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TESIS DOCTORAL

Implicación de los péptidos leptina, colecistocinina y ghrelina, y del derivado lipídico oleiletanolamida en la regulación del comportamiento alimentario en los teleósteos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

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IMPLICACIÓN DE LOS PÉPTIDOS LEPTINA, COLECISTOCININA Y GHRELINA, Y DEL DERIVADO LIPÍDICO OLEILETANOLAMIDA EN LA REGULACIÓN DEL COMPORTAMIENTO ALIMENTARIO EN LOS TELEÓSTEOS

MEMORIA PRESENTADA POR ANA BELÉN TINOCO PÉREZ PARA OBTENER EL TÍTULO DE DOCTOR EN BIOLOGÍA POR LA UNIVERSIDAD COMPLUTENSE DE MADRID

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INVOLVEMENT OF LEPTIN, CHOLECYSTOKININ, GHRELIN, AND THE LIPID-DERIVED OLEOYLETHANOLAMIDE IN THE REGULATION OF FEEDING BEHAVIOR IN TELEOSTS

THESIS DEFENDED BY ANA BELÉN TINOCO PÉREZ TO OBTAIN THE DOCTORATE DEGREE IN BIOLOGY BY COMPLUTENSE UNIVERSITY OF MADRID

MADRID, 2015

María Jesús Delgado Saavedra, Catedrática de la Universidad Complutense de Madrid, y Nuria de Pedro Ormeño, Profesora Titular de la Universidad Complutense de Madrid,

CERTIFICAN:

Que la presente Tesis Doctoral titulada **"Implicación de los péptidos leptina, colecistocinina y ghrelina, y del derivado lipídico oleiletanolamida en la regulación del comportamiento alimentario en los teleósteos"** presentada por Ana Belén Tinoco Pérez, licenciada en Ciencias del Mar, para optar al grado de Doctor por la Universidad Complutense de Madrid, ha sido realizada bajo su dirección y reúne todos los requisitos necesarios para proceder a su presentación y defensa pública.

Y para que conste a los efectos oportunos, firman el presente en Madrid a 3 de Febrero de 2015.

Dra. María Jesús Delgado Saavedra Dra. Nuria de Pedro Ormeño

La presente Tesis Doctoral ha sido realizada en el Departamento de Fisiología (Fisiología Animal II) de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid, bajo la codirección de la Dra. María Jesús Delgado Saavedra y de la Dra. Nuria de Pedro Ormeño.

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"Nosotros, los de entonces,

ya no somos los mismos"

Pablo Neruda

(Veinte poemas de amor y una canción desesperada, 1924)

A la Pepa que se fue,

a la Pepa que vino,

y a Miguel.

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ABREVIATURAS

5-HT	Serotonina (5-hidroxitriptamina)
AEA	Anandamida
CART	Transcrito inducido por la cocaína y anfetamina
CCK	Colecistocinina
CCK-8S	Octapéptido sulfatado de la colecistocinina
CT	*Circadian time*, tiempo circadiano
L/D	Luz/oscuridad
CRH	Hormona liberadora de corticotropina
DA	Dopamina
FAA	*Food anticipatory activity*, actividad anticipatoria al alimento
FAAH	Amidohidrolasa de ácidos grasos
FAEs	Aciletanolamidas, amidas de ácidos grasos
GH	Hormona de crecimiento
GPR119	Receptor huérfano acoplado a proteínas G 119
IP	Intraperitoneal
ICV	Intracerebroventricular
MCH	Hormona concentradora de melanina
NA	Noradrenalina
NAPEs	N-acilfostatidiletanolaminas
NAPE-PLD	Fosfolipasa-D dependiente de NAPE
NPY	Neuropéptido Y
OEA	Oleiletanolamida
PACAP	Polipéptido activador de la adenilato ciclasa
POMC	Proopiomelanocortina
PPARs	Receptores activados por proliferadores de peroxisomas
RnF	Grupo de peces alimentados diariamente a un horario aleatorio y mantenidos en luz constante
SF10	Grupo de peces alimentados diariamente a las 10:00 h y mantenidos en luz constante
SF22	Grupo de peces alimentados diariamente a las 22:00 h y mantenidos en luz constante
SNC	Sistema nervioso central
TRPV1	Receptor de potencial transitorio activado por vanilloides-1
VIP	Péptido intestinal vasoactivo
ZT	*Zeitgeber time*, tiempo del sincronizador

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I. RESUMEN GENERAL/GENERAL SUMMARY

Implicación de los péptidos leptina, colecistocinina y ghrelina, y del derivado lipídico oleiletanolamida en la regulación del comportamiento alimentario en los teleósteos

INTRODUCCIÓN

La ingesta en los peces, al igual que en el resto de los vertebrados, es un proceso complejo controlado por un sistema central, focalizado en el encéfalo, que funciona coordinadamente con un sistema periférico, formado por una amplia variedad de órganos, entre ellos el tracto gastrointestinal. Ambos sistemas se encuentran regulados por señales endógenas (metabólicas y neuroendocrinas) y externas o ambientales (fotoperiodo, temperatura, disponibilidad de alimento, etc.). Las señales endógenas se originan tanto en el sistema nervioso central como en los órganos periféricos, y pueden actuar tanto estimulando la ingesta, como factores orexigénicos, o inhibiéndola, como factores anorexigénicos (Volkoff, 2009a). La presente Tesis Doctoral se centra en el estudio de las señales endógenas que se sintetizan principalmente en la periferia, como el hígado (leptina) y el tracto gastrointestinal (colecistocinina, oleiletanolamida y ghrelina).

El péptido **leptina** está implicado en la regulación de la ingesta y el balance energético en los vertebrados (Copeland et al., 2011; Friedman, 2014). Su acción anorexigénica se ha observado en la mayoría de los peces estudiados (Copeland et al., 2011), si bien, su implicación en la señalización del estado nutricional depende de la especie estudiada, así como del régimen alimenticio empleado (Londraville et al., 2014). En los mamíferos, tanto los niveles circulantes como la expresión génica de leptina muestran un perfil rítmico diario con variaciones en función del horario de alimentación (Kalra et al., 2003; Schoeller et al., 1997; Xu et al., 1999). En los ciprínidos y los salmónidos se han identificado dos ortólogos de leptina (lep-a1 y lep-a2) que presentan incrementos posprandiales (Huising et al., 2006; Moen y Finn, 2013); sin embargo, hasta el momento el perfil diario de expresión de leptina sólo se ha estudiado en el salmón del Atlántico (*Salmo salar*) (Moen y Finn, 2013).

La **colecistocinina (CCK)** es una hormona clave en la regulación de la ingesta y de la fisiología digestiva de los vertebrados (Yu y Smagge, 2014). En los peces, al igual que en los mamíferos, la CCK también parece estar involucrada en la contracción de la vesícula biliar, la secreción de enzimas pancreáticas (Einarsson et al., 1997; Murashita et al., 2008a; Volkoff, 2006) y en la motilidad intestinal (Forgan y Forster, 2007; Olsson et al., 1999). Esta hormona actúa mediante su unión a dos subtipos de receptores específicos, CCKAR y CCKBR, que difieren en su distribución y su afinidad por los péptidos de la familia de la CCK/gastrina (Staljanssens et al., 2011; Yu y Smagge, 2014). Estos receptores de la CCK

han sido ampliamente estudiados en los mamíferos, sin embargo, hasta la fecha, la información existente acerca de los receptores de la CCK en los peces es muy limitada e incompleta.

La **ghrelina** es actualmente la única hormona con acción orexigénica de origen periférico en los vertebrados (Jönsson, 2013; Kaiya et al., 2013b). Su acción orexigénica en los teleósteos se ha demostrado en el carpín (Kang et al., 2011a) y la tilapia (*Oreochromis mossambicus*) (Riley et al., 2005); sin embargo, en la trucha arcoíris (*Oncorhynchus mykiss*) se han publicado tanto efectos orexigénicos, como anorexigénicos, e incluso ausencia de efecto sobre la ingesta (Jönsson, 2013). Además, la ghrelina en los vertebrados regula la secreción hipofisaria de la hormona de crecimiento (GH), el metabolismo lipídico y la actividad locomotora; aunque estas acciones parecen ser dependientes de la especie en estudio (Jönsson, 2013; Kaiya et al., 2013b; Keen-Rhinehart y Bartness, 2005).

La **oleiletanolamida (OEA)** es una amida de ácido graso con múltiples funciones en los mamíferos, entre otras, interviene en la regulación de la ingesta y el peso corporal, del metabolismo lipídico y de la actividad locomotora (Piomelli, 2013; Proulx et al., 2005). Su función anorexigénica se ha demostrado en base a la reducción de la ingesta que produce el tratamiento periférico con OEA en los roedores, así como a la movilización intestinal de la OEA que se observa en respuesta a la alimentación en los roedores y en la serpiente pitón (*Python molurus*) (Piomelli, 2013). En los peces, la anandamida (Valenti et al., 2005), y el precursor de la OEA, el ácido oleico (Librán-Pérez et al., 2012, 2014) intervienen en la regulación de la ingesta, pero en la actualidad se desconocen las posibles funciones de la OEA en estos vertebrados.

El objetivo general de la presente Tesis Doctoral es profundizar en el estudio de la regulación de la ingesta en peces, utilizando el carpín (*Carassius auratus*) y la trucha común (*Salmo trutta*) como modelos experimentales.

PRINCIPALES RESULTADOS

Capítulo 1. El sistema de la leptina en el carpín (*Carassius auratus*): distribución tisular, regulación por la alimentación, ritmicidad diaria y su sincronización al horario de alimentación

Artículo 1. *Leptin and leptin receptor expression in the goldfish (Carassius auratus). Regulation by food intake and fasting/overfeeding conditions.*
***Peptides* 2012, 34: 329-335**

Artículo 2. *Leptin expression is rhythmic in brain and liver of goldfish (Carassius auratus). Role of feeding time.*

***Gen Comp Endocrinol* 2014, 204: 239-247**

El primer objetivo estudia el patrón de expresión de las dos leptinas (*gLep-aI* y *gLep-aII*) y de su receptor (*gLepR*) en el encéfalo y en los tejidos periféricos del carpín. A continuación, con objeto de determinar si la leptina actúa como regulador de la ingesta a corto y/o largo plazo, se estudian los cambios posprandiales, el efecto del ayuno (1 semana) y de la sobrealimentación (2 semanas) en la expresión del sistema de la leptina en el encéfalo y el hígado. Finalmente, se investigan las posibles variaciones durante un ciclo completo de 24 horas en la expresión del sistema de la leptina, y el papel del horario de alimentación en la sincronización de los ritmos observados.

Los resultados muestran una amplia distribución de la *gLep-aI* tanto en el encéfalo como en los tejidos periféricos, mientras que la expresión de *gLep-aII* se localiza principalmente en el encéfalo; sugiriendo que ambos parálogos de la leptina podrían desempeñar diferentes funciones en el carpín. La amplia distribución de ambas leptinas, junto con la extensa expresión del receptor, sugiere que la leptina en el carpín puede desempeñar acciones pleiotrópicas, como se ha propuesto en otros vertebrados (Londraville et al., 2014). No se observan diferencias en la expresión del sistema de la leptina entre los carpines controles, los sobrealimentados y los mantenidos en ayuno, por lo que la leptina no parece actuar como señal adipostática en este teleósteo. Sin embargo, la expresión de *gLep-aI* en el hígado presenta un incremento significativo a las 9 horas tras la ingestión de alimento, como sucede en otros teleósteos (Huising et al., 2006; Moen y Finn, 2013; Zhang et al., 2013), y en concordancia con su papel como señal de saciedad posprandial en el carpín (De Pedro et al., 2006; Volkoff et al., 2003). Como era de esperar, la expresion hepática de *gLep-aI* en condiciones fotoperiódicas 12L/12D y alimentacion diaria a un horario fijo, muestra un ritmo diario acorde con la respuesta posprandial. Sin embargo, en el encéfalo el ritmo de expresión de *gLep-aII* no parece estar relacionado con la ingesta sugiriendo que en la regulación de la expresión de leptina intervienen mecanismos diferenciales en el encéfalo y en los órganos periféricos. Finalmente, en ausencia de la señal ciclo luz/oscuridad, el horario de alimentación por sí solo no es capaz de sincronizar esta expresión rítmica.

Capítulo 2. Colecistocinina y motilidad intestinal en el carpín (*Carassius auratus*). Mecanismos de acción y receptores implicados

Artículo 3. *The contractile effect of cholecystokinin (CCK-8S) on goldfish proximal intestine is mediated by cholecystokinin type A receptor.*
***Comp Biochem Physiol A* 2015, (En revisión)**

El objetivo de este estudio es investigar el posible papel de la CCK en la regulación de la motilidad intestinal en el carpín utilizando un sistema de cultivo *in vitro* en baño de órganos. En este modelo se emplearon aproximaciones farmacológicas con el fin de identificar posibles rutas de señalización y caracterizar los receptores implicados.

La adición al baño de órganos de CCK-8S (1 nM-1 μM) induce una respuesta contráctil en las preparaciones de intestino proximal, dependiente de la concentración de CCK-8S en el baño (EC_{50} 53,8), sugiriendo que la CCK puede intervenir en la regulación de la motilidad intestinal en el carpín, como sucede en los mamíferos (Wu et al., 2013). La contracción inducida por CCK no se modifica en presencia de atropina (100 μM) o tetrodotoxina (1 μM), resultados que revelan que este efecto contráctil es independiente del control colinérgico y del sistema entérico, por lo que parece tener lugar directamente en células musculares del intestino del carpín. La acción contráctil de la CCK-8S se reduce de forma dependiente de la concentración, en ausencia de calcio extracelular, poniendo de manifiesto la importancia de este catión en dicho efecto contráctil. La preincubación de segmentos intestinales con L365-260 (antagonista específico del CCKBR; 1 μM) no bloquea la acción contráctil de la CCK-8S. Sin embargo, cuando preincubamos con el antagonista selectivo del receptor CCKAR, devazepide (1 μM) esta acción contráctil se reduce un 30%, indicando que la contracción inducida por CCK-8S está mediada, al menos en parte, por su unión al receptor CCKAR. Además, se han obtenido las secuencias parciales de los dos receptores de la CCK en el carpín, CCKAR y CCKBR. Estos receptores de la CCK en el carpín presentan una distribución diferencial, encontrando al subtipo CCKBR en el encéfalo principalmente, mientras que el subtipo CCKAR se localiza principalmente en órganos digestivos (intestino, vesícula biliar e hígado), reforzando la propuesta de la implicación de este subtipo de receptor en las funciones gastrointestinales de la CCK.

Capítulo 3. La ghrelina en la regulación del comportamiento alimentario, el crecimiento y el metabolismo lipídico en la trucha común (*Salmo trutta*)

Artículo 4. *Ghrelin increases food intake, swimming activity and growth in juvenile Brown trout (Salmo trutta).*
***Physiol Behav* 2014, 124: 15-22**

La ghrelina promueve la ingesta, la ganancia de peso corporal y la adiposidad en la mayoría de los peces estudiados, sin embargo sus efectos en la trucha arcoíris parecen variar en función de la dosis, la vía de administración y el origen de la ghrelina exógena empleada (Jönsson, 2013). Con el fin de investigar las acciones de la ghrelina en otro salmónido, en primer lugar se determinan los efectos del tratamiento subcrónico de ghrelina (475 ng de ghrelina de trucha/g de peso corporal-PC) en la ingesta, el peso corporal, la actividad locomotora y el comportamiento agresivo de ejemplares salvajes juveniles (1 año) de trucha común. Además, se estudiaron las posibles interacciones entre la ghrelina y algunos reguladores centrales de la ingesta, como el neuropéptido Y (NPY) y las monoaminas.

Los peces con implantes de ghrelina muestran a los 7 días de tratamiento un incremento significativo de la ingesta, acorde con el papel orexigénico de esta hormona en la mayoría de los vertebrados, pero contrario a lo descrito en la trucha arcoíris (Jönsson, 2013; Kaiya et al., 2013b). Este incremento de la ingesta, así como del crecimiento, inducido por la ghrelina, no está acompañado por alteraciones en el contenido de los triglicéridos circulantes ni de la actividad lipasa en el hígado y el músculo. Sin embargo, no podemos descartar que el incremento de la actividad locomotora provocado por el tratamiento con ghrelina en la trucha común pueda estar enmascarando una posible acción lipogénica de esta hormona (Gao et al., 2012); como tampoco podemos descartar diferencias interespecíficas en teleósteos (Kaiya et al., 2008). Otro efecto observado tras el tratamiento con ghrelina en la trucha común fue el incremento de la actividad exploratoria en búsqueda de alimento, acción que corrobora la implicación de esta hormona en la inducción de la actividad anticipatoria en el carpín (Nisembaum et al., 2014). El tratamiento con ghrelina no modifica la actividad monoaminérgica en el encéfalo, ni la expresión del NPY en el hipotálamo. Finalmente, por primera vez en los peces, se ha señalado la posible implicación de esta hormona en la regulación del comportamiento agresivo, pues las truchas tratadas con ghrelina tienden a iniciar un mayor número de conflictos.

Capítulo 4. Regulación de la ingesta en los peces por un mediador lipídico. Acciones de la oleiletanolamida en el carpín (*Carassius auratus*)

Artículo 5. *Role of oleoylethanolamide as a feeding regulator in goldfish.*

***J Exp Biol* 2014, 217: 2761-2769**

El primer propósito de este estudio fue determinar el contenido endógeno de la OEA en el encéfalo y en los tejidos periféricos del carpín, y si el estado nutricional modifica los niveles de la OEA en estos tejidos. Posteriormente, analizamos el efecto de un tratamiento periférico agudo con OEA (5 μg/g PC) en la ingesta, la actividad locomotora y los niveles plasmáticos de glucosa y triglicéridos. Por último, se estudian las posibles interacciones a nivel central y periférico de este mediador lipídico con conocidos reguladores de la ingesta en los teleósteos.

La OEA está presente en todos los tejidos estudiados, tanto encefálicos como en la periferia, con niveles de OEA en los segmentos intestinales del carpín (bulbo intestinal e intestino proximal) similares a los descritos en el tracto gastrointestinal de los mamíferos (Fu et al., 2007). El ayuno de 48 horas, como sucede en los roedores y en la serpiente pitón, reduce el contenido de la OEA intestinal, recuperándose los valores basales cuando los carpines son realimentados. Estos resultados confirman el papel de la OEA como factor de saciedad en distintos vertebrados (Piomelli, 2013). En apoyo de esta hipótesis se observa una reducción de la ingesta dependiente del tiempo inducida por la administración periférica de la OEA. De forma concomitante a la reducción de la ingesta, a las 2 horas del tratamiento, se produce una disminución de la actividad locomotora y de los triglicéridos plasmáticos. Por último, nuestros datos revelan que las acciones de la OEA sobre la homeostasis energética en el carpín podrían estar mediadas, al menos en parte, por la inhibición de la expresión de ghrelina en el intestino, y por la estimulación de la actividad serotoninérgica en el telencéfalo, sin encontrar alteraciones de la expresión de la CCK intestinal, de la leptina en el encéfalo y el hígado, ni del NPY y la orexina-A hipotalámicos.

CONCLUSIONES

De modo global los resultados obtenidos en la presente Tesis Doctoral apuntan a la relevancia de los tejidos periféricos, mayoritariamente el tracto gastrointestinal, en el control de la ingesta en teleósteos. En resumen, proponemos las siguientes conclusiones principales:

• La leptina en el carpín regula la ingesta a corto plazo, actuando como señal de saciedad, independientemente del estado nutricional, sin poder ser considerada como una señal de adiposidad a largo plazo en este teleósteo. La amplia distribución de las leptinas y de su receptor en el encéfalo y en la periferia concuerda con la naturaleza pleiotrópica de estas leptinas en el carpín.

• La expresión de leptina (*gLep-aI* y *gLep-aII* en el hipotálamo, y *gLep-aI* en el hígado) muestra una ritmicidad diaria en los carpines expuestos a un ciclo 12L/12D y alimentados a un horario fijo diariamente. Sin embargo, en ausencia de este ciclo luz/oscuridad, el horario de alimentación por sí solo no es capaz de sincronizar esta expresión rítmica. La expresión diaria de leptina se regula de manera diferencial en el encéfalo y en los tejidos periféricos.

• El péptido CCK-8S regula la motilidad del intestino proximal del carpín induciendo una respuesta contráctil dependiente de concentración, independiente del control colinérgico y del sistema entérico, que puede producirse de forma directa en células musculares. Las aproximaciones farmacológicas realizadas y la distribución de los receptores de la CCK encontrada en el carpín apuntan al subtipo de receptor CCKAR como mediador del efecto contráctil observado.

• La ghrelina en la trucha común interviene en la regulación de la búsqueda de alimento y posiblemente también en el comportamiento agresivo, con efectos claros de incremento de la ingesta y del peso corporal, sin modificaciones del metabolismo lipídico hepático y muscular, ni de la actividad monoaminérgica encefálica ni de la expresión hipotalámica del NPY.

• Nuestros resultados demuestran, por primera vez en peces, la implicación del derivado lipídico OEA en la regulación de la ingesta a corto plazo, actuando como factor de saciedad, aparentemente mediante la modulación de la ghrelina intestinal y del sistema serotoninérgico en el telencéfalo del carpín. La implicación de la OEA en la regulación de la actividad locomotora y el metabolismo lipídico en el carpín apoya resultados previos en los mamíferos y nos permite sugerir un alto grado de conservación funcional evolutiva de esta aciletanolamida en la homeostasis energética.

Involvement of leptin, cholecistokinin, ghrelin, and the lipid-derived oleoylethanolamide in the regulation of feeding behavior in teleosts

INTRODUCTION

Feeding in fish, as in other vertebrates, is a complex phenomenon involving a central system, focused on the encephalon, working in coordination with a peripheral system made up for a broad variety of tissues, as the gastrointestinal tract. Both systems are regulated by endogenous (metabolic and neuroendocrine) and external (photoperiod, temperature, food availability, etc.) cues. Endogenous signals can be originated in central nervous system and in peripheral organs, and include appetite stimulators or orexigenic factors, and inhibitors or anorexigenic factors (Volkoff, 2009a). This Doctoral Thesis is focused on those endogenous signals mainly synthesized in peripheral tissues as liver (leptin) and gastrointestinal tract (cholecystokinin, oleoylethanolamide and ghrelin).

Leptin is a peptide that regulates food intake and energy balance in vertebrates (Copeland et al., 2011; Friedman, 2014). Leptin anorectic action has been reported in most studied fish (Copeland et al., 2011). However, its regulation by nutritional status depends on the feeding regime and seems to be species-specific (Londraville et al., 2014). In mammals, leptin circulating and expression levels follow a daily rhythmic profile superimposed by postprandial leptin peaks (Kalra et al., 2003; Schoeller et al., 1997; Xu et al., 1999). In cyprinids and salmonids it has been found two leptin type a paralogs genes (leptin-a1 and leptin-a2) which show postprandial increases (Huising et al., 2006; Moen and Finn, 2013). But at present, the daily expression profile of teleost leptin has been only studied in Atlantic salmon (*Salmo salar*) (Moen and Finn, 2013).

Cholecystokinin (CCK) plays a key role on feeding regulation and digestive physiology in vertebrates (Yu and Smagge, 2014). In fish, CCK is involved in the contraction of the gallbladder, the pancreatic enzyme secretion (Einarsson et al., 1997; Murashita et al., 2008a; Volkoff, 2006) and gastric motility (Forgan and Forster, 2007; Olsson et al., 1999), as in mammals The biological actions of CCK in vertebrates are mediated by two specific receptors, CCKAR and CCKBR, which differ in their distribution and affinities for CCK/gastrin peptides (Staljanssens et al., 2011; Yu and Smagge, 2014). The knowledge of CCK receptors in fish is scarce and uncompleted to date.

Ghrelin is currently the only described orexigenic hormone with peripheral origin in vertebrates (Jönsson, 2013; Kaiya et al., 2013b). In teleosts, ghrelin orexigenic action has been demonstrated in goldfish (Kang et al., 2011a) and tilapia (*Oreochromis mossambicus*) (Riley et al., 2005); whereas it has been published a wide variety of ghrelin

effects on feeding (orexigenic, anorexigenic and no changes) in rainbow trout (*Oncorhynchus mykiss*) (Jönsson, 2013). Besides feeding regulation, ghrelin is also involved in the growth hormone (GH) secretion from the pituitary, lipid metabolism and locomotor activity, although, all these actions seem to be species-specific (Jönsson, 2013; Kaiya et al., 2013b; Keen-Rhinehart and Bartness, 2005).

Oleoylethanolamide (OEA) is a fatty acid ethanolamide that regulates feeding, body weight, lipid metabolism and locomotion, among other actions, in mammals (Piomelli, 2013; Proulx et al., 2005). The role of OEA as a satiety signal is supported by the feeding reduction observed after peripheral administration in rodents and by the food-induced OEA intestinal mobilization also in rodents and in the Burmese python (*Python molurus*) (Piomelli, 2013). However, despite the confirmed role of anandamide (Valenti et al., 2005), and the OEA precursor, oleic acid (Librán-Pérez et al., 2012, 2014) on fish feeding regulation, it is yet unknown the possible role of OEA in fish.

The general objective of the present Doctoral Thesis is to deep into the study of feeding regulation in fish, using goldfish (*Carassius auratus*) and brown trout (*Salmo trutta*) as experimental models.

RESEARCH CONTENTS

Chapter 1. Leptin system in goldfish (*Carassius auratus*): tissular distribution, regulation by feeding, daily rhythmicity and its synchronization by scheduled feeding

Paper 1. Leptin and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions.
***Peptides* 2012, 34: 329-335**

Paper 2. Leptin expression is rhythmic in brain and liver of goldfish (*Carassius auratus*). Role of feeding time.
***Gen Comp Endocrinol* 2014, 204: 239-247**

Firstly, the expression pattern of the two leptins (*gLep-aI* and *gLep-aII*) and leptin receptor (*gLepR*) in goldfish brain and peripheral tissues was investigated. Secondly, it was studied the postprandial expression, the effect of fasting (1 week) and overfeeding (2 weeks) on such leptin system expression in liver and brain. Finally, 24-h variations in expression of this leptin system and their synchronization by scheduled feeding were investigated.

The *gLep-aI* was widely expressed in central and peripheral tissues, whereas *gLep-aII* was primarily expressed in brain, suggesting that both paralogs can play different roles

in goldfish. Moreover, the widely distribution of leptins, together with the ubiquitous expression of leptin receptor suggest that leptins may have pleiotropic actions in goldfish, as it is observed in other vertebrates (Londraville et al., 2014). No significant differences in the leptin system expression were found among control, overfed and fasting groups, suggesting that leptin is not acting as an adipostat signal in this teleost. However, hepatic *gLep-aI* expression significantly increased at 9-h postfeeding, supporting its role as a postprandial satiety signal in goldfish, in harmony with its anorexigenic effect in this species (De Pedro et al., 2006; Volkoff et al., 2003) and with previous data in fish (Huising et al., 2006; Moen and Finn, 2013; Zhang et al., 2013). As expected, goldfish under 12-h of light/12-h of dark (12L/12D) cycle and scheduled feeding showed a daily rhythm in liver *gLep-aI* expression that seems to be a postprandial response. However the *gLep-aII* expression rhythm in brain does not appear to be related with food intake, indicating that leptin expression in goldfish is differently regulated at central and peripheral level. Finally, under constant light and different feeding regimes, feeding time by itself was not enough to synchronize neither hepatic *gLep-aI* nor brain *gLep-aII* expression rhythms.

Chapter 2. Cholecystokinin and intestinal motility in goldfish (*Carassius auratus*). Mechanism of action and receptors

Paper 3. The contractile effect of cholecystokinin (CCK-8S) on goldfish proximal intestine is mediated by cholecystokinin type A receptor.
***Comp Biochem Physiol A* 2015, (Under review)**

The aim of this study was to investigate the putative role of CCK-8S in the regulation of intestinal motility in goldfish by the use of an *in vitro* organ bath system. Pharmacological approaches were used to identify possible signaling pathways and involved receptors.

The addition of CCK-8S (1 nM-1 μM) to the organ bath induced a significant contraction of the proximal intestine in a concentration-dependent manner, with an EC_{50} value of 53.8 nM. This result is in agreement with the role of CCK on gastrointestinal motility in mammals (Wu et al., 2013). This CCK-induced contraction was not inhibited by atropine (100 μM) or tetrodotoxin (1 μM), suggesting that the CCK-induced contractile effect is independent of cholinergic control, tetrodotoxin-insensitive, and probably occurs directly on intestine smooth muscle. The CCK-induced contraction was significantly reduced in absence of extracellular calcium, indicating a dependence of CCK-8S contractile effect on extracellular calcium. The CCKBR selective antagonist, L365-260 (1 μM) was

unable to block the CCK-induced contractile effect, whereas CCKAR selective antagonist, devazepide (1 μM), inhibited the 30% of the CCK-induced contraction; suggesting that the CCK-8S action on intestinal motility in goldfish is mediated, at least in part, by the CCKAR receptor subtype. Partial-lengths mRNAs encoding two CCK isoforms (CCKAR and CCKBR) were sequenced and phylogenetically analyzed. The goldfish CCK receptors CCKAR and CCKBR differ in their tissue distribution, being CCKBR more centrally distributed, whereas the CCKAR is mainly localized in digestive organs (intestine, gallbladder, liver), supporting the involvement of CCKAR in gastrointestinal functions of CCK in this teleost.

Chapter 3. Role of ghrelin in the regulation of feeding behavior, growth and lipid metabolism in brown trout (*Salmo trutta*)

Paper 4. Ghrelin increases food intake, swimming activity and growth in juvenile Brown trout (*Salmo trutta*).

***Physiol Behav* 2014, 124: 15-22**

Ghrelin promotes food intake, body weight gain and adiposity in the most studied fish, but its effects in rainbow trout seems to vary depending on doses, administration route and source of exogenous hormone (Jönsson, 2013). In order to investigate the ghrelin actions in other salmonid, the first goal was to determine the effects of subchronic treatment of ghrelin (at dose of 475 ng of rainbow trout ghrelin/g of body mass-BM) on feeding, body mass gain, swimming activity and aggressive behavior in one year old wild brown trout. Secondly, we aimed to deep into the knowledge of possible interplay among ghrelin and central feeding regulators, as neuropeptide Y (NPY) and monoamines.

The feeding and growth increase in brown trout after ghrelin implants for 7 days is in accordance with the orexigenic role of this hormone in the most vertebrates, but contrary to what happens in rainbow trout (Jönsson, 2013; Kaiya et al., 2013b). This increase in feeding and growth was not accompanied by alterations of triglycerides content and lipase activity in liver and muscle. However, it cannot be discarded that the ghrelin-enhanced locomotor activity might mask the hypothetical ghrelin lipogenic action (Gao et al., 2012), as well as species-specific actions (Kaiya et al., 2008). Moreover, an increased foraging activity was observed in brown trout, in accordance with ghrelin-induced food anticipatory activity in goldfish (Nisembaum et al., 2014). No interactions were found among ghrelin and brain monoaminergic activity and hypothalamic NPY expression. Finally, for the first time in fish, a trend towards a more aggressive behavior in ghrelin treated trouts was pointed out.

Chapter 4. Food intake regulation by a lipid mediator in fish. Actions of oleoylethanolamide in goldfish (*Carassius auratus*)

Paper 5*.* Role of oleoylethanolamide as a feeding regulator in goldfish.

***J Exp Biol* 2014, 217: 2761-2769**

The first purpose of the present study was to determine the endogenous content of OEA in goldfish brain and peripheral tissues, and whether this fatty acid ethanolamide is regulated by nutritional status. Then, it was analyzed the effects of acute OEA peripheral administration (at dose of 5 μg/g BM) on food intake, locomotor activity and plasma glucose and triglycerides. Finally, the possible interplay among this lipid and some known feeding regulators at both central and peripheral level was investigated.

All the studied tissues, both central and peripheral, showed a certain endogenous OEA content. The OEA content in the gastrointestinal segments (intestinal bulb and proximal intestine) was in the same range as in equivalent regions in mammals (Fu et al., 2007). As in rodents and Burmese python, OEA gastrointestinal content decreased after 48-h fasting, and subsequently returned to baseline levels following re-feeding in goldfish; supporting the involvement of this lipid mediator as a satiety factor in vertebrates (Piomelli, 2013). In accordance with this hypothesis, peripheral OEA administration produced a time-dependent inhibition of food intake. This anorexigenic effect was accompanied by a decrease of locomotor activity and circulating triglycerides at 2-h post-treatment. In addition, our results showed that OEA actions on energy homeostasis in goldfish would be mediated, at least in part, by inhibition of intestinal ghrelin expression and stimulation of serotoninergic activity in the telencephalon; without affecting the expression of intestinal CCK, brain and hepatic leptin, and brain NPY and orexin-A.

FINDINGS

In overall, the results obtained in the present Doctoral Thesis point to the relevance of the peripheral tissues, mostly gastrointestinal tract, in the control of food intake in fish. Briefly, the main findings are as follows:

• Leptin in goldfish acts regulating feeding at short-term, as a postprandial satiety signal, but apparently it is independent of the nutritional status. Then, it should not be considered as an adiposity signal at long-term in this teleost. The widespread distribution

of leptins and its receptor in both brain and peripheral tissues suggests pleiotropic actions of leptins in goldfish.

• Leptin expression (*gLep-aI* and *gLep-aII* in the hypothalamus, and *gLep-aI* in the liver) is rhythmic under a light/dark cycle and a scheduled feeding. Daily leptin expression rhythms are differentially regulated in brain and peripheral tissues, and such rhythms are not driven by feeding time by itself.

• The peptide CCK-8S regulates motility in goldfish proximal intestine strips, and evokes a non-cholinergic contractile and tetrodotoxin-insensitive concentration-dependent response directly on smooth muscle cells. Pharmacological approaches and distribution analysis of CCK receptors support that the contractile effect of CCK-8S might be mediated by the CCKAR receptor subtype.

• Ghrelin is involved in the regulation of foraging activity and probably aggressive behavior in wild brown trout, resulting in feeding increase and body weight gain, without modifications in liver and muscle lipid metabolism, brain monoaminergic activity and hypothalamic expression of NPY.

• For the first time in fish, our results demonstrate that the lipid-derived OEA is involved in the short-term regulation of food intake, as a satiety factor, apparently by modulation of intestinal ghrelin and telencephalic serotoninergic system. The involvement of OEA in the regulation of locomotor activity and lipid metabolism in goldfish, as in mammals, suggests a high conservation of OEA actions in energy balance throughout vertebrate phylogeny.

II. INTRODUCCIÓN

1. REGULACIÓN DE LA INGESTA EN PECES

Los mecanismos básicos que regulan el comportamiento alimentario se encuentran muy conservados en los vertebrados. En general, la regulación del apetito abarca un gran número de mecanismos complejos y multifactoriales. La ingesta en los peces, al igual que en el resto de los vertebrados, está controlada por un sistema central, focalizado en el encéfalo, que funciona coordinadamente con un sistema periférico, formado por tejidos periféricos, como el tracto gastrointestinal. Ambos sistemas se encuentran regulados por señales endógenas y externas o ambientales. Las señales endógenas (metabólicas y neuroendocrinas) tanto de origen central como periférico pueden ser de carácter orexigénico o anorexigénico. Las señales externas o ambientales (temperatura, fotoperiodo, presencia de alimento, etc.), y específicamente las que fluctúan de forma rítmica, aseguran una organización funcional temporal, generando de forma directa salidas rítmicas de carácter nervioso o endocrino, que inciden en la mayoría de funciones fisiológicas y comportamentales (De Pedro y Björnsson, 2001; Falcón et al., 2010; Gorissen et al., 2006; Hoskins y Volkoff, 2012).

En los últimos años, los teleósteos están siendo ampliamente empleados como modelos experimentales en estudios de regulación de la ingesta tanto en términos de fisiología básica como aplicada a la producción animal. Su fácil manejo y adaptabilidad a los protocolos de experimentación (Volkoff et al., 2009a) junto a su diversidad genómica, morfológica, comportamental y ecológica (Volff, 2005), les confieren un valor añadido frente a otros vertebrados. Entre los ciprínidos, el carpín o carpa dorada, *Carassius auratus*, es una especie muy utilizada en estudios de regulación de la ingesta, con requerimientos poco exigentes y buenas respuestas a la manipulación en el laboratorio (Hoskins y Volkoff, 2012). En la última década, estudios realizados en el carpín están aportando una valiosa información acerca del funcionamiento del sistema circadiano, pues este teleósteo presenta una gran plasticidad frente a señales sincronizadoras externas, como el fotoperiodo y el horario de alimentación (Aranda et al., 2001; Feliciano et al., 2011; Nisembaum et al., 2012; Sánchez-Vázquez et al., 2001; Vera et al., 2007). Además de los ciprínidos, los salmónidos son una familia ampliamente utilizada en estudios de fisiología animal, en gran parte debido a su interés comercial. Existen numerosos estudios en salmónidos que abordan los cambios fisiológicos derivados del paso de la vida en agua dulce a agua marina o esmoltificación (Dêbowski et al., 1999; McCormick, 1994) y sobre regulación de la ingesta en sus diferentes etapas de desarrollo (Hoskins y Volkoff, 2012). El salmónido estudiado en la presente Tesis Doctoral ha sido la trucha común o trucha

marrón, *Salmo trutta*. En esta especie se están realizando importantes esfuerzos para conocer y caracterizar su comportamiento, tanto alimentario como social, principalmente con la finalidad de lograr una producción suficiente que permita reforzar las amenazadas poblaciones salvajes (Brockmark y Johnsson, 2010; Kallio-Nyberg et al., 2010; Klemetsen et al., 2003).

A continuación se describe brevemente la información disponible hasta la fecha sobre los principales neuropéptidos, monoaminas y hormonas involucradas en la regulación de la ingesta en los teleósteos, clasificándolos en dos grandes grupos: señales estimuladoras u orexigénicas y señales inhibidoras o anorexigénicas. En el apartado II.1.2. de la presente Tesis Doctoral se han resumido las principales señales externas que influyen en el apetito de los teleósteos.

1.1. REGULACIÓN NEUROENDOCRINA

1.1.1. Señales orexigénicas

Como parte del sistema central de estimulación de la ingesta en peces, se ha descrito el papel de la apelina, la β-endorfina, el endocannabinoide anandamida (AEA), la galanina, la hormona de crecimiento (GH), el neuropéptido Y (NPY), la noradrenalina (NA), las orexinas, el péptido relacionado con Agouti y la hormona liberadora de tirotropina (Abbott y Volkoff, 2011; Cerdá-Reverter y Peter, 2003; De Pedro et al., 2001; Volkoff et al., 2009a). El número de factores orexigénicos periféricos conocido es muy limitado, concretándose por el momento únicamente el papel de la ghrelina (Volkoff et al., 2005). De entre todos estos factores orexigénicos, queremos destacar el papel del NPY, las orexinas y los sistemas monoaminérgico y endocannabinoide por su relación con la presente Tesis Doctoral. Además, en el apartado II.4. de la presente Memoria se describe más detalladamente el conocimiento actual sobre la ghrelina.

El NPY es uno de los neuropéptidos más abundantes en el encéfalo, mostrando una amplia distribución por todo el sistema nervioso central (SNC), además de en tejidos periféricos como el tracto gastrointestinal (Cerdá-Reverter et al., 2000; Kehoe y Volkoff, 2007; MacDonald y Volkoff, 2009; Narnaware y Peter, 2002; Silverstein et al., 1998). Ejerce un efecto estimulador de la ingesta tras el tratamiento intracerebroventricular (ICV) (López-Patiño et al., 1999; Narnaware et al., 2000; Silverstein et al., 2001), efecto que se revierte parcialmente con el pre-tratamiento con su antagonista (De Pedro et al., 2000; López-Patiño et al., 1999). Reforzando su papel orexigénico, se ha descrito que sus niveles de expresión en el encéfalo presentan cambios periprandiales (Narnaware et al., 2000;

Vera et al., 2007) y se incrementan en condiciones de ayuno (Narnaware et al., 2000; Narnaware y Peter, 2001; Silverstein et al., 1998). Se ha sugerido que su acción sobre la ingesta se produce, en parte, a través de la modulación de numerosos reguladores del apetito, tanto estimuladores (péptidos opioides, galanina, orexinas, GH y ghrelina) como inhibidores (hormona liberadora de corticotropina-CRH, transcrito inducido por la cocaína y anfetamina-CART, cortisol, hormona concentradora de melanina-MCH y leptina) (Volkoff et al., 2009a).

Las orexinas (orexina-A y orexina-B), también denominadas hipocretinas, se encuentran ampliamente distribuidas por el organismo, sin embargo las encontramos mayoritariamente en el encéfalo, y más específicamente en el hipotálamo (Kaslin et al., 2004; Xu y Volkoff, 2007; Yan et al., 2011), donde sus niveles incrementan tras el ayuno, como cabe esperar de un regulador orexigénico (Abbott y Volkoff, 2011; Nakamachi et al., 2006; Wall y Volkoff, 2013). La administración ICV de orexina-A estimula la ingesta en los teleósteos (Nakamachi et al., 2006; Volkoff et al., 1999; Yokobori et al., 2011), sugiriéndose que su acción esta mediada por la interacción con otros reguladores del apetito, como el NPY, la leptina, la ghrelina y la hormona liberadora de tirotropina (Abbott y Volkoff, 2011; Miura et al., 2007; Nisembaum et al., 2014; Volkoff et al., 2003; Yan et al., 2011).

El sistema monoaminérgico central está implicado en la regulación de la ingesta en los peces, produciendo tanto aumento como disminución de la misma. En cuanto a sus acciones estimulando el apetito, se ha descrito la activación del sistema noradrenérgico en el hipotálamo del carpín tras 7 días de ayuno (De Pedro et al., 2001). Otra vinculación de la NA con la regulación orexigénica del apetito se ha obtenido tras el tratamiento ICV con el antagonista del receptor adrenérgico α_2, que parece mediar el efecto orexigénico inducido por la galanina en el carpín (De Pedro et al., 1995a). Además, se ha descrito una reducción del metabolismo noradrenérgico tras un tratamiento subcrónico con melatonina (De Pedro et al., 2008) y agudo con leptina (De Pedro et al., 2006), ambos reguladores anorexigénicos de la ingesta.

La existencia de un sistema endocannabinoide funcional en el carpín se desconocía hasta la publicación del trabajo pionero de Valenti y colaboradores (2005). El contenido telencefálico de AEA en este teleósteo, incrementa significativamente tras 24 horas de ayuno, efecto que sugiere el papel orexigénico central de este cannabinoide. El tratamiento intraperitoneal (IP) sin embargo, produce una respuesta bifásica sobre la ingesta, pues dosis bajas la aumentan y dosis altas la reducen, sugiriéndose que su acción está mediada por el receptor CB_1. Posteriormente se ha sugerido, mediante ensayos de inmunohistoquímica, que el efecto orexigénico de la AEA sobre la ingesta en el carpín esta

mediado por el NPY (Cottone et al., 2009). En la dorada (*Sparus aurata*), se observó el mismo efecto orexigénico de la AEA administrada en el agua del tanque, sugiriéndose al igual que en el carpín la implicación del receptor CB_1 y del NPY en esta acción orexigénica (Piccinetti et al., 2010).

1.1.2. Señales anorexigénicas

Se ha propuesto la existencia de un número importante de reguladores anorexigénicos de la ingesta en teleósteos. A nivel central se ha caracterizado el papel de la CRH, el CART, la dopamina (DA) y la serotonina (5-hidroxitriptamina; 5-HT), la hormona liberadora de gonadotropina, la MCH, la hormona estimulante de los melanocitos, la neuromedina U, el péptido liberador de prolactina, el péptido relacionado con el gen de la calcitocina (CGRP), el polipéptido activador de la adenilato ciclasa (PACAP), las taquicininas, la urotensina I, y recientemente el *spexin* (Bernier y Peter, 2001a; Cerdá-Reverter et al., 2011; De Pedro et la., 1998a, 1998b; Kelly y Peter 2006; Matsuda et al., 2005a, 2008b; Volkoff et al., 2009a; Wonh et al., 2013). Son numerosas las señales anoréticas periféricas descritas hasta el momento actual, incluyendo la amilina, la colecistocinina (CCK), el cortisol, el estradiol, la insulina, la leptina, la melatonina, los péptidos liberadores de gastrina como la bombesina, el péptido similar al glucagón, el péptido intestinal vasoactivo (VIP) y la testosterona (Gregory y Wood, 1999; Himick y Peter, 1994a; López-Olmeda et al., 2006; Matsuda et al., 2005b; Pinillos et al., 2001; Soengas y Aldegunde, 2004; Thavanathan y Volkoff, 2006; Volkoff et al., 2009ª). De entre todos ellos, queremos destacar el papel de la CRH, la DA y la 5-HT, y el de los reguladores periféricos que actúan a nivel del tracto gastrointestinal. En los apartados II.2. y II.3. de la presente Memoria se presenta un resumen del conocimiento actual sobre la leptina y la CCK, por su relación directa con la presente Tesis Doctoral.

La CRH es un potente anorexigénico central, describiéndose su papel en la regulación de la ingesta en los peces por primera vez en el grupo de la Dra. Delgado (De Pedro et al., 1993, 1995b). Su acción anorética parece ser independiente de la activación del eje hipotálamo-hipófisi-interrenal, produciéndose principalmente a través de receptores específicos (Arai et al., 2001; Bernier y Peter, 2001b; De Pedro et al., 1997), aunque también están implicados receptores α_1-adrenérgicos y dopaminérgicos (De Pedro et al., 1998a). La CRH en el carpín interviene en los efectos orexigénicos del NPY (Bernier et al., 1998a) y en la acción anorexigénica de la hormona estimulante de melanocitos (Matsuda et al., 2008a) y de la 5-HT (De Pedro et al., 1998b; Ortega et al., 2013).

Como se ha descrito en el apartado anterior (II.1.1.1.) el sistema monoaminérgico puede regular la ingesta de manera dual, incrementándola o reduciéndola. Así, se han propuesto a la DA y la 5-HT como reguladores anorexigénicos, mediando la inhibición de la ingesta inducida por la CRH (De Pedro et al., 1997, 1998a, 1998b), y a la NA, que ejerce una acción inhibitoria del apetito a través del receptor adrenérgico α_1 (De Pedro et al., 1998a).

De entre los reguladores periféricos queremos destacar el papel de los que producen efectos anorexigénicos a nivel del tracto gastrointestinal, subrayando su papel como reguladores de la motilidad intestinal. Un gran número de evidencias relacionan la motilidad gastrointestinal con la regulación del apetito. En los vertebrados, el retraso en el vaciamiento gástrico se asocia con una menor ingesta (Tack et al., 2001; Wisen y Hellstrom, 1995). Así, la relajación del estómago junto con una disminución en la contractilidad antral y duodenal, y un aumento en la presión pilórica producen un retraso del vaciado gástrico, y la consecuente disminución de la ingesta (Little et al., 2007). Actualmente, el conocimiento sobre la motilidad gastrointestinal en los peces es muy limitado. Se ha descrito un patrón similar al complejo motor mioeléctrico de los mamíferos, característico de un estado interdigestivo, en el bacalao del Atlántico (*Gadus morhua*) (Karila y Holmgren, 1995). Y en cuanto al ritmo de ondas lentas, propio de un estado de digestión activa, su presencia o ausencia varía en función de la especie estudiada (Forgan y Foster, 2007; Holmberg et al., 2006; Velarde et al., 2009). En los peces, al igual que en los mamíferos, señales neuronales y hormonales modifican este ritmo de ondas lentas. Así, se han propuesto como excitadores de la motilidad gastrointestinal a la acetilcolina, la histamina, la 5-HT, las taquicininas, la ghrelina y la galanina; y como reguladores inhibidores al óxido nítrico, la melatonina, el VIP y el PACAP. Otros agentes potenciales reguladores, como la NA y la DA, la bombesina y la CCK, parecen ejercer diferentes efectos en función de la especie en estudio y de la sección del tracto gastrointestinal investigada (Forgan y Foster, 2007; Karila et al., 1993; Olsson, 2009; Olsson et al., 1999; Olsson y Holmgren, 2001; Velarde et al., 2009). Reguladores con demostrado papel anorexigénico en los peces tras su administración exógena, como el VIP y el PACAP (Matsuda et al., 2005a, 2005b), ejercen un efecto predominantemente inhibitorio sobre la motilidad intestinal (Holmberg et al., 2004; Matsuda et al., 2000; Olsson y Holmgren, 2000) y gástrica (Grove y Holmgren, 1992; Holmgren, 1983). Al igual que la melatonina, cuya administración IP produce una disminución de la ingesta (De Pedro et al., 2008; Pinillos et al., 2001) y cuya adición al cultivo *in vitro* de segmentos intestinales del carpín atenúa la contracción inducida por acetilcolina, además de modular

la estimulación serotoninérgica en el intestino de este teleósteo (Velarde et al., 2009, 2010).

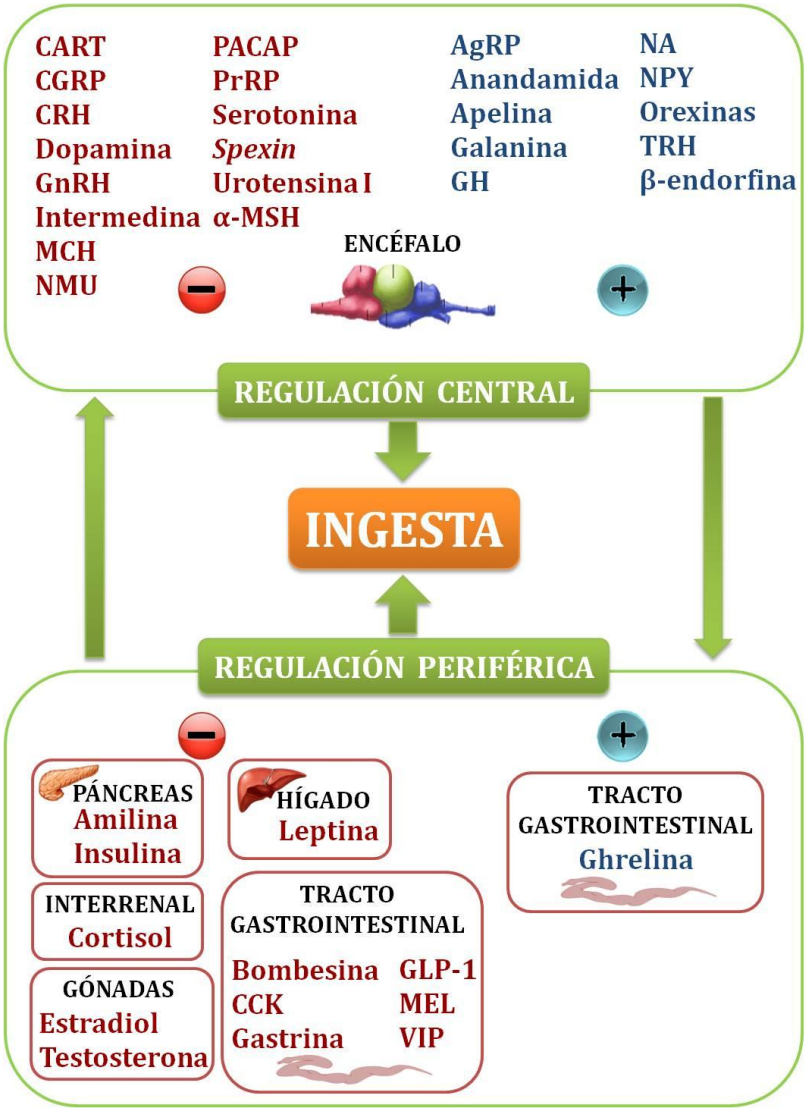


Figura 1. Esquema de los principales reguladores orexigénicos y anorexigénicos de la ingesta en peces. AgRP: péptido relacionado con Agouti; CART: transcrito inducido por cocaína y anfetamina; CCK: colecistocinina; CGRP: péptido relacionado con el gen de la calcitocina; CRH: hormona liberadora de corticotropina; GH: hormona de crecimiento; GLP-1: péptido similar al glucagón; GnRH: hormona liberadora de gonadotropinas; MCH: hormona concentradora de melanina; MEL: melatonina; α-MSH: hormona estimulante de los melanocitos; NA: noradrenalina; NMU: neuromedina U; NPY: neuropéptido Y; PACAP: polipéptido activador de la adenilato ciclasa; PrRP: péptido liberador de prolactina; TRH: hormona liberadora de tirotropina; VIP: péptido intestinal vasoactivo; ⊕: estimulación; ⊖: inhibición.

1.2. REGULACIÓN AMBIENTAL

Entre los factores externos que influyen en la alimentación de los teleósteos destacamos la temperatura, la calidad del agua, los factores sociales, el ciclo luz/oscuridad (ciclo L/D) y el acceso al alimento, particularmente cuando este se presenta de forma periódica.

La relación entre temperatura y alimentación se ha demostrado en un amplio número de especies, sobre todo en aquellas de interés comercial. En general, se admite que a medida que aumenta la temperatura también aumenta el consumo de alimento y el crecimiento (Bendiksen et al., 2002; Guijarro et al., 1999; Kehoe y Volkoff, 2008; Rozin y Mayer, 1961; Sunuma et al., 2007). Si bien, se ha observado que el mantenimiento de los animales a temperaturas muy superiores a las estimadas como óptimas puede provocar el efecto contrario, una disminución de la ingesta y del crecimiento (Hevroy et al., 2012; Kullgren et al., 2013). De forma paralela, la ingesta se reduce a bajas temperaturas. En los teleósteos los efectos estimuladores e inhibidores del apetito producidos por la temperatura parecen estar mediados, en parte, por modificaciones en los reguladores de la ingesta. Así, se han observado modificaciones en los niveles plasmáticos de ghrelina (Nieminen et al., 2003) y en la expresión génica del CART en el encéfalo (Kehoe y Volkoff, 2008) en función de la temperatura. Así mismo, se ha descrito que en los peces expuestos a bajas temperaturas los tratamientos con β-endorfina y galanina no resultan efectivos (Guijarro et al., 1999). Otros factores como la calidad del agua (salinidad, turbidez, pH, etc.), la forma y el color del tanque de cultivo, así como los factores sociales (densidad de población, jerarquías, etc.) también afectan a la ingesta en los peces (Lall y Tibbetts, 2009; Papoutsoglou et al., 2000; Tacon, 1995; Van der Salm et al., 2004), si bien, por el momento el conocimiento sobre cómo afectan estas señales externas a los reguladores de la ingesta es muy limitado.

Las respuestas del organismo a los neuropéptidos y hormonas reguladoras de la ingesta están sometidas a un doble control, homeostático y temporal (Berthoud, 2006; Guijarro et al., 2004; Kalsbeek et al., 2003; López et al., 2006; Thorpe et al., 2003; Vivas et al., 2011). Cualquier señal ambiental que varíe de forma cíclica es susceptible de generar un ritmo biológico. Estos ritmos se pueden clasificar en función de sus periodos, siendo los más conocidos aquellos con una periodicidad entre 20 y 28 h, denominados ritmos circadianos (Aschoff, 1981; Johnson et al., 1996). Entre todas las señales externas susceptibles de generar un ritmo circadiano destacamos:

- El ciclo L/D: el ciclo diario de salida y puesta de sol afecta tanto al comportamiento alimentario como a la actividad de los vertebrados. La información

luminosa, proveniente del ciclo L/D es captada por estructuras fotosensibles, como la retina, y conducida a centros encefálicos donde tiene lugar el "encarrilamiento" de los osciladores sincronizados al ciclo L/D (LEOs, *light entrainable oscillators*) (Mendoza y Challet, 2009; Sosniyenko et al., 2009).

- El horario de alimentación: la disponibilidad de alimento en el medio natural no es constante sino que está restringida a ciertas fases del ciclo L/D. Por ello, los animales han desarrollado la capacidad de predecir la hora de alimentación optimizando el proceso de alimentación de la forma más eficiente (Madrid et al., 2001). Así se ha propuesto la existencia de relojes u osciladores que, de forma específica, se "encarrilan" por el alimento (FEOs, *feeding entrainable oscillator*s) (Feliciano et al., 2011; Stephan, 2002). Este "encarrilamiento" por la alimentación en vertebrados se caracteriza por una mayor actividad locomotora que anticipa la llegada del alimento (FAA, *food anticipatory activity*). La FAA se ha observado tanto en los mamíferos como en los peces, y consiste en un incremento pronunciado de la actividad motora entre 1 y 3 horas previas al momento del día en que se suministra el alimento (Feliciano et al., 2011; Sánchez-Vázquez et al., 1997; Stephan, 2002).

En el caso de los teleósteos también cabe mencionar otros ritmos biológicos con un marcado papel en la regulación de la ingesta, como son los ritmos mareales (periodicidad de 12,4 h), los lunares (periodicidad de 28 días) y los estacionales (López-Olmeda y Sánchez-Vázquez, 2010). De todos ellos, los ritmos estacionales son los más conocidos, así se ha descrito que las variaciones estacionales de luz y temperatura desempeñan un papel muy importante en la regulación de la ingesta en los peces, pues algunas especies dejan de alimentarse en los meses más fríos, para alcanzar su máximo de ingesta y crecimiento en los más cálidos (Madrid et al., 2001).

2. LEPTINA

2.1. ESTRUCTURA

La leptina, producto del gen *ob* (obesidad), es una hormona sintetizada fundamentalmente por el tejido adiposo en los mamíferos (Zhang et al., 1994) e implicada en el control de la ingesta y la regulación del balance energético en la mayoría de los vertebrados estudiados, incluidos los peces (Friedman, 2014; Londraville et al., 2014).

Actualmente se conocen las secuencias de leptina en varias familias de teleósteos: *Tetraodontidae* (Kurokawa et al., 2005), *Salmonidae* (Angotzi et al., 2013; Frøiland et al., 2010; Murashita et al., 2008b; Rønnestad et al., 2010), *Cyprinidae* (Gorissen et al., 2009; Huising et al., 2006; Li et al., 2010; Tang et al., 2013), *Adrianichthydae* (Kurokawa y Murashita, 2009); *Ictaluridae* (Kobayashi et al., 2011), *Bagridae* (Gong, Y. et al., 2013), *Serranidae* (Zhang et al., 2013) y *Percidae* (He et al., 2013). En todos los casos la identidad de las secuencias de la leptina de peces con las leptinas de mamíferos es muy baja (< 30%), aunque en líneas generales la estructura terciaria de la leptina parece estar bastante conservada en los vertebrados, particularmente el puente disulfuro, propiedad única de la familia de las citoquinas de tipo I, y que configura su conformación tridimensional (Figura 2), (Gorissen et al., 2009; Londraville et al., 2014). En concordancia con el proceso de duplicación génica experimentada al inicio del linaje de los peces teleósteos (Hoegg et al., 2004; Taylor et al., 2003) se ha detectado la presencia de una duplicación del gen *ob* (Copeland et al., 2011; Denver et al., 2011), demostrándose la presencia de leptinas tipo a y tipo b en el medaka (*Oryzias latipes*) (Kurokawa y Murashita, 2009), el pez cebra (*Danio rerio*) (Gorissen et al., 2009) y en el mero de pintas naranjas (*Epinephelus coioides*) (Zhang et al., 2013). Además, se ha encontrado una segunda duplicación de la leptina-a (leptina-aI y aII) en ciprínidos y salmónidos (Angotzi et al., 2013; Huising et al., 2006; Rønnestad et al., 2010; Tang et al., 2013), y de la leptina-b (leptina-bI y bII) en salmónidos (Angotzi et al., 2013). En el inicio de la presente Tesis Doctoral no se conocían las secuencias de las leptinas del carpín, publicándose posteriormente en esta especie la presencia de dos secuencias de leptina similares a las leptina-aI y leptina-aII de la carpa común (*Cyprinus carpio*), respectivamente, y a la leptina-a del pez cebra (Copeland et al., 2011; Denver et al., 2011).

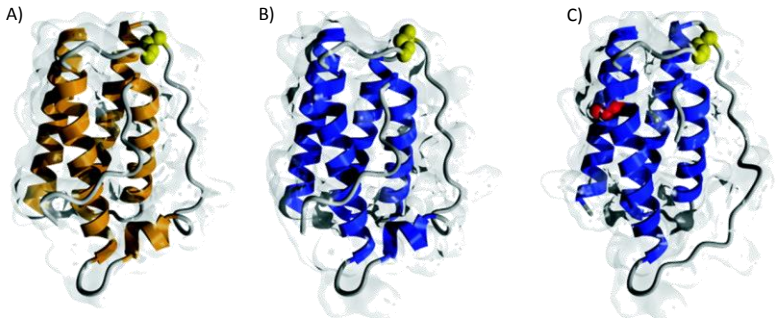


Figura 2. Estructura terciaria de la leptina humana (A), la leptina-a (B) y la leptina-b (C) del pez cebra (Gorissen et al., 2009). En amarillo se ha representado el puente disulfuro que estabiliza la estructura terciaria y en rojo la tercera cisteína de la leptina-b del pez cebra.

2.2. SÍNTESIS Y DISTRIBUCIÓN

La leptina en los mamíferos se sintetiza principalmente en el tejido adiposo desde donde se libera a la circulación general (Zhang et al., 1994). Parece existir una relación directa entre el nivel de adiposidad y los niveles circulantes de leptina, que actúa como señal periférica de saciedad informando al SNC del estado nutricional y las reservas energéticas del animal (Henry y Clarke, 2008). En los peces, sin embargo, la expresión de leptina en el tejido adiposo es reducida, siendo el hígado el órgano con mayor expresión de esta hormona en todas las especies investigadas (Copeland et al., 2011; He et al., 2013). Otras localizaciones también muestran una expresión importante de leptina, como el cerebro, las gónadas, el tracto gastrointestinal y el bazo, si bien el patrón de distribución de esta hormona en los peces varía notablemente dependiendo del tipo de leptina, la especie en estudio, el estado de desarrollo e incluso el sexo del animal (Rønnestad et al., 2010; Tang et al., 2013).

En los mamíferos los niveles circulantes de leptina y su expresión génica presentan variaciones a lo largo del día, determinadas, entre otros factores, por la ingestión de alimento (Gómez Abellán et al., 2011; Kalsbeek et al., 2001; Xu et al., 1999). De manera similar, se ha descrito cómo la ingesta modifica los niveles de expresión de las leptinas de teleósteos, observándose incrementos posprandiales de esta hormona en el hígado de la carpa común (Huising et al., 2006), del salmón del Atlántico (*Salmo salar*) (Moen y Finn, 2013) y del mero de pintas naranjas (Zhang et al., 2013), aunque esta relación parece depender de la especie (Yuan et al., 2014).

2.3. RECEPTORES

El receptor de leptina es una proteína transmembrana perteneciente a la familia de los receptores de citoquinas de tipo I, que utiliza la ruta de comunicación intracelular JAK/STAT (Peelman et al., 2014). No se identificó el receptor de leptina en un teleósteo hasta 2007, en el medaka marino (*Oryzias melastigma*) (Wong et al., 2007). En los mamíferos se han descrito seis isoformas del receptor de leptina: una clasificada como forma larga (LEPRb) de distribución hipotalámica y cinco clasificadas como formas cortas (LEPRa, LEPRc, LEPRd, LEPRe y LEPRf) de amplia distribución (Peelman et al., 2014). En los peces no parece estar tan definido el número de isoformas presentes, habiéndose descrito en algunos teleósteos una sola forma del receptor (Gong, Y. et al., 2013; Kurokawa et al., 2008; Kurokawa y Murashita, 2009; Liu et al., 2010; Zhang et al., 2013), tres isoformas en *Carassius carassius* (Cao et al., 2011), cuatro en la trucha arcoíris (*Oncorhynchus mykiss*) (Gong, N. et al., 2013) y cinco en el salmón del Atlántico (Rønnestad et al., 2010). Aunque la similitud de la secuencia del receptor en peces comparada con la de otros tetrápodos es muy baja (inferior al 40%), el análisis estructural de los receptores en los distintos vertebrados evidencia una notable conservación de ciertos dominios funcionales, como los dominios fibronectinas tipo II, un dominio de inmunoglobulina "C2-*like*", un par de residuos de triptófano/serina, los dos sitios de unión de la leptina y un dominio de unión a proteínas STAT (Cao et al., 2011; Gong, N. et al., 2013; Kurokawa y Murashita, 2009; Kurokawa et al., 2008; Liu et al., 2010; Rønnestad et al., 2010; Zhang et al., 2013).

Los análisis de distribución del receptor de leptina en los teleósteos revelan una elevada expresión en el hipotálamo, acorde con la distribución previamente observada en mamíferos, donde se expresa mayoritariamente en núcleos hipotalámicos relacionados con la regulación de la ingesta (Denver et al., 2011; Peelman et al., 2014; Zhang et al., 2013). También se han detectado niveles de expresión del receptor de leptina en otras regiones centrales, como el telencéfalo, el techo óptico, el cerebelo y la hipófisis, y en tejidos periféricos, como el hígado, el ovario, el riñón, el músculo, el tejido adiposo, el bazo, las branquias, la piel, el corazón y el riñón (Gong, N. et al., 2013; Kurokawa y Murashita, 2009; Kurokawa et al., 2008; Zhang et al., 2013).

2.4. ACTIVIDAD BIOLÓGICA

Posiblemente la única función de la leptina que se conserva a lo largo de la filogenia de los vertebrados es su acción anorexigénica (Londraville et al., 2014). Los

primeros estudios realizados administrando leptina en los peces no encontraron modificaciones de la ingesta ni del peso corporal en el salmón del Pacífico (*Oncorhynchus kisutch*) (Baker et al., 2000), el pez gato (*Ictalurus punctatus*) (Silverstein y Plisetskaya, 2000), ni en el perciforme *Lepomis cyanellus* (Londraville y Duvall, 2002). Sin embargo, estudios posteriores han demostrado que la administración aguda de leptina en el carpín reduce significativamente la ingesta (Aguilar et al., 2010; De Pedro et al., 2006; Murashita et al., 2008b; Volkoff et al., 2003), de forma similar a las acciones anoréticas descritas en los mamíferos. La administración crónica de leptina también reduce la ingesta y la ganancia de peso corporal (De Pedro et al., 2006). En esta acción anorética de la leptina subyacen interacciones con otros sistemas peptidérgicos hipotalámicos, inhibiendo a neuropéptidos orexigénicos y estimulando señales anorexigénicas. Aunque por el momento no se han estudiado en los peces todas las posibles interacciones descritas en los mamíferos, actualmente se conoce que la leptina revierte los efectos estimuladores de la ingesta inducidos por el NPY y la orexina-A; mientras que potencia los efectos anoréticos del CART y la CCK (Volkoff et al., 2003, 2009a). Estos resultados han sido confirmados por la reducción del ARNm del NPY y el incremento del ARNm del CART y la CCK tras el tratamiento agudo con leptina. Además, las catecolaminas también están implicadas en las acciones de la leptina, habiéndose encontrado reducción de las tasas de recambio adrenérgico y dopaminérgico en el hipotálamo tras la administración crónica de leptina (De Pedro et al., 2006).

Históricamente, se ha reconocido el papel de la leptina en la regulación del balance energético a largo plazo en los mamíferos, comunicando al SNC las reservas corporales de grasa (Friedman, 2014). Sin embargo, esta accion "adipostática" de la leptina no parece tener lugar en los peces, donde se han descrito mayoritariamente incrementos en el ARNm de leptina o de la proteína circulante, así como decrementos y la ausencia de modificaciones del sistema de la leptina en respuesta al ayuno (Londraville et al., 2014). La leptina en los mamíferos interviene en el balance energético a través de sus acciones metabólicas (Frühbeck y Salvador, 2000; Ramsay et al., 2004; Reidy y Weber, 2000). Una de las acciones más directas de la leptina en el metabolismo es la estimulación de las vías lipolíticas e inhibición de la lipogénesis (Reidy y Weber, 2000). La leptina en los peces también promueve la lipolisis, observándose una reducción de los niveles plasmáticos de triglicéridos y del contenido hepático de lípidos totales (De Pedro et al., 2006), así como de la lipoproteína lipasa y la esteroil CoA desaturasa-I hepáticas (Li et al., 2010); con aumentos en las proteínas de unión de ácidos grasos (FABP) y la carnitina palmitoil transferasa (Londraville y Duvall, 2002). La leptina juega también un papel relevante en el eje insulina-glucosa en los mamíferos, con una inhibición de la insulina, reducciones de la

glucogenólisis hepática y producción de glucosa, y un incremento en la captación de glucosa y glucogénesis en el músculo (Frühbeck y Salvador, 2000; Amitani et al., 2013). Las posibles acciones de la leptina en el metabolismo glucídico en los peces son más controvertidas, ya que se han descrito tanto incrementos como reducciones de la glucemia tras la administración exógena de leptina (Aguilar et al., 2010; De Pedro et al., 2006; Vivas et al., 2011).

La gran diversidad fisiológica, ecológica, endocrina, etc., que muestran los peces se refleja en la diversidad de funciones que parece tener la leptina en este grupo de vertebrados, algunas de las cuales coinciden con las desarrolladas por esta hormona en los mamíferos (Londraville et al., 2014). Así, y de forma muy resumida, destacamos el papel de la leptina como inmunomodulador, como factor de crecimiento durante el desarrollo, en la regulación de la reproducción y la actividad locomotora, así como su implicación en las respuestas adaptativas a diferentes agentes estresantes, como la hipoxia ambiental (Copeland et al., 2011; Londraville et al., 2014).

3. COLECISTOCININA

3.1. ESTRUCTURA

La CCK es una de las principales hormonas gastrointestinales descritas, aislada por primera vez del duodeno porcino como un péptido de 33 aminoácidos (Mutt y Jorpes, 1968). Esta hormona está muy relacionada estructuralmente con la gastrina, hormona gastrointestinal descubierta 4 años antes (Tracy y Gregory, 1964), aceptándose que ambas moléculas proceden de un ancestro común (Dupré y Tostivint, 2013).

La CCK se encuentra en una variedad de formas moleculares derivadas de un precursor prepro-CCK cuyo extremo carboxilo contiene la molécula biológicamente activa. En numerosas especies de peces se ha identificado la secuencia de la CCK, incluyendo las familias *Cyprinidae* (Peyon et al., 1998), *Paralichthyidae* (Kurokawa et al., 2003; Suzuki et al., 1999), *Salmonidae* (Jensen et al., 2001; Murashita et al., 2009), *Tetraodontidae* (Kurokawa et al., 2003), *Cupleidae* (Kamisaka et al., 2005), *Carangidae* (Murashita et al., 2006), *Pleuronectidae* (MacDonald y Volkoff, 2009), *Sciaenidae* (Webb et al., 2010), *Ictaluridae* (Peterson et al., 2012) y *Sparidae* (Micale et al., 2012). En muchos de los teleósteos estudiados se han detectado las secuencias CCKA y CCKB (Dupré y Tostivint, 2013; Micale et al., 2012; Murashita et al., 2009; Peterson et al., 2012), y una tercera secuencia de CCK en la trucha arcoíris (Jensen et al., 2001). La homología de las secuencias de teleósteos con las de otros vertebrados, incluyendo la CCK humana, es inferior al 50% (Micale et al., 2012). Sin embargo, los análisis de las secuencias de CCK de peces revelan el procesado de la pro-CCK en diferentes formas de octapéptidos sulfatados en el extremo C-terminal (CCK-8S) que difieren en el aminoácido de la posición 6 (asparagina, leucina o treonina) (Volkoff et al, 2005), y que mantienen un alto grado de conservación en la filogenia (Peterson et al., 2012; Vigna, 2000) (Figura 3).

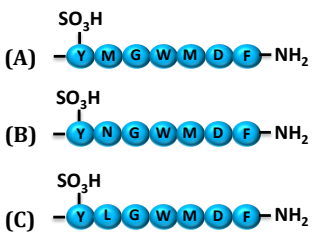


Figura 3. Secuencias de aminoácidos que componen el extremo carboxilo de la CCK en (A) humano, ratón, pollo y *Xenopus*; (B) trucha (CCK-N); y (C) carpín, lenguado y trucha (CCK-L) (modificado de Vigna, 2000).

3.2. SÍNTESIS Y DISTRIBUCIÓN

La CCK en los mamíferos se sintetiza principalmente en el tracto gastrointestinal, encontrando altas concentraciones del péptido en células enteroendocrinas de tipo I situadas en la mucosa del duodeno, del yeyuno, y del colon (Côté et al., 2014). Además, la CCK está muy vinculada al sistema nervioso, observándose a elevada concentración en el encéfalo y en el sistema entérico, localizada en neuronas del íleon y del colon, así como en los plexos submucoso y mientérico (Dockray, 2014).

En los peces, mediante técnicas inmunohistoquímicas y de PCR cuantitativa en tiempo real se ha descrito la presencia de los péptidos CCK-*like* en una amplia variedad de tejidos, aunque se observan principalmente en el tracto gastrointestinal y en el SNC (MacDonald y Volkoff, 2009; Micale et al., 2012; Peyon et al., 1998). En el tracto gastrointestinal de los teleósteos la mayor expresión de CCK se ha descrito en ciegos pilóricos, estómago e intestino anterior, si bien los patrones de expresión muestran una importante variación en función de la especie y de la secuencia de CCK (Kurokawa et al., 2003; Murashita et al., 2009). También se ha evidenciado expresión de la CCK en otros tejidos periféricos como el riñón, el músculo, el corazón, la piel, las gónadas y las branquias (Jensen et al., 2001; Kamisaka et al., 2005; MacDonald y Volkoff, 2009; Murashita et al., 2006, 2009; Webb et al., 2010).

En los peces, al igual que en los mamíferos, la ingesta modifica los niveles de CCK, habiéndose encontrado incrementos posprandiales tanto de la CCK plasmática (Jönsson et al., 2006) como de su expresión génica (Peterson et al., 2012; Peyon et al., 1998), expresión que disminuye en condiciones de ayuno (MacDonald y Volkoff, 2009; Murashita et al., 2006). No obstante, el efecto del ayuno en la expresión de la CCK en los teleósteos varía dependiendo del modelo de ayuno empleado y de la especie en estudio (MacDonald y Volkoff, 2009; Micale et al., 2012; Murashita et al., 2009).

3.3. RECEPTORES

Las acciones de la CCK están mediadas por receptores pertenecientes a la superfamilia de receptores acoplados a proteínas G con 7 dominios transmembrana (Staljanssens et al., 2011), a la que pertenecen otros receptores de péptidos con funciones gastrointestinales, como la motilina, la neuromedina U, la neurotensina y la ghrelina (Feighner et al., 1999; Fujii et al., 2000; Hosoya et al., 2000; Kojima et al., 2000; Smith et al., 1999; Vincent et al., 1999). Se han identificado dos subtipos principales del receptor de CCK en los vertebrados, el CCKAR y el CCKBR (también llamados CCK-1R y CCK-2R,

respectivamente), siendo el subtipo A la forma predominante en la periferia y el subtipo B en localizaciones centrales (Staljanssens et al., 2011).

La información existente sobre el receptor de CCK en los peces es muy limitada y derivada de estudios muy recientes. Actualmente sólo existen dos trabajos que estudien los receptores de CCK en los teleósteos, uno en seriola (*Seriola quinqueradiata*) (Furutani et al., 2013) y el otro en el salmón del Atlántico (Rathore et al., 2013). En *GenBank* podemos encontrar las secuencias del pez cebra (*GenBank ID*: CCKAR, XM_692401.5; CCKBR, XM_009304469.1) y de la tilapia (*Oreochromis mossambicus*) (*GenBank ID*: CCKAR, XM_005454409.1; CCKBR, XM_005457866.1), sin existir por el momento un estudio asociado a éstas. En el caso del receptor CCKAR se ha observado una alta homología de las secuencias tanto con otros peces (del 68% al 83%) como con los mamíferos (del 60% al 63%) (Furutani et al., 2013; Rathore et al., 2013). Este receptor presenta una distribución tisular similar a la descrita en los mamíferos (Staljanssens et al., 2011), con los mayores niveles de expresión asociados al tracto gastrointestinal y al páncreas (Furutani et al., 2013; Rathore et al., 2013). Se han descrito modificaciones posprandiales de la expresión de CCKAR en ciegos pilóricos y vesícula biliar (Furutani et al., 2013).

En el caso del CCKBR (o CCK-2R) de los teleósteos, en el único trabajo publicado hasta el momento, se ha detectado una segunda duplicación génica que ha originado los subtipos CCK-2R1 y CCK-2R2 (Rathore et al., 2013). Ambas secuencias muestran una similitud entre ellas del 90%, sugiriéndose que proceden de un evento reciente de duplicación genómica en los salmónidos (Moghadam et al., 2005; Rathore et al., 2013). La homología de las dos secuencias del receptor de salmónidos con las CCKBR de otros peces es del 70%, presentando una homología con el CCKBR de los mamíferos menor que la descrita para el subtipo A. En función del patrón de expresión de los dos subtipos B del salmón del Atlántico, se ha sugerido que el CCK-2R1 podría ser el ortólogo de los receptores de gastrina descritos en los mamíferos (debido a su expresión mayoritaria en el intestino), mientras que el CCK-2R2, de expresión mayoritaria en el cerebro, correspondería al receptor de CCK de los mamíferos, (Rathore et al., 2013; Staljanssens et al., 2011).

3.4. ACTIVIDAD BIOLÓGICA

La CCK se libera con la llegada del alimento al intestino, respuesta general en vertebrados (Aldman y Holmgren, 1995; Liddle et al., 1985), actuando como señal de saciedad a corto plazo tanto en los mamíferos como en los peces (Owyang y Heldsinger,

2011; Volkoff et al., 2005). En el carpín varios estudios han descrito una disminución de la ingesta tras la administración aguda y crónica del péptido CCK-8S, tanto administrado por vía IP como ICV (Himick y Peter, 1994b; Hoskins y Volkoff, 2012; Volkoff et al., 2003). Este efecto anorético también se ha observado con tratamientos periféricos (vía IP u oral) en otros teleósteos, tratándose de una acción específica bloqueada con antagonistas farmacológicos (Gélineau y Boujard, 2001; Penney y Volkoff, 2013; Rubio et al., 2008). El péptido CCK está implicado en las acciones anorexigénicas de otros reguladores de la ingesta en los peces. Así, la CCK podría mediar en parte las acciones de la leptina sobre la ingesta (Volkoff et al., 2003), además de actuar de forma sinérgica en los efectos anorexigénicos de la amilina (Hoskins y Volkoff, 2012; Thavanathan y Volkoff, 2006) y la apelina (Penney y Volkoff, 2013).

Una de las primeras acciones fisiológicas descritas para la CCK es su participación en la regulación de las secreciones y la motilidad gastrointestinal en vertebrados (Dufresne et al., 2006). En varias especies de teleósteos se ha demostrado su efecto estimulador de la contracción de la vesícula biliar (Aldman y Holmgren, 1995; Einarsson et al., 1997; Rajjo et al., 1988) y de las secreciones gástrica y pancreática (Murashita et al., 2008a; Volkoff, 2006). En relación con el efecto de la CCK sobre la motilidad gastrointestinal, en general se propone una influencia en el control del tránsito, reduciendo la tasa de vaciado gástrico (Olsson et al., 1999, Olsson y Holmgren, 2001), no obstante los escasos estudios publicados también muestran efectos estimuladores, dependiendo del modelo experimental empleado, la región del tracto gastrointestinal investigada y la especie en estudio. Así, se han encontrado efectos estimuladores contráctiles en preparaciones longitudinales del estómago cardíaco del bacalao del Atlántico (Jönsson et al., 1987) y en preparaciones circulares de la región pilórica del estómago del salmón real (*Oncorhynchus tshawytscha*) (Forgan y Forster, 2007), mientras que en la trucha arcoíris, la CCK-8S disminuye la frecuencia y la amplitud de las contracciones en la región cardíaca, sin afectar a la región pilórica del estómago (Olsson et al., 1999).

Otras acciones propuestas para la CCK sintetizada periféricamente incluyen la regulación neuroendocrina de la liberación de la GH en los teleósteos. Se ha descrito que la administración IP e ICV de la CCK-8S estimula la liberación de GH en la hipófisis del carpín (Himick et al., 1993), efecto que podría estar mediado por la somatostatina ya que la administración de CCK-8S disminuye la expresión de la prepro-somatostatina-I en el encéfalo de este teleósteo (Canosa et al., 2005; Canosa y Peter, 2004).

4. GHRELINA

4.1. ESTRUCTURA

La ghrelina es un péptido de 28 aminoácidos identificado por Kojima y colaboradores en 1999 como el ligando endógeno del receptor de la hormona de crecimiento. Este péptido cuenta con una modificación post-traduccional en el tercer aminoácido, serina, que presenta una esterificación por un ácido graso, siendo en la mayoría de los casos el ácido acil n-octanoico (Figura 4). La ghrelina se ha identificado en numerosas especies de vertebrados, habiéndose descrito diferentes formas de ghrelina que pueden variar tanto en el número de aminoácidos como en el ácido graso responsable de la esterificación de la serina (Kaiya et al., 2011), existiendo una alta conservación en los 7 primeros residuos N-terminales, responsables de la activación del receptor (Kojima et al., 1999; Matsumoto et al., 2001).

La estructura primaria de la ghrelina se ha determinado en diferentes especies de peces, como el carpín, la anguila japonesa (*Anguilla japonica*), la trucha arcoíris, la tilapia y el pez gato (Jönsson, 2013). En la mayoría de los teleósteos, además de la acilación en la serina, el extremo C-terminal se encuentra amidado (Kaiya et al., 2008; Kaiya et al., 2011). En el caso del carpín se han aislado recientemente 11 formas de ghrelina compuestas por un número diferente de aminoácidos (entre 14 y 19) con el extremo C-terminal generalmente libre (Miura et al., 2009). La principal forma circulante en esta especie es la secuencia octanoilada de 17 aminoácidos, aunque también se ha encontrado circulante una forma desacilada de ghrelina (Kaiya et al., 2008; Matsuda et al., 2006).

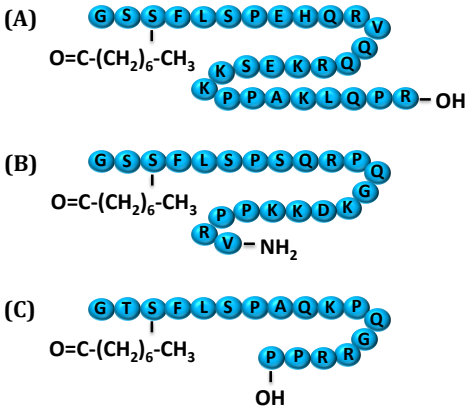


Figura 4. Estructura primaria de la ghrelina en (A) humano, (B) anguila y (C) carpín (forma octanoilada de 17 aminoácidos) (modificado de Kaiya et al., 2008 y Miura et al., 2009).

4.2. SÍNTESIS Y DISTRIBUCIÓN

La síntesis de ghrelina en todos los vertebrados estudiados se produce principalmente en las células endocrinas del estómago (Date et al., 2000; Jönsson, 2013; Rindi et al., 2002). En los peces que carecen de estómago, como el carpín, la mayor producción de ghrelina ocurre en el intestino (Unniappan et al., 2002). Una vez sintetizada la prepro-ghrelina tiene lugar un proceso de acilación que da lugar a la forma madura del péptido. La enzima responsable de esta reacción, la O-aciltransferasa (GOAT), miembro de la superfamilia de las O-aciltransferasas unidas a membrana (MBOATs), presenta su máxima expresión en la mucosa gástrica de los mamíferos (Gutierrez et al., 2008; Sakata et al., 2009; Yang et al., 2008) no conociéndose su distribución en los peces.

La ghrelina también se expresa en otros órganos y tejidos, aunque a niveles menores que en el estómago y el intestino. En los peces, por ejemplo, se ha encontrado ARNm de ghrelina a nivel periférico en el corazón, el páncreas, el hígado, el bazo y las branquias (Kaiya et al., 2008), y a nivel central principalmente en el hipotálamo y en el bulbo olfatorio (Jönsson, 2013; Kaiya et al., 2008).

La regulación de los niveles de ghrelina está estrechamente relacionada con la ingesta. De forma general en los vertebrados se ha detectado un aumento en los niveles circulantes de esta hormona inmediatamente antes de la ingestión de alimentos, experimentando posteriormente una caída posprandial (Kaiya et al., 2008, 2013b). También se han encontrado modificaciones periprandiales de la ghrelina circulante así como de su expresión génica en los peces, aunque esta relación entre la ghrelina y el estado nutricional resulta más ambigua a la vista de los diferentes resultados publicados en este grupo de vertebrados. En algunas especies el ayuno induce un incremento de los niveles circulantes de ghrelina y de la expresión hipotalámica e intestinal de prepro-ghrelina, como sucede en el carpín, la dorada, el pez cebra y la tilapia. En cambio, el ayuno produce un descenso en la ghrelina circulante en la trucha y la lota (*Lota lota*); sin alterar los niveles de ARNm de ghrelina en el intestino de la tilapia del Nilo (*Oreochromis niloticus*) y el bacalao del Atlántico (Jönsson, 2013; Volkoff et al., 2009a).

4.3. RECEPTORES

El receptor de ghrelina es miembro de la superfamilia de los receptores acoplados a proteínas G con 7 dominios transmembrana (Howard et al., 1996; McKee et al., 1997; Smith et al., 1999). En los mamíferos se han descrito las isoformas GHS-R1a, de 366 aminoácidos, que es la forma funcional, y la GHS-R1b, de 289 aminoácidos, cuya actividad

funcional no es bien conocida (Kaiya et al., 2013a). Ambas isoformas también existen en los vertebrados no mamíferos estudiados, aunque la complejidad de receptores existentes en los peces es mayor que la de los mamíferos debido a los procesos de duplicación del genoma y poliploidización que han experimentado algunos teleósteos. Así, en el caso del carpín, se ha descrito la isoforma del receptor GHS-Ra y sus subtipos 1a (GHS-R1a-1 y GHS-R1a-2) y 2a (GHS-R2a-1 y GHS-R2a-2). Todos los subtipos parecen ser funcionales a excepción del GHS-R2a-2, siendo la isoforma GHS-R1a la responsable de las acciones de la ghrelina en el metabolismo lipídico (Kaiya et al., 2010). Otra isoforma similar al GHS-R1a, GHS-R1a-LR (*like receptor*) se ha encontrado en un número limitado de peces pertenecientes a los órdenes *Perciformes*, *Salmoniformes*, *Gasterosteiformes* y *Tetraodontiformes* (Kaiya et al., 2013a). La isoforma GHS-R1b se ha observado en la trucha arcoíris, la tilapia y el pez gato (Kaiya et al., 2009a, 2009b; Small et al., 2009).

Generalmente, al igual que ocurre en los mamíferos (Gnanapavan et al., 2002; Guan et al., 1997; Howard et al., 1996), el receptor de ghrelina en los peces se expresa predominantemente en la hipófisis, seguido del cerebro, así como en los tejidos periféricos como el tracto gastrointestinal, el hígado y las gónadas, aunque su distribución parece depender de la especie y del subtipo de receptor estudiado (Jönsson, 2013; Kaiya et al., 2013a). En el carpín el subtipo 1a se detectó en el encéfalo, la hipófisis, el hígado, el intestino y el testículo, mientras que el 2a parece expresarse predominantemente en el encéfalo, el riñón y las gónadas de ambos sexos (Kaiya et al., 2010).

4.4. ACTIVIDAD BIOLÓGICA

La primera función descrita para la ghrelina fue la estimulación de la secreción de la GH en la hipófisis, siendo posteriormente identificada como un potente estimulador de la ingesta, incrementando la ganancia de peso corporal y la adiposidad en los mamíferos (Higgins et al., 2007; Kojima y Kankawa, 2005). Esta funcionalidad de la ghrelina también se ha demostrado de forma general en los peces (Jönsson, 2013; Kaiya et al., 2013a, 2013b). Es un potente orexigénico (tras administración aguda, IP e ICV) en el carpín (Kang et al., 2011a) y la tilapia (Riley et al., 2005). Sin embargo, en la trucha arcoíris se han publicado una variedad de efectos, orexigénico inducido por un tratamiento IP (Shepherd et al., 2007), anorexigénico en respuesta a un tratamiento ICV (Jönsson et al., 2010), o incluso sin efecto sobre la ingesta (Jönsson et al., 2007). Estas acciones de la ghrelina sobre la ingesta en los teleósteos y los mamíferos se ha sugerido que pueden ocurrir tanto de manera directa como indirecta (vía nervio vago) sobre núcleos cerebrales implicados en la regulación de la ingesta (Banks et al., 2002; Date et al., 2002; Lu et al., 2002; Pan et

al., 2006). Al igual que en los mamíferos (Cowley et al., 2003; Toshinai et al., 2003; Yamanaka et al., 2003), en el carpín también se ha implicado al NPY y la orexina-A en el efecto orexigénico de la ghrelina (Miura et al., 2006, 2007). A su vez, la ghrelina puede formar parte de los circuitos empleados por la melatonina, la MCH y la nesfatina-1 en la inhibición de la ingesta en esta especie (Jönsson, 2013).

En la actualidad existen pocos estudios que analicen los posibles efectos de tratamientos crónicos con ghrelina en los teleósteos, y con resultados variados. Así, se ha mostrado un incremento de la ingesta y de los depósitos hepáticos de grasa en la tilapia y el carpín tras 21 días de tratamiento con ghrelina (Kang et al., 2011b; Riley et al., 2005). Sin embargo, en la trucha arcoíris, 14 días de tratamiento reducen la ingesta, sin afectar a las reservas lipídicas abdominales, hepáticas y musculares (Jönsson et al., 2010). También se ha implicado a la ghrelina en la regulación de la homeostasis de la glucemia, existiendo en los peces una interacción bidireccional, ya que la administración aguda IP de glucosa aumenta significativamente los niveles de ARNm de ghrelina en el estómago (Riley et al., 2009), mientras que el tratamiento IP con ghrelina incrementa significativamente la glucosa plasmática (Schwandt et al., 2010). Otros datos a favor de la interacción ghrelina-glucosa es la expresión de ghrelina en los cuerpos de Brockman del pez cebra (Eom et al., 2013), y la activación de marcadores glucosensores en el cerebro de la trucha arcoíris tras la administración exógena de ghrelina (Polakof et al., 2011).

La ghrelina también parece estar implicada en el control de la actividad locomotora, si bien los resultados publicados son diversos dependiendo de la especie y la ruta de administración del péptido. Mientras que en la rata la administración central de ghrelina reduce la locomoción, y la periférica la aumenta (Jerlhag, 2008; Tang-Christensen et al., 2004), en los peces, concretamente en el carpín, ocurre lo contrario (Kang et al., 2011b; Yahashi et al., 2012). En la trucha arcoíris no parece estar involucrada en el control de la actividad locomotora (Jönsson et al., 2010).

Se han destacado otras funciones de la ghrelina, algunas de las cuales comparten los mamíferos y los teleósteos, como la regulación de la motilidad intestinal (Olsson et al., 2008; Peeters, 2005), la respuesta al estrés (Janzen et al., 2012; Stengel et al., 2011), en la funcionalidad reproductora (Muccioli et al., 2011; Unniappan y Peter, 2004) y del sistema circadiano (Lamont et al., 2013; Nisembaum et al., 2014).

5. OLEILETANOLAMIDA

5.1. ESTRUCTURA

Las aciletanolamidas (amidas de ácidos grasos, FAEs) son mediadores endógenos de naturaleza lipídica que incluyen, entre otros, a la oleiletanolamida (OEA), la AEA y la palmitoiletanolamida, (PEA) (Figura 5). La principal diferencia entre ellas reside en el ácido graso que incorporan en su posición amina, que en el caso de la OEA es el ácido oleico (Cadas et al., 1997; Schmid et al., 1996).

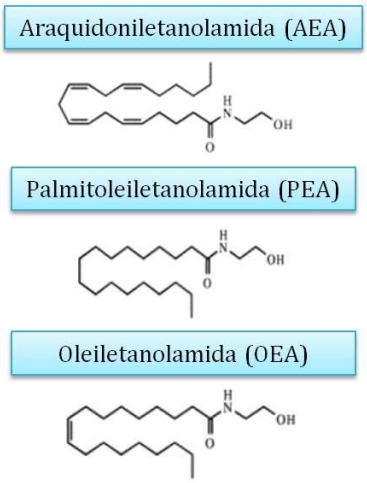


Figura 5. Estructura química de algunas aciletanolamidas (modificado de Lo Verme et al., 2005).

5.2. SÍNTESIS Y DISTRIBUCIÓN

La síntesis de OEA y otras FAEs es un proceso a demanda a partir de un precursor fosfolipídico de membrana. En los mamíferos, la ruta de síntesis consta de 2 reacciones bioquímicas. En la primera etapa se transfiere un grupo acilo a un grupo amino de la fosfatidiletanolamina catalizada por una N-aciltransferasa (NAT) dependiente de calcio cuya estructura molecular, en la actualidad, permanece sin identificar. Esta transferencia da lugar a la familia de las N-acilfostatidiletanolaminas (NAPEs). La segunda reacción es catalizada por una fosfolipasa-D dependiente de NAPE (NAPE-PLD), clonada e identificada como miembro de las zinc-metalohidrasas (Okamoto et al., 2004) que, en el caso de las NAPEs que contienen ácido oleico en su posición amina (N-oleil-fosfatidiletanolamina; NOPE), rendirán OEA por hidrólisis de su enlace fosfodiéster distal (Cadas et al., 1997; Schmid et al., 1996).

La degradación intracelular de la OEA se realiza por hidrólisis enzimática catalizada por las enzimas amidohidrolasa de ácidos grasos (FAAH; Cravatt et al., 1996) o la ácido amidasa de N-aciletanolamina (NAAA; Tsuboi et al., 2005), produciendo ácido oleico y etanolamida.

La OEA en los mamíferos estudiados presenta una amplia distribución tisular, encontrando en roedores los mayores niveles de esta FAE en el yeyuno, niveles intermedios en el tejido adiposo, el duodeno, el íleon, el pulmón, el estómago y el corazón; y bajas concentraciones en el músculo, el hígado, el colon, el riñón, el bazo (Fu et al., 2007). También se ha descrito la presencia de la OEA en distintas áreas encefálicas, como el tronco encefálico, el tálamo, el estriado, el cerebelo, la corteza cerebral, el hipotálamo y el hipocampo (Fu et al., 2007). El único vertebrado no mamífero en el que se han estudiado los niveles endógenos de la OEA en el tracto gastrointestinal es la serpiente pitón (*Phyton molurus*), donde los mayores niveles se localizan en el intestino delgado, seguido del estómago y el colon (Astarita et al., 2006).

Los niveles tisulares de OEA están regulados por la alimentación. Así, se ha demostrado que los niveles de OEA en el intestino delgado se reducen durante el ayuno, incrementando después de la realimentación en la rata (Fu et al., 2007; Petersen et al., 2006; Rodríguez de Fonseca et al., 2001), el ratón (Fu et al., 2007) y la serpiente pitón (Astarita et al., 2006). Esta movilización intestinal de OEA en respuesta a la alimentación parece iniciarse con la incorporación del ácido oleico derivado de la dieta a los enterocitos (en su zona apical) gracias a la acción de la translocasa de ácidos grasos CD36 (Drover et al., 2008), aumentando la producción de NAPEs con ácido oleico, precursoras de OEA (Fu et al., 2003; Guijarro et al., 2010; Schwartz, 2008, 2011). Además, se produce la activación de la NAPE-PLD, que acelera la liberación de OEA, así como la inhibición de la actividad FAAH, que favorece la acumulación de la OEA recién formada (Fu et al., 2007).

5.3. RECEPTORES

La OEA es un ligando endógeno del receptor activado por proliferadores de peroxisomas del subtipo alfa (PPAR-α), actuando como un agonista de alta afinidad, con valores de K_D e IC_{50} de aproximadamente 40 nM y 120 nM, respectivamente (Fu et al., 2003; Guzmán et al., 2004). El recetor PPAR-α es un factor de transcripción perteneciente a la superfamilia de receptores nucleares activados por ligandos, a la que también pertenecen los receptores de esteroides sexuales, glucocorticoides y hormonas tiroideas. Los PPARs se encuentran ampliamente distribuidos y controlan la expresión de genes

implicados en la síntesis y oxidación de ácidos grasos, estando involucrados en el almacenamiento de ácidos grasos en diferentes tejidos (principalmente el hígado, el corazón, el músculo y el riñón). En los peces se ha demostrado la existencia de las tres isoformas de PPARs conocidas en los mamíferos (PPAR-α, PPAR-β y PPAR-γ), con una distribución tisular similar (Carmona-Antoñanzas et al., 2014; Mimeault et al., 2006; Zheng et al., 2013).

La OEA, al igual que otras N-aciletanolaminas, puede activar otros receptores, como el ampliamente distribuido receptor de potencial transitorio activado por vanilloides-1, TRPV1 (Ahern, 2003; Almasi et al., 2008; Wang et al., 2005), y un receptor huérfano acoplado a proteínas G, el GPR119, con expresión mayoritaria en el tracto gastrointestinal (Godlewski et al., 2009; Overton et al., 2006). Aunque la expresión de ambos receptores, TRPV1 y GPR119, se ha demostrado en los peces (Fredriksson et al., 2003; Gau et al., 2013; Zimov y Yazulla, 2004), su posible papel en la regulación de la ingesta aún está por dilucidar.

5.4. ACTIVIDAD BIOLÓGICA

La movilización de la OEA intestinal en respuesta a la llegada del alimento (Astarita et al., 2006; Fu et al., 2007; Rodríguez de Fonseca et al., 2001) sugiere que esta molécula podría estar implicada en la regulación de la ingesta, actuando como un factor de saciedad. Dicha hipótesis ha sido confirmada en los roedores mediante estudios farmacológicos, que describen una reducción (dosis- y tiempo- dependientes) de la ingesta tras la administración periférica de OEA, cuyo efecto principal es prolongar el intervalo de tiempo entre comidas sucesivas (Fu et al., 2003; Gaetani et al., 2003; Nielsen et al., 2004; Oveisi et al., 2004; Rodríguez de Fonseca et al., 2001). En cuanto a los posibles mecanismos involucrados en su acción anorexigénica, diversas evidencias señalan que dicha acción está mediada por una estimulación vagal activadora del tronco cerebral y del hipotálamo (Fu et al., 2011; Gaetani et al., 2010; Rodríguez de Fonseca et al., 2001; Wang et al., 2005). Se ha demostrado en los roedores que la OEA activa la oxitocina en los núcleos paraventricular y supraóptico hipotalámicos (Gaetani et al., 2010; Romano et al., 2013), incrementa la expresión del CART en el núcleo paraventricular, y la actividad monoaminérgica hipotalámica (Serrano et al., 2011). A nivel periférico existen datos contradictorios en relación a los neuropéptidos gastrointestinales implicados, ya que algunos estudios en ratas han demostrado que la OEA incrementa los niveles plasmáticos de ghrelina y del péptido YY (Cani et al., 2004; Serrano et al., 2011), mientras que otros

estudios no encontraron dichas modificaciones asociadas a la reducción de ingesta inducida por la OEA (Proulx et al., 2005).

Además de su papel reductor de la ingesta a corto plazo, la OEA interviene en la regulación del peso corporal y del metabolismo lipídico. Se ha descrito que esta FAE reduce el peso corporal en roedores tanto tras un tratamiento subcrónico (1 semana; Rodríguez de Fonseca et al., 2001), como crónico (2 semanas, Fu et al., 2005; 4-5 semanas, Thabuis et al., 2010, 2011), atribuyéndose la reducción del peso observada no sólo a la menor ingesta inducida por la OEA, sino también a un efecto directo de esta FAE sobre el metabolismo lipídico (Lo Verme et al., 2005). En esta línea se ha propuesto que la OEA de forma general promueve la lipolisis e inhibe la lipogénesis (Matias et al., 2007; Thabuis et al., 2008; Pavón et al., 2010), disminuyendo los niveles de triglicéridos circulantes y aumentando los de ácidos grasos no esterificados y glicerol (Fu et al., 2005; Guzmán et al., 2004; Thabuis et al., 2010).

Otra función que parece desempeñar la OEA es la regulación de la actividad locomotora. Rodríguez de Fonseca y colaboradores (2001) observaron una reducción de la actividad locomotora coincidente en el tiempo con la reducción de la ingesta producida por la administración aguda de OEA en las ratas. Hasta el momento aún no se han dilucidado los mecanismos implicados en las acciones de la OEA sobre la actividad locomotora, pero la disminución de la actividad parece ser independiente de la acción hipofágica, ya que el tratamiento con capsaicina bloquea únicamente la acción anorética inducida por la OEA, sin afectar a la locomoción (Rodríguez de Fonseca et al., 2001). De manera cualitativa se ha observado que la administración IP de esta FAE disminuye la actividad ambulatoria y provoca una postura extendida en el suelo con los miembros posteriores extendidos, sin que este comportamiento pueda ser atribuido a dolor visceral (Proulx et al., 2005). La OEA, además, reduce en la rata las conductas estereotipadas como el aseo rostral (*grooming*) y la postura erguida en la prueba del campo abierto (Proulx et al., 2005).

Recientemente, se han descrito otras funciones de la OEA en los mamíferos, como su implicación en la consolidación de la memoria (Campolongo et al., 2009), modulación del estrés (Hill et al., 2009), estimulación del estado de alerta (Murillo-Rodríguez et al., 2007), inducción de muerte celular (Lueneberg et al., 2011), incremento de la liberación de dopamina (Murillo-Rodríguez et al., 2011), y se ha relacionado con la privación del sueño (Koethe et al., 2009) y el sistema circadiano (Murillo-Rodríguez et al., 2006). Hasta el momento se desconoce si la OEA también está implicada en algunas de estas funciones en los vertebrados no mamíferos.

6. ESPECIES DE ESTUDIO

6.1. EL CARPÍN DORADO (*Carassius auratus*)

El carpín dorado es un teleósteo de agua dulce perteneciente al Orden *Cypriniformes*, Familia *Cyprinidae*, Subfamilia *Cyprininae*. Los ciprínidos comprenden más de dos mil especies ampliamente distribuidas en el planeta, a excepción de Madagascar, Australia, Nueva Zelanda y América del Sur. El carpín comenzó a domesticarse en China hace más de mil años y se introdujo en Japón en el siglo XVI, desde donde fue exportado a Europa en el siglo XVII (Kottelat y Freyhof, 2007). Se cultiva principalmente como especie ornamental, aunque también son muy utilizados en investigación debido a su fácil manejo y sencillo mantenimiento, mostrando una rápida adaptación a las condiciones de laboratorio (Kottelat y Freyhof, 2007). Morfológicamente este ciprínido presenta un cuerpo macizo, con la aleta dorsal de base amplia y sin barbillones bucales. Originalmente de color pardo amarillento o verdoso con reflejos blanco-plateados por los costados y el vientre (Fotografía 1). Los adultos suelen medir entre 10 y 20 cm, pudiendo llegar a alcanzar 45 cm de longitud. El crecimiento por lo general es rápido, alcanzando la madurez sexual tiene a partir de los 8-10 meses, comenzando con puestas pequeñas, que a los 3-4 años pueden alcanzar los 170.000-380.000 huevos. La freza se produce entre los meses de mayo y julio, aunque puede variar dependiendo de la temperatura del agua (Muus y Dahlström, 1981).

Fotografía 1. Ejemplar adulto de carpín dorado.

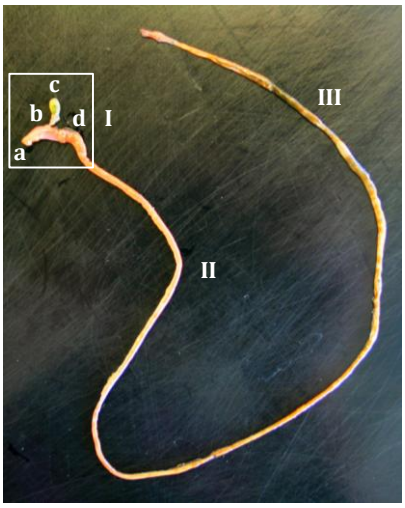
Es un animal omnívoro y bentónico, que en su hábitat natural se alimenta fundamentalmente de organismos planctónicos (pequeños crustáceos y moluscos), gusanos, larvas de insectos y plantas acuáticas (Muus y Dahlström, 1981). En condiciones de laboratorio selecciona para su dieta principalmente carbohidratos, seguido de grasas y proteínas (Sánchez-Vázquez et al., 1998). En su comportamiento alimentario, es característica la natación constante buscando y persiguiendo su comida, si bien en

condiciones de horarios fijos de alimentación presenta una marcada FAA (Aranda et al., 2001; Sánchez-Vázquez et al., 2001), como un aumento de la actividad locomotora en las 2-3 h previas a la hora de administración del alimento.

Carassius auratus es, en general, un teleósteo diurno, con máximos de actividad locomotora durante la fotofase del ciclo diario (Aranda et al., 2001), aunque también puede presentar patrones de actividad nocturna (Iigo y Tabata, 1996). En la última década se ha utilizado extensamente como modelo de experimentación en estudios cronobiológicos, en buena medida por la gran plasticidad que muestra su sistema circadiano. De forma particular nuestro grupo de investigación ha demostrado la relevancia de señales ambientales, como el fotoperiodo y el horario de alimentación, en el funcionamiento general de su sistema circadiano, y en particular en la sincronización de su actividad locomotora (Feliciano et al., 2011; Vera et al., 2007; Vivas et al., 2011).

6.1.1. El tracto gastrointestinal de *Carassius auratus*

El tracto digestivo del carpín se divide en las siguientes cuatro regiones: una porción anterior que incluye la boca y la faringe, el intestino anterior, el intestino medio y el intestino posterior (Fotografía 2) (Kapoor et al., 1975; McVay y Kaan, 1940). La parte más anterior del tubo digestivo está conectada con la musculatura de la cabeza y contiene los dientes faríngeos (cuatro filas a cada lado) unidos a los huesos faríngeos posteriores. El carpín, como la mayoría de los peces, no produce enzimas digestivas en esta región del tracto digestivo. El intestino anterior se inicia en el borde branquial posterior e incluye el esófago, el bulbo intestinal (que se extiende hasta la apertura del conducto biliar) y una parte del intestino propiamente dicho que se inicia justo tras la vesícula biliar al que se le denomina intestino proximal. La característica más notable de esta especie es la ausencia de estómago. Esófago e intestino se unen mediante el esfínter intestinal, que desemboca en el bulbo intestinal, región que no presenta diferencias histológicas con el intestino propiamente dicho, del que se diferencia por su mayor tamaño relativo y mayor grosor de las capas mucosas, lo que permite una gran expansión del mismo. Es en el bulbo intestinal donde se realiza el vertido de las secreciones biliares, marcando el inicio de la digestión enzimática. El intestino propiamente dicho se caracteriza por su gran longitud, típica de su alimentación, que permite distribuir el alimento a lo largo de toda la superficie intestinal, maximizando los procesos de digestión y absorción. El intestino se encuentra enormemente plegado y está separado completamente de la vejiga natatoria, ocupando la porción ventral del cuerpo del animal. La última porción intestinal es el recto, de menor grosor que el resto del tubo y sin pliegues, comunicándose con el exterior a través del ano.



Fotografía 2. El tracto digestivo de *Carassius auratus*. (I) Intestino anterior: (a) esófago, (b) bulbo intestinal, (c) vesícula biliar, (d) intestino proximal; (II) intestino medio; (III) intestino posterior.

6.2. LA TRUCHA COMÚN (*Salmo trutta*)

La trucha común es un teleósteo perteneciente al Orden *Salmoniforme*, Familia *Salmonidae*, Subfamilia *Salmoninae*. Es una especie autóctona de Europa, norte de África y Asia occidental, y tras haber sido introducida en al menos 24 países fuera de Europa, en la actualidad se puede afirmar que es una especie distribuida a nivel mundial (Klemetsen et al., 2003). Según la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) es probablemente la primera especie de teleósteo que se cultivó en cautividad, comenzando su cultivo en 1739 en Alemania. Actualmente, los mayores productores de trucha común son Rusia, seguido de Italia y Rumania, destinándose una gran parte de la producción a la repoblación de poblaciones silvestres. Es un pez de aspecto fusiforme, ligeramente comprimido lateralmente y dotado de un pedúnculo caudal ancho y fuerte. La aleta adiposa, característica común de la familia *Salmonidae*, es pequeña y surge más próxima al origen de la aleta caudal que del borde posterior de la aleta dorsal (Fotografía 3). Su morfología varía mucho en función del hábitat en que se encuentre, habiéndose descrito alrededor de 50 variedades fenotípicas (Behnke 1986; Pakkasmaa y Piironen, 2001), con diferencias en el color (tanto entre poblaciones como dentro de las mismas), en el tamaño (Ojanguren y Braña, 2003) y en algunos caracteres sexuales secundarios (Klemetsen et al., 2003).

Fotografía 3. Ejemplar adulto de trucha común.

En función del tipo de ciclo vital que desarrollen, los ejemplares de trucha común pueden clasificarse en dos grupos: los migradores y los residentes. Los individuos migradores se dirigen hacia el mar, donde permanecerán la mayor parte de su vida (son diádromos, anádromos), mientras que los residentes migran hacia zonas más bajas de los ríos (son potamodromos) (Hendry et al. 2003; Klemetsen et al., 2003). En poblaciones con acceso al mar, se ha descrito que la decisión de migrar al mar podría estar controlada por factores genéticos y ambientales (Giger et al., 2006; Jonsson, 1982; Wysujack et al., 2009). En otoño, tanto residentes como migradores regresan a las zonas de puesta para desovar. La edad de maduración varía en función del sexo y hábitat, así los residentes necesitan de 1 a 10 años, mientras que los ejemplares migradores tras 2-3 años en el mar están preparados para el desove (Klemetsen et al., 2003).

La trucha común es una especie carnívora oportunista, sin embargo durante su ciclo de vida, en función del tamaño, sexo y estado de desarrollo, se puede especializar temporalmente en determinados alimentos. En sus primeras etapas comen en la superficie y aguas poco profundas, mientras los adultos se alimentan en aguas profundas (Klemetsen et al., 2003). Durante sus primeros años de vida, la trucha común presenta un comportamiento territorial muy marcado con la finalidad de tener el mayor acceso a la comida (Johnsson et al., 1999), llegando incluso a formar grupos jerárquicos dependiendo de la densidad de población (Brockmark y Johnsson, 2010). En condiciones de laboratorio, presenta un patrón de actividad alimentaria característico de los salmónidos, mostrando dos incrementos estrechamente relacionados con las horas de luz, uno al inicio de la fase luminosa y otro al final (Boujard et al., 2007).

Los salmónidos, incluida la trucha común, han sido ampliamente utilizados en investigación, debido tanto a su interés comercial como ecológico, con el fin de optimizar las técnicas de cultivo para producción y dirigidas a la obtención de ejemplares para repoblación (Kallio-Nyberg et al., 2010; Klemetsen et al., 2003).

III. OBJETIVOS

Numerosos estudios realizados en especies pertenecientes a distintas clases de vertebrados confirman que los mecanismos básicos que regulan el comportamiento alimentario se encuentran muy conservados en la filogenia animal. De forma general se acepta que la ingestión de alimento en los peces está regulada por un sistema central que actúa en coordinación con un sistema periférico, integrando señales tanto endógenas como externas o ambientales. Sin embargo, a pesar de los recientes avances realizados en investigación, nuestro conocimiento actual sobre la regulación de la ingesta en los teleósteos aún es limitado, y sólo nos permite elaborar un modelo simplificado y relativamente incompleto.

En los próximos años se prevé que el sector acuícola desarrolle un gran potencial de crecimiento y, teniendo presente el estancamiento de la actividad extractiva, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) estima que antes de 2030 más del 65% de los alimentos acuáticos procederán de la acuicultura. En la industria del cultivo intensivo de peces el gasto en alimentación representa uno de los mayores costes económicos, por lo que la sostenibilidad de la producción piscícola requiere necesariamente del diseño de estrategias de alimentación más eficaces y que, además, minimicen el impacto medioambiental que pueden ocasionar estas explotaciones.

Dada la importancia, tanto desde el punto de vista científico como económico, que tiene el conocimiento y la comprensión de los sistemas que regulan el apetito en los peces, el objetivo general de la presente Tesis Doctoral es profundizar en el estudio de la regulación neuroendocrina de la ingesta en teleósteos, utilizando dos modelos animales comúnmente empleados en investigación, un ciprínido, el carpín y un salmónido, la trucha común. Para ello, hemos centrado nuestra atención en el estudio de señales periféricas que pueden desempeñar tanto funciones anorexigénicas como orexigénicas en estas especies, y cuyo origen es, mayoritariamente, el tracto gastrointestinal. Concretamente, hemos investigado distintos aspectos fisiológicos y comportamentales de varios reguladores neuroendocrinos, algunos de ellos más conocidos por su implicación en la alimentación de los peces, como la leptina, la ghrelina y la colecistocinina; y otros, como la oleiletanolamida, cuya participación en la regulación de la alimentación se ha descrito recientemente en los mamíferos pero se desconoce en los peces.

En el contexto de este objetivo general, en la presente Tesis Doctoral se abordan los siguientes objetivos específicos:

• Caracterizar varios componentes esenciales del sistema de leptina en el carpín, (los parálogos leptina-aI y aII, y su receptor) mediante el estudio de los patrones de distribución tisular central y periférica. Investigar los efectos de la alimentación sobre dicho sistema, analizando si la leptina actúa como una señal a corto y/o a largo plazo en la regulación de la ingesta en el carpín.

• Determinar el perfil diario de expresión de las leptinas y su receptor en el hígado y en el encéfalo del carpín en animales expuestos a condiciones fotoperiódicas 12L/12D y un horario fijo de alimentación diaria. Además investigamos el posible efecto sincronizador del horario de alimentación sobre los ritmos diarios de expresión de leptina observados.

• Estudiar la actividad de la CCK-8S en la regulación de la contractilidad intestinal del carpín en un modelo *in vitro* de baño de órganos. Mediante aproximaciones farmacológicas, investigar posibles rutas de señalización que pueden intervenir en la acción contráctil de este regulador y caracterizar los receptores implicados en dichas acciones.

• Determinar los posibles efectos de la administración subcrónica, mediante implantes, de ghrelina en la ingestión de alimento, el crecimiento, el metabolismo lipídico, la actividad locomotora y el comportamiento agresivo en ejemplares salvajes de trucha común, profundizando en las posibles interacciones con otros reguladores, como las monoaminas y el NPY.

• Investigar el papel de la OEA en peces, estudiando su distribución tisular y posible regulación por el estado nutricional. Evaluar el posible efecto de la administración periférica aguda de este mediador lipídico en la ingesta, la actividad locomotora y niveles de metabolitos plasmáticos, determinando las posibles interacciones con otros reguladores centrales (NPY, orexinas, monoaminas, leptina) y periféricos (CCK, leptina y ghrelina) de la ingesta en teleósteos.

Los dos últimos objetivos se han desarrollado dentro del programa de formación del personal investigador financiado por el Ministerio de Economía y Competitividad mediante la realización de estancias breves de Ana Belén Tinoco Pérez en centros de investigación extranjeros: en el Departamento de Ciencias Biológicas y Ambientales de la Universidad de Gotemburgo (Suecia), con la dirección del Dr. Jörgen I. Johnsson, y en el Departamento de Descubrimiento y Desarrollo de Fármacos del Instituto Italiano de Tecnología de Génova (Italia), con la dirección del Dr. Daniele Piomelli.

IV. RESULTADOS/RESULTS

Capítulo 1

El sistema de la leptina en el carpín (*Carassius auratus*): distribución tisular, regulación por la alimentación, ritmicidad diaria y su sincronización al horario de alimentación

1.1. *Leptin and leptin receptor expression in the goldfish (Carassius auratus). Regulation by food intake and fasting/overfeeding conditions.*
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1.2. *Leptin expression is rhythmic in brain and liver of goldfish (Carassius auratus). Role of feeding time.*
***General and Comparative Endocrinology* 2014, 204: 239-247**

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Leptins and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions

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ABSTRACT

Leptin is a hormone involved in feeding and body weight regulation in vertebrates, but the relationship between energy status and leptin has not been clearly established in fish. The aim of this study was to investigate in a teleost, the goldfish (*Carassius auratus*), the tissue expression pattern of two leptins (*gLep-aI* and *gLep-aII*) and leptin receptor (*gLepR*); and the effect of feeding on expression of these genes. Leptin system expression in goldfish was firstly analyzed in fish under overfeeding (2 weeks) or fasting (1 week), and secondly, at different postfeeding times (0, 3, 6, 9 and 12 h). Goldfish has two Lep-a paralog genes, *gLep-aI* was widely expressed in central and peripheral tissues, whereas *gLep-aII* was preferentially expressed in brain. This different distribution pattern of leptins suggests that they can play different physiological roles in goldfish. The *gLepR* mRNA was ubiquitous expressed, with the highest expression in the telencephalon and hypothalamus. No significant differences in the leptin system expression were found among control, overfed and fasting groups, suggesting an apparent lack of correlation between nutritional status and leptin system in goldfish. Hepatic expression of *gLep-aI* significantly increased 9 h after feeding time, while hypothalamic leptin system expression did not change after feeding. In summary, leptin in goldfish could signal short-term changes in food intake, as postprandial satiety, but seems to be independent of fasting/overfeeding conditions in this teleost. The widespread distribution of leptins and leptin receptor in goldfish strongly supports that this hormone may have pleitropic actions in fish.

1. Introduction

Leptin, a product of the obese (*ob*) gene discovered in mouse by Zhang et al. in 1994 [40], has been related with food intake and energy balance in mammals [8,32]. In fish, the presence of a leptin-like peptide was first evidenced by immuno-cross-reactivity [14], and its existence was certainly demonstrated after the finding by synteny of a leptin sequence in the pufferfish (*Takifugu rubripes* [19]). Since then, and in accordance with the well known genome duplication in teleosts, the presence of diverse leptin genes have been evidenced in several fish species [2,5]. Two leptins are present in some fish as the Japanese medaka (*Oryzias latipes*), *mLEP-A* and *mLEP-B* [17], and the zebrafish (*Danio rerio*), *leptin-a* and *leptin-b* [7]. However, certain fish species, as the pufferfish, could lack leptin-b because an ulterior gene loss [2]. In fact, the majority of the fish leptins cloned are leptin type a [2], with only one leptin-a to date reported in the Artic charr (*Salvelinus alpinus* [6]), the silver carp (*Hypophthalmichthys molitrix* [21]) and the grass carp (*Ctenopharyngodon idellus* [21]); and two leptin-a paralogs found in the common carp (*Cyprinus carpio*; *leptin-I* and *leptin-II* [11]) and in the Atlantic salmon (*Salmo salar*, *sLep-A1* and *sLep-A2* [30]). To date, differences in location, regulation and functions of such diverse fish leptin genes have been poorly studied, particularly in central tissues.

Fish leptins show less than 30% of similarity with tetrapods leptins, in accordance with the low level of primary sequence conservation of this peptide. However, it seems that the secondary and tertiary structure of the protein is highly conserved [5]. In contrast to mammals, and similarly to other ectotherm vertebrates, there is none or a very modest expression of leptin in fish adipose tissue [2,11]. The highest expression of leptins is found in the liver, an important adipose store in fish [6,11,16,19,27]. High levels of leptin expression have also been located in brain, gonads, gastrointestinal tract and spleen [7,16,17,30].

Leptins act through a variety of receptors (LepR) that have been identified in mammals [34], birds [10] and amphibians [3]. Several LepR forms exist in mammals, one long form found in several hypothalamic nuclei, and five widely distributed short isoforms [39]. In fish, several studies described LepR, and the central and peripheral distribution of its expression [1,17,18,22,30,38]. To date,

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only one LepR gene has been found in fish, and different isoforms have been identified in two species, the Atlantic salmon (*S. salar* [30]) and the crucian carp (*Carassius carassius* [1]), being the long form the only one that conserves all functionally important domains as in mammals [30,39].

Leptin and adiposity are closely related in mammals, where elevated circulating leptin levels signal positive energy balance, agreeing with the anorectic role of this hormone [5,8,32]. However, the relationship between energy status and leptin has not been clearly established in fish. Plasma leptin levels were reduced by 2 weeks fasting in green sunfish, *Lepomis cyanellus* [14] and burbot, *Lota lota* [28], but increased in rainbow trout (*Oncorhynchus mykiss*) fasted for 3 weeks, from the first week onwards [15]. Recently, different results have also been reported on the possible relationship between energy status and tissue leptin mRNA expression in fish. Short-term fasting (1 week) reduced liver *leptin-b* in zebrafish, but not modified *leptin-a* [7]. Atlantic salmon reared on a restricted feeding diet for 10 months showed a reduction in *sLepA1* mRNA expression in visceral adipose tissue, while hepatic *sLepA2* increased, without changes in plasma leptin levels [30]. Expression of both genes in liver and circulating leptin were raised in Atlantic salmon under 7-weeks diet restriction [35]. However, hepatic leptin expression in common carp was not affected by either 6-days or 6-weeks fasting, whereas an acute and transient postprandial increase was found [11]. Recent results in catfish (*Ictalurus punctatus*) have shown that prolonged fasting (30 days) did not modify mRNA leptin in liver and brain [16]. To date, little information exist on feeding regulation of the *LepR* expression in fish, finding a reduction [35] or not changes [30] in Atlantic salmon brain. In summary, all these studies in different fish species has led to inconsistent outcomes at present, and further investigation is required to clarify the leptin functions in fish and to deep into the knowledge of the evolution of leptin physiology.

In goldfish (*Carassius auratus*), injection of human leptin reduces food intake and body weight [4,36,37], but nothing is known about possible changes in expression of leptins and LepR by feeding status. Thus, in the present work we firstly studied the expression pattern of different *leptins* and *LepR* in goldfish central and peripheral tissues. Moreover, we have investigated the possible changes in leptin system expression induced by feeding. To this end, we studied the possible effect of fasting and/or overfeeding on leptins expression in peripheral (liver) and central tissues (hypothalamus), and on leptin receptor expression in brain (hypothalamus and telencephalon). Moreover, postfeeding changes of these genes in liver and hypothalamus were also investigated.

2. Materials and methods

2.1. Fish and housing

Goldfish (15–20 g, body weight, bw) were obtained from a commercial supplier and reared at 21 ± 2 °C in aquaria (60 l) with a constant flow of filtered water, under 12 h light:12 h dark (12L:12D) photoperiod (lights on at 08:00). Fish were fed once daily with a 1% bw with dry pellets (Sera Pond Bio-granulat, Sera Biogram, Germany) at 10:00. Fish were maintained under these conditions for at least 15 days prior to the experimental use. All the fish handling procedures were approved by the Animal Ethics Committee of Complutense University of Madrid, and were in accordance with standards specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and in the Guidelines of the European Union Council (2010/63/EU) for the use of research animals.

2.2. Sequences analysis

Available nucleotide sequences of goldfish *leptins* (GenBank ID: FJ534535 and FJ854572), and goldfish *LepR* (GenBank ID: EU911005) were compared with other vertebrate sequences using the *ClustalW* (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The phylogenetic trees were constructed based on amino acid difference (p-distance) with neighbor-joining algorithm using the *Clustalx2 2.0.12* software [20] and the *Dendroscope 2.5* software [12]. The reliability of the trees was assessed by bootstrapping using 1000 replications.

2.3. Experimental designs

2.3.1. Tissular distribution and expression of leptins and leptin receptor

For tissular distribution of goldfish leptins and leptin receptor transcripts, fish were immersed in iced water (4 °C) and sacrificed by decapitation at 10:00. The sampling day fish did not receive food, corresponding to 24-h fasting. Central tissues (retina, telencephalon, hypothalamus, optic tectum, cerebellum and pituitary gland) and peripheral tissues (heart, gill, muscle, spleen, adipose tissue, foregut, hindgut, liver, gonads and kidney) were quickly dissected out, frozen on dry ice and stored at −80 °C until RNA extraction.

2.3.2. Effect of fasting and overfeeding on leptin system

To determine the effect of different feeding regimes on *leptins* and *LepR* transcripts abundance, fish were divided into three experimental groups ($n = 5$/group): control feeding (1% bw per day at 10:00 for 15 days), overfeeding (6% bw per day, distributed in three meals at 08:30, 14:30 and 19:30, for 15 days), and fasting (food deprivation for the last 7 days of the experiment). Animals were weighed at days 1, 7 and 14, and the amount of food adjusted to the bw mean of the group. Fish did not receive food on sampling day. Fish were sacrificed at 10:00 as previously described, and telencephalon, hypothalamus and liver were quickly dissected, frozen and stored at −80 °C.

2.3.3. Postfeeding changes on leptin system

To investigate possible changes in the expression of *leptins* and *leptin receptor* after the last meal fish maintained for 2 weeks under housing conditions (2.1) were sacrificed at the following postfeeding times: 0, 3, 6, 9 and 12 h ($n = 8$/sampling point). For the first sampling, fish were sacrificed at 10:00, just before food delivery, and then, it corresponds to 24-h fasting. The hypothalamus and liver were quickly dissected out, frozen on dry ice and stored at −80 °C.

2.4. RNA isolation, cDNA synthesis and quantification of leptins and leptin receptor expression

Total RNA from goldfish tissues was extracted with Trizol (TRI® Reagent method, Sigma Chemical, Madrid, Spain) and treated with DNase (Promega, Madison, USA) at 37 °C for 40 min to eliminate genomic DNA. Then, 1 μg of RNA was retro-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA), excepting for the hypothalamus in the postfeeding changes experiment where 0.25 μg of RNA was retro-transcribed. The protocol was provided by the manufacturer and included the following steps: (1) preincubation of RNA with random primers (5 min at 65 °C); (2) addition of dNTPs, first strand buffer, DTT and RNAsin (2 min at 42 °C); (3) addition of the SuperScript II Reverse Transcriptase (50 min at 42 °C) and (4) inhibition of the enzyme (15 min at 70 °C).

Table 1
Primer sequences and corresponding accession numbers.

Target gene	Accession number	Primer	Sequence 5′ → 3′	Product (pb)
gLep-aI	FJ534535	Forward	AGCTCCTCATAGGGGATC	192
		Reverse	TAGATGTCGTTCTTTCCTTA	
gLep-aII	FJ854572	Forward	CTCCAAAAATCTTCATTGATCC	195
		Reverse	AGGATTTCATTCTATCTTTC	
gLepR	EU911005	Forward	GCTAGCTGTGTGCATCTTTCC	159
		Reverse	AGATCTCTCGCTGTGGACTGA	
18Sr RNA	EF100727	Forward	ATAGATTAAGAGGACGGCCGG	143
		Reverse	TGATCGTCTTCGAACCTCCGAC	
β-actin	AB039726	Forward	GGCCTCCCTGTCTATCTTCC	156
		Reverse	TTGAGAGGTTTGGGTTGGTC	

Relative expression of goldfish *leptins* and *LepR* was assessed by qRT-PCR in a CFX96^{TM} Real-Time System (Biorad Laboratories, Hercules, USA). Due to the similarity of both leptins sequences, specific primer design was hand made using an alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to identify and select divergent parts between them (Table 1). The *gLepR* primers (Table 1) were designed using the software *Primer3* [31]. Reactions were carried out in a final volume of 20 μl containing: 10 μl of iTaq SYBR Green Supermix (Biorad Laboratories, Hercules, USA), specific primers (0.4 μM; Sigma Chemical, Madrid, Spain) and 1 μl of the cDNA synthetized as above described. The protocol used to measure leptins expression was: 1 cycle at 95 °C for 3 min and 40 cycles consisting in 95 °C for 10 s, 55 °C for 30 s and 72 °C for 45 s. In the case of *gLepR*, a similar protocol was used with the exception of the annealing temperature that was 60 °C. The ΔΔCt method [24] was used to determine the relative mRNA expression. The 18Sr RNA (for the two first experiments) or β-actin (in the postfeeding changes experiment) of goldfish (GenBank ID: EF100727 and AB039726, respectively) were used as reference genes. The efficiency of the amplification for all genes studied was around 100%. Negative controls included replacement of cDNA by water and the use of non-retrotranscribed total RNA. At the end of each reaction a melting curve was performed (in the range of 65–95 °C with 0.5 °C increments). The specificity of the amplification reactions was confirmed by the melting temperature, the size of the obtained PCR products, and by cutting with restriction enzymes. To this end 2 μl of the PCR products were digested by selected restriction enzymes (AfI II: Takara Bio Inc., Otsu, Japan; and Nde II: Promega, Madison, USA) during 2 h at 37 °C. After that, PCR digested and non-digested products were visualized in an agarose gel to determine if the size of the products were the expected ones using a molecular weight marker (RBMM2, Real, Durviz, Paterna Spain).

2.5. Statistics

Statistics analyses were performed with the software *Statgraphics Plus version 5.1*. Significant differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keul's (SNK) multiple comparison test. When necessary, values were transformed (logarithmic transformation) to get a normal distribution and homogeneity of variances. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Phylogeny of leptins and leptin receptor

Available leptin sequences of goldfish codify for two complete proteins that were compared with other vertebrate leptins. During the preparation of the present manuscript, two articles on fish leptins phylogeny (including goldfish leptins sequences used in this work) have been published [2,5], and then we do not show the phylogenetic tree obtained. Each sequence of goldfish *leptin* showed high similarity (85%) with each one of the carp leptins (called carp *leptin-I* and carp *leptin-II*; GenBank ID: AJ830744 and AJ830745, respectively). Thus, we renamed the goldfish leptins sequences as suggested Copeland et al. [2]: goldfish *leptin type aI* (*gLep-aI*; GenBank ID: FJ534535), and goldfish *leptin type aII* (*gLep-aII*; GenBank ID: FJ854572).

The partial *gLepR* sequence encodes for a protein fragment of 206 amino acids. The phylogenetic tree supports the orthology among vertebrates (Fig. 1). This fragment of the goldfish leptin receptor shares an identity of 82% with the zebrafish (from the same Cyprinidae family) and lower identity with LepR from other fish families, as the Atlantic salmon (Salmonidae), the pufferfish and the marine medaka, *Oryzias melastigma* (Acanthopterygii), with respectively 45%, 39% and 38% values. Lower identity values were observed between *gLepR* and the *LepR* of tetrapods: western clawed frog (*Xenopus tropicalis*), chicken (*Gallus gallus*), pig (*Sus scrofa*) and human (29%, 34%, 33% and 34%), respectively.

3.2. Distribution of goldfish leptins and leptin receptor gene expression

Goldfish leptins showed a differential expression pattern (Fig. 2A). The *gLep-aI* was mainly expressed in gonads (ovary) and liver, showing moderated expression in other peripheral locations as kidney, foregut, hindgut and spleen, as well as in the brain, the neural retina and the pituitary gland. By contrast, *gLep-aII* was preferentially expressed in brain, particularly in optic tectum, cerebellum, telencephalon and hypothalamus, while lower expression of *gLep-aII* was detected in the gonads (ovary) and the liver. *gLep-aII* was not detected in the retina, pituitary gland, spleen, foregut,

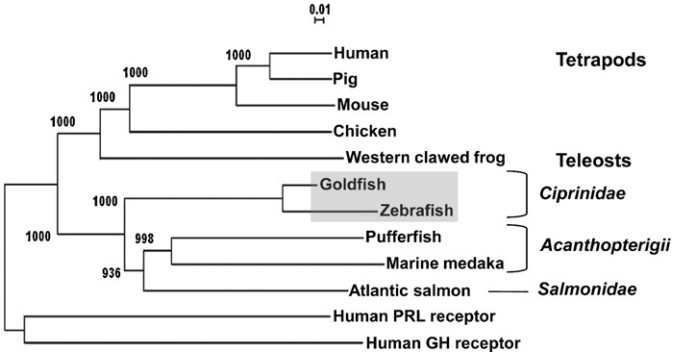

Fig. 1. Neighbor-joining phylogram of fish leptin receptor amino acid sequences. Branch lengths reflect evolutionary divergence. Prolactin and growth hormone receptors, from the same cytokine family of leptin receptor, were included as an outgroup. The GenBank ID of the used sequences were as follows: human, AAA93015.1; pig, ACT52816.1; chicken, BAA94292; mouse (*Mus musculus*), P48356; Western clawed frog, NP_001037866.1; zebrafish, NP_001106847; goldfish, ACG69477.1; pufferfish, BAG67079.1; marine medaka, ABC86922; Atlantic salmon, BAI23197.1; human prolactin receptor, P16471.1; and human growth hormone receptor, P10912.

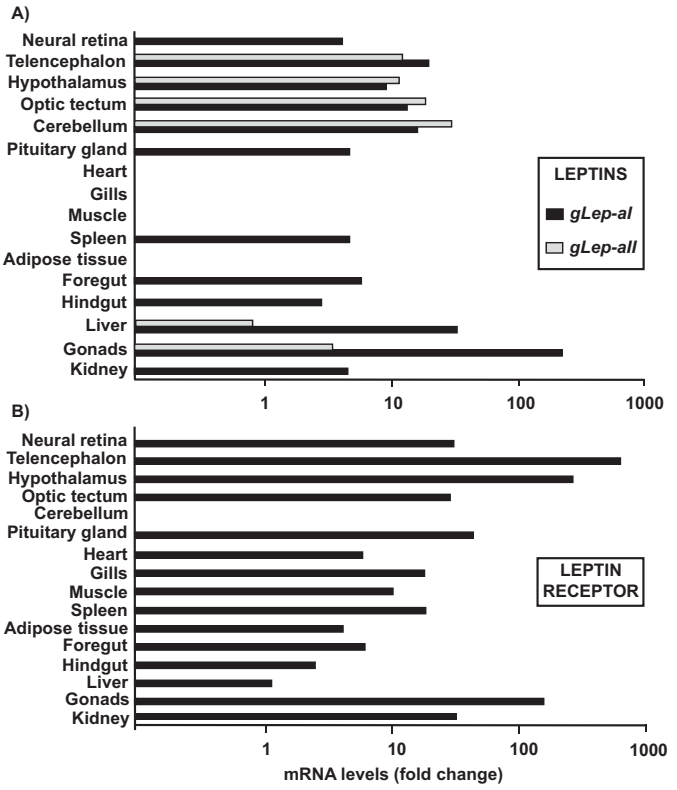

Fig. 2. Distribution pattern of (A) *gLep-aI*, *gLep-aII*, and (B) *gLepR* transcripts in goldfish tissues. Relative expression has been calculated from qRT-PCR data using the ΔΔCt method. Data represent the relative fold increase respect to the lowest mean expression (liver). Note logarithmic scale of the *x*-axis.

hindgut nor kidney (with 40 cycles of amplification). Indeed, no expression of leptins was detected (with 40 cycles of amplification) in the heart, gills, muscle and adipose tissue of goldfish.

The *gLepR* mRNA exhibited an abundant expression in the telencephalon, hypothalamus and gonads (ovary) of goldfish; moderated expression in the optic tectum, pituitary gland, retina, spleen, heart, gills, muscle, kidney, foregut and adipose tissue; and low expression in the hindgut and liver (Fig. 2B). The cerebellum did not show any *gLepR* expression (with 40 cycles of amplification).

3.3. Overfeeding and fasting on leptins and LepR mRNA expression in goldfish

The overfeeding of goldfish for 2 weeks significantly ($P < 0.05$) increased body weight compared with control and fasted fish, as expected (Table 2). However, 1 week of fasting did not produce statistically significant differences in body weight respect to the control group, although there was a tendency to decrease.

Fig. 3 summarizes the relative expression of leptin transcripts in the three experimental groups in liver and hypothalamus. In the liver the *gLep-aI* mRNA abundance showed a tendency to increase in both, the overfeeding and fasting groups, compared to control fish, although there were no statistically significant differences (Fig. 3A).

Table 2
Effect of overfeeding (2 weeks) and fasting (1 week) on goldfish body weight (g).

Day	Control	Overfed	Fasting
1	20.41 ± 0.73	20.40 ± 0.68	20.23 ± 0.39
7	21.22 ± 0.91	26.21 ± 1.52*	20.74 ± 0.57
14	20.66 ± 0.86	28.76 ± 1.71*	19.60 ± 0.57

Data are presented as mean ± S.E.M. ($n = 5$).
* $P < 0.05$.

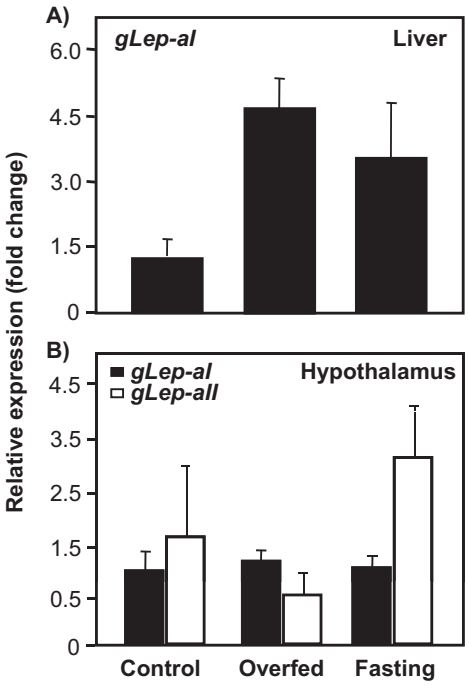

Fig. 3. Relative expression of goldfish leptins in (A) liver (*gLep-aI*) and (B) hypothalamus (*gLep-aI* and *gLep-aII*) after two weeks of overfeeding and 1 week of fasting. Relative expression has been calculated from qRT-PCR data using the ΔΔCt method. Data represent the mean ± S.E.M. ($n = 5$) of the relative fold change with respect to the control group.

The *gLep-aI* mRNA expression in hypothalamus was very similar in the three experimental groups (Fig. 3B). The *gLep-aII* mRNA was quantified only in the hypothalamus due to its low expression in the liver, and no statistically significant differences were found (Fig. 3B).

The *gLepR* expression in the hypothalamus and the telencephalon of goldfish under overfeeding or fasting is shown in Fig. 4. There were not statistically significant differences among

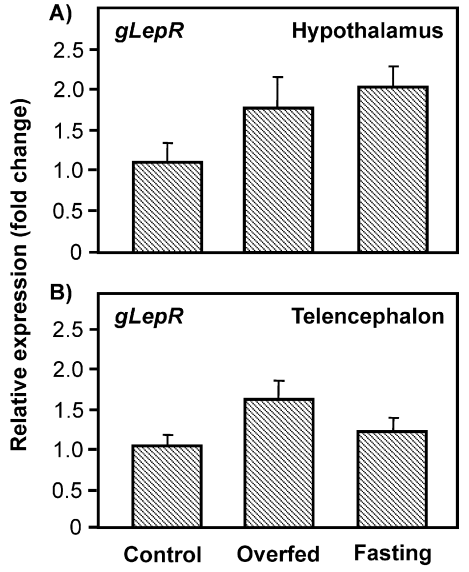

Fig. 4. Relative expression of goldfish *LepR* in (A) hypothalamus and (B) telencephalon after two weeks of overfeeding and 1 week of fasting. Relative expression has been calculated from qRT-PCR data using the ΔΔCt method. Data represent the mean ± S.E.M ($n = 5$) of the relative fold increase with respect to the control group.

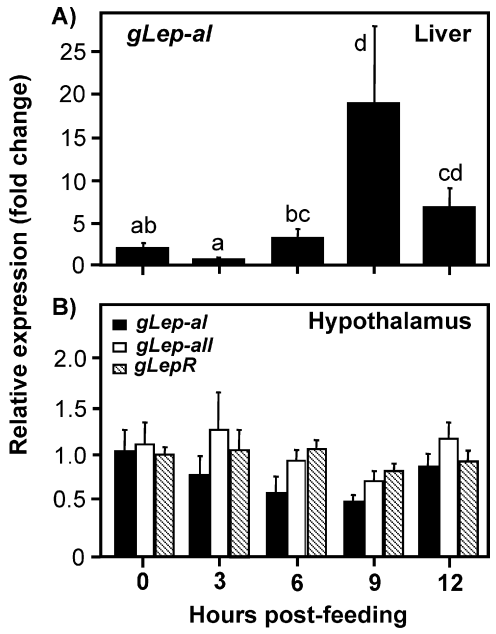

Fig. 5. Postfeeding changes of (A) *gLep-aI* in liver and (B) *gLep-aI*, *gLep-aII* and *gLepR* in hypothalamus in goldfish. Relative expression has been calculated from qRT-PCR data using the ΔΔCt method. Data represent the mean ± S.E.M. ($n = 8$) of the relative fold increase with respect to the control group. Significant differences among groups (ANOVA, $P < 0.05$) are indicated by different letters (SNK test).

the different feeding experimental conditions in any of the studied central tissues.

3.4. Postfeeding changes of leptin system expression in goldfish

Fig. 5 shows the postprandial changes in leptin mRNA expression in liver and hypothalamus of goldfish. The hepatic expression of *gLep-aI* showed a significant ($P < 0.05$) increase at 9 h after feeding time (Fig. 5A). This increase began to return to basal values at 12 h postfeeding, but the values does not yet matched the ones observed in the fish 24-h fasted (group sampled at 10:00, 0 h postfeeding).

The hypothalamic expression of *gLep-aI*, *gLep-aII* and *gLepR* in goldfish did not exhibit statistically significant differences at any studied times (0, 3, 6, 9 and 12 h) after feeding (Fig. 5B).

4. Discussion

Present results show that goldfish *leptins aI* and *aII* paralogs are differentially expressed, suggesting that they can play different roles in fish physiology. The increase in hepatic leptin expression 9 h after feeding supports its role as a postprandial satiety signal in fish, but the widely distribution of leptin system (leptins and LepR) found in central and peripheral tissues in goldfish suggests that this hormone may have pleiotropic actions.

Putative differences in location, regulation and function of the fish *leptin-a* genes are unknown to date. In goldfish *gLep-aI* was preferentially expressed in peripheral tissues, as gonads and liver (present results), the two main expression sites for this hormone in fish [7,17,30]. *gLep-aI* was also expressed in the brain locations studied in goldfish. Moreover, it is the only leptin gene found in the gut, kidney, spleen, pituitary and neural retina in this teleost. By contrast, *gLep-aII* was not so ubiquitous expressed in goldfish. It was mainly expressed in the brain, while in peripheral tissues was only present in the gonads and liver. This different distribution pattern of goldfish *gLep-aI* and *gLep-aII* reported here supports different physiological roles of these two genes.

Contrary to the well-known duplicity of leptin genes, only one leptin receptor gene has been found in all studied fish species till now [5,18,22]. However, alternative splicing of the LepR mRNA results in different isoforms in mammals [39] and also in some fish species (Atlantic salmon [30]; crucian carp [1]). The partial sequence of the available goldfish leptin receptor codifies for a region of the extracellular domain of the LepR. This region is common among the different receptor isoforms described in fish that differs in their transcellular and intracellular domains [1,30]. Thus, we cannot discard the presence of different isoforms of LepR in goldfish, although just one isoform has been reported in some fish (Japanese medaka [17]; and zebrafish [22]). A high *gLepR* expression was observed in hypothalamus and telencephalon of goldfish, as in pufferfish and zebrafish [18,22]. This *LepR* expression in the goldfish hypothalamus supports the anorectic effect of this hormone in this species [4,37]. However, in addition to its "classical" role on energy homeostasis, other leptin functions must be considered in fish since the widespread distribution of *LepR* throughout the brain (present results [18,22]). Moreover, the expression of leptin in the brain (present results [7,16,17,30,35]) indicates a possible autocrine/paracrine function of this hormone in teleost's central tissues.

A high expression of *gLep-aI* and *LepR* has been observed in goldfish pituitary gland, as in all studied teleosts [7,18,30] and also in other vertebrates [3,23,26], suggesting a role for leptin in the local regulation of the pituitary function. The presence of *gLep-aI* and *gLepR* transcripts in foregut and hindgut in goldfish (an stomachless specie, where foregut would be equivalent to the stomach in other fish) is in agreement with *LepR* expression observed in the Atlantic salmon stomach and midgut [30] and catfish stomach [16]. These data could suggest a signaling function of leptin in the gastrointestinal tract, similarly to the one described in mammals [9].

Leptins and leptin receptor transcripts have also been found in peripheral tissues no directly related with feeding, as gills and gonads. A high *gLepR* expression was detected in goldfish gills, as previously described in marine medaka [38], Japanese medaka [17], zebrafish [22] and crucian carp [1]. These results could be related to the leptin signaling for environmental hypoxia [1,30,38]. The high expression of *gLep-aI* and *gLepR* in goldfish gonads (ovary) also agrees with previous findings in the Atlantic salmon [30], zebrafish [7] and pufferfish [18], pointing to possible interactions between leptin and the reproductive physiology in fish [2,30], in agreement with its role in mammalian reproduction [25].

The differential distribution of leptins (present data [7,11,30]), together with the detection of leptin expression in other locations mainly related to feeding regulation in fish (goldfish hypothalamus, present data), makes necessary to investigate the possible effects of feeding conditions on leptin expression not only in liver, but also in central structures. With this aim, we investigated the existence of possible differences in *gLep-aI* (liver and hypothalamus), *gLep-aII* (hypothalamus) and *gLepR* (hypothalamus and telencephalon) expression under overfed (2 weeks) and fasting (1 week) conditions. The lack of significant changes among the experimental groups can suggest the absence of a direct relationship among the expression of leptin system (leptins and leptin receptors) and overfeeding/fasting in goldfish, at least under these feeding regimes. This apparent lack of correlation between nutritional status and tissue leptin mRNA expression is in agreement to that observed in other fish species. Liver leptin expression was not changed in fish under positive energy balance (zebrafish, 8-weeks overfed [29]; common carp, 6-weeks fed to satiation [11]), nor by short- or long-term fasting (common carp [11]). Similarly, 30 days of fasting did not affect leptin expression in the catfish brain and liver [16]. In zebrafish, hepatic levels of *leptin-a* transcripts were not changed after 1 week of fasting, but the *leptin-b*

(the minoritary isoform in this tissue) declined [7]. Only in Atlantic salmon there are evidences that leptin system can be sensitive to the metabolic status of fish [30,35]. On one hand, rationed feeding (60% for 10 months) reduced adipose tissue *sLepA1* and increased liver *sLepA2*, without changes in both, leptin receptor brain expression and circulating leptin [30]. On the other hand, a different regulation has been found in Atlantic salmon under 7-week restricted feeding (40%), with increases in both hepatic genes and circulating leptin, and decreases in brain expression of leptin receptor [35]. When interpreting this high variety of results in fish is important to consider the possible species-specific differences, the different life story stages, and the different experimental feeding conditions.

Our results suggest that leptin genes expression in goldfish appears to be independent of the feeding status, and leptin might not be acting as an adiposity signal at long-term, as recently proposed in salmonids [13]. This idea is also supported by the lack of correlation between body weight and leptin hepatic expression in goldfish (data not shown), neither between plasma leptin levels and the condition factor in rainbow trout [15]. It has been speculated that leptin may signal energy deficits under stress conditions in fish, such as chronic hypoxia, that compromise the fish survival more than deficits in nutrient availability [1,2]. These differences in the regulation of leptin by energy status between fish and mammals can be probably related to the highly different metabolic rates in ectotherms and endotherms, as recently suggested [2]. Moreover, it can not be discarded that feeding conditions used in the present study, although do not modify the messengers of the studied genes, they could alter their post-transcriptional regulation, modifying circulating leptin and/or its receptors. To date, the scarce studies in fish that simultaneously quantify transcripts and proteins [30,35] do not allow to establish a clear relationship among them. Indeed, the analysis of *gLepR* expression includes all putative isoforms that the goldfish could have. Then, no changes in translation *de novo* of *gLepR* cannot allow rule out a possible regulation of the alternative splicing of the messenger that results in unfunctional short-forms of the LepR.

The fact that feeding was followed by an increase in liver expression of *gLep-aI* supports a relationship between leptin and short-term food intake in goldfish, as in common carp [11] and mammals [33]. Thus, hepatic *gLep-aI* could promote postprandial satiety, in agreement with the rapid anorectic effect induced by mammalian leptin injection in this teleost [4,37]. This increase in hepatic *gLep-aI* expression could be also related with a decrease in energy demands during the inactive phase of these diurnal goldfish. In fact, a relationship between leptin and the circadian system has been recently suggested [36] and has to be further investigated.

Till our knowledge this is the first report measuring leptin expression under different feeding conditions in the hypothalamus of fish. None of the studied genes changed their expression levels in the hypothalamus of goldfish, suggesting that leptin-a paralogs could have a different regulation. It seems that the *gLep-aII*, more abundant in the central tissues, is not affected by feeding in goldfish. As above discussed, changes at no-transcriptional levels cannot be discarded. Moreover, *gLep-aI*, which increases in the liver 9 h after feeding, did not change in the hypothalamus of goldfish, indicating that the same leptin gene is differently regulated depending on the tissue. More studies have to be performed to understand if the two leptin-a genes have acquired different functions, and how they are regulated in fish.

In summary, the widespread distribution of leptins and leptin receptor in central and peripheral tissues in goldfish suggest that different physiological roles can be played by this hormone in fish, broadening the perspective of the functions of this hormone in vertebrates.

Acknowledgments

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References

[1] Cao Y-B, Xue JL, Wu L-Y, Jiang W, Hu P-N, Zhu J. The detection of 3 leptin receptor isoforms in crucian carp gill and the influence of fasting and hypoxia on their expression. Domest Anim Endocrinol 2011;41:74–80.
[2] Copeland DL, Duff RJ, Liu Q, Prokop J, Londraville RL. Leptin in teleost fishes: an argument for comparative study. Front Physiol 2011;2:26.
[3] Crespi EJ, Denver RJ. Leptin (ob gene) of the South African clawed frog *Xenopus laevis*. Proc Natl Acad Sci 2006;103:10092–7.
[4] De Pedro N, Martínez-Álvarez R, Delgado MJ. Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). J Endocrinol 2006;188:513–20.
[5] Denver RJ, Bonnett RM, Boorse GC. Evolution of leptin structure and function. Neuroendocrinology 2011;94:21–38.
[6] Froiland E, Murashita K, Jørgensen EH, Kurokawa T. Leptin and ghrelin in anadromous Arctic charr: cloning and change expressions during a seasonal feeding cycle. Gen Comp Endocrinol 2010;165:134–43.
[7] Gorissen M, Bernier NJ, Nabuurs SB, Flik G, Huising MO. Two divergent leptin paralogues in zebrafish (*Danio rerio*) that originate early in teleostean evolution. J Endocrinol 2009;201:329–39.
[8] Grill HJ. Leptin and the systems neuroscience of meal size control. Front Neuroendocrinol 2010;31:61–78.
[9] Guilmeau S, Buyse M, Tsocas A, Laigneau JP, Bado A. Duodenal leptin stimulates cholecystokinin secretion: evidence of a positive leptin–cholecystokinin feedback loop. Diabetes 2003;52:1664–72.
[10] Horev G, Einat P, Aharoni T, Eshdat Y, Friedman-Einat M. Molecular cloning and properties of the chicken leptin-receptor (CLEPR) gene. Mol Cell Endocrinol 2000;162:95–106.
[11] Huising MO, Geven EJ, Kruiswijk CP, Nabuurs SB, Stolte EH, Spanings FA, et al. Increased leptin expression in common carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. Endocrinology 2006;147:5786–97.
[12] Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R. BMC Bioinformatics 2007;8:460.
[13] Johnsen CA, Hagen Ø, Adler M, Jönsson E, Kling P, Bickerdike R, et al. Effects of feed, feeding regime and growth rate on flesh quality, connective tissue and plasma hormones in farmed Atlantic salmon (*Salmo salar L.*). Aquaculture 2011;318:343–54.
[14] Johnson RM, Johnson TM, Londraville RL. Evidence for leptin expression in fishes. J Exp Zool 2000;286:718–24.
[15] Kling P, Rønnestad I, Stefansson SO, Murashita K, Kurokawa T, Björnsson BT. A homologous salmonid leptin radioimmunoassay indicates elevated plasma leptin levels during fasting of rainbow trout. Gen Comp Endocrinol 2009:307–12.
[16] Kobayashi Y, Quiniou S, Booth NJ, Peterson BC. Expression of leptin-like peptide (LLP) mRNA in channel catfish (*Ictalurus punctatus*) is induced by exposure to *Edwardsiella ictaluri* but is independent of energy status. Gen Comp Endocrinol 2011;173:411–8.
[17] Kurokawa T, Murashita K. Genomic characterization of multiple leptin genes and a leptin receptor gene in the Japanese medaka, *Oryzias latipes*. Gen Comp Endocrinol 2009;161:229–37.
[18] Kurokawa T, Murashita K, Suzuki T, Uji S. Genomic characterization and tissue distribution of leptin receptor and leptin receptor overlapping transcript genes in the pufferfish, *Takifugu rubripes*. Gen Comp Endocrinol 2008;158:108–14.
[19] Kurokawa T, Uji S, Suzuki T. Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. Peptides 2005;26:745–50.
[20] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23:2947–8.
[21] Li GG, Liang XF, Xie Q, Li G, Yu Y, Lai K. Gene structure, recombinant expression and functional characterization of grass carp leptin. Gen Comp Endocrinol 2010;166:117–27.
[22] Liu Q, Chen Y, Copeland D, Ball H, Duff RJ, Rockich B, et al. Expression of leptin receptor gene in developing and adult zebrafish. Gen Comp Endocrinol 2010;166:346–55.
[23] Liu X, Dunn IC, Sharp PJ, Boswell T. Molecular cloning and tissue distribution of a short form chicken leptin receptor mRNA. Domest Anim Endocrinol 2007;32:155–66.
[24] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(\Delta\Delta C(T))$ method. Methods 2001;25:402–8.
[25] Máčajová M, Lamošová D, Zeman M. Role of leptin in farm animals: a review. J Vet Med A 2004;51:157–66.
[26] Morash BA, Imran A, Wilkinson D, Ur E, Wilkinson M. Leptin receptors are developmentally regulated in rat pituitary and hypothalamus. Mol Cell Endocrinol 2003;210:1–8.
[27] Murashita K, Uji S, Yamamoto T, Rønnestad I, Kurokawa T. Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol B 2008;150:377–84.

[28] Nieminen P, Mustonen A-M, Hyvärinene H. Fasting reduces plasma leptin- and ghrelin-immunoreactive peptide concentrations of the burbot (*Lota lota*) at 2 °C but not a 10 °C. Zool Sci 2003;20:1109–15.
[29] Oka T, Nishimura Y, Zang L, Hirano M, Shimada Y, Wang Z, et al. Diet-induced obesity in zebrafish shares common patophysiological pathways with mammalian obesity. BMC Physiol 2010;10:21.
[30] Rønnestad I, Nilsen TO, Murashita K, Angotzi AR, Moen AGG, Stefansson SO, et al. Leptin and leptin receptor genes in Atlantic salmon: cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. Gen Comp Endocrinol 2010;168:55–70.
[31] Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.
[32] Sahu A. Minireview: a hypothalamic role in energy balance with special emphasis on leptin. Endocrinology 2004;145:2613–20.
[33] Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, et al. Transient increase in obese gene expression after food intake or insulin administration. Nature 1995;377:527–9.
[34] Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, et al. Identification and expression cloning of a leptin receptor, OB-R. Cell 1995;83:1263–71.
[35] Trombley SA, Maugars G, Kling P, Björnsson BT, Schmitz M. Effects of long-term restricted feeding on plasma leptin, hepatic expression and leptin receptor expression in juvenile Atlantic salmon (*Salmo salar* L.). Gen Comp Endocrinol 2011.
[36] Vivas Y, Azpeleta C, Feliciano A, Velarde E, Isorna E, Delgado MJ, et al. Time-dependent effects of leptin on food intake and locomotor activity in goldfish. Peptides 2011;32:989–95.
[37] Volkoff H, Eykelbosh AJ, Peter RE. Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. Brain Res 2003;972:90–109.
[38] Wong M, Yu R, Ng P, Law S, Tsang A, Kong R. Characterization of a hypoxia-responsive leptin receptor (omLepR$_L$) cDNA from the marine medaka (*Oryzias melastigma*). Mar Pollut Bull 2007;54:797–803.
[39] Zabeau L, Lavens D, Peelman F, Eyckerman S, Vandekerckhove J, Tavernier J. The ins and outs of leptin receptor activation. FEBS Lett 2003;546:45–50.
[40] Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425–32.

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Leptin expression is rhythmic in brain and liver of goldfish (*Carassius auratus*). Role of feeding time

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ABSTRACT

Daily rhythms of feeding regulators are currently arousing research interest due to the relevance of the temporal harmony of endocrine regulators for growth and welfare in vertebrates. However, it is unknown the leptin circadian pattern in fish. The aim of this study is to investigate if leptin (*gLep-aI* and *gLep-aII*) expression is rhythmic in goldfish (*Carassius auratus*) liver and brain, and if such rhythms are driven by feeding time through a food entrainable oscillator. Fish maintained under 12-h light:12-h dark photoperiod and a scheduled feeding time showed 24-h locomotor activity and glycaemia rhythms. Moreover, hepatic *gLep-aI* and brain *gLep-aI* and *gLep-aII* expression were rhythmic with different daily profiles, showing a postprandial increase of leptin expression in the liver but not in the brain. Under constant light and different feeding regimes (scheduled fed at 10:00, 22:00 or randomly fed), feeding time synchronized daily rhythms in locomotor activity, glycaemia and clock gene expression (*gPer1a*, *gPer3* and *gCry3*), but the rhythmic expression of hepatic *gLep-aI* and brain *gLep-aII* only remained in fed fish at 10:00. In summary, daily rhythms of leptin expression in goldfish are differently regulated at central and peripheral level, and they are not directly driven by clock genes. The role of food entrained oscillators on leptin expression rhythms in fish remains to be demonstrated.

1. Introduction

Leptin is a peptide that regulates food intake and energy balance in mammals and fish (Copeland et al., 2011; Grill, 2010; Li, 2011). In mammals, leptin is mainly produced by the adipose tissue (Zhang et al., 1994) and encodes body fat reserves, acting as a short-term satiety signal (Denver et al., 2011; Grill, 2010; Li, 2011). In fish, leptin is synthesized mainly in the liver although it is also expressed in brain and other peripheral tissues (Frøiland et al., 2010; Huising et al., 2006; Kobayashi et al., 2011; Kurokawa and Murashita, 2009; Murashita et al., 2008; Tinoco et al., 2012). An anorectic action of leptin has been reported in most studied fish (Aguilar et al., 2010; De Pedro et al., 2006; Murashita et al., 2008; Vivas et al., 2011; Volkoff et al., 2003). However, its regulation by overfeeding and fasting conditions depends on the feeding regime and seems to be species-specific (Frøiland et al., 2010; Huising et al., 2006; Kobayashi et al., 2011; Rønnestad et al., 2010; Tinoco et al., 2012).

Since the discovery that alterations of the circadian system can affect energy balance (Bass and Takahashi, 2010; Froy, 2011; Kalsbeek et al., 2011), several studies have investigated possible interactions between feeding regulators and hormones involved in energy balance and the circadian system. This relationship seems to be bidirectional in mammals. Thus, on one hand, some feeding regulators (including leptin) can affect circadian clocks (Ando et al., 2011; Inyushkin et al., 2009; Prosser and Bergeron, 2003). On the other, it is reported a circadian dependence of synthesis and secretion (Feillet, 2010; Xu et al., 1999), and effects (Arble et al., 2011; Merino et al., 2008) of feeding regulators. In fish, leptin actions depend on the time of hormone administration, indicating a circadian dependence of leptin effects (Vivas et al., 2011).

Several studies show daily rhythmic profiles of this hormone in mammals, although it is unknown which physiological factor(s) entrains this diurnal variation. As a rule (with some exceptions), leptin circulating levels are higher during the night in nocturnal species, and during the day in diurnal ones (Cuesta et al., 2009; Kalsbeek et al., 2011; Karakas et al., 2005; Xu et al., 1999). Moreover, superimposed postprandial leptin peaks may interfere with leptin daily rhythms (Kalra et al., 2003; Schoeller et al., 1997; Xu

Abbreviations: 12L:12D, 12-h of light and 12-h of dark; 24L, constant light; CT, circadian time; FEO, food entrainable oscillator; SF, scheduled fed; RnF, randomly fed; ZT, zeitgeber time.

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et al., 1999). A daily leptin mRNA expression pattern has been found in rat adipose tissue (Xu et al., 1999) and human explants of adipose tissue (Gómez Abellan et al., 2011), but not in mice (Kennaway et al., 2013; Stütz et al., 2007). In addition, a daily rhythm in the hypothalamic expression of leptin receptor has been described in rodents (Ellis et al., 2008; Xu et al., 1999).

In fish, to our knowledge, the daily profile of leptin expression has been only studied in the Atlantic salmon (*Salmo salar*), in which daily changes of leptin mRNA (*lepa1*) have been found in the white muscle, belly flap, visceral adipose tissue and liver (Moen and Finn, 2013). In the common carp (*Cyprinus carpio*) hepatic leptin expression differs between two 12-h shifted studied time points (Klaren et al., 2013). Also in goldfish, two leptin genes have been cloned: leptin-aI (*gLep-aI*) and leptin-aII (*gLep-aII*), mainly expressed in peripheral tissues and brain, respectively (Tinoco et al., 2012). Hepatic *gLep-aI* expression in goldfish peaks at 9-h post-feeding, suggesting that leptin could be acting as a postprandial satiety signal (Tinoco et al., 2012).

Circadian clocks are the basis of the circadian system organization, and they are synchronized by external cues as the light–dark cycle, named as LEOs (Light Entrainable Oscillators; Hastings et al., 2007), and feeding-fasting cycles, known as FEOs (Food Entrainable Oscillators; Stephan, 2002). The molecular basis of endogenous oscillators is well conserved in vertebrates, and consist on transcriptional-translational loops of genes called "clock genes", which oscillate with a periodicity close to 24-h (Hastings et al., 2007; Idda et al., 2012). In goldfish clock genes expression rhythms have been reported in various central and peripheral locations, including the liver (Feliciano et al., 2011; Nisembaum et al., 2012; Velarde et al., 2009), the main site of leptin synthesis in this species (Tinoco et al., 2012). Moreover, it has been proved that such clock genes oscillations in the optic tectum, the hypothalamus and the liver can be synchronized by feeding time (Feliciano et al., 2011).

The aim of this study was to investigate if leptins are expressed in a daily rhythmic manner, and if they could be synchronized by scheduled feeding in goldfish through food entrained oscillators. To this end, the daily expression of *gLep-aI, gLep-aII* and *gLepR* (goldfish leptin receptor) was studied in the liver and the brain of fish maintained under a 12L:12D photoperiod (12-h of light and 12-h of dark) and a scheduled feeding. The synchronization of fish to the environmental conditions (photoperiod and feeding time) was assessed by the recording of locomotor activity daily rhythms. Daily changes in glycaemia were analyzed taking into account the reported relevance of glucose metabolism for leptin secretion and synthesis in mammals (Amitani et al., 2013; Wang et al., 1998). Since daily expression rhythms for both leptins occurred under these conditions, our next goal was to ascertain if such rhythms were functionally linked to a FEO. To this end, fish were maintained under constant light and different scheduled feeding. Then, daily variations in locomotor activity, glycaemia, and expression of clock genes (*gPer1*, *gPer3* and *gCry3*) and leptins in brain and liver were quantified throughout a 24-h cycle.

2. Materials and methods

2.1. Animals and housing

Goldfish (10–17 g body weight, bw) were purchased from a local supplier and kept in aerated, filtered water 60-l tanks at 22 ± 1 °C. Fish were maintained under a 12L:12D photoperiod (lights-on at 08:00) and fed once a day at 10:00 with a commercial pellet diet (1% bw per day; Sera Biogran, Heidelberg, Germany), for at least 2 weeks before experimentation. All the fish handling procedures comply with the international standards for the Care and Use of Laboratory Animals, were approved by the Animal Experiments Committee of the Complutense University of Madrid, and were in accordance with the Guidelines of the European Union Council (2010/63/EU) for the use of research animals.

2.2. Experimental designs

2.2.1. Experiment 1. Daily leptin expression under light–dark cycle and scheduled feeding

Goldfish were maintained under 12L:12D photoperiod and fed (1% bw per day) at zeitgeber time ZT 3 for 2 weeks. Locomotor activity was registered throughout all the experimental period. At the end, blood was sampled and fish were sacrificed every 3-h ($n = 8$ fish per sampling point) during a 24-h cycle at ZT 3, ZT 6, ZT 9, ZT 12, ZT 15, ZT 18, ZT 21, ZT 24 and ZT 3b (ZT 3 of the following day). On the day of sacrifice, fish also ate at ZT 3, with the exception of animals sampled at ZT 3 and ZT 3b. Hypothalamus and liver were quickly dissected and immediately frozen in dry ice and stored at −80 °C until use. Leptin expression (*gLeptin-aI* and *gLeptin-aII*) in both tissues, and leptin receptor expression *(gLepR*) in hypothalamus, were analyzed by quantitative PCR. Plasma glucose levels were determined using an enzymatic–colorimetric assay with a commercial kit (Spinreact, Girona, Spain) based in the oxidation of the glucose by the glucose oxidase.

2.2.2. Experiment 2. Effect of feeding time on daily leptin expression

Goldfish maintained under constant light (24 L) to eliminate the synchronizing effect of the light–dark cycle were divided into three experimental groups ($n = 30$ fish/group) fed (1% bw per day) at different times. Two groups were fed on schedule at 10:00 (SF10) or at 22:00 (SF22), and the third one was subjected to a random scheduled feeding regime (RnF; determined by software). Locomotor activity was registered throughout all the experiment (6 weeks). At the end, 24-h fasted fish were killed every 6-h during a 24-h cycle ($n = 6$ fish per sampling time) at the following circadian times: CT 0, CT 6, CT 12, CT 18 and CT 24 h. The CT 0 corresponds to the last meal time on the previous day before sampling in each group, i.e., 10:00 for the SF10; 22:00 for the SF22; and 13:00 for the RnF group. Blood was collected and optic tectum, hypothalamus and liver were quickly removed. Plasma and tissues samples were immediately frozen in dry ice and stored at −80 °C until use. Glucose content was measured in plasma; *gLeptin-aI* expression in liver and hypothalamus, and *gLeptin-aII* expression in liver, hypothalamus and optic tectum were quantified. Clock genes expression (*gPer1a*, *gPer3* and *gCry3*) was quantified in liver and optic tectum.

2.3. Locomotor activity recording

Locomotor activity was registered as previously described (Feliciano et al., 2011). Two infrared photocells (Omrom E3S-AD62, Japan), connected to a computer with specific software (Micronec, Spain), were placed in the aquarium walls. The photocells were positioned one below the automatic feeder and the other one in the opposite corner at the bottom. The aquaria walls were covered with opaque paper to minimize external interferences during the experiments. Data were analyzed using the chronobiology software EL TEMPS® (University of Barcelona, Spain).

2.4. Quantitative PCR analysis

Gene expression analysis was performed in a CFX96™ Real-Time System (Biorad, Hercules, CA, USA) as previously described (Nisembaum et al., 2012; Tinoco et al., 2012). Briefly, total RNA from goldfish tissues was extracted with Trizol (TRI® Reagent method, Sigma Chemical, St Louis, USA) and treated with DNase (Promega, Madison, WI, USA). Then, 1 μg of RNA (except for the hypothalamus in the first experiment where 0.25 μg was used)

was retro-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The quantitative PCR (qPCR) reactions were carried out in a 20 µl volume using: 0.1 µl of cDNA for 18S rRNA and 1 µl for all the other genes; iTaq™ SYBR® Green Supermix (Biorad, Hercules, CA, USA); and specific primers (0.4 µM, Sigma Chemical, Madrid, Spain; Supplementary data 1). Calibration curves were generated with serial dilutions of cDNA; all curves exhibited slopes close to −3.32 and efficiencies between 95% and 105%. The qPCR conditions for *gLep-aI*, *gLep-aII*, and 18S rRNA were: 1 cycle at 95 °C for 3 min and 40 cycles consisting in: 95 °C for 10 s, 55 °C for 30 s and 72 °C for 45 s. The protocol was similar for the other genes excepting the annealing temperature: 58 °C for all studied clock genes and 60 °C for *gLepR* and ß-actin. All the samples from each experimental group were analyzed in duplicate. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression using ß-actin or 18S rRNA as reference genes. Negative controls included replacement of cDNA by water and the use of non-retrotranscribed total RNA. The specificity of the amplification was confirmed by the melting temperature of qPCR products (measured at the end of all reactions) and by the size in an agarose gel.

2.5. Data analysis

Statistical differences in gene expression among the different sampling times were determined by one-way analysis of variance (ANOVA) followed by a post hoc (Student Newman Keuls, SNK) test using Statgraphics software. When necessary, values were transformed (logarithmic or square root transformation) to obtain a normal distribution and homogeneity of variances. Differences were considered significant when $p < 0.05$.

Daily (24-h) rhythms in gene expression were determined by fitting the data to a sinusoidal function by the least squares method described by Duggleby (1981). The formula used was $f(t) = M + A\cos(t\pi/12\text{-}\varphi)$, where $f(t)$ is the gene expression level at a given time, mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours, and φ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M, A, and φ, and their standard error (SE), the SE being based on the residual sum of squares in the least-squares fit (Duggleby, 1981). The zero-amplitude test, which indicates if the sinusoidal amplitude differs from 0 with a given probability (Koukkari and Sothern, 2006) was used to ascertain the significance of the rhythms. The time series data were considered to display a 24-h rhythm by ANOVA ($p < 0.05$) and by the zero-amplitude test with cosinor analysis ($p < 0.005$; Nisembaum et al., 2012).

3. Results

3.1. Experiment 1. Daily leptin expression under light–dark cycle and scheduled feeding

In the present study, goldfish maintained under 12L:12D photoperiod and fed at ZT 3 displayed a clear 24-h rhythmic pattern of locomotor activity, with high values during daytime and low activity during nighttime, as is can be observed in the average waveform of locomotor activity (Fig. 1A). The highest peak of activity was shown after lights on and prior to feeding time. The daily profile of glycaemia showed significant differences among sampling points and a 24-h significant rhythm. The maximum values in plasma glucose levels were observed after feeding, with minimum values throughout the nighttime (Fig. 1B). The *gLep-aI* expression in liver showed a statistically significant 24-h rhythm under 12L:12D photocycle and scheduled feeding (Fig. 1C, Table 1). The maximal transcript abundance was observed at the beginning of the dark phase (ZT 13.4 ± 0.9 h) and the amplitude of this rhythm was around 10-fold change. The *gLep-aI* peak arose at 9-h post feeding and remained low during the night when goldfish are inactive. The expression of *gLep-aII* in the liver was not enough abundant to be quantified.

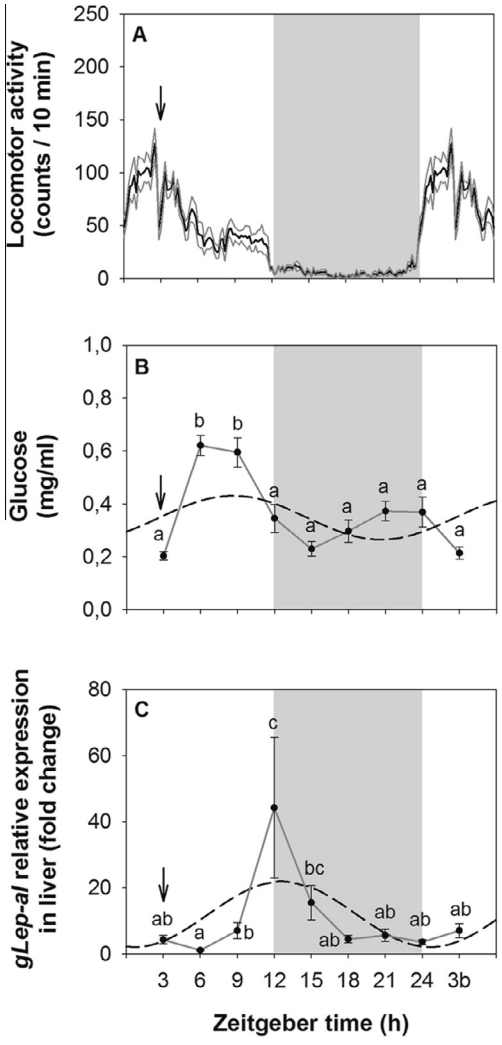

Fig. 1. Daily profiles of locomotor activity (A), plasma glucose levels (B) and *gLep-aI* expression in the liver (C) of goldfish maintained under 12L:12D and schedule fed at ZT 3. White and gray areas indicate light and dark conditions, respectively. The arrow indicates feeding time. Average waveform of locomotor activity was calculated for 5 consecutive days (black line represents the mean and gray line represents the SEM). Glucose levels and gene relative expression are showed as the mean ± SEM ($n = 8$). When significant (ANOVA, $p < 0.05$), differences among groups (SNK test) are indicated by different letters. Dash line represents the periodic sinusoidal functions obtained by cosinor (#, Zero-amplitude Test $p < 0.005$).

Table 1
Parameters defining the leptin expression rhythms in goldfish under 12L:12D photoperiod.

Tissue	Gene	Acrophase (ZT, h)	Amplitude (fold change)	Statistics
Liver	*gLep-aI*	13.4 ± 0.9	9.9 ± 4.1	#/*
Hypothalamus	*gLep-aI*	22.9 ± 1.1	0.4 ± 0.1	#/*
	gLep-aII	3.2 ± 1.4	0.5 ± 0.2	#/*

Parameters from sinusoidal functions are expressed as the value ± standard error (SE). NS: no significant.
Significant rhythm.
* $p < 0.05$ (ANOVA).

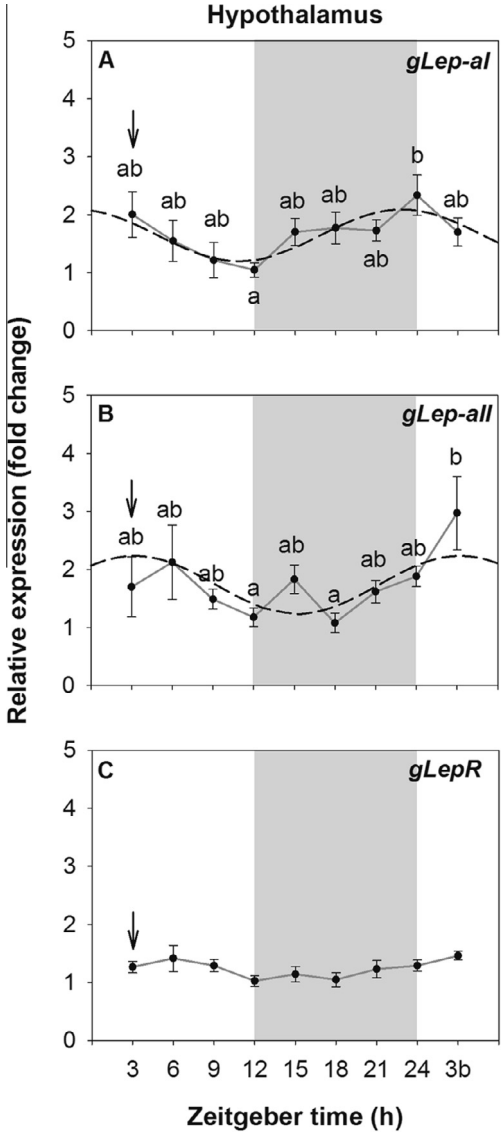

Fig. 2. Daily profiles of *gLep-aI* (A), *gLep-aII* (B) and *gLepR* (C) expression in the hypothalamus of goldfish maintained under 12L:12D and schedule fed at ZT 3. White and gray areas indicate light and dark conditions, respectively. The arrow indicates feeding time. Gene relative expression data are showed as the mean ± SEM ($n = 8$). When significant (ANOVA, $p < 0.05$), differences among groups (SNK test) are indicated by different letters. Dash line represents the periodic sinusoidal functions obtained by cosinor (#, Zero-amplitude Test $p < 0.005$).

The expression of both leptins, *gLep-aI* and *gLep-aII*, in the hypothalamus also showed a 24-h rhythm (Fig. 2A and B), with similar low amplitudes (0.5-fold change, Table 1). The acrophase of *gLep-aI* rhythm (ZT 22.9 ± 1.1 h) seems to be 4-h in advance compared to *gLep-aII* acrophase (ZT 3.2 ± 1.4 h). Compared to the rhythm of *gLep-aI* in liver, the hypothalamic *gLep-aI* rhythm presented a delayed acrophase (around 10 h) and lower amplitude (around 10 times; Fig. 1C, Fig. 2A). No significant differences were found for *gLepR* abundance in the hypothalamus throughout the 24-h period (Fig. 2C).

3.2. Experiment 2. Effect of feeding time on daily leptin expression

Under 24L, locomotor activity patterns exhibited a pronounced increase in the activity around 3-h before feeding time in both scheduled fed groups (SF10 and SF22; Fig. 3A and B), while randomly fed fish (RnF) presented a constant locomotor activity pattern during the 24-h cycle (Fig. 3C). Plasma glucose levels exhibited a significant 24-h rhythm in scheduled fed groups (Fig. 3D and E) with acrophases around 7–10 h. The amplitude in SF10 fish almost doubled the amplitude of SF22 group (0.11 ± 0.02 and 0.06 ± 0.02 mg/ml, respectively). No differences in glycaemia throughout the 24-h cycle were observed in the RnF group (Fig. 3F). The expression of *gLep-aI* in liver of fish from experiment 2 is shown in the Figs. 3G–I. The SF10 fish exhibited a significant rhythm of *gLep-aI* (Fig. 3G) with the maximal transcript expression at CT 7.3 ± 0.8 h, and around 2-fold change in the amplitude (Table 2). This hepatic *gLep-aI* rhythm (under 24L) presented an amplitude around 5-fold lower and a phase advance of 6-h compared to the rhythm observed under 12L:12D photocycle and in presence of food availability the sampling day (Fig. 1C). Leptin expression in the liver (*gLep-aI*) remains unmodified through the 24-h in the SF22 and RnF groups (Fig. 3H and I). The expression of *gLep-aII* in the liver was below the limit of quantification.

In contrast to hepatic leptin, the *gLep-aI* expression did not show daily variations in the hypothalamus of any experimental groups (SF10, SF22 and RnF; data not shown). The 24-h expression of *gLep-aII* was rhythmic in the optic tectum and the hypothalamus of SF10 fish (Fig. 4A and C), but not in the SF22 fish (Fig. 4B and D). The rhythms of *gLep-aII* in both hypothalamus and optic tectum showed similar acrophases (CT 5.1 ± 0.9 and 4.6 ± 0.9 h; Table 2).

The Figs. 5 and 6 show the daily expression patterns of the studied clock genes in the liver (Fig. 5) and the optic tectum (Fig. 6) of scheduled fed goldfish. All the studied genes (*gPer1a, gPer3* and *gCry3*) displayed significant 24-h rhythms in both tissues and in both scheduled fed groups (SF10 and SF22), except for the *gPer3* in the liver of fish fed at 22:00 (although there were daily significant differences by ANOVA, and the daily profile is similar to that of *gPer3* expression in SF10 fish; Table 3). The higher expression levels of *gPer1a* and *gPer3* took place around CT 22–24 h in the liver and the optic tectum of both SF groups (Figs. 5A–D and Figs. 6A–D). The maximal expression of *gCry3* occurred at CT 15–16 in both tissues and also in both SF experimental groups (Figs. 5E, F and 6E, F; Table 3).

4. Discussion

This study brings new information about circadian regulation of leptin expression, which is currently unclear in both, fish and mammals (Cuesta et al., 2009; Kennaway et al., 2013; Moen and Finn, 2013) and totally unknown in other vertebrates. Goldfish maintained under a light–dark cycle and a scheduled feeding displayed leptin expression rhythms (*gLep-aI* in the liver, and *gLep-aI* and *gLep-aII* in the hypothalamus). One point still under debate is whether such leptin expression rhythms are due to postprandial changes or could be driven by endogenous oscillators.

4.1. Lep-aI expression in the liver displays a clear rhythm that seems to be a postprandial response

The postprandial increase in hepatic *gLep-aI* (~10-fold) at the end of the light phase (9-h post-feeding) agrees with previous results in this species (Tinoco et al., 2012) and other teleosts, where leptin-a hepatic expression increases at 6 and 9-h postfeeding (common carp, Huising et al., 2006; Atlantic salmon, Moen and Finn, 2013; orange-spotted grouper, Zhang et al., 2013). These data, together with the anorectic role of this hormone reported in fish (Aguilar et al., 2010; De Pedro et al., 2006; Murashita et al., 2008; Vivas et al., 2011; Volkoff et al., 2003), support the role of this peptide as a short-term signal of feeding status in fish (Huising et al., 2006; Moen and Finn, 2013; Tinoco et al., 2012).

Studies in mammals suggest that different factors can be involved in the leptin daily rhythm such as photoperiod, hormones

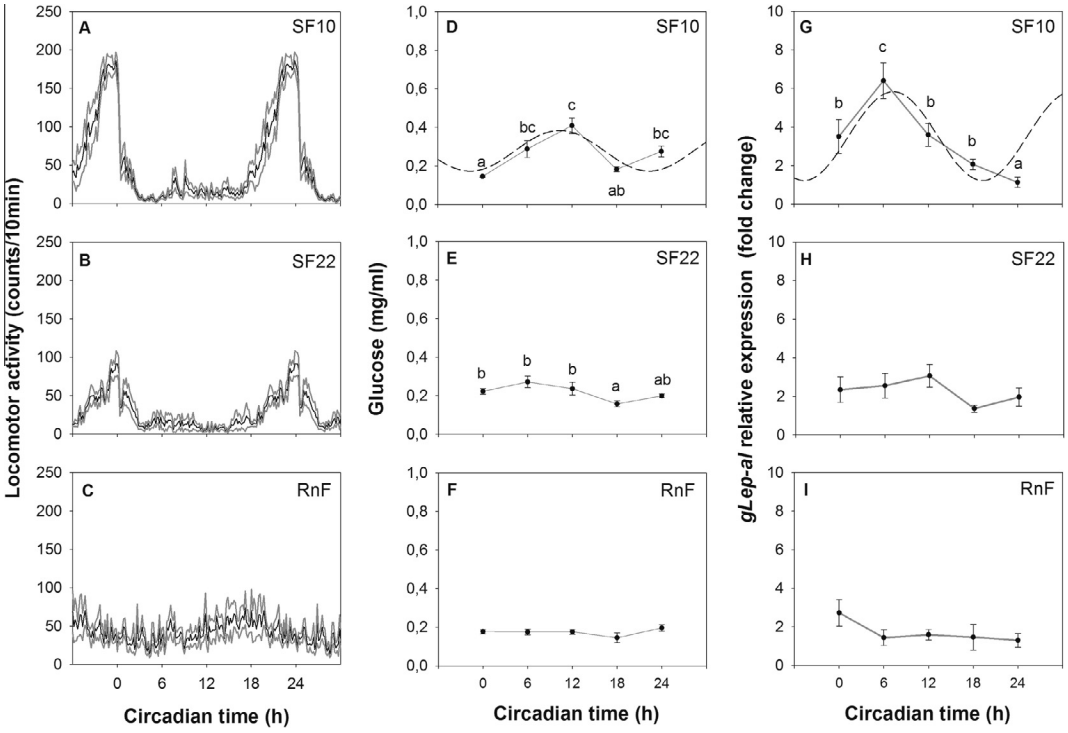

Fig. 3. Daily profiles of locomotor activity (A, B, C), plasma glucose levels (D, E, F) and *gLep-aI* expression in the liver (G, H, I) of goldfish maintained under 24L and fed at 10:00 (SF10), at 22:00 (SF22) or randomly (RnF). Fish were fasted for 24-h in the sampling day. Average waveforms of locomotor activity were calculated for 5 consecutive days (black line represents the mean and gray line represents the SEM). Glucose levels and gene relative expression data are showed as the mean ± SEM ($n = 6$). When significant (ANOVA, $p < 0.05$), differences among groups (SNK test) are indicated by different letters. Dash line represents the periodic sinusoidal functions obtained by cosinor (#, Zero-amplitude Test $p < 0.005$).

Table 2
Parameters defining the leptins expression rhythms in goldfish under constant light.

Tissue	Gene	Experimental group	Acrophase (CT, h)	Amplitude (fold change)	Statistics
Liver	*gLep-aI*	SF10	7.3 ± 0.8	2.3 ± 0.6	#/*
		SF22	7.9 ± 2.0	0.7 ± 0.4	NS
Hypothalamus	*gLep-aII*	SF10	4.6 ± 0.9	1.1 ± 0.3	#/*
		SF22	0.7 ± 8.4	0.2 ± 0.3	NS
Optic tectum	*gLep-aII*	SF10	5.1 ± 0.9	2.7 ± 0.7	#/*
		SF22	0.1 ± 9.3	0.6 ± 1.2	NS

Parameters from sinusoidal functions are expressed as the value ± standard error (SE). NS: no significant. SF10: scheduled fed at 10:00, SF22: scheduled fed at 22:00.
Significant rhythm.
* $p < 0.05$ (ANOVA).

(cortisol, insulin), and food derived metabolites (Cuesta et al., 2009; Feillet, 2010; Froy, 2011; Kalsbeek et al., 2001). Particularly glucose metabolism is a main determinant for leptin secretion and synthesis in these vertebrates (Amitani et al., 2013; Wang et al., 1998). The daily leptin expression and glycemia profiles in goldfish held under 12L:12D and fed at 10:00 are in accordance with this idea, because a postprandial glucose peak precedes the hepatic peak of leptin expression, as described in the common carp (Huising et al., 2006). Although it has not been measured, a postprandial increase in circulating leptin could be also expected. Nevertheless, the relationship between leptin and glucose is complex in fish, where both hypoglycemic and hyperglycemic effects of leptin have been described (Aguilar et al., 2010; Baltzegar et al., 2014; de Pedro et al., 2006; Vivas et al., 2011). In fact, such relationship between the glycemia increase and leptin expression induction was not observed in the hypothalamus of goldfish in the experiment 1, neither in brain and liver under 24L and 24-h fasted (experiment 2), where the rise in leptin expression anticipated glucose increase.

Other hormones and/or metabolites are probably involved in leptin daily rhythms in goldfish, as described in mammals (Cuesta et al., 2009; Feillet, 2010; Froy, 2011; Kalsbeek et al., 2001). Nevertheless, as it is below discussed (4.3), leptin expression rhythms in goldfish might be driven by endogenous oscillators, as suggested in some mammals (Kalsbeek et al., 2001; Simon et al., 1998).

Daily variations of leptin expression and ulterior expected changes in circulating levels could be also linked to locomotor activity rhythms. Animals under 12L:12D photoperiod and a scheduled feeding regime (experiment 1) exhibited a marked activity daily rhythm. The highest leptin expression in liver occurred at the end of the day. Thus, an increase in circulating leptin during the night could be expected in these animals, coinciding with the low nocturnal activity. This agrees with the locomotor

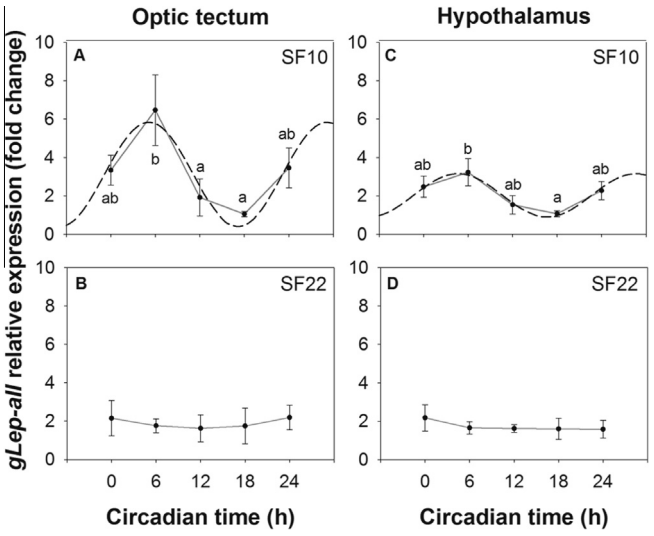

Fig. 4. Daily profiles of *gLep-aII* expression in the optic tectum (A, B) and hypothalamus (C, D) of goldfish maintained under 24L and fed at 10:00 (SF10) or at 22:00 (SF22). Fish were fasted for 24-h in the sampling day. Gene relative expression data are showed as the mean ± SEM ($n = 6$). When significant (ANOVA, $p < 0.05$), differences among groups (SNK test) are indicated by different letters. Dash line represents the periodic sinusoidal functions obtained by cosinor (#, Zero-amplitude Test $p < 0.005$).

activity decrease induced by leptin previously reported in this species (Vivas et al., 2011). Goldfish maintained under 24L and scheduled feeding (experiment 2) showed a daily pattern of locomotor activity that reflect the food anticipatory activity (an increase in the activity just several hours before food supply), as previously described (Sánchez-Vázquez and Madrid, 2001; Feliciano et al., 2011). Under these conditions, the daily profiles of hepatic and brain leptin expression are similar, and began to decrease some hours before FAA. Therefore, the lowest levels of circulating leptin could be expected when food anticipatory activity increase. The synchronization of leptin rhythmic expression in both tissues (liver and brain) would promote the activity increase linked to the food supply anticipation.

4.2. Leptin expression is differentially regulated in brain and peripheral organs

To our knowledge leptin expression rhythms in specific brain regions have not been previously studied in any vertebrate species, although the existence of leptin-a gene expression in brain has been recently reported in a few fish species (orange-spotted grouper, Zhang et al., 2013; Atlantic salmon, Rønnestad et al., 2010; Japanese medaka, Kurokawa and Murashita, 2009) including goldfish (Tinoco et al., 2012). In goldfish *gLep-aI* is mainly expressed in the liver, while *gLep-aII* is mainly located in brain regions, specifically in the hypothalamus and the optic tectum (Tinoco et al., 2012). Present results suggest that different mechanisms regulate brain and hepatic leptin expression in this teleost. The acrophases of 24-h *gLep-aI* and *gLep-aII* rhythms in the hypothalamus occurred before or around meal time (i.e. leptin increases are not postprandial responses), that is 12-h shifted compared to hepatic *gLep-aI*. Results from our second experiment confirm this differential regulation. The rhythm of *gLep-aI* in SF10 goldfish under 24L and 24-h fasting was clearly attenuated in liver (in comparison with fed fish under 12L:12D, experiment 1), and abolished in the hypothalamus (data not shown). Contrary to the liver, the amplitudes of the *gLep-aII* rhythms in the brain are similar in both experiments. In addition, postprandial changes were reported in the expression of *gLep-aI* in peripheral, but not in brain (neither *gLep-aI* nor *gLep-aII*; Tinoco et al., 2012), emphasizing different functions for central and peripheral leptins. Such results could be explained by leptin's pleiotropic nature, not only is it involved in feeding related functions (Baltzegar et al., 2014; Londraville et al., 2014; Kobayashi et al., 2011). Future studies are warranted on pleiotropic actions of leptin in fish, and particularly searching the role of centrally synthesized leptin.

The *gLepR* expression did not exhibit daily variations in goldfish hypothalamus in any of the studied experimental conditions. These results disagree with daily rhythms in the leptin receptor expression previously described in rat (Xu et al., 1999), mice (Kennaway et al., 2013) and Siberian hamster (Ellis et al., 2008). Previous studies in goldfish showed that leptin receptor expression is not modified by energy status (Rønnestad et al., 2010; Tinoco et al., 2012). Thus, it seems that leptin receptor expression does not change under different environmental and/or metabolic conditions in fish. Nevertheless, it cannot be discarded that it exists

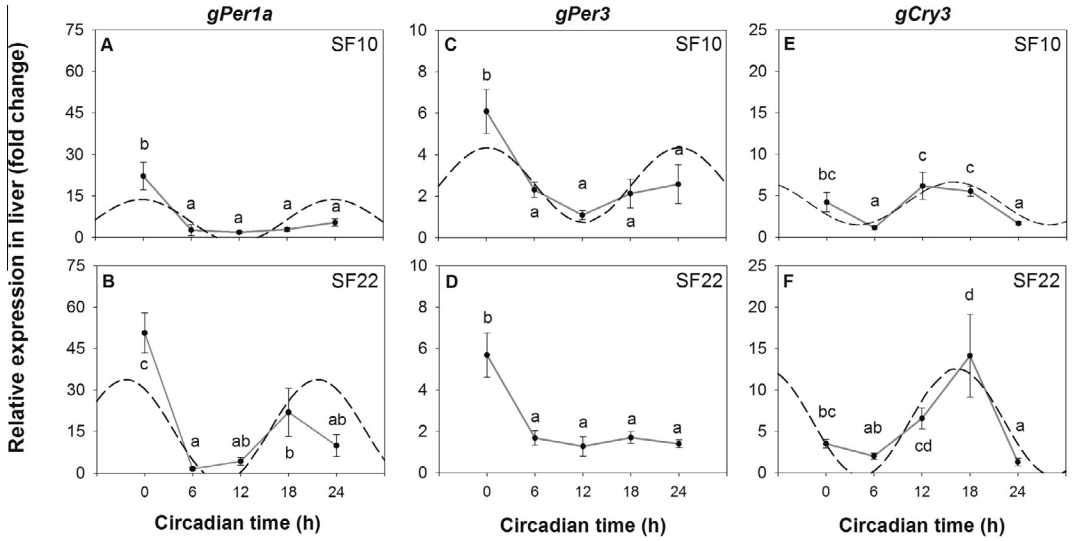

Fig. 5. Daily profiles of *gPer1a* (A, B), *gPer3* (C, D) and *gCry3* (E, F) in the liver of goldfish maintained under 24L and fed at 10:00 (SF10) or at 22:00 (SF22). Fish were fasted for 24-h in the sampling day. Gene relative expression data are showed as the mean ± SEM ($n = 6$). When significant (ANOVA, $p < 0.05$), differences among groups (SNK test) are indicated by different letters. Dash line represents the periodic sinusoidal functions obtained by cosinor (#, Zero-amplitude Test $p < 0.005$).

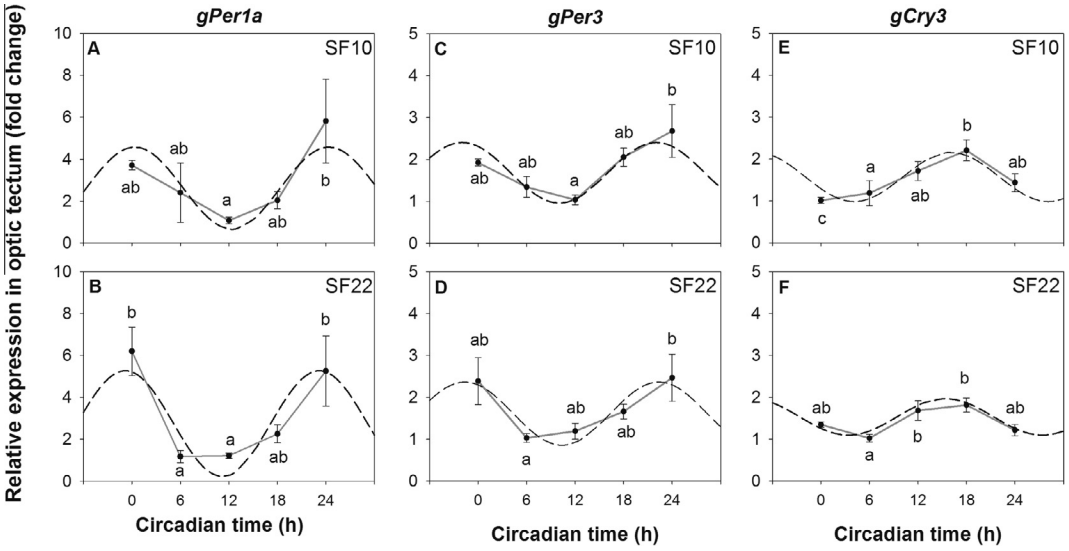

Fig. 6. Daily profiles of *gPer1a* (A, B), *gPer3* (C, D) and *gCry3* (E, F) in the optic tectum of goldfish maintained under 24L and fed at 10:00 (SF10) or at 22:00 (SF22). Fish were fasted for 24-h in the sampling day. Gene relative expression data are showed as the mean ± SEM ($n = 6$). When significant (ANOVA, $p < 0.05$), differences among groups (SNK test) are indicated by different letters. Dash line represents the periodic sinusoidal functions obtained by cosinor (#, Zero-amplitude Test $p < 0.005$).

Table 3
Parameters defining the clock genes expression rhythms in goldfish under constant light.

Tissue	Gene	Experimental group	Acrophase (CT)	Amplitude (fold change)	Statistics
Liver	*gPer1a*	SF10	23.8 ± 1.3	7.7 ± 2.4	#/*
		SF22	20.7 ± 1.1	18.4 ± 5.1	#/*
	gPer3	SF10	0.1 ± 1.3	1.8 ± 0.5	#/*
		SF22	24.0 ± 1.6	1.37 ± 0.5	*
	gCry3	SF10	15.9 ± 1.0	2.6 ± 0.7	#/*
		SF22	16.4 ± 0.8	6.4 ± 1.5	#/*
Optic tectum	*gPer1a*	SF10	0.3 ± 0.1.6	2.0 ± 0.7	#/*
		SF22	23.2 ± 1.1	2.5 ± 0.6	#/*
	gPer3	SF10	22.0 ± 1.2	0.7 ± 0.2	#/*
		SF22	22.4 ± 1.3	0.8 ± 0.2	#/*
	gCry3	SF10	16.1 ± 0.9	0.6 ± 0.2	#/*
		SF22	15.4 ± 0.9	0.4 ± 0.1	#/*

Parameters from sinusoidal functions are expressed as the value ± standard error (SE). SF10: scheduled fed at 10:00, SF22: scheduled fed at 22:00.
Significant rhythm.
* $p < 0.05$ (ANOVA).

possible splicing mechanisms as described in fish and mammals (Rønnestad et al., 2010; Zabeau et al., 2003) with functional consequences.

4.3. Is leptin expression driven by an endogenous food entrained oscillator?

The second experiment was designed to investigate if leptin expression rhythmicity under 12L:12D photoperiod could be driven by an endogenous oscillator(s) entrained by the scheduled feeding. To avoid masking effects of the light–dark cycle and food intake, leptin expression was studied in goldfish maintained under 24L and 24-h fasted, where the daily locomotor activity rhythm and the molecular clock-core functioning are synchronized to a scheduled feeding (Feliciano et al., 2011; Nisembaum et al., 2012). Under these conditions, leptin expression only was rhythmic in SF10 group (maintaining the previous scheduled feeding), but not in SF22 (food supply moved from 10:00 to 22:00). Then, feeding time by itself was unable to induce daily leptin expression rhythms in goldfish.

The clock genes *gPer1a*, *gPer3* and *gCry3* have been related with food synchronization in different tissues of goldfish related with the FEO network (liver, hypothalamus and optic tectum; Feliciano et al., 2011; Nisembaum et al., 2012). The fact that in the present study these clock genes presented similar daily profiles, acrophases and amplitudes in both scheduled feeding groups (SF10 and SF22) indicates that dysfunctional clockwork was not the reason for the absence of rhythmicity in leptin expression in SF22 goldfish. Nevertheless, it cannot be discarded that other clock genes might be affected in this SF22 group.

It is difficult to explain the differences in leptin mRNA rhythms found in SF10 and SF22 fish. Assuming a functioning molecular clock, such differences can be explained based on the feeding conditions in the days prior to the experiment. Recent studies suggest that some temporal events depend on learning and memories, and would not be direct outputs of a clock, although circadian oscillator could be necessary for such learning (Mulder et al., 2013; Silver et al., 2011). In this sense, one might speculate that fish could need the presence of a LD cycle to "learn" the upkeep of a leptin mRNA rhythm, and then, a leptin expression rhythm is detected under 12L:12D. On the contrary, in the absence of the LD cycle signaling, fish fed at 10:00 might sense a "metabolic status" resembling the previous one (because the feeding time is conserved), and then, they retained a leptin rhythm. In fact, such leptin mRNA rhythm

would not be a consequence of food entrained oscillator activity, justifying the absence of this leptin rhythm in the SF22 fish.

In conclusion, it seems that signaling by environmental (light–dark cycle, feeding time) and endogenous (metabolic cues) factors are involved in the daily leptin expression rhythms in central and peripheral organs of goldfish. Feeding time alone is not able to synchronize leptin rhythmic expression, and clock genes rhythms in the encephalon and the liver thus not ensure leptin expression rhythms. If the circadian system is important for the induction and maintenance of mRNA leptin rhythms, as occurs in mammals, remains to be elucidated in fish.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.06.006.

References

Aguilar, A.J., Conde-Sieira, M., Polakof, S., Miguez, J.M., Soengas, J.L., 2010. Central leptin treatment modulates brain glucosensing function and peripheral energy metabolism of rainbow trout. Peptides 31, 1044–1054.
Amitani, M., Asakawa, A., Amitani, H., Inui, A., 2013. The role of leptin in the control of insulin-glucose axis. Front. Neurosci. 7, 51.
Ando, H., Kumazaki, M., Motosugi, Y., Ushijima, K., Maekawa, T., Ishikawa, E., et al., 2011. Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice. Endocrinology 152, 1347–1354.
Arble, D.M., Vitaterna, M.H., Turek, F.W., 2011. Rhythmic leptin is required for weight gain from circadian desynchronized feeding in the mouse. PLoS One 6, e25079.
Baltzegar, D.A., Reading, B.J., Douros, J.D., Borski, R.J., 2014. Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. J. Endocrinol. 220, 61–72.
Bass, J., Takahashi, J.S., 2010. Circadian integration of metabolism and energetics. Science 330, 1349–1354.
Copeland, D.L., Duff, R.J., Liu, Q., Prokop, J., Londraville, R.L., 2011. Leptin in teleost fishes: an argument for comparative study. Front. Physiol. 2, 26.
Cuesta, M., Clesse, D., Pevet, P., Challet, E., 2009. From daily behavior to hormonal and neurotransmitters rhythms: comparison between diurnal and nocturnal rat species. Horm. Behav. 55, 338–347.
De Pedro, N., Martinez-Alvarez, R., Delgado, M.J., 2006. Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). J. Endocrinol. 188, 513–520.
Denver, R.J., Bonett, R.M., Boorse, G.C., 2011. Evolution of leptin structure and function. Neuroendocrinology 94, 21–38.
Duggleby, R.G., 1981. A nonlinear regression program for small computers. Anal. Biochem. 110, 9–18.
Ellis, C., Moar, K.M., Logie, T.J., Ross, A.W., Morgan, P.J., Mercer, J.G., 2008. Diurnal profiles of hypothalamic energy balance gene expression with photoperiod manipulation in the Siberian hamster, *Phodopus sungorus*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R1148–R1153.
Feillet, C.A., 2010. Food for thoughts: feeding time and hormonal secretion. J. Neuroendocrinol. 22, 620–628.
Feliciano, A., Vivas, Y., de Pedro, N., Delgado, M.J., Velarde, E., Isorna, E., 2011. Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (*Carassius auratus*). J. Biol. Rhythms 26, 24–33.
Frøiland, E., Murashita, K., Jørgensen, E.H., Kurokawa, T., 2010. Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. Gen. Comp. Endocrinol. 165, 136–143.
Froy, O., 2011. The circadian clock and metabolism. Clin. Sci. (Lond) 120, 65–72.
Gómez Abellan, P., Gómez Santos, C., Madrid, J.A., Milagro, F.I., Campion, J., Martínez, J.A., et al., 2011. Site-specific circadian expression of leptin and its receptor in human adipose tissue. Nutr. Hosp. 26, 1394–1401.
Grill, H.J., 2010. Leptin and the systems neuroscience of meal size control. Front. Neuroendocrinol. 31, 61–78.
Hastings, M., O'Neill, J.S., Maywood, E.S., 2007. Circadian clocks: regulators of endocrine and metabolic rhythms. J. Endocrinol. 195, 187–198.
Huising, M.O., Geven, E.J., Kruiswijk, C.P., Nabuurs, S.B., Stolte, E.H., Spanings, F.A., et al., 2006. Increased leptin expression in common Carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. Endocrinology 147, 5786–5797.
Idda, M.L., Bertolucci, C., Vallone, D., Gothilf, Y., Sánchez-Vázquez, F.J., Foulkes, N.S., 2012. Circadian clocks: lessons from fish. Prog. Brain Res. 199, 41–57.
Inyushkin, A.N., Bhumbra, G.S., Dyball, R.E., 2009. Leptin modulates spike coding in the rat suprachiasmatic nucleus. J. Neuroendocrinol. 21, 705–714.
Kalra, S.P., Bagnasco, M., Otukonyong, E.E., Dube, M.G., Kalra, P.S., 2003. Rhythmic, reciprocal ghrelin and leptin signaling: new insight in the development of obesity. Regul. Pept. 111, 1–11.
Kalsbeek, A., Fliers, E., Romijn, J.A., La Fleur, S.E., Wortel, J., Bakker, O., et al., 2001. The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels. Endocrinology 142, 2677–2685.
Kalsbeek, A., Scheer, F.A., Perreau-Lenz, S., La Fleur, S.E., Yi, C.X., Fliers, E., et al., 2011. Circadian disruption and SCN control of energy metabolism. FEBS Lett. 585, 1412–1426.
Karakas, A., Camsari, C., Serin, E., Gunduz, B., 2005. Effects of photoperiod and food availability on growth, leptin, sexual maturation and maintenance in the Mongolian gerbils (*Meriones unguiculatus*). Zoolog. Sci. 22, 665–670.
Kennaway, D.J., Varcoe, T.J., Voultsios, A., Boden, M.J., 2013. Global loss of Bmal1 expression alters adipose tissue hormones, gene expression and glucose metabolism. PLoS One 8, e65255.
Klaren, P.H., van Dalen, S.C., Atsma, W., Spanings, F.A., Hendriks, J., Flik, G., 2013. Voluntary timing of food intake increases weight gain and reduces basal plasma cortisol levels in common carp (*Cyprinus carpio* L.). Physiol. Behav. 122C, 120–128.
Kobayashi, Y., Quiniou, S., Booth, N.J., Peterson, B.C., 2011. Expression of leptin-like peptide (LLP) mRNA in channel catfish (*Ictalurus punctatus*) is induced by exposure to Edwardsiella ictaluri but is independent of energy status. Gen. Comp. Endocrinol. 173, 411–418.
Koukkari, W.L., Sothern, R.B., 2006. Chronobiometry: analyzing for rhythms, introducing biological rhythms. Springer, Netherlands, pp. 577–602.
Kurokawa, T., Murashita, K., 2009. Genomic characterization of multiple leptin genes and a leptin receptor gene in the Japanese medaka, *Oryzias latipes*. Gen. Comp. Endocrinol. 161, 229–237.
Li, M.D., 2011. Leptin and beyond: an odyssey to the central control of body weight. Yale J. Biol. Med. 84, 1–7.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔ C(T)) Method. Methods 25, 402–408.
Londraville, R.L., Macotela, Y., Duff, R.J., Easterling, M.R., Liu, Q., Crespi, E.J., 2014. Comparative endocrinology of leptin: assessing function in a phylogenetic context. Gen. Comp. Endocrinol. 203, 146–157.
Merino, B., Somoza, B., Ruiza-Cayo, M., Cano, V., 2008. Circadian rhythm drives the responsiveness of leptin-mediated hypothalamic pathway of cholecystokinin-8. Neurosci. Lett. 442, 165–168.
Moen, A.G., Finn, R.N., 2013. Short-term, but not long-term feed restriction causes differential expression of leptins in Atlantic salmon. Gen. Comp. Endocrinol. 183, 83–88.
Mulder, C., Van Der Zee, E.A., Hut, R.A., Gerkema, M.P., 2013. Time-place learning and memory persist in mice lacking functional per1 and per2 clock genes. J. Biol. Rhythms 28, 367–379.
Murashita, K., Uji, S., Yamamoto, T., Rønnestad, I., Kurokawa, T., 2008. Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 150, 377–384.
Nisembaum, L.G., Velarde, E., Tinoco, A.B., Azpeleta, C., de Pedro, N., Alonso-Gómez, A.L., et al., 2012. Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). Chronobiol. Int. 29, 665–673.
Prosser, R.A., Bergeron, H.E., 2003. Leptin phase-advances the rat suprachiasmatic circadian clock in vitro. Neurosci. Lett. 336, 139–142.
Rønnestad, I., Nilsen, T.O., Murashita, K., Angotzi, A.R., Gamst Moen, A.G., Stefansson, S.O., et al., 2010. Leptin and leptin receptor genes in Atlantic salmon: cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. Gen. Comp. Endocrinol. 168, 55–70.
Sánchez-Vázquez, F.J., Madrid, J.A., 2001. Feeding Anticipatory Activity, Food Intake in Fish. Blackwell Science Ltd, pp. 216–232.
Schoeller, D.A., Cella, L.K., Sinha, M.K., Caro, J.F., 1997. Entrainment of the diurnal rhythm of plasma leptin to meal timing. J. Clin. Invest. 100, 1882–1887.
Silver, R., Balsam, P.D., Butler, M.P., LeSauter, J., 2011. Food anticipation depends on oscillators and memories in both body and brain. Physiol. Behav. 104, 562–571.
Simon, C., Gronfier, C., Schlienger, J.L., Brandenberger, G., 1998. Circadian and ultradian variations of leptin in normal man under continuous enteral nutrition: relationship to sleep and body temperature. J. Clin. Endocrinol. Metab. 83, 1893–1899.
Stephan, F.K., 2002. The "other" circadian system: food as a zeitgeber. J. Biol. Rhythms 17, 284–292.
Stütz, A.M., Staszkiewicz, J., Ptitsyn, A., Argyropoulos, G., 2007. Circadian expression of genes regulating food intake. Obesity (Silver Spring) 15, 607–615.
Tinoco, A.B., Nisembaum, L.G., Isorna, E., Delgado, M.J., de Pedro, N., 2012. Leptins and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions. Peptides 34, 329–335.

Velarde, E., Haque, R., Iuvone, P.M., Azpeleta, C., Alonso-Gómez, A.L., Delgado, M.J., 2009. Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of period and cryptochrome transcripts in retina, liver, and gut. J. Biol. Rhythms 24, 104–113.

Vivas, Y., Azpeleta, C., Feliciano, A., Velarde, E., Isorna, E., Delgado, M.J., et al., 2011. Time-dependent effects of leptin on food intake and locomotor activity in goldfish. Peptides 32, 989–995.

Volkoff, H., Eykelbosh, A.J., Peter, R.E., 2003. Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. Brain Res. 972, 90–109.

Wang, J., Liu, R., Hawkins, M., Barzilai, N., Rossetti, L., 1998. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. Nature 393, 684–688.

Xu, B., Kalra, P.S., Farmerie, W.G., Kalra, S.P., 1999. Daily changes in hypothalamic gene expression of neuropeptide Y, galanin, proopiomelanocortin, and adipocyte leptin gene expression and secretion: effects of food restriction. Endocrinology 140, 2868–2875.

Zabeau, L., Lavens, D., Peelman, F., Eyckerman, S., Vandekerckhove, J., Tavernier, J., 2003. The ins and outs of leptin receptor activation. FEBS Lett. 546, 45–50.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425–432.

Zhang, H., Chen, H., Zhang, Y., Li, S., Lu, D., Zhang, H., et al., 2013. Molecular cloning, characterization and expression profiles of multiple leptin genes and a leptin receptor gene in orange-spotted grouper (*Epinephelus coioides*). Gen. Comp. Endocrinol. 181, 295–305.

Capítulo 2

Colecistocinina y motilidad intestinal en el carpín (*Carassius auratus*). Mecanismos de acción y receptores implicados

2.1. *The contractile effect of cholecystokinin (CCK-8S) on goldfish proximal intestine is mediated by cholecystokinin type A receptor.*

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The contractile effect of cholecystokinin (CCK-8S) on goldfish proximal intestine is mediated by cholecystokinin type A receptor.

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Tetrodotoxin

ABSTRACT

The gut-brain peptide cholecystokinin (CCK) plays a key role in digestive physiology of vertebrates. However, its role on gut motility in fish has not been explored in depth. In the present study, it is investigated the effect of the sulfated octapeptide of CCK (CCK-8S) on intestinal motility of a stomachless teleost, the goldfish (*Carassius auratus*) by using an in vitro system of isolated intestine. The addition of CCK-8S to the organ bath evoked a concentration-dependent contractile response in proximal gut strips. The contractions were tetrodotoxin- insensitive, indicating that they are independent on the enteric nervous system. The CCK8S induced gut contraction was extracellular calcium dependent, and it was not blocked by atropine, suggesting that probably uses a non-cholinergic pathway. Then, CCK-8S appears to act directly on smooth muscle tissue in the goldfish gut. Partial-lengths mRNAs encoding two CCK isoforms (CCKAR and CCKBR) were sequenced and phylogenetically analyzed. The structural analysis of these sequences suggest that both receptors belongs to the G-protein coupled receptor superfamily. The highest expression of goldfish CCKAR was observed along the whole intestine whereas the CCKBR gen was predominantly expressed in hypothalamus, vagal lobe and posterior intestine. Pharmacologically approaches by using devazepide (a selective CCKAR receptor antagonist) and L365,260 (a selective CCKBR receptor antagonist), and tissue distribution of mRNA specific CCK receptors suggest that the CCK-induced contractile response in goldfish proximal gut is mediated, at least in part, by the CCKAR receptor subtype.

1. Introduction

The gut-brain peptide cholecystokinin (CCK) is a member of the CCK-gastrin family, highly conserved in both invertebrates and vertebrates (Yu and Smagge, 2014) with a key role in digestive physiology of vertebrates, including fish (Olsson and Holmgren, 2011). It is synthesized as the 115-amino-acid precursor prepro-CCK polypeptide and enzymatically cleaved post-translationally to generate CCK/gastrin-like peptides. These biologically active peptides share similar carboxyl-terminus, being the biologically active octapeptides (CCK-8) the most abundant and conserved in vertebrates (Vigna, 2000; Chandra and Liddle, 2007). Partial and complete mRNA sequences of CCK have been reported for a number of fish species (rev. Micale et al., 2012). The sequence analyses of fish CCK cDNAs reveal cleavage sites for the processing of the pro-CCK into octapeptides, confirmed by chromatographic analysis (rev. Volkoff, 2005).

The CCK-like peptides have a widespread distribution within the gastrointestinal tract and the central and peripheral nervous system in both mammalian and non-mammalian species (Hur et al., 2013; Yu and Smagghe, 2014). In fact, two principal sources of CCK are reported in mammals, the endocrine I cells in the duodenal wall that are in contact with the lumen of the intestine, and peptidergic nerves both in the enteric nervous

system and in the central nervous system (Wu et al., 2013). In fish CCK/gastrin-like peptides have been demonstrated by means of RT-PCR, *in situ* hybridization or immunohistochemistry in the gut and nervous system of a high number of species (Himick and Peter, 1994; Peyon et al., 1998; McDonald and Volkoff, 2009; Murashita et al., 2009; Micale et al., 2012).

In mammals, CCK released in response to the presence of dietary lipids and peptides generates multiple effects on secretion and motility, including stimulation of pancreatic and gastric secretions, pyloric and gallbladder contraction and delay of gastric emptying (Dufresne et al., 2006; Staljanssens et al., 2011; Wu et al., 2013). Moreover, CCK is involved in feeding regulation (Chandra and Liddle, 2007; Tillner et al., 2014) and it seems to play a wide variety of physiological actions related to memory and learning, nociception, anxiety and major depression (Hebb et al., 2005). In fish, CCK-like peptides are involved in the regulation of food intake (Himick and Peter, 1994; Volkoff, 2005; Rubio et al., 2008; MacDonald and Volkoff, 2009) and glucose homeostasis (Polakof et al., 2011), but the role of these peptides in digestive functions remains far less known. As in mammals, CCK is released when meal is present in the intestine (Aldman y Holmgren, 1995; Murashita et al., 2009), and is involved in the contraction of the gallbladder and the pancreatic enzyme secretion (Rajjo et al., 1988; Aldman y Holmgren, 1995; Einarsson et al., 1997). The role of CCK in gastric motility in fish has been evidenced in only two studies in salmonids. The predominant effect of CCK-8 in the rainbow trout (*Oncorhynchus mykiss*) was to retard gastric emptying *in vivo*, and *in vitro* CCK-8 had either inhibitory or excitatory effects on the cardiac stomach, depending on the concentration and experimental approach (Olsson et al., 1999). In Chinook salmon (*Oncorhynchus tshawytscha*) a concentration-dependency of circular ring contractility in response to CCK-8 in cardiac stomach, pyloric stomach, pyloric sphincter and intestine was observed(Forgan and Forster, 2007).

The CCK exerts its biological effects through two physiologically and pharmacologically characterized specific receptors, namely CCKAR and CCKBR, in mammals (Noble et al, 1999). Both receptor subtypes are members of the seven transmembrane G protein-coupled receptors family, and show a high degree of mutual similarity with around 50% amino acid identity, suggesting that they share a common ancestor (Yu et al., 2014). CCKAR and CCKBR differ in their affinity for CCK/gastrin peptides and tissue distribution, being the CCKAR mainly localized in peripheral organs and discrete brain areas, whereas CCKBR is more centrally-distributed (Dufresne et al., 2006; Staljanssens et al., 2011). That distribution in mammals supports the involvement of CCKAR in gastrointestinal functions, while the brain CCKBR stimulation by CCK has been implicated in anxiety, analgesia, learning, memory, and dopamine-related behaviors (Staljanssens et al., 2011). The current knowledge of CCK receptors in fish is scarce and derived from recent studies in yellowtail (*Seriola quinqueradiata*) (Furutani et al., 2013) and Atlantic salmon (*Salmo salar*) (Rathore et al., 2013). An early study in goldfish characterized CCK/gastrin binding sites in brain and pituitary. Binding of ^{125}I-sulfated CCK octapeptide (^{125}I-CCK-8s) in tissue sections was found to be saturable, reversible, time dependent and displaceable by CCK/gastrin-like peptides (Himick et al., 1996). The phylogenetic analysis of fish CCK receptors revealed two clusters, the CCKAR and CCK-BR, and a third CCK receptor subtype in Atlantic salmon, the CCK2R1/gastrin, with one common ancestor gene (Rathore et al., 2013). The CCKAR mRNA levels increased in the gallbladder and pyloric caeca after feeding *in vivo,* and by CCK in cultured pyloric caeca from yellowtail, suggesting that CCKAR regulates digestion in yellowtail (Furutani et al., 2013).

The goldfish (*Carassius auratus*) is a teleost that have provided most of current knowledge regarding feeding regulation in fish (De Pedro and Björnsson, 2001; Volkoff et al., 2005). In this teleost, a 123-amino-acid CCK precursor is deduced, in which CCK-8 is contained near the C-terminus with an amidation and tyrosyl sulfation sites, and only one aminoacid substitution at position 5 of CCK is found compared to mammalian CCK-8 sequence (Peyon et al. 1998). Expression studies revealed the presence of preproCCK mRNA in the goldfish gastrointestinal tract, pituitary and a wide range of brain areas (Peyon et al., 1998), supporting the satiety role described for the sulfated form of CCK-8 in this species (Himick and Peter, 1994). The possible actions of CCK on digestive functions in goldfish remain to be unexplored to date. The goldfish belongs to the cyprinids, a group of fish which lacks a stomach, but gut motility and its control by several neuroendocrine regulators have been established *in vitro* in this species (Velarde et al. 2009, 2010, 2011; Nisembaum et al., 2013). However, no studies on possible regulation of intestinal motility by CCK have been previously reported. Then, the first objective of the present study was to investigate the effect of the sulfated octapeptide of CCK (CCK-8S) on goldfish proximal intestine motility in isolated tissue preparations, and to deep into the mechanism of action underlying such effect. Subsequently, the specificity of CCK effect on intestine motility was investigated by a pharmacological approach. Finally, the goldfish CCK receptors were partially sequenced and its distribution in peripheral and central tissues was characterized.

2. Materials and Methods

2.1. Animals and intestine strips preparations

Goldfish (*Carassius auratus*) with a body weight of 30-50 g obtained from a local commercial supplier in Madrid (Spain) were used in the study. Fish were kept in 60 L aquaria with filtered and aerated fresh water in a temperature-controlled room (21 ± 2ºC) under 12 h light:12 h dark (lights on at 08:00 h), and daily fed at 10:00 h on a commercial flake diet (1% body weight, Sera Pond, Heinserberg, Germany) for at least two weeks before the assays. The procedures were approved by the Animal Experimentation Committee of Complutense University, and performed according to the European Communities Council Directive (2010/63/UE).

Experiments were performed in an organ bath system with intestinal strips preparations following the procedure previously described (Velarde et al., 2009). Briefly, fish were killed at 1 h post-feeding, whole gut was removed and the luminal content was flushed out using physiological saline solution. Longitudinal segments (1 cm) of proximal intestine (the part of the intestine after the intestinal bulb) were immediately mounted in water-jacketed organ baths and attached to an isometric force transducer (LCM Systems Ltd., Cibertec, Madrid, Spain). Changes in force (mN) respect to baseline were recorded via an amplifier (Pre205, Cibertec, Spain) to a PC, using the data acquisition software (ADQ2C, CromaNec, Spain). As a control of an adequate tissue response the intestine preparations were tested with acetylcholine (10 µM) at both, the start and the end of the experimental procedures.

2.2. Drugs

The acetylcholine, CCK-8S ($Tyr[SO_3H]_{27}$ cholecystokinin fragment 26-33 amide) and atropine were purchased from Sigma-Aldrich (Madrid, Spain). Tetrodotoxin (TTX) was purchased from Abcam Biochemicals (Cambridge, UK), and the CCK receptors antagonists, devazepide and L365,260 were from Tocris Bioscience (Bristol, UK). The atropine was firstly dissolved in a small amount of hydrochloride acid 0,1N (final hydrochloride acid content of 0.08%), and the receptors antagonists were firstly dissolved in ethanol (final ethanol content ≤ 0.07%). All drugs were finally diluted in saline solution and prepared fresh before use.

2.3. Effects of CCK-8S on intestinal motility in goldfish

The effects of CCK-8S on goldfish intestinal motility were established by non-cumulative concentration-response curves by adding separately four CCK-8S increasing concentrations (1 nM, 10 nM, 100 nM and 1 µM) to the organ bath. The strips preparations were rinsed thrice with fresh saline solution between CCK-8S consecutive concentrations which were added at 20 min intervals, once the steady baseline was reached.

Effect of CCK-8S in calcium-free medium

The possible role of calcium on CCK-8S-induced contraction in intestine strips was tested by using a calcium-free saline solution at 25 °C (pH 7.8) containing (in mM): NaCl, 143; KCl, 2.5; $MgSO_4$, 0.8; $NaHCO_3$, 15; KH_2PO_4, 1; HEPES, 5; and glucose, 10. Paired intestine strips from the same fish (n=8) were used to compare contractile responses to CCK-8S (1 nM, 100 nM and 1 µM) in the presence or absence of extracellular calcium.

Effect of atropine and tetrodotroxin on CCK-8S-induced contraction

To determine the possible involvement of enteric neurons in the contractile effect of CCK-8S, we used the muscarinic non-selective antagonist atropine, and the voltage-gated sodium channel blocker tetrodotoxin (TTX). The proximal intestine strips were pre-incubated for 10 min with atropine (100 µM; n=6) or TTX (1 µM; n=6) before the addition of CCK-8S at the concentration that evoked the maximal contractile response (1 µM).

Effect of CCK antagonists on the CCK-8S-induced contraction

The receptor subtype involved in the CCK-8S-induced contraction in the goldfish proximal intestine was studied by incubation of isolated intestine strips in presence of two different antagonists of CCK receptors, the devazepide (a CCKAR antagonist), and the L365,260 (a CCKBR antagonist). The isolated strips were pre-incubated for 10 min in presence of increasing concentrations of devazepide (30 nM, 100 nM, 300 nM and 1 µM; n=6/concentration), or L365,260 (0.1 nM, 10 nM and 1 µM; n=4/concentration). Both antagonists were tested in non-cumulative curves. The CCK-8S (1 µM) was added to the organ bath after 10 min of incubation with the antagonist.

2.4. Partial cloning and distribution of CCK receptors in goldfish

Partial cDNA cloning of goldfish CCK receptors and phylogenetic analysis

To obtain a partial sequence of goldfish CCK receptors, total RNA from hypothalamus and gallbladder was isolated using TRI Reagent (Sigma-Aldrich, Madrid, Spain) and treated with RQ1 RNase-Free DNase (Promega, Madison, USA) according to the manufacturer's instructions. Then, an aliquot of 1 µg of total RNA was reverse transcribed (RT) to cDNA in a 25 µL reaction volume using random primers, RNase inhibitor and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). The reverse transcription reaction conditions consisted in 25ºC for 10 min, an extension of 50 min at 42ºC and a denaturalization step of 70ºC for 15 min.

The first strand cDNA fragments obtained were used as a template to amplify the CCK

Table 1.
Nucleotide sequence, amplicon size and purpose of the primers used in the present study.

Gene	Primer sequence (5'-3')	Amplicon size (pb)	Purpose
CCKAR	F1: TGGAGCGCTACAGTGCCAT R1: ACCAAGCGGCACATGT	187	cDNA cloning qPCR
	F1: TGGAGCGCTACAGTGCCAT R2: TGAGGCCATAAGCTGTCATCAT	282	cDNA cloning
CCKBR	F1: ACGACGGAGATGGCTGCTAC R1: CGGATCACTCGCTTCTTGGC	148	cDNA cloning qPCR
	F2: TGGCAGACCCGATCTCATGCCTA R1: CGGATCACTCGCTTCTTGGC	491	cDNA cloning
β-Actin	F: CAGGGAGTGATGGTTGGCAT R: AACACGCAGCTCGTTGTAGA	168	qPCR

F, forward primer; R, reverse primer

receptors genes using various sets of primers (Table 1) all obtained from Sigma-Aldrich (Madrid, Spain). Primers were hand-made designed by selecting convergent parts between the receptors nucleotide nucleotide sequences of *Danio rerio* (GenBank ID: CCK-1R, XM_692401.5; CCR-2R, XM_009304469.1, *Salmo salar* (GenBank ID: CCK-1R, JX017294.1; CCK-2R, JX017296.1) and *Oreochromis niloticus* (GenBank ID: CCK-1R, XM_005454409.1; CCK-2R, XM_005457866.1) using an alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The PCRs were performed in a 25 µl reaction volume containing 1.25 U of *Taq*DNA Polymerase recombinant, PCR Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mixture (all from Invitrogen, Carlsbad, USA), 0.2 µM of each forward and reverse primer and 1 µl of cDNA. Reaction conditions underwent an initial incubation at 94ºC for 3 min, followed by 40 cycles of 94ºC for 45 sec, 57ºC for 30 sec and 72ºC for 1 min, with a final extension step at 72ºC for 10 min. The RT reaction and all PCRs were carried out in an Eppendorf Mastercycler Gradient.

PCR products were electrophoresed on a 2% agarose gel. Single bands for each PCR were purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich, Madrid, Spain) and sequenced (Genomic Unity, Complutense University, Madrid, Spain). The confirmation of the nucleotide deduced sequences was obtained by carrying out a BLAST to compare each sequence with the sequences from the GenBank-NCBI data base.

Phylogenetic analysis was performed by aligning the goldfish CCKR sequences with those of other vertebrates retrieved from Genbank (NCBI) and Ensembl Genome using the Clustal-W2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/).
Alignment was performed using the partial fragment of the amino acid sequences for each protein which coincide with the obtained sequence for goldfish. A phylogenetic tree was constructed by the neighbor-joining method, with 1000 replicates for the bootstrap test. The FigTree v1.3.1 software (http://tree.bio.ed.ac.uk/software/figtree/) was used for the graphical representation.
Tissue distribution of CCK receptors in goldfish

To elucidate the expression pattern of CCKAR and CCKBR in goldfish tissues, samples of hypothalamus, telencephalon, vagal lobe, gallbladder, intestinal bulb, proximal and posterior intestine, hindgut, liver and muscle were collected. Total RNA extraction and reverse transcription were performed as above described. Then, a real-time or quantitative PCR (qPCR) was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, USA). The specific primers sequences used for target genes CCKAR and CCKBR and housekeeping gene β-actin are shown in Table 1, and were ordered to Sigma-Aldrich. Genes were amplified in qPCR runs using a 96-well plate loaded with 1 µl of cDNA and 0.5 µM of each forward and reverse primer in a final volume of 10 µl. Each qPCR run included a standard curve for the corresponding gene made of two replicates of four serial dilution points and water controls in order to ensure that the reagents were not contaminated. The qPCR cycling conditions consisted of a ramp of 95ºC for 30 sec and 40 cycles of a two-step amplification program (95ºC for 5 sec and 60ºC for 30 sec). A melting curve was systematically monitored (temperature gradient at 0.5ºC/5 sec from 65 to 95ºC) at the end of each run to confirm the specificity of the amplification reaction. All runs were performed using a CFX96™ Real-time System (Bio-Rad Laboratories, Hercules, USA). The qPCR products were checked by electrophoresis on a 2% agarose gel. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression.

2.5. Statistics

Differences in force (mN) generated by the intestine strips were integrated by data acquisition software (ADQ2C, CromaNec, Madrid, Spain). The results were expressed as mean ± SEM. Statistical differences were tested by one-way ANOVA followed by Tukey post-hoc test, with $p < 0.05$ considered as the statistically significant threshold. Statistics analyses were performed with the software Statgraphics Plus, version 5.1. The EC50 values were calculated by a non-linear regression of a 4-parameters logistic model using the SigmaPlot 11.0 program.

3. Results

3.1. Contractile response to CCK-8S and its mechanism of action in proximal intestine of goldfish

The effect of CCK-8S on isolated intestine strips of goldfish is shown in Figure 1. The addition of CCK-8S (1 nM to 1 µM) to the organ bath induced a significant contraction of the proximal intestine strips in a concentration-dependent manner. The EC_{50} value for the concentration-response curve corresponds to a CCK-8S concentration of 54 nM. The maximum intestinal contraction was achieved at 1 µM CCK-8S concentration, which induces a 12-fold increase in force. The role of calcium on such CCK-8S-induced contraction in intestine strips was studied in strips incubated in a Ca^{2+}-free saline solution (Fig. 1. dashed line). The addition of CCK-8S to the organ bath in absence of extracellular calcium induced a concentration-dependent contraction, but the EC50 increased more than 3 times (188 nM) compared to the EC50 value in control medium. Accordingly, the maximal contraction was reduced by a 34.1% compared to the contraction evoked in presence of extracellular calcium (Fig. 1).

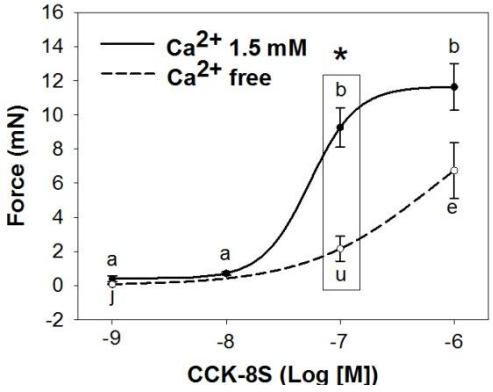

Fig. 1. Concentration-response curves of CCK-8S on contractile activity (mN) of goldfish isolated proximal intestine in presence (continuous line) or absence of extracellular calcium (dashed line). Results are expressed as mean ± SEM (n = 8). Different latin letters (for standard conditions) and different greek letters (for calcium-free conditions) indicate statistically significant differences ($p < 0.05$; ANOVA, Tukey post-hoc test) among different concentrations for each condition. * indicates statistical differences between presence and absence of extracellular calcium at the same CCK concentration ($p < 0.05$; ANOVA, Tukey post-hoc test).

To discard the possible involvement of cholinergic transmission in the CCK-8S induced contraction in intestinal strips, the organ bath intestine preparation was pre-incubated for 10 min in the presence of atropine, the general antagonist of muscarinic receptors. The atropine (100 µM) did not modify the contractile response of intestine strips evoked by CCK-8S (1 µM) (Fig. 2A). Moreover, the blockade of generation and conduction of action potentials obtained with the pre-incubation of intestine strips in the presence of tetrodotoxin (1µM) did not alter significantly the CCK-8S-induced contraction (Fig. 2B).

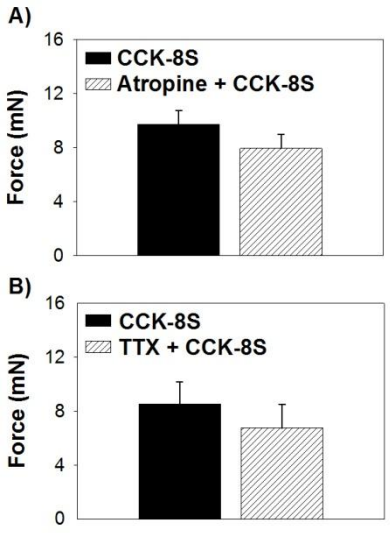

Fig. 2. Effects of preincubation during 10 min with (A) atropine (100 µM) and (B) tertrodotoxine (TTX) (1 µM) on CCK8S-evoked contraction (1 µM) of isolated proximal intestine from goldfish. Results are expressed as mean ± SEM (n = 6).

3.2. The CCK-8S-induced contraction in goldfish proximal intestine is mediated by the CCKAR receptor

Two different antagonists of CCK receptors were used to test the receptor subtype involved in the CCK-8S-induced contraction in the proximal intestine of goldfish. The Figure 3 shows two representative profiles of the contraction evoked by CCK-8S (1 µM) alone, and in the presence of the CCKAR antagonist, devazepide (1 µM). An inhibition of the maximal contraction induced by CCK-8S in the intestine strips pre-incubated with devazepide (1 µM) was observed. This inhibition was not observed at lower devazepide concentrations (from 30 to 300 nM), and reached around a 30% reduction in the presence of 1 µM of the antagonist in the organ bath (Fig. 4A). The pre-incubation of intestine strips for 10 min with the selective CCKBR antagonist, L365,260 (from 0.1 nM to 1µM) did not modify significantly the CCK-8S-induced contraction in the goldfish proximal intestine (Fig. 4B).

3.3. Two CCK receptors subtypes are expressed in goldfish tissues

Two partial fragments of 240 bp and 455 bp in length were cloned from the cDNA sequence of goldfish, CCKAR and CCKBR respectively, reporting the identification of the two subtypes of cholecystokinin receptors in goldfish tissues. Furthermore, we found two allelic variants of each gene, named CCKARv1 (GenBank accession no. **KP164828**) and CCKARv2 (GenBank accession no. **KP164829**), and CCKBRv1 (GenBank accession no **KP164830**) and CCKBRv2 (GenBank

accession no **KP164831**), different one another by one nucleotide base.

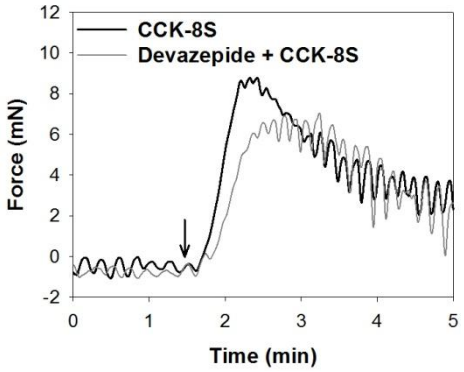

Fig. 3. Representative profile of the effect of CCK-1R antagonist, devazepide (1 μM) on the CCK-8S-evoked contraction (1 μM) of isolated proximal intestine from goldfish. The arrow indicates the time of CCK-8S addition.

The comparison among the deduced amino acid sequences from these cDNA fragments and those from other vertebrates retrieved from GenBank (NCBI) and Ensembl Genome is shown in a phylogenetic tree (Fig. 5), where only bootstrap values over 500 are included. The CCKAR and CCKBR clearly form two separated clades with 100% bootstrap support. In each clade, fish appear strongly separated from tetrapod species. In agreement with the concept of traditional taxonomy, goldfish CCK receptors amino acid sequences appear closely related to zebrafish, with a strong bootstrap support (943 for CCKAR and 984 for CCKBR) and 95.3% and 90% amino acid sequence identity for CCKAR and CCKBR, respectively. The highest sequence divergence was observed with mouse, sharing 82.5% and 45% amino acid sequence identity for CCKAR and CCKBR, respectively.

The Figure 6 shows the alignment among the amino acid sequences for the two receptor subtypes from goldfish, zebrafish and salmon. Regarding CCKAR, the partial peptide sequence obtained for goldfish corresponds with the amino acid positions 157 to 236 of salmon sequence, which are located between the transmembrane domains (TMD) 4 and 5. A detailed analysis demonstrates that the biologically functional residues of the cloned fragment (Lys 160, Ile 167, Trp 171, Met 200, Arg 202 and Leu 236) are conserved in both goldfish and salmon. Regarding the subtype CCKBR, the cloned fragment corresponds with the amino acid positions 181 to 334 of salmon sequence, comprising the peptide fragment from middle of TMD4 to the beginning of TMD6. All the functional motifs in salmon sequence (Trp 182, Met 189, Tyr 192, Ser 196, Cys 213, His 215, Phe 235, Pro 238, Leu 248, Tyr 254, Gln 258, Lys 329, Lys 330 and Arg 331) are conserved in goldfish.

3.4. Expression pattern of CCK receptors in goldfish

The distribution of goldfish CCKAR and CCKBR transcripts, and expression levels in various tissues of this teleost are shown in Figure 7. The highest expression of CCKAR was found in the proximal intestine, with high levels of mRNA expression along the whole intestinal tract (intestinal bulb, posterior gut and gallbladder). Low levels of CCKAR expression were found in the studied brain regions, liver and muscle (Fig. 7A). The goldfish CCKBR gen was predominantly expressed in hypothalamus, vagal lobe and posterior intestine, whereas faint expression of CCKBR was detected in telencephalon, and very faint expression in liver and muscle. The relative mRNA expression of this receptor subtype was almost no detectable in gallbladder, intestinal bulb and proximal intestine.

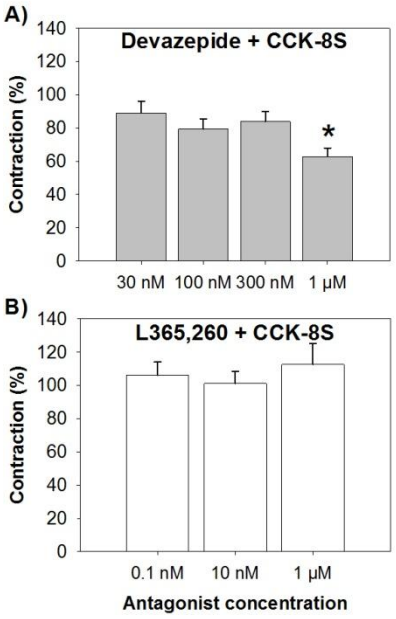

Fig. 4. Effects of CCK receptor antagonists on CCK-8S-evoked contraction (1 μM) of isolated proximal intestine from goldfish, preincubated 10 min with increasing concentrations of (A) L365,260 (n = 4/concentration) and (B) devazepide (n = 6/concentration). Results are expressed as percentage (mean ± SEM), considering as 100 % the maximal contraction induced by CCK (1 μM). * indicates statistical differences with the CCK-evoked contraction ($p < 0.05$; ANOVA, Tukey post-hoc test).

4. Discussion

The peptide CCK-8S induces a contractile response of the goldfish proximal intestine without modifications of spontaneous basal tone. This contraction is extracellular calcium dependent and seems to use a non-cholinergic pathway. Molecular distribution of mRNA specific CCK receptors and pharmacological approaches suggest that the CCK-8S-induced contractile response is mediated (at least in part) by the CCKAR receptor subtype, probably located in the muscle of the goldfish proximal intestine.

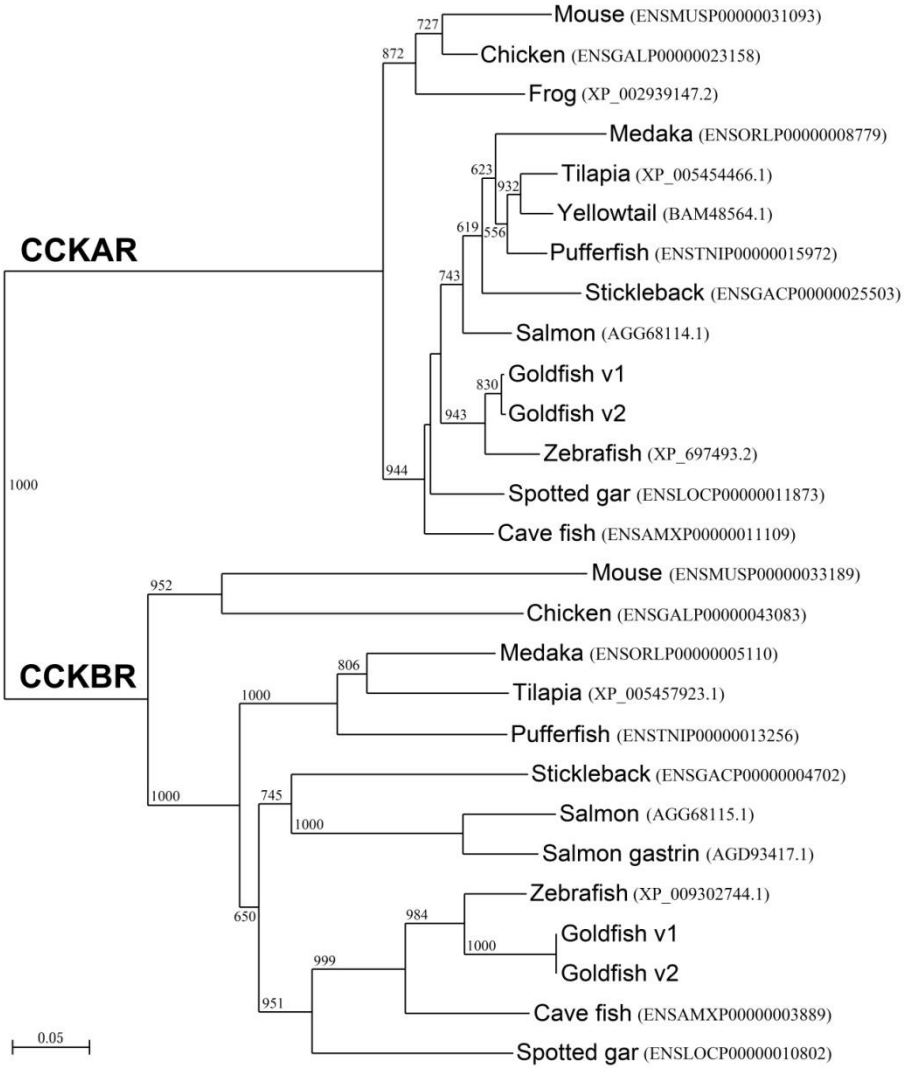

Fig. 5. Phylogenetic analysis of goldfish CCKAR and CCKBR. Partial peptide sequences were used to generate the dendrogram. The phylogenetic tree was generated by the Neighbor-Joining method using Clustal-W2 tool. Branch length is proportional to evolutionary distance. Number in branches indicates the robustness (significance) of each internal node after 1000 bootstrap repetitions. Numbers below 500 are omitted, indicating the low supported nodes. Peptide accession number for each species is given alone with the vernacular name.

The curve concentration-response obtained in goldfish, the EC_{50} value, and the identification of CCK receptors in the gut support the specificity of such effect of CCK-8S on intestine motility in this teleost. The magnitude of the contraction induced by the addition of CCK-8S to the organ bath is the same order than serotonin-induced contraction of intestinal strips in goldfish (Velarde et al., 2010), and lower than cholinergic contraction (Velarde et al., 2009). The increase in muscular tone is concentration-dependent (it is not observed at 10 nM lower concentrations) and reaches the maximum force with 10 µM CCK8-S concentrations. It is unknown the endogenous concentration of CCK-8 in fish intestine, but the EC_{50} value is into a similar affinity range to those reported in mammals (Dufresne et al., 2006). Overall. these data support the physiological role of the effect of CCK-8S demonstrated on this *in vitro* system in goldfish. In the present study we used commercially CCK-8S, as in previous studies that investigates gut motility in rainbow trout (Olsson et al., 1999) and Chinook salmon (Forgan y Foster, 2007). The C-terminus octapeptide of CCK is the most abundant form of CCK that is present in the gastrointestinal tract in mammals (Beinfeld 2003; McDonald, 2009), and the structure of this CCK-8 is well conserved during phylogeny (Johnsen, 1998, Staljanssens, 2011). In goldfish, it was described a protein of 123 amino acids, but it is suggested that the short form, CCK-8, is physiologically active, with the four-amino acid C-terminal consensus sequence (Trp-Met-Asp-Phe-NH_2) essential for receptor activation, being the third amino acid methionine, as in mammals (Peyon et al 1998). The CCK-8S is reported as the natural ligand with the highest affinity for CCKAR (Dufresne et al., 2006), and the sulphate ester is a critical determinant of the biological activity of this peptide in mammals (Noble et al., 1999).

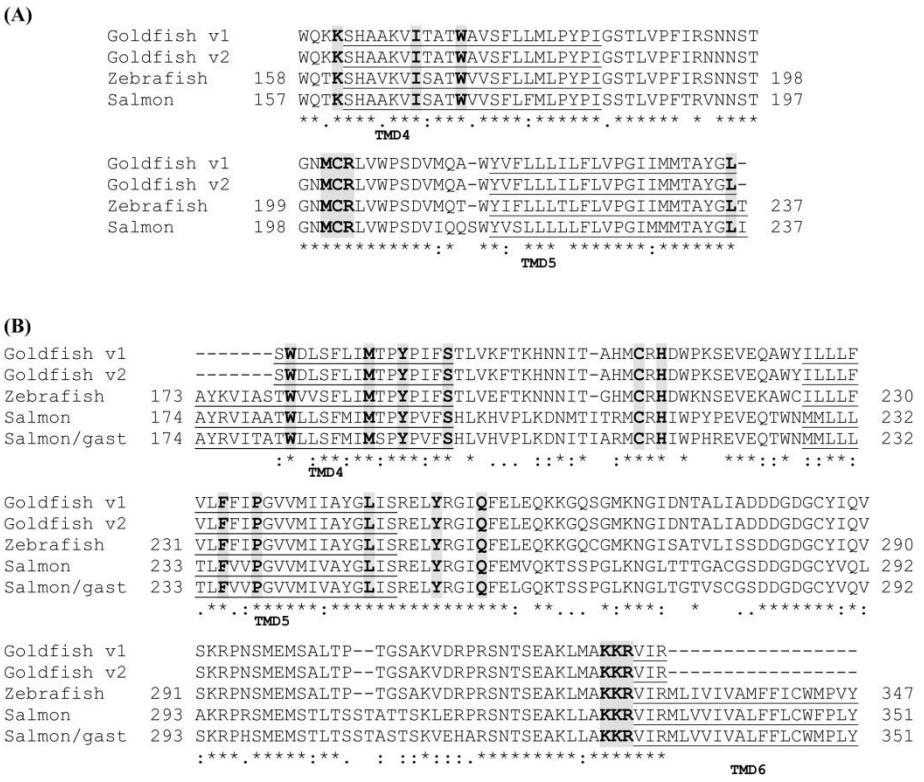

Fig. 6. Alignment of the two variants deduced (A) CCKAR v1 and CCKAR v2 and (B) CCKBR v1 and CCKBR v2 amino acid sequences of goldfish with the coincident fragments of zebrafish and salmon. Trans-membrane domains (TMD) are underlined and residues shaded represent functional motifs.

Gut motility in non-mammalian vertebrates as in mammals is controlled by the presence of food, autonomic nerves and gut peptides, including the CCK. In mammals, the CCK from the duodenum delay gastric emptying of food and affect the migrating motor complexes as well as gallbladder contractions (Dufresne et al., 2006). Present results in goldfish indicate a contractile action of CCK-8S without alterations of basal tone in proximal intestine strips preparations. The motility of proximal intestine in goldfish is well characterized, it exhibits a basal nitrergic tone (Velarde et al., 2011), and it is highly sensitive to cholinergic and serotoninergic contraction (Velarde et al 2009, 2010). Moreover, the proximal intestine shows the highest mRNA expression of CCKAR receptors (present results) and the highest frequency of CCK-producing cells in the perciform, the blacktip grouper (*Epinephelus fasciatus*) (Hur et al, 2013). However, the role of CCK on gut motility in fish remains largely unknown. Different effects were found in rainbow trout, depending on the concentration and experimental arrangement. The addition of CCK-8S on longitudinal strip preparations from the cardiac stomach increased the resting tension, but abolished the rhythmic contractions (Olsson et al., 1999). On the other hand, intra-arterial infusion of different concentrations of CCK-8S increased or decreased the frequency and amplitude of rhythmic contractions in cardiac stomach being unaffected the pyloric part of the stomach (Olsson et al., 1999). In the Chinook salmon it is described an increase in contractility (spikes per min) in response to CCK-8 (10^{-8} -10^{-6} M) in circular gut rings (Forgan and Foster, 2007), in agreement with results in the Atlantic cod (*Gadus morhua*) where longitudinal cardiac stomach strips were contracted by CCK (Jönsson et al., 1987). From a comparative view, salmonids, including the rainbow trout, have a well-developed stomach divided into a receptive, cardiac region leading into a narrower, muscular pyloric region which coordinates transfer of food to the intestine *via* the pyloric sphincter, while cyprinids as goldfish are stomachless teleost, and the proximal part of the intestine is often enlarged, forming a bulb-like structure serving storage purposes. Then, in spite of the general acceptance that CCK has inhibitory effects on gastric emptying in fish (rev. Olsson and Holmgren, 2011), the wide variety of responses reported suggest a more complex action of this peptide on fish gastrointestinal motility, as occurs in mammals, where CCK is relaxing sphincters but stimulating smooth muscle via cholinergic stimulation in gallbladder and small intestine (Grider 1994, Wu et al., 2013).

The effects of CCK on gastrointestinal tract motility are brought about either directly on smooth muscle cells or are mediated by neurons in the enteric nervous system (Daniel et al., 1989). In the regulation of gut motility acetylcholine and tachykinins are the main stimulators with different responses depending on the experimental set-up and the physiological state of the gut (Olsson and Holmgren, 2011). A cholinergic excitatory

innervation of the fish gut, as in most vertebrates, has long been established (Olsson, 2009), but the CCK-8S induced contraction in goldfish is atropine-resistant, indicating that the CCK-8S contraction is not under cholinergic control, and discard a mechanism involving CCK stimulation of vagal pathway. The dose of atropine used in the present study markedly inhibits cholinergic stimulation (Velarde et al., 2009), and abolish the serotonin-induced contraction (Velarde et al., 2010). In mammals, direct effects of CCK on smooth muscle

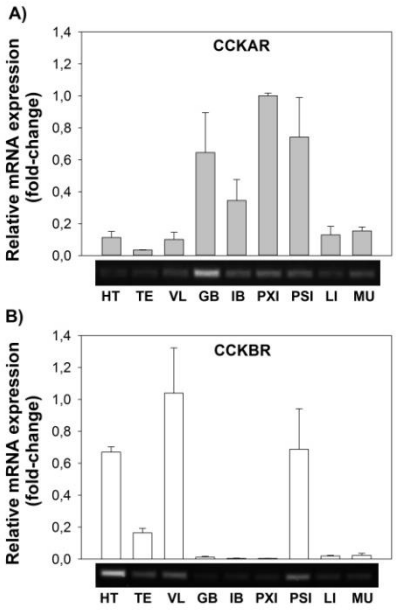

Fig. 7. Tissue distribution for (A) CCKAR and (B) CCKBR in goldfish. The cDNA fragments 187 and 148 pb respectively, were amplified by RT-PCR using β-actin as housekeeping gene and visualized on a 2% agarose gel . Data are expressed as mean ± SEM (n=2). HT: hypothalamus; TE: telencepahlon; VL: vagal lobe; GB: gallbladder; IB: intestinal bulb; PXI: proximal intestine; PSI: posterior intestine; LI: liver; MU: muscle.

cells seem to be invariably contractile whereas the neuraly-mediated may be contractile or relaxant actions (Grider, 1994). Olsson and coworkers (1999) by a combination of *in vivo* and *in vitro* approaches in rainbow trout conclude that the excitatory effects of CCK-8S at higher concentrations are at least partly mediated by cholinergic pathways. In goldfish we discard that CCK may stimulate cholinergic neurons, instead its contractile effect could be either direct (on the muscle) or indirect (non-cholinergic neuraly-mediated). But such possible indirect effect is also discarded because the CCK-8S-induced contraction were tetrodotoxin-insensitive, indicating that CCK-8S contractile effect is independent on the enteric nervous system. The fact that the basal myogenic rhythm was unaltered by CCK-8S discards the interstitial cells of Cajal as a direct target for this peptide, since the progression of the spontaneously occurring contractions in the teleost intestine is tetrodotoxin-sensitive (Karila y Holmgren, 1995). These results suggest that the site of CCK action in intestine of goldfish may be the smooth muscle cells, as occurs with melatonin effect on gut motility in this teleost (Velarde et al 2010). In support of a direct effect of CCK-8S on muscle cells in the goldfish intestine we found that this contractile effect is reduced in the absence of extracellular calcium, since the muscle contraction requires an increase in intracellular calcium concentration (either by influx from outside the cells through plasmatic membrane channels or by release from intracellular stores). A similar dependence on external calcium mobilization has been described in the cholinergic activation of gastric smooth muscle in rainbow trout (Aronsson and Holmgren 2000), in the melatonin-induced attenuation of cholinergic contraction (Velarde et al., 2009), and in the serotoninergic stimulation in proximal intestine of goldfish (Velarde et al.,2010). In mammals, the direct contractile effects of CCK have been evidenced by the use of tetrodotoxin, and the CCK receptors described on smooth muscle cells from different regions of the gut support such direct actions (Grider, 1994). However, to date it is unknown if the reported CCK effects on gastrointestinal motility in rainbow trout and Chinook salmon are direct on muscle cells or mediated by interneurons.

The specificity of the contractile response induced by CCK-8S in goldfish proximal intestine was examined by the pre-incubation of the intestine strips in the presence of CCK receptor antagonists. The obtained results indicate that the excitatory effect of CCK-8S is partially mediated by CCKAR receptor subtype, while the CCKBR receptor plays no significant role in the CCK regulated motility in goldfish intestine. Present results are the first that involves CCK receptors in the control of gut motility in fish, and agree with data in mammals where the CCKAR seems to be the subtype involved in gut motility regulation (Lee et al., 2013: Little et al., 2010; Staljanssens et al., 2011, Varga et al., 2004). The incomplete blockade of CCK-8S contraction by devazepide might suggest the existence of additional non-receptor mediated mechanisms underlying such CCK-8S contractile effect, or even the involvement of more than one receptor subtype, as suggested for the CCK-8S effects on the cardiac stomach in the rainbow trout (Olsson et al., 1999). Nevertheless, it cannot be discarded that pharmacology of devazepide, the most potent and widely studied among selective CCKAR antagonists in mammals (Varga et al., 2004; Berna et al., 2007; Yu 2014) would be slightly different in fish. In spite of the scarce knowledge available on fish CCK receptors pharmacology, devazepide treatment counteracts significantly CCK effects on food intake in coho salmon (*Oncorhynchus kisutch*) (Lohmus 2008).

Present study also reports the identification of both subtypes of CCK receptors (CCKAR and CCKBR) in goldfish. Comparisons and alignment analyses of the obtained sequences (GenBank and Ensembl databases) result in two separate clusters, that represent the two receptor subtypes described in vertebrates earlier named CCK-1R and CCK-2R (Rathore et al., 2013; Staljanssens et al., 2011). The high similarity of these two receptor genes in the vertebrate lineage lead to the

assumption that both CCKAR and CCKBR evolve from the same duplicated ancestor genes (Yu et al., 2014). Within each cluster, tetrapod and fish CCK receptors fall into a separate subcluster, indicating orthology among sequences. As expected, goldfish CCK receptors sequences appear more closely related with zebrafish sequences, sharing high similarity between them (95.3% for CCKAR and 90% for CCKBR). The lowest degree of similarity among teleosts is found with cavefish (*Astyanax fasciatus mexicanus*) CCKAR (86.8%) and tilapia (*Oreochromis mossambicus*) CCKBR (62%). This lower sequence identity for the CCKBR, and the higher length of their phylogenetic tree branches suggests that this CCKBR diversified more recently than CCKAR. Two transcript variants for each CCK receptor subtype in goldfish were demonstrated in this study, in agreement with the two CCKBR paralogues found in Atlantic salmon (CCK-2R1/gastrin, as ortholog of mammalian gastrin receptor, and CCK-2R2) (Rathore et al., 2013). In fact, Yu and co-workers (2014) recently suggest in many teleost such duplication for CCK2R gene, but not for CCK1R which seems to be present in only one copy in teleost. Our results in goldfish support such duplication for both CCK receptors subtypes. Duplicate genes are frequently found in teleosts as a result of the fish-specific whole-genome duplication that occurred about 350 million years ago (Hoegg et al., 2004; Taylor et al., 2003), and the additional duplication believed to have occurred in cyprinids and salmonids (Kurokawa y Murashita, 2009).

The partial sequences obtained in goldfish for both CCKAR and CCKBR include the peptide fragment containing the transmembrane domains 4 and 5, out of the 7 domains typical of the G-protein coupled receptor superfamily. Structural analysis of the sequences shows that all the functional motifs present in CCK receptors of mammals (Archer-Lahlou et al., 2005; Foucaud et al., 2008) and salmon (Rathore et al., 2013) are conserved in goldfish. Among these, human CCKAR residues in the second extracellular loop (ECL2) (Met and Arg) that account for the selectivity for sulfated versus nonsulfated ligand (Archer-Lahlou et al., 2005; Foucaud et al., 2008) are present in goldfish. Goldfish partial sequences conserved all the functional motifs involved in receptor-ligand interaction located between the positions 157 and 236 for salmon CCKAR sequence, and 181 and 334 for salmon CCKBR (Foucaud et al., 2008; Rathore et al., 2013). The bridge residue cysteine of ECL2 described in mammals (Miller y Gao, 2008; Rathore et al., 2013) is also conserved in both CCK receptor subtypes in goldfish.

The tissue expression pattern for the two CCK receptors genes cloned in goldfish is clearly different. The CCKAR is widely distributed by the organism, with the highest relative expression in the digestive organs (gallbladder, intestinal bulb, and in both segments of the intestine, proximal and posterior). In contrast, the CCKBR is mainly expressed in brain (vagal lobe, hypothalamus and telencephalon) with the presence of significant transcripts in the posterior intestine. This differential expression pattern for both receptor subtypes matches the one previously described in mammals (Dufresne et al., 2006; Noble et al., 1999) and Atlantic salmon (Rathore et al., 2013), supporting the functional specialization of each receptor subtype and the functional conservation of CCK receptors through the vertebrate phylogeny. Accordingly with this hypothesis, we provide present data supporting the functional implication of CCKAR, but not CCKBR, in the regulation of gut motility in goldfish. In mammals, the CCKAR is predominantly expressed in the gastrointestinal system, where it seems to mediate many digestive functions, including gut motility (Dufresne et al., 2006) and the transmission of sensory information from the gut to the brain (Varga et al., 2004). The CCKBR receptor which binds and responds to either gastrin or CCK with almost the same affinity or potency and discriminates poorly between sulfated and non-sulfated peptides (Dufresne et al., 2006) is mainly located in the central nervous system and participates in the neurobiology of anxiety, depression, psychosis, cognition, and nociception (Noble et al., 1999). Our results from pharmacological approaches in goldfish intestinal strips reinforce such functional specialization of CCK receptor subtypes, since only CCKAR, but not CCKBR, is involved in the contractile CCK-8S effect. Many studies in fish investigate the distribution of CCK peptides, but only a few studies localize expression of CCK receptors. A recent study in yellowtail shows that CCKAR is mainly expressed in the stomach, pyloric caeca, intestine, and gallbladder, and it mediates the secretion of digestive enzymes by CCK, as in mammals, supporting such functional conservation of CCKAR (Furutani et al, 2013). In the Atlantic salmon CCKAR mRNA is highly expressed in pancreas, gut and gallbladder, and *in vivo* and *in vitro* approaches suggest an involvement of this receptor in pancreatic regulation and gallbladder contractions by CCK (Rathore et al., 2013). The physiological functions of CCKBR in fish remain to be investigated.

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References

Aldman, G., Holmgren, S., 1995. Intraduodenal fat and amino acids activate gallbladder motility in the rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol. 100, 27-32.

Beinfeld MC (2003) Biosynthesis and processing of pro CCK: recent progress and future challenges. Life Sci. 72, 747–757.

Berna, M.J., Jensen, R.T., 2007. Role of CCK/gastrin receptors in gastrointestinal/metabolic diseases and results of human studies using gastrin/CCK receptor agonists/antagonists in these diseases. Curr Top Med Chem. 7, 1211-1231.

Chandra, R., Liddle, R.A., 2007. Cholecystokinin. Curr Opin Endocrinol Diabetes Obes. 14, 63-67.

De Pedro, N., Björnsson, T.B., 2001. Regulation of food intake by neuropeptides and hormones. In: Food intake in fish (Ed. Houlihan D., Boujard T., Jobling M.). Blackwell Science Oxford, pp 269-296.

Daniel, E.E., Collins, S.M., Fox, J.-A.E.T., Huizinga, J.D. 1989. Pharmacology of neuroendocrine peptides, In: Handbook of Physiology: The Gastrointestinal System, (Eds. S.G. Schultz S.G., Wood, J.D., Rauner B.B.), Vol. I, part 1, sec. 6, Am. Physiol. Soc., Bethesda, pp. 759–816.

Dufresne, M., Seva, C., Fourmy, D., 2006. Cholecystokinin and gastrin receptors. Physiol Rev. 86, 805-847.

Einarsson, S., Davies, P.S., Talbot, C., 1997. Effect of exogenous cholecystokinin on the discharge of the gallbladder and the secretion of trypsin and chymotrypsin from the pancreas of the Atlantic salmon, *Salmo salar* L. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 117, 63-67.

Forgan, L.G., Forster, M.E., 2007. Effects of potential mediators of an intestinal brake mechanism on gut motility in Chinook salmon (*Oncorhynchus tshawytscha*). Comp Biochem Physiol C Toxicol Pharmacol. 146, 343-347.

Furutani, T., Masumoto, T., Fukada, H., 2013. Molecular cloning and tissue distribution of cholecystokinin-1 receptor (CCK-1R) in yellowtail *Seriola quinqueradiata* and its response to feeding and in vitro CCK treatment. Gen Comp Endocrinol. 186, 1-8.

Grider, J.R., 1994. Role of cholecystokinin in the regulation of gastrointestinal motility. J Nutr. 124, 1334S-1339S.

Hebb, A.L.O., Poulin, J-F., Roach, S.P., Zacharko, R.M., Drolet, G. 2005. Cholecystokinin and endogenous opioid peptides: Interactive influence on pain, cognition, and emotion. Prog Neuro-Psychopharmacol Biol Psychiat 29, 1225- 1238.

Himick, B.A., Vigna, S.R., Peter, R.E. 1996. Characterization of cholecystokinin binding sites in goldfish brain and pituitary. Am J Physiol. 271, R137-R143.

Himick, B.A., Peter, R.E., 1994. CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. Am J Physiol. 267, R841-851.

Hur, S-W, Lee C-H, Lee S-H, Kim B-H, Kim, H-B, H-J Baek, Y-D Lee, 2013, Characterization of cholecystokinin-producing cells and mucus-secreting goblet cells in the blacktip grouper, Epinephelus fasciatus. Tissue Cell. 45,153– 157.

Johnsen, A.H., 1998. Phylogeny of the cholecystokinin/gastrin family. Front Neuroendocrinol. 19, 73-99.

Kurokawa, T., Suzuki, T., Hashimoto, H., 2003. Identification of gastrin and multiple cholecystokinin genes in teleost. Peptides. 24, 227-235.

Lee, J.H., Kim, S.Y., Kwon, Y.K., Kim, B.J., So, I., 2013. Characteristics of the cholecystokinin-induced depolarization of pacemaking activity in cultured interstitial cells of Cajal from murine small intestine. Cel Physiol Biochem. 31, 542-554.

Little, T.J., Gopinath, A., Patel, E., McGlone, A., Lassman, D.J., D'Amato, M., McLaughlin, J.T., Thompson. D.G., 2010. Gastric emptying of hexose sugars: role of osmolality, molecular structure and the CCK receptor. Neurogastroenterol Motil. 22:1183-1190.

MacDonald, E., Volkoff, H., 2009. Cloning, distribution and effects of season and nutritional status on the expression of neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and cholecystokinin (CCK) in winter flounder (*Pseudopleuronectes americanus*). Horm Behav. 56, 58-65.

Micale, V., Campo, S., D'Ascola, A., Guerrera, M.C., Levanti, M.B., Germana, A., Muglia, U., 2012. 3,4 Cholecystokinin in white sea bream: molecular cloning, regional expression, and immunohistochemical localization in the gut after feeding and fasting. Plos One. 7, e52428.

Murashita, K., Kurokawa, T., Nilsen, T.O., Ronnestad, I., 2009. Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): molecular cloning and tissue expression. Gen Comp Endocrinol. 160, 223-235.

Nisembaum L.G., Tinoco A.B., Moure A.L., Alonso-Gómez A.L., Delgado M.J., Valenciano A.I. 2013. The arylalkylamine-N-acetyltransferase (AA-NAT) acetylates dopamine in the digestive tract of goldfish: A role in intestinal motility. Neurochem Int. 62:873-880.

Noble, F., Wank, S.A., Crawley, J.N., Bradwejn, J., Seroogy, K.B., Hamon ,M., Roques, B.P. 1999. International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev. 51:745–781.

Olsson, C. Holmgren, S. 2011 Autonomic control of gut motility: A comparative view. Auton Neurosci.165 , 80–101.

Olsson, C., Aldman, G., Larsson, A., Holmgren, S., 1999. Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*. J Exp Biol. 202, 161-170.

Peyon, P., Lin, X.W., Himick, B.A., Peter, R.E., 1998. Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish. Peptides. 19, 199-210.

Polakof, S., Miguez, J.M., Soengas, J.L., 2011. Cholecystokinin impact on rainbow trout glucose homeostasis: possible involvement of central glucosensors. Regul. Pept. 172, 23–29.

Rajjo, I.M., Vigna, S.R., Crim, J.W., 1988. Actions of cholecystokinin-related peptides on the gallbladder of bony fishes in vitro. Comp Biochem Physiol C. 90, 267-273.

Rathore, R.M., Angotzi, A.R., Jordal, A.E., Ronnestad, I., 2013. Cholecystokinin receptors in Atlantic salmon: molecular cloning, gene expression, and structural basis. Physiol Rep. 1, e00069.

Rubio, V.C., Sánchez-Vázquez, F.J., Madrid, J.A., 2008. Role of cholecystokinin and its antagonist proglumide on macronutrient selection in European sea bass *Dicentrarchus labrax*, L. Physiol Behav. 93, 862–869.

Staljanssens, D., Azari, E.K., Christiaens, O., Beaufays, J., Lins, L., Van Camp, J., et al., 2011. The CCK(-like) receptor in the animal kingdom: functions, evolution and structures. Peptides. 32, 607-619.

Tillner, R., Rønnestad, I., Dhert, P., Ueberschär, B., 2014. The regulatory loop between gut cholecystokinin and tryptic enzyme activity in sea bass (*Dicentrarchus labrax*) larvae is influenced by different feeding regimes and trigger substances. Aquaculture. 420–421, 139–146.

Varga, G., Balint, A., Burghardt, B., D'Amato, M., 2004. Involvement of endogenous CCK and CCK1 receptors in colonic motor function. Br J Pharmacol. 141, 1275-1284.

Velarde, E., Alonso-Gómez, A.L., Azpeleta, C., Isorna, E., Delgado, M.J., 2009. Melatonin attenuates the acetylcholine-induced contraction in isolated intestine of a teleost fish. J Comp Physiol B. 179, 951-959.

Velarde E**.,** Delgado M.J., Alonso-Gómez A.L. 2010. Serotonin-induced contraction in isolated intestine from a teleost fish (*Carassius auratus*): characterization and interaction with melatonin. Neurogastroenterol Motil. 22, 364-373

Velarde, E., Alonso-Gómez, A.L., Azpeleta, C., Isorna, E., De Pedro, N., Delgado, M.J., 2011. Melatonin effects on gut motility are independent of the relaxation mediated by nitrergic system in the goldfish. Comp Biochem Physiol A. 159, 367-371.

Vigna, S.R., 2000. Evolution of the cholecystokinin and gastrin peptides and receptors. Am Zool. 40, 287-295.

Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Berniere, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen Comp Endocrinol. 142, 3–19.

Wu, T., Rayner, C.K., Young, R.L., Horowitz, M., 2013. Gut motility and enteroendocrine secretion. Curr Opinion Pharmacol. 13, 928–934.

Yu, N., Smagghe, G., 2014. CCK(-like) and receptors: Structure and phylogeny in a comparative perspective. Gen Comp Endocrinol. 209, 74-81.

Capítulo 3

La ghrelina en la regulación del comportamiento alimentario, el crecimiento y el metabolismo lipídico en la trucha común (*Salmo trutta*)

3.1. *Ghrelin increases food intake, swimming activity and growth in juvenile Brown trout (Salmo trutta).*

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Ghrelin increases food intake, swimming activity and growth in juvenile brown trout (*Salmo trutta*)

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HIGHLIGHTS

- Ghrelin increases food intake and growth in juvenile wild brown trout.
- Lipid metabolism and lipid deposition in liver/muscle were not modified by ghrelin.
- Ghrelin may increase the swimming and foraging activities in juvenile wild brown trout.
- Ghrelin could be implicated in aggressive behavior in juvenile wild brown trout.
- Ghrelin peripheral treatment (7 days) has no effect on brain monoamines and NPY mRNA.

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ABSTRACT

Several key functions of ghrelin are well conserved through vertebrate phylogeny. However, some of ghrelin's effects are contradictory and among teleosts only a limited number of species have been used in functional studies on food intake and foraging-related behaviors. Here we investigated the long-term effects of ghrelin on food intake, growth, swimming activity and aggressive contest behavior in one year old wild brown trout (*Salmo trutta*) using intraperitoneal implants. Food intake and swimming activity were individually recorded starting from day 1, and aggressive behavior was tested at day 11, after ghrelin implantation. Body weight and growth rate were measured from the beginning to the end of the experiment. Triglycerides and lipase activity in muscle and liver; monoaminergic activity in the telencephalon and brainstem; and neuropeptide Y (NPY) mRNA levels in the hypothalamus were analyzed. Ghrelin treatment was found to increase food intake and growth without modifying lipid deposition or lipid metabolism in liver and muscle. Ghrelin treatment led to an increased foraging activity and a trend towards a higher swimming activity. Moreover, ghrelin-treated fish showed a tendency to initiate more conflicts, but this motivation was not reflected in a higher ability to win the conflicts. No changes were observed in monoaminergic activity and NPY mRNA levels in the brain. Ghrelin is therefore suggested to act as an orexigenic hormone regulating behavior in juvenile wild brown trout. These actions are accompanied with an increased growth without the alteration of liver and muscle lipid metabolism and they do not seem to be mediated by changes in brain monoaminergic activity or hypothalamic expression of NPY.

1. Introduction

Ghrelin is a 28 amino acid peptide that was discovered in rat stomach by Kojima et al. [1] as the endogenous ligand to the growth hormone secretagogue-receptor (GHSR), and thus a potent stimulator of pituitary growth hormone (GH) release. A number of studies have identified GHSR, both at the gene transcript and protein levels, in hypothalamic appetite centers as well as in various peripheral tissues in several non-mammalian species such as chicken (*Gallus gallus*) [2], blackhead seabream (*Acanthopagrus schlegelii*) [3], rainbow trout (*Oncorhynchus mykiss*) [4], and goldfish (*Carassius auratus*) [5]. The ghrelin gene has been cloned in several fish species [6–9] (for more information see review: [10]). Although ghrelin is mainly produced in the stomach of vertebrates, it is also expressed in other tissues including the brain, intestine, pancreas, gall bladder, kidney and gills [1,11].

In mammals, ghrelin generally promotes food intake, body weight gain and adiposity through central and peripheral modes of action [12,13]. It has been suggested that ghrelin has an important role in meal initiation because it increases just before feeding, and decreases immediately after food intake [13]. Subsequent studies also suggest that ghrelin regulates non-homeostatic aspects of eating, as hedonic eating or reward-seeking behavior [14,15] and learning/memory processes [16]. In rats, the orexigenic action of ghrelin is mediated by the neuropeptide Y (NPY)/agouti-related protein in the brain [12,17,18].

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As in mammals, ghrelin stimulates GH secretion from the pituitary in fish [6,7,19,20]. Moreover, the role of ghrelin in the regulation of energy balance is supported by an orexigenic action of ghrelin under both acute and chronic treatments [9,21–24]. Both single central and peripheral ghrelin injections increased food intake in goldfish [9,24] by stimulating hypothalamic orexigenic peptides, such as NPY and orexin [21,22]. In orange-spotted grouper (*Epinephelus coioides*), a diet supplemented (8 weeks) with ghrelin increased food intake and growth by the NPY neuronal pathway [25], as in goldfish and mammals. In Mozambique tilapia (*Oreochromis mossambicus*) ghrelin increased food intake, and hence body weight gain and hepatic fat deposition after long-term (21 days) peripheral treatment [23]. In rainbow trout the effects of ghrelin treatment on food intake seem to vary depending on dose, route of administration and source of hormone [26,27]. Both short-term intracerebroventricular (icv) treatment and long-term (duration of 14 days) intraperitoneal (ip) treatment with homologous ghrelin decreased food intake in rainbow trout. The acute central action was likely mediated through the central anorexigenic corticotropin-releasing factor (CRF) system in the central nervous system (CNS) [26]. This is similar to data found in neonatal chicks, where central ghrelin injections suppress food intake via the CRF system [28]. On the other hand, in rainbow trout ip injections with heterologous ghrelin stimulated food intake [27], whereas ip injections with rainbow trout ghrelin (rtGHRL) had no effect on food intake in the short term [29]. The overall amino acid similarity between rat and rtGHRL is 39%, however, the first 7 N-terminal amino acids of ghrelin are identical between both species and this part is considered as the "active core". An in vitro assay showed similar bioactivity between rat ghrelin and rtGHRL [6], but how this translates to the in vivo situation in rainbow trout has not been explored in the same study. Moreover, in vivo data in chicken and zebrafish indicate that other regions than the N-terminal are part of the pharmacophore [30,31].

Studies have revealed that locomotor activity and/or emotional behavior are affected by food intake regulators. However, the few studies that have addressed the role of ghrelin on locomotor activity have yielded inconsistent results. In goldfish and rodents, a single icv and ip ghrelin treatment alters locomotor activity but the effects depend on the route of administration [32–34]. In rainbow trout, on the other hand, activity was unchanged after 14 days of ghrelin treatment [26]. Ghrelin may also alter behavior by interacting with brain monoamines [14,32], which are known to be involved in behavioral functions in both mammals and fish [35,36]. Ghrelin altered the dopaminergic system in mice after central administration [14,32]. Based on mammalian findings, Kang et al. [37] hypothesized that ghrelin may mediate locomotor activity via noradrenergic pathways, or through interaction with hypothalamic neuropeptides such as orexin in goldfish. However, to our knowledge nothing is known about the potential effects of peripheral ghrelin treatment on brain monoamine content in fish. The dopaminergic system mediates the GH-induced increase in swimming activity and foraging in rainbow trout [38,39]. The GH treatment also increases dominance, risk-taking and aggression, demonstrating an integrated action of GH on both physiology and behavior in fish [40,41]. However, despite the well-known general link between ghrelin and GH it is not known if ghrelin can modify aggressive behavior in fish similarly to GH.

Data on ghrelin effects on food intake in fish are somewhat inconsistent and only a limited number of fish species have been used in functional studies on food intake and swimming activity. Salmonids are suitable models for the study of energy balance regulation, both due to their importance in aquaculture and their seasonal and life-stage dependent changes in feeding behavior and appetite [42,43]. Brown trouts (*Salmo trutta*) spend their first 1–3 years in streams where they aggressively defend feeding territories [44] or form hierarchical groups, depending on population density [45,46]. The first aim of this study was to clarify the effects of ghrelin on feeding, growth, swimming activity and aggressive behavior in one year old wild brown trout. The second aim was to investigate if NPY and monoamines are involved in mediating these effects.

2. Materials and methods

The experiments were approved by the Ethical Committee for Animal Research in Gothenburg (License 8-2011), and comply with current laws in Sweden and the European Directive 2010/63/EU. The Gothenburg team has 25 years experience of behavioral and physiological studies on salmonids and care was taken to minimize stress to the fish during all phases of the study.

2.1. Experimental design

This research was carried out in two experiments starting on the 20th of April 2011 (experiment 1) and the 3rd of June 2011 (experiment 2). Wild brown trout (1 + year parr; 26 fish of 6.5–8.5 cm and 20 fish of 7.1–9.5 cm, experiments 1 and 2 respectively) were caught by electrofishing (LUGAB 1000, straight DC, 400 V; Lug AB, Sweden) in River Lerån (N 58° 2.510′, E 11° 54.991′; experiment 1) and Norumsån (N58° 2.589′, E11° 50.759′; experiment 2). The fish were brought into holding aquaria to acclimate at the Department of Biological and Environmental Sciences, University of Gothenburg, Sweden for 10 days. Then, 20 fish were randomly distributed to individual experimental 5 l aquaria with the bottom covered with sand and the walls partly covered with black plastic to reduce stress. The fish were held at 12°C and simulated natural photoperiod (using an electronic timer) and hand-fed once a day to satiety with calf liver (6.2 ± 0.26 mg of dry weight) as previously described [47]. After 5 days of acclimation, fish were anesthetized (2-phenoxyethanol, 0.5 ml/l; ICN Biomedicals Inc., Germany), weighed (body mass; BM; g) and measured (fork length; L; cm). Then, fish (n = 10/group) were ip implanted with or without rainbow trout ghrelin (rtGHRL) at a dose of 475 ng rtGHRL/g BM as described by Jönsson et al. [26]. The ghrelin used was the octanoylated (C-8) 23-amino acid form, synthesized by Peptide Institute Inc., Japan [6]. The treatment (ghrelin or control) of each fish was kept anonymous to the person observing. Food intake in both experiments, and swimming activity and aggressive behavior in experiment 1 were studied. The duration of the first experiment was based on Jönsson et al. [26]. Based on the results observed in experiment 1, we decided that a 7-day second experiment would be adequate for brain sampling since behavioral differences between treatment groups were evident. The treatment period was considered as sub-chronic. On day 12 (experiment 1) and 7 (experiment 2) fish were killed by an overdose of anesthetic (2-phenoxyethanol, 1 ml/l), followed by a blow to the head. The BM (g) and L (cm) were recorded, and liver was excised and weighed. Muscle (a square piece of 1 × 1 cm) was cut just behind the dorsal fin, and the hypothalamus, telencephalon and brainstem were quickly dissected out in experiment 2. Samples were immediately frozen on liquid nitrogen and stored at −80 °C.

2.2. Food intake, growth, and lipid metabolism studies

Food intake was individually recorded once daily (9:00 am) starting from day 1 after pellet implantation to days 9 and 6 for experiments 1 and 2, respectively. Each fish was initially presented with one piece of liver. If the fish ate within 60 s we continued feeding it until the animal refused three consecutive pieces of food and was considered to be satisfied. If the fish did not eat the food within a minute we moved to the next aquaria. All the observations were done in a random order among the fish each day.

Specific growth rate for weight, condition factor and hepatosomatic index were calculated as $SGRW = (\ln(BM_f / BM_i)100)/d$; $CF = (BM / L^3)100$; and $HSI = (W_{Li} / BM_f)100$, where BMi and BMf are the body mass initial and final, respectively, d is the number of days and W_{Li} is the liver weight.

The triglyceride (TG) content in liver and muscle (nmol/g of tissue) was analyzed following the protocol described by Schwartz and Wolins [48]. The lipoprotein lipase (LPL; nmol/μg of protein) activity was quantified with the Roar LPL activity assay kit (Roar Biomedical, USA). The method is based on the fluorometric quantification of the product of LPL-mediated lipolysis of an added substrate which is directly proportional to the LPL activity. The protein quantification was done using the BCA kit (Pierce, USA).

2.3. Behavioral tests: swimming activity and dyadic aggression

Swimming activity was individually recorded every second day until day 8 after treatment. The activity was studied for each fish for 3 min at 8:00 am (1 h before feeding time) and 2:00 pm (5 h after feeding time) using a video camera. To quantify the activity each tank was marked with 1 horizontal line and 3 vertical lines (Fig. 1). The position of the fish was recorded every 3 s. The proportion of time actively maintaining a position in the water column (holding) and in food intake position (the area in which the food fell into the tank, Fig 1), and the number of squares crossed as a measure of swimming activity were calculated for each individual. All the observations were done in a random order among the fish each day.

At day 9 post-treatment, size-matched pairs of fish (one control and one ghrelin; $n = 9$; maximum size difference 0.3 ± 0.1 cm) were transferred to a contest tank separated into two compartments by a dividing PVC-wall. The dyadic aggression test was done at day 11 to allow the fish to establish territories for 2 days before the contest. The procedure employed to quantify the aggressiveness was done as previously described by Neregård et al. [49]. The variables studied were: (1) time to start conflicts (the time elapsed between the removal of the dividing wall and the first aggressive interaction); (2) identity of initiator (of an interaction); (3) number of attacks + bites (rapid approach towards an individual who is displaced + contact including a bite); (4) circling (number of laps the fish circle around each other head to tail with erect fins); (5) conflict duration (time from the first interaction to the resolution of the conflict) and (6) identity of winner.

2.4. Brain amines quantification

The content of norepinephrine (NE), dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA) in the telencephalon and brainstem was quantified by HPLC coupled with coulometric electrochemical detection (Coulochem II, ESA). The tissues were homogenized by sonication in 100 μl of cold perchloric acid (0.3 N; Scharlau, Spain) containing sodium bisulfite (0.4 mM; Sigma Chemical, Spain) and ethylenediaminetetraacetic acid (EDTA, 0.4 mM; Sigma Chemical, Spain). The homogenate was centrifuged (13,000 rpm for 5 min) and the supernatant was injected into the HPLC system. The mobile phase (flow rate 1 ml per min) consisted of 10 mM phosphoric acid, 0.1 mM disodium EDTA, 0.4 mM sodium octanesulfonic acid (Sigma Chemical, Spain) and 3% of acetonitrile (Panreac, Spain), pH 3.1. The analytical column was C18, 125 × 4.6 mm internal diameter, 5 pm particle size. The oxidation potential was 200 mV and the signal from analytical cell was recorded with a sensitivity of 20 nA. The amount of monoamines in the samples was calculated as the area under peaks and expressed as pmol per mg of protein. Protein content was determined by the method of Lowry et al. [50], using serum bovine albumin (Sigma Chemical, Spain) as standard.

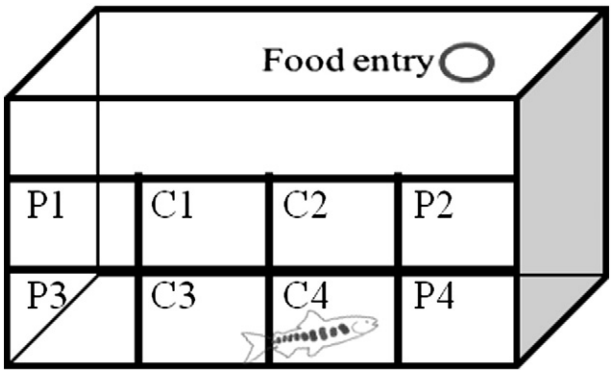

Fig. 1. Illustration of the aquaria sections used to analyze the holding position, swimming activity and food intake position.

2.5. NPY mRNA level quantification

To obtain the brown trout NPY partial sequence (*btNPY*), total RNA from the hypothalamus was extracted, treated with DNase, and transcribed according to the protocol used by Tinoco et al. [51]. Specific primers (Table 1) were designed using ClustalW alignment [52] of rainbow trout (*O. mykiss*, GenBank ID: AF203902) and Atlantic salmon (*Salmo salar*, GenBank ID: AB455539) NPY sequences, and the software *Primer3* [53]. The cDNA amplification was carried out by PCR with annealing temperatures increasing from 55 °C to 64 °C. The sizes of the obtained fragments were: 373 bp for F2R1; 364 bp for F2R2; 299 bp for F3R1; and 290 bp for F3R2. The PCR products of all trials were gel purified using the kit by GenElute™ (Sigma Chemical, Spain) and sequenced in the genomic unit of Complutense University of Madrid.

To analyze the ghrelin effect on hypothalamic NPY expression, total RNA from the hypothalamus was extracted and treated with DNase. Then, 0.3 μg of RNA was retro-transcribed and the expressions of *btNPY* mRNA and 18Sr RNA as reference gene (18Sr, GenBank ID: FJ710888) were analyzed by qRT-PCR using specifics primers (Table 1). The protocol used was the same as described by Tinoco et al. [51]. Annealing temperatures for qRT-PCR were: 65 °C for *btNPY* and 61 °C for 18Sr. Negative controls included replacement of cDNA by water and the use of non-retrotranscribed total RNA. The efficiency of the amplification for all genes studied was around 100% and the specificity of the amplification was confirmed by the melting temperature and the visualization of the obtained PCR products in agarose gel. The ΔΔCt method [54] was used to determine the relative mRNA expression (fold change) assigned with relative value of "1" to the control group.

2.5.1. Statistical analysis

Food intake, growth parameters, triglyceride content and lipase activity, monoamines and NPY mRNA levels were analyzed by independent t-tests. Activity data were analyzed using a generalized linear mixed models based on negative binomial distribution for count data and binomial distribution for proportion data, using log link functions. The model

Table 1
Primer sequences.

Target gene	Accession number		Primer sequences 5′ → 3′	Product (bp)
Primers used to obtain brown trout NPY sequence				
NPYf2	AF203902/ AB455539	Foward	gcagaacgcacagcagcagaaa	
NPYf3	AF203902/ AB455539	Foward	atgcatcctaacttgggtacctgg	
NPYr1	AF203902/ AB455539	Reverse	cacaacgagggttcatcatatctg	
NPYr2	AF203902/ AB455539	Reverse	ggttcatcatatctggactgtggg	
Primers for qRT-PCR				
btNPY	KC991202	Foward	gaaggctacccggtcaaac	112
		Reverse	cctctgccttgtgatgaggt	
18Sr rRNA	FJ710888	Foward	tggccgttcttagttggt	113
		Reverse	ctctaagaagttggacgccg	

included treatment (ghrelin/control) as fixed factor, day of treatment as a fixed within-subject factor, the interaction term between day and treatment, and experiment (first or second) as a random factor. The contest aggression data were analyzed with one-sample binomial test for categorical data (probability of winning or initiating a conflict), and Wilcoxon signed-rank test for the continuous data (time to initiation, aggression and circling frequencies). All statistical analyses were done using IBM SPSS Statistics 20 (IBM Corporation, USA).

3. Results

3.1. Effect of ghrelin on food intake, growth, and lipid metabolism

The rtGHRL treatment induced a significantly higher mean daily food intake ($p = 0.02$). The amount of ingested food was three times higher in ghrelin-treated fish than in control fish (Fig. 2A; experiments 1 and 2 combined). The SGRW, as an index of daily BM increase, was significantly higher (~two-fold) in ghrelin-treated compared with control fish ($p = 0.03$; Fig. 2). This effect is observed combining the results for both experiments for 7 days of treatment.

The discrete growth parameters, BM, L and CF, were not significantly modified after 7 days of ghrelin peripheral treatment (experiment 2; Table 2). Neither were there any significant differences after 12 days of treatment in experiment 1 (Table 2). However, ghrelin-treated fish showed higher values than the control group in all the growth parameters studied.

The HSI, hepatic TG content and LPL activity were not significantly altered by 7 or 12 days of ghrelin treatment (Table 2). There were no significant differences in the TG content or the LPL activity in muscle between the ghrelin-treated and control groups (Table 2).

3.2. Effect of ghrelin on behavior: swimming activity and aggression

Ghrelin treatment did not significantly influence the proportion of time actively maintaining a position in the water column (holding) or swimming activity (number of squares crossed) in the morning or afternoon (Fig. 3A–B, C–D, Table 3). However, there was a trend towards a higher swimming activity in ghrelin-treated compared to control-treated fish in the morning ($p = 0.074$; Fig. 3C, Table 3). Also, a trend to spend more time in the food intake position during the morning in ghrelin-treated fish was observed ($p = 0.098$; Fig. 3E, Table 3). Ghrelin-treated fish spent significantly more time that control fish in the food intake position during the afternoon ($p = 0.043$; Fig. 3F, Table 3). In addition, from the first to the last day of the feeding trials there was a general and significant increase in the time spent in the food intake position in the morning for all fish ($p = 0.034$; Fig. 3E, Table 3).

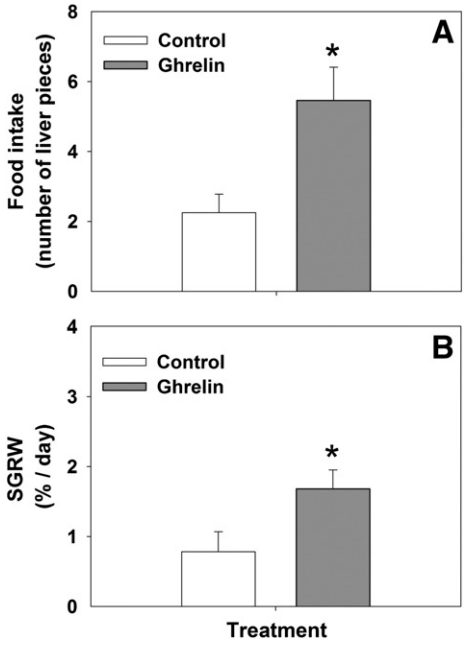

Fig. 2. Mean food intake and mean specific growth rate for weight (SGRW) in control and ghrelin treated fish for 7 days of treatment. Data are expressed as mean ± standard error (*: $p < 0.05$). Data from experiments 1 and 2 were combined.

Table 2
Somatic growth and lipid analysis in control- and ghrelin-treated brown trout.

	Control	Ghrelin	p-Value (*t*-test)
Experiment 1			
BM_i (g)	3.54 ± 0.28	3.45 ± 0.28	0.823
BM_f (g)	3.56 ± 0.22	4.02 ± 0.39	0.321
L_i (cm)	7.36 ± 0.21	7.23 ± 0.18	0.371
L_f (cm)	7.33 ± 0.18	7.40 ± 0.21	0.815
CF_i	0.88 ± 0.01	0.90 ± 0.01	0.224
CF_f	0.90 ± 0.03	0.97 ± 0.04	0.134
HSI (%)	0.90 ± 0.12	1.10 ± 0.11	0.232
Experiment 2			
BM_i (g)	5.41 ± 0.59	6.04 ± 0.47	0.248
BM_f (g)	5.49 ± 0.59	6.58 ± 0.52	0.146
L_i (cm)	8.16 ± 0.28	8.56 ± 0.19	0.256
L_f (cm)	8.24 ± 0.26	8.71 ± 0.19	0.159
CF_i	0.97 ± 0.16	0.95 ± 0.03	0.528
CF_f	0.96 ± 0.03	0.98 ± 0.02	0.537
HSI (%)	1.36 ± 0.08	1.58 ± 0.15	0.237
Liver TG (nmol/g)	386.22 ± 65.44	308.47 ± 29.77	0.329
Muscle TG (nmol/g)	209.89 ± 26.7	207.07 ± 37.88	0.953
Liver LPL (nmol/μg prot)	2.17 ± 0.25	2.51 ± 0.37	0.464
Muscle LPL (nmol/μg prot)	0.18 ± 0.01	0.20 ± 0.01	0.319

Initial and final body mass (BM_i; BM_f), fork length (L_i; L_f) and condition factor (CF_i; CF_f), hepatosomatic index (HSI), triglyceride content (TG) and lipoprotein lipase activity (LPL) in muscle and liver. Data are shown as mean ± SEM.

Ghrelin treatment did not significantly affect the agonistic behavior of brown trout after 11 days of treatment. There were no significant differences in the time to start the conflicts, the aggression (number of attacks + bites, and circling) and the conflict duration (data not shown). However, ghrelin-treated fish tended to initiate more conflicts than control fish (7 ghrelin vs 2 control; $p = 0.18$; Fig. 4), but this motivation was not reflected in the competitive ability since ghrelin-treated and control fish won an equal number of aggressive interactions (Fig. 4).

3.3. Effect of ghrelin on brain monoamines and NPY mRNA levels

The contents of NE, DA and 5-HT were not modified in the telencephalon and brainstem after 7 days of ghrelin peripheral treatment. However, there was a weak tendency towards lower 5-HIAA levels in ghrelin-treated fish compared with control fish in the telencephalon ($p = 0.090$; Table 4). The content of DOPAC in the samples was below the detection limit.

The partial sequence of NPY from brown trout has high similarity with the amino acid NPY sequences of other salmonids such as Atlantic salmon (GenBank ID: BAH24101; 100%) and rainbow trout (GenBank ID: AAQ13835; 97%). Peripheral ghrelin treatment for 7 days had no effect on mRNA levels of NPY in the hypothalamus (Table 4).

4. Discussion

Peripheral sub-chronic ghrelin treatment had a strong effect on food intake which was more than three times as high in ghrelin-treated fish than in controls, supporting an important orexigenic role of ghrelin in juvenile brown trout. This confirms the results reported in chronically ghrelin-treated Mozambique tilapia, orange-spotted grouper [23,25] and Atlantic salmon (Jönsson, unpublished data), and is also consistent

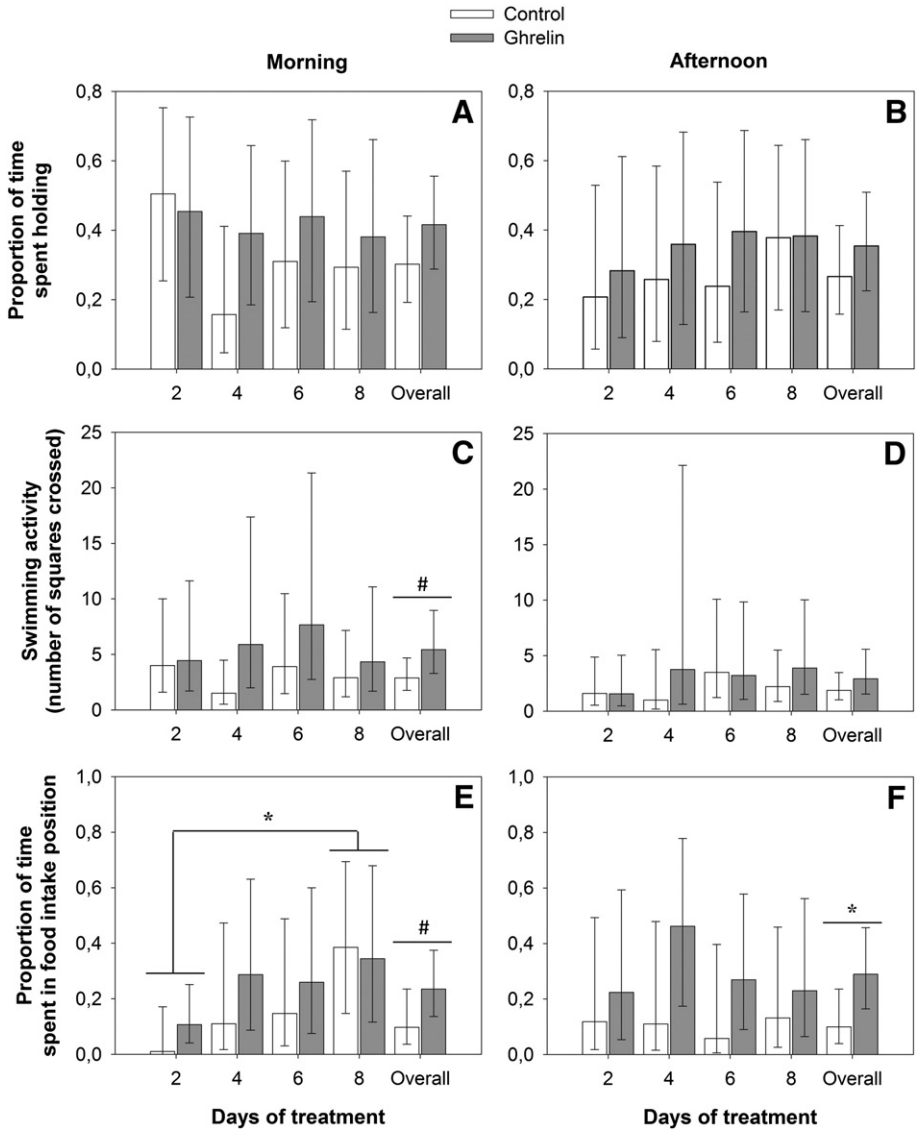

Fig. 3. Behavioral measurements over time for proportion of time spent holding (actively maintaining a position in the water column), swimming (number of squares crossed), and proportion of time spent in food intake position in the morning and in the afternoon, for 8 days in control- and ghrelin-treated fish. Data are expressed as estimated marginal mean ± 95% Wald confidence interval by day/group and overall mean value for the whole period for each group. (*: $p < 0.05$; #: $p < 0.1$).

with more short term (hours after treatment) studies in goldfish using homologous ghrelin [9,22,24]. These results also agree with the stimulatory effect of ghrelin on food intake described in mammals [12,13]. In rainbow trout, however, single ip-injected rtGHRL had no effect on food intake [29]. Moreover, both a 2-week peripheral treatment and a short-term icv treatment with rtGHRL decreased daily food intake in rainbow trout [26]. The previously described orexigenic role of ghrelin in rainbow trout (acute treatment) was probably due to heterologous ghrelin being used [27]. Despite using the same route of administration and type of ghrelin that Jönsson and coworkers used [26], we observed an opposite effect of this hormone on food intake in juvenile brown trout. This difference could be due to the different life stages of the two species of salmonids, or as a result of the long-time domestication process experienced by rainbow trout. However, species-specific effects of ghrelin on appetite as has been described for some other functions cannot be ruled out [11].

Our results show a clear effect of ghrelin on growth rate, which more than doubled in ghrelin-treated fish suggesting that ghrelin stimulates body weight gain in brown trout through increased food intake. However, the ghrelin treatment probably did not last long enough to generate a significant difference in body mass and condition factor. It is generally accepted that ghrelin increases body mass in mammals due to increased food intake and reduction in fat utilization, promoting fat storage [13]. In fish, this hypothesis is supported to some extent by previous studies on Mozambique tilapia [23], goldfish [37] and orange-spotted grouper [25]. In rainbow trout, however, the anorexigenic effect of ghrelin instead resulted in decreased growth rate after 14 days [26]. In this study, ghrelin did not affect HSI, which can be used as an index of fat incorporation into the liver, muscle and liver fat content or uptake (as measured by LPL activity). A possible explanation for this is that the increased physical activity in the ghrelin-treated fish antagonized the lipogenic action of ghrelin. Thus, it seems that the increased growth rate of brown trout as in grouper [25] and males of goldfish [37] was mainly due to the stimulation of food intake by ghrelin. In addition to species-specific functions, explanations for the sometimes inconsistent effects of ghrelin in fish may involve differences in experimental design, developmental/life stage, as well as seasonal changes in behavior, appetite, growth and adiposity.

We also observed that ghrelin-treated fish tended to be more active than control fish in the morning before feeding time, which is in line with a recent short-term study on goldfish where there was an increase in locomotor activity after icv-ghrelin treatment [37]. However, after ip-

Table 3
Parameters of linear mixed model analysis.

	Interaction	F	df	p-Value
Morning (8 am)				
Proportion of time spent holding	D	0.982	3; 68	0.407
	T	1.440	1; 68	0.234
	T × D	0.522	3; 68	0.669
Swimming activity (number of squares crossed)	D	0.508	3; 68	0.678
	T	3.299	1; 68	0.074
	T × D	0.651	3; 68	0.651
Proportion of time spent in food intake position	D	3.056	3; 68	0.034
	T	2.822	1; 68	0.098
	T × D	0.746	3; 68	0.528
Afternoon (2 pm)				
Proportion of time spent holding	D	0.352	3; 68	0.788
	T	0.810	1; 68	0.371
	T × D	0.126	3; 68	0.944
Swimming activity (number of squares crossed)	D	0.759	3; 67	0.521
	T	0.987	1; 67	0.324
	T × D	0.420	3; 67	0.740
Proportion of time spent in food intake position	D	0.251	3; 67	0.860
	T	4.256	1; 67	0.043
	T × D	0.290	3; 67	0.833

Day (D), treatment (T), interaction between day and treatment (T × D), observed F-statistic (F) and degrees of freedom (df).

Table 4
Brain amines content and hypothalamic neuropeptide Y expression level in control- and ghrelin-treated brown trout.

	Tissue	Control	Ghrelin	p-Value (*t*-test)
NA (pmol/mg prot)	Telencephalon	61.74 ± 8.59	45.03 ± 5.43	0.110
DA (pmol/mg prot)		11.65 ± 3.21	12.09 ± 2.32	0.912
5HT (pmol/mg prot)		25.72 ± 1.06	21.98 ± 2.19	0.167
5HIAA (pmol/mg prot)		6.97 ± 0.74	5.36 ± 0.52	0.090
5HIAA/5HT (%)		27.36 ± 2.96	28.91 ± 7.01	0.851
NA (pmol/mg prot)	Brainstem	22.49 ± 2.09	20.91 ± 2.44	0.627
DA (pmol/mg prot)		11.78 ± 2.59	12.32 ± 2.45	0.882
5HT (pmol/mg prot)		18.67 ± 1.27	16.70 ± 1.73	0.373
5HIAA (pmol/mg prot)		3.13 ± 0.25	2.89 ± 0.38	0.610
5HIAA/5HT (%)		16.99 ± 1.24	17.14 ± 1.13	0.936
NPY mRNA (fold change)	Hypothalamus	1.16 ± 0.27	0.83 ± 0.17	0.274

Noradrenaline (NA), dopamine (DA), serotonine (5HT), indolacetic acid (5HIAA), the ratio between serotonin metabolite and serotonin (5HIAA/5HT) and neuropeptide Y expression levels (NPY mRNA). Data are shown as mean ± SEM.

ghrelin injections activity decreased in goldfish [34,37]. Moreover, in rainbow trout, a 14-day treatment with rtGHRL (ip) had no effect on swimming activity [26]. In mammals, the effect of ghrelin on physical activity depends on the administration route and dose. Ghrelin icv-treatment decreased activity [33], while peripheral treatment increased locomotor activity and accumbal DA-overflow [32]. On the other hand, central ghrelin treatment increased the locomotor activity when the ghrelin injection was specifically targeted to the nucleus accumbens [14]. The mechanisms responsible for ghrelin's different actions on locomotor activity among the same, or among different, species are still unclear. Our results also showed that ghrelin-treated fish spend more time in the feeding position (i.e. close to the food inlet), suggesting that ghrelin stimulates appetite and the motivation to feed. Similar effects have been found in mammals, i.e. Siberian hamsters (*Phodopus sungorus*), where ghrelin treatment increased foraging and hoarding behaviors [55] but, to our knowledge, this is the first study showing the direct effect of ghrelin on foraging activity in fish. An indirect link between ghrelin and foraging activity has been suggested in smallmouth bass (*Micropterus dolomieu*) in which plasma ghrelin levels in males are elevated near the time when they stop guarding their fry and begin to actively forage again [56]. In addition, it has been demonstrated that exogenous GH increases swimming activity and foraging in rainbow trout [38,39,57]. It is possible that ghrelin alters swimming activity and foraging indirectly by increasing GH levels in brown trout. However, this picture is inconclusive as ghrelin had no long-term in vivo effect on circulating GH levels in tilapia [23] or rainbow trout [26]. More studies are needed to clarify the potential short- and long-term effects of ghrelin on GH secretion in brown trout. Moreover, it has been suggested that ghrelin may have a beneficial effect on memory retention in rats [16]. Thus, ghrelin-induced effects on learning and memory could potentially have influenced the behavioral results, but additional specially designed experiments are required to separate such effects from purely motivational influences.

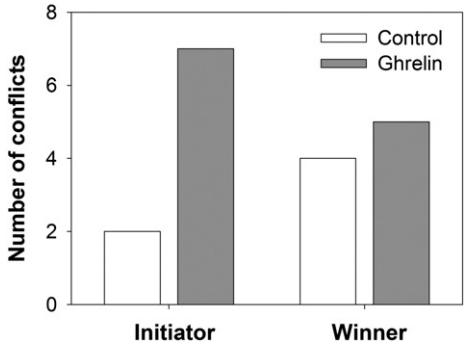

Fig. 4. Frequencies of control- and ghrelin-treated fish initiating and winning dyadic aggression conflicts staged between nine size-matched pairs consisting of one control- and one ghrelin-treated fish. The dyadic conflicts were conducted on day 11 post-treatment.

For the first time, the possible role of ghrelin on aggressive contest behavior in fish was investigated. Ghrelin-treated fish showed a tendency to initiate more conflicts than the control group, but the results were not significant, potentially due to low statistical power since only nine pairs were tested. Similarly, Riley et al. [23] made the observation that ghrelin-treated Mozambique tilapia seemed to feed more aggressively, and it is well established that GH increases dominance, risk-taking and aggression in salmonids [40,41]. It is possible that ghrelin can have similar effects, e.g. we found no effects of ghrelin on competitive ability (i.e. the ability to win dyadic contests), which is concordant with previous studies in rainbow and brown trout in which GH treatment increased aggression but, not the probability of winning contests [49,58]. More studies are necessary to understand the motivational effect of ghrelin on aggression and to what extent this effect is mediated by GH.

It has been demonstrated in rodents that the peripheral ghrelin signal is transmitted to the CNS via vagal or splanchnic afferents [59]. The orexigenic neuropeptides orexin and NPY are implicated as the central regulators of food intake in response to ghrelin in goldfish [21,22]. In rainbow trout, where ghrelin has an anorexigenic effect, this is likely mediated by CRH in the CNS [26]. In this study, contrary to what happens in rainbow trout [26] we observed that ghrelin stimulated food intake. Therefore, the brain NPY mRNA levels together with the monoaminergic activity were quantified. Ghrelin treatment for 7 days did not modify the monoaminergic activity in the telencephalon and brainstem. However, it is possible that the low sample size reduced the statistical power to detect treatment effects. In rodents, central or peripheral administration of ghrelin induced dopamine-overflow in nucleus accumbens [14,32]. There is no published data on the effect of ghrelin treatment on monoamine content in fish; but in rainbow trout the brain's dopaminergic system has been found to mediate the behavioral effects of GH [38,39]. Further studies are needed to elucidate if there is a link between ghrelin, dopamine and other brain monoamines in fish. In mammals, NPY in hypothalamic appetite centers mediates the effect of ghrelin on food intake [17,18]. In agreement with this, it was

demonstrated that the orexigenic action of ghrelin is mediated by the NPY neuronal pathway in goldfish [22]. A single icv, but not ip-injection, of ghrelin increased brain NPY expression levels (2 h post-injection) in goldfish [22], and a ghrelin-supplemented diet for 8 weeks increased hypothalamic NPY mRNA levels in orange-spotted grouper [25]. In our study, hypothalamic NPY mRNA expression was not modified after 7 days of ghrelin treatment indicating that the stimulatory effect of ghrelin on food intake via NPY occurs at the posttranslational level or through other modes of action. For example, it is possible that ghrelin stimulates food intake by altering NPY release, without the stimulation of its synthesis, as was suggested after a single peripheral injection of ghrelin in goldfish [22]. This study is a first investigation of a possible mechanism of action of ghrelin in brown trout. The orexigenic effect that was found warrants further studies aimed at examining the possible involvement of other orexigenic regulators, e.g. orexin, as suggested in goldfish [21].

5. Conclusions

In summary, we have demonstrated that ghrelin induces behavioral changes in foraging activity and food intake in brown trout resulting in increased growth, without any alteration in lipid metabolism or lipid deposition in liver and muscle. Ghrelin-treated fish also tended to be more active than control-treated fish. In addition, a possible relation between ghrelin and aggression may be indicated, but needs to be addressed in future studies. The lack of effect of ghrelin on central NPY and monoaminergic activity warrants further investigations into the mechanisms of action of ghrelin on appetite and behavior in brown trout.

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References

[1] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1994;402:656–60.
[2] Geelissen SM, Beck IM, Darras VM, Kuhn ER, Van der Geyten S. Distribution and regulation of chicken growth hormone secretagogue receptor isoforms. Gen Comp Endocrinol 2003;134:167–74.
[3] Chan CB, Cheng CH. Identification and functional characterization of two alternatively spliced growth hormone secretagogue receptor transcripts from the pituitary of black seabream *Acanthopagrus schlegeli*. Mol Cell Endocrinol 2004;214:81–95.
[4] Kaiya H, Mori T, Miyazato M, Kangawa K. Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*. Comp Biochem Physiol A 2009;153:438–50.
[5] Kaiya H, Miura T, Matsuda K, Miyazato M, Kangawa K. Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2a in goldfish, *Carassius auratus*. Mol Cell Endocrinol 2010;327:25–39.
[6] Kaiya H, Kojima M, Hosoda H, Moriyama S, Takahashi A, Kawauchi H, et al. Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. Endocrinology 2003;144:5215–26.
[7] Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T, Grau EG, et al. Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. J Endocrinol 2003;176:415–23.
[8] Murashita K, Kurokawa T, Nilsen TO, Rönnestad I. Ghrelin, cholesystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): molecular cloning and tissue expression. Gen Comp Endocrinol 2009;160:223–35.
[9] Unniappan S, Lin X, Cervini L, Rivier J, Kaiya H, Kangawa K, et al. Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology 2002;143:4143–6.
[10] Kaiya H, Miyazato M, Kangawa K. Recent advances in the phylogenetic study of ghrelin. Peptides 2011;32:2155–74.
[11] Kaiya H, Miyazato M, Kangawa K, Peter RE, Unniappan S. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp Biochem Physiol A 2008;149:109–28.
[12] Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–8.
[13] Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407:908–13.
[14] Jerlhag E, Egecioglu E, Dickson SL, Andersson M, Svensson L, Engel JA. Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward. Addict Biol 2006;11:45–54.
[15] Perelló M, Sakata I, Birnbaum S, Chuang JC, Osborne-Lawrence S, Rovinsky SA, et al. Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. Biol Psychiatry 2010;67:880–6.
[16] Carlini VP, Monzon ME, Varas MM, Cragnolini AB, Schioth HB, Scimonelli TN, et al. Ghrelin increases anxiety-like behavior and memory retention in rats. Biochem Biophys Res Commun 2002;299:739–43.
[17] Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti related protein mRNA levels and body weight in rats. Diabetes 2001;50:2438–43.
[18] Seoane LM, Lopez M, Tovar S, Casanueva FF, Senaris R, Dieguez C. Agouti related peptide, neuropeptide Y and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. Endocrinology 2003;144:544–51.
[19] Picha ME, Strom CN, Riley LG, Walker AA, Won ET, Johnstone WM, et al. Plasma ghrelin and growth hormone regulation in response to metabolic state in hybrid striped bass: effects of feeding, ghrelin and insulin-like growth factor-I on in vivo and in vitro GH secretion. Gen Comp Endocrinol 2009;161:365–72.
[20] Ran X, Li W, Lin H. Rat ghrelin stimulates GH release and GH mRNA expression in the pituitary of orange-spotted grouper, *Epinephelus coioides*. Fish Physiol Biochem 2004;30:95–102.
[21] Miura T, Maruyama K, Shimakura S, Kaiya H, Uchiyama M, Kangawa K, et al. Regulation of food intake in the goldfish by interaction between ghrelin and orexin. Peptides 2007;28:1207–13.
[22] Miura T, Maruyama K, Shimakura S, Kaiya H, Uchiyama M, Kangawa K, et al. Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. Neurosci Lett 2006;407:279–83.
[23] Riley LG, Fox BK, Kaiya H, Hirano T, Grau EG. Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*. Gen Comp Endocrinol 2005;142:234–40.
[24] Unniappan S, Canosa LF, Peter RE. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology 2004;79:100–8.
[25] Gao YJ, Tian LX, Yang HJ, Liang GY, Yue YR, Liu YJ. The influence of ghrelin and des-ghrelin on feed intake, growth performance and hypothalamic NPY mRNA expression of grouper *Epinephelus coioides*. Aquaculture 2012;364–365:19–24.
[26] Jönsson E, Kaiya H, Björnsson BTh. Ghrelin decreases food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) through the central anorexigenic corticotropin-releasing factor system. Gen Comp Endocrinol 2010;166:39–46.
[27] Shepherd BS, Johnson JK, Silverstein JT, Parhar IS, Vijayan MM, McGuire A, et al. Endocrine and orexigenic actions of growth hormone secretagogues in rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol A 2007;146:390–9.
[28] Saito ES, Kaiya H, Tachibana T, Tomonaga S, Denbow DM, Kangawa K, et al. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul Pept 2005;125:201–8.
[29] Jönsson E, Forsman A, Einarsdottir IE, Kaiya H, Ruohonen K, Björnsson BTh. Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. Comp Biochem Physiol A 2007;147:1116–24.
[30] Kitazawa T, Kaiya H, Taneike T. Contractile effect of ghrelin-related peptides on the chicken gastrointestinal tract in vitro. Peptides 2007;28:617–24.
[31] Olsson C, Holbrook JD, Bompadre G, Jönsson E, Hoyle CHV, Sanger GJ, et al. Identification of genes for the ghrelin and motilin receptors and a novel related gene in fish, and stimulation of intestinal motility in zebrafish (*Danio rerio*) by ghrelin and motilin. Gen Comp Endocrinol 2008;155:217–26.
[32] Jerlhag E. Systemic administration of ghrelin induces conditioned place preference and stimulates accumbal dopamine. Addict Biol 2008;13:358–63.
[33] Tang-Christensen M, Vrang N, Ortmann S, Bidlingmaier M, Horvath TL, Tschöp M. Central administration of ghrelin and agouti-related protein (83-132) increases food intake and decreases spontaneous locomotor activity in rats. Endocrinology 2004;145:4645–52.
[34] Yahashi S, Kang KS, Kaiya H, Matsuda K. GHRP-6 mimics ghrelin-induced stimulation of food intake and suppression of locomotor activity in goldfish. Peptides 2012;34:324–8.
[35] Mason ST. Catecholamines and behaviour. Cambridge: Cambridge University Press; 1984.
[36] Winberg S, Nilsson G. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. Comp Biochem Physiol C 1993;106:597–614.
[37] Kang KS, Yahashi S, Matsuda K. The effects of ghrelin on energy balance and psychomotor activity in a goldfish model: an overview. Int J Pept 2011;2011:171034.
[38] Johansson V, Winberg S, Jönsson E, Hall D, Björnsson BTh. Peripherally administered growth hormone increases brain dopaminergic activity and swimming in rainbow trout. Horm Behav 2004;46:436–43.
[39] Jönsson E, Johansson V, Björnsson BTh, Winberg S. Central nervous system actions of growth hormone on brain monoamine levels and behavior of juvenile rainbow trout. Horm Behav 2003;43:367–74.

[40] Johnsson JI, Winberg S, Sloman KA. Social interactions. In: Sloman KA, Wilson RW, Balshine S, editors. Behav Physiol Fish, vol. 24. New York: Elsevier Academic Press; 2006. p. 151–96.
[41] Jönsson E, Björnsson BTh. Physiological functions of growth hormone in fish with special reference to its influence on behaviour. Fish Sci 2002;68:742–74.
[42] Kadri S, Thorpe JE, Metcalfe NB. Anorexia in one-sea-winter Atlantic salmon (*Salmo salar*) during summer, associated with sexual maturation. Aquaculture 1997; 151:405–9.
[43] Metcalfe NB, Thorpe JE. Anorexia and defended energy levels in overwintering juvenile salmon. J Anim Ecol 1992;61:175–81.
[44] Johnsson JI, Nöbbelin F, Bohlin T. Territorial competition among wild brown trout fry: effects of ownership and body size. J Fish Biol 1999;54:469–72.
[45] Jenkins TM. Social structure position choice and microdistribution of two trout species (*Salmo trutta* and *Salmo gairdneri*) resident in mountain streams. Anim Behav Monogr 1969;2:57–123.
[46] Kalleberg H. Observations in a stream tank of territoriality and competition in juvenile salmon and trout (*Salmo salar* L. and *S. trutta* L.). Rept Instr Freshw Res Drottningholm 1958;39:55–98.
[47] Brockmark S, Adriaenssens B, Johnsson JI. Less is more: density influences the development of behavioural life skills in trout. Proc R Soc B 2010;277:3035–43.
[48] Schwartz DM, Wolins NE. A simple and rapid method to assay triacylglycerol in cells and tissues. J Lipid Res 2007;48:2514–20.
[49] Neregård L, Sundt-Hansen L, Björnsson BTh, Johnsson JI. Growth hormone affects behaviour of wild brown trout *Salmo trutta* in territorial owner-intruder conflicts. J Fish Biol 2008;73:2341–51.
[50] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[51] Tinoco AB, Nisembaum LG, Isorna E, Delgado MJ, de Pedro N. Leptins and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions. Peptides 2012;34:329–35.
[52] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23:2947–8.
[53] Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.
[54] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(\Delta\Delta C(T))$ method. Methods 2001; 25:402–8.
[55] Keen-Rhinehart E, Bartness TJ. Peripheral ghrelin injections stimulate food intake, foraging, and food hoarding in Siberian hamsters. Am J Physiol Regul Integr Comp Physiol 2004;288:R716–22.
[56] Hanson KC, Abizaid A, Cooke SJ. Causes and consequences of voluntary anorexia during the parental care period of wild male smallmouth bass (*Micropterus dolomieu*). Horm Behav 2009;56:5039.
[57] Jönsson E, Johansson IJ, Björnsson BTh. Growth hormone increases predation exposure of rainbow trout. Proc R Soc Lond B Biol Sci 1996;263:647–51.
[58] Jönsson E, Johnsson JI, Björnsson BTh. Growth hormone increases aggressive behavior in juvenile rainbow trout. Horm Behav 1998;33:9–15.
[59] Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 2002;123:1120–8.

Capítulo 4

Regulación de la ingesta en los peces por un mediador lipídico. Acciones de la oleoiletanolamida en el carpín (*Carassius auratus*)

4.1. *Role of oleoylethanolamide as a feeding regulator in goldfish.*
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RESEARCH ARTICLE

Role of oleoylethanolamide as a feeding regulator in goldfish

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ABSTRACT

Oleoylethanolamide (OEA) is a bioactive lipid mediator, produced in the intestine and other tissues, which is involved in energy balance regulation in mammals, modulating feeding and lipid metabolism. The purpose of the present study was to investigate the presence and possible role of OEA in feeding regulation in goldfish (*Carassius auratus*). We assessed whether goldfish peripheral tissues and brain contain OEA and their regulation by nutritional status. OEA was detected in all studied tissues (liver, intestinal bulb, proximal intestine, muscle, hypothalamus, telencephalon and brainstem). Food deprivation (48 h) reduced intestinal OEA levels and levels increased upon re-feeding, suggesting that this compound may be involved in the short-term regulation of food intake in goldfish, as a satiety factor. Next, the effects of acute intraperitoneal administration of OEA on feeding, swimming and plasma levels of glucose and triglycerides were analysed. Food intake, swimming activity and circulating triglyceride levels were reduced by OEA 2 h post-injection. Finally, the possible interplay among OEA and other feeding regulators (leptin, cholecystokinin, ghrelin, neuropeptide Y, orexin and monoamines) was investigated. OEA actions on energy homeostasis in goldfish could be mediated, at least in part, through interactions with ghrelin and the serotonergic system, as OEA treatment reduced ghrelin expression in the intestinal bulb, and increased serotonergic activity in the telencephalon. In summary, our results indicate for the first time in fish that OEA could be involved in the regulation of feeding, swimming and lipid metabolism, suggesting a high conservation of OEA actions in energy balance throughout vertebrate evolution.

KEY WORDS: Fatty acid ethanolamide, Food intake, Locomotor activity, Monoamines, Triglycerides, *Carassius auratus*

INTRODUCTION

Energy homeostasis in animals is tightly regulated by a complex network of signals adjusting food intake to satisfy metabolic and nutritional requirements. The gastrointestinal tract is involved in feeding regulation in vertebrates through both neuronal and humoral mechanisms. Among these peripheral signals originating in the gastrointestinal tract, lipid-derived messengers such as oleoylethanolamide (OEA) can play a significant role in the regulation of energy balance, as shown by several studies in mammals (Lo Verme et al., 2005; Thabuis et al., 2008; Piomelli, 2013). OEA is a fatty acid ethanolamide (FAE), a structural analogue of the endocannabinoid arachidonoylethanolamide (anandamide) but does not activate the cannabinoid receptors (Rodríguez de Fonseca et al., 2001). This FAE acts as an endogenous ligand for peroxisome proliferator-activated receptor alpha (PPAR-α) (Rodríguez de Fonseca et al., 2001; Fu et al., 2003). In addition to binding to this nuclear receptor, its effects may also be mediated at least in part by the transient receptor potential vanilloid subtype 1 (TRPV1) (Ahern, 2003; Almási et al., 2008) and an orphan G-protein coupled receptor (GPR119) (Overton et al., 2006).

OEA has been detected in different peripheral tissues and brain in mammals (Fu et al., 2007; Izzo et al., 2010). Nutrient status regulates OEA mobilization in a tissue-specific manner. In the small intestine, OEA levels decrease during food deprivation and increase upon re-feeding in rat (Rodríguez de Fonseca et al., 2001; Petersen et al., 2006) and mice (Fu et al., 2007). A feeding-induced OEA mobilization in small intestine of the Burmese python (*Python molurus*) has also been described (Astarita et al., 2006a). By contrast, OEA levels increase in liver, pancreas and fat in response to fasting, and no changes were observed in other peripheral tissues (stomach, colon, lung, heart, muscle and kidney) or in brain structures (brainstem, hypothalamus, cerebellum, cortex, thalamus and striatum) in rats (Fu et al., 2007; Izzo et al., 2010). The periprandial fluctuations of OEA found in small intestine suggest that this lipid amide may contribute to the regulation of feeding behaviour, possibly acting as a satiety signal. Pharmacological studies in rodents support this idea, as systemic administration of OEA causes a dose- and time-dependent suppression of food intake by prolonging the interval between successive meals (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Gaetani et al., 2003; Cani et al., 2004; Nielsen et al., 2004). This response is not due to stress, malaise or aversion, although the anorectic effect of OEA is accompanied by a suppression of locomotor activity in mammals (Rodríguez de Fonseca et al., 2001; Proulx et al., 2005). In rats, OEA injection was followed by reductions in ambulation and in spontaneous activity in the open field, and by an increase in the time that rats pushed their abdomen against the floor with splayed hindlimbs (Proulx et al., 2005). Nevertheless, it has been suggested that OEA modulates feeding and locomotion through distinct mechanisms, because the anorectic action, but not its effect on movement, was abrogated after capsaicin treatment (Rodríguez de Fonseca et al., 2001).

The molecular mechanisms involved in the anorectic effect of OEA have been partially elucidated in mammals. It is known that OEA-induced hypophagia is mediated by the stimulation of vagal sensory nerves that in turn stimulate the brainstem and hypothalamus (Rodríguez de Fonseca et al., 2001; Wang et al., 2005; Fu et al., 2011). Anorectic actions of OEA can be mediated through the modulation of central and peripheral signals involved in feeding regulation. It has been described that this FAE suppresses feeding by activating hypothalamic oxytocin transmission (Gaetani et al., 2010; Romano et al., 2013). Moreover, interactions between OEA and hypothalamic monoamines and cocaine- and amphetamine-regulated transcript (CART) have also been suggested (Serrano et al., 2011). At the peripheral level, some gastrointestinal neuropeptides are modified by OEA administration, although contradictory data have been published in rats. On the one hand, reductions in gut peptides, such as peptide YY and ghrelin, have

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List of symbols and abbreviations

5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine (serotonin)
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
FAE	fatty acid ethanolamide
GHRL	ghrelin
i.p.	intraperitoneal
M_b	body mass
MS-222	tricaine methanesulphonate
NA	noradrenaline
NPY	neuropeptide Y
OEA	oleoylethanolamide
PPAR-α	peroxisome proliferator-activated receptor alpha

been described after OEA administration (Cani et al., 2004; Serrano et al., 2011). On the other hand, Proulx et al. reported that OEA reduces food intake without causing peripheral changes in several gastrointestinal peptides, included peptide YY and ghrelin (Proulx et al., 2005).

In addition to its short-term effects on feeding, OEA has also been implicated in the control of body mass and lipid metabolism. Subchronic (1 week) and chronic (2 or more weeks) administration of this FAE decreased food intake accompanied by a marked inhibition of body mass gain in rodents (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Guzmán et al., 2004; Fu et al., 2005). It has been proposed that the effect of OEA on body mass is due not only to the feeding decrease but also to a direct effect on lipid metabolism (Lo Verme et al., 2005). Specifically, OEA promotes lipolysis and inhibits lipogenesis in important metabolic tissues such as liver, adipose tissue, muscle and gut (Thabuis et al., 2008; Pavón et al., 2010).

Accumulating evidence indicates that basic mechanisms controlling feeding behaviour are generally conserved among vertebrates. Fish are a valuable experimental model because they show a remarkable diversity that makes them attractive for the study of the evolution of feeding regulation systems in vertebrates (Hoskins and Volkoff, 2012). As in other vertebrates, food intake in fish is regulated by a complex interplay among hormones, neuropeptides and monoaminergic systems, acting at the central and peripheral level. Goldfish, *Carassius auratus* (Linnaeus 1758), is one of the most studied teleost species with regard to feeding regulation (Volkoff et al., 2009). Neuropeptide Y (NPY), orexins and ghrelin are examples of powerful orexigenic factors in this species, whereas cholecystokinin (CCK) and leptin act as anorexic signals (de Pedro and Björnsson, 2001; Volkoff et al., 2009). Dopamine (DA) and serotonin (5-HT) systems have been found to inhibit food intake, while noradrenaline (NA) stimulates it (de Pedro et al., 1998a; de Pedro et al., 1998b). Moreover, interactions between monoaminergic systems and other feeding regulators have been previously reported in goldfish (de Pedro et al., 1998a; de Pedro et al., 2006; de Pedro et al., 2008).

The involvement of FAEs in the control of food intake in fish was reported for the first time by Valenti et al. (Valenti et al., 2005). They demonstrated that the goldfish brain contains the cannabinoid CB_1 receptor, the endocannabinoids anandamide and 2-arachidonoylglycerol, as well as an enzymatic activity similar to the mammalian FAAH (fatty acid amide hydrolase). Intraperitoneal (i.p.) administration of anandamide stimulated food intake at low doses in this species. In agreement with the orexigenic role of anandamide, fasting increased its levels in the telencephalon. Similar results were observed in the sea bream *Sparus aurata* (Piccinetti et al., 2010), with brain anandamide and 2-arachidonoylglycerol raised by 24 h of food deprivation, and a food intake increase induced by anandamide administration. However, to date, nothing is known about whether other FAEs, such as OEA, are involved in food intake regulation in fish. As FAEs, particularly OEA, have been linked to diet and it is known that dietary lipids reduce feeding (Librán-Pérez et al., 2012; Librán-Pérez et al., 2014), this FAE might have an important role in the regulation of feeding and body composition in fish, valuable information for fields such as aquaculture.

The present study was aimed at investigating the presence and possible role of OEA in food intake in fish, using the cyprinid *C. auratus* as an experimental model. First, we assessed whether goldfish peripheral tissues and brain contain OEA and whether this compound is regulated by nutritional status. Thus, OEA levels in liver, intestinal bulb, proximal intestine, muscle, hypothalamus, telencephalon and brainstem of goldfish, fed or following 48 h of the food deprivation, with or without re-feeding, were measured. Next, we analysed the effects of acute OEA administration on food intake, locomotor activity and plasma glucose and triglycerides in this species. Finally, we studied the possible interplay among this FAE and some known feeding regulators in this teleost. With this objective, gene expression of peripheral (leptin, CCK and ghrelin) and central (leptin, NPY and orexin) signals and brain activity of monoaminergic systems were analysed after OEA administration under two feeding conditions: fed and following 24 h food deprivation.

RESULTS

Experiment 1: effects of fasting and feeding on OEA content

Endogenous OEA was detected in all tissues of *C. auratus* studied, both central and peripheral. The OEA content in the intestinal bulb and proximal intestine was almost 5 and 3 times higher than the values observed in muscle and liver, respectively (Fig. 1). In the brain, the highest OEA content was observed in the brainstem, almost 3 and 6 times higher than in the hypothalamus and telencephalon, respectively (Table 1). The OEA levels in the brainstem were comparable to those found in the gastrointestinal tract.

Fig. 1 shows the OEA content in peripheral tissues in fed, fasted (48 h) and fasted (48 h) + re-fed fish 30 and 120 min after feeding. OEA levels at 30 min were markedly decreased ($P<0.05$) after food deprivation for 48 h in intestinal bulb (58%), proximal intestine (45%) and muscle (56%). OEA levels returned to baseline after re-feeding in the three tissues. A similar pattern (decreased OEA content in the fasted group and back to baseline levels with re-feeding) was observed at 120 min in these tissues, though without statistically significant differences (Fig. 1A,B,D). No such changes were observed in liver among the different experimental groups at either of the studied time intervals (Fig. 1C).

The OEA content in the brain (hypothalamus, telencephalon and brainstem) under different feeding conditions is reported in Table 1. Fasting for 48 h significantly ($P<0.05$) increased the OEA content in the telencephalon compared with fed fish 30 min after food intake, and re-feeding did not cause a return to baseline levels. No such differences were observed in the hypothalamus and brainstem at any sampling time analysed (30 and 120 min).

Experiment 2: effects of OEA on food intake and locomotor activity

Fig. 2A–C shows food intake during discrete and cumulative intervals after acute i.p. injection of either vehicle or OEA at doses of 5 μg g^{-1} body mass (M_b) in goldfish. Food intake was

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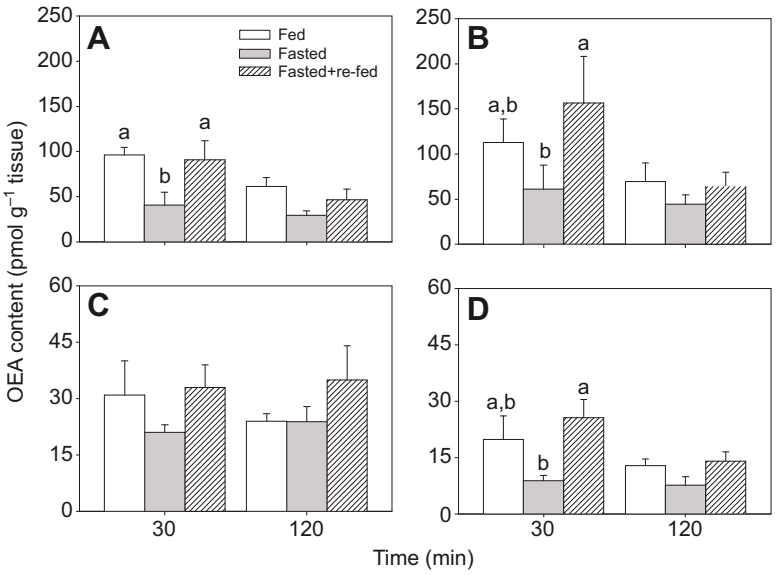

Fig. 1. Effect of feeding conditions on oleoylethanolamide (OEA) content in goldfish peripheral tissues. OEA content in fed, fasted (48 h) and fasted (48 h) + re-fed fish 30 and 120 min after feeding in: (A) intestinal bulb, (B) proximal intestine, (C) liver and (D) muscle. Data are expressed as means + s.e.m. Different letters indicate significant differences (P<0.05) among experimental groups for the same time period.

significantly reduced compared with the control group during the 0–2 h interval (P<0.001; Fig. 2A), but not during the discrete interval 2–8 h (Fig. 2B). Cumulative food intake 8 h after injection was significantly decreased (P<0.05; Fig. 2C) in OEA-treated fish with respect to control fish. These reductions were around 72% at 2 h and 29% at 8 h after the OEA treatment.

The i.p. administration of OEA (5 μg g^{-1} M_b) significantly decreased swimming activity (around 35%) 2 h post-injection (P<0.05; Fig. 2D). A similar trend of decreased swimming was observed during the 2–8 h interval (36%; Fig. 2E) and 0–8 h interval (31%, Fig. 2F), although this reduction in locomotor activity was not statistically significant.

Experiment 3: effects of OEA on plasma metabolites, gene expression of feeding regulators and monoaminergic system

Plasma triglyceride levels were significantly reduced 2 h after OEA i.p. treatment (5 μg g^{-1} M_b) under fasted (24 h) and fed conditions (P<0.005; Fig. 3A). A trend towards higher plasma triglyceride levels was observed in fed fish compared with 24 h food-deprived animals. There were no significant differences in glycaemia in fish treated with OEA relative to the control group (Fig. 3B). Plasma glucose levels were lower in 24 h fasted fish (both control and OEA treated) than in fed fish 2 h post-feeding (P<0.005). There was no interaction between the treatment (vehicle or OEA injection) and feeding conditions (fasted or fed) for both metabolites studied.

Fig. 4 summarizes the results of OEA treatment on gene expression of peripheral feeding regulators. The two-way ANOVA pointed to an interaction between treatment and feeding conditions (P<0.05) in ghrelin (*gGHRL*) gene expression in goldfish intestinal bulb. OEA i.p. treatment reduced *gGHRL* mRNA levels in goldfish intestinal bulb 2 h post-injection in fed fish, but not in 24 h-fasted fish (P<0.05; Fig. 4A). The expression of goldfish CCK (*gCCK*) in the intestinal bulb (Fig. 4B) and goldfish leptin-aI (*gLep-aI*) in the liver (Fig. 4C) was not modified by OEA treatment and/or different feeding conditions in any of the studied groups.

Analysis of the effects of peripheral OEA treatment on central feeding regulators revealed no significant differences in the expression of hypothalamic goldfish leptins (*gLep-aI* and *gLep-aII*), goldfish orexin (*gOrexin*) and goldfish NPY (*gNPY*) 2 h post-injection in both fed and 24 h-fasted fish (Table 2).

Table 3 shows the hypothalamic and telencephalic levels of monoamines (NA, DA and 5-HT) and their metabolites [3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindole acetic acid (5-HIAA)], as well as the monoaminergic turnover (DOPAC/DA and 5-HIAA/5-HT) after i.p. administration of vehicle or OEA (5 μg g^{-1} M_b) in fasted and fed goldfish 2 h post-injection. Feeding conditions modified the hypothalamic NA content regardless of treatment (vehicle or OEA injection), with the highest levels in fed fish compared with 24 h fasted fish (P<0.05; Table 3). No differences by OEA treatment or feeding condition were found in the level of monoamines DA and 5-HT and their main oxidative metabolites (DOPAC and 5-HIAA), and the DOPAC/DA and 5-HIAA/5-HT ratios in goldfish hypothalamus (Table 3). In the telencephalon, a significant (P<0.05) effect of feeding conditions on NA and 5-HIAA content and 5-HIAA/5-HT ratio was observed,

Table 1. OEA content in goldfish brain 30 and 120 min post-feeding

Tissue	Post-feeding time (min)	Fed (pmol g^{-1})	Fasted (48 h) (pmol g^{-1})	Fasted + re-fed (pmol g^{-1})
Hypothalamus	30	47.3±3.0	49.5±1.6	41.4±2.7
	120	43.1±2.3	42.2±2.7	47.0±5.7
Telencephalon	30	20.8±1.8^{a}	28.4±2.1^{b}	26.0±2.2^{a,b}
	120	21.0±1.7^{a,b}	16.6±3.1^{b}	26.0±2.0^{a}
Brainstem	30	130.5±12.1	123.7±9.1	100.5±5.1
	120	96.8±13.3	115.4±9.2	99.4±9.5

Data (pmol g^{-1} tissue) are expressed as means ± s.e.m. Different letters indicate significant differences (P<0.05) among experimental groups for the same time period.
OEA, oleoylethanolamide.

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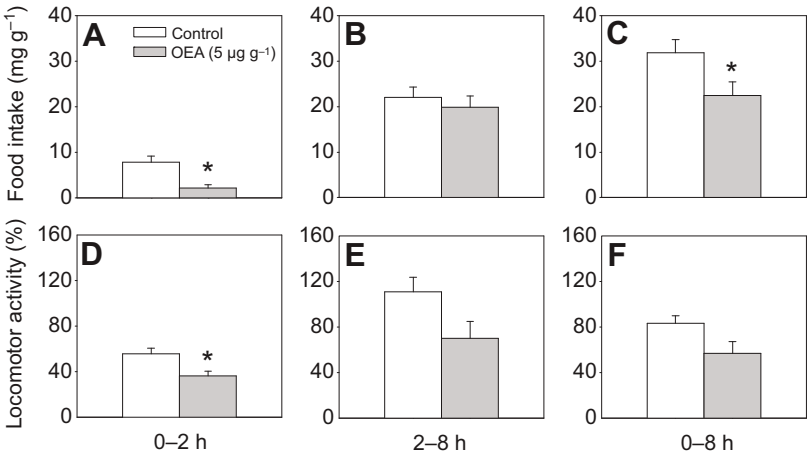

Fig. 2. Effect of OEA on goldfish food intake and locomotor activity. Food intake (A–C) and locomotor activity (D–F) 0–2 h (A,D), 2–8 h (B,E) and 0–8 h (C,F) after i.p. administration of vehicle alone (control group) or OEA (5 μg g^{-1} M_b). Data are expressed as means + s.e.m. *P<0.05 versus control group.

with lower values in 24 h fasted fish compared with fed fish. The NA content and 5-HIAA/5-HT ratio 2 h post-injection were significantly increased (P<0.05 and P<0.005, respectively) by OEA treatment in both fed and fasted goldfish. The DA and 5-HT telencephalic content was not significantly modified by either treatment or feeding condition in any of the studied experimental groups.

DISCUSSION

The present findings indicate for the first time in fish a potential role of OEA as a lipid-derived satiety factor. The intestinal OEA levels were downregulated during short-term fasting, suggesting that this lipid amide could be involved in the short-term regulation of food intake in goldfish. In support of this hypothesis, i.p. administration of OEA produced a time-dependent inhibition of food intake, accompanied by a decrease of locomotor activity and triglyceride plasma levels. These actions of OEA could be mediated through the modulation of peripheral (ghrelin) and central (monoamines) signals.

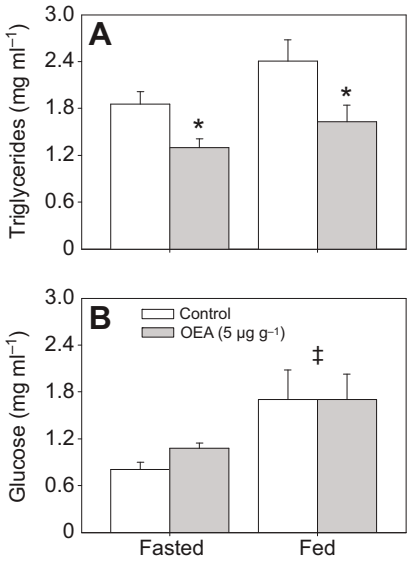

Fig. 3. Effect of OEA on goldfish plasma triglycerides and glucose. Plasma levels of triglycerides (A) and glucose (B) 2 h after i.p. administration of vehicle alone (control group) or OEA (5 μg g^{-1}) in fed and 24 h food-deprived goldfish. Data are expressed as means + s.e.m. *P<0.05 between control and OEA treatments; ‡P<0.05 between fasted and fed groups.

Regulation of OEA levels by feeding

We have reported the presence of endogenous OEA in both peripheral tissues and brain of goldfish. Gastrointestinal segments (intestinal bulb and proximal intestine) in goldfish fed daily showed similar OEA levels to those previously reported in equivalent regions in fed rats (Fu et al., 2007). OEA was also found in other peripheral tissues (liver and muscle), as well as in brain structures (telencephalon, hypothalamus and brainstem), with lower levels in fish than in rats.

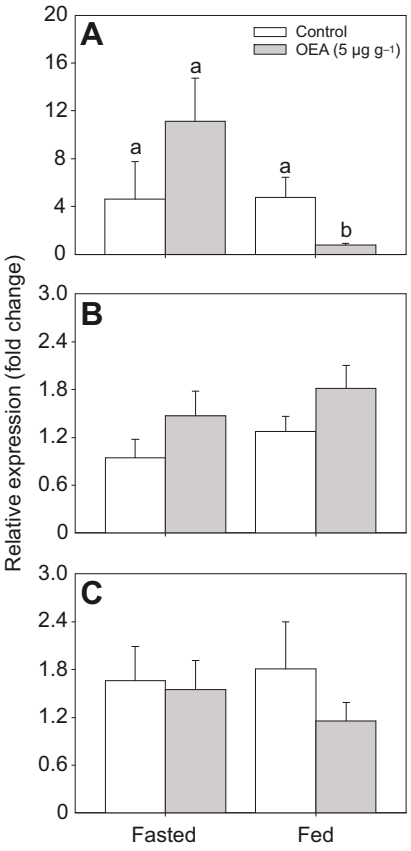

Fig. 4. Effect of OEA on expression of goldfish peripheral feeding regulators. The relative expression of genes encoding (A) ghrelin (*gGHRL*) and (B) cholecystokinin (*gCCK*) in intestinal bulb, and (C) leptin-al (*gLep-al*) in liver 2 h after i.p. administration of vehicle alone (control group) or OEA (5 μg g^{-1}) in fed and 24 h food-deprived goldfish. Data are expressed as means + s.e.m. Different letters indicate significant differences (P<0.05) among experimental groups.

Table 2. Relative expression of feeding regulators in goldfish hypothalamus 2 h after i.p. administration of OEA

	Fasted (24 h)		Fed	
Gene	Control	OEA	Control	OEA
gLep-aI	1.04±0.13	1.49±0.29	1.08±0.06	1.24±0.12
gLep-aII	1.07±0.18	1.66±0.33	1.29±0.22	1.23±0.09
gOrexin	1.66±0.49	1.70±0.44	2.28±0.48	3.81±0.54
gNPY	3.31±1.36	5.32±2.51	1.07±0.41	1.67±0.41

OEA was given at 5 μg g^{-1} body mass (M_b). Data are expressed as means ± s.e.m.
gLep-aI, goldfish leptin-aI; *gLep-aII*, goldfish leptin-aII; *gOrexin*, goldfish orexin; *gNPY*, goldfish neuropeptide Y.

Feeding promotes OEA mobilization in the small intestine of studied species, such as rats (Rodríguez de Fonseca et al., 2001; Petersen et al., 2006; Fu et al., 2007), mice (Fu et al., 2007) and Burmese pythons (Astarita et al., 2006a). Our results also support this hypothesis in fish, as intestinal OEA content decreased after 48 h of fasting, and subsequently returned to baseline levels following re-feeding. We cannot confirm intestinal biosynthesis of this FAE in goldfish, as we did not measure the enzymatic activities responsible of OEA synthesis. Similar downregulation of OEA levels has also been observed in goldfish muscle, but not in rats (Fu et al., 2007), and the possible physiological significance of this response in fish remains unknown. The time course of changes in OEA levels in goldfish intestine and muscle indicates higher levels of this lipid amide at 30 min than at 120 min, suggesting that OEA is a rapid satiety signal. In fact, the decrease in OEA content following fasting was rapidly reverted by re-feeding (after 10 min) in rats (Fu et al., 2007). The fact that fasting induces upregulation of OEA content in other peripheral tissues, such as liver, pancreas, spleen and adipose tissue in rats (Fu et al., 2007; Izzo et al., 2010), but not in fish liver (present results) agrees with the downregulation of lipogenesis in liver induced by food deprivation (Pérez-Jiménez et al., 2012) and suggests that nutrient availability regulates OEA mobilization in a tissue-specific manner.

In the brain, the existing evidence in rats does not support a major role for OEA, as there are no fasting/re-feeding-induced changes (Fu et al., 2007; Izzo et al., 2010). Similar results have been found in goldfish hypothalamus and brainstem, but not in the telencephalon, where fasting increased OEA levels, in disagreement with its anorectic role. Fasting also increased anandamide levels in goldfish telecenphalon (Valenti et al., 2005), but this FAE increases food intake (Valenti et al., 2005). Thus, this similar response to fasting of OEA and anandamide does not appear to be in agreement with the opposite effect of these two FAEs. This conflicting result in goldfish suggests there are other roles of OEA in the telencephalon. In accordance with this, other functions of OEA have been described in mammals, such as in memory consolidation, stress, sleep–wake cycle, cellular viability and circadian system (for review, see Sarro-Ramírez et al., 2013).

Effects of OEA on food intake, locomotor activity and plasma metabolites

This is the first report documenting possible actions of OEA in fish. We found that i.p.-administered OEA (5 μg g^{-1} M_b) exerted an inhibitory effect on food intake 2 and 8 h post-injection in goldfish. This result is consistent with previous reports in mammals in which peripheral treatment with OEA was found to reduce food intake at similar dosages (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Cani et al., 2004; Nielsen et al., 2004). The fact that the feeding decrease was observed during the first 2 h after OEA injection, but not during the next discrete interval (2–8 h), suggests that this lipid amide acts for a short time in goldfish. OEA can modify food intake in the first 20 or 30 min post-injection in mammals (Cani et al., 2004; Serrano et al., 2011). Nevertheless, such early changes in feeding intake by FAEs can be extended for some hours, as in the present study. Thus, the OEA-induced decrease of cumulative food intake observed 8 h post-injection in goldfish would reflect the inhibitory action of OEA over a short time (2 h), which is maintained at least 8 h after the treatment. Moreover, the hypophagic actions of OEA appear to depend on the feeding state of the animal. In free-feeding rats, this lipid mediator increased the latency of feeding onset without changes in meal size, while OEA both delayed feeding onset and reduced meal size in food-deprived rats (Gaetani et al., 2003). Our experimental model to study the anorectic effect of OEA utilized 24 h food-deprived goldfish, indicating that OEA reduces feeding induced by fasting, but it is still unknown whether other feeding behaviour parameters, such as latency, post-meal interval or meal frequency, could be modified by OEA in fish. Several lines of evidence in mammals support the idea that OEA decreases food intake by activating PPAR-α receptor. In summary, mice lacking PPAR-α do not respond to OEA (Fu et al., 2003);

Table 3. Brain changes in monoaminergic system in goldfish 2 h after i.p. administration of OEA

	Fasted (24 h)		Fed	
	Control	OEA	Control	OEA
Hypothalamus				
NA (pmol mg^{-1} protein)	50.95±3.37	52.79±2.97	62.66±7.53‡	68.77±6.34‡
DA (pmol mg^{-1} protein)	56.45±3.44	43.40±1.24	52.96±4.53	61.01±10.24
DOPAC (pmol mg^{-1} protein)	1.75±0.19	1.97±0.45	1.95±0.30	1.71±0.20
DOPAC/DA (%)	3.13±0.31	4.46±0.98	3.98±0.76	3.95±1.38
5-HT (pmol mg^{-1} protein)	116.35±8.56	82.45±12.00	109.38±9.09	136.31±17.51
5-HIAA (pmol mg^{-1} protein)	21.76±2.06	21.93±3.28	23.58±1.72	28.34±2.54
5-HIAA/5-HT (%)	18.95±1.75	24.41±2.48	21.85±1.00	22.16±2.26
Telencephalon				
NA (pmol mg^{-1} protein)	46.35±3.68	61.71±5.05*	65.08±6.51‡	74.29±6.10*,‡
DA (pmol mg^{-1} protein)	17.49±5.86	12.24±1.35	12.60±1.30	14.84±1.33
5-HT (pmol mg^{-1} protein)	49.30±11.84	38.69±1.70	43.59±3.17	41.92±2.84
5-HIAA (pmol mg^{-1} protein)	12.42±2.01	13.05±0.72	15.03±1.81‡	18.12±1.25‡
5-HIAA/5-HT (%)	27.10 ±1.78	33.93±2.08*	34.70±3.48‡	43.70±3.48*,‡

OEA was given at 5 μg g^{-1} M_b. Data are expressed as means ± s.e.m. *P<0.05 between control and OEA treatment; ‡P<0.05 between fasted and fed groups.
NA, noradrenaline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid.

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PPAR-α agonists have anorectic actions similar to OEA (Astarita et al., 2006b); and OEA stimulates the transcription of various PPAR-α target genes (Fu et al., 2003). The existence of the PPAR subtypes α, β and γ has been demonstrated in fish (Mimeault et al., 2006; Zheng et al., 2013; Carmona-Antoñanzas et al., 2014), but to date it is unknown whether these nuclear receptors could be involved in the effects of OEA in these vertebrates. In addition, TRPV1 and GPR119 receptors have been shown to be involved in the feeding suppression actions of OEA in rodents (Ahern, 2003; Overton et al., 2006; Almási et al., 2008), although genetic removal of either TRPV1 or GPR119 has no effect on OEA-induced hypophagia (Piomelli, 2013). Molecular studies have also demonstrated the expression of TRPV1 and GPR119 receptors in fish species (Fredriksson et al., 2003; Gau et al., 2013), but the physiological roles of these receptors have not yet been elucidated.

Given that the metabolic precursor of OEA is oleic acid, it is important to point out that central or peripheral administration of oleic acid causes satiety effects in fish, probably mediated by fatty acid-sensing systems through different mechanisms related to fatty acid metabolism (Librán-Pérez et al., 2012; Librán-Pérez et al., 2014). Thus, it cannot be ruled out that the OEA mobilization in fish is induced by oleic acid in the intestine, as it has been suggested in mammals (Piomelli, 2013).

Present results suggest that OEA may play a role in the regulation of locomotor activity in fish, as reported in mammals (Rodríguez de Fonseca et al., 2001; Proulx et al., 2005). In both cases, the anorectic effect of OEA was accompanied by a significant reduction of locomotor activity. Rodríguez de Fonseca et al. (Rodríguez de Fonseca et al., 2001) suggested that the two responses are unrelated because the feeding decrease elicited by OEA was eliminated after selective degeneration of sensory fibres by capsaicin treatment, but not the reduction in locomotor activity. The possible interactions of OEA regulation of feeding and swimming activity in fish have not been studied to date. At least two possibilities could be addressed: on the one hand, the anorectic action of OEA might be due to the reduction of locomotor behaviour induced by this lipid amide; on the other hand, a decrease in activity might be related to a decrease in searching behaviour, as a direct consequence of the satiety effect of OEA. We cannot draw conclusions on the independence of these effects, based on the present results, but previous studies in goldfish have suggested that feeding and locomotor activity can be independently regulated by other anorectic hormones, such as leptin (Vivas et al., 2011) and melatonin (Azpeleta et al., 2010).

A significant decrease in triglyceride plasma levels after OEA injection in goldfish is in accordance with the general role of peripheral OEA in increasing fat utilization in mammals (Thabuis et al., 2008; Pavón et al., 2010; Piomelli, 2013). Systemic administration of OEA in rats stimulated lipolysis in adipocytes, decreasing circulating triglycerides and rapidly increasing the circulating non-esterified fatty acids and glycerol (Guzmán et al., 2004; Fu et al., 2005). Similar results were observed after incubation of rat adipocytes in the presence of OEA, suggesting that this lypolitic action of OEA involves the PPAR-α receptor (Guzmán et al., 2004). Moreover, an enhanced fatty acid oxidation was also found in muscle, heart and liver cells of rats and mice (Guzmán et al., 2004). As mentioned above, the effects of OEA might be mediated, at least in part, by oleate and its effects on fatty acid-sensing systems (Librán-Pérez et al., 2012; Librán-Pérez et al., 2014). The reduction in triglycerides does not seem to be due to the reduction in food intake induced by OEA, as this effect was not observed in the pair-fed group in rats (Guzmán et al., 2004). The fact that the decrease in triglycerides in goldfish also occurred in the group that had not received food after OEA injection also supports such a hypothesis in fish. All these findings together suggest that OEA would play an important role in lipid metabolism in mammals and probably in fish.

A 24 h fast reduced glycaemia in goldfish, as expected (Polakof et al., 2012), and this was not affected by OEA treatment. Similar results in rats have shown that OEA administration does not modify blood glucose levels (Guzmán et al., 2004; Fu et al., 2005). However, some experiments *in vitro* have suggested that OEA may be involved in glucose metabolism regulation, as it inhibits insulin-stimulated glucose uptake in isolated rat adipocytes (González-Yanes et al., 2005). This possible inhibitory action of OEA on insulin actions in fish deserves to be investigated.

Interplay between OEA and other feeding regulators

The action of OEA on energy homeostasis in goldfish could be mediated by interactions with ghrelin, as the present results show reductions in ghrelin mRNA levels in the intestinal bulb induced by OEA. Ghrelin is a well-known orexigenic signal in fish that can also increase locomotor activity and lipid deposition in some species (Jönsson, 2013). Thus, OEA might reduce food intake and locomotor activity by decreasing gastrointestinal synthesis of ghrelin. Taking into consideration that OEA inhibits adipogenesis in mammals, and the adipogenic effect of ghrelin in mammals and fish (Thabuis et al., 2008; Jönsson, 2013), it is tempting to speculate that the action of OEA on lipid metabolism could be mediated, at least in part, by a reduction in ghrelin. A decrease in ghrelin expression by OEA was observed only in fed goldfish, but not in 24 h food-deprived fish, suggesting that the OEA–ghrelin interaction could depend on the energy status of the animals. This dependence also seems to occur in mammals, although the results vary. On the one hand, the decrease in circulating ghrelin induced by OEA occurs in fasted rats but not in fed rats (Cani et al., 2004). On the other hand, no changes in plasma ghrelin in fasted rats have been reported (Proulx et al., 2005; Serrano et al., 2011). This apparent discrepancy between the present results in fish and previous results in mammals might arise from species-specific differences, different physiological conditions (such as reproductive stage) and differences in experimental approaches (quantification of mRNA versus plasma levels, duration of fasting imposed on the animals, etc.).

To study whether the anorectic effect of OEA implies modulation of the secretion of other anorectic signals from the gastrointestinal tract in fish, we analysed the expression of CCK in the intestinal bulb of goldfish injected with this lipid amide. In the present study, OEA did not modify CCK expression, supporting previous data in mammals indicating that it is unlikely that CCK mediates the effects of OEA on food intake (Proulx et al., 2005). In fact, the primary contribution of OEA to normal feeding is in the regulation of satiety (delaying feeding onset and prolonging the time between meals), while CCK contributes to the process of satiation or meal termination by reducing meal size (Gaetani et al., 2003).

The unaltered hepatic and hypothalamic leptin expression in OEA-injected fish suggests that the reductions in food intake, locomotor activity and triglycerides induced by this FAE in goldfish cannot be directly attributed to an activation of leptin, an anorectic signal that also induces hypoactivity and lipolytic actions in this teleost (Vivas et al., 2011). The independence of the effects of OEA from leptin agrees with a previous finding in mammals, where OEA reduces both feeding and circulating lipids in obese Zucker rats lacking functional leptin receptors (Fu et al., 2005).

Because the OEA effect is associated with the activation of brain regions involved in feeding regulation, in the present study we

examined whether peripheral administration of OEA induced changes in the expression of hypothalamic neuropeptides. There were no changes in the expression of NPY and orexin, two important orexigenic peptides in goldfish (Volkoff et al., 2009), following OEA injection. A previous study in rats (Serrano et al., 2011) demonstrated that OEA failed to modulate hypothalamic expression of NPY and AgRP (agouti-related protein) in experimental conditions (fed and 24 h fasted) similar to those of the present study. These data support the hypothesis that these orexigenic peptides in hypothalamus do not play a critical role in the anorectic effect of OEA in fish and mammals, although interactions between OEA and other orexigenic and anorexigenic neuropeptides, such as CART and oxytocin (Serrano et al., 2011; Gaetani et al., 2010), cannot be ruled out.

The central neurotransmitters recruited by peripheral OEA to inhibit food intake in rats have been studied previously (Serrano et al., 2011). The hypothalamic content of NA and DA increased after OEA injection, with a decrease in DOPAC/DA and without modifications to the serotonergic system. These effects were found only with the highest dose (20 mg kg^{-1}) of OEA, but not with 5 mg kg^{-1}. No changes were observed in goldfish hypothalamic monoamines (NA, DA and 5-HT), metabolites (DOPAC and 5-HIAA) and turnover (DOPAC/DA and 5-HT/5-HIAA) following OEA administration. These differences could be the consequence of different experimental approaches such as OEA dose (5 mg kg^{-1} in fish versus 20 mg kg^{-1} in rats) and time post-injection (2 h in fish versus 1 h in rats). Given the telencephalon is involved in the regulation of feeding and swimming in fish (Lin et al., 2000; Wilson and McLaughlin, 2010), the increases in NA, 5-HIAA and 5-HIAA/5-HT ratio induced by OEA in this brain region are potentially very interesting. The fact that these effects of OEA were similar in fed and fasted fish allows us to disregard the possibility that drug-induced feeding changes could be the cause of these monoaminergic neurotransmission alterations. As serotonin reduces feeding and swimming activity in fish (de Pedro et al., 1998b; Kuz'mina and Garina, 2013), the inhibitory effect of OEA on food intake and locomotor activity in goldfish could be mediated by serotonergic activation. The NA increase in goldfish telencephalon could not explain the OEA anorectic action, considering that this monoamine stimulates feeding in fish (de Pedro et al., 1998a; de Pedro et al., 2001). The possible cross-talk between OEA and telencephalic NA could be related to other functions of OEA. In mammals it has been proposed that OEA facilitates memory consolidation through noradrenergic activation of the amygdala (Campolongo et al., 2009). Recent results in rats have suggested that noradrenergic neurons are involved in the circuit responsible for the activation of hypothalamic oxytocin, which mediates the food intake inhibition induced by peripheral OEA administration (Romano et al., 2013). The identification of a functional link between OEA and brain NA is an intriguing question and future studies should examine all these possible interactions.

In conclusion, our results indicate for the first time in fish that OEA may be involved in the regulation of feeding, swimming and lipid metabolism, suggesting a high conservation of OEA actions in energy balance throughout vertebrate evolution.

MATERIALS AND METHODS

Animals

Experiments were performed with goldfish (*C. auratus*). Animals were obtained from a commercial supplier and reared at 21±2°C in aquaria (60 l) with a constant flow of filtered water, under a 12 h light:12 h dark photoperiod (lights on at 08:00 h). The aquaria walls were covered with opaque paper to minimize external interference during the experiments. Fish were fed once daily with 1% M_b commercial dry pellets (32.1% crude protein, 5% crude fat, 1.9% crude fibre, 5.1% humidity and 6.8% crude ash; Sera Biogram, Heinsberg, Germany) at 10:00 h. Animals were maintained under these conditions for at least 15 days prior to experimental use.

All the fish handling procedures comply with international standards for the Care and Use of Laboratory Animals, were approved by the Animal Experiments Committee of the Complutense University of Madrid and were in accordance with the Guidelines of the European Union Council (2010/63/EU) for the use of research animals.

OEA administration

OEA (Sigma Chemical, Madrid, Spain) was dissolved in 5% Tween 20, 5% polyethyleneglycol (Sigma Chemical) and 90% teleost saline (20 mg Na_2CO_3/100 ml of 0.6% NaCl). Fish (24 h food deprived) were anaesthetized in water containing tricaine methanesulphonate (MS-222, 0.14 g l^{-1}; Sigma Chemical). Immediately after the loss of equilibrium, fish were weighed and injected at feeding time (10:00 h). Goldfish were not fed for 24 h prior to injections (advisable conditions to test anorexigenic regulators). The i.p. injections were performed using 1 ml syringes and 0.3 mm Microlance needles (Lab-Center, Madrid, Spain), close to the ventral midline posterior to the pelvic fins (de Pedro et al., 2006). Fish were i.p. injected with 10 µl vehicle g^{-1} M_b alone (control group) or containing OEA (5 µg g^{-1} M_b, experimental group). The OEA dose was chosen based on studies previously reported in mammals (Cani et al., 2004; Fu et al., 2003; Nielsen et al., 2004; Rodríguez de Fonseca et al., 2001; Serrano et al., 2011). After the i.p. injections, fish were transferred to the experimental aquaria with anaesthetic-free water, where swimming activity and equilibrium were recovered within 1–2 min.

Experiment 1: effects of fasting and feeding on OEA content

Fish (12.02±0.47 g M_b) were divided into three groups (N=16 fish/group): control (fish were fed 1% M_b at 10:00 h), fasted (animals were food deprived for 48 h) and fasted + re-fed [fish were fasted for 48 h and re-fed (1% M_b) at 10:00 h]. Fish were killed by anaesthetic overdose (MS-222; 0.28 g l^{-1}) followed by spinal section 30 and 120 h after feeding (10:30 h and 12:00 h). Liver, intestinal bulb, proximal intestine (the first centimetre post-intestinal bulb), muscle and brain (hypothalamus, telencephalon and brainstem) were dissected on ice, immersed in liquid nitrogen and immediately stored at −80°C until posterior analysis. These tissues were chosen in accordance with previous studies in mammals and python (Astarita et al., 2006a; Fu et al., 2007), and taking into account the central relevance of the hypothalamus and telencephalon in feeding regulation in fish (Volkoff et al., 2009). Tissues were then weighed and homogenized in a methanol (Thermo Fisher Scientific, Milano, Italy) solution spiked with the deuterated analogue of OEA ([$^2H_4$]-OEA; Cayman Chemical, Ann Arbor, MI, USA), used as internal standard (IS) and mixed with chloroform (Thermo Fisher Scientific) and water (1:2:1). The FAEs in the samples were fractioned by open-bed silica gel column chromatography, as previously described (Cadas et al., 1997). Briefly, the lipid extracts were reconstituted in chloroform and loaded onto small columns packed with silica gel G (60 Å 230–400 Mesh ASTM; Whatman, Clifton, NJ, USA). FAEs were eluted with a chloroform/methanol 9:1 (v/v) solution. Eluates were dried under N_2 and reconstituted in 0.1 ml of acetonitrile with 0.1% of formic acid (Sigma Chemical). Samples were then analysed by LC-MS/MS on a Xevo-TQ triple quadruple mass spectrometer coupled with a UPLC chromatographic system. Standard curves for OEA were prepared in the 1 nmol l^{-1} to 10 µmol l^{-1} range. OEA and its deuterated analogue were loaded on a reversed phase BEH C18 column (50×2.1 mm inner diameter, 1.7 µm particle size) operated at 0.5 ml min^{-1} flow rate. Analytes were eluted from the column using a linear gradient of acetonitrile in water (both added with 0.1% formic acid). The column and the UPLC-MS/MS system were purchased from Waters Inc. (Milford, PA, USA). Quantification of analytes was performed by monitoring the following MRM (multiple reaction monitoring) transitions (parent m/z>daughter m/z, collision energy eV): OEA 326>62, 20; [$^2H_4$]-OEA 330>66, 20. OEA content in the samples was calculated from the analyte to IS peak area ratio and expressed as pmol mg^{-1} tissue.

Experiment 2: effects of OEA on food intake and locomotor activity

Fish (15.67±0.52 g M_b) were divided into two groups (N=16 fish/group): i.p. injected with vehicle or OEA (5 µg g^{-1} M_b). Immediately, individual goldfish were placed alone in 5 l aquaria. Pre-weighed food was supplied in excess (3% M_b) 10 min after fish were injected, and any remaining food was collected after 2 h. New, pre-weighed food (5% M_b) was added to the aquaria and any remaining food was collected at 8 h post-injection. Food intake was measured during the discrete intervals 0–2 and 2–8 h, the sum of which represents the cumulative interval 0–8 h, as previously described (de Pedro et al., 1998b).

Locomotor activity was recorded in groups of six fish (29.51±0.54 g M_b) in tanks of 60 l (N=6 tanks/group), after i.p. injection of vehicle or OEA (5 µg g^{-1} M_b). Swimming was recorded by using infrared photocells (OMRON E3SAD12, Osaka, Japan) fixed on the aquaria wall, as previously described (Azpeleta et al., 2010). The activity values registered in each tank, 2 and 8 h after vehicle or OEA injection, were expressed as a percentage with respect to the locomotor activity recorded at the same time periods in the same tank the day prior to treatment.

Experiment 3: effects of OEA on plasma metabolites, gene expression of feeding regulators and monoaminergic system

Fish (16.98±0.58 g M_b; N=8 fish/group) were i.p. injected with vehicle or OEA (5 µg g^{-1} M_b) at the scheduled feeding time (10:00 h), and maintained under two feeding conditions: fed (1% M_b) or food deprived (24 h). Two hours after injection, fish were anaesthetized and blood was taken by heparinized syringes from the caudal vein. Then, animals were killed by anaesthetic overdose (MS-222; 0.28 g l^{-1}) followed by spinal section, and tissues sampled. Brain (hypothalamus and telencephalon) and peripheral tissues (liver and intestinal bulb) were dissected on ice, immersed in liquid nitrogen and immediately stored at −80°C until posterior analysis. Feeding regulators and tissues studied were chosen considering previous studies in mammals on interactions between OEA and other feeding signals (Serrano et al., 2011; Gaetani et al., 2010), the relevance of these compounds in feeding regulation in fish and their main sites of synthesis and action in fish (Volkoff et al., 2009).

Plasma was obtained after centrifugation (4 min at 6000 rpm) and stored at −80°C until biochemical analysis. Plasma glucose and triglyceride levels were determined using an enzymatic/colorimetric method with commercial kits (GOD-POP and GPO-POD, respectively; Spinreact, Girona, Spain).

The mRNA levels of leptin-aI, leptin-aII, NPY and orexin in hypothalamus; leptin-aI in liver; and CCK and GHRL in intestinal bulb were measured. Feeding regulator gene expression was quantified by quantitative PCR (qPCR) using the goldfish β-actin as a reference gene (no differences between saline- and OEA-injected fish were observed). Total RNA was extracted using Trizol (Sigma Chemical). After DNase treatment (Promega, Madison, WI, USA), total RNA (from 0.25 to 0.8 µg depending on the tissue) was retro-transcribed (SuperScript II Reverse Transcriptase; Invitrogen, Carlsbad, CA, USA). Gene expression analysis was performed in a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The qPCR reactions were developed in a 20 µl volume using iTaq SYBR Green Supermix (Bio-Rad). Specific primers (Sigma Chemical; supplementary material Table S1) and qPCR conditions employed for *β-actin*, *gLep-aI* and *gLep-aII* were as previously described (Tinoco et al., 2012). For the other genes, qPCR conditions were similar, but with annealing temperatures of 60°C (*gCCK*) and 65°C (*gNPY*, *gOrexin* and *gGHRL*). All samples were analysed in duplicate. Calibration curves for each gene were generated with serial dilutions of cDNA; all curves exhibited slopes close to −3.32 and efficiencies between 95% and 105%. Negative controls included replacement of cDNA by water and the use of non-retrotranscribed total RNA. The specificity of the amplification reactions was confirmed by the melting temperature of qPCR products (measured at the end of all reactions) and by the size in an agarose gel. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative expression (fold change).

The content of NA, DA, DOPAC (a major DA metabolite), 5-HT and 5-HIAA (a major 5-HT metabolite) in hypothalamus and telencephalon were quantified by HPLC (Agilent 1100, Madrid, Spain) with coulometric detection (ESA Coulochem II, Chelmsford, MA, USA) as previously described (de Pedro et al., 2008). Briefly, the tissues were sonicated in 100 µl of cold perchloric acid (0.3 mol l^{-1}; Scharlab, Sentmenat, Spain) containing 0.4 mmol l^{-1} sodium bisulphate and 0.4 mmol l^{-1} EDTA disodium salt dihydrate (Sigma Chemical). The homogenate was centrifuged (13,000 rpm for 5 min) and the supernatant was injected into the HPLC system. The mobile phase (flow rate 1 ml min^{-1}) consisted in 10 mmol l^{-1} phosphoric acid, 0.1 mmol l^{-1} disodium EDTA, 0.4 mmol l^{-1} sodium octanesulphonic acid (Sigma Chemical) and 3% acetonitrile (Panreac, Barcelona, Spain), pH 3.1. Separation was performed using a reversed phase C18 analytical column, 125×4.6 mm internal diameter, 5 µm particle size (Teknokroma, Barcelona, Spain). The oxidation potential was 200 mV and the signal from the analytical cell was recorded with a sensitivity of 20 nA. Acquisition and integration of chromatograms were performed with Clarity Chromatography Station software (Micronec, Madrid, Spain). Protein content was determined by the method of Lowry et al. (Lowry et al., 1951). The amount of monoamines in the samples was calculated as the area under the peak and expressed as pmol mg^{-1} protein. Metabolite/monoamine ratios are used as an index of monoaminergic activity.

Statistical analyses

Results are expressed as means ± s.e.m. Food intake and swimming activity data were analysed by Student's t-test to ascertain statistical differences between controls and OEA-treated fish in each time period. Plasma glucose and triglyceride levels, feeding regulator mRNA and monoamine content were analysed by two-way ANOVA, using treatment and feeding condition as independent factors. Tukey multiple range test were performed for multi-group comparisons only for significant interactions. One-way ANOVA followed by Tukey test was used to evaluate the effects of fasting and feeding on OEA content. When necessary, values were transformed (logarithmic or square root transformation) to obtain a normal distribution and homogeneity of variances. A Kruskall–Wallis non-parametric test was used to analyse statistical differences in telencephalic content of DA and *gNPY* hypothalamic expression. Analyses were conducted using IBM SPSS Statistics 19 (IBM Corporation, Armonk, NY, USA) and differences were considered statistically significant at P<0.05.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.B.T. and N.D.P. conceived and designed the experiments, and interpreted the findings. E.I. and M.J.D. collected and analysed the data from experiments carried out in Complutense University of Madrid. A.A. and D.P. collected and analysed the data from experiments carried out in Italian Institute of Technology. All authors drafted and revised the article.

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Supplementary material

Supplementary material available online at
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References

Ahern, G. P. (2003). Activation of TRPV1 by the satiety factor oleoylethanolamide. *J. Biol. Chem.* **278**, 30429-30434.

Almási, R., Szoke, E., Bölcskei, K., Varga, A., Riedl, Z., Sándor, Z., Szolcsányi, J. and Petho, G. (2008). Actions of 3-methyl-N-oleoyldopamine, 4-methyl-N-oleoyldopamine and N-oleoylethanolamide on the rat TRPV1 receptor in vitro and in vivo. *Life Sci.* **82**, 644-651.

Astarita, G., Rourke, B. C., Andersen, J. B., Fu, J., Kim, J. H., Bennett, A. F., Hicks, J. W. and Piomelli, D. (2006a). Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (*Python molurus*). *Am. J. Physiol.* **290**, R1407-R1412.

Astarita, G., Di Giacomo, B., Gaetani, S., Oveisi, F., Compton, T. R., Rivara, S., Tarzia, G., Mor, M. and Piomelli, D. (2006b). Pharmacological characterization of

hydrolysis-resistant analogs of oleoylethanolamide with potent anorexiant properties. *J. Pharmacol. Exp. Ther.* **318**, 563-570.
Azpeleta, C., Martínez-Alvarez, R. M., Delgado, M. J., Isorna, E. and de Pedro, N. (2010). Melatonin reduces locomotor activity and circulating cortisol in goldfish. *Horm. Behav.* **57**, 323-329.
Cadas, H., di Tomaso, E. and Piomelli, D. (1997). Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J. Neurosci.* **17**, 1226-1242.
Campolongo, P., Roozendaal, B., Trezza, V., Cuomo, V., Astarita, G., Fu, J., McGaugh, J. L. and Piomelli, D. (2009). Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. *Proc. Natl. Acad. Sci. USA* **106**, 8027-8031.
Cani, P. D., Montoya, M. L., Neyrinck, A. M., Delzenne, N. M. and Lambert, D. M. (2004). Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide. *Br. J. Nutr.* **92**, 757-761.
Carmona-Antoñanzas, G., Tocher, D. R., Martínez-Rubio, L. and Leaver, M. J. (2014). Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene* **534**, 1-9.
de Pedro, N. and Björnsson, T. (2001). Regulation of food intake by neuropeptides and hormones. In *Food Intake in Fish* (ed. D. Houlihan, T. Boujard and M. Jobling), pp. 267-296. Oxford: Blackwell Science Ltd.
de Pedro, N., Delgado, M. J., Pinillos, M. L. and Alonso-Bedate, M. (1998a). Alpha1-adrenergic and dopaminergic receptors are involved in the anoretic effect of corticotropin-releasing factor in goldfish. *Life Sci.* **62**, 1801-1808.
de Pedro, N., Pinillos, M. L., Valenciano, A. I., Alonso-Bedate, M. and Delgado, M. J. (1998b). Inhibitory effect of serotonin on feeding behavior in goldfish: involvement of CRF. *Peptides* **19**, 505-511.
de Pedro, N., Delgado, M. J. and Alonso-Bedate, M. (2001). Fasting and hypothalamic catecholamines in goldfish. *J. Fish Biol.* **58**, 1404-1413.
de Pedro, N., Martínez-Alvarez, R. and Delgado, M. J. (2006). Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). *J. Endocrinol.* **188**, 513-520.
de Pedro, N., Martínez-Alvarez, R. M. and Delgado, M. J. (2008). Melatonin reduces body weight in goldfish (*Carassius auratus*): effects on metabolic resources and some feeding regulators. *J. Pineal Res.* **45**, 32-39.
Fredriksson, R., Höglund, P. J., Gloriam, D. E., Lagerström, M. C. and Schiöth, H. B. (2003). Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives. *FEBS Lett.* **554**, 381-388.
Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodríguez De Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G. et al. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* **425**, 90-93.
Fu, J., Oveisi, F., Gaetani, S., Lin, E. and Piomelli, D. (2005). Oleoylethanolamide, an endogenous PPAR-alpha agonist, lowers body weight and hyperlipidemia in obese rats. *Neuropharmacology* **48**, 1147-1153.
Fu, J., Astarita, G., Gaetani, S., Kim, J., Cravatt, B. F., Mackie, K. and Piomelli, D. (2007). Food intake regulates oleoylethanolamide formation and degradation in the proximal small intestine. *J. Biol. Chem.* **282**, 1518-1528.
Fu, J., Dipatrizio, N. V., Guijarro, A., Schwartz, G. J., Li, X., Gaetani, S., Astarita, G. and Piomelli, D. (2011). Sympathetic activity controls fat-induced oleoylethanolamide signaling in small intestine. *J. Neurosci.* **31**, 5730-5736.
Gaetani, S., Oveisi, F. and Piomelli, D. (2003). Modulation of meal pattern in the rat by the anorexic lipid mediator oleoylethanolamide. *Neuropsychopharmacology* **28**, 1311-1316.
Gaetani, S., Fu, J., Cassano, T., Dipasquale, P., Romano, A., Righetti, L., Cianci, S., Laconca, L., Giannini, E., Scaccianoce, S. et al. (2010). The fat-induced satiety factor oleoylethanolamide suppresses feeding through central release of oxytocin. *J. Neurosci.* **30**, 8096-8101.
Gau, P., Poon, J., Ufret-Vincenty, C., Snelson, C. D., Gordon, S. E., Raible, D. W. and Dhaka, A. (2013). The zebrafish ortholog of TRPV1 is required for heat-induced locomotion. *J. Neurosci.* **33**, 5249-5260.
González-Yanes, C., Serrano, A., Bermúdez-Silva, F. J., Hernández-Dominguez, M., Páez-Ochoa, M. A., Rodríguez de Fonseca, F. and Sánchez-Margalet, V. (2005). Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. *Am. J. Physiol. Endocrinol. Metab.* **289**, E923-E929.
Guzmán, M., Lo Verme, J., Fu, J., Oveisi, F., Blázquez, C. and Piomelli, D. (2004). Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-alpha). *J. Biol. Chem.* **279**, 27849-27854.
Hoskins, L. J. and Volkoff, H. (2012). The comparative endocrinology of feeding in fish: insights and challenges. *Gen. Comp. Endocrinol.* **176**, 327-335.
Izzo, A. A., Piscitelli, F., Capasso, R., Marini, P., Cristino, L., Petrosino, S. and Di Marzo, V. (2010). Basal and fasting/refeeding-regulated tissue levels of endogenous PPAR-alpha ligands in Zucker rats. *Obesity (Silver Spring)* **18**, 55-62.
Jönsson, E. (2013). The role of ghrelin in energy balance regulation in fish. *Gen. Comp. Endocrinol.* **187**, 79-85.
Kuz'mina, V. V. and Garina, D. V. (2013). The effects of peripherally injected serotonin on feeding and locomotor activities in carp *Cyprinus carpio* L. *Inland Water Biol.* **6**, 62-69.
Librán-Pérez, M., Polakof, S., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2012). Evidence of a metabolic fatty acid-sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. *Am. J. Physiol.* **302**, R1340-R1350.
Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2014). Central administration of oleate or octanoate activates hypothalamic fatty acid sensing and inhibits food intake in rainbow trout. *Physiol. Behav.* **129**, 272-279.
Lin, X., Volkoff, H., Narnaware, Y., Bernier, N. J., Peyon, P. and Peter, R. E. (2000). Brain regulation of feeding behavior and food intake in fish. *Comp. Biochem. Physiol.* **126A**, 415-434.
Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ Δ C(T)) Method. *Methods* **25**, 402-408.
Lo Verme, J., Gaetani, S., Fu, J., Oveisi, F., Burton, K. and Piomelli, D. (2005). Regulation of food intake by oleoylethanolamide. *Cell. Mol. Life Sci.* **62**, 708-716.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
Mimeault, C., Trudeau, V. L. and Moon, T. W. (2006). Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARbeta) mRNA levels in male goldfish (*Carassius auratus*). *Toxicology* **228**, 140-150.
Nielsen, M. J., Petersen, G., Astrup, A. and Hansen, H. S. (2004). Food intake is inhibited by oral oleoylethanolamide. *J. Lipid Res.* **45**, 1027-1029.
Overton, H. A., Babbs, A. J., Doel, S. M., Fyfe, M. C., Gardner, L. S., Griffin, G., Jackson, H. C., Procter, M. J., Rasamison, C. M., Tang-Christensen, M. et al. (2006). Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metab.* **3**, 167-175.
Pavón, F. J., Serrano, A., Romero-Cuevas, M., Alonso, M. and Rodríguez de Fonseca, F. (2010). Oleoylethanolamide: a new player in peripheral control of energy metabolism. Therapeutic implications. *Drug Discov. Today Dis. Mech.* **7**, e175-e183.
Pérez-Jiménez, A., Cardenete, G., Hidalgo, M. C., García-Alcázar, A., Abellán, E. and Morales, A. E. (2012). Metabolic adjustments of *Dentex dentex* to prolonged starvation and refeeding. *Fish Physiol. Biochem.* **38**, 1145-1157.
Petersen, G., Sørensen, C., Schmid, P. C., Artmann, A., Tang-Christensen, M., Hansen, S. H., Larsen, P. J., Schmid, H. H. and Hansen, H. S. (2006). Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. *Biochim. Biophys. Acta* **1761**, 143-150, discussion 141-142.
Piccinetti, C. C., Migliarini, B., Petrosino, S., Di Marzo, V. and Carnevali, O. (2010). Anandamide and AM251, via water, modulate food intake at central and peripheral level in fish. *Gen. Comp. Endocrinol.* **166**, 259-267.
Piomelli, D. (2013). A fatty gut feeling. *Trends Endocrinol. Metab.* **24**, 332-341.
Polakof, S., Panserat, S., Soengas, J. L. and Moon, T. W. (2012). Glucose metabolism in fish: a review. *J. Comp. Physiol. B* **182**, 1015-1045.
Proulx, K., Cota, D., Castañeda, T. R., Tschöp, M. H., D'Alessio, D. A., Tso, P., Woods, S. C. and Seeley, R. J. (2005). Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. *Am. J. Physiol.* **289**, R729-R737.
Rodríguez de Fonseca, F., Navarro, M., Gómez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodríguez, E., Giuffrida, A., LoVerme, J., Gaetani, S. et al. (2001). An anorexic lipid mediator regulated by feeding. *Nature* **414**, 209-212.
Romano, A., Potes, C. S., Tempesta, B., Cassano, T., Cuomo, V., Lutz, T. and Gaetani, S. (2013). Hindbrain noradrenergic input to the hypothalamic PVN mediates the activation of oxytocinergic neurons induced by the satiety factor oleoylethanolamide. *Am. J. Physiol.* **305**, E1266-E1273.
Sarro-Ramírez, A., Sánchez-López, D., Tejeda-Padrón, A., Frías, C., Zaldívar-Rae, J. and Murillo-Rodríguez, E. (2013). Brain molecules and appetite: the case of oleoylethanolamide. *Cent. Nerv. Syst. Agents Med. Chem.* **13**, 88-91.
Serrano, A., Pavón, F. J., Tovar, S., Casanueva, F., Señarís, R., Diéguez, C. and de Fonseca, F. R. (2011). Oleoylethanolamide: effects on hypothalamic transmitters and gut peptides regulating food intake. *Neuropharmacology* **60**, 593-601.
Thabuis, C., Tissot-Favre, D., Bezelgues, J. B., Martin, J. C., Cruz-Hernandez, C., Dionisi, F. and Destaillats, F. (2008). Biological functions and metabolism of oleoylethanolamide. *Lipids* **43**, 887-894.
Tinoco, A. B., Nisembaum, L. G., Isorna, E., Delgado, M. J. and de Pedro, N. (2012). Leptins and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions. *Peptides* **34**, 329-335.
Valenti, M., Cottone, E., Martinez, R., de Pedro, N., Rubio, M., Viveros, M. P., Franzoni, M. F., Delgado, M. J. and Di Marzo, V. (2005). The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake. *J. Neurochem.* **95**, 662-672.
Vivas, Y., Azpeleta, C., Feliciano, A., Velarde, E., Isorna, E., Delgado, M. J. and de pedro, N. (2011). Time-dependent effects of leptin on food intake and locomotor activity in goldfish. *Peptides* **32**, 989-995.
Volkoff, H., Unniappan, S. and Kelly, S. P. (2009) The endocrine regulation of food intake. In *Fish Neuroendocrinology* (ed. N. J. Bernier, G. Van der Kraak, A. P. Farrel, and C. J. Brauner), pp. 421-265. London: Academic Press Ltd.
Wang, X., Miyares, R. L. and Ahern, G. P. (2005). Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *J. Physiol.* **564**, 541-547.
Wilson, A. M. and McLaughlin, R. (2010). Foraging behaviour and brain morphology in recently emerged brook charr, *Salvelinus fontinalis*. *Behav. Ecol. Sociobiol.* **64**, 1905-1914.
Zheng, J. L., Luo, Z., Zhu, Q. L., Tan, X. Y., Chen, Q. L., Sun, L. D. and Hu, W. (2013). Molecular cloning and expression pattern of 11 genes involved in lipid metabolism in yellow catfish *Pelteobagrus fulvidraco*. *Gene* **531**, 53-63.

V. DISCUSIÓN

1. EL SISTEMA DE LA LEPTINA

1.1. Distribución tisular de leptinas y su receptor en el carpín

Actualmente, gracias a los grandes avances en la tecnología genómica, se han clonado los genes de la leptina y de su receptor en todos los grupos de vertebrados, a excepción de las aves (Londraville et al., 2014). Los datos sobre el sistema de la leptina en el carpín aportados en la presente Tesis Doctoral han contribuido también a dicho conocimiento en un contexto fisiológico comparado.

En concordancia con el proceso de duplicación génica experimentado al comienzo del linaje de los teleósteos (hace aproximadamente 300 millones de años), el gen *ob* se encuentra duplicado en los peces, existiendo leptina del tipo a y del tipo b (Gorissen et al., 2009; Kurokawa y Murashita, 2009; Zhang et al., 2013). Posteriormente, una segunda duplicación en algunas especies (carpa común, carpín, salmón) resultó en la tetraploidización de algunos de sus genes. De ahí la existencia de leptina-aI y aII en los ciprínidos y en el salmón del Atlántico (Huising et al., 2006; Rønnestad et al., 2010; Tang et al., 2013), y leptina-bI y bII, descritas únicamente en salmónidos hasta la fecha (Angotzi et al., 2013). En el caso del carpín se ha evidenciado la existencia de dos parálogos de leptina tipo a, *gLep-aI* y *gLep-aII*, con una alta homología (80% de identidad de aminoácidos), sin haberse detectado por el momento la existencia de leptinas del tipo b en esta especie. Nuestros resultados muestran que las dos leptinas (aI y aII) expresadas en el carpín presentan un diferencial patrón de distribución tisular, de forma similar al descrito en otros teleósteos (Douros et al., 2014; Kurokawa y Murashita, 2009; Rønnestad et al., 2010; Tang et al., 2013; Zhang et al., 2013), sugiriendo que ambos parálogos pueden desempeñar diferentes funciones en los peces. A diferencia de la bien conocida duplicacion del gen *ob* en teleósteos, hasta la fecha sólo se ha encontrado una única secuencia del receptor de la leptina en todos las especies de peces estudiadas (Denver et al., 2011; Kurokawa et al., 2008; Liu et al., 2010), con diferentes isoformas (Cao et al., 2011; Rønnestad et al., 2010; Zhang et al., 2013) que derivan de un único gen por *splicing* alternativo, al igual que se ha descrito en los mamíferos (Peelman et al., 2014). La secuencia parcial del receptor de la leptina obtenida en el carpín codifica para una región del dominio extracelular común a todas las isoformas descritas (Cao et al., 2011; Rønnestad et al., 2010), por lo que no es posible determinar a cual de ellas corresponde.

Desde un punto de vista fisiológico, y teniendo presente la enorme diversidad en la ecología, fisiología y ciclos biológicos de los distintos organismos que expresan esta

hormona, es fácil imaginar que la leptina va a presentar una gran diversidad funcional. La mayoría de los estudios realizados en vertebrados se han centrado en la función de la leptina en la homeostasis energética, constatando también su papel en la función reproductora, interacciones con el sistema inmune, desarrollo larvario, termogénesis y estrés (Copeland et al., 2011; Londraville et al., 2014; Mácajová et al., 2004). Los altos niveles de expresión del receptor de la leptina en áreas encefálicas relacionadas con la regulacion de la ingesta en los peces, como el hipotálamo y el telencéfalo (Lin et al., 2000; Volkoff, 2009a; Wilson y McLaughlin, 2010), apoyan el papel anorexigénico de esta hormona descrito previamente en teleósteos (Chisada et al., 2013; De Pedro et al., 2006; Murashita et al., 2008b; Volkoff et al., 2003), así como en el resto de los vertebrados (Copenland et al., 2011; Londraville et al., 2014), siendo probablemente una de las funciones de la leptina más conservadas.

La expresión simultánea del receptor de la leptina y de la propia leptina en múltiples localizaciones (hipófisis, telencéfalo, hipotálamo, techo óptico, bazo, intestino, hígado, gónadas y riñón) en el carpín, además de sugerir funciones de tipo autocrino/paracrino para esta hormona, plantea la posibilidad de una fucionalidad mucho más amplia que la regulación de la ingesta en este teleósteo. Así, el elevado nivel de expresion de la leptina y del receptor en las gónadas del carpín sugiere una implicación en la reproducción en esta especie, como se ha sugerido en otros peces, donde podría intervenir estimulando el eje reproductor (Frøiland et al., 2010; Peyon et al., 2001, 2003; Weil et al., 2003), al igual que en los mamíferos (Copeland et al., 2011; Londraville et al., 2014). El intestino es otra diana periférica de relevancia en términos de expresión del receptor de la leptina en el carpín, donde además observamos expresión de leptina del tipo aI, coincidiendo con los resultados obtenidos en el salmón del Atlántico, que muestran niveles significativos de leptina (aI y aII) y del receptor en el estómago, ciegos pilóricos e intestino (Rønnestad et al., 2010). Ambos trabajos apoyan un posible papel de la leptina como reguladora del tracto gastrointestinal en teleósteos, al igual que en mamíferos, donde actua de forma sinérgica con la CCK en la regulación de la ingesta a corto plazo, la secrecion pancreática exocrina y la absorción duodenal de proteínas y grasas derivadas del alimento (Guilmeau et al., 2004). También se han sugerido acciones de la leptina a nivel hipofisario en base a la presencia tanto de la leptina como de su receptor en la hipófisis del carpín (presente Tesis Doctoral), en otras especies de peces (Gong, Y. et al., 2013; Gorissen et al., 2009; Kurokawa y Murashita, 2009; Rønnestad et al., 2010; Zhang et al., 2013), y en otros vertebrados (Crespi y Denver, 2006; Huang et al., 2014; Morash et al., 2003). En este sentido, se ha relacionado a la leptina con el estrés y el eje hipotálamo-hipofisis-interrenal, demostrando en la carpa común que la leptina inhibe la secreción

hipofisaria de la adrenocorticotropina (Gorissen et al., 2012). En esta funcionalidad como regulador hipofisario, recientemente se ha demostrado que la leptina estimula la síntesis y secreción de prolactina en la hipófisis de la tilapia (Douros et al., 2014). Por último, cabe destacar un posible papel de la leptina en la señalización de la hipoxia ambiental (Cao et al., 2011; Wong et al., 2007), relacionado con la presencia de la leptina y su receptor en las branquias de algunos peces (Cao et al., 2011; Kurokawa et al., 2008; Liu et al., 2010; Rønnestad et al., 2010; Wong et al., 2007).

En definitiva, los resultados obtenidos en este trabajo avalan la naturaleza pleiotrópica de la leptina en los teleósteos, y ponen de manifiesto la necesidad de realizar más estudios filogenéticos que analicen los genes de leptina en un mayor número de especies pertenecientes a diferentes grupos taxonómicos de vertebrados, así como más estudios fisiológicos sobre las distintas leptinas, incluyendo la leptina-b, que ayudarán a contestar preguntas aún no resueltas, como por ejemplo qué ortólogo de la leptina es el homólogo de la leptina de tetrápodos, cuál es el origen de la función de la leptina y su evolución en los vertebrados.

1.2. Leptinas y regulación del estado energético en el carpín

A pesar de la conservación de las acciones anoréticas de la leptina en los vertebrados, las funciones de esta hormona en la regulación de la homeostasis energética parecen diferir considerablemente entre peces y mamíferos. Estudios previos en teleósteos, en coincidencia con nuestros resultados, han demostrado una elevada expresión de leptina en el hígado (Frøiland et al., 2010; Gorissen et al., 2009; He et al., 2013; Huising et al., 2006; Kobayashi et al., 2011; Kurokawa et al., 2005, 2008; Kurokawa y Murashita, 2009). Este alto nivel de expresión hepática de leptina coincide con que el hígado es el principal lugar de síntesis y almacenamiento de lípidos en muchos peces (Henderson and Tocher, 1987; McClelland et al., 1995), incluyendo el carpín, en el que la escasa grasa perivisceral muestra niveles de expresión muy bajos o nulos para los dos tipos de leptina; a diferencia de lo que sucede en los mamíferos, donde esta hormona se sintetiza mayoritariamente en el tejido adiposo (Zhang et al., 1994).

En los mamíferos existe una clara relación entre leptina y adiposidad, de manera que esta hormona actuaría como una señal de las reservas corporales de grasa, informando al SNC que llevaría a cabo los ajustes fisiológicos necesarios para garantizar la supervivencia en periodos de ayuno, interviniendo por tanto en la regulación del balance energético a largo plazo (Friedman, 2014; Grill et al., 2010). Sin embargo, este modelo

"adipostático" no resulta tan evidente en los peces, donde existen una gran variedad de resultados publicados. Por un lado, se ha demostrado un incremento de los niveles de expresión de leptina durante periodos de ayuno (desde días a semanas) en la trucha arcoíris (Kling et al., 2009), la trucha alpina (*Salvelinus alpinus*) (Frøiland et al., 2010), el salmón del Atlántico (Trombley et al., 2012), el mero de pintas naranjas (Zhang et al., 2013) y el lenguado chileno (*Paralichthys adspersus*) (Fuentes et al., 2013), con reducciones de leptina asociadas a la alimentación (Trombley et al., 2012; Fuentes et al., 2013). Por otro, exiten evidencias de una falta de correlación entre el estado nutricional y el sistema de la leptina (ligando y receptor) en algunos teleósteos. Así, modificaciones nutricionales, como periodos de ayuno y/o sobrealimentación, no producen alteraciones significativas del sistema de la leptina en el pez cebra (Oka et al., 2010), la carpa común (Huising et al., 2006), el pez gato (Kobayashi et al., 2011), la tilapia (Shpilman et al 2014) y el carpín (presente Tesis Doctoral). Aunque todos los datos parecen apuntar a que la leptina no actuaría como señal adipostática a largo plazo en los peces, no podemos descartar que se estén produciendo alteraciones de la leptina a nivel post-transcripcional, que modifiquen al receptor y/o los niveles circulantes de la hormona. Además, hay que tener en cuenta las diferencias entre especies, así como los distintos diseños experimentales realizados (periodo de ayuno y sobrealimentación utilizado, tejidos estudiados, hora del día y estación del año en la que se obtenían las muestras de tejidos, etc.).

Esta marcada diferencia entre teleósteos y mamíferos en el sistema de la leptina puede ser entendida en base a las diferentes tasas metabólicas entre poiquilotermos y homeotermos, y a su diferente regulación del metabolismo energético a largo plazo (Copeland et al., 2011). En esta línea se ha sugerido que la leptina en los vertebrados no mamíferos, y específicamente en los teleósteos, podría actuar señalizando déficits energéticos en situaciones de estrés, como los que se generan en condiciones de hipoxia (Bernier et al., 2012; Cao et al., 2011; Zhang et al., 2013) y de adaptación hiperosmótica (Douros et al., 2014). Como respuesta a estas condiciones adversas estresantes, en las que hay mayores requerimientos energéticos, se produce un incremento de leptina que actuaría modificando el gasto y regulando la liberación de los lípidos almacenados, garantizando así la supervivencia de los animales (Copeland et al., 2011; Londraville et al., 2014).

A pesar de la ausencia de correlación entre la leptina y el estado nutricional a largo plazo en el carpín, en la presente Tesis Doctoral hemos puesto de manifiesto un posible papel de la leptina en la señalización a corto plazo, si tenemos en cuenta el incremento

posprandial de *gLep-aI* que se produce en el hígado a las 9 horas después de la ingestión de alimento. Estos resultados coinciden con datos previos en otros teleósteos, como la carpa común (Huising et al., 2006), el salmón del Atlántico (Moen y Finn, 2013) y el mero de pintas naranjas (Zhang et al., 2013). Además, están en concordancia con la acción de la leptina como factor de saciedad que se observa tras su administración exógena, y que produce una rápida disminución de la ingesta en este ciprínido (De Pedro et al., 2006; Volkoff et al., 2003). Por el contrario, un estudio reciente en *Schizothorax prenanti* (Yuan et al., 2014) no ha encontrado cambios en la leptina a nivel hepático relacionados con la ingesta, sugiriendo que la respuesta posprandial de leptina puede ser específica de especie.

El hecho de que no se hayan encontrado modificaciones en la expresión hipotalámica de leptina (*gLep-aI* y *gLep-aII*) como respuesta a la ingestión de alimento, como observamos en el hígado, podría indicarnos que la leptina puede regularse de manera diferente en el encéfalo y en la periferia. Además, el hecho de que la expresión de *gLep-aII* no se modificase en el hipotálamo en respuesta a la alimentación, aun cuando se expresa mayoritariamente en el encéfalo, reafirma la posibilidad, expuesta en el apartado anterior, de que los dos parálogos de la leptina podrían estar involucradas en diferentes funciones en el carpín, además de que podrían presentar una diferente regulación.

1.3. Ritmicidad diaria de la leptina en el hígado y el hipotálamo del carpín y sincronización al horario de alimentación

Diferentes evidencias nos han llevado a pensar en la existencia de un ritmo diario de leptina en el carpín, como ha sido demostrado previamente en los mamíferos (Cuesta et al., 2009; Gómez Abellán et al., 2011; Kalsbeek et al., 2011; Karakas et al., 2005; Xu et al., 1999). Por un lado, las acciones de la leptina en el carpín dependen de la hora de administración, indicando una dependencia circadiana de los efectos de esta hormona (Vivas et al., 2011). Por otro, en la presente Tesis Doctoral hemos encontrado que el incremento posprandial de leptina hepática comienza a retornar a niveles basales a las 12 horas tras la ingesta, coincidiendo con el inicio de la escotofase y la fase de menor actividad de esta especie, durante la que se producirá una disminución de la demanda energética. Por ello nos planteamos investigar si las diferentes leptinas se expresan siguiendo un patrón rítmico y si son sincronizadas por el horario de alimentación. En condiciones fotoperiódicas de 12L/12D y con un horario fijo de alimentación a las 10:00 h, observamos un ritmo diario en la expresión de leptina en el hígado y el hipotálamo. El ritmo diario en la expresión hepática de *gLep-aI* presenta su acrofase al inicio de la fase

oscura (ZT 13,4 ± 0,9 h), en concordancia con el incremento posprandial observado en otros teleósteos (Huising et al., 2006; Moen y Finn, 2013; Zhang et al., 2013). Si comparamos con el ritmo de leptina-aI en el hipotálamo, vemos que la acrofase tiene lugar aproximadamente 12 h después, mostrando además una amplitud 10 veces menor, por lo que no parece que el ritmo observado en el hipotálamo esté influenciado por la ingestión de alimento. Estos resultados sugieren, como hemos comentado anteriormente, que la leptina parece regularse mediante mecanismos diferentes en el encéfalo y en la periferia.

Como datos complementarios, que aportaron más información sobre la posible regulación del ritmo diario de la expresión de leptina, se cuantificó el patrón diario de la actividad locomotora y de la glucemia en las mismas condiciones experimentales. El patrón de actividad en el carpín ha sido extensamente empleado como control de la sincronización de los peces a las condiciones ambientales (Feliciano et al., 2011; Vera et al., 2007). En presencia de fotoperiodo 12L/12D y alimentación en la fase luminosa a las 10:00 h, los carpines muestran un marcado ritmo diario de actividad, con valores máximos durante la fotofase y mínimos durante la escotofase, confirmando el patrón diurno de este ciprínido en las condiciones mencionadas (Feliciano et al., 2011; Nisembaum et al., 2012; Sánchez-Vázquez y Madrid, 2001; Vera et al., 2007). Al comparar los ritmos de leptina hepática y actividad locomotora comprobamos que el pico máximo de la expresión de leptina precede a la disminución de la actividad natatoria observada durante toda la fase oscura. A pesar de no haber medido los niveles circulantes de leptina, cabría esperar un incremento en los mismos posteriormente al incremento en la expresión, coincidiendo por tanto con la fase inactiva de los peces. Estos datos apoyarían la reducción en la actividad natatoria que produce la inyección IP de leptina en el carpín (Vivas et al., 2011). Es posible que también exista una relación entre los ritmos diarios de leptina y glucosa, teniendo presente que el metabolismo de la glucosa es uno de los principales determinantes de la síntesis y secreción de la leptina en mamíferos (Amitani et al., 2013; Wang et al., 1998). Los datos de la presente Tesis en el carpín apoyan esta hipótesis, ya que el máximo posprandial de glucosa plasmática precede al mostrado por la leptina, de forma similar a los datos descritos en la carpa común (Huising et al., 2006). No obstante, la relación leptina-glucosa es compleja ya que la leptina a su vez regula la glucemia en los peces, aunque sus efectos no parecen tan claros, al haberse descrito tanto respuestas hiper- como hipoglucemiantes (Aguilar et al., 2010; Baltzegar et al., 2014; De Pedro et al., 2006; Vivas et al., 2011). Otros factores podrían contribuir a la expresión rítmica de leptina, como sugieren diversos estudios en mamíferos, que han implicado a diferentes hormonas (cortisol e insulina) y metabolitos derivados de la ingestión de alimento (Cuesta et al., 2009; Feillet, 2010; Froy, 2011; Kalsbeek et al., 2001; Schoeller et al., 1997).

Con el fin de estudiar el efecto del horario de alimentación en la ritmicidad diaria de la expresión de leptina mantuvimos a los peces en luz constante (anulando el efecto sincronizador del ciclo L/D), recibiendo la ración diaria de comida a diferentes horarios, 10:00 h (grupo SF10), 22:00 h (grupo SF22) y con alimentación aleatoria (grupo RnF; ausencia de ambos sincronizadores). Dichas condiciones experimentales han sido utilizadas con éxito previamente por nuestro grupo de investigación, demostrando que los ritmos diarios de actividad locomotora y de genes reloj (*gPer1a*, *gPer3* y *gCry3*) en tejidos centrales y periféricos se sincronizan al horario de alimentación en ausencia de un ciclo L/D (Feliciano et al., 2011; Nisembaum et al., 2012). De forma similar, en la presente Tesis Doctoral hemos encontrado perfiles rítmicos similares de la actividad y de la expresión de los genes reloj mencionados en el hígado y el techo óptico en los grupos SF10 y SF22, ritmos que desaparecen en el grupo con alimentación aleatoria. Estos resultados confirman que existe un reloj funcional en el carpín que, en ausencia de un ciclo L/D, es sincronizado por el horario de alimentación, que actúa como un potente *zeitgeber* en este teleósteo. Sin embargo, el hecho de que el ritmo diario de la expresión de leptina en el hígado y en los tejidos centrales (hipotálamo y techo óptico) se observe en los peces alimentados a las 10:00 h, pero no a las 22:00 h, sugiere que el horario de alimentación por sí solo no es capaz de inducir los ritmos diarios de expresión de leptina en el carpín. A pesar de que existe consenso acerca de la existencia de variaciones diarias en los niveles de leptina en vertebrados (Huising et al., 2006; Manzar y Hussain, 2011; Moen y Finn, 2013; Zhang et al., 2013), actualmente no se han definido los mecanismos que subyacen a estas oscilaciones diarias de la expresión de leptina. Numerosas señales se han postulado como posibles sincronizadores de los ritmos de leptina en mamíferos, de origen endógeno, como la insulina, los glucocorticoides, la melatonina, la ghrelina, las orexinas, el NPY y los metabolitos derivados de la ingesta (Cuesta et al, 2009; Kalra et al., 2003; Kalsbeek et al, 2001; Feillet et al., 2010; Froy et al, 2011; Manzar y Hussain, 2011), y de origen exógeno, como el ciclo L/D (Li et al., 2012), la temperatura (Manzar y Hussain, 2011) y el horario de alimentación (Patton y Mistlberger, 2013; Xu et al., 1999).

Asumiendo un funcionamiento correcto del reloj endógeno, las diferencias encontradas en los ritmos de leptina entre los grupos SF10 y SF22 cabría buscarlas en las condiciones de aclimatación previas (ciclo 12L/12D y horario fijo de alimentación a las 10:00 h). A medida que se va conociendo mejor el funcionamiento del reloj endógeno, así como la generación y regulación de los ritmos, nuevas evidencias señalan que algunos eventos rítmicos dependen del aprendizaje y la memoria, incluso en ausencia de los mecanismos clásicos de transcripción-traducción del reloj (Silver et al., 2011). En este sentido, se han observado evidencias de la existencia de un oscilador metabólico (basado

en ciclos de utilización de energía, oxidación-reducción) que se acoplaría al oscilador genético (basado en mecanismos de transcripción-traducción de genes) manteniendo una sincronía entre el ambiente y los procesos bioquímicos internos (Bass y Takahashi, 2010; Silver et al., 2011). Si ocurriera algo similar en los peces, podemos especular que en el grupo SF10 los animales, a pesar de perder la señal ambiental rítmica ciclo L/D, conservan una señal metabólica (horario de alimentación a las 10:00 h tanto durante el periodo experimental como en el previo de aclimatación) que les permite "recordar" el ritmo diario de leptina que se expresa en condiciones 12L/12D. Sin embargo, los peces alimentados a las 22:00 h y en luz constante han sufrido una modificación de todas las señales, externas (ausencia de ciclo L/D, y alimentación desplazada 12 h con respecto a su horario habitual) e internas (metabólicas también desplazadas 12 h), lo que no les habría permitido "recordar" cuándo debe generarse el ritmo de leptina, justificando la ausencia de expresión rítmica de esta hormona en el grupo SF22.

Otras consideraciones a tener en cuenta a la hora de interpretar nuestros resultados es que, aunque los ritmos de la actividad locomotora y de la expresión de genes reloj indican que las diferencias en la expresión de leptina en los grupos SF10 y SF22 no pueden ser explicadas desde el punto de vista de un reloj disfuncional, no podemos descartar que otros genes reloj no estudiados en este trabajo pudieran estar afectados en el grupo SF22, dando como resultado la ausencia de ritmo de leptina en este grupo. Resulta también interesante constatar que las amplitudes de los genes reloj estudiados fueron significativamente mayores en el hígado que en el encéfalo, confirmando que el hígado es un tejido con una capacidad robusta de sincronización al horario de alimentación en el carpín (Feliciano et al., 2011), como se ha demostrado en los mamíferos (Damiola et al., 2000; Kornmann et al., 2007; Stokkan et al., 2001).

Por tanto, los resultados de los ritmos de expresión de leptina y su posible sincronización al horario de alimentación en el carpín sugieren que tanto las señales ambientales (ciclo luz/oscuridad y horario de alimentación) como las endógenas (metabólicas) están involucradas en la expresión rítmica de leptina. El hecho de que el horario de alimentación por sí solo no sea capaz de sincronizar los ritmos diarios de leptina, y que estos no parezcan depender exclusivamente de la ritmicidad de los genes reloj estudiados, pone de manifiesto una vez más la complejidad del sistema circadiano, y la necesidad de investigar en profundidad los mecanismos de interacción de la alimentación con procesos de aprendizaje y memoria.

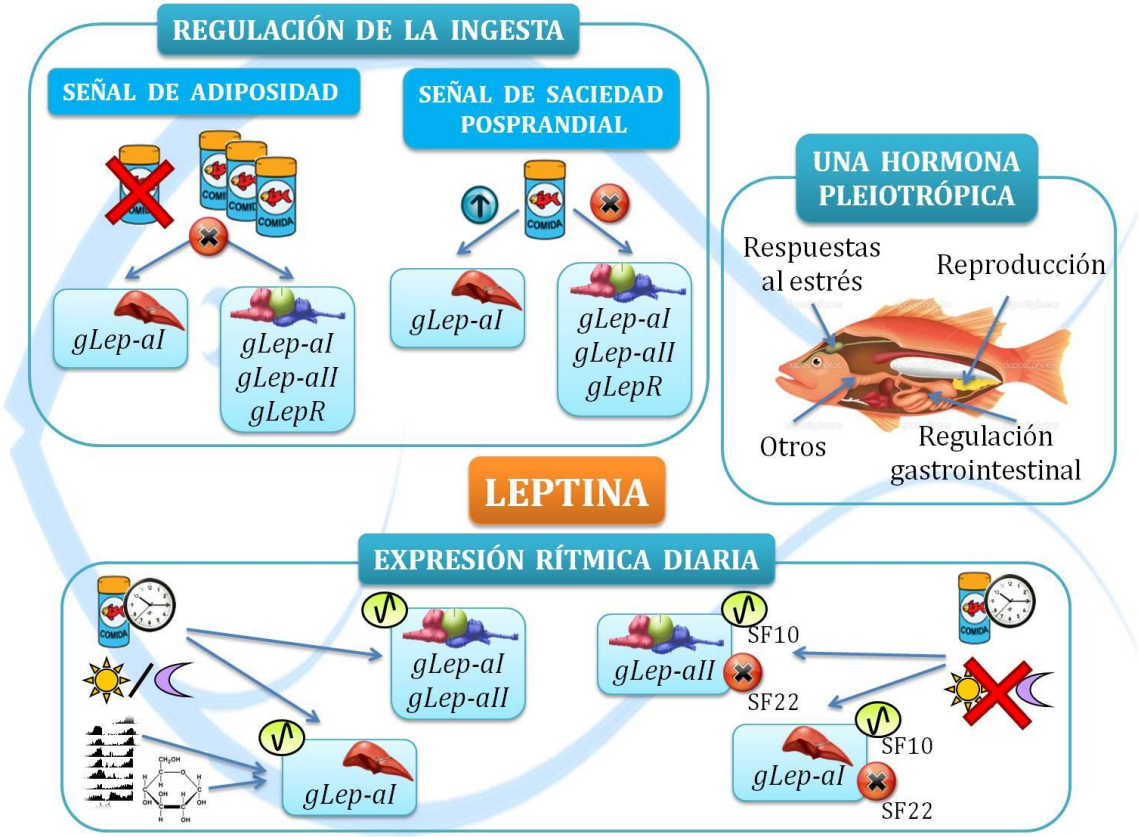

Figura 6. Representación esquemática de la funcionalidad de la leptina en el carpín. SF10: carpines alimentados a las 10:00 h en condiciones de luz constante; SF22: carpines alimentados a las 22:00 h en condiciones de luz constante; : ayuno; : sobrealimentación; : horario fijo de alimentación; : ciclo 12L/12D; : ritmo; : sin cambios; incremento.

2. COLECISTOCININA Y MOTILIDAD INTESTINAL

2.1. Motilidad intestinal en el carpín. Efectos y mecanismos de acción de la CCK

El intestino desempeña un papel esencial en la regulación de la homeostasis metabólica, ya que proporciona información clave al cerebro en relación con el alimento ingerido/digerido, configurando el denominado eje intestino-cerebro. Mediante su capacidad de detección de nutrientes, producción endocrina y regulación de la motilidad genera un complejo sistema de señalización esencial en el proceso de alimentación. Numerosos estudios previos relacionan motilidad gastrointestinal con la regulación del apetito, siendo el tránsito intestinal un factor determinante en el control de la ingesta realizado por la ruta de señalización intestino-cerebro (Dockray, 2014). Son varias las hormonas intestinales que, producidas de forma local, intervienen en este complejo sistema de regulación. En la presente Tesis Doctoral nuestro interés en este contexto se ha centrado en la CCK, cuya síntesis y efectos en el organismo tienen lugar en ambas localizaciones, el cerebro y el tracto gastrointestinal (Côté, 2014). En los teleósteos, de forma general, y en el carpín en concreto, se conocen bien las acciones anorexigénicas, periféricas y centrales, producidas por la CCK (Himick y Peter, 1994b; Hoskins y Volkoff, 2012; Volkoff et al., 2003), así como las interacciones de este péptido con otros agentes reguladores del apetito (Volkoff, 2006). Sin embargo, en la actualidad se desconoce si posibles acciones en la motilidad intestinal subyacen a dicho efecto anorexigénico, siendo una cuestión de particular interés en este ciprínido que carece de estómago. La motilidad intestinal, que implica contracciones y relajaciones del músculo liso gastrointestinal necesarias para el transporte, la mezcla y el procesado del alimento ingerido, está sometida a un complejo control neural y endocrino que garantice su funcionamiento óptimo (Wu et al., 2013), representando, por tanto, un objetivo idóneo para evaluar acciones de la CCK. Por todo ello, en la presente Tesis Doctoral hemos abordado el estudio de las posibles acciones del péptido biológicamente activo de la CCK (CCK-8S) en la motilidad intestinal del carpín mediante una aproximación *in vitro*.

La herramienta metodológica empleada en este estudio es el baño de órganos acoplado a un transductor isométrico, que permite registrar cambios en la contractilidad del músculo intestinal ante la presencia en el medio de cultivo de distintos agentes reguladores. Este procedimiento se emplea habitualmente en farmacología experimental para caracterizar las respuestas contráctiles del músculo liso a fármacos y/o la estimulación eléctrica, proporcionando resultados fiables con un alto grado de reproducibilidad y precisión. Nuestro grupo de investigación posee una amplia

experiencia en el manejo de este sistema *in vitro*, habiendo demostrado su eficacia en el análisis de la regulación de la motilidad del intestino proximal del carpín (Nisembaum et al., 2013; Velarde et al., 2009, 2010, 2011). Esta aproximación *in vitro* genera información valiosa complementaria a los estudios *in vivo*, y tiene claras ventajas frente a otras alternativas *in vitro* utilizadas en estudios previos del control de la motilidad gastrointestinal en peces (Olsson y Holmgren, 2001).

La adición de CCK-8S (forma mayoritaria de la CCK circulante) al baño de órganos genera una respuesta contráctil en segmentos del intestino proximal del carpín sin afectar al tono basal espontáneo que se registra en estas preparaciones (Velarde et al., 2009). El incremento generado en la fuerza de contracción es dependiente de la concentración de CCK-8S, no observándose a concentraciones inferiores a 10^{-8} M, muestra un perfil sigmoideo clásico, y valores de EC_{50} en un intervalo de afinidad similar a los descritos en mamíferos (Dufresne et al., 2006). Estos datos apoyan la relevancia fisiológica de este efecto del péptido CCK-8S en la motilidad del intestino proximal y, aunque no hay datos en la bibliografía acerca de la concentración endógena de la CCK en el intestino anterior de este teleósteo, los valores de EC_{50} están dentro de un orden de magnitud esperable para estos reguladores (Olsson y Holmgren, 2011; Wu et al., 2013). Este estudio es el primero publicado en los ciprínidos, aunque en los salmónidos se ha sugerido que la CCK-8S interviene en el control del vaciamiento gástrico en la trucha arcoíris (Olsson et al., 1999) y en el salmón real (Forgan y Foster, 2007). A pesar del uso de distintos enfoques experimentales (*in vitro* e *in vivo*), distintas regiones (zona anterior del estómago, esfínter pilórico), y la variedad de respuestas obtenidas (excitadoras e inhibidoras), el consenso general parece apuntar a que la CCK, además de estimular la contracción de la vesícula biliar (Murashita et al, 2008a), enlentece el vaciado gástrico (Volkoff, 2006). Sin embargo, esta conclusión resulta cuestionable porque la CCK-8S en la trucha no afecta a la región posterior (pilórica) del estómago (Olsson et al., 1999), cuyo papel es esencial en la regulación del tránsito gastro-intestinal. Estudios realizados en los mamíferos ponen de manifiesto esta acción dual de la CCK, relajando los esfínteres (esofágico inferior y esfínter de Oddi) pero contrayendo la musculatura lisa a través de la estimulación colinérgica en la vesícula biliar y en el intestino delgado (Grider, 1994; Wu et al., 2013). Los aumentos de la fuerza contráctil inducidos por la CCK-8S en segmentos longitudinales de intestino proximal del carpín coinciden con los publicados por Jönsson y colaboradores (1987) en el bacalao del Atlántico, donde segmentos gástricos se contraen por el péptido humano CCK-8S. Con la aproximación experimental utilizada en el carpín, teniendo en cuenta que se ha estudiado la zona más anterior del intestino en este teleósteo carente de estómago, y que el ritmo espontáneo de ondas lentas no se ve afectado, proponemos (aunque no podemos

concluirlo de forma absoluta) que el incremento en la tensión intestinal inducido por CCK-8S podría favorecer la propulsión del alimento en sentido antero-posterior, sin descartar una participación en la facilitación de un mezclado local del alimento con secreciones digestivas. Es importante tener presente que la actividad contráctil del intestino ha de garantizar el avance del alimento ingerido a una velocidad óptima que permita también una óptima exposición y mezclado con las secreciones digestivas.

En el intestino de los mamíferos se plantea que la acción de la CCK puede ser directa sobre el músculo liso, mediada neuralmente, o de ambos tipos, dependiendo de la región intestinal en estudio, tratándose en general de un efecto contráctil cuando es directo sobre las células de músculo liso, mientras que las acciones mediadas neuralmente pueden ser contráctiles o relajantes, dependiendo del tipo de neurotransmisor liberado (Grider, 1994). En la presente Tesis Doctoral nos propusimos investigar si el efecto contráctil de la CCK-8S en el carpín se ejercía directamente sobre células de músculo liso o vía neuronal. El sistema nervioso entérico, en líneas generales, mantiene una organización funcional similar en todos los vertebrados, pero con una menor complejidad en los peces, donde la mayoría de los somas neuronales se encuentran en el plexo mientérico situado entre las dos capas musculares, circular y longitudinal, con axones que se proyectan a ambas capas, vasos sanguíneos, células y glándulas endocrinas de las capas mucosa y submucosa (Olsson, 2009). Teniendo presente esta organización anatómico funcional del intestino, nuestros resultados apoyan una acción directa de la CCK-8S sobre la capa de músculo liso, e independiente del sistema nervioso entérico, al no verse modificada la contracción inducida por la CCK por la presencia de tetrodotoxina en el baño de órganos. De forma similar, el efecto contráctil de la sustancia P en el estómago (Jensen et al., 1993) y la acción inhibitoria del péptido relacionado con el gen de la calcitocina (CGRP) en el intestino del bacalao del Atlántico también son directos e independientes de tetrodotoxina (Shahbazi et al., 1998). A favor de esta idea se ha demostrado la presencia de los receptores de CCK en las células de músculo liso de distintas regiones del intestino de mamíferos (Grider, 1994). Además, los datos obtenidos descartan la mediación de terminaciones colinérgicas en el efecto contráctil de la CCK-8S, que no es modificado por el bloqueo farmacológico con el antagonista muscarínico, atropina. Este mecanismo difiere del descrito para la estimulación serotoninérgica contráctil en segmentos intestinales de este teleósteo, que es bloqueada por la misma concentración de atropina que la utilizada en el presente estudio (Velarde et al., 2010). Sin duda, la transmisión colinérgica desempeña una función esencial en la regulación de la motilidad gastrointestinal de los vertebrados, incluyendo los teleósteos (Dockray, 2014; Olsson y Holmgren, 2011), pero es necesario considerar que algunas de las distintas funciones de la CCK pueden actuar

independientemente del sistema colinérgico. En este sentido, la CCK liberada por la llegada del alimento parece utilizar rutas vagales colinérgicas que median su función anorexigénica (Dockray, 2014), pero puede actuar de forma directa en las células musculares (estimulada por determinada composición del quimo) para controlar el tránsito del quimo en tramos anteriores del tracto gastrointestinal con el fin de garantizar un nivel adecuado de digestión.

Las aproximaciones farmacológicas que utilizan antagonistas han permitido obtener información más precisa acerca de las funciones de la CCK (Dufresne, 2006; Noble et al., 1999). Los resultados que se presentan en esta Memoria en relación con la adición de antagonistas de receptores de CCK al baño de órganos indican que el efecto contráctil inducido por la CCK-8S está mediado, en parte, por receptores del subtipo A (véase más adelante), y la abundancia de este subtipo de receptor en el intestino del carpín apoya esta propuesta. También en los mamíferos se han descrito acciones paracrinas de la CCK a través de receptores CCKAR (Berna et al., 2007; Dufresne et al., 2006; Strader y Woods, 2005). Como el bloqueo producido por el antagonista específico de los receptores CCKAR no es completo, planteamos al menos dos posibles explicaciones. Por un lado, puede que parte del efecto contráctil no esté mediado específicamente por los receptores de la CCK, y por otro, cabe la posibilidad de que los antagonistas empleados, diseñados comercialmente para los receptores de la CCK de los mamíferos, no sean efectivos en los receptores del carpín. Esta limitación en la disponibilidad de antagonistas comerciales específicos para peces ha sido puesta de manifiesto en estudios previos (Velarde et al., 2010).

2.2. Receptores de CCK en el carpín. Filogenia y distribución

En la presente Tesis Doctoral se muestran las secuencias parciales de dos isoformas (CCKAR y CCKBR) del receptor de la CCK en el carpín, mostrando el análisis filogenético de las mismas un alto valor (872 para CCKAR y 952 para CCKBR en 1000 repeticiones estadísticas) en la ramificación de estas dos secuencias en relación a las de tetrápodos, indicando ortología entre secuencias. Estas dos secuencias se sitúan cada una en un *cluster* diferente, representando a cada subtipo de receptor descrito en vertebrados, denominados CCK-1R y CCK-2R (Rathore et al., 2013; Staljanssens et al., 2011). Como esperábamos, ambas secuencias obtenidas en el carpín se sitúan junto a las de otro ciprínido, el pez cebra, presentando una alta similitud entre ellas, 95,3% para CCKAR y 90% para CCKBR. El menor porcentaje de similitud entre teleósteos lo observamos en el receptor tipo A con el pez de las cuevas (*Astyanax fasciatus mexicanus*) (86,8%) y el

receptor tipo B con la tilapia (62%). Por otro lado, la elevada similitud de la secuencia CCKAR en los teleósteos (> 86%) indica un alto grado de conservación de este subtipo de receptor, a diferencia del CCKBR, cuya menor similitud y mayor longitud de las ramificaciones en el dendrograma, sugieren que este subtipo podría ser anterior al CCKAR.

Con las secuencias parciales obtenidas en el carpín parece plausible la duplicación de ambas secuencias del receptor de la CCK en esta especie, de forma similar a lo obtenido en el salmón del Atlántico, donde se han encontrado dos parálogos del receptor tipo B, denominados CCK-2R1/gastrina (ortólogo del receptor de gastrina de mamíferos) y CCK-2R2 (Rathore et al., 2013), estos dos parálogos del receptor B de salmón del Atlantico muestran además similitud entre las dos variantes de secuencias de CCKBR en el carpín (61,5% y 62,5% con CCK-2R1/gastrina y CCK-2R2 de salmón, respectivamente). La existencia de estas variantes se justifica por la tetraploidización génica experimentada por estos peces debido a la duplicación del genoma completo que tuvo lugar hace 350 millones de años en el linaje de los teleósteos (Hoegg et al., 2004; Taylor et al., 2003), y su repetición posterior en los ciprínidos y los salmónidos (Kurokawa y Murashita, 2009; Moghadam et al., 2005).

La mayor divergencia entre secuencias de los receptores de la CCK la encontramos al comparar las secuencias del carpín con las de mamíferos, por ejemplo con las de ratón (*Mus musculus*), con el que los porcentajes de similitud alcanzan el 82,5% para CCKAR, y apenas el 45% para CCKBR. Estos datos apoyan la propuesta del elevado grado de conservación del receptor de la CCK tipo A, frente a la filogenia del receptor de la CCK tipo B cuya identidad de aminoácidos ronda la mitad que para el CCKAR. A pesar de estos porcentajes tan dispares, ambas secuencias parciales de los receptores de la CCK en el carpín conservan todos los dominios funcionales descritos en la secuencia de mamíferos (Archer-Lahlou et al., 2005; Foucaud et al., 2008). Esta conservación de dominios funcionales también se ha demostrado en el único estudio publicado en peces hasta la actualidad (Rathore et al., 2013), a partir de las secuencias completas del receptor de la CCK del salmón del Atlántico, donde se ha determinado que todos los dominios funcionales y estructurales de mamíferos se conservan en este teleósteo, excepto los residuos triptófano 39 y glutamina 40 del receptor de CCK tipo A que afectan a la interacción con el extremo N-terminal de la CCK. En el carpín las secuencias obtenidas presentan los dominios 4 y 5 completos, de los 7 dominios transmembrana característicos de los receptores acoplados a proteínas G, así como la cisteína del segundo bucle extracelular responsable de la formación del puente disulfuro (Miller y Gao, 2008; Rathore et al., 2013). Además, también están presentes los dos aminoácidos más importantes que

confieren la selectividad del CCKAR por la forma sulfatada de la CCK frente a la no sulfatada, la metionina 195 y la arginina 97 de dicho bucle (Archer-Lahlou et al., 2005; Foucaud et al., 2008), metionina 200 y arginina 202 en el carpín. Las secuencias parciales del capín también comparten con el salmón y los mamíferos todos los dominios funcionales implicados en la interacción péptido-receptor entre las posiciones 157 y 236 de la secuencia de salmón para el CCKAR, y entre las 181 y 334 para el CCKBR (Archer-Lahlou et al., 2005; Foucaud et al., 2008; Rathore et al., 2013).

El patrón de distribución de las dos secuencias clonadas del receptor de la CCK del carpín es claramente diferente. La secuencia CCKAR está ampliamente distribuida por todo el organismo, si bien los niveles más elevados de expresión se localizan en el tracto gastrointestinal. La secuencia CCKBR, sin embargo, se expresa mayoritariamente en el encéfalo (hipotálamo, telencéfalo y lóbulo vagal), con niveles significativos también en el intestino posterior. Podemos considerar inexistente la presencia de transcritos de la CCKBR en el resto del tracto gastrointestinal, el hígado y el músculo del carpín. Este patrón diferencial de distribución de la expresión de ambos subtipos de receptores coincide con el descrito en los mamíferos (Dufresne et al., 2006; Lacourse et al., 1997; Lay et al., 2000) y en el salmón del Atlántico (Rathore et al., 2013), apoyando no sólo la especialización funcional de cada subtipo de receptor en el carpín, como se ha propuesto en los mamíferos (Dufresne et al., 2006), sino también la conservación funcional de los receptores de la CCK en la filogenia de los vertebrados. A favor de esta hipótesis de la especialización funcional de los receptores de la CCK, aportamos los resultados de la presente Memoria en relación con la implicación funcional del CCKAR, pero no del CCKBR, en la motilidad intestinal del carpín, discutidos anteriormente.

La presencia del receptor de la CCK subtipo A en el hígado del carpín corrobora datos previos en seriola (Furutani et al., 2013), sin que por el momento se le haya atribuido alguna función en este tejido. Glaser y colaboradores (1997) detectaron niveles significativos de expresión del CCKBR/gastrina en células del epitelio de la vesícula biliar de la rata, reforzando su implicación en la regulación de la secreción biliar. Sin embargo, otros no han detectado expresión de ninguno de los dos receptores de la CCK en el hígado del ratón (Lacourse et al., 1997; Lay et al., 2000). El hígado de los peces (como mencionamos anteriormente), especialmente en los ciprínidos, tiene notables diferencias respecto al de los mamíferos, siendo el principal órgano de reserva de grasa y el que presenta mayores niveles de expresión de leptina en estos teleósteos (He et al., 2013; Huising et al., 2006; Kurokawa et al., 2005; Murashita et al., 2008b; datos de la presente Tesis Doctoral). Teniendo presente la sinergia de las acciones anoréticas de la leptina y la

CCK en el carpín (Volkoff et al., 2003), y la implicación del receptor de la CCK subtipo A en la síntesis de leptina en el hígado propuesta en los mamíferos (Tsunoda et al., 2003), resulta tentador especular acerca de una posible regulación de la síntesis de la leptina en el hígado del carpín mediada por CCK a través de receptores de tipo CCKAR, estimulando a la realización de nuevas investigaciones encaminadas a dilucidar esta cuestión.

La finalidad de incluir el músculo esquelético en el barrido de tejidos en nuestro diseño experimental fue la de contar con un tejido control en el que cabría esperar la nula expresión de CCKAR y CCKBR, como sucede en la seriola (Furutani et al., 2013). Sin embargo, hemos encontrado una expresión del subtipo CCKAR en el músculo del carpín. Esta diferencia puede deberse a que nuestro método de amplificación sea más sensible que el empleado en la seriola, aunque no podemos descartar diferencias entre especies. No obstante, en la bibliografía publicada en los mamíferos no se ha investigado la posible presencia de este subtipo de receptor de la CCK en el músculo esquelético, aunque si se ha descrito en músculo liso de la vesícula biliar y en la musculatura del esfínter pilórico. Sería interesante confirmar la presencia de estos receptores en el músculo esquelético y su posible significado funcional, en el contexto de diferencias relativas funcionales del receptor CCKAR en distintos tipos de músculos.

Nuestro estudio revela la presencia de ambos subtipos de receptores en el encéfalo del carpín, si bien el CCKBR parece ser el receptor mayoritario en las estructuras analizadas, patrón que coincide con el único estudio realizado en peces, en el salmón del Atlántico (Rathore et al., 2013), y con los datos publicados en los mamíferos (Dufresne et al., 2006; Staljanssens et al., 2011). La funcionalidad del receptor cerebral de la CCK de tipo A es menos conocida en los mamíferos, habiéndose relacionado con procesos de nocicepción, memoria y aprendizaje; sin embargo se ha estudiado en profundidad la funcionalidad del receptor cerebral CCKBR, que asume funciones similares a las del tipo A, y además está implicado en las respuestas al estrés y en los trastornos de ansiedad y pánico (Berna et al., 2007; Hebb et al., 2005), y en la acción anorexigénica de la CCK (Frommelt et al., 2013). La información disponible a cerca de los receptores de la CCK en el encéfalo de teleósteos es muy limitada y se restringe a la detección de expresión en el encéfalo completo en la seriola y el salmón del Atlántico (Furutani et al., 2013; Rathore et al., 2013). Por otro lado, hasta la actualidad, las únicas acciones de la CCK estudiadas a nivel central son la reducción de la ingesta (Himick y Peter, 1994b; Hoskins y Volkoff, 2012; Kang et al., 2010; Volkoff et al., 2003) y la liberación de la GH hipofisaria (Canosa y Peter, 2004; Canosa et al., 2005). Teniendo en cuenta la presencia mayoritaria del receptor de CCK tipo B en el encéfalo, y específicamente en el hipotálamo, área implicada en la

regulación de la ingesta, parece factible que este receptor CCKBR sea el implicado en la acción anorética de la CCK a nivel central, aunque no podemos descartar una posible participación del subtipo CCKAR. Aproximaciones farmacológicas utilizando antagonistas selectivos para estos subtipos de receptores pueden contribuir al conocimiento en profundidad de los receptores implicados en la regulación de la ingesta por la CCK en los teleósteos (Gélineau y Boujard, 2001; Rubio et al., 2008). Alternativamente, el análisis de los posibles cambios en los niveles de expresión de ambos subtipos de receptores en el hipotálamo, asociados a alteraciones del estado nutricional de los peces, también contribuiría a profundizar en esta interesante cuestión.

Por último, es importante destacar que la presencia de receptores de la CCK tanto en localizaciones encefálicas, como en distintas zonas del tracto gastrointestinal del carpín nos permite proponer, como se ha sugerido previamente en los mamíferos (Dufresne et al., 2006), que la CCK puede estar desempeñando funciones de tipo autocrino/paracrino en estas localizaciones, donde se ha encontrado expresión de la CCK (Peyon et al., 1998).

3. REGULACIÓN DEL BALANCE ENERGÉTICO POR GHRELINA Y OLEILETANOLAMIDA

3.1. Ghrelina en la trucha común. Efectos comportamentales y acciones en la ingesta

El notable incremento de la ingesta y la tasa de crecimiento estándar tras la administración periférica subcrónica de ghrelina en la trucha común apoya claramente el papel orexigénico de esta hormona descrito en otros vertebrados (Jönsson, 2013; Kaiya et al., 2008; Nakazato et al., 2001; Tschöp et al., 2000). Estudios previos realizados en otros teleósteos han demostrado un incremento de la ingesta tras la administración aguda de ghrelina, tanto IP como ICV (Miura et al., 2006; Unniappan et al., 2002, 2004), así como en tratamientos crónicos con administración periférica mediante bomba micro-osmótica (Riley et al., 2005) y administración oral mediante suplemento de ghrelina en la dieta (Gao et al., 2012). Sin embargo, este efecto orexigénico no ha podido confirmarse en la trucha arcoíris cuando se administra la ghrelina homóloga de trucha (Jönsson et al., 2007, 2010), a pesar de que estudios previos en la misma especie y con el mismo diseño experimental pero administrando ghrelina de rata encontraron un incremento de la ingesta (Shepherd et al., 2007). Ante estos resultados cabe plantearse que las acciones de la ghrelina sobre el apetito en este salmónido dependen del tipo de ghrelina empleada (homóloga *versus* heteróloga), además de otros factores a tener en cuenta como la dosis, vía de administración (IP *versus* ICV), etc. En la presente Tesis Doctoral empleamos la misma ghrelina de trucha y la misma vía de administración (IP mediante implantes) en la trucha común que la utilizada por Jönsson y colaboradores (2010) en la trucha arcoíris encontrando, sin embargo, un incremento significativo del apetito en la trucha común. Actualmente desconocemos las posibles causas que justifiquen estas diferencias entre los dos salmónidos estudiados, si bien es necesario tener presente que la trucha arcoíris es una especie que ha experimentado en las últimas décadas procesos importantes de domesticación, y la consiguiente selección de aquellos individuos que presentan un mayor tamaño (Huntingford, 2004), lo que sin duda puede haber modificado la fisiología de esta especie. Así, se han demostrado cambios fisiológicos de la GH en salmónidos domesticados (Fleming et al., 2002; Tymchuk et al., 2009), y teniendo en cuenta la relación entre la ghrelina y la GH en los vertebrados (Ahmed y Harvey, 2002; Fox et al., 2007; Kojima et al., 1999; Picha et al., 2009), resulta tentador especular sobre una consecuente modificación también en el sistema de la ghrelina en estos salmónidos domesticados, que justificaría las diferencias encontradas entre la trucha común de origen salvaje utilizada en la presente Tesis Doctoral y la trucha arcoíris.

Asociado al incremento de la ingesta y el peso corporal, la ghrelina estimula también el almacenamiento de grasa en los mamíferos (Tschöp et al., 2000) y en algunos teleósteos, como la tilapia (Riley et al., 2005), los carpines hembras (Kang et al., 2011b) y en la trucha arcoíris (Jönsson et al., 2007). Sin embargo, nosotros no observamos dicho efecto lipogénico de la ghrelina en la trucha común, al no encontrar modificaciones significativas en ninguno de los parámetros analizados del metabolismo lipídico en el hígado y el músculo. Estos resultados coinciden con la ausencia de efecto lipogénico de la ghrelina descrita en carpines machos (Kang et al., 2011b), y sugieren que el mayor crecimiento de los peces (trucha común y carpín) tratados con la ghrelina se debería principalmente a una mayor ingestión de alimento y no a posibles acciones lipogénicas de esta hormona. Sin embargo, en el carpín no podemos descartar que la ghrelina pueda haber generado un efecto lipogénico, pero que habría sido enmascarado por el incremento de la actividad locomotora (y consecuente mayor consumo de energía) que muestran estos peces inyectados con ghrelina, dando como resultado final que las reservas lipídicas no se vean modificadas, como sucede en el mero de pintas naranjas (Gao et al., 2012). Tampoco podemos descartar diferencias entre especies (Kaiya et al., 2008), así como diferencias entre los diseños experimentales, el estado de desarrollo y el sexo de los animales, e incluso diferencias estacionales que deriven en cambios en el comportamiento alimentario, el crecimiento y el grado de adiposidad (Kestemont y Baras, 2001).

Los principales resultados obtenidos sobre la actividad natatoria en truchas comunes tratadas con ghrelina indican que esta hormona parece incrementar la actividad locomotora 1 hora antes de la llegada de la comida, así como la denominada "posición de ingesta" (zona del acuario bajo del comedero en la que los peces esperaban la llegada de alimento), sugiriendo un incremento en la actividad destinada a la búsqueda de alimento. Otras evidencias de esta relación de la ghrelina con la actividad de búsqueda de alimento en los peces han sido obtenidas recientemente en nuestro grupo de investigación, donde la administración de ghrelina en el carpín induce una FAA (Nisembaum et al., 2014), al igual que sucede en los mamíferos (Blum et al., 2009; LeSauter et al., 2009). Aunque de forma indirecta, también en la lubina de boca pequeña (*Micropterus dolomieu*) se ha sugerido dicha relación, ya que los niveles de ghrelina circulante aumentan significativamente justo después de la época de cría, cuando los adultos comienzan de nuevo a alimentarse activamente (Hanson et al., 2009). Además del efecto motivacional de la ghrelina por la obtención de alimento, no podemos descartar que, tal y como se ha descrito en los mamíferos (Carlini et al., 2002), esta hormona esté potenciando procesos de aprendizaje y retención de memoria en los peces, lo que también podría haber influido en los resultados comportamentales obtenidos. Cabe destacar en este punto una idea interesante sugerida

en los mamíferos, relacionada con el papel de la ghrelina en la ingestión del alimento y el gasto energético. La ghrelina podría desencadenar inicialmente una sensación de apetito, incrementando la actividad locomotora encaminada a la búsqueda de alimento y su ingestión; y posteriormente desempeñaría más una función de ahorro energético, suprimiendo gastos no esenciales, como la actividad física espontánea (Castañeda et al., 2005; Healy et al., 2011; Keen-Rhinehart y Bartness, 2005). Aunque esta posibilidad es interesante, en la actualidad no hay estudios que la avalen en los peces.

Otro punto innovador del presente trabajo ha sido el estudio del efecto de la ghrelina en el comportamiento agresivo en teleósteos. Esta primera aproximación se llevó a cabo en base a estudios previos realizados en los salmónidos que indicaban un comportamiento agresivo potenciado (dominancia, comportamientos de riesgo y agresión) tras el tratamiento con la GH (Johnsson, 2006; Jönsson et al., 1998; Jönsson y Björnsson, 2002; Neregård et al., 2008; Riley et al., 2005). En nuestro estudio, los peces tratados con ghrelina tienden a iniciar un mayor número de conflictos, aunque este comportamiento no se vea posteriormente reflejado en un incremento del número de victorias. Estos resultados están en armonía con la teoría de partida que sugiere una implicación de esta hormona en el comportamiento agresivo, posiblemente mediado por un incremento de la GH. En efecto, se ha demostrado en ambas truchas, común y arcoíris, un incremento de la agresividad tras el tratamiento con la GH, sin que esta mayor agresividad produzca como resultado un incremento de las habilidades competitivas, y por lo tanto un aumento del número de victorias (Jönsson et al., 1998; Neregård et al., 2008). Consideramos que estos resultados abren una nueva línea de estudio del papel de la ghrelina en el comportamiento animal, siendo de gran interés profundizar en los mecanismos implicados en los efectos de la ghrelina sobre el comportamiento agresivo en los peces.

3.2. Distribución tisular de la OEA en el carpín. Regulación por el estado nutricional

Los resultados obtenidos en el capítulo 4 de la presente Tesis Doctoral muestran, por primera vez en peces niveles endógenos de la OEA, tanto en los tejidos periféricos como en el encéfalo, indicando una amplia distribución de esta FAE en el carpín, concordante con los datos previos en los roedores (Fu et al., 2007). El contenido gastrointestinal (bulbo intestinal e intestino proximal) de OEA cuantificado en el carpín fue similar al observado en el duodeno y el estómago de rata (Fu et al., 2007), tejidos que podríamos considerar equivalentes teniendo en cuenta que *Carassius auratus* es un ciprínido, y como tal carece de estómago. Además, los cambios inducidos por el ayuno son

similares a los observados en el intestino delgado de los roedores y los ofidios (Fu et al., 2007; Petersen et al., 2006; Rodríguez de Fonseca et al., 2001). En los peces un ayuno de 48 h disminuye significativamente el contenido intestinal de la OEA, recuperándose los valores basales cuando los peces se realimentan. Es destacable la rapidez con la que se produce esta respuesta (~ 30 min), lo que junto al hecho de que los niveles de OEA son mayores a los 30 minutos que a los 120 minutos, sugiere que la OEA actúa como una señal rápida de saciedad. En los mamíferos la reducción de la OEA intestinal después de un ayuno es revertida rápidamente (10 min) por la realimentación (Fu et al., 2007). Con los datos actuales en peces no podemos confirmar que se esté produciendo una síntesis de OEA en el intestino en respuesta a la alimentación, ya que aún no se ha medido la actividad de las enzimas encargadas de su síntesis y degradación, como si se ha realizado en otros vertebrados (Astarita et al., 2006; Fu et al., 2007; Petersen et al., 2006; Rodríguez de Fonseca et al., 2001). En este sentido, nuevos experimentos encaminados al análisis de la actividad enzimática de la NAPE-PLD y la FAAH en los teleósteos serían muy interesantes en aras de despejar esta incógnita. Considerando que el precursor metabólico de la OEA es el ácido oleico, es importante señalar que la administración central o periférica de este ácido tiene un efecto anorético en la trucha, probablemente mediado por los sistemas sensores de ácidos grasos, a través de diferentes mecanismos relacionados con el metabolismo lipídico (Librán-Pérez et al., 2012, 2014). Por tanto, no puede descartarse que la movilización intestinal de la OEA encontrada en los peces esté siendo inducida por el ácido oleico derivado de la dieta, como se ha sugerido en los mamíferos (Piomelli, 2013).

A nivel periférico detectamos niveles de OEA también en el músculo y en el hígado del carpín, aunque únicamente se observó una respuesta al ayuno en el músculo, similar a la descrita a nivel intestinal. Estos resultados difieren de los observados en la rata, donde el ayuno provoca un incremento en el contenido de esta FAE en el hígado, el páncreas, el bazo y el tejido adiposo (Fu et al., 2007; Izzo et al., 2010), sin alteraciones significativas en el músculo (Fu et al., 2007). Esta discrepancia podría ser debida a diferencias interespecíficas, por ejemplo el hígado en los teleósteos tiene una importante función como reservorio de grasa relevando al tejido adiposo de esta función (Bianco et al., 2013; Mandrioli et al., 2012), presentando una respuesta lipogénica ante situaciones de ayuno (Pérez-Jiménez et al., 2012).

La expresión de OEA a nivel central muestra un patrón de distribución tisular similar al descrito en las ratas (Fu et al., 2007), con los mayores niveles en el tronco encefálico. Los datos obtenidos hasta la fecha en los roedores no mostraron

modificaciones cerebrales de la OEA tras el ayuno (Fu et al., 2007; Izzo et al., 2010), sugiriendo que la OEA no parece ser relevante en la regulación de la ingesta a nivel central. Resultados similares encontramos en el hipotálamo y el tronco encefálico del carpín, pero no en el telencéfalo, donde el contenido de OEA incrementa tras un ayuno de 48 horas. Esta respuesta al ayuno es aparentemente opuesta a la que cabría esperar de un regulador anorexigénico, como así se ha descrito en el carpín para otra FAE, la AEA (Valenti et al., 2005), regulador orexigénico tanto en los peces como en los mamíferos (Borrelli y Izzo, 2009; Valenti et al., 2005). Por tanto, a la espera de nuevos estudios hay que considerar que la OEA puede desempeñar otras funciones en el telencéfalo, tejido que en los teleósteos se ha relacionado con la regulación de la actividad locomotora, la memoria espacial, la búsqueda de alimento y el uso del hábitat (Lin et al., 2000; Wilson y McLaughlin, 2010). En los mamíferos, la OEA se ha relacionado también con la consolidación de la memoria, el estrés, el ciclo sueño-vigilia, el sistema circadiano y la viabilidad celular (Sarro-Ramírez et al., 2013).

3.3. Regulación de la ingesta y de la actividad locomotora por la OEA en el carpín

Este capítulo de la presente Tesis Doctoral aporta los primeros resultados sobre los posibles efectos de la administración exógena de OEA en peces. Hemos podido confirmar en el carpín la conocida acción anorexigénica de la OEA en los roedores (Fu et al., 2003; Gaetani et al., 2003; Nielsen et al., 2004; Oveisi et al., 2004; Proulx et al., 2005; Rodríguez de Fonseca et al., 2001; Serrano et al., 2011). En los mamíferos el efecto de la OEA aparece desde los primeros 20-30 min de su administración y se prolonga hasta las 2-3 horas posteriores (Cani et al., 2004; Serrano et al., 2011). En el carpín, la administración IP de OEA reduce la ingesta durante el intervalo 0-2 h post-tratamiento, pero no durante el intervalo siguiente de 2-6 h, sugiriendo que esta FAE actúa a tiempos cortos. De hecho, la disminución significativa observada en el intervalo acumulativo 0-8 h en el carpín es probablemente el resultado del marcado descenso de la ingesta que experimentan los peces durante las 2 primeras horas. Estos resultados confirman el papel de la OEA como factor de saciedad a tiempos cortos, discutido anteriormente. El efecto anorético de la OEA en los mamíferos parece depender de las condiciones de alimentación a las que están sometidos los animales. Así, en las ratas con libre acceso al alimento, el tratamiento con OEA retrasa el inicio de la ingesta, sin afectar al tamaño de la ingesta; mientras que en los animales en condiciones de ayuno de 24 horas la OEA además de retrasar el inicio de la ingestión de alimento, reduce también el tamaño de la ingesta (Gaetani et al., 2003).

Resulta por tanto muy interesante investigar en un futuro que parámetros del comportamiento alimentario en los peces son afectados específicamente por esta FAE.

Junto a la reducción de la ingesta, la administración de OEA también reduce la actividad locomotora total en el carpín, lo que nos plantea la siguiente cuestión, ¿puede haber una relación de dependencia entre ambos parámetros? Es decir, ¿un menor nivel de actividad repercute en una menor posibilidad de conseguir alimento?; y viceversa, ¿la menor ingestión de alimento puede determinar una menor actividad de los peces, a fin de disminuir el consumo de energía ante la restricción alimentaria? Aunque apenas existe información disponible al respecto, Rodríguez de Fonseca y colaboradores (2001) sugirieron que ambas respuestas a la administración de OEA son independientes en la rata, ya que ratas tratadas con capsaicina e inyectadas con OEA presentan una reducción de la actividad locomotora, sin alteraciones de la ingesta. Dicha disociación es posible que también exista en los peces, como se ha sugerido para otras hormonas anorexigénicas, como la melatonina (Azpeleta et al., 2010) y la leptina (Vivas et al., 2011) en el carpín. En ambos estudios se demostró que el efecto reductor de la actividad locomotra es independiente del efecto anorético producido por estas hormonas. Igualmente, datos previos en este teleósteo apoyan la existencia de una regulación independiente de ambos parámetros, la actividad locomotora y la actividad alimentaria (Sánchez-Vázquez et al., 1996).

El análisis de los parámetros metabólicos muestra una reducción de los niveles plasmáticos de triglicéridos tras la administración de OEA, lo que sugiere un efecto lipolítico similar al descrito previamente en los mamíferos (Pavón et al., 2010 Piomelli, 2013; Thabuis et al., 2010). La OEA produce en las ratas una disminución de los triglicéridos circulantes junto con un aumento de los ácidos grasos no esterificados y del glicerol (Fu et al., 2005; Guzmán et al., 2004). La reducción de los triglicéridos por la inyección de OEA en los peces se produjo tanto en los animales alimentados como en los que no recibieron comida, demostrando que la disminución de los triglicéridos circulantes no es una consecuencia directa de la reducción en la ingesta inducida por la OEA, como se ha propuesto en los mamíferos (Guzmán et al., 2004). Por último, como mencionamos anteriormente, no podemos descartar que los efectos de la OEA en la regulación lipídica puedan estar mediados, al menos en parte, por el oleato y su papel en la funcionalidad de los sistemas sensores de ácidos grasos, descritos en la trucha arcoíris (Librán-Pérez et al., 2012, 2014).

Las acciones de la OEA sobre el metabolismo de los glúcidos están menos definidas que en el caso de la regulación lipídica. Los escasos estudios disponibles muestran, por un

lado, una disminución de la captación de glucosa (estimulada por insulina) tras el tratamiento con OEA en cultivos de hepatocitos de rata (González-Yanes et al., 2005), pero no de adipocitos (Guzmán et al., 2004); y por otro, también en la rata, no se han encontrado modificaciones en la glucemia tras el tratamiento IP con OEA (Fu et al., 2005; Guzmán et al., 2004). Nuestros resultados en el carpín, donde la glucemia tampoco se modifica por el tratamiento IP con OEA, parecen indicar la falta de efecto de esta FAE, cuando es administrada perifericamente, sobre el metabolismo de los glúcidos. No obstante, con los datos existentes en la actualidad no es posible trazar una hipótesis clara acerca de la posible implicación de la OEA en este aspecto.

Actualmente desconocemos qué tipo de receptores están implicados en las acciones fisiológicas de la OEA en el carpín. Como posibles candidatos, dada su relevancia en la regulación de la ingesta en los mamíferos, cabe destacar a los receptores PPAR-α (Fu et al., 2003), TPRV-1 y GPR119 (Ahern, 2003; Almási et al., 2008; Overton et al., 2006). En los peces se ha demostrado la existencia de los tres tipos de receptores: PPAR, los subtipos α, β and γ (Fredriksson et al., 2003; Gau et al., 2013); TRPV1 y GPR119 (Carmona-Antoñanzas et al., 2014; Mimeault et al., 2006; Zheng et al., 2013), aunque por el momento no se tiene información sobre su posible funcionalidad en los peces y su relación con la OEA. En este contexto de búsqueda de mecanismos implicados en las acciones de la OEA, otro posible objetivo a dilucidar es la implicación del nervio vago, ya que se ha descrito un bloqueo por capsaicina del efecto anóretico de la OEA en la rata (Rodríguez de Fonseca et al., 2001).

Estos primeros resultados sobre los efectos de la OEA en peces han contribuido significativamente al estudio de esta FAE en un contexto filogenético que resulta de gran interés, teniendo en cuenta que únicamente se había estudiado hasta la fecha en roedores y en la pitón. Con los datos disponibles hasta el momento, podemos sugerir que las acciones de la OEA sobre el balance energético parecen estar altamente conservadas a lo largo de la filogenia de los vertebrados.

3.4. Interacciones ghrelina-OEA con otros reguladores de la alimentación en los peces

La regulación de la ingesta es el resultado de la interrelación e integración hipotalámica del conjunto de señales orexigénicas y anorexigénicas producidas por el organismo ante diferentes estímulos endógenos y/o ambientales. Así, la conducta alimentaria no se ve promovida o inhibida por la acción puntual de un determinado

neuropéptido u hormona, sino por el balance entre las actividades de numerosos reguladores, así como por las interacciones entre ellos (De Pedro y Björnsson, 2001; Gorissen et al., 2006; Hoskins y Volkoff et al., 2012; Volkoff et al., 2009a). Desde un punto de vista experimental es necesario, sin embargo, abordar en primer lugar cada factor regulador de la ingesta de forma independiente y, caracterizados sus efectos, estudiar las posibles interacciones con otros reguladores. Como se indicó en la introducción de esta Memoria, el NPY y las orexinas son importantes reguladores orexigénicos a nivel central en los peces (López-Patiño et al., 1999; Nakamachi et al., 2006; Narnaware et al., 2001; Volkoff et al., 2009a), formando parte de los circuitos neuronales utilizados por otros reguladores de la ingesta, siendo de forma general estimulados por reguladores orexigénicos e inhibidos por reguladores anorexigénicos (Hoskins y Volkoff et al., 2012; Volkoff et al., 2009a). En el caso concreto de la ghrelina son conocidas sus interacciones a nivel hipotalámico tanto con el NPY como con la orexina-A en el carpín (Miura et al., 2006, 2007) y con el NPY en el mero de pintas naranjas (Gao et al., 2012). El hecho de que en nuestro estudio la ghrelina estimule la ingesta sin modificaciones en la expresión génica de NPY hipotalámico sugiere que el efecto orexigénico de la ghrelina vía NPY podría ocurrir a nivel postraduccional o a través de otros modos de acción. Por ejemplo, Miura y colaboradores (2006) sugirieron en el carpín que la ghrelina puede modificar la liberación de NPY, sin implicar una estimulación de su síntesis, de acuerdo con trabajos previos de mamíferos (Shintani et al., 2001). En relación con la OEA, parece que ninguno de estos péptidos orexigénicos (NPY y orexinas) desempeñan un papel relevante como mediadores hipotalámicos del efecto anorético de esta FAE, resultado que concuerda con los datos obtenidos por Serrano y colaboradores (2011) en ratas, donde tampoco se encontraron modificaciones hipotalámicas de la expresión génica de NPY tras la administración de OEA.

La ghrelina también puede formar parte de los circuitos que utilizan otros reguladores de la ingesta como la melatonina, la MCH y la nesfatina-1 para inhibir la ingesta en peces (Jönsson, 2013), por lo que nos planteamos investigar la posible interacción entre la OEAy la ghrelina en el carpín. Nuestros resultados de disminución de los niveles de expresión de ghrelina en el bulbo intestinal a las 2 h del tratamiento con OEA sugieren que el efecto anorético de esta FAE puede implicar la reducción intestinal de esta señal orexigénica, y apoyan la existencia de un circuito OEA-ghrelina sugerido en los mamíferos (Cani et al., 2004; Serrano et al., 2011). No obstante, el estado energético del animal puede modular estas interacciones, ya que la disminución de los niveles plasmáticos de ghrelina tras la administración periférica de OEA se produce en ratas en ayuno durante 24 horas, pero no en ratas alimentadas (Cani et al., 2004; Serrano et al.,

2011), También en el carpín hemos encontrado que las modificaciones de ghrelina inducidas por la OEA pueden variar según el estado nutricional, observándose únicamente en peces alimentados, pero no en ayuno, similar a los resultados de Proulx y colaboradores (2005) que no encontraron cambios en la ghrelina circulante en ratas en ayuno. Otros factores a tener en cuenta en la interpretación de estos resultados son los derivados de las diferencias en los diseños experimentales. Por ejemplo, la forma de alimentación, *ad libitum* (Cani et al., 2004; Serrano et al., 2011) *versus* una vez al día (datos de la presente Tesis Doctoral); los tiempos de muestreo post-inyección, menores de 2 horas (Cani et al., 2004; Proulx et al., 2005) *versus* 2 y 6 horas (Serrano et al., 2011; datos de la presente Tesis Doctoral); la hora del día en que se realizan los tratamientos, al inicio de la fase luminosa (Serrano et al., 2011; datos de la presente Tesis Doctoral) *versus* inicio fase oscura (Proulx et al., 2005); o el tipo de análisis de ghrelina realizado, niveles circulantes (Cani et al., 2004; Proulx et al., 2005; Serrano et al., 2011) *versus* expresión génica (datos de la presente Tesis Doctoral). Por último, considerando las acciones adipogénicas de la ghrelina en mamíferos y peces, así como su efecto estimulador de la actividad locomotora (Jönsson, 2013; Kang et al., 2011a; Thabuis et al., 2008; resultados de la presente Tesis Doctoral), resulta tentador especular que la ghrelina puede mediar, al menos en parte, las acciones de la OEA en el metabolismo lipídico y la actividad locomotora descritas en la presente Tesis Doctoral.

La OEA y la CCK son dos reguladores anorexigénicos que se sintetizan a nivel intestinal en respuesta a la ingestión del alimento en los mamíferos y los teleósteos (Buffa et al., 1976; Fu et al., 2007; Himeno et al., 1983; Jönsson et al., 2006; Rodríguez de Fonseca et al., 2001; resultados de la presente Tesis Doctoral). Con el fin de estudiar si el efecto anorético de la OEA implica un incremento en la secreción gastrointestinal de CCK, analizamos la expresión de este neuropéptido en el bulbo intestinal de los carpines tratados con OEA. La ausencia de modificaciones en la expresión génica de CCK apoya la hipótesis de que la CCK probablemente no está involucrada en los efectos anoréticos de la OEA, como se ha descrito en las ratas (Proulx et al., 2005). Esta ausencia de interacción concuerda con la diferente forma en que ambos reguladores gastrointestinales actúan sobre el comportamiento alimentario en los mamíferos, donde la OEA induce saciedad principalmente por un retraso del inicio de la ingesta y prolongando el intervalo entre comidas; mientras que la CCK acelera el proceso de finalización de la misma, reduciendo la cantidad total de alimento ingerido (Gaetani et al., 2003).

Otra posible interacción funcional entre reguladores analizada en la presente Tesis Doctoral implica a la OEA y a la leptina, en base a su común participación en la regulación

de la homeostasis energética en los mamíferos (Grill, 2010; Henry y Clarke, 2008; Reidy y Weber, 2000). En los peces, la administración de leptina reduce la ingesta y la actividad locomotora e incrementa la lipolisis (Aguilar et al., 2010; Chisada et al., 2013; De Pedro et al., 2006; Murashita et al., 2008b; Vivas et al., 2011; Volkoff et al., 2003), efectos similares a los descritos en la presente Tesis Doctoral tras la administración periférica de OEA en el carpín. Sin embargo, estas acciones fisiológicas de la OEA no pueden atribuirse a una activación del sistema de la leptina, ya que no hemos encontrado modificaciones en la expresión de esta hormona ni en el hígado ni en el hipotálamo tras la administración de OEA en el carpín. Otros datos que no apoyan una posible mediación de la leptina en los efectos de la OEA se han obtenido en las ratas Zucker obesas que, aunque carecen de los receptores funcionales de la leptina, la OEA reduce su ingesta, el contenido hepático de lípidos y los niveles plasmáticos de colesterol y triglicéridos (Fu et al., 2005).

Entre los circuitos cerebrales que pueden mediar las acciones de la ghrelina y la OEA nos pareció interesante estudiar los sistemas monoaminérgicos, debido a su implicación tanto en la regulación de la ingesta como de la actividad locomotora en los peces (De Pedro et al., 1998, 1998b, 2001; Kuz'mina y Garina, 2013). Resultados previos en los mamíferos han demostrado modificaciones del sistema dopaminérgico por la ghrelina en el núcleo accumbens (Jerlhag, 2008; Jerlhag et al., 2006). Y en la trucha arcoíris se ha relacionado al sistema dopaminérgico con el incremento de la actividad natatoria y de la busqueda de alimento inducida por la GH (Johansson et al., 2004; Jönsson et al., 2003). Sin embargo, en la trucha común (datos de la presente Tesis Doctoral) no se han encontrado modificaciones significativas de la neurotransmisión noradrenérgica, dopaminérgica ni serotoninérgica por el tratamiento con ghrelina en ninguna de las regiones cerebrales estudiadas. A partir de la escasa información disponible en la actualidad, no es posible proponer una relación clara entre los efectos de la ghrelina en los peces y el sistema monoaminérgico cerebral, requiriéndose la realización de nuevos diseños experimentales para su clarificación.

En relación con las posibles interacciones entre la OEA y los sistemas de neurotransmisión monoaminérgicos, nuestros resultados en el carpín no apoyan esta posible relación a nivel hipotalámico, a diferencia de los resultados obtenidos por Serrano y colaboradores (2011) en la rata, donde la OEA incrementa el contenido hipotalámico de la NA y la DA. Entre ambos estudios hay diferencias metodológicas, como el tiempo post-inyección de muestreo (2 horas en el carpín *versus* 1 hora en la rata) y la dosis de OEA (5 mg/kg en el carpín *versus* 20 mg/kg en la rata), que podrían justificar los distintos resultados. Por otro lado, en el telencéfalo, tejido con una importante implicación en la

regulación de la ingesta y la actividad locomotora en peces (Lin et al., 2000; Wilson y McLaughlin, 2010), los incrementos encontrados de la NA, el ácido 5-hidroxiindol acético (5-HIAA) y la ratio 5-HT/5-HIAA en el carpín resultan muy interesantes. Si consideramos la acción inhibidora de la 5-HT en la ingesta y la actividad locomotora en peces (De Pedro et al., 1998b; Kuz'mina y Garina, 2013; Mennigen et al., 2009), cabe la posibilidad de que el efecto reductor de la OEA sobre estos comportamientos pueda estar mediado por el sistema serotoninérgico a nivel telencefálico. El incremento observado en la actividad serotoninérgica (ratio 5-HIAA/5-HT) telencefálica confirmaría dicha hipótesis en el carpín. Más difícil de explicar es el incremento telencefálico de la NA inducido por la administración de OEA observado en el carpín, teniendo presente el papel orexigénico de esta catecolamina a nivel hipotalámico (De Pedro et al., 1995a, 2001). Es posible que esta interacción entre la NA y la OEA esté relacionada con otras funciones de esta FAE, aunque estos aspectos aún no han sido investigados en este grupo de vertebrados. En los mamíferos por ejemplo se ha puesto de manifiesto que una activación noradrenérgica en la amígdala media la consolidación de la memoria inducida por OEA (Campolongo et al., 2009). Por último, cabe considerar que la interacción OEA-NA puede formar parte de circuitos cerebrales más complejos, como ha sido previamente sugerido por Romano y colaboradores (2013) en los mamíferos. Estos autores proponen que la OEA podría estimular la neurosecreción de oxitocina en el núcleo paraventricular a través de un incremento previo de la liberación de NA en el núcleo del tracto solitario, produciendo como resultado final una inhibición de la ingesta. El hecho de que las modificaciones en el sistema serotoninérgico y noradrenérgico encontradas en el telencéfalo se produzcan tanto en peces en ayuno como en alimentados nos permite descartar la posibilidad de que dichas alteraciones en la neurotransmisión monoaminérgica fueran una consecuencia de los cambios agudos en la ingestión de alimento que produce la OEA.

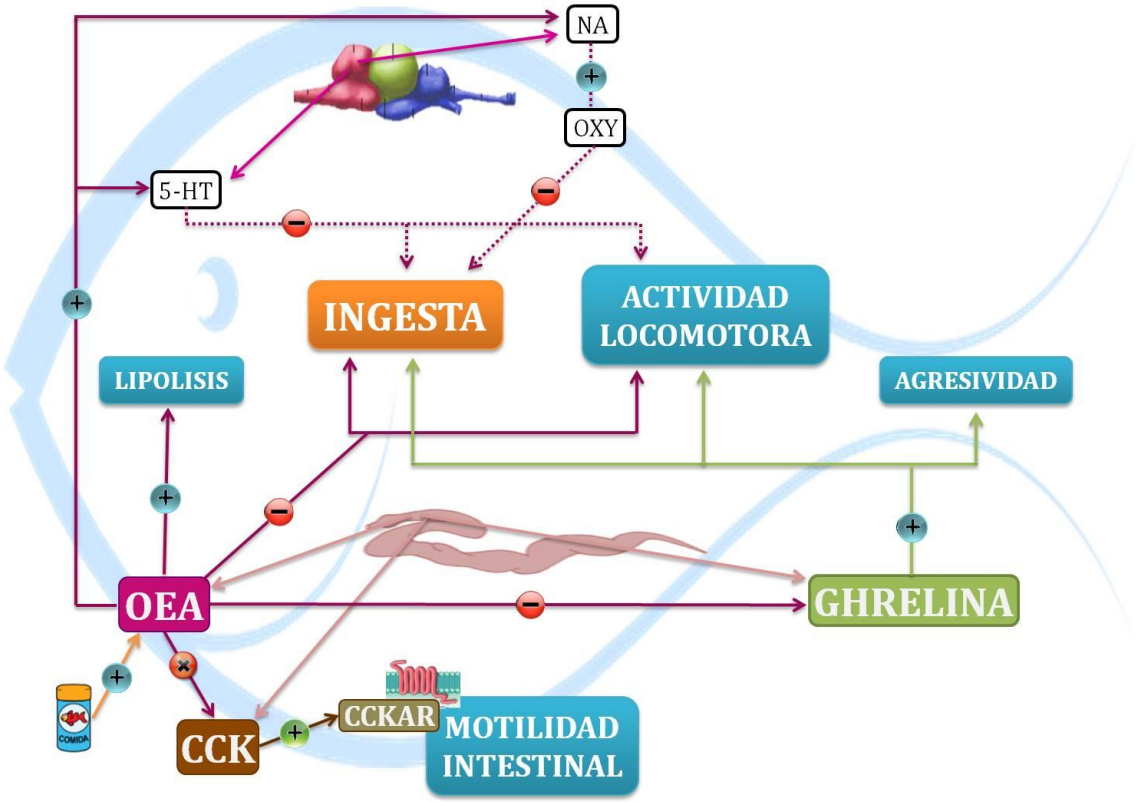

Figura 7. Representación esquemática de los principales resultados obtenidos en la presente Tesis Doctoral en relación con las acciones de la ghrelina, la colecistocinina y la OEA en los peces. Las líneas punteadas indican que esas acciones tienen lugar de manera indirecta. 5-HT: serotonina; CCK: colecistocinina; CCKAR: receptor de CCK del subtipo A; NA: noradrenalina; OEA: oleiletanolamida; OXY: oxitocina; ⊕: estimulación; ⊖: inhibición; ⊗: sin efecto.

VI. CONCLUSIONES/FINDINGS

A partir de los resultados obtenidos en la presente Tesis Doctoral proponemos las siguientes conclusiones:

1. La amplia distribución tisular encontrada para las leptinas y su receptor en el encéfalo y localizaciones periféricas del carpín sugiere que, además de su conocido papel en la homeostasis energética, pueden desempeñar otras funciones en este teleósteo. El diferente patrón tisular de expresión hallado para ambos parálogos, aI y aII, apunta a una especialización funcional de estas moléculas.

2. Los resultados mostrados en la presente Memoria no avalan una relación directa entre el sistema de la leptina (leptinas y receptor) y el estado nutricional del carpín, ni su funcionalidad como señal de adiposidad a largo plazo, al menos en relación a su expresión génica. Los cambios posprandiales observados en los niveles de expresión de la leptina-aI en el hígado del carpín indican que esta hormona puede intervenir en la regulación de la ingesta como señal de saciedad a corto plazo.

3. La expresión de leptina-aI en el hígado y el hipotálamo, y de leptina-aII en el hipotálamo del carpín presenta un perfil rítmico diario en carpines expuestos a un ciclo 12L/12D y alimentados diariamente a un horario fijo. Diversos factores ambientales (fotoperiodo y horario de alimentación) y/o endógenos (metabólicos) podrían estar implicados en la regulación del ritmo de expresión de la leptina en el carpín, sin que por el momento podamos definir su relevancia, pues en ausencia del ciclo L/D, el horario de alimentación por sí sólo no es capaz de sincronizar esta expresión rítmica.

4. En la regulación de la expresión de leptina intervienen mecanismos diferenciales en el encéfalo y en órganos periféricos, ya que el perfil del ritmo de expresión de esta hormona presenta un incremento posprandial en el hígado, pero no en el cerebro. Estos resultados corroboran la naturaleza pleiotrópica de esta hormona, que muy probablemente no sólo está implicada en funciones relacionadas con la alimentación.

5. El péptido CCK-8S induce en preparaciones *in vitro* de intestino proximal de carpín una respuesta contráctil dependiente de calcio, independiente del control colinérgico y del sistema entérico, tratándose posiblemente de una acción directa sobre células musculares. Las curvas concentración-respuesta obtenidas apoyan una función fisiológica para la CCK en la regulación de la motilidad del intestino proximal de este teleósteo.

6. El carpín posee, al menos, dos subtipos de receptores de CCK, denominados CCKAR y CCKBR, pertenecientes a la superfamilia de receptores acoplados a proteínas G con 7 dominios transmembrana. La distribución diferencial de estos subtipos (el CCKAR asociado al tracto intestinal, y el CCKBR con distribución amplia, pero preferentemente central), junto a los estudios farmacológicos revelan que las acciones contráctiles de la CCK en el intestino proximal del carpín parecen estar mediadas por el subtipo CCKAR.

7. La ghrelina es un potente regulador orexigénico en juveniles salvajes de trucha común, ya que su administración subcrónica periférica estimula la ingesta y la actividad relacionada con la búsqueda de alimento, generando una mayor tasa de crecimiento, sin alteraciones del metabolismo lipídico hepático y muscular. Esta acción orexigénica no parece estar mediada por cambios en la expresión de NPY en el hipotálamo, ni en el contenido encefálico de monoaminas.

8. El hecho de que las truchas tratadas con ghrelina sean más activas y tiendan a iniciar más conflictos que los individuos controles podría indicar una posible implicación de esta hormona en la regulación del comportamiento en esta especie, apuntando hacia un posible papel en el desarrollo de conductas agresivas.

9. Por primera vez en los peces se demuestra una distribución amplia (central y periférica) de la OEA, apoyando la relevancia de este mensajero lipídico endógeno en la filogenia de los vertebrados. Los niveles de OEA en el intestino del carpín son regulados por la alimentación, mostrando un rápido incremento posprandial que sugiere que esta molécula interviene en la regulación de la ingesta como señal rápida de saciedad.

10. La administración periférica de OEA en el carpín produce efectos anorexigénicos, lipolíticos e hipolocomotores, acciones que parecen estar mediadas por interacciones con otros reguladores, como la ghrelina y el sistema serotoninérgico. Estos resultados ponen de manifiesto que este mediador lipídico actúa como un regulador endógeno multifuncional también en teleósteos, y nos permiten proponer un alto grado de conservación funcional evolutiva de la OEA en la homeostasis energética.

From the results obtained in the present Doctoral Thesis we propose the following conclusions:

1. The widespread distribution of leptins and leptin receptor in the brain and peripheral tissues of goldfish suggests that, in addition to its well known role on energy homeostasis, leptin system may play pleiotropic actions in this teleost. The different tissue expression pattern observed for both leptins paralogs, aI and aII, points out the functional specialization of these leptins in goldfish.

2. The results shown in this Doctoral Thesis do not support a direct relationship between the leptin system (leptins and leptin receptor) expression and nutritional status in goldfish. Then, it should not be considered as an adiposity signal at long-term in this teleost. The postprandial changes of leptin-aI hepatic expression indicate that this hormone can signal short-term changes in food intake, as postprandial satiety signal.

3. Environmental (light/dark cycle and scheduled feeding) and endogenous (metabolic) signals are involved in the daily expression rhythm of leptin-aI in the liver and the hypothalamus, and leptin-aII in the hypothalamus of goldfish. Feeding time by itself is not able to synchronize this leptin daily rhythmic expression.

4. A postprandial increase of leptin expression occurs in the liver, but not in the brain, suggesting that different mechanisms regulate brain and hepatic leptin expression in goldfish. These results give support to the pleiotropic actions of leptin, which seems to be not only involved in feeding-related functions.

5. The CCK-8S peptide exerts a contractile response in an *in vitro* assay of isolated proximal intestine of goldfish. This effect is dependent of extracellular calcium, independent of cholinergic control, insensitive to tetrotodoxin, and then, probably occurs directly on smooth muscle cells. The concentration-response curves obtained support a physiological role of CCK in the regulation of proximal intestinal motility in this teleost.

6. Two CCK receptors paralogs, named CCKAR and CCKBR, belonging to the superfamily of G-protein coupled receptors that contain seven transmembrane domains, are presented in goldfish. Pharmacological approaches and the differential distribution of both CCK receptors (CCKAR is mainly expressed in gastrointestinal tract, while CCKBR is widely distributed, being primarily expressed in brain) support that the contractile effect of CCK-8S on the proximal intestine might be mediated by the CCKAR receptor subtype.

7. Ghrelin is a powerful orexigenic gut hormone in juvenile wild brown trout, since peripheral sub-chronic ghrelin administration strongly stimulates food intake and foraging activity. This orexigenic effect results in an increased growth, without any alteration in liver and muscle lipid metabolism, hypothalamic NPY expression, nor brain monoaminergic activity.

8. The foraging activity increase and the tendency to start more conflicts found in ghrelin-treated trouts, suggest a possible involvement of this hormone in the behavior regulation in this species, pointing to a possible role in the development of aggressive behaviors.

9. For the first time in fish, it has been reported a wide distribution (in brain and peripheral tissues) of endogenous OEA, supporting the relevance of this endogenous lipid messenger throughout vertebrate evolution. The intestinal OEA levels display a rapid postprandial increase, suggesting that this lipid amide can be involved in the short-term regulation of food intake in goldfish, as a rapid satiety signal.

10. Peripheral OEA administration produces anorexigenic, lipolytic effects and hipolocomotion in goldfish. These actions seem to be mediated by interactions with other regulators as ghrelin and serotoninergic system. These results reveal that this lipid mediator act as a multifunctional endogenous regulator in fish, and propose a high conservation of OEA actions in energy balance throughout vertebrate phylogeny.

VII. BIBLIOGRAFÍA/REFERENCES

Abbott, M., Volkoff, H. (2011). Thyrotropin Releasing Hormone (TRH) in goldfish (*Carassius auratus*): role in the regulation of feeding and locomotor behaviors and interactions with the orexin system and cocaine- and amphetamine regulated transcript (CART). *Horm Behav* **59**, 236-245.

Aguilar, A.J., Conde-Sieira, M., Polakof, S., Míguez, J.M., Soengas, J.L. (2010). Central leptin treatment modulates brain glucosensing function and peripheral energy metabolism of rainbow trout. *Peptides* **31**, 1044-1054.

Ahern, G.P. (2003). Activation of TRPV1 by the satiety factor oleoylethanolamide. *J Biol Chem* **278**, 30429-30434.

Ahmed, S., Harvey, S. (2002). Ghrelin: a hypothalamic GH-releasing factor in domestic fowl (*Gallus domesticus*). *J Endocrinol* **172**, 117-125.

Aldman, G., Holmgren, S. (1995). Intraduodenal fat and amino acids activate gallbladder motility in the rainbow trout, *Oncorhynchus mykiss*. *Gen Comp Endocrinol* **100**, 27-32.

Almasi, R., Szoke, E., Bolcskei, K., Varga, A., Riedl, Z., Sandor, Z., et al. (2008). Actions of 3-methyl-N-oleoyldopamine, 4-methyl-N-oleoyldopamine and N-oleoylethanolamide on the rat TRPV1 receptor in vitro and in vivo. *Life Sci* **82**, 644-651.

Amitani, M., Asakawa, A., Amitani, H., Inui, A. (2013). The role of leptin in the control of insulin-glucose axis. *Front Neurosci* **7**, 51. doi: 10.3389/fnins.2013.00051.

Angotzi, A.R., Stefansson, S.O., Nilsen, T.O., Rathore, R.M., Rønnestad, I. (2013). Molecular cloning and genomic characterization of novel Leptin-like genes in salmonids provide new insight into the evolution of the Leptin gene family. *Gen Comp Endocrinol* **187**, 48-59.

Arai, M., Assil, I.Q., Abou-Samra, A.B. (2001). Characterization of three corticotropin-releasing factor receptors in catfish: a novel third receptor is predominantly expressed in pituitary and urophysis. *Endocrinology* **142**, 446-454.

Aranda, A., Madrid, J.A., Sánchez-Vázquez, F.J. (2001). Influence of light on feeding anticipatory activity in goldfish. *J Biol Rhythms* **16**, 50-57.

Archer-Lahlou, E., Tikhonova, I., Escrieut, C., Dufresne, M., Seva, C., Pradayrol, L., et al. (2005). Modeled structure of a G-protein-coupled receptor: the cholecystokinin-1 receptor. *J Med Chem* **48**, 180-191.

Aschoff, J. (1981). A survey on biological rhythms. In: Biological Rhythms (Ed. Aschoff, J.), pp. 3-10. *Springer Plenum Press*, New York.

Astarita, G., Rourke, B.C., Andersen, J.B., Fu, J., Kim, J.H., Bennett, A.F., et al. (2006). Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (*Python molurus*). *Am J Physiol Regul Integr Comp Physiol* **290**, R1407-1412.

Azpeleta, C., Martínez-Álvarez, R.M., Delgado, M.J., Isorna, E., de Pedro, N. (2010). Melatonin reduces locomotor activity and circulating cortisol in goldfish. *Horm Behav* **57**, 323-329.

Baker, D.M., Larsen, D.A., Swanson, P., Dickhoff, W.W. (2000). Long-term peripheral treatment of immature coho salmon (*Oncorhynchus kisutch*) with human leptin has no clear physiologic effect. *Gen Comp Endocrinol* **118**, 134-138.

Baltzegar, D.A., Reading, B.J., Douros, J.D., Borski, R.J. (2014). Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. *J Endocrinol* **220**, 61-72.

Banks, W.A., Tschöp, M., Robinson, S.M., Heiman, M.L. (2002). Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* **302**, 822-827.

Bass, J., Takahashi, J.S. (2010). Circadian integration of metabolism and energetics. *Science* **330**, 1349-1354.

Behnke, R.J. (1986). Brown trout. *Trout* **27**, 42–47.

Bendiksen, E.Å., Jobling, M., Arnesen, A.M. (2002). Feed intake of Atlantic salmon parr *Salmo salar* L. in relation to temperature and feed composition. *Aquac Res* **33**, 525-532.

Berna, M.J., Tapia, J.A., Sancho, V., Jensen, R.T. (2007). Progress in developing cholecystokinin (CCK)/gastrin receptor ligands that have therapeutic potential. *Curr Opin Pharmacol* **7**, 583-592.

Bernier, N.J., Bedard, N., Peter, R.E. (2004). Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. *Gen Comp Endocrinol* **135**, 230-240.

Bernier, N.J., Gorissen, M., Flik, G. (2012). Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp. *J Exp Biol* **215**, 2273-2282.

Bernier, N.J., Peter, R.E. (2001a). Appetite-suppressing effects of urotensin I and corticotropin-releasing hormone in goldfish (*Carassius auratus*). *Neuroendocrinology* **73**, 248-260.

Bernier, N.J., Peter, R.E. (2001b). The hypothalamic-pituitary-interrenal axis and the control of food intake in teleost fish. *Comp Biochem Physiol B Biochem Mol Biol* **129**, 639-644.

Berthoud, H.R. (2006). Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance. *Obesity* **14**,1S-4S.

Bhatti, S.F., De Vliegher, S.P., Van Ham, L., Kooistra, H.S. (2002). Effects of growth hormone-releasing peptides in healthy dogs and in dogs with pituitary-dependent hyperadrenocorticism. *Mol Cell Endocrinol* **197**, 97-103.

Bianco, C., Sirri, R., Sarli, B., Ostanello, F., Bonaldo, A., de Vico, G., et al. (2013). How severe is the lipid accumulation in fish liver? A fractal answer. *Fractal Lab J* **2**, 3. ISSN: 2280-3769.

Blum, I.D., Patterson, Z., Khazall, R., Lamont, E.W., Sleeman, M.W., Horvath, T.L., et al. (2009). Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. *Neuroscience* **164**, 351-359.

Borrelli, F., Izzo, A.A. (2009). Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. *Best Pract Res Clin Endocrinol Metab* **23**, 33-49.

Boujard, T., Ramezi, J., Vandeputte, M., Labbé, L., Mambrini, M. (2007). Group feeding behavior of brown trout is a correlated response to selection for growth shaped by the environment. *Behav Genet* **37**, 525-534.

Brockmark, S., Johnsson, J.I. (2010). Reduced hatchery rearing density increases social dominance, postrelease growth, and survival in brown trout (*Salmo trutta*). *Can J Fish Aquatic Sci* **67**, 288-295.

Buffa, R., Solcia, E., Go, V.L. (1976). Immunohistochemical identification of the cholecystokinin cell in the intestinal mucosa. *Gastroenterology* **70**, 528-532.

Cadas, H., di Tomaso, E., Piomelli, D. (1997). Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci* **17**, 1226-1242.

Campolongo, P., Roozendaal, B., Trezza, V., Cuomo, V., Astarita, G., Fu, J., et al. (2009). Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. *Proc Natl Acad Sci U S A* **106**, 8027-8031.

Cani, P.D., Montoya, M.L., Neyrinck, A.M., Delzenne, N.M., Lambert, D.M. (2004). Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide. *Br J Nutr* **92**, 757-761.

Canosa, L.F., Peter, R.E. (2004). Effects of cholecystokinin and bombesin on the expression of preprosomatostatin-encoding genes in goldfish forebrain. *Regul Pept* **121**, 99-105.

Canosa, L.F., Unniappan, S., Peter, R.E. (2005). Periprandial changes in growth hormone release in goldfish: role of somatostatin, ghrelin, and gastrin-releasing peptide. *Am J Physiol Regul Integr Comp Physiol* **289**, R125-133.

Cao, Y.B., Xue, J.L., Wu, L.Y., Jiang, W., Hu, P.N., Zhu, J. (2011). The detection of 3 leptin receptor isoforms in crucian carp gill and the influence of fasting and hypoxia on their expression. *Domest Anim Endocrinol* **41**, 74-80.

Carlini, V.P., Monzon, M.E., Varas, M.M., Cragnolini, A.B., Schioth, H.B., Scimonelli, T.N., et al. (2002). Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem Biophys Res Commun* **299**, 739-743.

Carmona-Antoñanzas, G., Tocher, D.R., Martínez-Rubio, L., Leaver, M.J. (2014). Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene* **534**, 1-9.

Castañeda, T.R., Jürgens, H., Wiedmer, P., Pfluger, P., Diano, S., Horvath, T.L., et al. (2005). Obesity and the neuroendocrine control of energy homeostasis: the role of spontaneous locomotor activity. *J Nutr* **135**, 1314-1319.

Cerdá-Reverter, J.M., Agulleiro, M.J., R, R.G., Sánchez, E., Ceinos, R., Rotllant, J. (2011). Fish melanocortin system. *Eur J Pharmacol* **660**, 53-60.

Cerdá-Reverter, J.M., Anglade, I., Martínez-Rodríguez, G., Mazurais, D., Muñoz-Cueto, J.A., Carrillo, M., et al. (2000). Characterization of neuropeptide Y expression in the brain of a perciform fish, the sea bass (*Dicentrarchus labrax*). *J Chem Neuroanat* **19**, 197-210.

Cerdá-Reverter, J.M., Peter, R.E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology* **144**, 4552-4561.

Chisada, S.-I., Kurokawa, T., Murashita, K., Rønnestad, I., Taniguchi, Y., Toyoda, A., et al. (2013). Leptin receptor-deficient (knockout) medaka, *Oryzias latipes*, show chronical up-regulated levels of orexigenic neuropeptides, elevated food intake and stage specific effects on growth and fat allocation. *Gen Comp Endocrinol* **195**, 9-20.

Copeland, D.L., Duff, R.J., Liu, Q., Prokop, J., Londraville, R.L. (2011). Leptin in teleost fishes: an argument for comparative study. *Front Physiol* **2**, 26. doi: 10.3389/fphys.2011.00026.

Côté, C.D., Zadeh-Tahmasebi, M., Rasmussen, B.A., Duca, F.A., Lam, T.K. (2014). Hormonal signaling in the gut. *J Biol Chem* **289**, 11642-11649.

Cottone, E., Guastalla, A., Pomatto, V., Campantico, E., Palermo, F., Magni, A.M., et al. (2009). Interplay of the endocannabinoid system with neuropeptide Y and corticotropin-releasing factor in the goldfish forebrain. *Ann N Y Acad Sci* **1163**, 372-375.

Cowley, M.A., Smith, R.G., Diano, S., Tschöp, M., Pronchuk, N., Grove, K.L., et al. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* **37**, 649-661.

Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A., Gilula, N.B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**, 83-87.

Crespi, E.J. and Denver, R.J. (2006). Leptin (ob gene) of the South African clawed frog *Xenopus laevis*. *Proc Natl Acad Sci U S A* **103**, 10092-10097.

Cuesta, M., Clesse, D., Pevet, P., Challet, E. (2009). From daily behavior to hormonal and neurotransmitters rhythms: comparison between diurnal and nocturnal rat species. *Horm Behav* **55**, 338-347.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* **14**, 2950-2961.

Date, Y., Kojima, M., Hosoda, H., Sawaguchi, A., Mondal, M.S., Suganuma, T., et al. (2000). Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* **141**, 4255-4261.

Date, Y., Nakazato, M., Hashiguchi, S., Dezaki, K., Mondal, M.S., Hosoda, H., et al. (2002). Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* **51**, 124-129.

Dêbowski, P., Dobosz, S., Robak, S. (1999). Estimation of smoltification of hatchery-reared sea trout (*Salmo trutta morpha trutta* L.) based on body morphology. *Arch Pol Fish* **7**, 257–266.

De Pedro, N., Alonso-Gómez, A.L., Gancedo, B., Delgado, M.J., Alonso-Bedate, M. (1993). Role of corticotropin-releasing factor (CRF) as a food intake regulator in goldfish. *Physiol Behav* **53**, 517-520.

De Pedro, N., Alonso-Gómez, A.L., Gancedo, B., Valenciano, A.I., Delgado, M.J., Alonso-Bedate, M. (1997). Effect of α-helical-CRF[9-41] on feeding in goldfish: Involvement of cortisol and catecholamines. *Behav Neurosci* **111**, 398-403.

De Pedro, N., Björnsson, T. (2001). Regulation of food intake by neuropeptides and hormones. In: Food Intake in Fish (Eds. Houlihan, D., Boujard, T., Jobling, M.), pp. 267-296. *Blackwell Science Ltd*, Oxford.

De Pedro, N., Céspedes, M.V., Delgado, M.J., Alonso-Bedate, M. (1995a). The galanin-induced feeding stimulation is mediated via alpha 2-adrenergic receptors in goldfish. *Regul Pept* **57**, 77-84.

De Pedro, N., Delgado, M.J., Alonso-Bedate, M. (2001). Fasting and hypothalamic catecholamines in goldfish. *J Fish Biol* **58**, 1404-1413.

De Pedro, N., Delgado, M.J., Pinillos, M.L., Alonso-Bedate, M. (1998a). Alpha1-adrenergic and dopaminergic receptors are involved in the anoretic effect of corticotropin-releasing factor in goldfish. *Life Sci* **62**, 1801-1808.

De Pedro, N., Gancedo, B., Alonso-Gómez, A.L., Delgado, M.J., Alonso-Bedate, M. (1995b). CRF effect on thyroid function is not mediated by feeding behavior in goldfish. *Pharmacol Biochem Behav* **51**, 885-890.

De Pedro, N., López-Patiño, M.A., Guijarro, A.I., Pinillos, M.L., Delgado, M.J., Alonso-Bedate, M. (2000). NPY receptors and opioidergic system are involved in NPY-induced feeding in goldfish. *Peptides* **21**, 1495-1502.

De Pedro, N., Martínez-Álvarez, R., Delgado, M.J. (2006). Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). *J Endocrinol* **188**, 513-520.

De Pedro, N., Martínez-Álvarez, R.M., Delgado, M.J. (2008). Melatonin reduces body weight in goldfish (*Carassius auratus*): effects on metabolic resources and some feeding regulators. *J Pineal Res* **45**, 32-39.

De Pedro, N., Pinillos, M.L., Valenciano, A.I., Alonso-Bedate, M., Delgado, M.J. (1998b). Inhibitory effect of serotonin on feeding behavior in goldfish: involvement of CRF. *Peptides* **19**, 505-511.

Denver, R.J., Bonett, R.M., Boorse, G.C. (2011). Evolution of leptin structure and function. *Neuroendocrinology* **94**, 21-38.

Dockray, G.J. (2014). Gastrointestinal hormones and the dialogue between gut and brain. *J Physiol* **592**, 2927-2941.

Douros, J.D., Baltzegar, D.A., Breves, J.P., Lerner, D.T., Seale, A.P., Gordon Grau, E., et al. (2014). Prolactin is a major inhibitor of hepatic Leptin A synthesis and secretion: Studies utilizing a homologous Leptin A ELISA in the tilapia. *Gen Comp Endocrinol* **207**, 86-93.

Drover, V.A., Nguyen, D.V., Bastie, C.C., Darlington, Y.F., Abumrad, N.A., Pessin, J.E., et al. (2008). CD36 mediates both cellular uptake of very long chain fatty acids and their intestinal absorption in mice. *J Biol Chem* **283**, 13108-13115.

Dufresne, M., Seva, C., Fourmy, D. (2006). Cholecystokinin and gastrin receptors. *Physiol Rev* **86**, 805-847.

Dupré, D., Tostivint, H. (2013). Evolution of the gastrin-cholecystokinin gene family revealed by synteny analysis. *Gen Comp Endocrinol* **195**, 164-173.

Einarsson, S., Davies, P.S., Talbot, C. (1997). Effect of exogenous cholecystokinin on the discharge of the gallbladder and the secretion of trypsin and chymotrypsin from the pancreas of the Atlantic salmon, *Salmo salar* L. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **117**, 63-67.

Ellis, C., Moar, K.M., Logie, T.J., Ross, A.W., Morgan, P.J., Mercer, J.G. (2008). Diurnal profiles of hypothalamic energy balance gene expression with photoperiod manipulation in the Siberian hamster, *Phodopus sungorus*. *Am J Physiol Regul Integr Comp Physiol* **294**, R1148-1153.

Eom, J., Hong, M., Cone, R.D., Song, Y. (2013). Zebrafish ghrelin is expressed in pancreatic endocrine cells and regulated by metabolic state. *Biochem Biophys Res Commun* **439**, 115-120.

Falcón, J., Migaud, H., Munoz-Cueto, J.A., Carrillo, M. (2010). Current knowledge on the melatonin system in teleost fish. *Gen Comp Endocrinol* **165**, 469-482.

Feighner, S.D., Tan, C.P., McKee, K.K., Palyha, O.C., Hreniuk, D.L., Pong, S.S., et al. (1999). Receptor for motilin identified in the human gastrointestinal system. *Science* **284**, 2184-2188.

Feillet, C.A. (2010). Food for thoughts: feeding time and hormonal secretion. *J Neuroendocrinol* **22**, 620-628.

Feliciano, A., Vivas, Y., de Pedro, N., Delgado, M.J., Velarde, E., Isorna, E. (2011). Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (*Carassius auratus*). *J Biol Rhythms* **26**, 24-33.

Fleming, I.A., Agustsson, T., Finstad, B., Johnsson, J.I., Björnsson, B.T. (2002). Effects of domestication on growth physiology and endocrinology of Atlantic salmon (*Salmo salar*). *Can J Fish Aquat Sci* **59**, 1323-1330.

Forgan, L.G., Forster, M.E. (2007). Effects of potential mediators of an intestinal brake mechanism on gut motility in Chinook salmon (*Oncorhynchus tshawytscha*). *Comp Biochem Physiol C Toxicol Pharmacol* **146**, 343-347.

Foucaud, M., Archer-Lahlou, E., Marco, E., Tikhonova, I.G., Maigret, B., Escrieut, C., et al. (2008). Insights into the binding and activation sites of the receptors for cholecystokinin and gastrin. *Regul Pept* **145**, 17-23.

Fox, B.K., Riley, L.G., Dorough, C., Kaiya, H., Hirano, T., Grau, E.G. (2007). Effects of homologous ghrelins on the growth hormone/insulin-like growth factor-I axis in the tilapia, *Oreochromis mossambicus. Zool Sci* **24**, 391-400.

Fredriksson, R., Höglund, P.J., Gloriam, D.E., Lagerström, M.C., Schioth, H.B. (2003). Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives. *FEBS Lett* **554**, 381-388.

Friedman, J. (2014). 20 years of leptin: leptin at 20: an overview. *J Endocrinol* **223**, 1-8.

Frøiland, E., Murashita, K., Jørgensen, E.H., Kurokawa, T. (2010). Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. *Gen Comp Endocrinol* **165**, 136-143.

Frommelt, L., Lembke, V., Hofmann, T., Goebel-Stengel, M., Mönnikes, H., Wiedenmann, B., et al. (2013). The CCKB antagonist CI988 reduces food intake in fasted rats via a dopamine mediated pathway. *Peptides* **39**, 111-118.

Froy, O. (2011). The circadian clock and metabolism. *Clin Sci (Lond)* **120**, 65-72.

Frühbeck, G., Salvador, J. (2000). Relation between leptin and the regulation of glucose metabolism. *Diabetologia* **43**, 3-12.

Fu, J., Astarita, G., Gaetani, S., Kim, J., Cravatt, B.F., Mackie, K., et al. (2007). Food intake regulates oleoylethanolamide formation and degradation in the proximal small intestine. *J Biol Chem* **282**, 1518-1528.

Fu, J., Dipatrizio, N.V., Guijarro, A.I., Schwartz, G.J., Li, X., Gaetani, S., et al. (2011). Sympathetic activity controls fat-induced oleoylethanolamide signaling in small intestine. *J Neurosci* **31**, 5730-5736.

Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodríguez De Fonseca, F., et al. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* **425**, 90-93.

Fu, J., Oveisi, F., Gaetani, S., Lin, E., Piomelli, D. (2005). Oleoylethanolamide, an endogenous PPAR-alpha agonist, lowers body weight and hyperlipidemia in obese rats. *Neuropharmacology* **48**, 1147-1153.

Fuentes, E.N., Safian, D., Einarsdottir, I.E., Valdés, J.A., Elorza, A.A., Molina, A., et al. (2013). Nutritional status modulates plasma leptin, AMPK and TOR activation, and mitochondrial biogenesis: Implications for cell metabolism and growth in skeletal muscle of the fine flounder. *Gen Comp Endocrinol* **186**, 172-180.

Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Habata, Y., Hinuma, S., et al. (2000). Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. *J Biol Chem* **275**, 21068-21074.

Furutani, T., Masumoto, T., Fukada, H. (2013). Molecular cloning and tissue distribution of cholecystokinin-1 receptor (CCK-1R) in yellowtail *Seriola quinqueradiata* and its response to feeding and in vitro CCK treatment. *Gen Comp Endocrinol* **186**, 1-8.

Gaetani, S., Fu, J., Cassano, T., Dipasquale, P., Romano, A., Righetti, L., et al. (2010). The fat-induced satiety factor oleoylethanolamide suppresses feeding through central release of oxytocin. *J Neurosci* **30**, 8096-8101.

Gaetani, S., Oveisi, F., Piomelli, D. (2003). Modulation of meal pattern in the rat by the anorexic lipid mediator oleoylethanolamide. *Neuropsychopharmacology* **28**, 1311-1316.

Gao, Y.-J., Tian, L.-X., Yang, H.-J., Liang, G.-Y., Yue, Y.-R., Liu, Y.-J. (2012). The influence of ghrelin and des-ghrelin on feed intake, growth performance and hypothalamic NPY mRNA expression of grouper *Epinephelus coioides*. *Aquaculture* **364–365**, 19-24.

Gau, P., Poon, J., Ufret-Vincenty, C., Snelson, C.D., Gordon, S.E., Raible, D.W., et al. (2013). The zebrafish ortholog of TRPV1 is required for heat-induced locomotion. *J Neurosci* **33**, 5249-5260.

Gélineau, A., Boujard, T. (2001). Oral administration of cholecystokinin receptor antagonists increase feed intake in rainbow trout. *J Fish Biol* **58**, 716-724.

Giger, T., Excoffier, L., Day, P.J., Champigneulle, A., Hansen, M.M., Powell, R., et al. (2006). Life history shapes gene expression in salmonids. *Curr Biol* **16**, R281-282.

Glaser, S.S., Rodgers, R.E., Phinizy, J.L., Robertson, W.E., Lasater, J., Caligiuri, A., et al. (1997). Gastrin inhibits secretin-induced ductal secretion by interaction with specific receptors on rat cholangiocytes. *Am J Physiol* **273**, G1061-1070.

Gnanapavan, S., Kola, B., Bustin, S.A., Morris, D.G., McGee, P., Fairclough, P., et al. (2002). The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* **87**, 2988-2991.

Godlewski, G., Offertaler, L., Wagner, J.A., Kunos, G. (2009). Receptors for acylethanolamides-GPR55 and GPR119. *Prostag Oth Lipid M* **89**, 105-111.

Gómez Abellán, P., Gómez Santos, C., Madrid, J.A., Milagro, F.I., Campion, J., Martínez, J.A., et al. (2011). Site-specific circadian expression of leptin and its receptor in human adipose tissue. *Nutr Hosp* **26**, 1394-1401.

Gong, N., Einarsdottir, I.E., Johansson, M., Björnsson, B.T. (2013). Alternative splice variants of the rainbow trout leptin receptor encode multiple circulating leptin-binding proteins. *Endocrinology* **154**, 2331-2340.

Gong, Y., Luo, Z., Zhu, Q.L., Zheng, J.L., Tan, X.Y., Chen, Q.L., et al. (2013). Characterization and tissue distribution of leptin, leptin receptor and leptin

receptor overlapping transcript genes in yellow catfish *Pelteobagrus fulvidraco*. *Gen Comp Endocrinol* **182**, 1-6.

González-Yanes, C., Serrano, A., Bermúdez-Silva, F.J., Hernández-Dominguez, M., Páez-Ochoa, M.A., Rodríguez de Fonseca, F., et al. (2005). Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. *Am J Physiol Endocrinol Metab* **289**, E923-929.

Gorissen, M., Bernier, N.J., Manuel, R., de Gelder, S., Metz, J.R., Huising, M.O., et al. (2012). Recombinant human leptin attenuates stress axis activity in common carp (*Cyprinus carpio* L.). *Gen Comp Endocrinol* **178**, 75-81.

Gorissen, M., Bernier, N.J., Nabuurs, S.B., Flik, G., Huising, M.O. (2009). Two divergent leptin paralogues in zebrafish (*Danio rerio*) that originate early in teleostean evolution. *J Endocrinol* **201**, 329-339.

Gorissen, M., Flik, G., Huising, M. (2006). Peptides and proteins regulating food intake: a comparative view. *Anim Biol* **56**, 447-473.

Gregory, T.R., Wood, C.M. (1999). The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. *Physiol Biochem Zool* **72**, 286-295.

Grider, J.R. (1994). Role of cholecystokinin in the regulation of gastrointestinal motility. *J Nutr* **124**, 1334S-1339S.

Grill, H.J. (2010). Leptin and the systems neuroscience of meal size control. *Front Neuroendocrinol* **31**, 61-78.

Grove, D.J., Holmgren, S. (1992). Intrinsic mechanism controlling cardiac stomach volumen of the rainbow trout (*Oncorhynchus mykiss*) following gastric distension. *J Exp Biol* **163**, 49-63.

Guan, X.M., Yu, H., Palyha, O.C., McKee, K.K., Feighner, S.D., Sirinathsinghji, D.J., et al. (1997). Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* **48**, 23-29.

Guijarro, A.I., Delgado, M.J., Pinillos, M.L., López-Patiño, M.A., Alonso-Bedate, M., de Pedro, N. (1999). Galanin and β-endorphin as feeding regulators in cyprinids: effect of temperature. *Aquac Res* **30**, 483-489.

Guijarro, A.I., de Pedro, N., Martínez-Álvarez, R.M., Isorna, E., López-Patiño, M.A., Alonso-Gómez, A.L. et al. (2006). Ghrelin, leptin and neuropeptide Y in the cyprinid *Tinca tinca*. Daily and seasonal changes. *International 5th Symposium on Fish Endocrinology* (Castellón). P38.

Guijarro, A.I., Fu, J., Astarita, G., Piomelli, D. (2010). CD36 gene deletion decreases oleoylethanolamide levels in small intestine of free-feeding mice. *Pharmacol Res* **61**, 27-33.

Guilmeau, S., Buyse, M., Bado, A. (2004). Gastric leptin: a new manager of gastrointestinal function. *Curr Opin Pharmacol* **4**, 561-566.

Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., et al. (2008). Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci USA* **105**, 6320-6325.

Guzmán, M., Lo Verme, J., Fu, J., Oveisi, F., Blázquez, C., Piomelli, D. (2004). Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-alpha). *J Biol Chem* **279**, 27849-27854.

Hanson, K.C., Abizaid, A., Cooke, S.J. (2009). Causes and consequences of voluntary anorexia during the parental care period of wild male smallmouth bass (*Micropterus dolomieu*). *Horm Behav* **56**, 503-509.

He, S., Liang, X.F., Li, L., Huang, W., Shen, D., Tao, Y.X. (2013). Gene structure and expression of leptin in Chinese perch. *Gen Comp Endocrinol* **194**, 183-188.

Healy, J.E., Bateman, J.L., Ostrom, C.E., Florant, G.L. (2011). Peripheral ghrelin stimulates feeding behavior and positive energy balance in a sciurid hibernator. *Horm Behav* **59**, 512-519.

Hebb, A.L., Poulin, J.F., Roach, S.P., Zacharko, R.M., Drolet, G. (2005). Cholecystokinin and endogenous opioid peptides: interactive influence on pain, cognition, and emotion. *Prog Neuropsychopharmacol Biol Psychiatry* **29**, 1225-1238.

Henderson, R.J., Tocher, D.R. (1987). The lipid composition and biochemistry of freshwater fish. *Prog Lipid Res* **26**, 281-347.

Hendry, A.P., Bohlin, T., Jonsson, B., Berg, O. (2003) To sea or not to sea? Anadromy versus non-anadromy in salmonids. In: Evolution illuminated: salmon and their relatives (Eds. Hendry, A.P., Stearns, S.C.), pp. 92-125. *Oxford University Press*, Oxford.

Henry, B.A. and Clarke, I.J. (2008). Adipose tissue hormones and the regulation of food intake. *J Neuroendocrinol* **20**, 842-849.

Hevrøy, E.M., Waagbø, R., Torstensen, B.E., Takle, H., Stubhaug, I., Jørgensen, S.M., et al. (2012). Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. *Gen Comp Endocrinol* **175**, 118-134.

Higgins, S.C., Gueorguiev, M., Korbonits, M. (2007). Ghrelin, the peripheral hunger hormone. *Ann Med* **39**, 116-136.

Hill, M.N., Miller, G.E., Carrier, E.J., Gorzalka, B.B., Hillard, C.J. (2009). Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. *Psychoneuroendocrinology* **34**, 1257-1262.

Himeno, S., Tarui, S., Kanayama, S., Kuroshima, T., Shinomura, Y., Hayashi, C., et al. (1983). Plasma cholecystokinin responses after ingestion of liquid meal and intraduodenal infusion of fat, amino acids, or hydrochloric acid in man: analysis with region specific radioimmunoassay. *Am J Gastroenterol* **78**, 703-707.

Himick, B.A., Golosinski, A.A., Jönsson, A.C., Peter, R.E. (1993). CCK/gastrin-like immunoreactivity in the goldfish pituitary: regulation of pituitary hormone secretion by CCK-like peptides *in vitro*. *Gen Comp Endocrinol* **92**, 88-103.

Himick, B.A., Peter, R.E. (1994a). Bombesin acts to suppress feeding behavior and alter serum growth hormone in goldfish. *Physiol Behav* **55**, 65-72.

Himick, B.A., Peter, R.E. (1994b). CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. *Am J Physiol* **267**, 841-851.

Hoegg, S., Brinkmann, H., Taylor, J.S., Meyer, A. (2004). Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J Mol Evol* **59**, 190-203.

Holmberg, A., Olsson, C., Holmgren, S. (2006). The effects of endogenous and exogenous nitric oxide on gut motility in zebrafish *Danio rerio* embryos and larvae. *J Exp Biol* **209**, 2472-2479.

Holmberg, A., Schwerte, T., Pelster, B., Holmgren, S. (2004). Ontogeny of the gut motility control system in zebrafish *Danio rerio* embryos and larvae. *J Exp Biol* **207**, 4085-4094.

Holmgren, S. (1983). The effects of putative non-adrenergic, non-cholinergic autonomic transmitters on isolated strips from the stomach of the rainbow trout. *Salmo gairdneri*. *Comp Biochem Physiol C* **74**, 229-238.

Hoskins, L.J., Volkoff, H. (2012). The comparative endocrinology of feeding in fish: insights and challenges. *Gen Comp Endocrinol* **176**, 327-335.

Hosoya, M., Moriya, T., Kawamata, Y., Ohkubo, S., Fujii, R., Matsui, H., et al. (2000). Identification and functional characterization of a novel subtype of neuromedin U receptor. *J Biol Chem* **275**, 29528-29532.

Howard, A.D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., et al. (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273**, 974-977.

Huang, G., Li, J., Wang, H., Lan, X., Wang, Y. (2014). Discovery of a novel functional leptin protein (LEP) in zebra finches: evidence for the existence of an authentic avian leptin gene predominantly expressed in the brain and pituitary. *Endocrinology* **155**, 3385-3396.

Huising, M.O., Geven, E.J., Kruiswijk, C.P., Nabuurs, S.B., Stolte, E.H., Spanings, F.A., et al. (2006). Increased leptin expression in common Carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. *Endocrinology* **147**, 5786-5797.

Huntingford, F.A. (2004). Implications of domestication and rearing conditions for the behaviour of cultivated fishes. *Journal of Fish Biology* **65**, 122-142.

Iigo, M., Tabata, M. (1996). Circadian rhythms of locomotor activity in the goldfish *Carassius auratus*. *Physiol Behav* **60**, 775-781.

Izzo, A.A., Piscitelli, F., Capasso, R., Marini, P., Cristino, L., Petrosino, S., et al. (2010). Basal and fasting/refeeding-regulated tissue levels of endogenous PPAR-alpha ligands in Zucker rats. *Obesity* **18**, 55-62.

Janzen, W.J., Duncan, C.A., Riley, L.G. (2012). Cortisol treatment reduces ghrelin signaling and food intake in tilapia, *Oreochromis mossambicus*. *Domest Anim Endocrinol* **43**, 251-259.

Jensen, J., Karila, P., Jönsson, A.C., Aldman, G., Holmgren, S. (1993). Effects of substance P and distribution of substance P-like immunoreactivity in nerves supplying the stomach of the cod, *Gadus morhua*. *Fish Physiol Biochem* **12**, 237-247.

Jensen, H., Rourke, I.J., Møller, M., Jønson, L., Johnsen, A.H. (2001). Identification and distribution of CCK-related peptides and mRNAs in the rainbow trout, *Oncorhynchus mykiss*. *Biochim Biophys Acta* **1517**, 190-201.

Jerlhag, E. (2008). Systemic administration of ghrelin induces conditioned place preference and stimulates accumbal dopamine. *Addict Biol* **13**, 358-363.

Jerlhag, E., Egecioglu, E., Dickson, S.L., Andersson, M., Svensson, L., Engel, J.A. (2006). Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward. *Addict Biol* **11**, 45-54.

Johnson, C.H., Golden, S.S., Ishiura, M., Kondo, T. (1996). Circadian clocks in prokaryotes. *Mol Microbiol* **21**, 5-11.

Johnsson, J.I., Nöbbelin, F., Bohlin, T. (1999). Territorial competition among wild brown trout fry: effects of ownership and body size. *J Fish Biol* **54**, 469-472.

Johnsson, J.I., Winberg, S., Sloman, K.A. (2006). Social interactions. In: Fish Physiology, Behaviour and Physiology of Fish (Eds. Sloman, K.A., Wilson, R.W., Balshine, S.), Vol. 24, pp. 151–196. *Elsevier Academic Press*, New York.

Jönsson, A.C., Holmgren, S., Holstein, B. (1987). Gastrin/CCK-like immunoreactivity in endocrine cells and nerves in the gastrointestinal tract of the cod, *Gadus morhua*, and the effect of peptides of the gastrin/CCK family on cod gastrointestinal smooth muscle. *Gen Comp Endocrinol* **66**, 190-202.

Jonsson, B. (1982). Diadromous and resident trout *Salmo trutta*: is their difference due to genetics? *Oikos* **38**, 297–300.

Jönsson, E. (2013). The role of ghrelin in energy balance regulation in fish. *Gen Comp Endocrinol* **187**, 79-85.

Jönsson, E., Björnsson, B.T. (2002). Physiological functions of growth hormone in fish with special reference to its influence on behaviour. *Fish Sci* **68** Suppl 1, 742-748.

Jönsson, E., Forsman, A., Einarsdottir, I.E., Egnér, B., Ruohonen, K., Björnsson, B.T. (2006). Circulating levels of cholecystokinin and gastrin-releasing peptide in rainbow trout fed different diets. *Gen Comp Endocrinol* **148**, 187-194.

Jönsson, E., Forsman, A., Einarsdottir, I.E., Kaiya, H., Ruohonen, K., Björnsson, B.T. (2007). Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. *Comp Biochem Physiol A Mol Integr Physiol* **147**, 1116-1124.

Jönsson, E., Johnsson, J.I., Björnsson, B.T. (1998). Growth hormone increases aggressive behavior in juvenile rainbow trout. *Horm Behav* **33**, 9-15.

Jönsson, E., Kaiya, H., Björnsson, B.T. (2010). Ghrelin decreases food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) through the central anorexigenic corticotropin-releasing factor system. *Gen Comp Endocrinol* **166**, 39-46.

Kaiya, H., Kangawa, K., Miyazato, M. (2013a). Ghrelin receptors in non-mammalian vertebrates. *Front Endocrinol* **4**, 81. doi: 10.3389/fendo.2013.00081.

Kaiya, H., Kangawa, K., Miyazato, M. (2013b). What is the general action of ghrelin for vertebrates?-comparisons of ghrelin's effects across vertebrates. *Gen Comp Endocrinol* **181**, 187-191.

Kaiya, H., Miura, T., Matsuda, K., Miyazato, M., Kangawa, K. (2010). Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2a in goldfish, *Carassius auratus*. *Mol Cell Endocrinol* **327**, 25-39.

Kaiya, H., Miyazato, M., Kangawa, K. (2011). Recent advances in the phylogenetic study of ghrelin. *Peptides* **32**, 2155-2174.

Kaiya, H., Miyazato, M., Kangawa, K., Peter, R.E., Unniappan, S. (2008). Ghrelin: a multifunctional hormone in non-mammalian vertebrates. *Comp Biochem Physiol A Mol Integr Physiol* **149**, 109-128.

Kaiya, H., Mori, T., Miyazato, M., Kangawa, K. (2009a). Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*. *Comp Biochem Physiol A Mol Integr Physiol* **153**, 438-450.

Kaiya, H., Riley, L.G., Janzen, W., Hirano, T., Grau, E.G., Miyazato, M., et al. (2009b). Identification and genomic sequence of a ghrelin receptor (GHS-R)-like receptor in the Mozambique tilapia, *Oreochromis mossambicus*. *Zool Sci* **26**, 330-337.

Kallio-Nyberg, I., Jutila, E., Koljonen, M.-L., Koskiniemi, J., Saloniemi, I. (2010). Can the lost migratory *Salmo trutta* stocks be compensated with resident trout stocks in coastal rivers? *Fish Res* **102**, 69-79.

Kalra, S.P., Bagnasco, M., Otukonyong, E.E., Dube, M.G., Kalra, P.S. (2003). Rhythmic, reciprocal ghrelin and leptin signaling: new insight in the development of obesity. *Regul Pept* **111**, 1-11.

Kalsbeek, A., Fliers, E., Romijn, J.A., La Fleur, S.E., Wortel, J., Bakker, O., et al. (2001). The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels. *Endocrinology* **142**, 2677-2685.

Kalsbeek, A., Ruiter, M., La Fleur, S.E., Van Heijningen, C., Buijs, R.M. (2003). The diurnal modulation of hormonal responses in the rat varies with different stimuli. *J. Neuroendocrinology* **15**, 1144-1155.

Kalsbeek, A., Scheer, F.A., Perreau-Lenz, S., La Fleur, S.E., Yi, C.X., Fliers, E., et al. (2011). Circadian disruption and SCN control of energy metabolism. *FEBS Lett* **585**, 1412-1426.

Kamisaka, Y., Drivenes, O., Kurokawa, T., Tagawa, M., Rønnestad, I., Tanaka, M., et al. (2005). Cholecystokinin mRNA in Atlantic herring, *Clupea harengus*-molecular cloning, characterization, and distribution in the digestive tract during the early life stages. *Peptides* **26**, 385-393.

Kang, K.S., Yahashi, S., Azuma, M., Matsuda, K. (2010). The anorexigenic effect of cholecystokinin octapeptide in a goldfish model is mediated by the vagal afferent and subsequently through the melanocortin- and corticotropin-releasing hormone-signaling pathways. *Peptides* **31**, 2130-2134.

Kang, K.S., Yahashi, S., Matsuda, K. (2011a). Central and peripheral effects of ghrelin on energy balance, food intake and lipid metabolism in teleost fish. *Peptides* **32**, 2242-2247.

Kang, K.S., Yahashi, S., Matsuda, K. (2011b). The effects of ghrelin on energy balance and psychomotor activity in a goldfish model: an overview. *Int J Pept* **2011**, Article ID 171034, 9 pages. doi: 10.1155/2011/171034.

Kapoor, B.G., Smit, H., Verighina, I.A. (1975). The alimentary canal and digestion in teleost. *Adv Mar Biol* **13**, 109-230.

Karakas, A., Camsari, C., Serin, E., Gündüz, B. (2005). Effects of photoperiod and food availability on growth, leptin, sexual maturation and maintenance in the Mongolian gerbils (*Meriones unguiculatus*). *Zool Sci* **22**, 665-670.

Karila, P., Holmgren, S. (1995). Enteric reflexes and nitric oxide in the fish intestine. *J Exp Biol* **198**, 2405-2412.

Karila, P., Jönsson, A.C., Jensen, J., Holmgren, S. (1993). Galanin-like immunoreactivity in extrinsic and intrinsic nerves to the gut of the Atlantic cod, *Gadus morhua*, and the effect of galanin on the smooth muscle of the gut. *Cell Tissue Res* **271**, 537-544.

Kaslin, J., Nystedt, J.M., Östergård, M., Peitsaro, N., Panula, P. (2004). The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. *J Neurosci* **24**, 2678-2689.

Keen-Rhinehart, E. and Bartness, T.J. (2005). Peripheral ghrelin injections stimulate food intake, foraging, and food hoarding in Siberian hamsters. *Am J Physiol Regul Integr Comp Physiol* **288**, R716-722.

Kehoe, A.S., Volkoff, H. (2007). Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol A Mol Integr Physiol* **146**, 451-461.

Kehoe, A.S., Volkoff, H. (2008). The effects of temperature on feeding and expression of two appetite-related factors, neuropeptide Y and cocaine- and amphetamine-regulated transcript, in Atlantic cod, *Gadus morhua*. *J World Aquac Soc* **39**, 790-796.

Kelly, S.P., Peter, R.E. (2006). Prolactin-releasing peptide, food intake, and hydromineral balance in goldfish. *Am J Physiol Regul Integr Comp Physiol* **291**, R1474-1481.

Kestemont, P., Baras, E. (2001). Environmental factors and feed intake: mechanisms and interactions. In: Food Intake in Fish (Eds. Houlihan, D., Boujard, T., Jobling, M.), pp. 131-156. *Blackwell Science Ltd*, Oxford.

Klemetsen, A., Amundsen, P.A., Dempson, J.B., Jonsson, B., Jonsson, N., O'Connell, M.F., et al. (2003). Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* L.: a review of aspects of their life histories. *Ecol Freshw Fish* **12**, 1-59.

Kobayashi, Y., Quiniou, S., Booth, N.J., Peterson, B.C. (2011). Expression of leptin-like peptide (LLP) mRNA in channel catfish (*Ictalurus punctatus*) is induced by exposure to *Edwardsiella ictaluri* but is independent of energy status. *Gen Comp Endocrinol* **173**, 411-418.

Koethe, D., Schreiber, D., Giuffrida, A., Mauss, C., Faulhaber, J., Heydenreich, B., et al. (2009). Sleep deprivation increases oleoylethanolamide in human cerebrospinal fluid. *J Neural Transm* **116**, 301-305.

Kojima, M., Haruno, R., Nakazato, M., Date, Y., Murakami, N., Hanada, R., et al. (2000). Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66 (FM3). *Biochem Biophys Res Commun* **276**, 435-438.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656-660.

Kojima, M,. Kangawa, K. (2005). Ghrelin: structure and function. *Physiol Rev* **85**, 495-522.

Kornmann, B., Schaad, O., Reinke, H., Saini, C., Schibler, U. (2007). Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb Symp Quant Biol* **72**, 319-330.

Kottelat, M., Freihof, J. (2007). Handbook of european freshwater fishes. (Eds. *Kottelat, M.,* Freihof, J.), pp.78-148 *Publications Kottelat*, Cornol.

Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., et al. (2013). The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). *Comp Biochem Physiol A Mol Integr Physiol* **164**, 44-53.

Kurokawa, T., Murashita, K. (2009). Genomic characterization of multiple leptin genes and a leptin receptor gene in the Japanese medaka, *Oryzias latipes*. *Gen Comp Endocrinol* **161**, 229-237.

Kurokawa, T., Murashita, K., Suzuki, T., Uji, S. (2008). Genomic characterization and tissue distribution of leptin receptor and leptin receptor overlapping transcript genes in the pufferfish*, Takifugu rubripes*. *Gen Comp Endocrinol* **158**, 108-114.

Kurokawa, T., Suzuki, T., Hashimoto, H. (2003). Identification of gastrin and multiple cholecystokinin genes in teleost. *Peptides* **24**, 227-235.

Kurokawa, T., Uji, S., Suzuki, T. (2005). Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. *Peptides* **26**, 745-750.

Kuz'mina, V.V., Garina, D.V. (2013). The effects of peripherally injected serotonin on feeding and locomotor activities in carp *Cyprinus carpio* L. *Inland Water Biol* **6**, 62-69.

Lacourse, K.A., Lay, J.M., Swanberg, L.J., Jenkins, C., Samuelson, L.C. (1997). Molecular structure of the mouse CCK-A receptor gene. *Biochem Biophys Res Commun* **236**, 630-635.

Lall, S.P., Tibbetts, S.M. (2009). Nutrition, feeding, and behavior of fish. *Vet Clin North Am Exot Anim Pract* **12**, 361-372.

Lamont, E.W., Bruton, J., Blum, I.D., Abizaid, A. (2013). Ghrelin receptor-knockout mice display alterations in circadian rhythms of activity and feeding under constant lighting conditions. *Eur J Neurosci* **39**, 207-217.

Lay, J.M., Jenkins, C., Friis-Hansen, L., Samuelson, L.C. (2000). Structure and developmental expression of the mouse CCK-B receptor gene. *Biochem Biophys Res Commun* **272**, 837-842.

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D.W., Silver, R. (2009). Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc Natl Acad Sci USA* **106**, 13582-13587.

Li, A.J., Wiater, M.F., Oostrom, M.T., Smith, B.R., Wang, Q., Dinh, T.T., et al. (2012). Leptin-sensitive neurons in the arcuate nuclei contribute to endogenous feeding rhythms. *Am J Physiol Regul Integr Comp Physiol* **302**, R1313-1326.

Li, G.G., Liang, X.F., Xie, Q., Li, G., Yu, Y., Lai, K. (2010). Gene structure, recombinant expression and functional characterization of grass carp leptin. *Gen Comp Endocrinol* **166**, 117-127.

Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M.A., Míguez, J.M., Soengas, J.L. (2014). Central administration of oleate or octanoate activates hypothalamic fatty acid sensing and inhibits food intake in rainbow trout. *Physiol Behav* **129**, 272-279.

Librán-Pérez, M., Polakof, S., López-Patiño, M.A., Míguez, J.M., Soengas, J.L. (2012). Evidence of a metabolic fatty acid-sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. *Am J Physiol Regul Integr Comp Physiol* **302**, R1340-1350.

Liddle, R.A., Goldfine, I.D., Rosen, M.S., Taplitz, R.A., Williams, J.A. (1985). Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *J Clin Invest* **75**, 1144-1152.

Lin, X., Volkoff, H., Narnaware, Y., Bernier, N.J., Peyon, P., Peter, R.E. (2000). Brain regulation of feeding behavior and food intake in fish. *Comp Biochem Physiol A Mol Integr Physiol* **126**, 415-434.

Little, T.J., Horowitz, M., Feinle-Bisset, C. (2007). Modulation by high-fat diets of gastrointestinal function and hormones associated with the regulation of energy intake: implications for the pathophysiology of obesity. *Am J Clin Nutr* **86**, 531-541.

Liu, Q., Chen, Y., Copeland, D., Ball, H., Duff, R.J., Rockich, B., et al. (2010). Expression of leptin receptor gene in developing and adult zebrafish. *Gen Comp Endocrinol* **166**, 346-355.

Lo Verme, J., Gaetani, S., Fu, J., Oveisi, F., Burton, K., Piomelli, D. (2005). Regulation of food intake by oleoylethanolamide. *Cell Mol Life Sci* **62**, 708-716.

Londraville, R.L., Duvall, C.S. (2002). Murine leptin injections increase intracellular fatty acid-binding protein in green sunfish (*Lepomis cyanellus*). *Gen Comp Endocrinol* **129**, 56-62.

Londraville, R.L., Macotela, Y., Duff, R.J., Easterling, M.R., Liu, Q., Crespi, E.J. (2014). Comparative endocrinology of leptin: Assessing function in a phylogenetic context. *Gen Comp Endocrinol* 203, 146-157.

López, A., Rol de Lama, M.A., Madrid, J.A. (2006). Ritmos biológicos en la nutrición y el metabolismo. En: Cronobiología basica y clínica (Eds. Madrid, J.A., Rol de Lama, A.), pp. 514-553. *Editec@red*, Madrid.

López-Olmeda, J.F., Madrid, J.A., Sánchez-Vázquez, F.J. (2006). Melatonin effects on food intake and activity rhythms in two fish species with different activity patterns: Diurnal (goldfish) and nocturnal (tench). *Comp Biochem Physiol A Mol Integr Physiol* **144**, 180-187.

López-Olmeda, J.F. and Sánchez-Vázquez, F.J. (2010). Feeding rhythms in fish: from behavioral to molecular approach. In: Biological Clock in Fish (Eds. Kulczykowska, E., Popek, W., Kapoor B.G.), pp. 155-183. *Science Publishers*, Enfield.

López-Patiño, M.A., Guijarro, A.I., Isorna, E., Delgado, M.J., Alonso-Bedate, M., de Pedro, N. (1999). Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (*Carassius auratus*). *Eur J Pharmacol* **377**, 147-153.

Lu, S., Guan, J.L., Wang, Q.P., Uehara, K., Yamada, S., Goto, N., et al. (2002). Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. *Neurosci Lett* **321**, 157-160.

Lueneberg, K., Domínguez, G., Arias-Carrión, O., Palomero-Rivero, M., Millán-Aldaco, D., Morán, J., et al. (2011). Cellular viability effects of fatty acid amide hydrolase inhibition on cerebellar neurons. *Int Arch Med* **4**, 28.

Mácajová, M., Lamosová, D., Zeman, M. (2004). Role of leptin in farm animals: a review. *J Vet Med A Physiol Pathol Clin Med* **51**, 157-166.

MacDonald, E., Volkoff, H. (2009). Cloning, distribution and effects of season and nutritional status on the expression of neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and cholecystokinin (CCK) in winter flounder (*Pseudopleuronectes americanus*). *Horm Behav* **56**, 58-65.

Madrid, J.A., Boujard, T., Sánchez-Vázquez, F.J. (2001). Feeding rhythms. In: Food Intake in Fish. (Eds. Houlihan, D.F., Boujard, T., Jobling, M.), pp 189-215. *Blackwell Science*, Oxford.

Mandrioli, L., Sirri, R., Gatta, P.P., Morandi, F., Sarli, G., Parma, L., et al. (2012). Histomorphologic hepatic features and growth performances of juvenile Senegalese sole (*Solea senegalensis*) fed isogenetic practical diets with variable protein/lipid levels. *J Appl Ichthyol* **28**, 628-632.

Manzar, D., Hussain, M.E. (2011). Leptin rhythmicity and its relationship with other rhythm markers. *Biol Rhythm Res* **42**, 163-180.

Matias, I., Gonthier, M.P., Petrosino, S., Docimo, L., Capasso, R., Hoareau, L., et al. (2007). Role and regulation of acylethanolamides in energy balance: focus on adipocytes and beta-cells. *Br J Pharmacol* **152**, 676-690.

Matsuda, K., Kashimoto, K., Higuchi, T., Yoshida, T., Uchiyama, M., Shioda, S., et al. (2000). Presence of pituitary adenylate cyclase-activating polypeptide (PACAP) and its relaxant activity in the rectum of a teleost, the stargazer, *Uranoscopus japonicus*. *Peptides* **21**, 821-827.

Matsuda, K., Kojima, K., Shimakura, S., Wada, K., Maruyama, K., Uchiyama, M., et al. (2008a). Corticotropin-releasing hormone mediates alpha-melanocyte-stimulating hormone-induced anorexigenic action in goldfish. *Peptides* **29**, 1930-1936.

Matsuda, K., Maruyama, K., Miura, T., Uchiyama, M., Shioda, S. (2005a). Anorexigenic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: feeding-induced changes in the expression of mRNAs for PACAP and its receptors in the brain, and locomotor response to central injection. *Neurosci Lett* **386**, 9-13.

Matsuda, K., Maruyama, K., Nakamachi, T., Miura, T., Uchiyama, M., Shioda, S. (2005b). Inhibitory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) on food intake in the goldfish, *Carassius auratus*. *Peptides* **26**, 1611-1616.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Shimakura, S., Uchiyama, M., et al. (2006). Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. *Peptides* **27**, 2321-2325.

Matsuda, K., Nakamura, K., Shimakura, S., Miura, T., Kageyama, H., Uchiyama, M., et al. (2008b). Inhibitory effect of chicken gonadotropin-releasing hormone II on food intake in the goldfish, *Carassius auratus*. *Horm Behav* **54**, 83-89.

Matsumoto, M., Hosoda, H., Kitajima, Y., Morozumi, N., Minamitake, Y., Tanaka, S., et al. (2001). Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. *Biochem Biophys Res Commun* **287**, 142-146.

McClelland, G., Weber, J.-M., Zwingelstein, G., Brichon, G. (1995). Lipid composition of tissue and plasma in two mediterranean fishes, the gilt-head sea bream (*Chrysophyrys auratus*) and the European seabass (*Dicentratchus labrax*). *Can. J. Fish. Aquat. Sci*. **52**, 161-170

McCormick, S. (1994). Ontogeny and evolution of salinity tolerance in anadromous salmonids: Hormones and heterochrony. *Estuaries* **17**, 26-33.

McKee, K.K., Tan, C.P., Palyha, O.C., Liu, J., Feighner, S.D., Hreniuk, D.L., et al. (1997). Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics* **46**, 426-434.

McVay, J.A., Kaan, H.W. (1940). The digestive tract of *Carassius auratus*. *Biol Bull* **78**, 53-67.

Mendoza, J., Challet, E. (2009). Brain clocks: from the suprachiasmatic nuclei to a cerebral network. *Neuroscientist* **15**, 477-488.

Mennigen, J.A., Harris, E.A., Chang, J.P., Moon, T.W., Trudeau, V.L. (2009). Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. *Regul Pept* **155**, 99-104.

Micale, V., Campo, S., D'Ascola, A., Guerrera, M.C., Levanti, M.B., Germana, A., et al. (2012). Cholecystokinin in white sea bream: molecular cloning, regional expression, and immunohistochemical localization in the gut after feeding and fasting. *PLoS One* **7**, e52428.

Miller, L.J., Gao, F. (2008). Structural basis of cholecystokinin receptor binding and regulation. *Pharmacol Ther* **119**, 83-95.

Mimeault, C., Trudeau, V.L., Moon, T.W. (2006). Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-

activated receptor beta (PPARbeta) mRNA levels in male goldfish (*Carassius auratus*). *Toxicology* **228**, 140-150.

Miura, T., Maruyama, K., Kaiya, H., Miyazato, M., Kangawa, K., Uchiyama, M., et al. (2009). Purification and properties of ghrelin from the intestine of the goldfish, *Carassius auratus*. *Peptides* **30**, 758-765.

Miura, T., Maruyama, K., Shimakura, S., Kaiya, H., Uchiyama, M., Kangawa, K., et al. (2006). Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. *Neurosci Lett* **407**, 279-283.

Miura, T., Maruyama, K., Shimakura, S., Kaiya, H., Uchiyama, M., Kangawa, K., et al. (2007). Regulation of food intake in the goldfish by interaction between ghrelin and orexin. *Peptides* **28**, 1207-1213.

Moen, A.G., Finn, R.N. (2013). Short-term, but not long-term feed restriction causes differential expression of leptins in Atlantic salmon. *Gen Comp Endocrinol* **183**, 83-88.

Moghadam, H.K., Ferguson, M.M., Danzmann, R.G. (2005). Evolution of Hox clusters in Salmonidae: a comparative analysis between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *J Mol Evol* **61**, 636-649.

Morash, B.A., Imran, A., Wilkinson, D., Ur, E., Wilkinson, M. (2003). Leptin receptors are developmentally regulated in rat pituitary and hypothalamus. *Mol Cell Endocrinol* **210**, 1-8.

Muccioli, G., Lorenzi, T., Lorenzi, M., Ghe, C., Arnoletti, E., Raso, G.M., et al. (2011). Beyond the metabolic role of ghrelin: a new player in the regulation of reproductive function. *Peptides* **32**, 2514-2521.

Murashita, K., Fukada, H., Hosokawa, H., Masumoto, T. (2006). Cholecystokinin and peptide Y in yellowtail (*Seriola quinqueradiata*): molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting. *Gen Comp Endocrinol* **145**, 287-297.

Murashita, K., Fukada, H., Ronnestad, I., Kurokawa, T., Masumoto, T. (2008a). Nutrient control of release of pancreatic enzymes in yellowtail (*Seriola quinqueradiata*): involvement of CCK and PY in the regulatory loop. *Comp Biochem Physiol A Mol Integr Physiol* **150**, 438-443.

Murashita, K., Kurokawa, T., Nilsen, T.O., Rønnestad, I. (2009). Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): molecular cloning and tissue expression. *Gen Comp Endocrinol* **160**, 223-235.

Murashita, K., Uji, S., Yamamoto, T., Rønnestad, I., Kurokawa, T. (2008b). Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol B Biochem Mol Biol* **150**, 377-384.

Murillo-Rodríguez, E., Desarnaud, F., Próspero-García, O. (2006). Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat. *Life Sci* **79**, 30-37.

Murillo-Rodríguez, E., Palomero-Rivero, M., Millán-Aldaco, D., Arias-Carrión, O., Drucker-Colin, R. (2011). Administration of URB597, oleoylethanolamide or palmitoylethanolamide increases waking and dopamine in rats. *PLoS One* **6**, e20766.

Muus, B.J., Dahlström, P. (1981). Los peces de agua dulce de España y Europa, pp. 132-135. *Ed. Omega*, Madrid.

Mutt, V., Jorpes, J.E. (1968). Structure of porcine cholecystokinin-pancreozymin. 1. Cleavage with thrombin and with trypsin. *Eur J Biochem* **6**, 156-162.

Nakamachi, T., Matsuda, K., Maruyama, K., Miura, T., Uchiyama, M., Funahashi, H., et al. (2006). Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. *J Neuroendocrinol* **18**, 290-297.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., et al. (2001). A role for ghrelin in the central regulation of feeding. *Nature* **409**, 194-198.

Narnaware, Y.K., Peter, R.E. (2001). Neuropeptide Y stimulates food consumption through multiple receptors in goldfish. *Physiol Behav* **74**, 185-190.

Narnaware, Y.K., Peyon, P.P., Lin, X., Peter, R.E. (2000). Regulation of food intake by neuropeptide Y in goldfish. *Am J Physiol Regul Integr Comp Physiol* **279**, R1025-1034.

Neregård, L., Sundt-Hansen, L., Björnsson, B.T., Johnsson, J.I. (2008). Growth hormone affects behaviour of wild brown trout *Salmo trutta* in territorial owner–intruder conflicts. *J Fish Biol* **73**, 2341-2351.

Nielsen, M.J., Petersen, G., Astrup, A., Hansen, H.S. (2004). Food intake is inhibited by oral oleoylethanolamide. *J Lipid Res* **45**, 1027-1029.

Nieminen, P., Mustonen, A.M., Hyvärinen, H. (2003). Fasting reduces plasma leptin-and ghrelin-immunoreactive peptide concentrations of the burbot (*Lota lota*) at 2 degrees C but not at 10 degrees C. *Zool Sci* **20**, 1109-1115.

Nisembaum, L.G., de Pedro, N., Delgado, M.J., Isorna, E. (2014). Crosstalking between the "gut-brain" hormone ghrelin and the circadian system in the goldfish. Effects on clock gene expression and food anticipatory activity. *Gen Comp Endocrinol* **205**, 287-295.

Nisembaum, L.G., Tinoco, A.B., Moure, A.L., Alonso-Gómez, A.L., Delgado, M.J., Valenciano, A.I. (2013). The arylalkylamine-N-acetyltransferase (AANAT) acetylates dopamine in the digestive tract of goldfish: a role in intestinal motility. *Neurochem Int* **62**, 873-880.

Nisembaum, L.G., Velarde, E., Tinoco, A.B., Azpeleta, C., de Pedro, N., Alonso-Gómez, A.L., et al. (2012). Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). *Chronobiol Int* **29**, 665-673.

Noble, F., Wank, S.A., Crawley, J.N., Bradwejn, J., Seroogy, K.B., Hamon, M., et al. (1999). International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. *Pharmacol Rev* **51**, 745-781.

Ojanguren, A.F., Braña, F. (2003). Effects of size and morphology on swimming performance in juvenile brown trout (*Salmo trutta* L.). *Ecol Freshw Fish* **12**, 241-246.

Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., et al. (2010). Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiol* **10**, 21.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., Ueda, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem* **279**, 5298-5305.

Olsson, C. (2009). Autonomic innervation of the fish gut. *Acta Histochem* **111**, 185-195.

Olsson, C., Aldman, G., Larsson, A., Holmgren, S. (1999). Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss. J Exp Biol* **202**, 161-170.

Olsson, C., Holbrook, J.D., Bompadre, G., Jönsson, E., Hoyle, C.H., Sanger, G.J., et al. (2008). Identification of genes for the ghrelin and motilin receptors and a novel related gene in fish, and stimulation of intestinal motility in zebrafish (*Danio rerio*) by ghrelin and motilin. *Gen Comp Endocrinol* **155**, 217-226.

Olsson, C., Holmgren, S. (2000). PACAP and nitric oxide inhibit contractions in the proximal intestine of the atlantic cod, *Gadus morhua*. *J Exp Biol* **203**, 575-583.

Olsson, C., Holmgren, S. (2001). The control of gut motility. *Comp Biochem Physiol A Mol Integr Physiology* **128**, 479-501.

Olsson, C., Holmgren, S. (2011). Autonomic control of gut motility: a comparative view. *Auton Neurosci* **165**, 80-101.

Ortega, V.A., Lovejoy, D.A., Bernier, N.J. (2013). Appetite-suppressing effects and interactions of centrally administered corticotropin-releasing factor, urotensin I and serotonin in rainbow trout (*Oncorhynchus mykiss*). *Front Neurosci* **7**, 196. doi: 10.3389/fnins.2013.00196.

Oveisi, F., Gaetani, S., Eng, K.T., Piomelli, D. (2004). Oleoylethanolamide inhibits food intake in free-feeding rats after oral administration. *Pharmacol Res* **49**, 461-466.

Overton, H.A., Babbs, A.J., Doel, S.M., Fyfe, M.C., Gardner, L.S., Griffin, G., et al. (2006). Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metab* **3**, 167-175.

Owyang, C., Heldsinger, A. (2011). Vagal control of satiety and hormonal regulation of appetite. *J Neurogastroenterol Motil* **17**, 338-348.

Pakkasmaa, S., Piironen, J. (2001). Morphological differentiation among local trout (*Salmo trutta*) populations. *Biol J Linnean Soc* **72**, 231-239.

Pan, W., Tu, H., Kastin, A.J. (2006). Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. *Peptides* **27**, 911-916.

Papoutsoglou, S.E., Mylonakis, G., Miliou, H., Karakatsouli, N.P., Chadio, S. (2000). Effects of background color on growth performances and physiological responses of scaled carp (*Cyprinus carpio* L.) reared in a closed circulated system. *Aquac Eng* **22**, 309-318.

Patton, D.F., Mistlberger, R.E. (2013). Circadian adaptations to meal timing: neuroendocrine mechanisms. *Front Neurosci* **7**, 185. doi: 10.3389/fnins.2013.00185.

Pavón, F.J., Serrano, A., Romero-Cuevas, M., Alonso, M., Rodríguez de Fonseca, F. (2010). Oleoylethanolamide: a new player in peripheral control of energy metabolism. Therapeutic implications. *Drug Discov Today Dis Mech* **7**, e175-e183.

Peelman, F., Zabeau, L., Moharana, K., Savvides, S.N., Tavernier, J. (2014). 20 years of leptin: insights into signaling assemblies of the leptin receptor. *J Endocrinol* **223**, T9-23.

Peeters, T.L. (2005). Ghrelin: a new player in the control of gastrointestinal functions. *Gut* **54**, 1638-1649.

Penney, C.C., Volkoff, H. (2013). Peripheral injections of cholecystokinin, apelin, ghrelin and orexin in cavefish (*Astyanax fasciatus mexicanus*): Effects on feeding and on

the brain expression levels of tyrosine hydroxylase, mechanistic target of rapamycin and appetite-related hormones. *Gen Comp Endocrinol* **196**, 34-40

Pérez-Jiménez, A., Cardenete, G., Hidalgo, M.C., García-Alcázar, A., Abellán, E., Morales, A.E. (2012). Metabolic adjustments of *Dentex dentex* to prolonged starvation and refeeding. *Fish Physiol Biochem* **38**, 1145-1157.

Petersen, G., Sørensen, C., Schmid, P.C., Artmann, A., Tang-Christensen, M., Hansen, S.H., et al. (2006). Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. *Biochim Biophys Acta* **1761**, 143-150.

Peterson, B.C., Waldbieser, G.C., Riley, L.G., Jr., Upton, K.R., Kobayashi, Y., Small, B.C. (2012). Pre- and postprandial changes in orexigenic and anorexigenic factors in channel catfish (*Ictalurus punctatus*). *Gen Comp Endocrinol* **176**, 231-239.

Peyon, P., Lin, X.W., Himick, B.A., Peter, R.E. (1998). Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish. *Peptides* **19**, 199-210.

Peyon, P., Vega-Rubín de Celis, S., Gómez-Requeni, P., Zanuy, S., Pérez-Sánchez, J., Carrillo, M. (2003). In vitro effect of leptin on somatolactin release in the European sea bass (*Dicentrarchus labrax*): dependence on the reproductive status and interaction with NPY and GnRH. *Gen Comp Endocrinol* **132**, 284-292.

Peyon, P., Zanuy, S., Carrillo, M. (2001). Action of leptin on in vitro luteinizing hormone release in the European sea bass (*Dicentrarchus labrax*). *Biol Reprod* **65**, 1573-1578.

Piccinetti, C.C., Migliarini, B., Petrosino, S., Di Marzo, V., Carnevali, O. (2010). Anandamide and AM251, via water, modulate food intake at central and peripheral level in fish. *Gen Comp Endocrinol* **166**, 259-267.

Picha, M.E., Strom, C.N., Riley, L.G., Walker, A.A., Won, E.T., Johnstone, W.M., et al. (2009). Plasma ghrelin and growth hormone regulation in response to metabolic state in hybrid striped bass: effects of feeding, ghrelin and insulin-like growth factor-I on in vivo and in vitro GH secretion. *Gen Comp Endocrinol* **161**, 365-372.

Pinillos, M.L., de Pedro, N., Alonso-Gómez, A.L., Alonso-Bedate, M., Delgado, M.J. (2001). Food intake inhibition by melatonin in goldfish (*Carassius auratus*). *Physiol Behav* **72**, 629-634.

Piomelli, D. (2013). A fatty gut feeling. *Trends Endocrinol Metab* **24**, 332-341.

Polakof, S., Míguez, J.M., Soengas, J.L. (2011). Ghrelin effects on central glucosensing and energy homeostasis-related peptides in rainbow trout. *Domest Anim Endocrinol* **41**, 126-136.

Proulx, K., Cota, D., Castañeda, T.R., Tschöp, M.H., D'Alessio, D.A., Tso, P., et al. (2005). Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. *Am J Physiol Regul Integr Comp Physiol* **289**, R729-737.

Rajjo, I.M., Vigna, S.R., Crim, J.W. (1988). Actions of cholecystokinin-related peptides on the gallbladder of bony fishes in vitro. *Comp Biochem Physiol C* **90**, 267-273.

Ramsay, T.G., Bush, J.A., McMurtry, J.P., Thivierge, M.C., Davis, T.A. (2004). Peripheral leptin administration alters hormone and metabolite levels in the young pig. *Comp Biochem Physiol A Mol Integr Physiol* **138**, 17–25.

Rathore, R.M., Angotzi, A.R., Jordal, A.-E.O., Rønnestad, I. (2013). Cholecystokinin receptors in Atlantic salmon: molecular cloning, gene expression, and structural basis. *Physiol Rep* **1**, e00069.

Reidy, S.P., Weber, J. (2000). Leptin: an essential regulator of lipid metabolism. *Comp Biochem Physiol A Mol Integr Physiol* **125**, 285-298.

Riley, L.G., Fox, B.K., Kaiya, H., Hirano, T., Grau, E.G. (2005). Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*. *Gen Comp Endocrinol* **142**, 234-240.

Riley, L.G., Jr., Walker, A.P., Dorough, C.P., Schwandt, S.E., Grau, E.G. (2009). Glucose regulates ghrelin, neuropeptide Y, and the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*. *Comp Biochem Physiol A Mol Integr Physiol* **154**, 541-546.

Rindi, G., Necchi, V., Savio, A., Torsello, A., Zoli, M., Locatelli, V., et al. (2002). Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochem Cell Biol* **117**, 511-519.

Rodríguez de Fonseca, F., Navarro, M., Gómez, R., Escuredo, L., Nava, F., Fu, J., et al. (2001). An anorexic lipid mediator regulated by feeding. *Nature* **414**, 209-212.

Romano, A., Potes, C.S., Tempesta, B., Cassano, T., Cuomo, V., Lutz, T., et al. (2013). Hindbrain noradrenergic input to the hypothalamic PVN mediates the activation of oxytocinergic neurons induced by the satiety factor oleoylethanolamide. *Am J Physiol Endocrinol Metab* **305**, E1266-1273.

Rønnestad, I., Nilsen, T.O., Murashita, K., Angotzi, A.R., Gamst Moen, A.G., Stefansson, S.O., et al. (2010). Leptin and leptin receptor genes in Atlantic salmon: Cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. *Gen Comp Endocrinol* **168**, 55-70.

Rozin, P., Mayer, J. (1961). Regulation of food intake in the goldfish. *Am J Physiol* **201**, 968-974.

Rubio, V.C., Sánchez-Vázquez, F.J., Madrid, J.A. (2008). Role of cholecystokinin and its antagonist proglumide on macronutrient selection in European sea bass *Dicentrarchus labrax,* L. *Physiol Behav* **93**, 862-869.

Sakata, I., Yang, J., Lee, C.E., Osborne-Lawrence, S., Rovinsky, S.A., Elmquist, J.K., et al. (2009). Colocalization of ghrelin O-acyltransferase and ghrelin in gastric mucosal cells. *Am J Physiol Endocrinol Metab* **297**, E134-141.

Sánchez-Vázquez, F.J., Aranda, A., Madrid, J.A. (2001). Differential effects of meal size and food energy density on feeding entrainment in goldfish. *J Biol Rhythms* **16**, 58-65.

Sánchez-Vázquez, F.J., Madrid, J.A. (2001). Feeding anticipatory activity In: Food intake in fish (Eds: Houlihan, D., Boujard, T., Jobling, M.), pp. 216-232. *Blakwell Science*, Oxford.

Sánchez-Vázquez, F.J., Madrid, J.A., Zamora, S., Iigo, M., Tabata, M. (1996). Demand feeding and locomotor circadian rhythms in the goldfish, *Carassius auratus*: dual and independent phasing. *Physiol Behav* **60**, 665-674.

Sánchez-Vázquez, F.J., Madrid, J.A., Zamora, S., Tabata, M. (1997). Feeding entrainment of locomotor activity rhythms in the goldfish is mediated by a feeding-entrainable circadian oscillator. *J Comp Physiol A* **181**, 121-132.

Sánchez-Vázquez, F.J., Yamamoto, T., Akiyama, T., Madrid, J.A., Tabata, M. (1998). Selection of macronutrients by goldfish operating self-feeders. *Physiol Behav* **65**, 211-218.

Sarro-Ramírez, A., Sánchez-López, D., Tejeda-Padrón, A., Frias, C., Zaldivar-Rae, J., Murillo-Rodríguez, E. (2013). Brain molecules and appetite: the case of oleoylethanolamide. *Cent Nerv Syst Agents Med Chem* **13**, 88-91.

Schmid, H.H., Schmid, P.C., Natarajan, V. (1996). The N-acylation-phosphodiesterase pathway and cell signalling. *Chem Phys Lipids* **80**, 133-142.

Shahbazi, F., Karila, P., Olsson, C., Holmgren, S., Conlon, J.M., Jensen, J. (1998). Primary structure, distribution, and effects on motility of CGRP in the intestine of the cod *Gadus morhua*. *Am J Physiol* **275**, 19-28.

Shintani, M., Ogawa, Y., Ebihara, K., Aizawa-Abe, M., Miyanaga, F., Takaya, K., et al. (2001). Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* **50**, 227-232.

Schoeller, D.A., Cella, L.K., Sinha, M.K., Caro, J.F. (1997). Entrainment of the diurnal rhythm of plasma leptin to meal timing. *J Clin Invest* **100**, 1882-1887.

Schwandt, S.E., Peddu, S.C., Riley, L.G. (2010). Differential roles for octanoylated and decanoylated ghrelins in regulating appetite and metabolism. *Int J Pept* **2010**, Article ID 275804, 6 pages. doi: http://dx.doi.org/10.1155/2010/275804.

Schwartz, G.J. (2011). Gut fat sensing in the negative feedback control of energy balance--recent advances. *Physiol Behav* **104**, 621-623.

Schwartz, G.J., Fu, J., Astarita, G., Li, X., Gaetani, S., Campolongo, P., et al. (2008). The lipid messenger OEA links dietary fat intake to satiety. *Cell Metab* **8**, 281-288.

Serrano, A., Pavón, F.J., Tovar, S., Casanueva, F., Señarís, R., Diéguez, C., et al. (2011). Oleoylethanolamide: effects on hypothalamic transmitters and gut peptides regulating food intake. *Neuropharmacology* **60**, 593-601.

Shepherd, B.S., Johnson, J.K., Silverstein, J.T., Parhar, I.S., Vijayan, M.M., McGuire, A., et al. (2007). Endocrine and orexigenic actions of growth hormone secretagogues in rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol A Mol Integr Physiol* **146**, 390-399.

Shintani, M., Ogawa, Y., Ebihara, K., Aizawa-Abe, M., Miyanaga, F., Takaya, K., et al. (2001). Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* **50**, 227-232.

Shpilman, M., Hollander-Cohen, L., Ventura, T., Gertler, A., Levavi-Sivan, B. (2014). Production, gene structure and characterization of two orthologs of leptin and a leptin receptor in tilapia. *Gen Comp Endocrinol* **207**, 74-85.

Silver, R., Balsam, P.D., Butler, M.P., LeSauter, J. (2011). Food anticipation depends on oscillators and memories in both body and brain. *Physiol Behav* **104**, 562-571.

Silverstein, J.T., Bondareva, V.M., Leonard, J.B., Plisetskaya, E.M. (2001). Neuropeptide regulation of feeding in catfish, *Ictalurus punctatus*: a role for glucagon-like peptide-1 (GLP-1)? *Comp Biochem Physiol B Biochem Mol Biol* **129**, 623-631.

Silverstein, J.T., Breininger, J., Baskin, D.G., Plisetskaya, E.M. (1998). Neuropeptide Y-like gene expression in the salmon brain increases with fasting. *Gen Comp Endocrinol* **110**, 157-165.

Silverstein, J.T. and Plisetskaya, E.M. (2000). The effects of NPY and insulin on food intake regulation in fish. *Amer Zool* **40**, 296-308.

Small, B.C., Quiniou, S.M., Kaiya, H. (2009). Sequence, genomic organization and expression of two channel catfish, *Ictalurus punctatus*, ghrelin receptors. *Comp Biochem Physiol A Mol Integr Physiol* **154**, 451-464.

Smith, R.G., Palyha, O.C., Feighner, S.D., Tan, C.P., McKee, K.K., Hreniuk, D.L., et al. (1999). Growth hormone releasing substances: types and their receptors. *Horm Res* **51** Suppl 3, 1-8.

Soengas, J.L., Aldegunde, M. (2004). Brain glucose and insulin: effects on food intake and brain biogenic amines of rainbow trout. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **190**, 641-649.

Sosniyenko, S., Hut, R.A., Daan, S., Sumova, A. (2009). Influence of photoperiod duration and light-dark transitions on entrainment of Per1 and Per2 gene and protein expression in subdivisions of the mouse suprachiasmatic nucleus. *Eur J Neurosci* **30**, 1802-1814.

Staljanssens, D., Azari, E.K., Christiaens, O., Beaufays, J., Lins, L., Van Camp, J., et al. (2011). The CCK(-like) receptor in the animal kingdom: functions, evolution and structures. *Peptides* **32**, 607-619.

Stengel, A., Wang, L., Tache, Y. (2011). Stress-related alterations of acyl and desacyl ghrelin circulating levels: mechanisms and functional implications. *Peptides* **32**, 2208-2217.

Stephan, F.K. (2002). The "Other" Circadian System: Food as a Zeitgeber. *J Biol Rhythms* **17**, 284-292.

Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y., Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science* **291**, 490-493.

Strader, A.D., Woods, S.C. (2005). Gastrointestinal hormones and food intake. *Gastroenterology* **128**, 175-191.

Sunuma, T., Amano, M., Yamanome, T., Furukawa, K., Yamamori, K. (2007). Self-feeding activity of a pleuronectiform fish, the barfin flounder. *Aquaculture* **270**, 566-569.

Suzuki, T., Kurokawa, T., McVey, D.C. (1999). Sequence and expression analyses of cholecystokinin (CCK) precursor cDNA in the Japanese flounder (*Paralichthys olivaceus*). *Fish Physiol Biochem* **21**, 73-80.

Tack, J., Caenepeel, P., Fischler, B., Piessevaux, H., Janssens, J. (2001). Symptoms associated with hypersensitivity to gastric distention in functional dyspepsia. *Gastroenterology* **121**, 526-535.

Tacon, A.G.J. (1995). Application of nutrient requirement data under practical conditions: special problems of intensive and semi-intensive fish farming systems. *J Appl Ichthyol* **11**, 205-214.

Tang, Y., Yu, J., Li, H., Xu, P., Li, J., Ren, H. (2013). Molecular cloning, characterization and expression analysis of multiple leptin genes in Jian carp (*Cyprinus carpio var. Jian*). *Comp Biochem Physiol B Biochem Mol Biol* **166**, 133-140.

Tang-Christensen, M., Vrang, N., Ortmann, S., Bidlingmaier, M., Horvath, T.L., Tschöp, M. (2004). Central administration of ghrelin and agouti-related protein (83-132) increases food intake and decreases spontaneous locomotor activity in rats. *Endocrinology* **145**, 4645-4652.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y. (2003). Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res* **13**, 382-390.

Thabuis, C., Destaillats, F., Lambert, D.M., Muccioli, G.G., Maillot, M., Harach, T., et al. (2011). Lipid transport function is the main target of oral oleoylethanolamide to reduce adiposity in high-fat-fed mice. *J Lipid Res* **52**, 1373-1382.

Thabuis, C., Destaillats, F., Landrier, J.F., Tissot-Favre, D., Martin, J.C. (2010). Analysis of gene expression pattern reveals potential targets of dietary oleoylethanolamide in reducing body fat gain in C3H mice. *J Nutr Biochem* **21**, 922-928.

Thabuis, C., Tissot-Favre, D., Bezelgues, J.B., Martin, J.C., Cruz-Hernandez, C., Dionisi, F., et al. (2008). Biological functions and metabolism of oleoylethanolamide. *Lipids* **43**, 887-894.

Thavanathan, R., Volkoff, H. (2006). Effects of amylin on feeding of goldfish: interactions with CCK. *Regul Pept* **133**, 90-96.

Thorpe, A.J., Mullet, M.A., Wang, C., Kotz, C.M. (2003). Peptides that regulate food intake. Regional, metabolic and circadian specificity of lateral hypothalamic orexin A feeding stimulation. *Am J Physiol Regul Integr Comp Physiol* **284**, R1409-1417.

Toshinai, K., Date, Y., Murakami, N., Shimada, M., Mondal, M.S., Shimbara, T., et al. (2003). Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* **144**, 1506-1512.

Tracy, H.J., Gregory, R.A. (1964). Physiological properties of a series of synthetic peptides structurally related to gastrin I. *Nature* **204**, 935-938.

Trombley, S., Maugars, G., Kling, P., Björnsson, B.T., Schmitz, M. (2012). Effects of long-term restricted feeding on plasma leptin, hepatic leptin expression and leptin receptor expression in juvenile Atlantic salmon (*Salmo salar* L.). *Gen Comp Endocrinol* **175**, 92-99.

Tschöp, M., Smiley, D.L., Heiman, M.L. (2000). Ghrelin induces adiposity in rodents. *Nature* **407**, 908-913.

Tsuboi, K., Sun, Y.X., Okamoto, Y., Araki, N., Tonai, T., Ueda, N. (2005). Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. *J Biol Chem* **280**, 11082-11092.

Tsunoda, Y., Yao, H., Park, J., Owyang, C. (2003). Cholecystokinin synthesizes and secretes leptin in isolated canine gastric chief cells. *Biochem Biophys Res Commun* **310**, 681-684.

Tymchuk, W.E., Beckman, B., Devlin, R.H. (2009). Altered expression of growth hormone/insulin-like growth factor I axis hormones in domesticated fish. *Endocrinology* **150**, 1809-1816.

Unniappan, S., Canosa, L.F., Peter, R.E. (2004). Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. *Neuroendocrinology* **79**, 100-108.

Unniappan, S., Lin, X., Cervini, L., Rivier, J., Kaiya, H., Kangawa, K., et al. (2002). Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. *Endocrinology* **143**, 4143-4146.

Unniappan, S., Peter, R.E. (2004). In vitro and in vivo effects of ghrelin on luteinizing hormone and growth hormone release in goldfish. *Am J Physiol Regul Integr Comp Physiol* **286**, R1093-1101.

Valenti, M., Cottone, E., Martínez, R., de Pedro, N., Rubio, M., Viveros, M.P., et al. (2005). The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake. *J Neurochem* **95**, 662-672.

Van der Salm, A.L., Martínez, M., Flik, G., Wendelaar Bonga, S.E. (2004). Effects of husbandry conditions on the skin colour and stress response of red porgy, *Pagrus pagrus*. *Aquaculture* **241**, 371-386.

Velarde, E., Alonso-Gómez, A.L., Azpeleta, C., Isorna, E., Delgado, M.J. (2009). Melatonin attenuates the acetylcholine-induced contraction in isolated intestine of a teleost fish. *J Comp Physiol B* **179**, 951-959.

Velarde, E., Alonso-Gómez, A.L., Azpeleta, C., Isorna, E., de Pedro, N., Delgado, M.J. (2011). Melatonin effects on gut motility are independent of the relaxation mediated by the nitrergic system in the goldfish. *Comp Biochem Physiol A Mol Integr Physiol* **159**, 367-371.

Velarde, E., Delgado, M.J., Alonso-Gómez, A.L. (2010). Serotonin-induced contraction in isolated intestine from a teleost fish (*Carassius auratus*): characterization and interactions with melatonin. *Neurogastroenterol Motil* **22**, e364-373.

Vera, L.M., de Pedro, N., Gómez-Milán, E., Delgado, M.J., Sánchez-Muros, M.J., Madrid, J.A., et al. (2007). Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. *Physiol Behav* **90**, 518-524.

Vigna, S.R. (2000). Evolution of the cholecystokinin and gastrin peptides and receptors. *Am Zool* **40**, 287-295.

Vincent, J.P., Mazella, J., Kitabgi, P. (1999). Neurotensin and neurotensin receptors. *Trends Pharmacol Sci* **20**, 302-309.

Vivas, Y., Azpeleta, C., Feliciano, A., Velarde, E., Isorna, E., Delgado, M.J., et al. (2011). Time-dependent effects of leptin on food intake and locomotor activity in goldfish. *Peptides* **32**, 989-995.

Volff, J.N. (2005). Genome evolution and biodiversity in teleost fish. *Heredity* **94**, 280-294.

Volkoff, H. (2006). The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish. *Comp Biochem Physiol A Mol Integr Physiol* **144**, 325-331.

Volkoff, H., Bjorklund, J.M., Peter, R.E. (1999). Stimulation of feeding behavior and food consumption in the goldfish, *Carassius auratus*, by orexin-A and orexin-B. *Brain Res* **846**, 204-209.

Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P., et al. (2005). Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* **142**, 3-19.

Volkoff, H., Eykelbosh, A.J., Peter, R.E. (2003). Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. *Brain Res* **972**, 90-109.

Volkoff, H., Unniappan, S. and Kelly, S. P. (2009a) The endocrine regulation of food intake. In: Fish Neuroendocrinology (Eds. Bernier, N.J., Van der Kraak, G., Farrel, A.P., Brauner, C. J.), pp. 421-265. *Academic Press Ltd*, London.

Volkoff, H., Xu, M., MacDonald, E., Hoskins, L. (2009b). Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: notes on actions and responses to nutritional, environmental and reproductive changes. *Comp Biochem Physiol A Mol Integr Physiol* **153**, 8-12.

Wall, A., Volkoff, H. (2013). Effects of fasting and feeding on the brain mRNA expressions of orexin, tyrosine hydroxylase (TH), PYY and CCK in the Mexican blind cavefish (*Astyanax fasciatus mexicanus*). *Gen Comp Endocrinol* **183**, 44-52.

Wang, J., Liu, R., Hawkins, M., Barzilai, N., Rossetti, L. (1998). A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* **393**, 684-688.

Wang, X., Miyares, R.L., Ahern, G.P. (2005). Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *J Physiol* **564**, 541-547.

Webb Jr, K.A., Khan, I.A., Nunez, B.S., Rønnestad, I., Holt, G.J. (2010). Cholecystokinin: Molecular cloning and immunohistochemical localization in the gastrointestinal tract of larval red drum, *Sciaenops ocellatus* (L.). *Gen Comp Endocrinol* **166**, 152-159.

Weil, C., Le Bail, P.Y., Sabin, N., Le Gac, F. (2003). In vitro action of leptin on FSH and LH production in rainbow trout (*Onchorynchus mykiss*) at different stages of the sexual cycle. *Gen Comp Endocrinol* **130**, 2-12.

Wilson, A.M., McLaughlin, R. (2010). Foraging behaviour and brain morphology in recently emerged brook charr, *Salvelinus fontinalis*. *Behav Ecol Sociobiol* **64**, 1905-1914.

Wisen, O., Hellström, P.M. (1995). Gastrointestinal motility in obesity. *J Intern Med* **237**, 411-418.

Wong, M.K., Sze, K.H., Chen, T., Cho, C.K., Law, H.C., Chu, I.K., et al. (2013). Goldfish spexin: solution structure and novel function as a satiety factor in feeding control. *Am J Physiol Endocrinol Metab* **305**, E348-366.

Wong, M.M., Yu, R.M., Ng, P.K., Law, S.H., Tsang, A.K., Kong, R.Y. (2007). Characterization of a hypoxia-responsive leptin receptor (omLepR(L)) cDNA from the marine medaka (*Oryzias melastigma*). *Mar Pollut Bull* **54**, 797-803.

Wu, T., Rayner, C.K., Young, R.L., Horowitz, M. (2013). Gut motility and enteroendocrine secretion. *Curr Opin Pharmacol* **13**, 928-934.

Wysujack, K., Greenberg, L.A., Bergman, E., Olsson, I.C. (2009). The role of the environment in partial migration: food availability affects the adoption of a migratory tactic in brown trout *Salmo trutta*. *Ecol Freshw Fish* **18**, 52-59.

Xu, B., Kalra, P.S., Farmerie, W.G., Kalra, S.P. (1999). Daily changes in hypothalamic gene expression of neuropeptide Y, galanin, proopiomelanocortin, and adipocyte leptin gene expression and secretion: effects of food restriction. *Endocrinology* **140**, 2868-2875.

Xu, M., Volkoff, H. (2007). Molecular characterization of prepro-orexin in Atlantic cod (*Gadus morhua*): cloning, localization, developmental profile and role in food intake regulation. *Mol Cell Endocrinol* **271**, 28-37.

Yahashi, S., Kang, K.S., Kaiya, H., Matsuda, K. (2012). GHRP-6 mimics ghrelin-induced stimulation of food intake and suppression of locomotor activity in goldfish. *Peptides* **34**, 324-328.

Yamanaka, A., Beuckmann, C.T., Willie, J.T., Hara, J., Tsujino, N., Mieda, M., et al. (2003). Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* **38**, 701-713.

Yan, A., Zhang, L., Tang, Z., Zhang, Y., Qin, C., Li, B., et al. (2011). Orange-spotted grouper (*Epinephelus coioides*) orexin: molecular cloning, tissue expression, ontogeny, daily rhythm and regulation of NPY gene expression. *Peptides* **32**, 1363-1370.

Yang, J., Zhao, T.J., Goldstein, J.L., Brown, M.S. (2008). Inhibition of ghrelin O-acyltransferase (GOAT) by octanoylated pentapeptides. *Proc Natl Acad Sci U S A* **105**, 10750-10755.

Yokobori, E., Kojima, K., Azuma, M., Kang, K.S., Maejima, S., Uchiyama, M., et al. (2011). Stimulatory effect of intracerebroventricular administration of orexin A on food intake in the zebrafish, *Danio rerio*. *Peptides* **32**, 1357-1362.

Yu, N., Smagghe, G. (2014). CCK(-like) and receptors: Structure and phylogeny in a comparative perspective. *Gen Comp Endocrinol* **209**, 74-81.

Yuan, D., Wang, T., Zhou, C., Lin, F., Chen, H., Wu, H., et al. (2014). Leptin and cholecystokinin in *Schizothorax prenanti*: molecular cloning, tissue expression, and mRNA expression responses to periprandial changes and fasting. *Gen Comp Endocrinol* **204**, 13-24.

Zhang, H., Chen, H., Zhang, Y., Li, S., Lu, D., Zhang, H., et al. (2013). Molecular cloning, characterization and expression profiles of multiple leptin genes and a leptin receptor gene in orange-spotted grouper (*Epinephelus coioides*). *Gen Comp Endocrinol* **181**, 295-305.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432.

Zheng, J.L., Luo, Z., Zhu, Q.L., Tan, X.Y., Chen, Q.L., Sun, L.D., et al. (2013). Molecular cloning and expression pattern of 11 genes involved in lipid metabolism in yellow catfish *Pelteobagrus fulvidraco*. *Gene* **531**, 53-63.

Zimov, S., Yazulla, S. (2004). Localization of vanilloid receptor 1 (TRPV1/VR1)-like immunoreactivity in goldfish and zebrafish retinas: restriction to photoreceptor synaptic ribbons. *J Neurocytol* **33**, 441-452.