the putative FA sensing systems present in liver do no respond directly to decreased levels of circulating FA levels. In mammals, evidence indicates that FA sensing in the hypothalamus triggers the CNS to modify hepatic energy metabolism to maintain a proper balance of metabolites and energy (Lam 2010). The downstream mechanisms are likely based on sympathetic and parasympathetic systems providing innervation to the liver via splanchnic nerve and vagus nerve, respectively (Morgan *et al*. 2004; Migrenne *et al.* 2006). We have obtained similar effects in liver of rainbow trout after ICV administration of oleate or octanoate (Librán-Pérez *et al.* 2015a), and sympathetic fibers coming from the CNS innervate gastrointestinal tissues in rainbow trout (Burnstock 1959; Seth and Axelsson 2010). We may therefore hypothesize that the detection of low FA levels by hypothalamus induced metabolic changes in liver through the modulation of the sympathetic pathway. In fact, changes noted in liver parameters are comparable to those noted in the same species in response to increased catecholamine levels (Fabbri *et al.* 1998).

In BB changes induced by SDZ treatment in circulating FA levels were clearly reflected in changes in the same parameters, as demonstrated by the decrease observed

in the levels of FA, triglyceride or total lipid. These changes are in the same direction as those also observed in hypothalamus in the same experiment (Librán-Pérez *et al.* 2014b), and they are also the converse from those previously observed in the same tissue after increasing circulating levels of FA (Librán-Pérez *et al.* 2012, 2013b, 2015a) supporting that they are functional.

The FA sensing system related to FA metabolism in BB partially responded to decreased circulating levels of FA since increased activities of FAS and CPT-1 (the latter is striking since both enzymes participate in opposite metabolic pathways) as well as decreased mRNA abundance of ACC and ACLY as well as increased abundance of FAS occurred in fish treated with SDZ. More important, most of these changes were the opposite to those previously described in the same tissues in response to increased levels of FA (Librán-Pérez *et al.* 2012, 2013b, 2015a) such as for FAS activity, and mRNA abundance of ACLY, CPT1b or FAS. The FA-sensing system related to FAD/CD36 binding and subsequent modulation of transcription factors (PPARα, LXRα, and SREBP1c) displayed no changes in any parameter. Therefore, this system is not apparently involved in the response of BB to decreased levels of FA in contrast to the situation observed when circulating FA levels increase where a clear response was noted (Librán-Pérez *et al.* 2012, 2013b, 2015a). The FA-sensing system related to the mitochondrial activity and the potential of the K_{ATP} channel displayed few changes in BB. In fact, while no apparent changes occurred for the components of K_{ATP} (Kir6.x-like and SUR-like) only UCP2a mRNA displayed an increase suggesting an increased oxidation capacity for FA. Interestingly, this increased potential is the converse of that noted when FA levels increased in the same species (Librán-Pérez *et al.* 2012, 2013b, 2015a) but the weak response do not allow us to suggest that this system is partially responding to decreased levels of FA. Overall, the decrease in circulating levels of FA activate one of the FA sensing systems (FA metabolism) already described in BB of the same species responding to increased levels of FA whereas the other two systems apparently respond only to increased FA levels. As for the physiological role of the response, we can only speculate that it could relate to the secretion of insulin since its release from pancreas is enhanced in response to increased levels of circulating FA in mammals (MacDonald *et al.* 2006) and a similar response has been suggested in fish (Barma *et al.* 2006). In mammals, insulin release from pancreas is also related to hypothalamic FA sensing (Obici *et al.* 2002; Caspi *et al.* 2007; Diano and Horvath 2012), and in mammals parasympathetic activation stimulates, whereas sympathetic activation inhibits insulin secretion (Migrenne *et al.* 2006; Diano and Horvath 2012). Therefore, it seems reasonable that the sensing in hypothalamus of decreased FA levels also resulted in increased sympathetic outflow to the BB with the resultant lower insulin production and release.

The HPI axis is probably involved in the response of liver and BB to lowered FA levels

We have observed in the same experiment that SDZ treatment induced the activation of the HPI axis resulting in increased circulating levels of cortisol (Librán-Pérez *et al.* 2014b). This allowed us to suggest that the fall in circulating levels of FA sensed in the hypothalamus induced a counter-regulatory response to restore circulating FA levels through the activation of peripheral energy metabolism elicited by HPI axis activation. If rainbow trout liver is involved in this response, we would expect similar changes to those observed in physiological situations in which cortisol levels are high, such as during stress-induced conditions (López-Patiño *et al.* 2014). This seems to be the case, either for parameters related to lipid or glucose metabolism. As for lipid metabolism, the fish treated with SDZ displayed decreased levels of FA and triglyceride, decreased lipogenic potential (FAS activity and G6PDH mRNA abundance), and decreased potential of FA oxidation (UCP2a mRNA abundance), which are in agreement with results obtained under high cortisol levels conditions in the same (López-Patiño *et al.* 2014) or other (Vijayan *et al.* 1990) fish species. These changes clearly support the activation of the liver lipolytic potential trying to restore circulating FA levels though the involvement of adipose tissue lipolysis cannot be discarded. Since plasma FA levels in the group treated with SDZ were lower than in control group, this means that the enhanced lipolysis did not have time enough to restore levels. As for glucose metabolism, fish treated with SDZ displayed lower glycogen stores, decreased capacity for use of exogenous glucose (GK mRNA abundance), decreased potential for glycolysis (PK activity and mRNA abundance), and increased capacity of glucose release (G6Pase activity). Again, all these changes are comparable to those described under high cortisol levels conditions in the same (Morales *et al.* 1990; Vijayan and Moon 1992; López-Patiño *et al*. 2014) and other (Schwalme and Mackay 1991; Reubush and Heath 1996) fish species.

Many of the effects of SDZ treatment in liver and BB were counteracted by the presence of metyrapone resulting in values in parameters similar to those of the control group. This molecule is an inhibitor of cortisol synthesis, and therefore of the HPI axis in fish (Bernier and Peter 2001; Dindia *et al.* 2013). In this experiment, metyrapone treatment counteracted the effects of SDZ on several steps of the HPI axis, such as plasma cortisol levels, mRNA abundance of transcripts related to cortisol synthesis in head kidney, and mRNA abundance of corticotrophin releasing factor and corticotrophin releasing factor binding protein in hypothalamus (Librán-Pérez *et al.* 2014b). The effects of metyrapone treatment in liver restoring parameters to values close to those of controls are in agreement with the action of metyrapone antagonizing the effects of cortisol in several parameters assessed in liver of catfish such as citrate synthase, G6PDH, and lactate dehydrogenase activities (Tripathi and Verma 2003). If changes elicited by SDZ treatment disappeared when the HPI axis function was blocked this reinforces that the

HPI is involved in the counter-regulatory response to the decrease in circulating FA levels. How does this hypothesis match with the hypothetical sympathetic modulation of liver and BB response?. The regulation of the HPI axis is complex and involves several factors including catecholamines (Wendelaar Bonga 1997; Bernier 2006). Intra-head kidney interactions between interrenal cells producing cortisol and chromaffin cells producing catecholamines have been observed in rainbow trout (Reid *et al.* 1998) allowing us to suggest that the sympathetic outflow to the liver and/or BB could occur in parallel to the activation of HPI.

In summary, the decrease elicited by SDZ treatment in circulating FA levels elicited in liver and BB of rainbow trout two clearly differential responses. In liver, the changes observed were not compatible with a direct FA-sensing response but they are compatible with high cortisol physiological situations, as during stress conditions. Therefore, we may suggest that the detection of FA decrease in hypothalamus elicits a counter-regulatory response in liver resulting in an activation of lipolysis to restore FA levels in plasma. The activation of these metabolic changes in liver could be attributable to the activation of the HPI axis and/or to the action of sympathetic/parasympathetic pathways. In BB changes in circulating FA levels induce changes in several parameters compatible with the function of FA sensing systems informing about the decrease in circulating levels to match these changes with the production and release of pancreatic hormones, probably insulin.

3.9 TRABAJO EXPERIMENTAL Nº9

Insulin modulation of fatty acid sensing in rainbow trout

INTRODUCTION

Hypothalamic neurons in mammals respond to increases in plasma levels of long-chain fatty acid (LCFA), but not short-chain (SCFA) or medium-chain (MCFA) fatty acids (FA) through several mechanisms (López *et al.,* 2007; Benoit *et al.,* 2009; Duca and Yue, 2014) . This response is modulated by peripheral hormones including leptin, ghrelin or insulin (Blouet and Schwartz, 2009). Insulin has been reported to modulate hypothalamic FA-sensitive neurons resulting in an enhancement of the FA-sensing response related to increased pro-opio melanocortin (POMC)/cocaine and amphetamine-related transcript (CART) expression and decreased neuropeptide Y (NPY)/agouti-related protein (AgRP) expression ultimately leading to decreased food intake (Schwartz *et al.,* 2000; Blouet and Schwartz, 2009; De Morentin *et al.,* 2011). These FA-sensing mechanisms in peripheral tissues such as liver and endocrine pancreas also respond to nutrients and hormones (Kim *et al.,* 2007).

In fish, in previous studies (Librán-Pérez *et al.,* 2012, 2013, 2014, 2015; Soengas, 2014) we have characterized in rainbow trout the presence and functioning in hypothalamus, liver and Brockmann bodies (BB, main accumulation of pancreatic endocine tissue in fish) of FA sensor systems, which unlike mammals respond to changes in liver not only in LCFA such as oleate (OL) but also in MCFA like octanoate (OCT) (Soengas, 2014). These mechanisms, comparable in general with those described in mammals, 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, which are related to the control of food intake (hypothalamus), hormone release (BB) or metabolic homeostasis (liver). To date, there is no evidence in fish regarding the possible endocrine modulation of these FA-sensing systems. However, the cells in those FA-sensing areas are presumably the same than those involved in glucosensing, for which we demonstrated in rainbow trout the existence of a modulatory role of different hormones involved in food intake regulation including insulin (Polakof *et al.,* 2011b; Soengas, 2014). Despite increased levels of circulating FA elicit insulin release in fish (Barma *et al.,* 2006), information about insulin action on lipid metabolism is scarce and contradictory (Caruso *et al.,* 2008; Zhuo *et al.,* 2014) and no evidence is yet available regarding its possible modulatory role on FA sensing systems. In rainbow trout, the expression of insulin receptors has been demonstrated in different tissues including hypothalamus and liver (Caruso *et al.,* 2008, 2010). Also, in this species, insulin administration modulates brain glucosensing system

(Polakof *et al.,* 2007a, 2008c; Conde-Sieira *et al.,* 2010) and inhibits food intake (Soengas and Aldegunde, 2004). Therefore, we aimed to evaluate if the response of FA-sensing systems in hypothalamus, liver and BB of rainbow trout to raised levels of OL or OCT is modified in the presence of insulin.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12:12-h light-dark photoperiod in dechlorinated tap water at 15 °C. Fish weight was 100 ± 2 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Following 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks. Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. On the day of experiment, a first set of fish were anaesthetized in tanks with 2-phenoxyethanol (Sigma, 0.2% v/v), and weighed. Then, 15 fish per group received intraperitoneally (IP) 10 $mL.Kg^{-1}$ injection of saline solution alone (control), or containing insulin (2 mg bovine $insulin.kg^{-1}$ body mass, insulin from Sigma Chemical, ref: I-5500), OL (0,3 mg/kg), OCT (0,3 mg/kg), insulin + OL, or insulin + OCT. To safely deliver FA, they were solubilized in 45% hydroxypropyl-β-cyclodextrin (HBP) to a final concentration of 17 mM as previously described (Librán-Pérez *et al.,* 2012). Bovine insulin has been used in many studies in rainbow trout inducing the same effects than those elicited by homologous hormone (Plagnes-Juan *et al.,* 2008; Polakof *et al.,* 2009; Jin *et al.,* 2014). After 6h of teratment, fish were anaesthetized in tanks with 2-phenoxyethanol (Sigma, 0.2% v/v). 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. Fish were sacrificed by decapitation and hypothalamus, liver and BB were taken, snap-frozen, and stored at -80 °C. 9 fish per group were used to assess enzyme activities and metabolite levels whereas the remaining 6 fish were used for the assessment of mRNA levels by qRT-PCR.

A second set of fish was used to evaluate changes in food intake after intraperitoneal administration of FA and insulin. Food intake was registered for 5 days before treatment (to define basal line data) and then 6 and 24 h after IP treatment with saline alone (control, C; n=10 for each time point) or containing insulin (I; n=10 for each time point), OL (n=10 for each time point), OCT (n=10 for each time point), insulin and OL (I+OL; n=10 for each time point), or insulin and OCT (I+OCT; n=10 for each time point) with the same concentrations described above. After feeding, 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 previously described as the difference from the feed offered (De Pedro *et al.,* 1998; Polakof *et al.,* 2008a, 2008c). The experiment was repeated three times, and therefore results are shown as the mean ± SEM of the data obtained in three different tanks per treatment.

Assessment of metabolite levels and enzyme activities

Levels of FA, total lipids, triglyceride, and glucose in plasma were determined enzymatically using commercial kits (Wako Chemicals for FA, Spinreact for total lipid and triglyceride, and Biomérieux for glucose) adapted to a microplate format.

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 FA, total lipids, and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples. Tissue glycogen levels were assessed as previously described (Librán-Pérez *et al.,* 2012, 2013a). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) and tissue glucose levels was determined with a commercial kit (Biomérieux, Spain).

Samples for enzyme activities were homogenized by ultrasonic disruption in 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000 g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan) and microplates. Reaction rates of

enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT-1 activity, of 5,5´-Dithiobis(2-nitrobenzoic acid)-CoA complex at 412 nm. The reactions were started by the addition of supernatant (15 µl) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma, USA) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ATP-citrate lyase (ACLY, *EC* 4.1.3.8), fatty acid synthase (FAS, *EC* 2.3.1.85), malonyl CoA decarboxylase (MCD, *EC* 4.1.1.9), and carnitine palmitoyltransferase-1 (CPT-1, *EC* 2.3.1.21) activities were determined following available methods (Alvarez *et al.,* 2000; Polakof *et al.,* 2011a; Zhou *et al.,* 2011; Ditlecadet and Driedzic, 2012) .

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies) and treated with RQ1-DNAse (Promega). 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), in a total PCR reaction volume of 15 µl, containing 50-500 nM of each primer. mRNA abundance of acetyl-CoA carboxylase (ACC), ACLY, CART, CPT-1, FAT/CD36, FAS, inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), HOAD, liver X receptor α (LXRα), NPY, POMC-A1, PPARα, SREBP1c, SUR-like, and mitochondrial uncoupling protein 2a (UCP2a) were determined as described in the same species (Ducasse-Cabanot *et al.,* 2007; Kolditz *et al*., 2008; Polakof *et al.,* 2008a,b, 2010a; Cruz-Garcia *et al.,* 2009; Lansard *et al.,* 2009; Conde-Sieira *et al.,* 2010; Figueiredo-Silva *et al.,* 2012c; Librán-Pérez *et al.,* 2013a). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1. Relative quantification of the target gene transcript was done using β-actin gene expression as reference (Olsvik *et al.,* 2005), 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 (Pfaffl, 2001) with the Relative Expression Software tool (REST). 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: $R = [(E_{\text{target gene}})^{\Delta CT_{\text{Target gene}}(\text{mean control- mean unknown sample})}] / [(E_{EF1\alpha})^{\Delta CT_{EF1\alpha}(\text{mean control- mean unknown sample})}]$ 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.

Table 1 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
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	GenBank	NM_001124627
CPT-1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	GenBank	AF327058
CPT-1b	CCCTAAGCAAAAAGGGTCTTCA	CATGATGTCACTCCCGACAG	GenBank	AF606076
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
SUR-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	Sigenae	tcce0019d.e.20_3.1.s.om.8
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	GenBank	DQ295324
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	GenBank	NM_ 001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	Sigenae	tcbk0010c.b.21_5.1.om.4

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; 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; SUR-like, sulfonylurea receptor-like; UCP2a, mitochondrial uncoupling protein 2a.

Statistics

Comparisons among groups were carried out with one-way ANOVA followed by a Student–Newman–Keuls test, and differences were considered statistically significant at $P< 0.05$. When necessary, data were log-transformed to fulfil the conditions of the analysis of variance.

RESULTS

Changes in food intake are shown in Fig. 1. I, OL, and OCT treatment decreased food intake after 6h whereas I treatment alone decreased FI after 6 and 24h compared with controls. The combined treatment of OCT with I resulted in lower FI compared with OCT alone after 6 or 24h of treatment whereas a non significant trend was observed for OL.

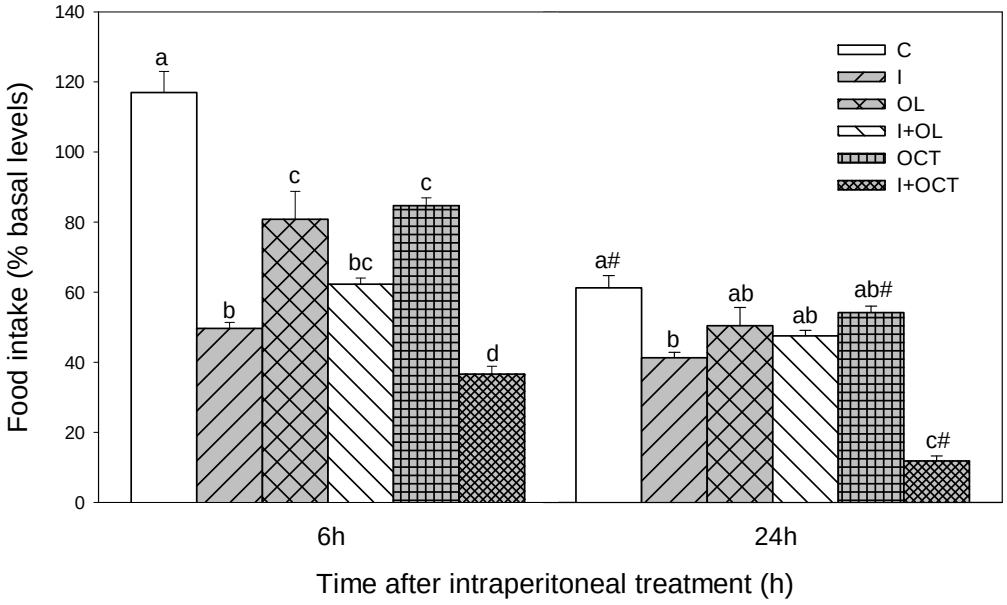

Fig. 1. Food intake in rainbow trout 6 and 24h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT). Different letters indicate significant differences ($P<0.05$) among treatments within each time. #, significantly different ($P<0.05$) from 6h at the same treatment. The results are shown as mean + S.E.M. of the results obtained in three different tanks in which 10 fish were used per group in each tank.

Levels of plasma metabolites are shown in Fig. 2. Treatment with OL or OCT increased levels of FA compared with the remaining groups (Fig. 2A). Triglyceride leves increased after OCT treatment compared with I treatment (Fig. 2B). Total lipid levels were lower in I+OL, OCT, and I+OCT groups compared with control (Fig. 2C). Glucose levels were lower in insulin-treated groups (alone or in combination with OL or OCT) than in the remaining groups whereas levels in OCT group were higher than any other group (Fig. 2D).

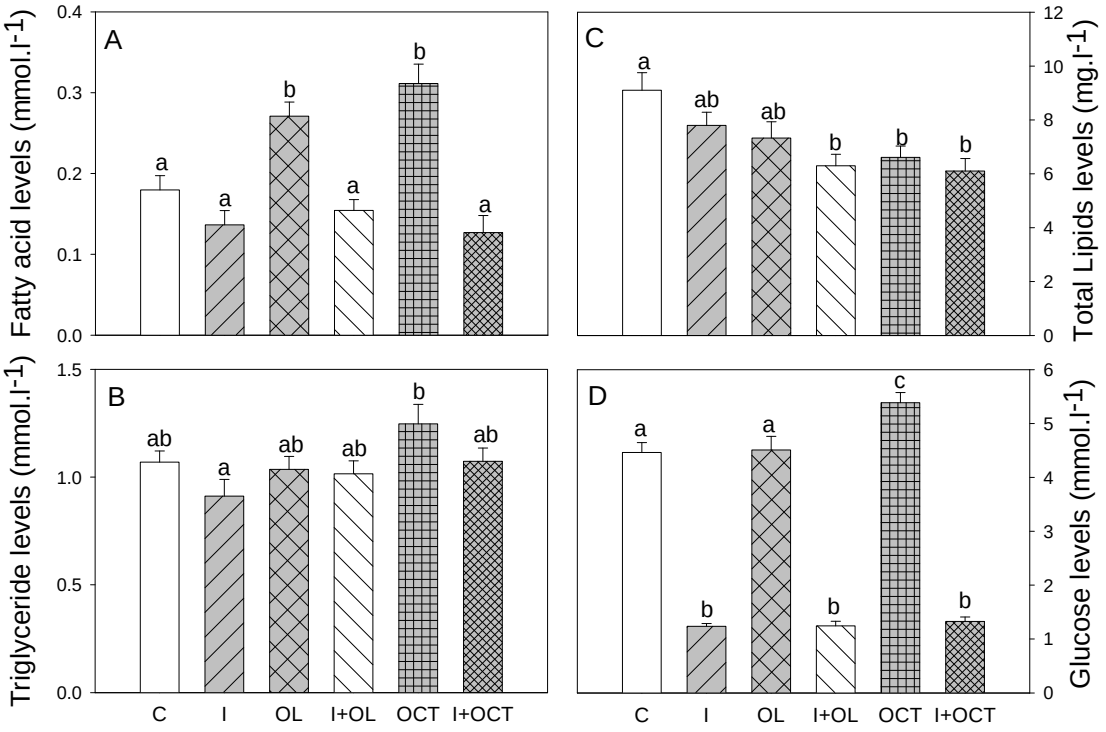

Fig. 2. Levels of FA (A), triglyceride (B), total lipid (C), and glucose (D) in plasma of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT). Each value is the mean + SEM of n = 15 fish per treatment. Different letters indicate significant differences (P<0.05) from different groups.

Metabolite levels and enzyme activities assessed in hypothalamus are shown in Fig. 3. FA levels (Fig. 3A) increased in all groups treated with OL or OCT alone or in combination with I compared with controls. Triglyceride levels (Fig. 3B) were lower in the OL than in control, I, and OCT groups whereas OCT was also higher than I+OL and I+OCT groups. There were no significant differences in total lipid levels (Fig. 3C). CPT-1 activity (Fig. 3D) decreased after IP treatment with I+OL or OCT compared with control and I groups. FAS activity (Fig. 3E) increased after IP treatment with OL compared with C, I+OL, OCT and I+OCT groups. ACLY activity (Fig. 3F) increased after IP treatment with I+OCT compared with I, OL and OCT treatments.

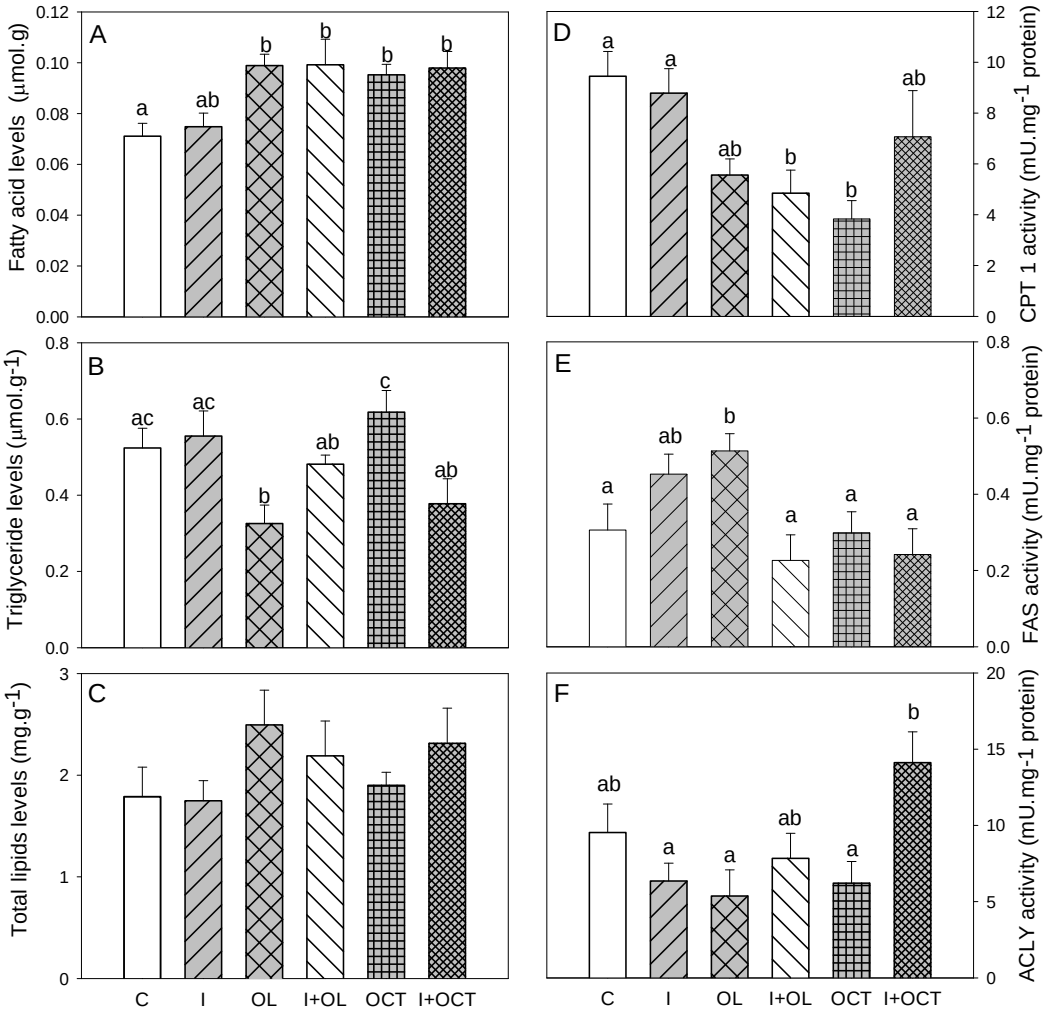

Fig. 3. Levels of FA (A), triglyceride (B), and total lipid (C), and activities of CPT-1 (D), FAS (E), and ACLY (F) in hypothalamus of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT). Each value is the mean + SEM of n = 9 fish per treatment. Different letters indicate significant differences (P<0.05) from different groups.

Metabolite levels and enzyme activities assessed in liver are shown in Fig. 4. FA levels (Fig. 4A) decreased after treatment with I or I+OL compared with controls. The levels of triglyceride (Fig. 4B) and total lipid (Fig. 4C) decreased in groups treated with I (alone or in combination with FA) compared with controls whereas triglyceride levels in the I group were also lower than those of OL and OCT groups (Fig. 4B). Glucose levels were lower in the groups treated with I (alone or in combination with OL or OCT) compared with the other groups (Fig. 4D). Glycogen levels decreased in all treated groups compared with controls whereas levels in the insulin-treated groups (alone or in combination with OL or OCT) were lower than those in the OL and OCT groups (Fig. 4E). CPT-1 activity was lower in the I+OCT than in control, OL, and OCT groups (Fig. 4F). The

activity of FAS (Fig. 4G) decreased after I, OL or OCT treatment compared with controls. Finally, no significant changes were noted for MCD activity (data not shown).

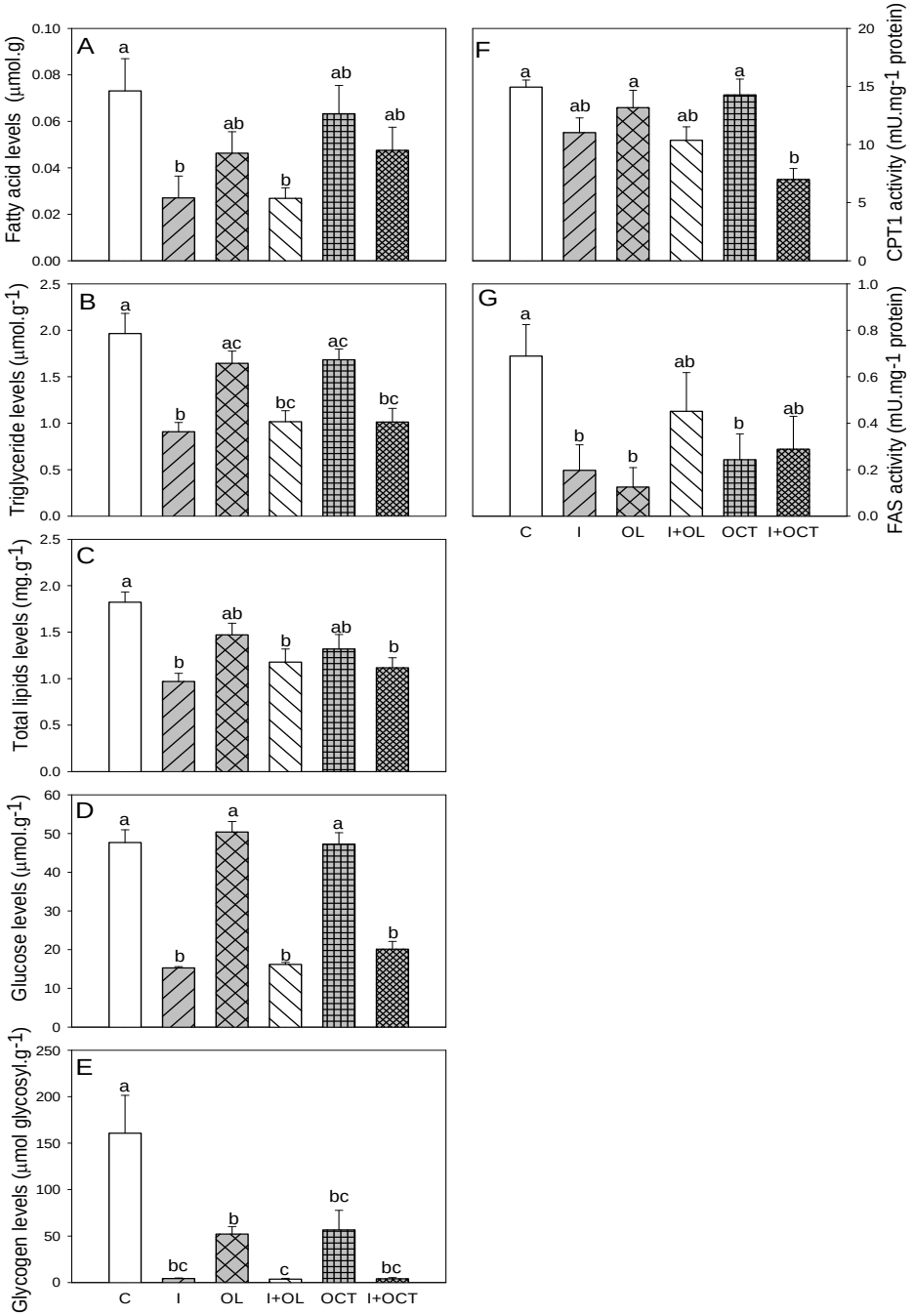

Fig. 4. Levels of FA (A), triglyceride (B), total lipid (C), glucose (D), and glycogen (E), and activities of CPT-1 (F), and FAS (G) in liver of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT). Each value is the mean + SEM of n = 9 fish per treatment. Different letters indicate significant differences (P<0.05) from different groups.

Metabolite levels and enzyme activities assessed in BB are shown in Fig. 5. FA levels (Fig. 5A) were higher in the I group than in any other group (except OCT) whereas levels were also higher than in controls in OL, I+Ol, and OCT groups. Triglyceride levels (Fig. 5B) increased after OL, I+OL or OCT treatment compared with controls. CPT-1 activity in the OL group was lower than that of I group (Fig. 5D). FAS activity (Fig. 5E) increased after I+OCT treatment compared with I treatment. Finally, no significant changes were noted for total lipid levels (Fig. 5C).

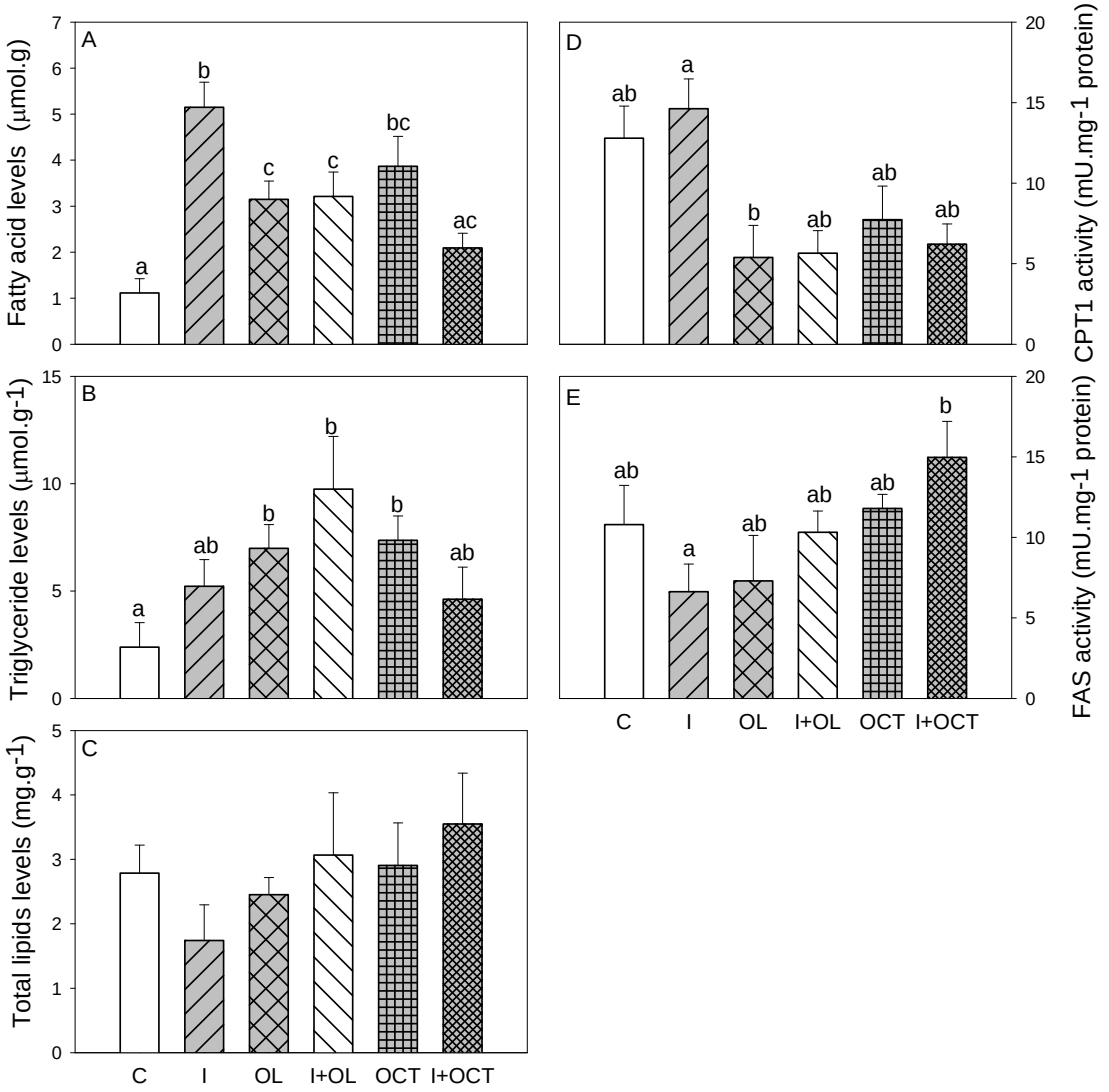

Fig. 5. Levels of FA (A), triglyceride (B), and total lipid (C), and activities of CPT1 (D) and FAS (E) in Brockmann bodies of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT). Each value is the mean + SEM of n = 9 fish per treatment. Different letters indicate significant differences (P<0.05) from different groups.

Changes in mRNA abundance of transcripts assessed in hypothalamus are shown in table 2. FAT/CD36 value was higher in the OL group than in controls. ACC levels decreased after I or I+OCT treatments compared with control and OCT groups. ACLY values decreased after treatment with I, OL, I+OL, and I+OCT compared with controls. Values of FAS were lower in the OL group than in control group. UCP2a mRNA abundance was higher in I+OL and OCT groups than in controls. The values for LXRα decreased after treatment with I or I+OCT compared with control and OCT groups. PPARα mRNA abundance was lower in I+OL and I+OCT groups compared with OL and OCT groups. SREBP1c mRNA abundance decreased after treatment with OL and I+OCT compared with control. The treatment with I+OCT resulted in lower mRNA abundance than in control for CART, control and I for NPY, and I, I+OL, and OCT for POMC. Finally, no significant changes were noted for levels of CPT-1c, HOAD, and Kir6.x-like.

Table 2 mRNA levels in hypothalamus of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT).

	C	I	OL	I+OL	OCT	I+OCT
Fatty acid transport						
FAT/CD36	1.00±0.07a	1.24±0.12ab	1.33±0.11b	1.72±0.12ab	1.41±0.20ab	1.36±0.13ab
Fatty acid metabolism						
ACC	1.00±0.11a	0.69±0.05b	0.87±0.14ab	0.79±0.06ab	0.97±0.12a	0.64±0.07b
ACLY	1.00±0.14a	0.61±0.09b	0.63±0.10b	0.57±0.10b	0.85±0.12ab	0.59±0.05b
CPT-1c	1.00±0.04	0.83±0.09	0.77±0.11	0.72±0.08	0.66±0.06	0.77±0.12
FAS	1.00±0.09a	0.95±0.10ab	0.70±0.06b	0.95±0.04ab	0.89±0.07ab	0.81±0.09ab
Mitochondrial uncoupling						
UCP2a	1.00±0.09a	1.26±0.24ab	1.52±0.33ab	2.15±0.52b	1.92±0.43b	1.76±0.25ab
K_{ATP} channel						
Kir6.x-like	1.00±0.07	0.99±0.14	0.80±0.12	1.05±0.15	0.77±0.11	0.98±0.08
Transcription factors						
LXRα	1.00±0.09a	0.74±0.09b	0.83±0.13ab	1.06±0.17ab	1.02±0.12a	0.72±0.07b
PPARα	1.00±0.12ab	0.85±0.07ab	1.35±0.23a	0.90±0.06b	1.19±0.18a	0.80±0.05b
SREBP1c	1.00±0.10a	0.78±0.07ab	0.61±0.07b	0.79±0.05ab	0.82±0.11ab	0.61±0.05b
Neuropeptides						
CART	1.00±0.09a	0.79±0.07ab	0.72±0.20ab	0.73±0.09ab	0.77±0.13ab	0.57±0.07b
NPY	1.00±0.07a	0.99±0.20a	0.87±0.15ab	0.70±0.15ab	0.71±0.17ab	0.54±0.12b
POMC-A1	1.00±0.07ab	1.22±0.13a	0.99±0.15ab	1.30±0.21a	1.39±0.14a	0.60±0.11b
LXRα	1.00±0.09a	0.74±0.09b	0.83±0.13ab	1.06±0.17ab	1.02±0.12a	0.72±0.07b

Data represent mean ± SEM 5 (gene expression) measurements. Gene expression results are referred to control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) from different groups.

Changes in mRNA abundance of transcripts assessed in liver are shown in table 3. FAT/CD36 values increased compared with controls for I, I+OL and I+OCT groups whereas levels were lower for OCT; values after treatment with I+OL or I+OCT were also higher than the remaining groups (except I and OL for the I+OCT group). ACLY values were higher in the I+OCT group compared with control, I, and I+OL groups. FAS values were higher in I, I+OL, and I+OCT than in the other groups. HOAD values in the group treated with OL were higher than the remaining groups (except control). UCP2a mRNA abundance in the group treated with OCT was higher than control, I, and I+OCT groups whereas value in the I group was lower than any other group. Kir6.x-like value was higher after treatment with I, I+OL, OCT, and I+OCT compared with control. SUR-like mRNA abundance in the group treated with I+OL was higher than any other group whereas value in the I group was lower than other groups (except I+OCT). LXRα value in the groups treated with OL, I+OL, and I+OCT was higher than control and OCT groups whereas in groups treated with I+OL value was also higher than the value of all other groups (except OL and I+OCT). PPARα mRNA abundance increased after treatment with I and I+OL compared with controls whereas value of the I+OL group was also higher than all other groups (except I and I+OCT). Finally, SREBP1c mRNA abundance increased after treatment with insulin (alone or in combination with OL or OCT) compared with the other groups.

Table 3 mRNA levels in liver of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT).

	C	I	OL	I+OL	OCT	I+OCT
Fatty acid transport						
FAT/CD36	1.00±0.1ad	1.73±0.21b	1.52±0.22ab	2.35±0.30c	0.87±0.11d	2.00±0.20bc
Fatty acid metabolism						
ACLY	1.00±0.07a	1.10±0.17a	1.63±0.23ab	1.06±0.13a	1.65±0.31ab	1.91±0.28b
FAS	1.00±0.07a	4.78±0.73b	1.12±0.11a	6.61±0.79b	0.74±0.12a	2.96±0.41b
HOAD	1.00±0.14ab	0.76±0.07a	1.30±0.10b	0.89±0.10a	0.94±0.10a	0.89±0.14a
Mitochondrial uncoupling						
UCP2a	1.00±0.12a	0.54±0.09b	1.26±0.14ac	1.17±0.11ac	1.51±0.13c	1.06±0.13a
K_{ATP} channel						
Kir6.x-like	1.00±0.09a	1.84±0.27b	1.17±0.11ab	2.62±0.42b	1.91±0.27b	1.94±0.26b
SUR-like	1.00±0.13a	0.62±0.08b	0.92±0.06a	1.31±0.09c	0.97±0.04a	0.88±0.12ab
Transcription factors						
LXRα	1.00±0.11a	1.27±0.17ab	1.56±0.17bc	1.97±0.20c	0.96±0.10a	1.53±0.05bc
PPARα	1.00±0.08a	1.90±0.25bc	1.52±0.21ab	2.33±0.37c	1.42±0.14ab	2.20±0.30abc
SREBP1c	1.00±0.07a	3.30±0.38b	1.65±0.27a	3.82±0.30b	1.25±0.13a	2.11±0.22b

Data represent mean ± SEM 5 (gene expression) measurements. Gene expression results are referred to control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) from different groups.

Changes in mRNA abundance of transcripts assessed in BB are shown in table 4. FAT/CD36 mRNA abundance was higher in the I+OL group than in I and OCT groups. CPT-1a mRNA abundance increased after OL treatment compared with the remaining groups (except I+OL). CPT-1b mRNA abundance was lower after treatment with I, I+OCT and I+OCT than the other groups. FAS value in the I+OL group was higher than control and I groups. HOAD value after OL treatment increased when compared with all other groups (except I+OL) whereas I+OCT value was also higher than that of OL group. UCP2a mRNA abundance increased after I+OCT treatment compared with controls. Kir6.x-like value increased after treatment with I, I+OL, OCT, and I+OCT compared with controls whereas values in the I+OL group were also higher than those of control and OL groups. LXRα value in OL and I+OCT was higher than that of controls, I and OCT whereas I+OCT value was higher than all other groups (except OL and I+OL). PPARα value was higher in the I+OL group than in all other groups. Finally, no significant differences were noted for ACLY and SREBP1c mRNA abundance.

Table 4 mRNA levels in Brockmann bodies of rainbow trout 6h after intraperitoneal administration of 10 mL·Kg^{-1} body weight of saline alone (control, C) or containing 2 mg bovine insulin·kg^{-1} body mass (I), or 300 µg.kg^{-1} oleate (OL) or insulin+oleate (I+OL) or 300 µg.kg^{-1} octanoate (OCT), or insulin+octanoate (I+OCT).

	C	I	OL	I+OL	OCT	I+OCT
Fatty acid transport						
FAT/CD36	1.00±0.20ab	0.80±0.15 a	1.40±0.34ab	1.72±0.28 b	0.77±0.09 a	1.40±0.47ab
Fatty acid metabolism						
ACLY	1.00±0.29	0.98±0.33	1.65±0.44	1.63±0.67	1.57±0.44	1.71±0.65
CPT-1a	1.00±0.14 a	0.72±0.08 a	9.88±2.7 b	4.37±1.73ab	0.95±0.18 a	3.11±1.16 a
CPT-1b	1.00±0.26 a	0.33±0.08 b	1.25±0.27 a	0.30±0.06 b	0.88±0.23 a	0.16±0.02 b
FAS	1.00±0.16 a	1.04±0.08 a	1.48±0.08ab	2.21±0.47 b	1.29 ±0.2ab	1.68±0.28ab
HOAD	1.00±0.14ac	0.59 ±0.08 a	2.25±0.37 b	1.58±0.24bc	1.18±0.26ac	1.23±0.36ac
Mitochondrial uncoupling						
UCP2a	1.00 ±0.08 a	1.21±0.12ab	1.51±0.14ab	1.49±0.41ab	1.86±0.34ab	2.13±0.35 b
K_{ATP} channel						
Kir6.x-like	1.00±0.17 a	1.84±0.10bc	1.17±0.18ab	2.62±0.27 c	1.92±0.24bc	1.93±0.22bc
Transcription factors						
LXRα	1.00±0.18 a	1.27±0.08ab	1.56±0.16bc	1.97±0.25 c	0.96±0.08 a	1.53±0.26bc
PPARα	1.00±0.09 a	0.76±0.09 a	1.11±0.20 a	2.63 ±0.66 b	0.89±0.17 a	0.84±0.16 a
SREBP1c	1.00±0.35	0.84±0.27	1.01±0.16	0.96±0.24	0.42±0.07	0.95±0.42

Data represent mean ± SEM 5 (gene expression) measurements. Gene expression results are referred to control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) from different groups.

DISCUSSION

Effects of treatment with FA or insulin alone

The treatment with OL or OCT alone induced in plasma and the tissues assessed changes in parameters related to FA sensing that in general are in agreement with those previously observed under similar experimental conditions *in vivo* (Librán-Pérez *et al.,* 2012, 2013a) thus validating the experimental design used. Furthermore, the decrease observed in food intake in fish treated with OL or OCT alone is also in agreement with that previously observed (Librán-Pérez *et al.,* 2012).

The treatment with insulin alone compared with controls induced changes confirmatory of previous studies carried out in the same or other fish species regarding the action of insulin. The decrease observed in food intake is in agreement with the known anorectic role of this hormone in the same species (Soengas and Aldegunde, 2004). In plasma, the decrease observed in glucose levels is in agreement with the well known hypoglycaemic effect of this hormone in rainbow trout (Plagnes-Juan *et al.,* 2008; Polakof *et al.,* 2010a; Jin *et al.,* 2014) whereas the decrease (though non significant) in plasma FA levels is also in agreement with that previously observed *in vivo* in the same species (Albalat *et al.,* 2007 ; Polakof *et al.,* 2010a). In liver, insulin treatment resulted in decreased levels of FA, triglyceride, glucose, and glycogen, and increased mRNA abundance of FAT/CD36, FAS, PPARα, and SREBP1c. In general, these changes, are in agreement with those previously observed *in vivo* in the same (Plagnes-Juan *et al.,* 2008; Polakof *et al.,* 2010a, 2011a; Sánchez-Gurmaches *et al.,* 2012; Jin *et al.,* 2014) and other fish species (Soengas and Aldegunde, 2004; Zhuo *et al.,* 2014) after insulin treatment, which together with those parameters not assessed in comparable studies before (such as decreased mRNA abundance of UCP2a or SUR-like) are indicative of enhanced lipogenic potential and decreased potential of FA oxidation. Altogether, these results validate again the experimental design used.

On the other hand, changes in other parameters after insulin treatment had not been evaluated in comparable studies in fish before. In this way, it is important to emphasize that while several studies described the effects of insulin treatment on lipid metabolism in liver (see above) there are no references available in fish for insulin effects on lipid metabolism in hypothalamus. In hypothalamus decreased ACC mRNA abundance, and ACLY activity and mRNA abundance were noted after insulin treatment, as indicative of decreased lipogenic potential. A similar decrease in lipogenic capacity had been observed in the same tissue in response to increased levels of FA resulting in an anorectic situation in rainbow trout (Librán-Pérez *et al.,* 2012). Since insulin is also anorectic *per se*, is not surprising to see the nature of these changes in hypothalamus.

Insulin treatment modified the FA-sensing response of tissues to increased levels of oleate or octanoate

When fish were co-treated with insulin and FA we aimed to evaluate whether or not the hormone was able to modify the response of putative FA-sensing systems to increased levels of circulating FA. *A priori*, considering the anorectic role of insulin in this species (Soengas and Aldegunde, 2004) we expected that the effects of the hormone would be a potentiation of the effect of the FA alone, which are also anorectic in this species (Librán-Pérez *et al.,* 2012).

The decrease in food intake induced by treatment with FA or insulin alone was further enhanced by simultaneous treatment of insulin with OL or OCT, with the effect being more important (and significant) with OCT. These changes agree with our hypothesis that the anorectic effects of enhanced levels of LCFA and MCFA could be further modulated by the action of insulin. These changes in food intake could be also related to the changes observed in the mRNA abundance of those hypothalamic neuropeptides involved in the metabolic regulation of food intake (Blouet and Schwartz, 2010). Accordingly, changes observed in mRNA abundance of CART after treatment with OCT, and in NPY after treatment with OL or OCT were further enhanced in the presence of insulin thus supporting changes noted in food intake. It is important to emphasize that the modulation by insulin of the response to OCT is specific of rainbow trout since no anorectic effects of this MCFA have been observed in mammals (Blouet and Schwartz, 2010).

As for parameters related to FA sensing, in hypothalamus very few significant changes were noted when comparing the effects of insulin and FA co-treatment with those of FA alone. In general, the effects of insulin in the modulation of FA-sensing in hypothalamus were very scarce, and no general trend could be concluded. How these results match with the changes already observed in food intake, which showed a clear potentiation of the effect of FA alone by insulin? Certainly, the regulation of food intake does not depend only on FA-sensing systems (Soengas, 2014) thus suggesting the existence of some interaction with other metabolic sensors of nutrients like glucose or amino acids, which could be also affected by insulin treatment, as suggested in mammals (Blouet and Schwartz, 2010; Polakof *et al*., 2011b; Morrison *et al.,* 2012).

In liver, we had previously observed that FA-sensing was presumably associated with previous hypothalamic FA sensing (Librán-Pérez *et al.,* 2015a) in a way that most of the changes displayed by these FA-sensing systems in liver in response to increased levels of OL or OCT were the result of previous hypothalamic FA sensing. The activation of those hypothalamic neurons would result in changes in the production of neuropeptides (leading to the decrease observed in food intake) as well as in increased outflow arriving

to the liver via vagal and/or splanchnic afferents. In general, most of the changes observed indicate that insulin treatment potentiates the effect of both OL and OCT alone, and this includes parameters related to the FA-sensing metabolism based on FA metabolism (CPT-1 activity, and mRNA abundance of ACLY and FAS), transport through FAT/CD36 and subsequent modulation of transcription factors (mRNA abundance of FAT/CD36, LXRα, PPARα, and SREBP1c) and mitochondrial activity (mRNA abundance of Kir6.x-like and SUR-like though only after OL treatment). In general, insulin is therefore potentiating the effects of FA alone in liver. If FA sensing in this tissue is indirect, how these results match with the absence of changes observed in most parameters assessed in hypothalamus? Lipid sensing in the brain of mammals triggers a negative feedback system to regulate hepatic glucose production and maintain glucose homeostasis (Duca and Yue, 2014). If insulin is inducing a further activation of those hypothalamic FA-sensing systems, a strong inhibitory signal would therefore arrive to the liver resulting in a potentiation of the responses elicited by FA alone.

In BB, we had previously reported that FA-sensing systems are present in this tissue responding directly to changes in circulating levels of OL and OCT (Librán-Pérez *et al.,* 2012, 2013, 2014) though since *in vitro* results were not exactly comparable to those observed *in vivo* we had hypothesized that FA sensing capacity in BB could be influenced by endocrine systems like insulin, and could be also affected by previous hypothalamic FA sensing (Librán-Pérez *et al.,* 2015a). In the present study, we obtained information regarding the modulatory action of insulin on such FA-sensing response. In general, the results were more similar to those observed in liver than in hypothalamus since almost all significant effects observed are in the sense of potentiating the action of FA alone. Thus, the FA-sensing system based on FA metabolism, which was activated by OL or OCT, was further activated in the presence of insulin as demonstrated by changes observed in FAS activity (OL) and mRNA abundance. The activation of the FA-sensing system based on transport through FAT/CD36 by OL or OCT was also apparently potentiated by the presence of insulin based on changes noted in mRNA abundance of FAT/CD36, LXRα, and PPARα (OL). Finally, the response of the FA-sensing system associated with mitochondrial activity to treatment with OL or OCT was also potentiated in the presence of insulin as demonstrated by the changes observed in mRNA abundance of Kir6.x-like and UCP2a (OCT). As a whole, the signals produced by insulin treatment are therefore reinforcing those induced by FA alone though, compared with liver, the amount of significant differences is reduced. As in the case of liver, we can only speculate that an enhanced insulin action in hypothalamus is inducing increased signaling into BB reinforcing the anabolic nature of the response to increased circulating levels of FA. Why is insulin inducing this response in BB? We had previously hypothesized that changes in circulating levels of FA could be integrated in BB through FA sensing systems to modulate insulin release in this tissue (Librán-Pérez *et al.,* 2012, 2013, 2014). If circulating levels of insulin

increased, this can be interpreted as a signal of anabolic state thus inducing an increased metabolic responses associated with that status including FA sensing.

In conclusion, we provide for the first time in fish, and in a non-mammalian vertebrate, evidence for the possible endocrine modulation of FA sensing in rainbow trout by a hormone involved in the control of metabolism such as insulin. Insulin has been demonstrated to modulate the activity of neurons involved in FA sensing in mammals. In rainbow trout, our results suggest that the modulatory role of this hormone on the responses of hypothalamic FA sensing systems to changes in circulating levels of OL or OCT was of minor importance in contrast to the mammalian model. However, this is in contrast with the effects observed by insulin treatment on food intake, clearly suggesting a potential effect of FA (especially OCT) on the anorectic effects of FA. The effect was especially relevant for OCT in contrast with the mammalian model where this FA is not involved in the anorectic effect elicited by FA-sensing. In contrast, the results obtained in liver and BB clearly support the modulatory action of insulin on the FA–sensing capacity of peripheral tissues like liver and BB where the responses to OL or OCT alone observed in parameters related to FA sensing were further potentiated by insulin. These results suggest that the changes observed in the activity of neurons involved in the production of anorexigenic and orexigenic factors ultimately leading to a further decrease in food intake are translated (presumably through sympathetic or vagal outflow) into the liver and BB to further potentiate the effects of FA alone.

3.10 TRABAJO EXPERIMENTAL Nº10

Effects on fatty acid sensing, food intake regulation and cellular signaling pathways in hypothalamus and liver of rainbow trout fed with a lipid-enriched diet

INTRODUCTION

In previous studies carried out in the teleost fish model rainbow trout (*Oncorhynchus mykiss* Walbaum), we have characterized in hypothalamus, liver and Brockmann bodies (BB, main accumulation of pancreatic endocrine tissue in this species) the presence and functioning 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 are related to 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*., 2011c; 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*., 2011a). 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 changes in dietary lipid levels that could be related to changes observed in parameters related to FA-sensing and the control of food intake.

MATERIALS AND METHODS

Experimental diets

Two fish meal based diets (Table 1) 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, the difference in lipid level was compensated for by adding non-digestible cellulose in diet LF. 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 1.

Table 1. 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 ($kJ \cdot g^{-1}$) of 23.7, 39.6 and 17.7 for protein, fat and carbohydrates, respectively.

Fish and experimental conditions

Rainbow trout were obtained from the INRA experimental fish farm facilities of Donzacq (Landes, France) and acclimatised two weeks prior to start of the experiment under laboratory conditions and 12:12-h light-dark photoperiod in 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) 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 different tanks 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 different tanks 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.

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 FA, triglyceride, and glucose in plasma were determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany, for FA, and Biomérieux, Grenoble, France, for triglyceride and glucose) adapted to a microplate format. 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 FA 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. mRNA abundance of ACC, ACLY, AgRP, AMPKα1, CART, CPT-1, FAS, FAT/CD36, HOAD, Kir6.x-like, lLXRα, NPY, POMC-A1, PPARα, SREBP1c, and UCP2a 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*., 2008b,2010c,2011a; Figueiredo-Silva *et al*., 2012c; Sánchez-Gurmaches *et al*., 2012; Craig and Moon 2013; MacDonald *et al*., 2014). Sequences of the forward and reverse primers used for each gene expression are shown in Table 2.

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.

Table 2. 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
AMPK-α1	ATCTTCTTCACGCCCCAGTA	GGGAGCTCATCTTTGAACCA	GenBank	HQ40367
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; AMPKα-1, 5'-AMP-activated protein kinase subunit α-1; 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.

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. Differences were considered statistically significant at $P<0.05$.

RESULTS

No mortality was observed throughout the 4-week feeding trial. Body weight, growth rate, and feed intake values are shown in table 3. 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.

Table 3. 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.

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

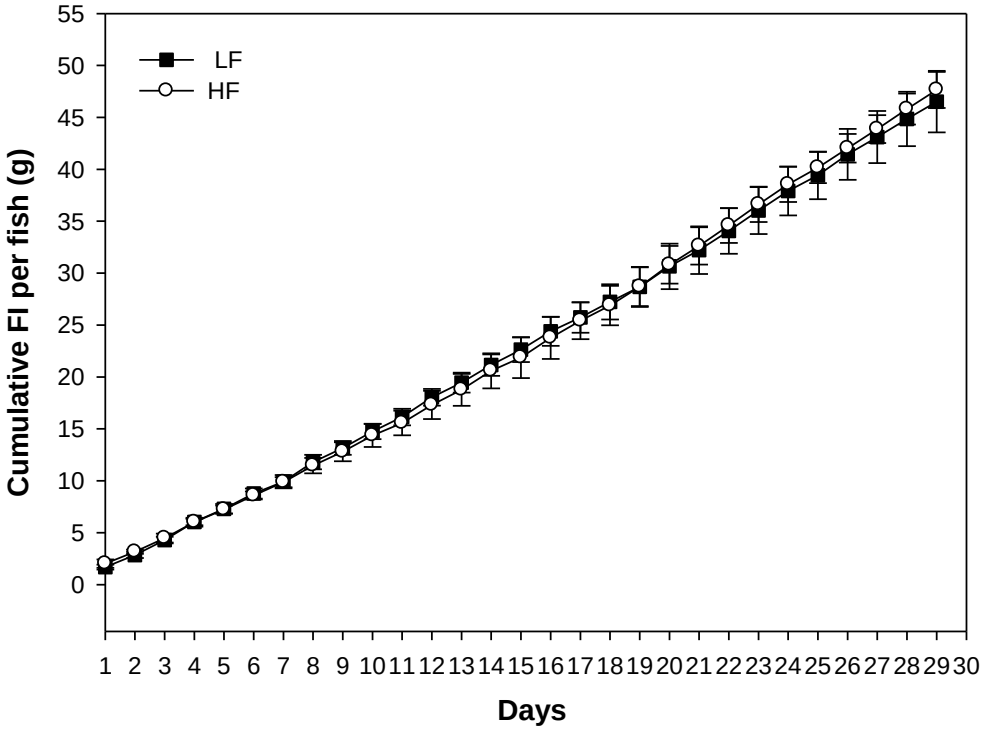

Fig. 1. Cumulative food intake (FI) in rainbow trout fed during 4 weeks with low fat (LF) or high fat (HF) diets. Data are means ±SD of 5 different tanks (containing 20 fish each) per tank. No significant difference between diets (P≥0.05) was noted.

Metabolite levels in plasma, hypothalamus and liver 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).

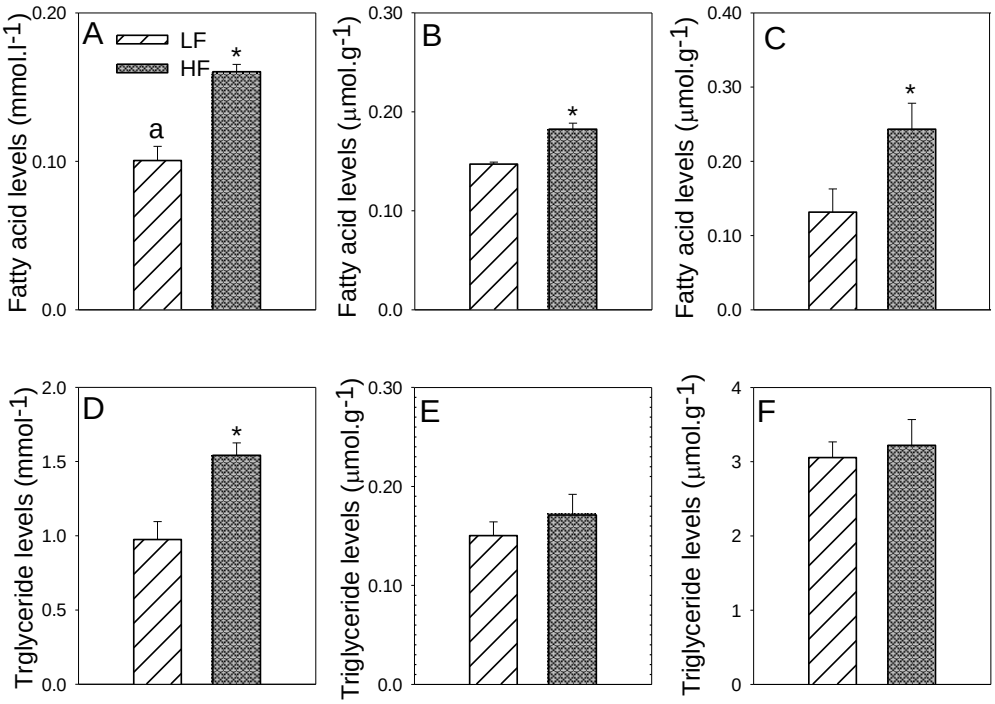

Fig. 2. Levels of non-esterified fatty acids and triglycerides in plasma (A), hypothalamus (B), and liver (C), and levels of triglyceride in plasma (D), hypothalamus (E), and liver (F) of rainbow trout 6h after the last meal with low fat (LF) or high fat (HF) diets. Each value is the mean + SEM of n = 9 fish per diet. *, significantly different ($P<0.05$) from fish fed with the LF diet.

Fig. 3 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 noted 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 noted after 1 and 6h (Fig. 3B).

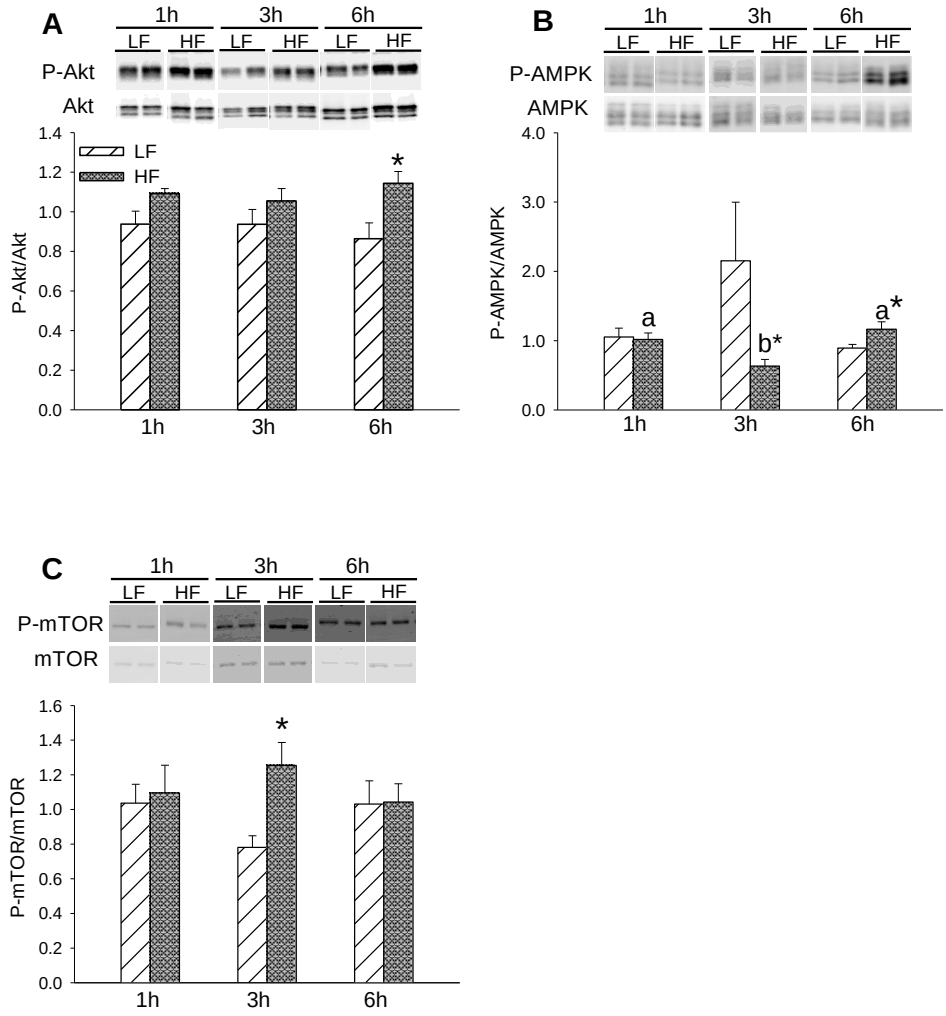

Fig. 3. Western blot analysis of Akt (A), AMPK (B), and mTOR (C) phosphorylation in hypothalamus of rainbow trout 1h, 3h and 6h after the last meal with low fat (LF) or high fat (HF) diets. 20 µg of total protein per lane were loaded on the gel respectively. Western blots were performed on 6 individual 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.

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.

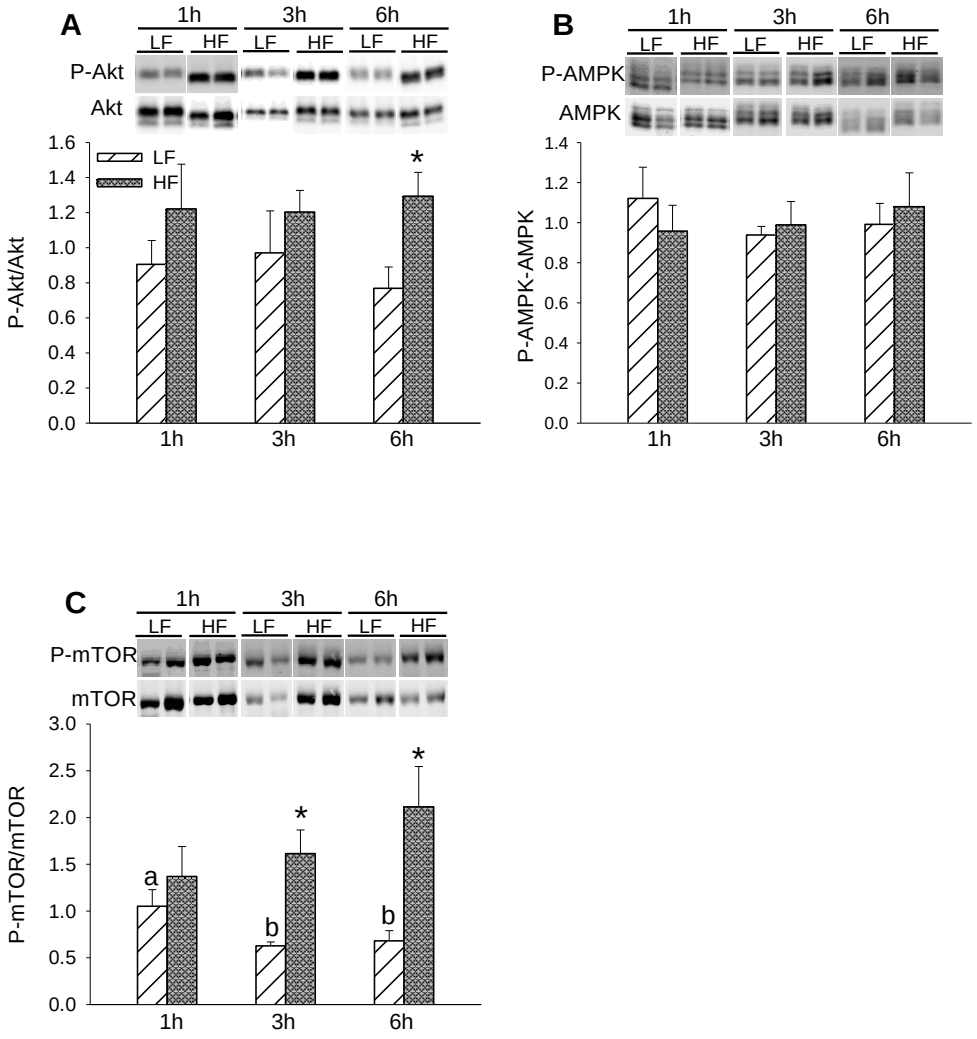

Fig.4. Western blot analysis of Akt (A), AMPK (B), and mTOR (C) phosphorylation in liver of rainbow trout 1h, 3h and 6h after the last meal with low fat (LF) or high fat (HF) diets. 20 µg of total protein per lane were loaded on the gel respectively. Western blots were performed on 6 individual samples 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.

Changes in mRNA abundance of transcripts assessed in hypothalamus 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. 5N), NPY (Fig. 5O), and POMC-A1 (Fig. 5P) 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 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), AMPKα1 (Fig. 5L), and AgRP (Fig. 5M).

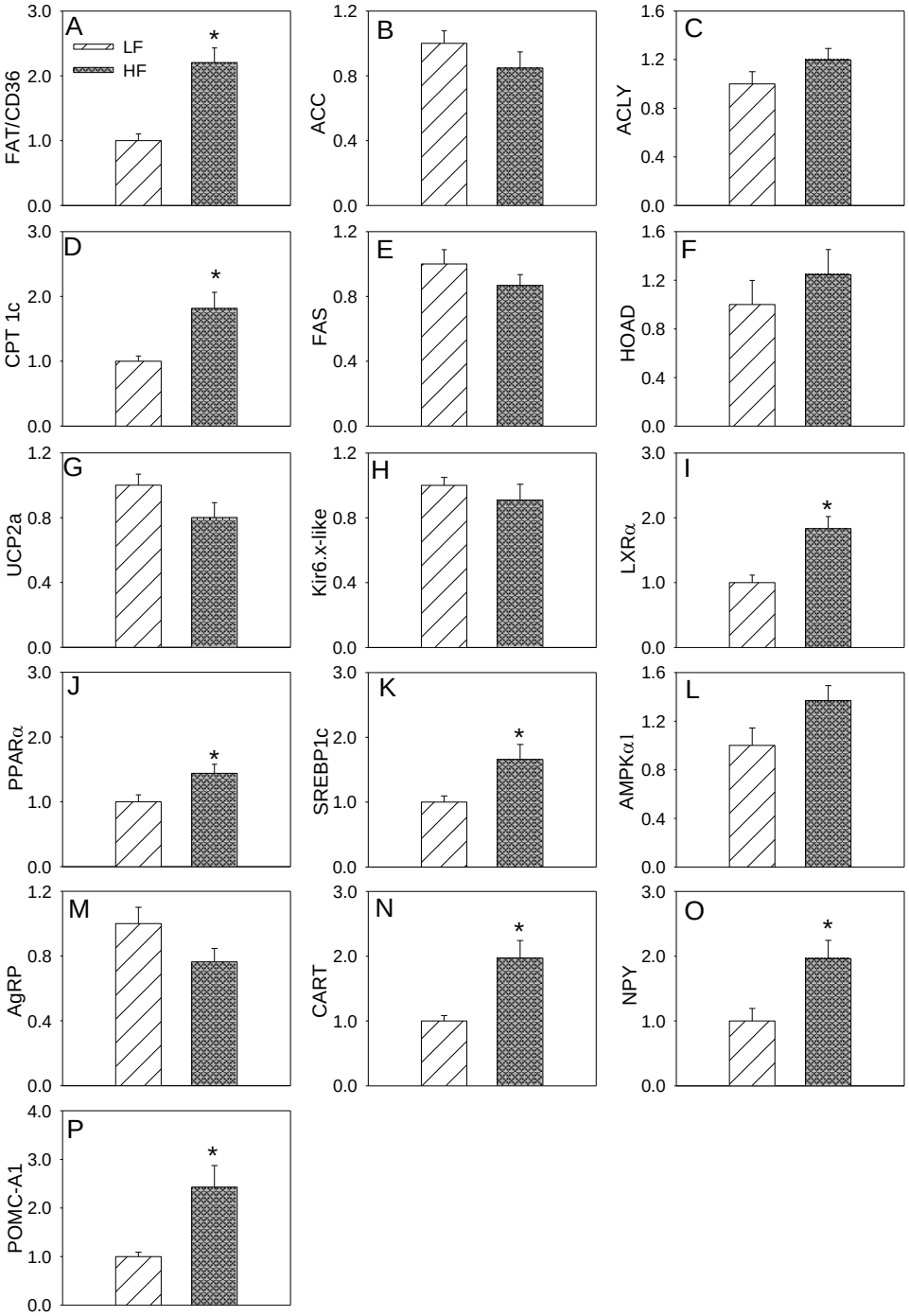

Fig. 5. Relative mRNA abundance of FAT/CD36 (A), ACC (B), ACLY (C), CPT 1c (D), FAS (E), HOAD (F), UCP2a (G), Kir6.x-like (H), LXRα (I), PPARα (J), SREBP1c (K), AMPKα1 (L), AgRP (M), CART (N), NPY (O) and POMC-A1 (P) in hypothalamus of rainbow trout 6 h after the last meal with low fat (LF) or high fat (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.

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), PPARα (Fig. 6J), and AMPKα1 (Fig. 6L) 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).

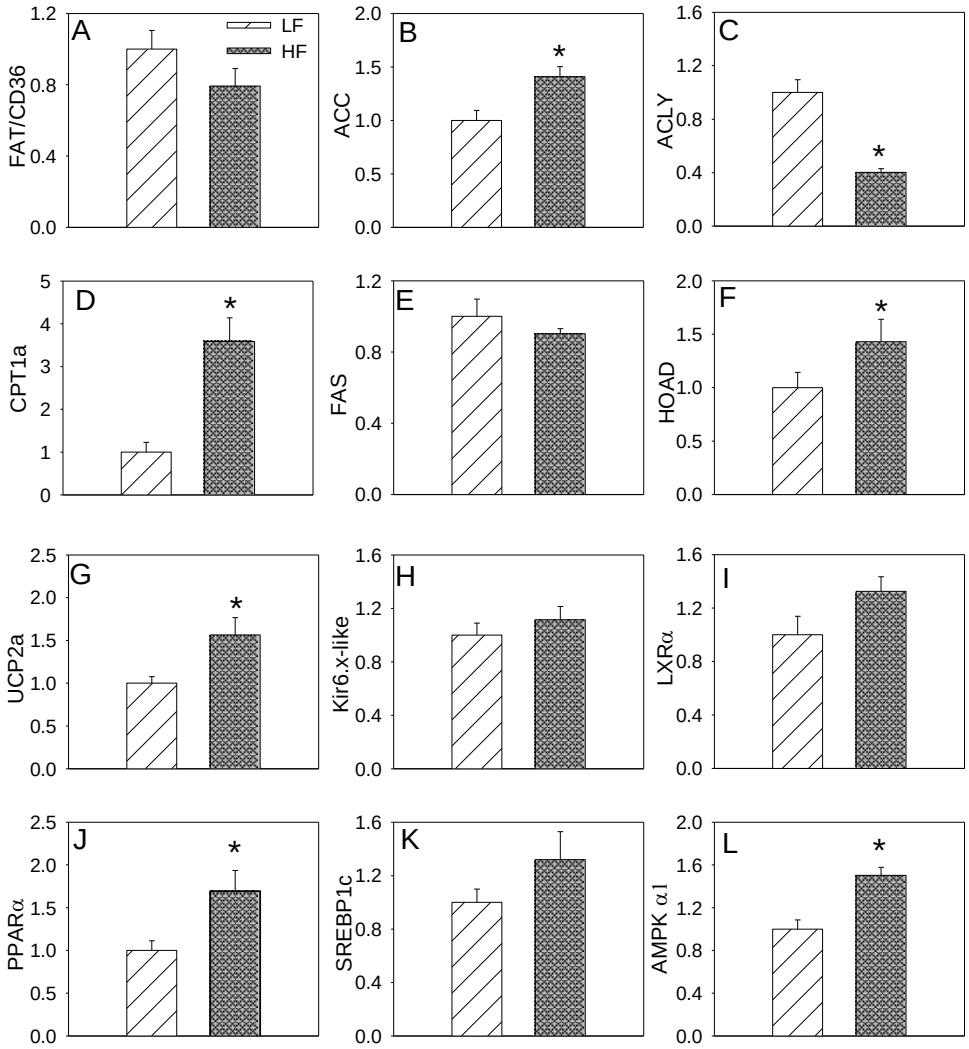

Fig. 6. Relative mRNA abundance of FAT/CD36 (A), ACC (B), ACLY (C), CPT1a (D), FAS (E), HOAD (F), UCP2a (G), Kir6.x-like (H), LXRα (I), PPARα (J), SREBP1c (K), and AMPKα1 (L) in liver of rainbow trout 6 h after the last meal o with low fat (LF) or high fat (HF) diets. Data represent mean + SEM of 6 measurements. 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.

DISCUSION

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 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). In the latter studies, the specific single FA injections activated the three systems, 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 the oleate and octanoate induced changes in the FA sensing systems related to FA metabolism and mitochondrial activity 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 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.This specificity in the response of FA-sensing systems in hypothalamus is comparable to that observed in rats 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, 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).

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 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 for15 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 in the fatty acid composition of the lipids used for preparing the HF diet (a mixture of fish oil and rapeseed oil in this study). 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 NPY increased and no changes were noted in 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 increased anorexigenic potential 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. It seems that increased levels of oleate in mammals (López *et al*., 2007), and oleate and octanoate in rainbow trout (Librán-Pérez *et al*., 2012; 2014a) are able to inhibit food intake whereas this did not occur here by increases in the dietary total lipid level. 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 could also help to explain the observed results in food intake.

Effects on integrative energy and nutrient sensors and cellular signaling pathways

In mammals central and peripheral AMPK is activated when circulating levels of FA, which has been associated with down-regulation of FA synthesis and increased FA oxidation (Florant and Healy, 2012). Since fish fed with the HF diet increased plasma levels of FA, an increased activation of AMPK is reasonable.

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 activation of AMPK was noted 6h after meal based on the increase in the phosphorylation status in hypothalamus and the increased mRNA abundance in liver. Considering that AMPK activation leads to the inhibition of energy-consuming biosynthetic pathways, 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 feeding rainbow trout with a diet rich in carbohydrates also resulted in increased mRNA abundance of AMPKα1 and SREBP1c in liver (Craig and Moon, 2013) whereas in 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 liver Akt phosphorylation state in 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*., 2011a), liver and muscle (Polakof *et al*., 2010c) 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.

4. DISCUSIÓN GENERAL

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Al igual que el resto de animales los peces se alimentan para satisfacer sus requerimientos energéticos y nutricionales, y mantener así su homeostasis energética. El aumento de la toma de alimentos y la recuperación del peso perdido tras un período de ayuno es un ejemplo simple pero convincente de la regulación de la ingesta (Kennedy, 1953). La ingesta de alimento en vertebrados, entre ellos los peces, es un proceso complejo en el cual se encuentran involucrados tanto factores endocrinos centrales como periféricos cuyas acciones están moduladas por variables intrínsecas y extrínsecas (Volkoff *et al.*, 2009).

Los mecanismos básicos de regulación de la ingesta en peces son similares a los descritos en vertebrados. En presencia de alimento, un animal recibe tanto estímulos externos (visuales y olfativos), como estímulos internos (niveles de hormonas, metabolitos y factores nerviosos). Existen diversos tejidos periféricos como el TGI, el tejido adiposo, el hígado y el páncreas que participan en el control de la alimentación, siendo el hipotálamo y más concretamente el núcleo arcuato (NLT en peces) el encargado de integrar todas estas señales tanto a largo plazo como a corto plazo (Valassi *et al*., 2008; Volkoff *et al*., 2009). Generalmente se acepta que la ingesta de alimento está bajo el control del sistema central de alimentación en combinación con un sistema periférico de saciedad (Konturek *et al.,* 2005). El conjunto de neuropéptidos orexigénicos y anorexigénicos constituye el eje del sistema central de la ingesta de alimento, mientras que el sistema periférico de saciedad involucraría varios péptidos y hormonas gastrointestinales.

Los niveles de ácidos grasos cirulantes, es una de esas señales cuya información llega al centro integrador de la ingesta provocando cambios en la misma (Marty *et al.*, 2007). En mamíferos, el mecanismo más aceptado a través del cual se detectan LCFA en el cerebro es de naturaleza metabólica. El aumento de los niveles de ácidos grasos induce un incremento en los niveles de malonil-CoA que inhibe la actividad de la carnitina palmitoil transferasa 1 (CPT-1) encargada de transportar los ácidos grasos hasta el interior de la mitocondria para su oxidación (López *et al.,* 2005, 2007). Además del metabolismo de ácidos grasos se han descrito otros mecanismos de detección de ácidos grasos en el hipotálamo como son: i) a través de la unión con el transportador de ácidos grasos FAT/CD36 (Le Foll *et al.,* 2009), ii) la activación de la proteína quinasa C tipo θ (PKC-θ) (Blouet y Schwartz, 2010) y iii) la producción mitocondrial de especies reactivas de oxígeno (ROS) por intercambio de electrones que dan como resultado la inhibición de la actividad del canal de potasio dependiente de ATP (K^+_{ATP}) (Blouet y Schwartz, 2010).

El aumento de los niveles de LCFA en mamíferos da lugar a una inhibición de la expresión de AgRP y NPY y un aumento de la expresión de POMC y CART , lo que provoca por tanto una inhibición en la ingesta de alimento (López *et al*., 2005). La relación entre el sistema sensor de ácidos grasos y el control de la ingesta de alimento ha sido también demostrada por el efecto anorexigénico de la inhibición de la enzima ácido graso sintetasa (FAS) (Loftus *et al*., 2000; Gao y Lane, 2003; Hu *et al*., 2011).

Primeras evidencias de un sistema sensor de ácidos grasos en peces

Hasta el momento no había estudios que demostrasen la existencia de mecanismos sensores de ácidos grasos en peces. Por lo tanto usando la trucha arco iris como modelo de pez teleósteo, en los trabajo Nº1 y Nº2 tras un incremento en los niveles circulantes de ácidos grasos como el oleato (LCFA) o el octanoato (MCFA), por primera vez se ha demostrado la existencia de componentes de mecanismos sensores de ácidos grasos tanto a nivel central (hipotálamo) como a nivel periférico (cuerpos de Brockmann e hígado), similares a los descritos en mamíferos.

En hipotálamo de mamíferos, el mecanismo sensor de FA relativo al metabolismo de FA se activa por LCFA pero no por MCFA o SCFA (López *et al.,* 2007). Esta situación parece ser diferente en trucha arco iris ya que en general el tratamiento con octanoato induce efectos similares a los producidos por el oleato, esto puede deberse a que los peces teleósteos contienen cantidades considerables de MCFA (Davis *et al.,* 1999; Trushenski, 2009) y no tienen preferencia en la oxidación de los MCFA en comparación con los LCFA (Figueiredo-Silva *et al.,* 2012), situación que contrasta con la de los mamíferos (OOyama *et al.,* 2009). No obstante, el incremento en los niveles de FA *in vivo* mediante tratamientos intraperitoneales a nivel hipotalámico produce resultados que se asemejan a los observados previamente en mamíferos tras tratamientos similares con oleato, dándose un incremento en los niveles de malonil Co-A y un descenso en niveles de ARNm de ACLY, ACO y CS. Si tenemos en cuenta otros posibles mecanismos sensores de ácidos grasos descritos en mamíferos como la vía relacionada con el transporte de ácidos grasos mediante el transportador FAT/CD36 (Nordrum *et al*., 2003), podemos observar que en hipotálamo de trucha arco iris tras el tratamiento con oleato no sólo se produce un incremento en los niveles de ARNm de FAT/CD36, sino que también en los de PPARα y SREBP1c con el subsiguiente descenso en los niveles de expresión de LXR (Cruz-García *et al.,* 2009) al igual que ocurre en mamíferos (Wolfgang *et al.,* 2007). Otra vía descrita en mamíferos es la asociada a la actividad mitocondrial con la consecuente inhibición del canal de potasio dependiente de ATP (K_{ATP}) (Blouet y Schwartz, 2010). En trucha arco iris, al igual que ocurre en mamíferos, a nivel hipotalámico observamos una clara inhibición en la expresión de Kir6.x-like y SUR-like que son los componentes del canal K_{ATP} .

En cuanto a la ingesta de alimento, se produce un descenso en ésta tras el tratamiento IP con oleato y octanoato, así como tras el tratamiento con C75 (inhibidor de FAS) que se contrarresta con la presencia de TOFA (inhibidor de ACC) al igual que ocurre en mamíferos (Loftus *et al.,* 2000; Gao y Lane, 2003; Hu *et al*., 2011). Todo esto sugiere la existencia de un mecanismo sensor de FA en trucha arco iris involucrado en el control de la ingesta de alimento. Al igual que en mamíferos, a nivel hipotalámico, hay un descenso en los niveles de expresión de ARNm de NPY y un incremento en los niveles de expresión de POMC (López *et al.,* 2005; López *et al.,* 2007) tras el tratamiento con oleato coincidiendo con la inhibición de la ingesta de alimento , sin embargo tras el tratamiento con octanoato no observamos diferencias en la expresión de estos neuropéptidos.

En cerebro posterior de trucha arco iris, el tratamiento con FA no induce cambios en los niveles de FA y si comparamos con los resultados obtenidos en parámetros relativos al metabolismo de los ácidos grasos en hipotálamo, observamos una ausencia de respuesta en muchos de los parámetros analizados al contrario que en mamíferos donde el cerebro posterior posee mecanismos sensores de FA basados en el metabolismo de los ácidos grasos (Migrenne *et al*., 2007). Tampoco se observan cambios en parámetros relativos al transporte de los ácidos grasos, ni a la vía asociada a la actividad mitocondrial.

Los resultados obtenidos en BB, tras el tratamiento con oleato y octanoato son en algunos casos similares a los observados en hipotálamo, como el descenso en los niveles de ARNm de FAS y CPT 1c tras el tratamiento con ambos ácidos grasos, lo que sugiere que el metabolismo de FA puede estar involucrado en la capacidad sensora de FA en BB. También los cambios relacionados con el potencial de transporte en BB son similares a los observados en hipotálamo, sin embargo la vía asociada a la actividad mitocondrial no se ve afectada.

En hígado, el tratamiento con oleato y octanoato induce una reducción del potencial glicolítico reflejado en el descenso en la actividad enzimática de PK y un incremento tras el tratamiento con oleato del potencial gluconeogénico reflejado en el aumento de la actividad enzimática de FBPasa. Además se produce un descenso en los niveles de ARNm de GK con ambos FA. El incremento en los niveles de glucosa y glucógeno en hígado (sólo con el tratamiento con oleato) sugiere que los FA estimulan el metabolismo de la glucosa. Con ambos tratamientos el potencial lipogénico se inhibe en hígado, quedando reflejado en un descenso en la actividad enzimática de FAS y ACLY y un descenso en los niveles de ARNm de FAS. Además la actividad de G6PDH en presencia del oleato y los niveles de ARNm de G6PDH en presencia de cualquiera de los dos ácidos grasos no muestran descensos similares a los observados en otros parámetros lipogénicos. Con ambos FA se da un incremento en la actividad enzimática y en los niveles de ARNm de MCD y un descenso en los niveles de mRNA de CPT1a, CPT1b y CS. No se producen cambios en los niveles de mRNA de FAT/CD36 y FATP1, por lo que el potencial de transporte de FA no se ve afectado. Los resultados proporcionan evidencias claras de

que ambos FA, incluso cuando las concentraciones son bajas, desempeñan un papel clave en la regulación de varios componentes de sistemas sensores de ácidos grasos presentes en hígado de trucha arco iris.

Dado que se sabe que, en trucha arco iris, un incremento en los niveles de lípidos en la dieta induce una hiperglucemia, reduciendo el potencial glucolítico y lipídico, e incrementando el potencial gluconeogénico en hígado (Gélineau *et al*., 2001; Panserat *et al*., 2002; Figueiredo-Silva *et al*., 2012a,b), se administró oralmente un tratamiento compuesto por una serie de aceites de pescado (FO) y se evaluaron en hígado parámetros relativos al metabolismo de la glucosa y al metabolismo lipídico con el fin de evaluar si el suministro de ácidos grasos produce una mejora en la tolerancia a la glucosa. El cambio más relevante en parámetros relativos al metabolismo de la glucosa fue un drástico descenso en los niveles de ARNm de GK, lo que coincide con estudios llevados a cabo en ratas (Jump *et al.,* 1994). Sin embargo, no se observaron diferencias en la actividad enzimática de GK, lo que sugiere que el efecto es solo a nivel molecular. Tras el tratamiento, también se produjo un incremento en los niveles de ARNm de FAS y G6PDH lo que coincide con la idea de que un incremento en el potencial lipogénico afecta al uso de la glucosa como se ha sugerido en otros estudios en peces (Panserat *et al.,* 2002, 2009; Polakof *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Figueiredo-Silva *et al.,* 2012bc; Kamalam *et al.,* 2012).

Comportamiento de los sistemas sensores de ácidos grasos

Tras demostrar en experimentos *in vivo* la existencia de sistemas sensores de ácidos grasos en hipotálamo, cuerpos de Brockmann e hígado de trucha arco iris que responden ante incrementos en los niveles de oleato y octanoato, quisimos saber si el efecto de los FA en dichos tejidos era un efecto directo o estaba mediado por cambios en otros sistemas endocrinos, si los cambios eran dependientes de la concentración de FA o si la respuesta en BB e hígado quizás era consecuencia de la previa detección a nivel hipotalámico seguida de transmisión de dicha activación por la vía vagal y simpática (Migrenne *et al.,* 2006; Blouet y Schwartz; 2010). Para ello, se desarrollaron los trabajos experimentales Nº3 y Nº4 en los cuales se incubaron *in vitro* (en ausencia de factores externos) hipotálamos, BB e hígados de trucha arco iris.

Los resultados obtenidos en hipotálamo *in vitro* tras tratamientos con concentraciones crecientes de oleato y octanoato son similares a los obtenidos *in vivo* observándose una respuesta dosis-dependiente que suele ser igual para oleato u octanoato para la mayoría de los parámetros analizados. Además se estimulan los niveles de ARNm de los péptidos anorexigénicos (POMC y CART) mientras que los niveles de NPY disminuyen tras el tratamiento con oleato. Estos cambios se corresponden con la reducción en la ingesta observada *in vivo* tras el tratamiento con oleato. Usando

inhibidores específicos de parámetros relativos a los mecanismos sensores de ácidos grasos, todos los antagonistas en presencia de oleato u octanoato contrarrestan la acción de los FA para varios de los parámetros medidos. Estos resultados obtenidos en hipotálamo de trucha arco iris permiten sugerir que este tejido tiene capacidad para detectar directamente cambios en los niveles de MCFA y LCFA a través de mecanismos relativos al metabolismo de FA, transporte mediante FAT/CD36 y actividad mitocondrial comparables a los de mamíferos pero con la diferencia de la capacidad de respuesta al octanoato de la trucha arco iris.

En BB, el tratamiento *in vitro* con oleato y octanoato produce un incremento en los niveles de FA, triglicéridos y lípidos totales de forma similar al producido en hipotálamo, pero diferente al descrito en mamíferos donde las células pancreáticas no responden al incremento en los niveles de MCFA como el octanoato (MacDonald *et al.,* 2008). Sin embargo, aunque los resultados son similares a los observados en hipotálamo, la mayoría de los cambios en los parámetros medidos *in vitro* en BB son en general diferentes a los previamente observados *in vivo*. Esto puede ser debido a que la acción de los FA en los mecanismos sensores de ácidos grasos en BB observados *in vivo* puedan estar influenciados por otros sistemas endocrinos como la insulina.

En hígado, los resultados obtenidos en la mayoría de los parámetros evaluados tras el tratamiento *in vitro* con oleato y octanoato son en general diferentes a los previamente observados *in vivo.* Se produce un incremento en las actividades enzimáticas de ACLY y G6PDH y en los niveles de ARNm de FAS y ACC por lo que hay una activación del potencial lipogénico. Hay una respuesta dosis-dependiente al incremento de los niveles de oleato u octanoato siendo además similar para ambos. Por otra parte, se sabe que los nervios vago y esplácnico están presentes en la trucha arco iris, aunque no está claro si las ramas de dichos nervios llegan al hígado (Burnstock, 1959; Seth y Axelsson, 2010). Los resultados observados en el hígado después del tratamiento *in vivo* con FA podrían ser el resultado de la activación previa hipotalámica que llegaría por vías nerviosas al hígado y el páncreas (Havel, 2001; Blouet y Schwartz, 2010) y que no se observaría en el estudio *in vitro* debido a que el tejido hepático estaría fuera de la influencia del hipotálamo. Por otra parte, en los mamíferos la liberación de insulina pancreática también está relacionada con la detección de FA en hipotálamo (Obici *et al*, 2002 ; Caspi *et al*, 2007) por lo que no se puede descartar que un mecanismo similar está también presente en la trucha arco iris. Estos resultados nos permiten hipotetizar que la capacidad sensora de FA observada *in vivo* en hígado podría ser de naturaleza indirecta.

La similitud entre los resultados obtenidos *in vivo* e *in vitro* en hipotálamo de trucha arco iris nos hacen plantearnos que el efecto de los FA en este tejido sea directo. Esta hipótesis se vio apoyada tras el tratamiento a nivel central con oleato y octanoato (trabajo experimental nº5) en el caso de los mecanismos relacionados con el metabolismo de FA (siendo en general más rápida la activación para el caso del oleato) y

la actividad mitocondrial, mientras que en el caso del mecanismo asociado al transporte de ácidos grasos existen otros factores que interaccionan con el mismo, de modo que la respuesta obtenida *in vivo* sería debida a una acción indirecta. Los niveles de ARNm de FAT/CD36 y SREBP1c tras el tratamiento *in vitro* y a nivel central con oleato son similares pero diferentes a los observados tras el tratamiento IP donde se produce un aumento de los niveles de FAT/CD36 que es comparable al suscitado en el hígado de la misma especie tras el tratamiento con insulina (Sánchez-Gurmaches *et al*., 2012). Esta situación sería también comparable a la observada en el hipotálamo de mamíferos donde los niveles de ARNm de FAT/CD36 se incrementan tras una alimentación con dietas ricas en grasas (Moullé *et al.,* 2013), situación que se sabe que provoca un aumento de los niveles de insulina en plasma. Se produce un descenso en la ingesta de alimento con ambos FA que además coincide con los cambios que se observan en la expresión de NPY, POMC y CART en el hipotálamo y que a su vez coincide con los resultados previamente descritos en esta misma especie tras la alimentación con dietas ricas en grasas (Figueiredo-Silva *et al.,* 2012b). Parece pues que la activación de los mecanismos sensores de ácidos grasos hipotalámicos es directa en el caso de los mecanismos relacionados con el metabolismo de ácidos grasos y la actividad mitocondrial mientras que en el caso del mecanismo asociado al transporte de ácidos grasos existirían otros factores que estarían interaccionando y los resultados obtenidos tras el tratamiento IP podrían deberse a una acción indirecta.

Los nervios vago y esplácnico están presentes en el tracto gastrointestinal de trucha arco iris (Burnstock 1959; Seth y Axelsson, 2010) y las proyecciones hipotalámicas que terminan en el complejo dorsal-vagal provocan un flujo de información eferente a varios efectores del balance energético como el hígado, el páncreas y el tejido adiposo de una forma similar a la sugerida en mamíferos (Obici *et al.,* 2001; Blouet y Schwartz, 2010). Además hormonas como la insulina tienen un efecto en el metabolismo de los FA en peces (Barma *et al*. 2006; Caruso and Sheridan 2011). Con estos conocimientos previos y debido a la diferencia de resultados en BB e hígado tras el tratamiento *in vivo* y tras el tratamiento *in vitro*, nos planteamos evaluar si esta diferencia se debe a una posible interacción con otros sistemas. Para ello se llevó a cabo un tratamiento a nivel central con oleato y octanoato (trabajo experimental Nº6) que indujo cambios en varios parámetros relativos al mecanismo sensor de FA en ambos tejidos periféricos. En BB, los resultados son en general diferentes a los observados tras el tratamiento IP, con sólo unas pocas coincidencias. Si la capacidad sensora de FA en BB depende de la capacidad sensora previa de FA en hipotálamo, los resultados tras el tratamiento ICV deberían ser similares a los observados tras el tratamiento IP lo que nos lleva a sugerir que la capacidad sensora directa observada en BB tras el tratamiento *in vitro*, se modifica *in vivo* por los efectos de sistemas endocrinos periféricos inducidos por cambios en los niveles circulantes de FA. En contraste con los resultados obtenidos en BB, muchos de los parámetros analizados en hígado tras el tratamiento ICV con oleato y octanoato muestran coincidencias

relacionadas con el metabolismo de los FA previamente descritos tras el tratamiento IP y diferencias con respecto a los cambios observados *in vitro*. Por lo tanto, la capacidad sensora de FA en hígado de trucha arco iris parece ser indirecta y podría ser consecuencia de la previa capacidad sensora del hipotálamo seguida de un flujo de información eferente a nivel vagal y/o simpático.

Respuesta de los sistemas sensores de ácidos grasos frente al descenso de los niveles circulantes de ácidos grasos e implicación del eje hipotálamo-hipófisis-interrenal

En los trabajos anteriores observamos que el aumento en los niveles de ácidos grasos como el oleato o el octanoato en plasma provocaba la estimulación de los sistemas sensores de ácidos grasos en hipotálamo, BB e hígado en paralelo a cambios en la expresión hipotalámica de factores orexigénicos y anorexigénicos y la inhibición de la ingesta. Por el contrario, hasta el momento no se ha estudiado en peces qué respuesta se desencadena cuando se produce un descenso en los niveles de ácidos grasos plasmáticos. Por ello, evaluamos si el descenso en los niveles de ácidos grasos circulantes (inducido farmacológicamente mediante el tratamiento con SDZ WAG 994) produce una inhibición en los sistemas sensores de ácidos grasos hipotalámicos y ello se refleja en una activación de la ingesta (trabajo experimental Nº7). Si la respuesta que se observa es producto de dicha caída, debería ser contrarrestada si además del tratamiento con el fármaco se trata a los animales con un preparado (intralipid) que produce un incremento en los niveles de ácidos grasos circulantes. Además, también evaluamos en hipotálamo, BB e hígado, la posible participación del eje hipotálamo-hipófisis-interrenal (HPI), equivalente al eje hipotálamo-hipófisis-adrenal (HPA) en mamíferos, mediante el tratamiento con SDZ en presencia de metirapona que es un inhibidor de eje HPI en peces (Bernier y Peter, 2001; Dindia *et al.,* 2013).

El descenso en los niveles circulantes de FA indujo un claro incremento en la ingesta de alimento, que se encuentra asociada al descenso en el potencial anorexigénico en el hipotálamo y a cambios en parámetros relacionados con el mecanismo sensor de ácidos grasos. Se produce un descenso de la actividad de FAS y HOAD, así como de la expresión de CPT1c, CPT1d y MCD, y un incremento en la expresión de FAS, también se observa un descenso significativo en la expresión de FAT/CD36 en paralelo al descenso en los niveles de ácidos grasos y un incremento en los niveles de ARNm de UCP2a. Además el tratamiento con intralipid contrarresta dichos cambios. El descenso en los niveles de ácidos grasos aparentemente induce una respuesta de regulación en trucha arco iris, en la cual la activación del eje HPI está probablemente involucrada, ya que la expresión de distintas proteínas involucradas en la síntesis de cortisol en riñón cefálico, como son 11βH, 3βHSD, P450scc y StAR se incrementa de manera significativa tras el tratamiento con SDZ, y estos valores se contrarrestan cuando tratamos a los animales con SDZ junto a intralipid. En trucha arco iris un incremento en la expresión de dichos genes se

corresponde con un incremento en los niveles de cortisol en plasma (Alderman *et al.,* 2012; Aluru y Vijayan*,* 2006) que también observamos en el presente estudio. Además, hay un incremento en la expresión de CRF y CRFBP, que en la misma especie cambian normalmente en paralelo a los niveles de cortisol en plasma (Jeffrey *et al.,* 2014) y, una vez más, la presencia de intralipid contrarresta dicho aumento. En definitiva, estos resultados nos permiten sugerir que el descenso en los niveles circulantes de ácidos grasos activa el eje HPI. Esta activación probablemente no se encuentra relacionada con el control de la ingesta de alimento a través del sistema sensor de ácidos grasos pero sí con la modulación de la lipólisis en los tejidos periféricos para restablecer los niveles de ácidos grasos en plasma. Esta respuesta contrareguladora iniciada en el hipotálamo, probablemente mediante cambios en la expresión de CRF, puede llegar a los tejidos periféricos como el hígado donde se desencadenarían cambios de forma coincidente. Sin embargo, no podemos excluir la posibilidad de que i) la caída de los ácidos grasos en plasma sea detectada fuera del cerebro de modo que la información se transmita al hipotálamo mediante una vía nerviosa aferente o un factor humoral, o ii) que otras hormonas lipolíticas en peces como GH, glucagón o catecolaminas (Harmon *et al.,* 1993; O´Connor *et al.,* 1993; Fabbri *et al.,* 1998; Albalat *et al.,*2005; Sangiao-Alvarellos *et al.,* 2006) puedan estar involucradas en la respuesta contra-reguladora frente a la caída en los niveles circulantes de ácidos grasos.

En hígado y BB (trabajo experimental Nº8) el descenso de los niveles circulantes de FA induce dos respuestas claramente diferentes. En hígado, los cambios observados no son compatibles con una capacidad sensora directa de FA, ya que los resultados obtenidos apenas difieren de los obtenidos cuando incrementamos los niveles de FA. Sin embargo, son compatibles con situaciones fisiológicas de niveles de cortisol elevados durante condiciones de estrés. En mamíferos, hay evidencias que indican que la detección de FA en el hipotálamo activa el SNC con el fin de modificar el metabolismo energético hepático para mantener un equilibrio adecuado de los niveles de metabolitos y el *status* energético (Lam 2010). Por lo tanto , podemos sugerir que la capacidad de detectar el descenso de FA en el hipotálamo provoca una respuesta contra-reguladora en hígado que resultaría en una activación de la lipólisis para restablecer los niveles de FA en el plasma. La activación de estos cambios metabólicos en el hígado podría ser atribuible a la activación del eje HPI y/o a la acción de vías simpáticas/parasimpáticas.

En BB el descenso en los niveles circulantes de FA induce cambios contrarios a los observados tras un incremento en los niveles circulantes de FA en varios parámetros del mecanismo sensor de FA relativo al metabolismo de los FA. Sin embargo, los sistemas sensores de FA relativos al transporte de FA y la actividad mitocondrial apenas se ven afectados por este descenso. En mamíferos, la liberación de insulina desde el páncreas también está relacionada con la capacidad sensora de FA en el hipotálamo (Obici *et al.,* 2002; Caspi *et al.,* 2007; Diano y Horvath., 2012), y la activación parasimpática estimula, mientras que la activación simpática inhibe la secreción de insulina (Migrenne *et al.,*

2006; Diano y Horvath, 2012). Por lo tanto , parece razonable que la detección en el hipotálamo del descenso en los niveles de FA pueda producir un aumento de flujo simpático hacia los BB con la resultante disminución de la producción y liberación de insulina al igual que ocurre en mamíferos (MacDonald *et al.,* 2006).

Papel de la insulina en la modulación de la respuesta sensora de ácidos grasos

Se sabe en mamíferos que la respuesta ante el incremento de los niveles de ácidos grasos mediante los diferentes mecanismos sensores de FA está modulada por varias hormonas periféricas como la lepina, ghrelina e insulina (Blouet y Schwartz, 2010). La insulina produce una modulación en las neuronas hipotalámicas sensoras de FA lo que resulta en un aumento de la respuesta sensora de FA que conlleva un incremento en la expresión de POMC y CART y un descenso en la expresión de NPY y AgRP siendo el resultado final la inhibición de la ingesta de alimento (Schwartz *et al.,* 2000; Blouet y Schwartz, 2010; de Morentin *et al.,* 2011). En peces aunque se sabe que el incremento de los niveles circulantes de FA provoca la liberación de insulina (Barma *et al*., 2006), la información sobre la implicación de la insulina en el metabolismo lipídico es escasa y contradictoria (Caruso y Sheridan, 2011; Zhuo *et al.,* 2014). Teniendo en cuenta la información previa y los resultados obtenidos en los trabajos experimentales anteriores, en los cuales parece existir una capacidad sensora de FA indirecta mediada por sistemas endocrinos periféricos, evaluamos en el trabajo experimental Nº9 el posible papel modulador de la insulina en la actividad sensora de FA en hipotálamo, BB e hígado de trucha arco iris. El tratamiento con insulina induce un descenso en la ingesta de alimento que coincide con el conocido papel anoréctico de esta hormona en trucha arco iris (Soengas y Aldegunde, 2014). Este descenso se incrementa con el tratamiento simultáneo con los ácidos grasos, siendo aún más marcado en el caso del octanoato y coincidiendo con los cambios observados en los niveles de ARNm de CART y NPY. Sin embargo, los parámetros relativos al sistema sensor de FA hipotalámico no muestran apenas cambios tras el co-tratamiento con los FA con respecto al tratamiento solo con insulina. Por ello, los cambios en la regulación de la ingesta podrían deberse a la interacción con otros nutrientes como glucosa o aminoácidos, que también podrían estar afectados por el tratamiento con insulina (Blouet y Schwartz, 2010; Polakof *et al*., 2011a; Morrison *et al.,* 2012).

En hígado, en los trabajos experimentales anteriores, observamos los cambios en los mecanismos sensores de FA en hígado serían el resultado de la capacidad sensora hipotalámica previa. En general, los resultados obtenidos muestran que el tratamiento con insulina potencia el efecto tanto del oleato como del octanoato y esto incluye parámetros relativos al sistema sensor de FA basado en el metabolismo de los FA, en el transporte de FA a través de FAT/CD36 y a la actividad mitocondrial. Si la insulina induce una mayor activación de los sistemas sensores de FA en hipotálamo, puede ser que

llegue una fuerte señal inhibitoria al hígado que resultaría en una potenciación de las respuestas producidas por los FA solos.

En el trabajo experimental Nº1 describimos que posiblemente los sistemas sensores presentes en BB responderían directamente a cambios en los niveles circulantes de LCFA y MCFA, sin embargo, los resultados obtenidos en el trabajo experimental Nº3 tras el tratamiento *in vitro* con oleato y octanoato no eran comparables con los obtenidos *in vivo*, por lo que hipotetizamos que la capacidad sensora de FA en BB podría estar influenciada por sistemas endocrinos como la insulina y que podría verse afectada por la detección previa de FA a nivel hipotalámico. La insulina potencia el efecto de los FA en los BB, tanto en los parámetros relativos al mecanismo sensor de FA basado en el metabolismo de los FA, como en el basado en el transporte de FA, así como el basado en la actividad mitocondrial. Al igual que en el caso del hígado, solo podemos especular que la potenciación de la acción de la insulina en hipotálamo induciría un incremento de las señales a los BB reforzando la naturaleza anabólica de la respuesta al incremento de los niveles circulantes de FA. Un aumento en los niveles circulantes de insulina se podría interpretar como una señal de *status* anabólico y por lo tanto inducir un incremento en las respuestas metabólicas asociadas a la situación, incluyendo la capacidad sensora de FA.

Efectos de una dieta rica en lípidos en los sistemas sensores de ácidos grasos, en la regulación de la ingesta de alimento y las vías de señalización celular

Dado que se ha observado una reducción en la ingesta de alimento tras alimentar peces como la lubina (Boujard *et al.,* 2004) o la trucha arco iris (Gélineau *et al*., 2001; Figueiredo-Silva *et al.,* 2012a) con dietas ricas en lípidos, cabe esperar cambios en los sistemas sensores de FA en peces alimentados con dietas que contienen diferentes niveles de lípidos. En trucha arco iris, existen evidencias sobre la presencia y funcionamiento de AMPK en hígado (Craig y Moon, 2011, 2013; Polakof *et al*., 2011c; Fuentes *et al.,* 2013) y músculo (Craig y Moon, 2013; Magnoni *et al.,* 2014) pero no hay información en peces de la respuesta de AMPK a cambios en los niveles de nutrientes como los ácidos grasos, que sí se ha demostrado en mamíferos (Hardie y Ashford, 2014). Además, las proteínas implicadas en la señalización celular como mTOR y Akt parecen estar involucradas en la regulación nutricional del metabolismo de carbohidratos y lípidos en peces. Sin embargo, hasta el momento no existían estudios en peces que demostrasen la respuesta del sistema sensor de FA, así como la respuesta de estas proteínas cuando son alimentados con dietas compuestas por diferentes concentraciones de lípidos.

Por lo tanto, en el trabajo experimental Nº10, tras alimentar a los animales con una dieta rica (HF) o pobre (LF) en lípidos durante un mes, se estudió la respuesta de la ingesta de alimento, de la expresión de los neuropéptidos anorexigénicos y orexigénicos,

de parámetros relativos a los mecanismos sensores de FA en hipotálamo e hígado, del estado de fosforilación del sensor de energía intracelular AMPK así como la respuesta de proteínas implicadas en la señalización celular como mTOR y Akt.

En el hipotálamo, el mecanismo sensor basado en el metabolismo de los FA y de la actividad mitocondrial no parece verse afectado por la dieta HF. Sin embargo, el mecanismo basado en el transporte de FA mediante FAT/CD36 y la posterior modulación de los factores de transcripción sí parece ser activado por la dieta HF. Si comparamos estos resultados con los obtenidos en el trabajo experimental Nº1, tras el incremento de los niveles circulantes de FA, vemos que son claramente diferentes. Sin embargo, es interesante comparar estos resultados con los obtenidos tras producir un descenso de los niveles de FA inducido farmacológicamente con SDZ WAG 994 en el trabajo experimental Nº7, en el cual los sistemas basados en el metabolismo de FA y en la actividad mitocondrial solo respondían parcialmente frente al descenso de los niveles circulantes de FA. Por lo tanto, los sistemas sensores de FA están aparentemente diseñados para responder a cambios en los niveles de FA específicos como el oleato y octanoato en peces, pero no está claro que respondan a los cambios en los niveles globales de FA de un modo inespecífico como los que inducen las dietas utilizadas en este estudio, y esta respuesta parcial observada se debería probablemente a la presencia en la dieta HF de ácidos grasos capaces de inducir una respuesta en los mecanismos sensores de FA (oleato y octanoato).

En hígado, los parámetros relativos al sistema sensor de FA muestran aparentemente una respuesta parcial tras la alimentación con la dieta HF. El hígado respondería a variaciones en los niveles de FA específicos como el oleato y octanoato (básicamente como una respuesta indirecta a los efectos notados en hipotálamo), de modo que la respuesta cuando incrementamos los niveles de FA totales es en general comparable a la observada en esta especie cuando la sometemos a tratamientos con aceite de pescado como en el trabajo experimental Nº2.

No se observan diferencias en la ingesta de alimento si comparamos peces alimentados con HF o LF y en otros estudios llevados a cabo en trucha arco iris no se observan cambios en la ingesta de alimento en peces alimentados con dietas ricas en aceite de pescado durante 9 semanas (Figueiredo-Silva *et al.,* 2012a), sin embargo, en la misma especie alimentada durante 15 semanas con una dieta enriquecida con aceite de pescado sí hubo un descenso en la ingesta de alimento para los peces alimentados con esta dieta HF (Gèlineau *et al*., 2001). Por lo tanto, la ausencia de diferencias en la ingesta de alimento en el presente estudio podría deberse a que el tiempo empleado no fue suficiente (solo 4 semanas) y también a la diferencia en la composición lipídica usada para preparar la dieta HF en este estudio. En cuanto a los neuropéptidos involucrados en la regulación de la ingesta de alimento, el balance global es un incremento en el potencial anorexigénico de los peces alimentados con la dieta HF. Sin embargo, este aumento en el

potencial anorexigénico no es suficiente para inducir los cambios esperables en la ingesta de alimento. La explicación podría ser que sólo ciertos FA específicos como el oleato y octanoato en el caso de la trucha arco iris, inducirían cambios en la ingesta mientras que ello no ocurriría si se incrementara la composición total de lípidos en la dieta, al igual que ocurre en mamíferos (López *et al.,* 2007).

Pasadas 6 horas de la última toma de alimento, en los peces alimentados con la dieta HF se observa una aparente activación de AMPK. En mamíferos, el incremento en el estado de fosforilación de AMPK en hipotálamo inhibe la actividad de ACC lo que resulta en un descenso de la actividad de FAS y un aumento en la de CPT1 (Chari *et al*., 2010). En este estudio, en hipotálamo, el descenso de los niveles de ARNm de ACC y FAS no es significativo, pero hay un claro incremento en los niveles de ARNm de CPT 1c mientras que en hígado hay un incremento en los niveles de ARNm de ACC y CPT1a pero no hay cambios en FAS. Por otra parte, se sabe que AMPK ejerce un control negativo sobre la señalización de mTOR en mamíferos (de Morentin *et al.,* 2011). En este estudio, el incremento de la fosforilación de mTOR en hipotálamo tras pasar 3 horas de la ultima toma de alimento con la dieta HF coincide con el descenso de la fosforilación de AMPK en el mismo tiempo. Por lo que, en general AMPK responde al incremento en la disponibilidad de FA. El estado de fosforilación de mTOR y Akt tiende a ser más alto en los peces alimentados con la dieta HF que con la dieta LF tanto en hipotálamo como en hígado y el incremento es significativo pasadas 6 horas de la última toma de alimento en el caso de mTOR y Akt en hígado y Akt en hipotálamo y pasadas 3 horas en el caso de mTOR en hipotálamo. No existen estudios en hipotálamo de peces con los que podamos comparar estos resultados, pero en trucha arco iris un incremento en los niveles de nutrientes se ve reflejado en un incremento en la fosforilación de mTOR y Akt en hígado (Lansard *et al.,* 2009; Skiba-Cassy *et al.,* 2009; Mennigen *et al.,* 2012). En mamíferos, parámetros relativos al sistema sensor de FA se relacionan con estas vías de señalización celular, así Akt induce la expresión de SREBP1 que a su vez induce un aumento en la expresión de sus genes diana como FAS y ACLY. En el presente estudio, en hipotálamo se observa un aumento simultáneo en la fosforilación de Akt y la expresión de ARNm de SREBP1c pero no se observan cambios significativos en la expresión de FAS y ACLY. Por lo tanto, aunque en su conjunto podemos sugerir que la mayor disponibilidad de nutrientes, en este caso lípidos, induce una activación general de las vías de señalización celulares relacionadas con mTOR y Akt indicativas del estado anabólico experimentado por los peces, los cambios observados en los parámetros relacionados con los mecanismos sensores de FA no reflejan directamente la influencia de las vías de señalización evaluadas, lo que sugiere la existencia de una interacción más compleja.

5. CONCLUSIONES/CONCLUSIONS

5. CONCLUSIONES

1. Se han obtenido evidencias sobre la presencia en la trucha arco iris de sistemas sensores de ácidos grasos en el hipotálamo, cuerpos de Brockmann e hígado que responden ante cambios en los niveles circulantes de ácidos grasos.

2. Los mecanismos por los cuales los ácidos grasos se detectan en hipotálamo, cuerpos de Brockmann e hígado parecen ser similares a los propuestos en mamíferos, basados en 1) el metabolismo de los ácidos grasos; 2) la actividad mitocondrial, mediante la inhibición del canal de potasio dependiente de ATP (K^+_{ATP}); 3) el transporte de ácidos grasos a través del transportador FAT/CD36. A diferencia de mamíferos, estos sistemas se activan en respuesta a incrementos en los niveles de ácidos grasos de cadena media como el octanoato y larga como el oleato, mientras que en mamíferos sólo lo hacen frente a cambios en los niveles circulantes de ácidos grasos de cadena larga.

3. Las evidencias obtenidas *in vivo* e *in vitro*, en general, sugieren que la acción de oleato y octanoato sobre el hipotálamo es directa mientras que en los cuerpos de Brockmann y en hígado sería indirecta, de modo que pudiera deberse a la interacción con sistemas endocrinos o ser el resultado de la activación previa de los sensores hipotalámicos.

4. El incremento de los niveles de oleato u octanoato tanto a nivel periférico como a nivel central produce una inhibición de la ingesta de alimento que se puede relacionar tanto con la activación de los sistemas sensores como con cambios en la expresión de factores orexigénicos (NPY y AgRP) y anorexigénicos (POMC y CART) que van a regular la ingesta de alimento.

5. El descenso en los niveles circulantes de ácidos grasos inducido farmacológicamente produce un incremento en la ingesta, que no coincide en general con los cambios observados en los sistemas sensores de ácidos grasos centrales y periféricos. Asimismo, se activa el eje hipotálamo-hipófisis-interrenal, lo que probablemente esté relacionado con la potenciación de la lipólisis en los tejidos periféricos para restablecer los niveles de ácidos grasos en plasma.

6. El tratamiento con insulina potencia la respuesta de los sistemas sensores de ácidos grasos ante el incremento en los niveles de oleato u octanoato, fundamentalmente a nivel hepático y en menor medida en los cuerpos de Brockmann. Por el contrario, apenas influye sobre la actividad sensora hipotalámica, lo que no coincide con la

potenciación del efecto anoréctico de los ácidos grasos y los cambios observados en la expresión de neuropéptidos orexigénicos y anorexigénicos.

7. Alimentar los peces con una dieta enriquecida en lípidos durante 4 semanas no resulta en diferencias en la ingesta aunque sí se observan cambios en la expresión de neuropéptidos orexigénicos y anorexigénicos. Los sistemas sensores de ácidos grasos en hipotálamo e hígado solo se activan parcialmente, lo que nos permite sugerir que estarían aparentemente diseñados para responder frente a incrementos en los niveles de ácidos grasos específicos. El incremento de lípidos en la dieta produce asimismo en hipotálamo e hígado la activación del sensor de energía intracelular AMPK y las proteínas implicadas en la señalización celular Akt y mTOR.

A modo de conclusión general, los resultados de la presente Tesis Doctoral ponen de manifiesto por primera vez en peces (y en vertebrados no mamíferos), la existencia a nivel central y periférico de sistemas sensores de ácidos grasos que se activarían en respuesta a incrementos en los niveles circulantes de ácidos grasos específicos (oleato y octanoato) y que solo responden parcialmente frente a incrementos o descensos en los niveles totales de ácidos grasos. La actividad de dichos sistemas sensores estaría relacionada con el control de la ingesta de alimento (hipotálamo), la homeostasis metabólica (hígado) o la liberación de hormonas (cuerpos de Brockmann) y son necesarios estudios posteriores para elucidar los mecanismos implicados en dichas funciones.

5. CONCLUSIONS

1. Evidence of fatty acid sensing systems has been obtained in rainbow trout hypothalamus, Brockmann bodies and liver responding to changes in circulating fatty acid levels.

2. The mechanisms through wich fatty acids are sensed in hypothalamus, Brockmann bodies and liver appear to be similar to those suggested in mammals, based in 1) fatty acid metabolism; 2) mitochondrial activity, through inhibition of the potassium-dependant ATP channel (K^+_{ATP}); transport of fatty acids through FAT/CD36. Unlike mammals, these systems are activated in response to the increase in the levels of medium-chain fatty acids like octanoate and long-chain fatty acids like oleato whereas in mammals only respond to increase the levels of long-chain fatty acids.

3. Evidences obtained *in vivo* and *in vitro,* in general suggest that the increase of circulating oleate or octanoate levels in rainbow trout is directly sensed in hypothalamus while in Brockmann bodies and liver the fatty acid sensing capacity would be of indirect nature. It can be a consequence of hypothalamic fatty acid sensing and/or can be attributed to the interaction with other endocrine systems.

4. The increase in the levels of oleate or octanoate, either at peripheral or central levels elicited an inhibition in food intake. This inhibition can be associated with the activation of the fatty acid-sensing systems and with changes in the expression of the orexigenic (AgRP and NPY) and anorexigenic factors (POMC and CART) involved in the regulation of food intake.

5. The decrease in circulating levels of fatty acids induced by pharmacological treatment resulted in a clear increase in food intake, wich does not agree with the changes observed in the central and peripheral fatty acid sensing systems. The HPI axis becomes activated inducing a counter-regulatory response in rainbow trout through modulation of lipolysis in peripheral tissues to restore fatty acid levels in plasma.

6. Insulin treatment potentiates, especially in liver and in a lower extent in BB, the response of the fatty acid systems to be increase in levels of oleate or octanoate. The effects of insulin in the modulation of fatty acid sensing in hypothalamus were very scarce, which is in contrast with the enhancement of the anorectic effect of fatty acid, and the changes observed in the expression of anorexigenic and orexigenic neuropeptides.

7. Feeding fish for 4 weeks with a lipid-enriched diet did not result in differences in food intake but induced changes in the expression of orexigenic and anorexigenic neuropeptides. The fatty acid-sensing systems in hypothalamus and liver also respond partially to the lipid-enriched diet. Therefore, the fatty acid-sensing systems are apparently designed to respond to changes in the levels of specific fatty acids. The enhanced availability of lipid is inducing in hypothalamus and liver a general activation of the of intracellular energy sensor AMPK and of the proteins involved in cellular signaling pathways related to mTOR and Akt.

As a general conclusion, the results obtained in the present PhD Thesis show for the first time in fish (and in a non-mammals vertebrate) the existence, at central and peripheral locations, a fatty acid-sensing systems, which are activated in response to increases in circulating levels of specific fatty acids (oleate and octanoate) but only partially respond to increases or decreases in the levels of total fatty acids. The activity of these sensing systems would be related to the control of food intake (hypothalamus), metabolic homeostasis (liver) or hormone release (BB). Further studies are necessary to elucidate the mechanisms involved in these functions.

6. RESUMEN TESIS/THESIS SUMMARY

6. RESUMEN/THESIS SUMMARY

RESUMEN TESIS

Trabajo experimental Nº1: **Presencia de un sistema metabólico sensor de ácidos grasos en hipotálamo y cuerpos de Brockmann de trucha arco iris**

En mamíferos se ha descrito la existencia de mecanismos sensores que informan de la disponibilidad de nutrientes como la glucosa y ácidos grasos, que le permiten al cerebro modular la adquisición y el gasto de energía y las funciones metabólicas periféricas como un medio para controlar la homeostasis energética general. Además, también en mamíferos, existen neuronas especializadas en el hipotálamo que son capaces de detectar cambios en los niveles plasmáticos de ácidos grasos de cadena larga (LCFA), pero no de cadena corta (SCFA) o de cadena media (MCFA) y que juegan un papel fundamental en el control nervioso de la homeostasis energética. En cuanto a los sistemas sensores de nutrientes en mamíferos, en el caso concreto de los ácidos grasos se conocen varios mecanismos sensores, dependientes del metabolismo de ácidos grasos, transporte de ácidos grasos, actividad mitocondrial y la protein kinasa C. Como resultado de la acción de estos sistemas en hipotálamo de mamíferos, se desencadenan cambios en la expresión de factores orexigénicos y anorexigénicos con la consecuente regulación de la ingesta. Un incremento en los niveles de LFCA en mamíferos, producen una inhibición de la expresión de AgRP y de NPY y un incremento en la expresión de POMC y de CART.

No existen estudios en vertebrados no mamíferos sobre la presencia de mecanismos de detección de ácidos grasos y su posible relación con el control de ingesta de alimento. El metabolismo energético de los peces es diferente al de mamíferos, ya que son relativamente intolerantes a la glucosa, por lo tanto los lípidos y aminoácidos tienen más importancia en su metabolismo. Los peces usan como principal fuente de energía los lípidos para números procesos fisiológicos. Además, se ha observado una reducción de la ingesta en peces alimentados con dietas con alto contenido lipídico, lo que sugiere la presencia en peces de un mecanismo sensor de lípidos que regula la ingesta. En trucha arco iris se ha demostrado la existencia de glucosensores tanto a nivel central (hipotálamo, cerebro posterior) como a nivel periférico (BB) implicados en el control de la ingesta y en la secreción hormonal. Teniendo en cuenta todo esto, y usando la trucha arco iris como modelo de pez teleósteo, nuestro objetivo fue evaluar la presencia de componentes de mecanismos sensores de ácidos grasos y su respuesta ante un incremento de los niveles circulantes de ácidos grasos mediante la administración intraperitoneal de un ácido graso de cadena larga como el oleato (LFCA) y otro de cadena media como el octanoato (MFCA).

Por tanto, en un primer experimento, tras un mes de aclimatación, 12 peces por grupo se inyectaron intraperitonealmente con 10 ml/kg de solución salina (control) o conteniendo oleato (60 o 300 µg/kg) u octanoato (60 o 300 µg/kg). Pasadas 6 horas se

tomaron muestras de sangre, hipotálamo, cerebro posterior y cuerpos de Brockmann de los peces con el fin de evaluar la posible existencia de mecanismos sensores de ácidos grasos. Para ello, se evaluaron parámetros relacionados con el metabolismo de los ácidos grasos, actividades enzimáticas y niveles de ARNm mediante qRT-PCR. Posteriormente se realizó un segundo experimento, en este caso 8 peces por grupo se inyectaron intraperitonealmente con las mismas soluciones y concentraciones que en el experimento anterior y tras 6 y 24 horas se evaluó la ingesta. Para demostrar la relación entre el metabolismo sensor de ácidos grasos y el control de ingesta de alimento en un tercer set de peces se evaluó la ingesta tras 6 y 24 horas de la administración intraperitoneal de 10 ml/kg de solución salina al grupo control, 5 mg/Kg de C75 (inhibidor de FAS), 5 mg/Kg de TOFA (inhibidor de ACC) o 5mg/Kg de C75 y TOFA.

Los resultados obtenidos validan el diseño experimental, ya que el tratamiento con ambas concentraciones de oleato y octanoato produce un incremento en los niveles circulantes de FA, de triglicéridos y de lípidos totales pero no produce cambios en los niveles de glucosa. Además este incremento en los niveles circulantes de FA se refleja en el hipotálamo con un incremento de los niveles de FA y lípidos totales y un descenso en los niveles de triglicéridos. En hipotálamo de mamíferos, el mecanismo sensor de FA relativo al metabolismo de FA se activa por LCFA pero no por MCFA o SCFA. Esta situación parece ser diferente en trucha arco iris ya que en general el tratamiento con octanoato induce efectos similares a los producidos por el oleato. No obstante, con ambos tratamientos de FA los resultados obtenidos a nivel hipotalámico se asemejan a los de mamíferos, dándose un incremento en los niveles de malonil Co-A y un descenso en los niveles de ARNm de ACLY, ACO y CS. Como diferencia entre ambos tratamientos, cabe destacar que se produce un descenso en los niveles de ARNm de CTP1c tras el tratamiento con oleato, que no ocurre tras el tratamiento con el octanoato, esto puede deberse a que éste último no necesita CPT1 para entrar en la mitocondrial al igual que ocurre en mamíferos. En cuanto a la ingesta de alimento, se produce un descenso con ambos tratamientos, así como tras el tratamiento con C75 que se contrarresta en presencia de TOFA. Todo esto sugiere la existencia de un mecanismo sensor de FA en trucha arco iris involucrado en el control de ingesta de alimento. Al igual que en mamíferos, a nivel hipotalámico hay un descenso en los niveles de expresión de ARNm de NPY y un incremento en los niveles de expresión de POMC tras el tratamiento con oleato coincidiendo con la inhibición de la ingesta de alimento. Sin embargo, tras el tratamiento con octanoato, no observamos diferencias en la expresión de estos neuropéptidos. En cerebro posterior de trucha arco iris, el tratamiento con FA no induce cambios en los niveles de FA y si comparamos con los resultados obtenidos en parámetros relativos al metabolismo de los ácidos grasos en hipotálamo, observamos una ausencia de respuesta en muchos de los parámetros analizados. Los resultados obtenidos en BB en algunos casos son similares a los observados en hipotálamo, como el descenso en los niveles de

ARNm de FAS y CPT 1c tras el tratamiento con ambos ácidos grasos, lo que sugiere que el metabolismo de FA puede estar involucrado en la capacidad sensora de FA en BB.

Si tenemos en cuenta otros posibles mecanismos sensores de ácidos grasos descritos en mamíferos como la vía relacionada con el trasporte de ácidos grasos mediante el transportador FAT/CD36, podemos observar que en hipotálamo de trucha arco iris tras el tratamiento con oleato no sólo se produce un incremento en los niveles de ARNm de FAT/CD36, sino que también en los de PPAR α y SREBP1c con el subsiguiente descenso en los niveles de expresión de LXR al igual que ocurre en mamíferos. En cerebro posterior no hemos observado cambios en dichos parámetros mientras que en BB ambos tratamientos producen cambios similares a los ocurridos en hipotálamo.

Otro mecanismo sensor de ácidos grasos descrito en mamíferos es el asociado a la actividad mitocondrial, con la inhibición del canal de potasio dependiente de ATP (K_{ATP}). En trucha arco iris, al igual que ocurre en mamíferos, observamos a nivel hipotalámico una clara inhibición en la expresión de Kir6.x-like y SUR-like que son componentes del canal K_{ATP}. Sin embargo, esta inhibición no se observa en cerebro posterior y BB.

Estos resultados, prueban, por primera vez en peces, la existencia de capacidad sensora de ácidos grasos en hipotálamo y BB ante el incremento de los niveles circulantes de LCFA y MCFA que puede estar asociada con el control de ingesta de alimento.

Trabajo experimental Nº2: **Efectos del tratamiento con ácidos grasos en solitario o con un conjunto de estos en la respuesta del metabolismo hepático lipídico y de la glucosa. Posible presencia de mecanismos sensores de ácidos grasos**

En peces, el hígado juega un papel muy importante en el metabolismo de los FA y su interacción con la glucosa está parcialmente caracterizada. En trucha arco iris, un incremento en los niveles de lípidos en la dieta induce hiperglucemia, reduciendo el potencial glucolítico y lipídico, e incrementando el potencial gluconeogénico en hígado. En un primer experimento, se administró a truchas arco iris oralmente un tratamiento compuesto por una serie de aceites de pescado (FO) para evaluar si el suministro agudo de ácidos grasos en la dieta produce una mejora en la tolerancia a la glucosa.

Como hemos visto en el trabajo experimental Nº1, la administración intraperitoneal de oleato y octanoato en trucha arco iris, produce en hipotálamo y BB la activación de ciertos componentes pertenecientes a un sistema sensor de ácidos grasos. Por ello, en un segundo experimento, se administró a truchas arco iris mediante inyecciones IP uno de los ácidos grasos presentes en la dieta de aceites de pescado mencionada anteriormente como es el oleato (60 o 300 $\mu g.Kg^{-1}$) y octanoato (60 o 300 $\mu g.Kg^{-1}$) que se usó como control negativo ya que no estaba presente en la dieta del

experimento anterior, con el fin de determinar la posible presencia de mecanismos sensores de ácidos grasos en hígado.

Los resultados obtenidos validaron ambos diseños experimentales, ya que la administración oral de FO produjo un incremento en plasma de los niveles de triglicéridos y la administración IP de oleato y octanoato provocó un incremento en los niveles plasmáticos de ácidos grasos, lípidos totales y triglicéridos.

Tras la administración oral de FO, el cambio más relevante en parámetros relativos al metabolismo de la glucosa fue un drástico descenso en los niveles de ARNm de GK, lo que coincide con estudios llevados a cabo en ratas. Sin embargo, no se observaron diferencias en la actividad enzimática de GK, lo que sugiere que el efecto es solo a nivel molecular. Tras el tratamiento, también se produjo un incremento en los niveles de ARNm de FAS y G6PDH, lo que coincide con la idea de que un incremento en el potencial lipogénico afecta al uso de la glucosa como se ha sugerido en otros estudios. El incremento en el potencial lipogénico hepático acompañado por un descenso en el potencial de oxidación de FA en este estudio se reflejó en una reducción en los niveles de ARNm de CPT1a y CPT1b.

En el segundo experimento, tras el tratamiento IP con oleato u octanoato, se produjo una reducción del potencial glicolítico reflejado en el descenso en la actividad enzimática de PK y un incremento tras el tratamiento con oleato del potencial gluconeogénico reflejado en el aumento de la actividad enzimática de FBPasa.

Además, al igual que en el experimento anterior, se produjo un descenso en los niveles de ARNm de GK con ambos FA. El incremento en los niveles de glucosa y glucógeno en hígado (sólo con el tratamiento de oleato) sugiere que los FA estimulan el metabolismo de la glucosa con el fin de ser acumulados en vez de ser redirigidos al ciclo de los ácidos tricarboxílicos (TCA). Con ambos tratamientos el potencial lipogénico se inhibió en hígado quedando reflejado en un descenso en la actividad enzimática de FAS y ACLY y un descenso en los niveles de ARNm de FAS. Además la actividad de G6PDH en presencia de oleato y los niveles de ARNm de G6PDH en presencia de cualquiera de los dos FA no mostraron descensos similares a los observados en otros parámetros lipogénicos. Con ambos FA se dio un incremento en la actividad enzimática y en los niveles de ARNm de MCD y un descenso en los niveles de mRNA de CPT1a, CPT1b y CS, coincidiendo con los cambios observados en los niveles de mRNA de CPTs tras la administración oral de FO. La administración IP de estos FA no produjo cambios en los niveles de mRNA de FAT/CD36 y FATP1, por lo que el potencial de transporte de FA no se vio afectado.

Puesto que la respuesta metabólica a los FA administrados por separado, al oleato u octanoato, fue similar, una interacción entre FA y glucosa en lugar de FA por sí sola es probable.

Aunque la administración intraperitoneal de oleato y de octanoato no produjo un aumento en la lipogénesis ni tampoco redujo los niveles de glucosa en plasma, como se observó tras el tratamiento oral con FO, los resultados proporcionan evidencias claras de que ambos FA, incluso cuando las concentraciones son bajas, desempeñan un papel clave en la regulación de varios componentes de sistemas sensores de ácidos grasos presentes en hígado de trucha arco iris.

Trabajo experimental Nº3: **La capacidad sensora de oleato y octanoato en trucha arco iris es directa en hipotálamo y cuerpos de Brockmann**

La capacidad sensora de ácidos grasos en mamíferos se lleva a cabo mediante cuatro mecanismos: 1) metabolismo de los ácidos grasos mediante el cual se inhibe la CPT-1 encargada de transferir los ácidos grasos al interior de la mitocondria para su oxidación; 2) transporte de los ácidos grasos mediante el transportador FAT/CD36 y la subsiguiente modulación de factores de transcripción; 3) translocación y activación de la protein quinasa C-δ ; 4) producción mitocondrial de especies reactivas de oxígeno (ROS). Un incremento en los niveles de LCFA produce cambios en la actividad de estos sistemas en hipotálamo de mamíferos con la subsiguiente reducción de la ingesta, inhibición de los factores orexigénicos e incremento de los factores anorexigénicos. Además, se sabe que la detección a nivel central de glucosa y FA están relacionadas a través de una vía vagal y simpática para la regulación homeostática de la glucosa afectando a la liberación de insulina en páncreas, aunque también existe una regulación directa de la liberación de insulina desde las células β-pancreáticas.

Como hemos visto en el trabajo experimental Nº1, la administración IP con oleato (LCFA) o octanoato (MCFA) en trucha arco iris provoca una inhibición de la ingesta de alimento e induce en hipotálamo una respuesta compatible con la capacidad sensora de FA en la cual el metabolismo de FA, transporte mediado por FAT/CD36 y la actividad mitocondrial parecen estar involucrados, siendo estos mecanismos similares a los sugeridos en mamíferos, pero con la diferencia de que la trucha arco iris tiene la capacidad de detectar cambios en niveles de MCFA. Los resultados obtenidos en BB sugieren la presencia de componentes de los mecanismos sensores de FA basados en el metabolismo de los FA y en el transporte de los FA mediante FAT/CD36. No sabemos si el efecto de los FA en hipotálamo y BB es un efecto directo o está mediado por cambios en otros sistemas endocrinos, tampoco si los cambios son dependientes de la concentración de FA o si la respuesta en BB quizás sea consecuencia de la vía vagal y simpática relacionada con el hipotálamo de forma similar a la descrita en mamíferos.

Para responder a estas preguntas se llevaron a cabo dos experimentos *in vitro.* En el primero se incubaron durante 1 hora fragmentos de hipotálamo y BB de trucha arco iris

en medio Hanks modificado con glucosa (2mM) y en presencia de diferentes concentraciones de oleato u octanoato (1, 10 o 100 µM). En el segundo se incubaron durante 1 hora fragmentos de hipotálamo en medio Hanks modificado con glucosa (2mM) y con 100 µM de oleato o 100 µM de octanoato solo o en presencia de una serie de inhibidores de parámetros relativos a la capacidad sensora de ácidos grasos en mamíferos como son: 40 $\mu g.mL^{-1}$ de C75 , 10 µM de etomoxir, 1 µM de trolox, 20 µM de genipin, 500 µM de diazoxide, 5 µM de triacsin C, 50 nM de SSO o 40 $\mu g.mL^{-1}$ de TOFA. Se evaluaron parámetros relacionados con los mecanismos sensores de ácidos grasos y se determinó la expresión de neuropéptidos relacionados con la ingesta, con el fin de evaluar si existe un efecto directo de los FA en hipotálamo y BB de trucha arco iris.

En cuanto a los resultados, en hipotálamo el tratamiento con oleato u octanoato se refleja en un aumento en los niveles de FA, triglicéridos y lípidos totales. En la mayoría de los parámetros para ambos FA los cambios son similares a los previamente observados *in vivo* (trabajo experimental Nº1) y hay una respuesta dosis-dependiente, que suele ser igual para ambos tratamientos en los niveles de FA, lípidos totales, actividades enzimáticas de ACLY, FAS y HOAD y en niveles de ARNm de ACLY, FAS, NPY, PPARα, LXRα, CS y Kir6.x-like, y solo tras el tratamiento con oleato para niveles de ARNm de CPT1d y POMC. El hecho de que estos resultados sean similares a los observados *in vivo,* nos sugiere que la acción de los FA sobre los mecanismos sensores de FA en hipotálamo de trucha arco iris podría ser directa y no estar mediada por otros sistemas endocrinos o nutrientes. Observamos que en presencia de oleato u octanoato se estimulan los niveles de ARNm de los péptidos anorexigénicos (POMC y CART) mientras que los niveles de NPY disminuyen tras el tratamiento con oleato. Estos cambios corresponden con la reducción en la ingesta observada *in vivo* tras el tratamiento con oleato.

En BB, tras el tratamiento con ambos FA se produce un incremento en los niveles de FA, triglicéridos y lípidos totales de forma similar al producido en hipotálamo, pero diferente al descrito en mamíferos donde las células pancreáticas no responden a los MCFA como el octanoato. Sin embargo, aunque los resultados son similares a los observados en hipotálamo, la mayoría de los cambios en los parámetros medidos *in vitro* en BB son en general diferentes a los observados previamente *in vivo,* tales como niveles de lípidos totales, actividades enzimáticas de ACLY, FAS, HOAD (oleato), y niveles de ARNm de ACLY, FAS, SREBP1c, PPARα, LXRα, FAT/CD36 (oleato), CS, Kir6.x-like, SUR-like, CPT1a, y CPT1b. Esto puede ser debido a que la acción de los FA en los mecanismos sensores de ácidos grasos en BB observados *in vivo* puedan estar influenciados por otros sistemas endocrinos como la insulina.

Con respecto a los resultados obtenidos en hipotálamo usando inhibidores específicos de parámetros relativos a los mecanismos sensores de ácidos grasos, todos los antagonistas en presencia de oleato u octanoato contrarrestan la acción de los FA para varios de los parámetros medidos. Los efectos son comparables para ambos ácidos a

excepción del trolox que es más efectivo contrarrestando los efectos del octanoato y del diazoxide para el oleato. Los inhibidores relativos al mecanismo basado en el metabolismo de los ácidos grasos como C75, etomoxir, triacsin C y TOFA generalmente contrarrestan los efectos de ambos ácidos grasos en parámetros relativos a este mecanismo. Los inhibidores relativos al transporte de los FA y la actividad mitocondrial como trolox, genipin, SSO y diazoxide generalmente también contrarrestan los efectos de ambos ácidos en parámetros relacionados con el mecanismo. Los efectos del oleato y del octanoato en la expresión de NPY y POMC son contrarrestados en presencia de trolox, genipin, SSO y diazoxide.

En general, las respuestas observadas *in vitro* en hipotálamo de trucha arco iris nos permiten sugerir que este tejido tiene capacidad para detectar directamente cambios en los niveles de MCFA y LCFA a través de mecanismos relativos al metabolismo de FA, transporte mediante FAT/CD36 y actividad mitocondrial comparables a los de mamíferos, pero con la diferencia de la capacidad de repuesta al octanoato de la trucha arco iris. Con los resultados obtenidos tras la incubación de BB podemos hipotetizar que la capacidad sensora de FA previamente descrita *in vivo* en este tejido está influenciada por otros sistemas neuroendocrinos.

Trabajo experimental Nº4: **Respuesta de los mecanismos sensores de ácidos grasos hepáticos en trucha arco iris tras el tratamiento *in vitro* con oleato u octanoato**

El metabolismo hepático de trucha arco iris, así como el de mamíferos, responde de forma diferente a exposiciones a corto plazo o a largo plazo con FA. Sin embargo, mientras que la información que existe sobre la respuesta en peces a exposiciones a altos niveles de FA a largo plazo es considerable, apenas hay información sobre la repuesta ante exposiciones agudas con FA y en particular de los mecanismos por los cuales los FA ejercen su efecto.

En previos estudios se demostró en trucha arco iris, que la administración aguda de oleato (LCFA) u octanoato (MCFA) activa componentes de los mecanismos sensores de FA en hipotálamo, BB e hígado de trucha arco iris. Además, en hígado de trucha arco iris, tras el tratamiento intraperitoneal con oleato u octanoato se produce un incremento en la acumulación de glucosa y glucógeno, el potencial lipogénico disminuye y aumenta el catabolismo citoplasmático de los FA como se observó en el trabajo experimental Nº2. Sin embargo, no sabemos si el efecto de los FA en hígado es un efecto directo o está mediado por cambios en otros sistemas endocrinos, tampoco si los cambios son dependientes de la concentración de FA, o si la respuesta quizás sea consecuencia de la activación de la vía vagal y simpática relacionada con el hipotálamo de forma similar a la descrita en mamíferos.

Por lo tanto, se ha evaluado *in vitro* (en ausencia de mecanismos de regulación extrahepática) si el hígado responde a cambios en la concentración de FA de forma similar a lo previamente observado *in vivo.* Para ello, se incubaron durante una hora fragmentos de hígado de trucha arco iris en medio Hanks modificado con glucosa (2 mM) y en presencia de concentraciones crecientes de oleato u octanoato (1, 10 o 100 µM) y se evaluaron parámetros relacionados con los mecanismos sensores de ácidos grasos.

Los resultados obtenidos en la mayoría de los parámetros evaluados tras la incubación *in vitro* son, en general, diferentes a los observados previamente *in vivo.* Se produce un incremento en las actividades enzimáticas de ACLY y G6PDH y en los niveles de ARNm de FAS y ACC, por lo que hay una activación del potencial lipogénico. Esta activación no es comparable a lo ocurrido tras el tratamiento con FA *in vivo,* en el cual se producía una inhibición en hígado del potencial lipogénico. La vía del catabolismo citoplasmático de FA, que se ve incrementada por la presencia de oleato y de octanoato *in vivo*, se inhibe *in vitro*, representado por el descenso en la actividad enzimática de MCD. El potencial de oxidación en la mitocondria aparentemente disminuye basándonos en el descenso de la actividad de HOAD y de los niveles de ARNm de UCP2a.

Hay un incremento de los niveles de glucosa y glucógeno en hígado solo tras el tratamiento con oleato, lo que sugiere que los FA estimulan el metabolismo de la glucosa para su acumulación para el ciclo de los ácidos tricarboxílicos. Este incremento en los niveles de glucógeno es comparable al incremento que se produce también en trucha arco iris cuando se alimenta con dietas ricas en grasas. Se produce un aumento en las actividades enzimáticas de FBPasa y G6Pasa en presencia de los FA por lo que se ve activado el potencial gluconeogénico. Tanto la actividad de GK como los niveles de ARNm se incrementan tras el tratamiento con ambos FA. Estos cambios son contrarios a los observados previamente *in vivo,* pero son de nuevo similares a los producidos en trucha arco iris cuando se alimenta con dietas ricas en grasas.

En resumen, observamos que varios componentes de diferentes mecanismos sensores de FA en hígado de trucha arco iris tras su incubación *in vitro* presentan una respuesta dosis-dependiente al incremento de los niveles de oleato u octanoato siendo además similar para ambos. Sin embargo, en general, estos cambios no coinciden con los previamente observados *in vivo* en el trabajo experimental Nº2 en hígado de la misma especie. Estos resultados nos permiten hipotetizar, que la capacidad sensora de FA observada *in vivo* podría ser de naturaleza indirecta, atribuyéndose a la interacción con otros sistemas endocrinos.

Trabajo experimental Nº5: **Efectos del tratamiento intracerebroventricular con oleato y octanoato en la ingesta de alimento y los sistemas sensores de ácidos grasos hipotalámicos en trucha arco iris**

En el hipotálamo de mamíferos existen neuronas especializadas capaces de detectar cambios en los niveles plasmáticos de LCFA pero no de SCFA o MCFA a través de cuatro mecanismos diferentes: 1) metabolismo de los FA; 2) transporte de FA; 3) actividad mitocondrial; 4) activación de la protein quinasa δ. En peces, gracias a los estudios previos realizados, hemos obtenido evidencias de que tras el tratamiento *in vivo* con oleato u octanoato se produce una inhibición de la ingesta con los subsiguientes cambios en los niveles de ARNm de neuropéptidos específicos y cambios en varios parámetros relacionados con el metabolismo de ácidos grasos, transporte de ácidos grasos mediado por FAT/CD36 y actividad mitocondrial similares a los descritos en mamíferos, pero con la diferencia principal de que en mamíferos esos sistemas no responden frente a ácidos grasos de cadena media como el octanoato. Cuando la administración es *in vitro* la respuesta de dichos sistemas es similar a la descrita antes *in vivo,* lo que permite hipotetizar que la acción de dichos ácidos grasos es directa sobre el hipotálamo.

Por lo tanto, el objetivo de este trabajo será comprobar si tras el tratamiento ICV con FA en trucha arco iris, se observan los mismos resultados que en estudios previos, tanto con tratamientos intraperitoneales, como *in vitro*, y poder así corroborar fehacientemente la hipótesis de una acción directa de los ácidos grasos sobre el hipotálamo. Para ello, se llevaron a cabo dos experimentos. En el primero, tras un mes de aclimatación, 10 peces por grupo se inyectaron intraperitonealmente con 1 µL de solución salina (control) o conteniendo oleato (1M) u octanoato (1M) y tras 6 y 24 horas se evaluó la ingesta. En el segundo experimento, 30 peces por grupo fueron inyectados con solución salina (control), oleato u octanoato con las mismas concentraciones que en el primer experimento. Pasadas 2 horas y 6 horas se tomaron muestras de sangre y de hipotálamo de 15 peces por grupo y por tiempo. Se evaluaron parámetros relacionados con el metabolismo de los ácidos grasos, actividades enzimáticas y niveles de ARNm mediante qRT-PCR.

Los resultados indican que con ambos ácidos grasos se produce un descenso en la ingesta de alimento en los dos tiempos, siendo más acusado tras 24 horas. Además la expresión de neuropéptidos está en general relacionada con los cambios en la ingesta, produciéndose una inhibición de NPY y una activación de POMC y CART, por lo que este descenso probablemente está mediado por un mecanismo sensor central de ácidos grasos. En cuanto a los niveles de glucosa y lactato en plasma, no se observaron diferencias con respecto al grupo control y tampoco en los niveles de ácidos grasos, triglicéridos y lípidos totales, lo que valida el diseño experimental.

En hipotálamo, el tratamiento produjo un aumento en los niveles de FA y de triglicéridos tras 2 horas de la administración con oleato y tras 6 horas con octanoato. En los niveles de lípidos totales no se observaron diferencias significativas con respecto al grupo control tras 2 horas, sin embargo, tras 6 horas se observó un incremento tras el tratamiento con ambos FA, siendo significativamente mayor en el caso del oleato. En cuanto a las actividades enzimáticas hubo un descenso en la actividad de FAS tras el tratamiento con ambos ácidos grasos a las 2 horas, mientras que se produjo un incremento tras el tratamiento con octanoato a las 6 horas. En la actividad de ACLY, se pudo observar que prácticamente se mantiene la misma respuesta en ambos periodos de tiempo, exceptuando en el tratamiento con octanoato, en el que se observaron valores elevados pasadas 2 horas de la administración. Cabe destacar que la actividad de CPT-1 disminuyó con ambos tratamientos y transcurridos los dos tiempos.

Tras el tratamiento con oleato, se observó una disminución en los niveles de ARNm con respecto al grupo control de FAT/CD36 (2h y 6h), ACC (2h), ACLY (2h), CS (2h), FAS (2h), Kir6.x-like (2h y 6h), SUR-like (2h y 6h), LXRα (2h y 6h) y NPY (2h) y se produjo un incremento en los niveles de ARNm de CS y MCD. Tras el tratamiento con octanoato, se produjo un descenso en los niveles de ARNm de ACC (2h), Kir6.x-like (2h) y SUR-like (2h y 6h) y un incremento en los niveles de ARNm de CPT-1c (2h), CS (6h), MCD (2h) y UCP2 (2h).

En su conjunto, los datos obtenidos parecen indicar que el tratamiento intracerebroventricular en trucha arco iris con oleato u octanoato produce un descenso en la ingesta de alimento, que además coincide con los cambios que se observan en la expresión de factores orexigénicos (NPY) y anorexigénicos (POMC) en el hipotálamo. Además, el tratamiento activa el sistema sensor relacionado con el metabolismo de ácidos grasos, siendo en general más rápida la activación para el caso del oleato. Las respuestas son comparables a las descritas previamente *in vivo*. Los parámetros relacionados con el sistema sensor de ácidos grasos mediado por la actividad mitocondrial muestran en general los mismos resultados que los descritos previamente tras tratamiento *in vivo,* con lo que podríamos hipotetizar que la acción de los FA sobre el hipotálamo es directa y no está mediada por otros sistemas endocrinos. Sin embargo, el sistema sensor relacionado con el transporte de ácidos grasos no responde en general al tratamiento ICV con oleato, por lo que podría ser que existan otros factores que estén interaccionando con este sistema.

Trabajo experimental Nº6: **La administración central de oleato y octanoato afecta a los sistemas sensores de ácidos grasos en hígado y cuerpos de Brockmann de trucha arco iris**

En hipotálamo de mamíferos, la capacidad de detectar cambios en los niveles circulantes de ácidos grasos de cadena larga, se lleva a cabo por cuatro mecanimos diferentes : 1) metabolismo de FA; 2) transporte de FA; 3) actividad mitocondrial; 4) activación de la protein quinasa δ. En peces, gracias a los estudios previos realizados, hemos observado que los mecanismos sensores de ácidos grasos son similares a los de mamíferos con la diferencia de que también responden ante cambios en los niveles circulantes de ácidos grasos de cadena media como el octanoato. La activación de estos sistemas a nivel hipotalámico produce una inhibición de la ingesta de alimento. Además de con la ingesta, la detección a nivel central de ácidos grasos está relacionada con el flujo vagal y simpático para la regulación de la homeostásis que va a afectar a la liberación de hormonas pancreáticas y a la producción endógena de glucosa en el hígado.

El objetivo de este trabajo será comprobar si tras el tratamiento ICV con FA en trucha arco iris, se observan los mismos resultados que en estudios previos tras tratamientos intraperitoneales en hígado y BB, poder así corroborar la hipótesis de que la acción de los ácidos grasos sobre estos tejidos está relacionada con la interacción con otros sistemas. Para ello, tras un mes de aclimatación, 15 peces por grupo se inyectaron intraperitonealmente con 1 µL de solución salina (control) o conteniendo oleato (1M) u octanoato (1M) y tras 6 y horas se tomaron muestras de hígado y de BB. Se evaluaron parámetros relacionados con el metabolismo de los ácidos grasos, actividades enzimáticas y niveles de ARNm mediante qRT-PCR.

El tratamiento ICV con oleato y octanoato, produce en BB cambios en varios parámetros relativos al metabolismo de los FA, como actividades de ACLY, CPT-1 y CS y niveles de ARNm de FAS, ACLY, CPT1a, FAT/CD36 y PPAR. Sin embargo, pocos de estos cambios coinciden con los observados tras el tratamiento IP. Estos resultados, apoyan la idea de que la capacidad sensora de FA observada en BB tras el tratamiento in *vitro* se modifica bajo condiciones *in vivo* con el tratamiento IP debido a la interacción con los efectos producidos por los sistemas endocrinos periféricos inducidos por los cambios en los niveles circulantes de FA.

En hígado, a diferencia de BB, la mayoría de los resultados obtenidos tras el tratamiento ICV con oleato y octanoato son similares a los obtenidos previamente tras el tratamiento IP. Sin embargo, son diferentes a los obtenidos *in vitro*. Esto incluye, niveles de ácidos grasos, lípidos totales, actividades enzimáticas de FAS, ACLY (octanoato), MCD (oleato) y HOAD, y niveles de expresión de ARNm de ACC, ACLY, CPT1b y MCD (oleato). Hay evidencias que indican que la detección de FA a nivel hipotalámico activa el SNC con

el fin de modificar el metabolismo energético hepático para mantener un equilibrio adecuado de los niveles de metabolitos y el status energético.

En su conjunto, los datos parecen indicar que en trucha arco iris el tratamiento central con oleato u octanoato induce cambios en varios parámetros relativos a los mecanismos sensores de FA en tejidos periféricos como BB e hígado. Como los resultados obtenidos en BB son completamente diferentes a los obtenidos tras el tratamiento IP, podría ser que se debiera a la interacción con hormonas periféricas como la insulina. Sin embargo, en hígado tras el tratmiento ICV con oleato u octanoato los resultados son similares a los del tratamiento IP pero diferentes a los observados *in vitro* (donde el hígado no esta bajo la inluencia hipotalámica) por lo que la detección de FA en este tejido parece estar relacionada con la capacidad sensora previa en el hipotálamo.

Trabajo experimental Nº7: **Respuesta hipotalámica al descenso en los niveles de ácidos grasos en trucha arco iris. Posible implicación del eje hipotálamo-hipófisis-interrenal**

En los trabajos experimentales anteriores hemos visto que en peces el aumento en los niveles de ácidos grasos circulantes estimula los sistemas sensores de ácidos grasos en hipotálamo en paralelo a cambios en la expresión de factores orexigénicos y anorexigénicos y la inhibición de la ingesta. Hasta el momento no se ha estudiado en peces qué respuesta se desencadena cuando se produce un descenso en los niveles de ácidos grasos plasmáticos. Por lo tanto, el objetivo general del presente estudio, es evaluar en la trucha arco iris si el descenso en los niveles de ácidos grasos circulantes (inducido farmacológicamente mediante el tratamiento con SDZ WAG 994) produce una inhibición en los sistemas sensores de ácidos grasos a nivel hipotalámico y ello se refleja en una activación de la ingesta. Si la respuesta que se observa es producto de dicha caída, debería ser contrarrestada si además del tratamiento con el fármaco se trata a los animales con un preparado (intralipid) que produce un incremento en los niveles de ácidos grasos circulantes. Además también evaluamos la posible participación del eje hipotálamo-hipófisis-interrenal (HPI), equivalente al eje hipotálamo-hipófisis-adrenal (HPA) en mamíferos, mediante el tratamiento con SDZ en presencia de metirapona, que es un inhibidor de eje HPI en peces.

Para ello, se realizaron 2 experimentos. En el primer experimento se administró intraperitonealmente 10 mL/Kg de solución salina sola (0,6% NaCl) a los ejemplares pertenecientes al grupo control, o conteniendo SDZ WAG 994 (Tocris, 60 $\mu g.Kg^{-1}$) a los individuos del grupo SDZ, o conteniendo SDZ WAG 994 junto a intralipid a los individuos del grupo SDZ+IL. Pasadas 6 y 24 horas de los tratamientos se evaluó la ingesta. En el segundo experimento, a 15 ejemplares por grupo se les administró intraperitonealmente 10 $mL.Kg^{-1}$ de solución salina sola (control, C), o conteniendo SDZ (SDZ; Tocris, 60 $\mu g.Kg^{-1}$),

metirapona (M; Sigma, 1 mg.Kg^{-1}), SDZ y metirapona (SDZ+M), o SDZ e intralipid (SDZ+IL). Pasadas 6 horas de los tratamientos, se procedió a la recogida de sangre, hipotálamo y riñón cefálico para el posterior análisis de metabolitos, actividades enzimáticas y abundancia de ARNm.

En cuanto a los resultados, en general, el tratamiento con SDZ fue efectivo al inducir no solo un descenso en los niveles circulantes de ácidos grasos, sino también un descenso en los niveles de triglicéridos y lípidos totales y la presencia de intralipid logró contrarrestar la acción del SDZ. La ingesta de alimento se estimula claramente en los peces tratados con SDZ. Esto nos permite sugerir, por primera vez en peces, que el hipotálamo detecta el descenso en los niveles de ácidos grasos desencadenando una respuesta orexigénica e incrementando la ingesta de alimento. En hipotálamo, el tratamiento con SDZ afecta al sistema sensor de ácidos grasos relacionado con el metabolismo de ácidos grasos ya que se produce un descenso de la actividad de FAS y HOAD, así como de la expresión de CPT1c, CPT1d y MCD, y un incremento en la expresión de FAS. Además, la presencia de intralipid contrarresta dichos cambios. Sin embargo, otros parámetros que muestran cambios cuando se produce un incremento en los niveles circulantes de oleato u octanoato, como son las actividades de CPT-1 y ACLY, y la expresión de ACC, ACLY y CS no muestran ningún cambio en el presente estudio, por lo que parece que el sistema basado en el metabolismo de los ácidos grasos responde al menos parcialmente frente al descenso en los niveles circulantes de ácidos grasos. El sistema sensor de ácidos grasos relacionado con el transporte de ácidos grasos a través de FAT/CD36 también parece responder parcialmente, ya que la mayoría de los parámetros no muestran cambios significativos. En cuanto al sistema sensor de ácidos grasos dependiente de la actividad mitocondrial, sólo hay cambios en UCP2a. La expresión de los péptidos anorexigénicos (POMC y CART) muestra un claro descenso en el hipotálamo de los peces tratados con SDZ, y dichos cambios se contrarrestaron en presencia de intralipid, sin embargo, no observamos cambios significativos en la expresión de NPY, que desciende cuando se incrementan los niveles de ácidos grasos.

El tratamiento con SDZ induce un aumento en los niveles circulantes de cortisol, que se contrarresta en presencia de intralipid. Esto nos permite sugerir que la activación del eje HPI está involucrada en la respuesta frente al descenso en los niveles de ácidos grasos. La expresión de proteínas involucradas en la síntesis de cortisol en riñón cefálico, como son 11βH, 3βHSD, P450scc y StAR, se incrementa de manera significativa tras el tratamiento con SDZ, y estos valores se contrarrestan cuando tratamos a los animales con SDZ junto a intralipid. El tratamiento con SDZ incrementa los niveles de mRNA de CRF y CRFBP en hipotálamo, que en la misma especie cambian normalmente en paralelo a los niveles de cortisol en plasma. El tratamiento con metirapona fue efectivo al contrarrestar el incremento en los niveles de cortisol producido por el tratamiento con SDZ. Además, el tratamiento conjunto de SDZ+M induce cambios en parámetros a nivel plasmático, de manera que los resultados son comparables a los del grupo control y diferentes a los del

grupo SDZ, tales como los niveles en plasma de ácidos grasos, triglicéridos y lípidos totales. Esta vuelta a la normalidad es también evidente en parámetros evaluados en hipotálamo donde los niveles de ácidos grasos y triglicéridos disminuyen en el grupo SDZ y vuelven a los valores normales en el grupo SDZ+M. En los parámetros relacionados con el eje HPI se produjo un bloqueo efectivo del mismo como lo demuestra el fuerte descenso observado en la expresión de las cuatro proteínas relacionadas con el metabolismo de cortisol estudiadas en riñón cefálico en el grupo SDZ+M. Estos cambios están asociados con los cambios observados en los niveles de cortisol en plasma, que fueron similares a los observados en el grupo control y más bajos que los del grupo tratado con SDZ.

Por lo tanto, parece que la disminución de los niveles de ácidos grasos circulantes mediante el tratamiento con SDZ produce un descenso en los niveles de ácidos grasos a nivel hipotalámico, pero solo algunos de los parámetros involucrados en el sistema sensor de ácidos grasos en el hipotálamo varían de forma acorde. Además, induce un claro incremento en la ingesta de alimento que se encuentra asociada al descenso en el potencial anorexigénico en el hipotálamo y a cambios en parámetros relacionados con el mecanismo sensor de ácidos grasos en el hipotálamo. El descenso en los niveles de ácidos grasos aparentemente induce una respuesta contra-reguladora en trucha arco iris, en la cual la activación del eje HPI está probablemente involucrada.

Trabajo experimental Nº8: **Respuesta metabólica de los cuerpos de Brockmann e hígado al descenso de los niveles circulantes de ácidos grasos en trucha arco iris; posible implicación del eje hipotálamo-hipófisis-interrenal (HPI)**

En los vertebrados, los niveles circulantes de ácidos grasos descienden durante la fase post-abosrtiva para ser restaurados después de las comidas. Se produce una respuesta contra-reguladora para restablecer los niveles de ácidos grasos que ha sido asociada con la activación del eje hipotálamo-hipófisis-adrenal (HPA). En peces, en los trabajos experimentales previos, demostramos la existencia a nivel central y periférico de sistemas sensores de ácidos grasos que responden ante el incremento en los niveles circulantes de ácidos grasos. En tejidos periféricos de trucha la arco iris, como los cuerpos de Brockmann (BB), probablemente esta repuesta se module por la interacción con otros sistemas endocrinos. En hígado, la capacidad sensora de ácidos grasos parece estar relacionada con la capacidad sensora previa de ácidos grasos en hipotálamo. En el trabajo experimental Nº7, observamos que tras la disminución de los niveles de ácidos grasos circulantes mediante el tratamiento con SDZ se observa un claro incremento de la ingesta y se desencadena una respuesta contra-reguladora en hipotálamo de trucha arco iris, en la que probablemente esté implicado el eje hipotálamo-hipófisis-interrenal (HPI). Sin

embargo, no sabemos cual es la posible participación del eje HPI en la respuesta metabólica en los tejidos periféricos de peces como el hígado o los BB:

Por ello, a 15 ejemplares por grupo se les administró intraperitonealmente 10 $mL.Kg^{-1}$ de solución salina sola (control, C), o conteniendo SDZ (SDZ; Tocris, 60 $\mu g.Kg^{-1}$), metirapona (M; Sigma, 1 $mg.Kg^{-1}$), o SDZ y metirapona (SDZ+M). Pasadas 6 horas de los tratamientos, se procedió a la recogida de sangre, BB e hígado para el posterior análisis de metabolitos, actividades enzimáticas y abundancia de ARNm.

En BB, el mecanismo sensor de ácidos grasos basado en el metabolismo de los ácidos grasos responde parcialmente al descenso de los niveles circulantes de ácidos grasos observándose un incremento en las actividades enzimáticas de FAS y ACLY y un descenso en los niveles de ARNm de ACC y ACLY. Sin embargo, los sistemas sensores de ácidos grasos basados en el transporte de ácidos grasos y la actividad mitocondrial, apenas se ven afectados por el tratamiento con SDZ.

La respuesta en hígado es claramente diferente a la observada en BB. Se produce un descenso en los niveles de ácidos grasos, triglicéridos y también en el potencial lipogénico (actividad de FAS y niveles de ARNm de G6PDH) en respuesta al descenso en los niveles circulantes de ácidos grasos. Estos resultados apoyan claramente la idea de una activación del potencial lipogénico en hígado para intentar restablecer los niveles de ácidos grasos. En general, los resultados apenas difieren de los obtenidos cuando se incrementan los niveles de ácidos grasos, sin embargo, son compatibles con situaciones fisiológicas de niveles de cortisol elevados durante condiciones de estrés.

La mayoría de los efectos que el tratamiento con SDZ produce en ciertos parámetros en hígado y BB, se contrarrestan por el tratamiento con metirapona, afectando a los niveles de cortisol en plasma, a la abundancia en los niveles de ARNm de los transcritos relativos a la síntesis de cortisol en riñón cefálico y niveles de ARNm de CRF y CRFBP en hipotálamo.

En conjunto, estos resultados sugieren que la capacidad de detectar el descenso de los niveles de ácidos grasos en hipotálamo provoca una respuesta contrareguladora en hígado que resultaría en una activación de la lipogénesis para restablecer los niveles de ácidos grasos en plasma, que a su vez podría se el resultado de la activación del eje HPI. A su vez, esta detección a nivel hipotalámico de los ácidos grasos, podría producir una aumento de flujo simpático hacia los BB con el resultante descenso de la producción y liberación de insulina del mismo modo que se ha descrito en mamíferos.

Trabajo experimental Nº9: **Efecto de la insulina en el sistema sensor de ácidos grasos en trucha arco iris**

En mamíferos las hormonas periféricas, como la leptina, ghrelina e insulina, modulan la repuesta de los diferentes mecanismos sensores de ácidos grasos ante el incremento de los niveles de éstos. En este sentido, se sabe que la insulina produce una modulación a nivel hipotalámico que conlleva un aumento en la expresión de POMC y CART y un descenso en la expresión de NPY y AgRP produciéndose finalmente una inhibición de la ingesta. En los trabajos experimentales anteriores, caracterizamos en trucha arco iris la presencia y funcionamiento en hipotálamo, hígado y cuerpos de Brockmann (BB) de sistemas sensores de ácidos grasos que están relacionados con el control de la ingesta (hipotálamo), secreción hormonal (BB) u homeostásis metabólica (hígado). Sin embargo, hasta la fecha no hay estudios en peces que muestren evidencias sobre la posible modulación endocrina de estos sistemas sensores de ácidos grasos. En trucha arco iris, se ha demostrado la expresión de receptores de insulina en diferentes tejidos, como hipotálamo e hígado y además su administración produce una modulación del sistema glucosensor en cerebro e inhibe la ingesta.

En el presente trabajo experimental, queremos evaluar si la respuesta de los sistemas sensores de ácidos grasos en hipotálamo, BB e hígado de trucha arco iris frente al incremento en los niveles circulantes de oleato y octanoato se modifica en presencia de insulina. Para ello, se llevaron a cabo 2 experimentos. En el primero, 15 animales por grupo se inyectaron IP con solución salina (control), o con insulina (2 mg insulina-bovina.kg^{-1} masa) , oleato (0,3 mg/kg), octanoato (0,3 mg/kg), insulina + oleato, o insulina + octanoato. Tras 6 horas de tratamiento, se tomaron muestras de sangre, hipotálamo, BB e hígado para el posterior análisis de metabolitos, actividades enzimáticas y expresión de genes relativos al sistema sensor de ácidos grasos. En el segundo experimento, 10 peces por grupo se inyectaron intraperitonealmente con los mismos tratamientos y las mismas concentraciones que en el primer experimento para evaluar la ingesta 6 horas y 24 horas después del tratamiento.

Los resultados observados indican que no solo la insulina tiene un papel anoréctico en trucha arco iris, sino que además potencia el efecto de los ácidos grasos, observándose un mayor descenso en la ingesta de alimento cuando el tratamiento es simultáneo con el oleato o con el octanoato y coincidiendo con los cambios observados en la expresión de CART y NPY. Sin embargo, los parámetros relativos al sistema sensor de ácidos grasos en hipotálamo no muestras apenas cambios con el tratamiento simultáneo de insulina y ácidos grasos.

En hígado, se observa una potenciación del efecto de los ácidos grasos en los animales tratados con el tratamiento simultáneo de insulina+oleato o insulina+octanoato.

Este co-tratamiento afecta a parámetros relativos al sistema sensor de ácidos grasos basado en el metabolismo de los ácidos grasos, el transporte de los ácidos grasos y la actividad mitocondrial. En BB, los resultados tras el tratamiento simultáneo de la insulina con los ácidos grasos, son similares a los obtenidos en hígado. La insulina potencia el efecto del oleato y del octanoato en los 3 mecanismos sensores de ácidos grasos.

Por tanto, estos resultados nos proporcionan evidencias, por primera vez en peces, del efecto de la insulina sobre los mecanismos sensores de ácidos grasos tanto a nivel central como a nivel periférico. El tratamiento con insulina potencia la inhibición de la ingesta inducido por el tratamiento con ácidos grasos. Que este hecho no se vea reflejado en los parámetros relativos al mecanismo sensor de ácidos grasos en hipotálamo se puede deber a la interacción con otros nutrientes como la glucosa o aminoácidos. El efecto de la insulina a nivel periférico potenciando la acción de los ácidos grasos podría ser debido a una previa activación a nivel hipotalámico.

Trabajo experimental Nº10: **Efectos en el sistema sensor de ácidos grasos, la regulación de la ingesta y las vías de señalización celular en hipotálamo e hígado de trucha arco iris alimentada con una dieta rica en lípidos**

En peces, tras la alimentación con dietas ricas en lípidos se produce un descenso en la ingesta de alimento. Además en trucha arco iris, existen evidencias de la presencia y funcionamiento de sensores de energía intracelular como AMPK y proteínas implicadas en la señalización celular como mTOR y Akt. En los trabajos experimentales anteriores hemos caracterizado en hipotálamo, hígado y cuerpos de Brockmann, la presencia y funcionamiento de sistemas sensores de ácidos grasos cuya activación produce un descenso en la ingesta de alimento. Sin embargo, hasta la fecha, no hay información en ningún tejido de peces sobre como responden estos sistemas sensores de ácidos grasos así como las proteínas de señalización celular y los sensores de energía intracelular a cambios en los niveles de nutrientes como los ácidos grasos en la dieta. Por lo tanto, el objetivo del trabajo experimental Nº10 fue determinar en trucha arco iris alimentada con una dieta pobre en lípidos (LF) y una dieta rica en lípidos (HF) si la respuesta de la ingesta de alimento, de la expresión de neuropéptidos hipotalámicos y de los sistemas sensores de ácidos grasos en hipotálamo e hígado es similar a la observada en los trabajos experimentales anteriores cuando incrementábamos los niveles de ácidos grasos mediante inyección, así como determinar si el estado de fosforilación de AMPK, Akt y mTOR muestra cambios en hipotálamo e hígado en respuesta a los diferentes niveles de lípidos de la dieta.

Para ello, se alimentó con una dieta LF y una dieta HF (cinco réplicas por grupo) hasta saciedad durante 4 semanas dos veces al día y se evaluó la ingesta de alimento diariamente. Finalizado este perido, 1 ,3 y 6 horas después de la última toma de alimento se tomaron muestras de sangre, hipotálamo e hígado para el posterior análisis de metabolitos, y expresión de genes relativos al sistema sensor de ácidos grasos tras 6 horas y análisis del estado de fosforilación de AMPK, Akt y mTOR en los tres tiempos.

En cuanto a los resultados, la ingesta de alimento no se vió afectada por las diferentes dietas, quizás debido a que 4 semanas no es tiempo suficiente para ver su efecto o también pudioera deberse a la diferencia en la composición lipídica usada para preparar la dieta HF en este estudio. El balance global de los resultados obtenidos en la expresión de los neuropéptidos involucrados en la regulación de la ingesta de alimento, es un incremento en el potencial anorexigénico de los peces alimentados con la dieta HF. En el hipotálamo, el mecanismo sensor basado en el metabolismo de los FA y de la actividad mitocondrial no parece verse afectado por la dieta. Sin embargo, el mecanismo basado en el transporte de FA mediante FAT/CD36 y la posterior modulación de los factores de transcripción si parece activarse por la dieta HF. En hígado, los parámetros relativos al sistema sensor de FA muestran aparentemente una respuesta parcial tras la alimentación con la dieta HF. Pasadas 6 horas de la última toma de alimento, en los peces alimentados con la dieta HF se observa una aparente activación de AMPK. También se observa un incremento de la fosforilación de mTOR en hipotálamo tras pasar 3 horas de la ultima toma de alimento con la dieta HF, lo que coincide con el descenso de la fosforilación de AMPK en el mismo tiempo. El estado de fosforilación de mTOR y Akt tiende a ser más alto en los peces alimentados con la dieta HF que con la dieta LF tanto en hipotálamo como en hígado y el incremento es significativo pasadas 6 horas de la última toma de alimento en el caso de mTOR y Akt en hígado y Akt en hipotálamo y pasadas 3 horas en el caso de mTOR en hipotálamo.

Aunque en su conjunto parece que la mayor disponibilidad de nutrientes, en este caso lípidos, induce una activación general de las vías de señalización celulares relacionadas con mTOR y Akt, los cambios observados en los parámetros relacionados con el mecanismo sensor de ácidos grasos no reflejan directamente la influencia de las vías de señalización evaluadas, lo que sugiere que podría existir una interacción más compleja.

THESIS SUMMARY

In mammals, detection of substrates such as glucose and fatty acids allows the brain to modulate energy intake and expenditure and peripheral metabolic functions as a means of controlling overall energy homeostasis. Evidence in mammals demonstrate that specialized neurons within the hypothalamus (and possibly brainstem) are able to detect changes in plasma levels of long-chain fatty acid, but not short- or medium-chain FA, thus contributing to nervous control of energy homeostasis. The most accepted mechanism through which long-chain fatty acids are sensed in brain is of metabolic nature. Thus, increased fatty acid levels in plasma induced enhanced malonyl-CoA levels and subsequent inhibition of carnitine palmitoyltransferase 1 to import fatty acid-CoA into the mitochondria for oxidation. However, fatty acid metabolism only accounts for part of fatty acid sensing in hypothalamus, since alternative mechanisms have been suggested to be present, such as 1) binding to fatty acid translocase (FAT/CD36), and further modulation of transcription factors 2) fatty acid-induced activation of novel protein kinase C (PKC) isoforms, 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. Changes in the activity of those systems in mammalian hypothalamus have been associated, through not completely understood mechanisms, with the regulation of food intake through changes in the expression of orexigenic and anorexigenic factors.

Fish use lipids as a major energy source supporting numerous physiological, developmental, and reproductive events. Furthermore, a reduced food intake has been observed in several fish species fed with high fat diets or containing high fat stores suggesting that lipid sensor mechanisms regulating food intake may be present in fish. However, there are no studies in which the effect of particular fatty acids on food intake were evaluated.

In this context, the major objectives of the present Doctoral Thesis are to obtain direct evidence for the existence of fatty acid sensing systems in hypothalamus, Brockmann Bodies and liver of the rainbow trout *Oncorhynchus mykiss*, as well as to study in depth their mechanisms and physiological implications. To achieve this general objective, several specific aims were established:

1) To evaluate the presence in brain and Brockmann bodies of putative components of fatty acid sensing mechanisms and the response either at metabolic level or in the regulation in food intake to increased levels of circulating medium-chain fatty acids like octanoate as long-chain fatty acids like oleate *(Experimental approach Nº1).*
2) To evaluate the response of hepatic lipid and glucose metabolism to an acute treatment of a mixture fish oil or single fatty acid administration *(Experimental approach Nº2).*

3) To evaluate *in vitro* (in the absence of external influences) whether or not hypothalamus and BB respond to changes in fatty acid concentration in a way similar to that previously observed *in vivo (Experimental approach Nº3).*

4) To evaluate *in vitro* (in the absence of extrahepatic regulatory mechanisms) whether liver responds to changes in fatty acid concentration in a way similar to that previously observed *in vivo (Experimental approach Nº4).*

5) To evaluate the effects of intracerebroventricular treatment with oleate or octanoate on food intake, and in parameters related to putative fatty acid sensing systems in hypothalamus. If fatty acid are directly sensed in hypothalamus, central administration should elicit effects similar to those of intraperitoneal treatment *(Experimental approach Nº5).*

6) To evaluate the effects of intracerebroventricular treatment with oleate or octanoate in parameters related to putative fatty acid sensing systems in Brockmann bodies and liver. If fatty acid sensing in those tissues is related to previous fatty acid sensing in hypothalamus then changes observed in parameters after intracerebroventricular treatment should be similar to those previously observed after intraperitoneal treatment. In contrast, if changes after intracerebroventricular treatment are not similar to those observed after intraperitoneal treatment then a hypothetical interaction with other endocrine systems would be more likely *(Experimental approach Nº6).*

7) To assess if a decrease in levels of circulating fatty acids result in a inhibition of fatty acid-sensing systems in hypothalamus, which could be associated with increased food intake. If the response observed is due to the fall in fatty acid levels, it should be counteracted in the presence of intralipid (a lipid emulsion). Furthermore, we also assessed the possible involvement of the hypothalamus-pituitary-interrenal (HPI) axis in the response *(Experimental approach Nº7).*

8) To evaluate the peripheral (liver and BB) counter-regulatory response to a pharmacologically-induced reduction in fatty acid circulating levels in rainbow trout *(Experimental approach Nº8).*

9) To evaluate if food intake and the response of fatty acid sensing systems in hypothalamus, liver and Brockmann bodies to raised levels of oleate or octanoate are modified in the presence of insulin *(Experimental approach Nº9).*

10) To determine if 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 acids were raised by injection and 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 changes in dietary lipid levels that could be related to changes observed in parameters related to fatty acid-sensing and the control of food intake *(Experimental approach Nº10).*

Experimental approach Nº1: "**A metabolic fatty acid sensing mechanism is present in hypothalamus and Brockmann bodies of rainbow trout".**

Enhanced lipid levels inhibit food intake in fish but no studies have characterized the possible mechanisms involved. We hypothesize that the presence of fatty acid sensing mechanisms could be related to the control of food intake. Accordingly, we evaluated in hypothalamus, hindbrain and Brockmann bodies of rainbow trout changes in parameters related to fatty acid metabolism, transport of FA, nuclear receptors and transcription factors involved in lipid metabolism, and components of the K_{ATP} channel after IP administration of different doses of oleate (long-chain fatty acid, LCFA) or octanoate (medium-chain fatty acid, MCFA). The increase in circulating LCFA or MCFA levels elicited an inhibition in food intake and induced in hypothalamus a response compatible with fatty acid sensing in which fatty acid metabolism, binding to FAT/CD36 and mitochondrial activity are apparently involved, which is similar to that suggested in mammals except for the apparent capacity of rainbow trout to detect changes in MCFA levels. Changes in those hypothalamic pathways can be related to the control of food intake since food intake was inhibited when FA metabolism was perturbed (using FAS or ACC inhibitors) and changes in mRNA levels of specific neuropeptides such as NPY and POMC were also noticed. This response seems exclusive of the hypothalamus, since the other centre controlling food intake (hindbrain) was unaffected by treatments. The results obtained in BB suggest that at least two of the components of a putative fatty acid sensing system (based on fatty acid metabolism and binding to FAT/CD36) could be present. Therefore, the present study provides, for the first time in fish, evidence for a specific role for FA (MCFA and LCFA) as metabolic signals in hypothalamus and Brockmann bodies where the detection of those FA can be associated with the control of food intake and hormone release.

***Experimental approach Nº2:* "Effects of a mixture or single fatty acids treatment on the response of hepatic lipid and glucose metabolism. Possible presence of fatty acid-sensing systems".**

To assess the hypothesis that an acute dietary FA supply may improve glucose tolerance in rainbow trout, we orally administered fish with fish oil (FO; 10 $ml.kg^{-1}$, one time), which were then subjected to a glucose tolerance test and sampled 6h after injection. Parameters related to glucose and lipid metabolism were then assessed. The results suggest that when both nutrients were administered at the same time, an increased potential for lipogenesis occurred concomitantly with a lower level of glycemia. In a second experiment we administered intraperitoneally a single FA present in the FO mixture such as oleate (60 or 300 $\mu g.kg^{-1}$) whereas octanoate (60 or 300 $\mu g.kg^{-1}$) was used as negative control (absent from the FO). However, the effects of both FA were similar in reducing the potential of lipid synthesis and oxidation, and in enhancing the potential of glucose synthesis and glycogenesis. Differences found between FO and single FA administration show that response to FA was dependent on the treatment (mixture vs. single FA) but also comply with the idea that an interaction between FA and glucose rather than FA alone are in the origin of the results reported. The administration of individual FA such as oleate and octanoate failed in enhancing lipogenesis and reducing plasma glucose levels and thus in explaining results obtained with FO. However, results provide evidence that FA even provided at a low dose play a key role in the regulation of several putative components of a FA sensing system present in rainbow trout liver.

***Experimental approach Nº3: "In vitro* evidences of direct fatty acid-sensing capacity in hypothalamus and Brockmann bodies of rainbow trout".**

In a previous study we provided evidence for the presence in hypothalamus and BB of rainbow trout of fatty acid-sensing systems responding to changes in levels of oleate (LCFA) or octanoate (MCFA). Since those effects could be attributed to an indirect effect, in the present study we evaluated *in vitro* if hypothalamus and BB respond to changes in FA in a way similar to that observed *in vivo*. In a first set of experiments, we evaluated in hypothalamus and BB exposed to increased oleate or octanoate concentrations changes in parameters related to FA metabolism, FA transport, nuclear receptors and transcription factors, reactive oxygen species (ROS) effectors, components of the K_{ATP} channel, and (in hypothalamus) neuropeptides related to food intake. In a second set of experiments, we evaluated in hypothalamus the response of those parameters to oleate or octanoate in the presence of inhibitors of fatty acid sensing components. The responses observed *in vitro* in hypothalamus are comparable to those previously observed *in vivo* and specific inhibitors counteracted in many cases the effects of FA. These results support the capacity of rainbow trout hypothalamus to directly sense

changes in MCFA or LCFA levels. In BB increased concentrations of oleate or octanoate induced changes that in general were comparable to those observed in hypothalamus supporting direct FA sensing in this tissue. However, those changes were not coincident with those observed *in vivo* allowing us to suggest that the FA sensing capacity of BB previously characterized *in vivo* is influenced by other neuroendocrine systems.

***Experimental approach Nº4: "*Response of hepatic fatty acid-sensing systems in rainbow trout to *in vitro* oleate or octanoate treatment".**

In a previous study we provided evidence for the presence in liver of rainbow trout of fatty acid (FA) sensing systems responding to changes in levels of oleate (LCFA) or octanoate (MCFA). Since those effects could be attributed to an indirect effect, we have evaluated in the present study *in vitro* (in the absence of extrahepatic regulatory mechanisms) whether or not liver responds to changes in FA concentration in a way similar to that previously observed *in vivo*. Accordingly, liver slices were exposed to increased oleate or octanoate concentrations to evaluate changes in parameters related to FA metabolism, FA transport, nuclear receptors and transcription factors, ROS effectors, and glucose metabolism. The responses observed *in vitro* in liver were in general not coincident with those previously observed *in vivo* allowing us to suggest that FA sensing capacity of liver *in vivo* is of indirect nature and could be related among other reasons to an interaction with other endocrine systems and/or to FA sensing in hypothalamus.

***Experimental approach Nº5: "*Effects of oleate or octanoate intracerebroventricular treatment on food intake and hypothalamic fatty acid systems in rainbow trout".**

If levels of fatty acids like oleate and octanoate are directly sensed through different FA sensing systems in hypothalamus of rainbow trout, intracerebroventricular (ICV) administration of FA should elicit effects similar to those previously observed after intraperitoneal (IP) treatment. Accordingly, we observed after ICV treatment with oleate or octanoate decreased food intake accompanied in hypothalamus by reduced potential of lipogenesis and FA oxidation, and decreased potential of ATP-dependent inward rectifier potassium channel (K^+_{ATP}). Those changes support direct FA sensing through mechanisms related to FA metabolism and mitochondrial activity. The FA sensing through binding to FAT /CD36 and subsequent expression of transcription factors appears to be also direct but an interaction with peripheral hormones cannot be rejected. Moreover, decreased expression of NPY and increased expression of POMC were observed in parallel with the activation of FA sensing systems and decreased food intake. These results allow

us to suggest the involvement of at least these peptides in controlling the decreased food intake noted after oleate and octanoate treatment in rainbow trout.

Experimental approach Nº6: **"Fatty acid sensing systems are affected by central oleate or octanoate administration in liver and Brockmann bodies of rainbow trout".**

Intracerebroventricular treatment with oleate or octanoate induced in BB and liver of rainbow trout changes in several parameters related to FA sensing through FA metabolism, FA transport through FA translocase (FAT/CD36) and mitochondrial activity. Changes observed in BB were completely different to those observed in previous studies after treatment with the same FA either aftes *in vivo* or *in vitro* treatments. Therefore, FA sensing in BB is apparently direct and not related to previous FA sensing in hypothalamus, but it could be influenced by changes in the levels of peripheral hormones like insulin. In contrast, results obtained after ICV treatment with FA in liver were comparable to those observed after IP treatment but different to those observed after *in vitro* treatment. Therefore, FA sensing in liver is apparently indirect and be the consequence of previous hypothalamic FA sensing followed by vagal and/or sympathetic outflow.

Experimental approach Nº7: **"Hypothalamic response to decreased levels of fatty acid in rainbow trout. Possible involvement of the hypothalamus-pituitary-interrenal axis".**

We hypothesize that a decrease in circulating levels of FA in rainbow trout would result in the inhibition of putative hypothalamic FA sensing systems with concomitant changes in the expression of orexigenic and anorexigenic factors ultimately leading to a stimulation of food intake. To assess this hypothesis, we lowered circulating FA levels treating fish with SDZ WAG 994 (SDZ), a selective A1 adenosine receptor agonist that inhibits lipolysis. In additional groups, we also evaluated if the presence of intralipid was able to counteract changes induced by SDZ treatment, and the possible involvement of the hypothalamus-pituitary-interrenal (HPI) axis by treating fish with SDZ in the presence of metyrapone, which decreases cortisol synthesis in fish. The decrease in circulating levels of FA in rainbow trout induced a clear increase in food intake that was associated with the decrease of the anorexigenic potential in hypothalamus (decreased POMC-A1 and CART mRNA abundance), and with changes in several parameters related to putative FA-sensing mechanisms in hypothalamus. Intralipid treatment counteracted these changes. SDZ treatment also induced increased cortisol levels and the activation of different components of the HPI axis whereas these changes disappeared in the presence of intralipid or metyrapone. These results suggest that the HPI axis is involved in a counter-regulatory response in rainbow trout to restore FA levels in plasma.

Experimental approach Nº8: **"Brockmann bodies and liver metabolic response to decreased circulating fatty acid levels in rainbow trout; involvement of the hypothalamus-pituitary-interrenal (HPI) axis".**

We previously demonstrated in rainbow trout that the decrease in circulating levels of fatty acid FA induced by treating fish with SDZ induced a counter-regulatory response in which the activation of the HPI axis was likely involved. This activation, probably not related to the control of food intake through FA sensor systems but to the modulation of lipolysis in peripheral tissues, liver and BB, would target the restoration of FA levels in plasma. To assess this hypothesis, we lowered circulating FA levels by treating fish with SDZ alone, or SDZ in the presence of metyrapone (an inhibitor of cortisol synthesis). In liver, the changes observed were not compatible with a direct FA-sensing response but with a stress response, which allows us to suggest that the detection of a FA decrease in the hypothalamus elicits a counter-regulatory response in liver, resulting in an activation of lipolysis to restore FA levels in plasma. The activation of these metabolic changes in liver could be attributable to the activation of the HPI axis and/or to the action of sympathetic pathways. In contrast, in BB, changes in circulating FA levels induce changes in several parameters compatible with the function of FA sensing systems informing about the decrease in circulating FA levels.

Experimental approach Nº9: **"Insulin modulation of fatty acid sensing in rainbow trout".**

We aimed to evaluate if the response of FA-sensing systems in hypothalamus, liver and BB of rainbow trout to raised levels of oleate or octanoate is modified in the presence of insulin. Then, 15 fish per group received intraperitoneally 10 $mL.Kg^{-1}$ injection of saline solution alone (control), or containing insulin (2 mg bovine $insulin.kg^{-1}$ body mass, insulin), oleate (300 µg/kg), octanoate (300 µg/kg), insulin + oleate, or insulin + octanoate. Our results suggest that the modulatory role of insulin on the responses of hypothalamic FA sensing systems to changes in circulating levels of oleate or octanoate was of minor importance in contrast to the mammalian model. However, this is in contrast with the effects observed by insulin treatment on food intake, clearly suggesting a potential effect of FA (especially octanoate) on the anorectic effects of FA, allowing us to suggest the existence of an interaction between insulin treatment and other nutrient sensors, such as those involved in glucose and amino acid sensing. In contrast, the results obtained in liver and BB clearly support the modulatory action of insulin on the FA–sensing capacity of peripheral tissues like liver and BB where the responses already observed in parameters related to FA sensing were further potentiated by insulin. These results suggest that the changes observed in the activity of neurons involved in the production of anorexigenic and orexigenic factors ultimately leading to a further decrease in food intake are

translated (presumably through sympathetic or vagal outflow) into the liver and BB to further potentiate the effects of FA alone.

Experimental approach Nº10: **"Effects on fatty acid sensing, food intake regulation and cellular signaling pathways in hypothalamus and liver of rainbow trout fed with a lipid-enriched diet".**

In previous studies carried out in the rainbow trout, we have characterized in hypothalamus and liver the presence and functioning of FA-sensing systems whose activation ultimately lead to decreased food intake. Since a reduced food intake has been observed after feeding fish like 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. There is evidence in rainbow trout for the presence and functioning of AMPK and proteins involved in cellular signaling like mTOR and Akt but to date there is no information in any fish tissue regarding the response of these sensors and proteins to changes in the levels of nutrients like fatty acids. The aim of this study in rainbow trout fed with low fat (LF) or high-fat (HF) diets was to determine if the response of food intake, mRNA abundance of hypothalamic neuropeptides, 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 and to determine if the phosphorylation state of AMPK, Akt and mTOR display changes in hypothalamus and liver in response to changes in dietary lipid levels. 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 allowing us to suggest that FA-sensing response in fish to increased levels of FA is more dependent on the presence of specific FA rather than to the global increase in FA. In hypothalamus and liver, AMPK, Akt and mTOR are generally activated in fish fed the HF diet, suggesting an enhanced response of the cellular signaling pathways to the increased availability of FA.

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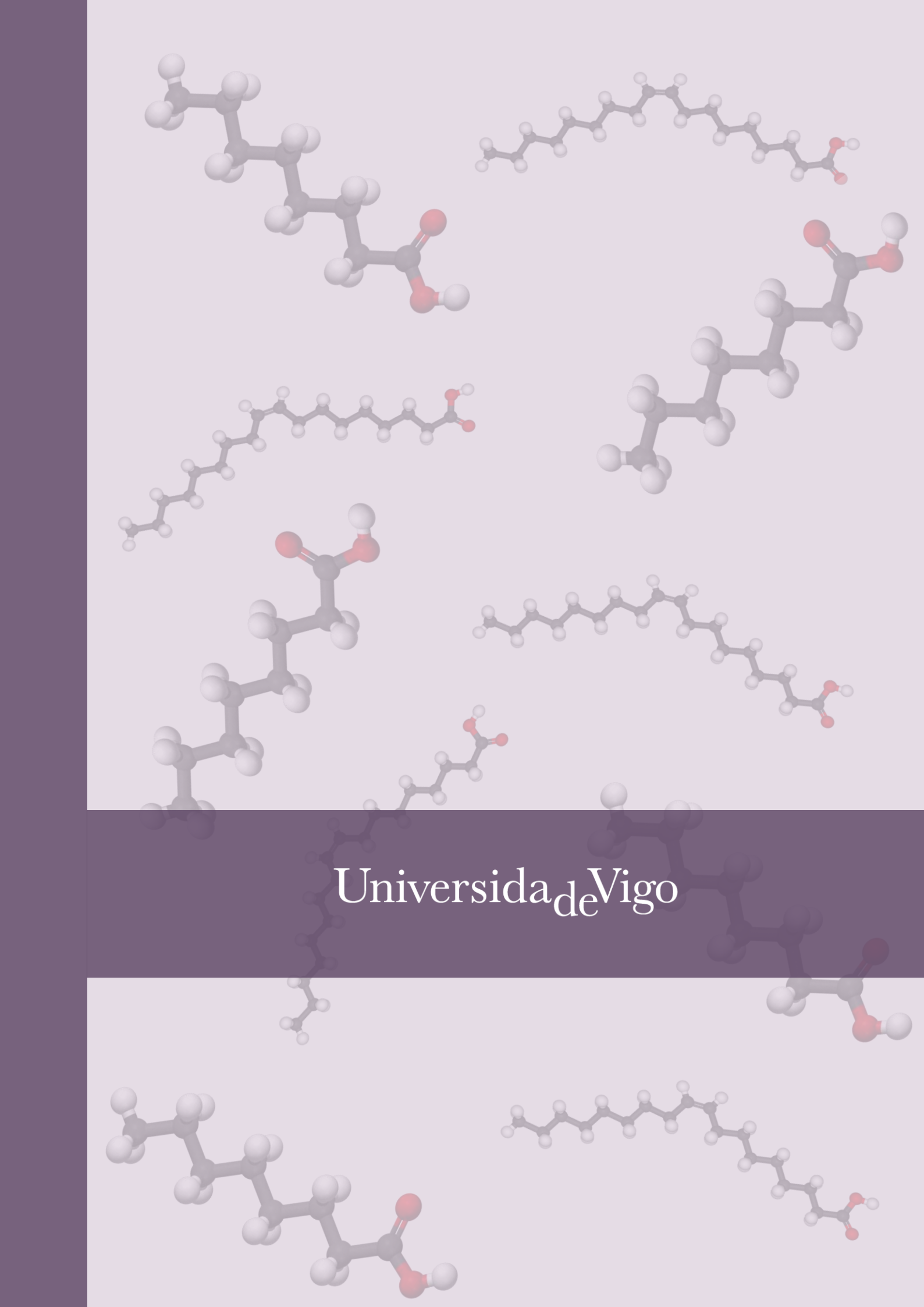

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