Paper de leptina i grelina sobre el teixit adipós i estudi de la proteolisi muscular en peixos

Role of leptin and ghrelin on adipose tissue and study of muscle proteolysis in fish

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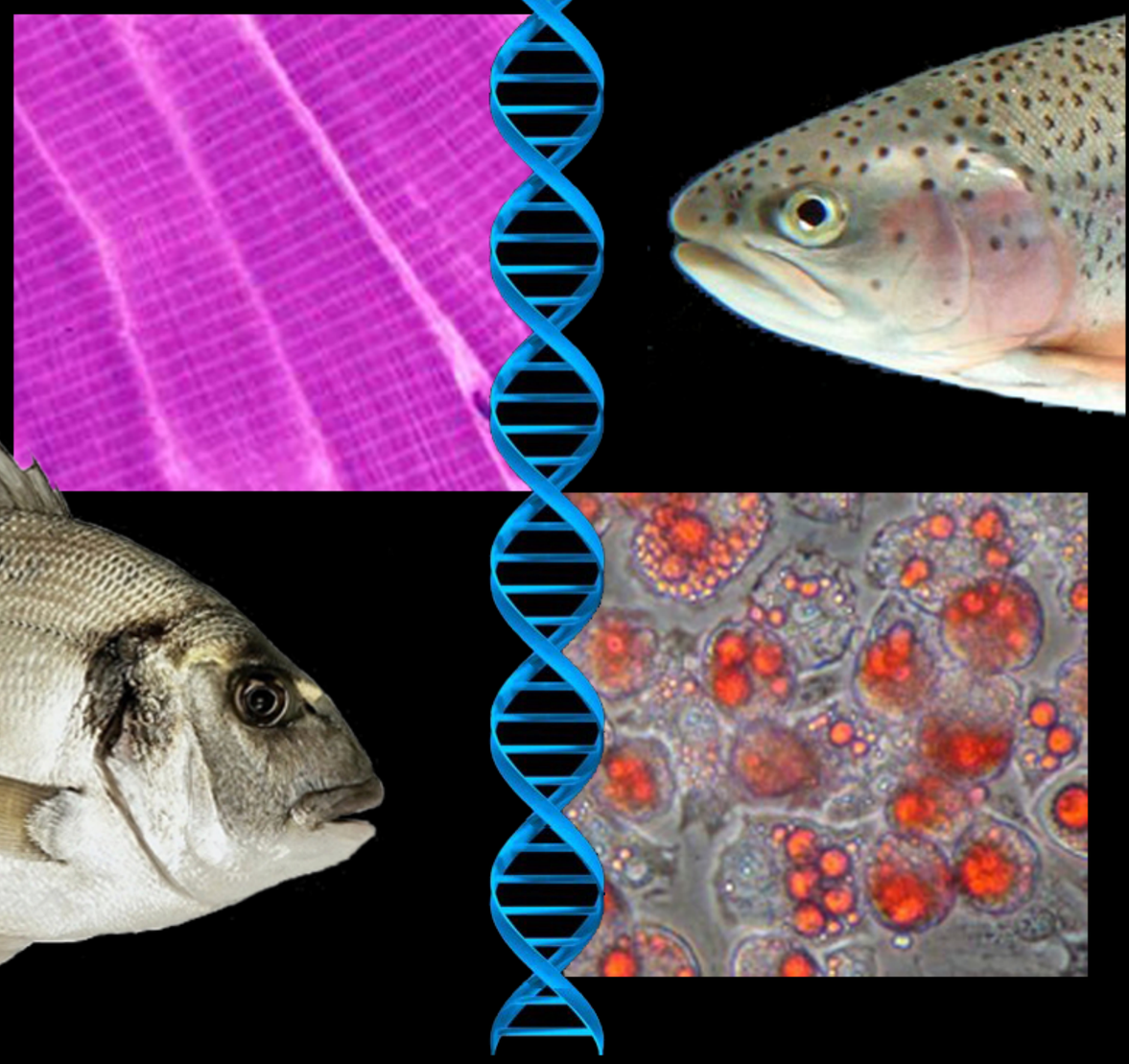

Cristina Salmerón Salvador

Tesi Doctoral

Gener 2014

FACULTAT DE BIOLOGIA

DEPARTAMENT DE FISIOLOGIA I IMMUNOLOGIA

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Paper de leptina i grelina sobre el teixit adipós i estudi de la proteòlisi muscular en peixos.

Memòria presentada per

Cristina Salmerón Salvador

Per optar al grau de

Doctora per la Universitat de Barcelona

Tesi realitzada sota la direcció de la Dra. Encarnación Capilla Campos del Departament de Fisiologia i Immunologia, Facultat de Biologia.

Adscrita al Departament de Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Programa d'Aqüicultura.

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Barcelona, Gener 2014

Agraïments

Agraïments

Aquesta tesi ha estat possible gràcies a moltes persones i institucions, que han dipositat la seva confiança per a que realitzés aquest gran projecte científic de fisiologia de peixos.

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notícies, però com són peixos, això a ningú l'importa perquè la seva vida té poc valor". Per mi sí que tenen molt valor les seves vides, per aquest motiu en tots els experiments de la present tesi he intentat aplicar dos principis bàsics, que els animals estiguessin correctament anestesiats i sacrificats, i que els experiments es realitzessin amb cura per evitar repeticions.

Aquesta tesi ha estat realitzada gràcies al suport econòmic de diverses institucions, i a nivell personal gràcies a una beca del setè programa marc de la Unió Europea (projecte Lifecycle).

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Introducció general

1. Importància de l'aqüicultura

L'aqüicultura es defineix com el cultiu d'organismes aquàtics, i inclou peixos, crustacis, mol·luscs i vegetals aquàtics. Aquest cultiu implica algun tipus d'intervenció en el procés de cria per augmentar la producció, com ara l'estoc regular, alimentació, protecció contra els depredadors, etc., i comporta també la propietat individual o col·lectiva dels organismes que es cultiven (Working Party of Experts of Aquaculture, 1988). Tot i que l'aqüicultura fa centenars, fins i tot milers d'anys que es practica, no va ser fins a mitjans del segle XX quan va començar a tenir major impacte econòmic i mediàtic.

La pesca de captura i l'aqüicultura van subministrar al món aproximadament uns 154 milions de tones de producte el 2011, dels quals 131 milions es van destinar a l'alimentació. El 2011, l'aqüicultura va produir uns 63,6 milions de tones, on la contribució de l'aqüicultura continental i la marina va ser de 44,3 i 19,3 milions de tones, respectivament (Figura 1) (FAO, 2012).

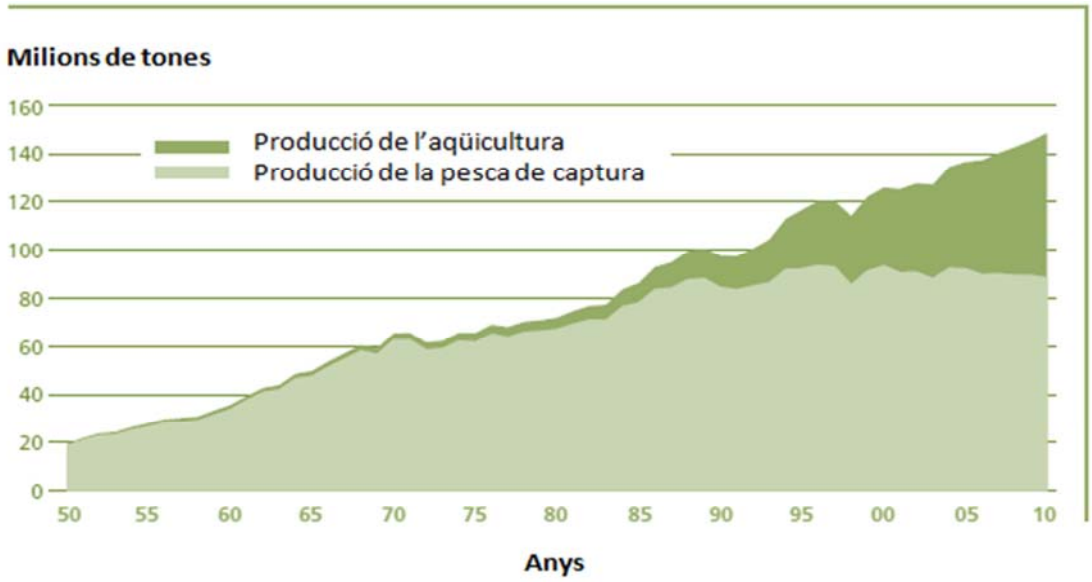


Figura 1. Producció mundial de l'aqüicultura i la pesca de captura. FAO, 2012.

En les tres últimes dècades (1980-2010), la producció mundial de peixos d'aqüicultura s'ha multiplicat per 12, a un índex mig anual del 8,8%. Aproximadament 600 espècies es cultiven actualment en captivitat en uns 190 països per a la seva producció aqüícola (FAO, 2012). El 2010, la composició de la producció aqüícola mundial va ser: peixos d'aigua dolça (56,4%, 33,7 milions de tones), mol·luscs (23,6%, 14,2 milions de tones), crustacis (9,6%, 5,7 milions de tones), peixos diàdroms (6,0%, 3,6 milions de tones), peixos marins (3,1%, 1,8 milions de tones) i altres animals aquàtics (1,4%, 814.300 tones).

El 2011, la producció d'aqüicultura de la Unió Europea va arribar als 1,26 milions de tones; el 75% d'aigua de mar, el 20,4% d'aigua dolça i el 4,6% d'aigua salobre. Els peixos van representar 647.156 tones del total, on la truita irisada (*Oncorhynchus mykiss*), el salmó Atlàntic (*Salmo salar*) i l'orada (*Sparus aurata*) van ser les tres espècies més cultivades amb 176.983, 171.034 i 98.840 tones, respectivament. A Espanya, les principals espècies d'aqüicultura produïdes varen ser: el musclo (*Mytilus* sp.), l'orada, la truita irisada, el llobarro (*Dicentrarchus labrax*) i el turbot (*Psetta maxima*), amb 212.556, 19.430, 16.818, 14.270 i 7.970 tones, respectivament (Figura 2) (APROMAR-ESACUA, 2013).

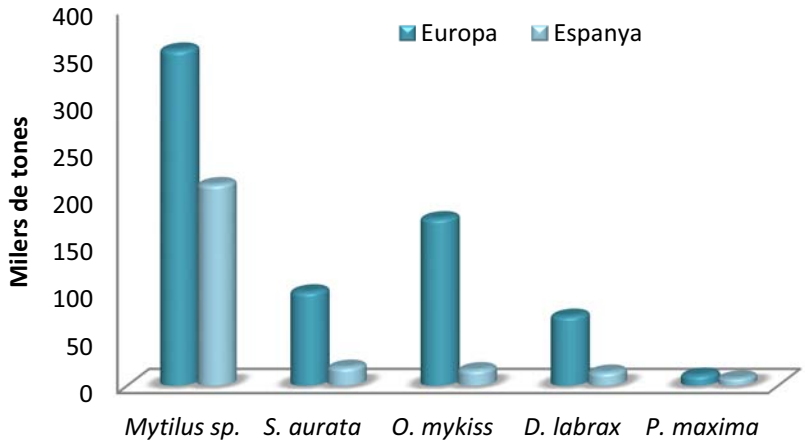


Figura 2. Producció total de productes d'aqüicultura per espècies el 2011 a Europa i Espanya. Dades de l'informe APROMAR-ESACUA, 2013.

Tot i el continu increment en els darrers anys, es calcula que per assolir la demanda, la producció mundial d'aliments ha de créixer en un 70% entre el 2010 i el 2050 (FAO, 2009). En relació als productes marins, més de la meitat del total que es consumeix al món avui en dia prové de granges aqüícoles, i el 2030 aquesta proporció serà de mitjana superior al 65%. La producció de l'aqüicultura marina (el 46,9% del total el 2011 (APROMAR-ESACUA, 2013)) té al seu favor que el 70% de la superfície de la Terra és aigua, i que per tant no requereix consum d'aigua dolça. A més a més, els animals aquàtics, són més eficients convertidors de l'aliment que els vertebrats terrestres, i les seves taxes de reproducció són diversos ordres de magnitud més altes que les d'aquests. Per aquesta raó, l'aqüicultura pot tenir un paper important en el futur per evitar la fam i la desnutrició humana, ja que proporciona proteïnes, olis essencials, com els olis poliinsaturats omega-3, vitamines i minerals que poden ajudar a millorar la salut i la qualitat de vida. En aquest sentit, algunes espècies, com és el cas de l'orada, tenen una producció actual procedent d'aqüicultura a nivell mundial que representa ja el 95,4% del total (Figura 3), i a Espanya del 92,4% (APROMAR-ESACUA, 2013).

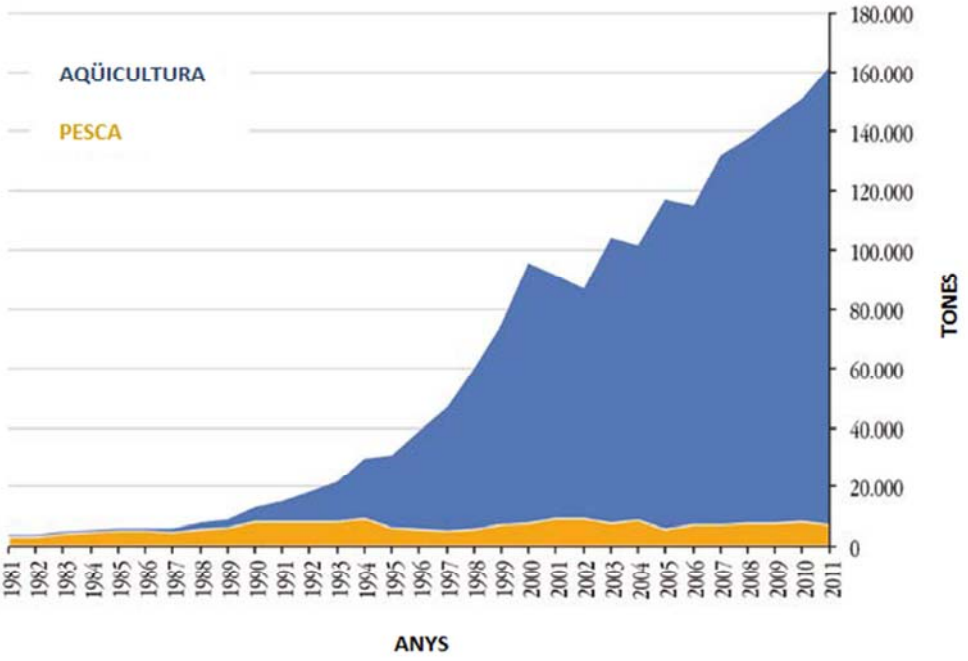


Figura 3. Producció mundial d'orada per l'aqüicultura i la pesca de captura. APROMAR-ESACUA, 2013.

L'aqüicultura però ha estat durament criticada, entre altres coses per l'explotació d'espècies marines per a produir aliment pels propis peixos o mariscs d'aqüicultura, com les anxoves, les sardines, el verat, l'arengada i la maire utilitzats per produir farina i oli de peix, i que ja es pesquen per sobre del seu límit biològic de seguretat; i també, en part per desconeixement, per l'apreciació de que els productes d'aqüicultura tenen menor qualitat que els seu homòlegs salvatges. Aquestes crítiques han impulsat a trobar solucions als diversos problemes com ara la recerca de fonts alternatives de farines i olis de peix per tal de desenvolupar una aqüicultura sostenible i de qualitat, i aquest èxit depèn, en gran mesura, de la recerca científica en aquests camps. No obstant, estudis enfocats a comprendre els factors relacionats amb la qualitat del producte, en base a les seves propietats organolèptiques, són encara relativament escassos.

El fet que la truita i l'orada es trobin entre les espècies més produïdes en aigües continental i marina, respectivament, i per tant consumides, a Europa i Espanya, les converteixen en una excel·lent elecció per a estudiar la seva biologia i millorar-ne la seva qualitat com a producte d'aqüicultura. El nostre grup de recerca té una àmplia experiència en la utilització d'aquestes espècies per a recerca bàsica en fisiologia, de manera que disposem de les eines necessàries pel seu estudi. Per aquestes raons, l'orada i la truita irisada seran els models de peixos cultivats utilitzats en la present tesi doctoral.

1.1. Biologia de les espècies d'estudi

Orada

L'orada (*Sparus aurata*) té un cos oval i aplanat lateralment, amb un cap gran amb el perfil arquejat i els ulls petits. Té una coloració gris platejada, amb una taca fosca al principi de la línia lateral estesa fins al marge superior de l'opercle, on continua a sota amb una banda rogenca. Mostra una característica banda daurada entre els ulls, que bordeja ambdues àrees fosques. L'aleta caudal forcada i amb els extrems negres. Pot arribar a fer uns 57 cm de longitud (Figura 4).

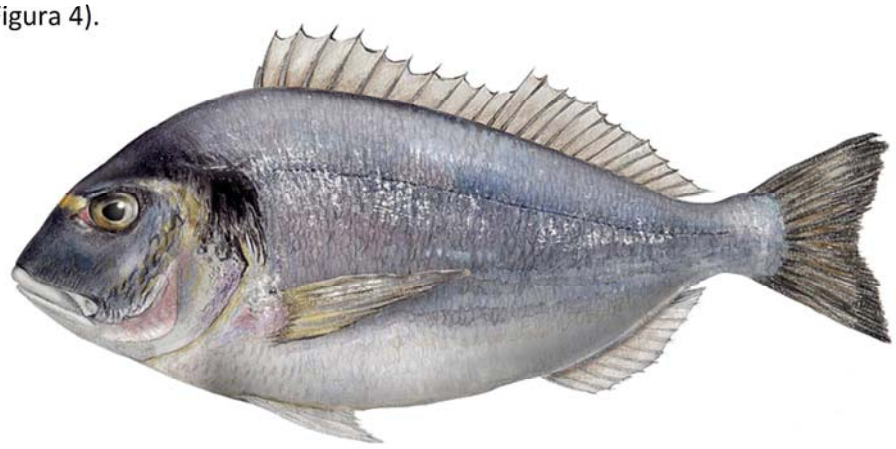


Figura 4. Orada (*Sparus aurata*).

L'orada es pot trobar comunament per tot el Mediterrani, tot i que la seva presència és menys freqüent en l'est i el sud-est Mediterrani i molt estranya en el Mar Negre. També es troba en les costes orientals de l'Oceà Atlàntic des de les Illes Britàniques fins a Cap Verd i al voltant de les Illes Canàries. És una espècie bentopelàgica (comportament demersal), que es troba en ambients costaners. Habita a les praderies marines, fons rocosos i sorrencs, així com a la zona de rompents i en profunditats d'uns 30 m, tot i que els adults es poden trobar fins a 150 m de profunditat. És una espècie eurihalina, i sovint entra en aigües salobres de llacunes i aiguamolls, i també tolera amplies variacions de temperatura. És una espècie sedentària, que es troba solitària, o formant petites agregacions. És principalment carnívora (mol·luscs, particularment musclos que pot aixafar fàcilment, crustacis i peixos), però accessòriament herbívora. Pel que fa a la seva biologia reproductiva, és una espècie hermafrodita protàndrica; la majoria dels individus són mascles durant els dos primers anys (20-30 cm) i posteriorment es tornen femelles (33-40 cm). El seu període de posta acostuma a ser des de desembre a abril, quan la temperatura de l'aigua és de 13-17ºC. Poden arribar a viure més de 10 anys (APROMAR-ESACUA, 2013; Basurco et al., 2011).

Truita irisada

La truita irisada (*Oncorhynchus mykiss*) té un cos allargat i fusiforme, amb la bora de l'aleta adiposa de color negre. Té una coloració de blau a verd oliva, amb una banda irisada rosada al llarg de la línia lateral i platejada per sota d'aquesta. El llom, els costats, el cap i les aletes estan cobertes per petits punts negres. La seva coloració pot variar amb l'hàbitat, la mida, i la condició sexual, passant de fosc intens a brillant i platejat. En estat salvatge no acostuma a mesurar més de 40 cm, tot i que pot arribar a 1,2 m i pesar fins a 24 Kg (Figura 5).

Figura 5. Truita irisada (*Oncorhynchus mykiss*).

És nativa de les conques que drenen el Pacífic a Nord Amèrica, des d'Alaska a Mèxic. Des del 1874 ha estat introduïda en les aigües de tots els continents excepte l'Antàrtida, amb propòsits d'oci per a la pesca esportiva i per l'aqüicultura. És una espècie que pot ocupar molt hàbitats diferents, que van des d'un cicle de vida anàdrom (viuen en l'oceà però fresen en rius i corrents amb fons de grava, fluxos ràpids i ben oxigenats); fins a habitar de forma permanent en llacs, on qualsevol població d'aquesta espècie és capaç d'emigrar al mar, sent capaces d'adaptar-se a tot tipus d'aigües. En el seu hàbitat, els adults són generalistes, alimentant-se d'invertebrats i peixos de mida petita, mentre que els alevins s'alimenten de zooplàncton. És una espècie que tolera un ampli rang de temperatures (0-27ºC), però la fresa i el creixement tenen lloc en un marge més estret (9-14ºC). La temperatura i la disponibilitat d'aliment influencien el creixement i la maduració, fent que l'edat de maduració sexual varií; tot i que generalment hi arriben als 3-4 anys. La soca anàdroma de l'espècie (coneguda con cap d'acer o "steelhead") acostuma a viure uns 11 anys, remuntant els rius per primera vegada per reproduir-se en el tercer any de vida, pel que acostumen a reproduir-se unes 8 vegades a la vida. La soca no anàdroma acostuma a viure un màxim de 6 anys, reproduint-se per tant només 3 vegades a la vida. En un cicle de vida anàdrom tenen un ràpid creixement, assolint els 7-10 Kg en 3 anys, mentre que en aigua dolça només arriben als 4,5 Kg en el mateix temps. (APROMAR-ESACUA, 2013; FAO, 2005-2012).

1.2. La qualitat en el producte d'aqüicultura

El producte final d'aqüicultura té uns requisits de qualitat indispensables per a ser comercialitzat. En general, un producte té una bona o correcta qualitat quan és segur per a la salut dels seus consumidors i satisfà les seves demandes i expectatives. El terme qualitat és difícil de definir i es compon de molts atributs diferents, incloent una àmplia gamma de característiques nutricionals i organolèptiques del producte (Grigorakis, 2007). Els tres elements principals que influeixen en el consum i l'acceptabilitat del peix són: el color, la textura i el gust. Un dels handicaps que té l'aqüicultura és l'apreciació per part dels consumidors, que opina que aquests peixos són de menor qualitat que els seus homòlegs salvatges (Grigorakis et al., 2003). Aquestes valoracions negatives van enfocades principalment a la qualitat (textura) del filet (Periago et al., 2005), així com al contingut de greix, sobretot perivisceral, el qual s'ha demostrat que sol ser més elevat en les espècies cultivades que en les salvatges (Alasalvar et al., 2002; Grigorakis, 2007).

Múscul

Al voltant del 70% del cos d'un peix és múscul, i és aquesta part la que té un valor més elevat en el mercat perquè és la que consumim (Figura 6). Els consumidors volen que el peix sigui fresc i que la seva carn tingui una textura ferma. La textura de la carn ve determinada per diversos factors tals com la cel·lularitat muscular i el nombre i mida de les fibres musculars (Hurling, 1996; Johnston et al., 2000; Periago et al., 2005); així com pel contingut de greix (Grigorakis et al., 2003). D'altra banda, en espècies com l'orada, el contingut de greix intramuscular sembla afectar també directament el color del filet (Grigorakis et al., 2003). Els diferents paràmetres relacionats amb la textura es poden veure afectats per: la dieta (Alami-Durante et al., 2010; Kiessling et al., 1991), el fotoperíode (Johnston et al., 2004), la temperatura (Johnston et al., 1998; López-Albors et al., 2008) i l'exercici (Ibarz et al., 2011; Totland et al., 1987) entre d'altres. A més a més, tots els processos que afectin el recanvi proteic; tant el creixement (síntesi proteica) com la degradació muscular (proteòlisi), acabaran repercutint també en la qualitat del filet.

Figura 6. Filets d'orada.

Quan un animal mor, s'inicia el procés de degradació muscular, degut inicialment a l'acidificació del medi per la glucòlisi anaeròbica *post-mortem*, afavorint primerament l'acció d'enzims propis de la cèl·lula (proteases) i posteriorment el creixement de bacteris, els quals també contribuiran a fer malbé el producte. L'acció dels enzims endògens, durant la proteòlisi muscular *post-mortem*, s'associa directament a la degradació de les proteïnes que componen les fibres musculars, provocant la tenderització o estovament de la carn (Bond and Warner, 2007; Huff-Lonergan et al., 1996; Huff-Lonergan et al., 2010; Kemp et al., 2010; Koohmaraie, 1996). La contribució de la proteòlisi en aquest procés està regulada principalment per: la quantitat de proteases presents en el múscul en el moment del sacrifici, la durada de l'estovament de la carn *post-rigor* i l'activitat de les proteases durant aquest període (Koohmaraie and Geesink, 2006).

Per tant, tenint en compte que es pretén obtenir un filet fresc i amb una bona textura és molt important conèixer la regulació d'aquestes proteases en el múscul, per tal de minimitzar els seus efectes negatius sobre la qualitat de la carn.

Teixit adipós

Quan un peix rep un excés d'energia en la dieta, aquesta s'acumula en forma de reserves com el greix. Aquest greix, segons l'espècie és emmagatzemat principalment al voltant de les vísceres (Figura 7), al fetge, i també al múscul o a nivell subcutani (Sheridan and Kao, 1998). A part de la importància del contingut lipídic a nivell del múscul, pel seu efecte en la qualitat de la carn, en les espècies que es comercialitzen senceres, la quantitat de greix perivisceral és també molt rellevant. En aquest procés d'emmagatzematge d'energia és molt important el paper que juga la nutrició, així com també certes hormones i factors de creixement.

Figura 7. Greix perivisceral a la truita irisada.

La majoria dels peixos d'aqüicultura són carnívors, amb alts requeriments de proteïna i amb una limitada tolerància als carbohidrats en la seva dieta, a diferència dels mamífers.

Malauradament, la proteïna que s'utilitza en les dietes per aqüicultura prové principalment de farina de peix i, tal com l'oli de peix, també utilitzat en els pinsos per a peixos, aquest és un recurs car i amb un important impacte ecològic. Per aquest motiu, la recerca de fonts alternatives de farines i olis de peix és clau per tal de desenvolupar una aqüicultura sostenible i de qualitat. Aquestes fonts alternatives són: ingredients d'origen vegetal, subproductes animals o organismes unicel·lulars (Tacon and Metian, 2008; Watanabe, 2002), però han de ser assequibles i d'alta qualitat (FAO, 2012). Per tal de reduir la quantitat de farina de peix en les dietes, sense comprometre els requeriments nutritius de l'animal, les dietes d'avui en dia tenen un elevat percentatge de lípids, els quals en molts casos provenen a més de productes vegetals. Els avantatges dels lípids són que tenen una alta digestibilitat i són més del doble d'energètics que els carbohidrats i les proteïnes. A més, les dietes altes en greixos poden ajudar a maximitzar l'energia procedent d'aquests lípids, permetent estalviar proteïna per a creixement (Sargent and Tacon, 1999). D'altra banda, un dels principals problemes que pot causar una alta incorporació de lípids en la dieta dels peixos és que en excés, l'energia pot acabar acumulant-se en forma de greix, alterar el contingut lipídic i el metabolisme d'àcids grassos (en relació amb els canvis en les activitats enzimàtiques) i perjudicar la qualitat organolèptica (en especial si el lípid és d'origen vegetal) i variar per tant, les seves propietats alimentàries (Benedito-Palos et al., 2008; Benedito-Palos et al., 2009; Bouraoui et al., 2011; Cruz-Garcia et al., 2011a; Sitjà-Bobadilla et al., 2005). Tot això, pot arribar a afectar directament la salut i el benestar de l'animal.

Per tant, tots els estudis encarats a conèixer millor el funcionament i la regulació del creixement i el metabolisme del teixit adipós, poden ajudar a trobar solucions per minimitzar l'excés de greix en aquelles espècies de peixos en que això no sigui apreciat, i garantir una millor qualitat del producte d'aqüicultura.

2. Múscul esquelètic

El múscul esquelètic, també conegut com múscul estriat per la seva morfologia estriada quan s'observa en el microscopi, s'utilitza principalment per la locomoció i el manteniment de la postura, però també com a reservori proteic i energètic. El múscul esquelètic està sota les ordres del sistema nerviós central i pot produir contraccions ràpides i anaeròbiques (múscul blanc), importants pels moviments bruscos d'escapament, així com contraccions lentes, sostingudes i aeròbiques (múscul vermell), que són les encarregades de la natació continuada. A nivell energètic, el múscul vermell és doncs preferiblement oxidatiu mentre que el múscul blanc és més glicolític (Luther et al., 1995).

2.1. Estructura del múscul

L'estructura general de la musculatura lateral del peix difereix molt de l'estructura dels músculs esquelètics d'altres vertebrats. El peix té un filet segmentat, on cada segment és un múscul separat anomenat miòtom. Cada miòtom està limitat per teixit connectiu intramuscular, classificat com a miocomata o miosepte de col·lagen, el qual actua com a punt de fixació per a les fibres musculars (Figura 8A) (Bremner and Hallett , 1985; Love, 1970). Els miòtoms no són simples làmines de múscul, sinó que tridimensionalment estan disposats en angle respecte la línia del cos, amb la vora interior més a prop de la part davantera del cos i la vora exterior més a prop de la cua. Això fa que la vora que mira cap a l'exterior del miòtom tingui forma de "W" i que interiorment es formin estructures semblants a cons (Figura 8B) (Katz, 2002).

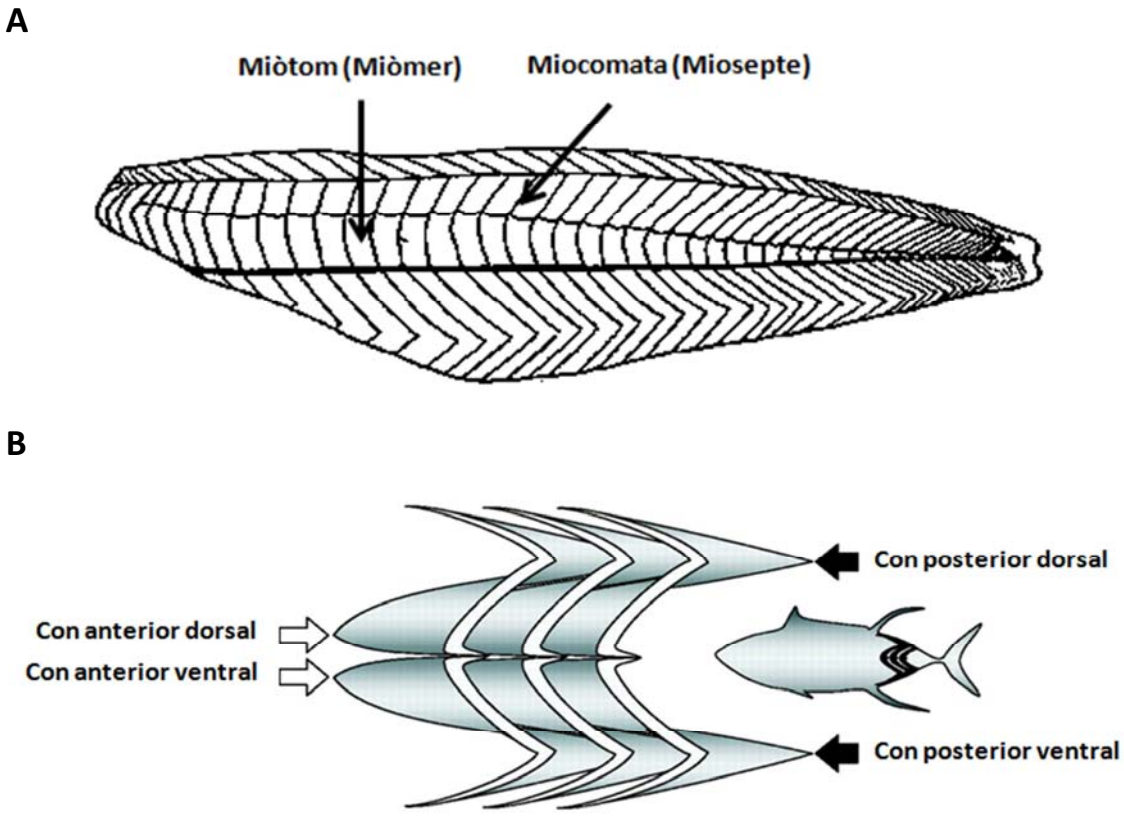


Figura 8. (A) Estructura d'un filet de peix després de l'eliminació de l'esquelet. La miocomata s'indica amb línies negres i separa cada segment muscular (miòtom). Adaptada de Love, 1970. **(B)** Estructura dels miòtoms en vista lateral. Aquest diagrama mostra tres miòtoms, cadascun separat per l'eliminació de dos miòtoms intermitjos per mostrar la naturalesa imbricada dels cons. La silueta del peix indica l'orientació dels miòtoms dins del filet. Cada con anterior s'indica amb una fletxa blanca, i cada con posterior amb una fletxa negra. Adaptada de Katz, 2002.

Cada miòtom es compon de diverses cèl·lules anomenades fibres musculars que s'orienten aproximadament en paral·lel a l'eix del cos, tot i que aquesta distribució varia quan ens allunyem de la superfície del cos. En la majoria de teleostis, les fibres musculars segueixen trajectòries helicoïdals. Els salmònids són una excepció en què les trajectòries no són helicoïdals, si no que s'estenen de la part anterior a la posterior dels cons miotomals, o des d'aquests cap a la columna vertebral (Alexander, 1969).

Les fibres musculars són cèl·lules multinucleades grans formades per la fusió de moltes cèl·lules (mioblasts). Cada fibra conté varies miofibril·les paral·leles dividides en unitats contràctils anomenades sarcòmers (Figura 9) (Sadava et al., 2008). El fet que els sarcòmers es repeteixen periòdicament, dóna a les miofibril·les dels vertebrats l'aspecte estriat. Dos tipus de proteïnes del miofilament conformen els sarcòmers, els monòmers d'actina i les molècules de miosina, generant un patró de bandes i línies (Figura 9). Els filaments de miosina i part dels filaments d'actina formen la banda-A, mentre que la resta dels filaments d'actina formen la banda-I. Tots els filaments de miosina de la banda-A estan connectats l'un a l'altre a través d'una estructura formant la línia o banda-M. Els filaments d'actina estan connectats a través del disc-Z (línia-Z) formant la banda-I dels sarcòmers adjacents. L'estructura de la línia-Z està disposada perpendicular a les miofibril·les i connecta els sarcòmers veïns.

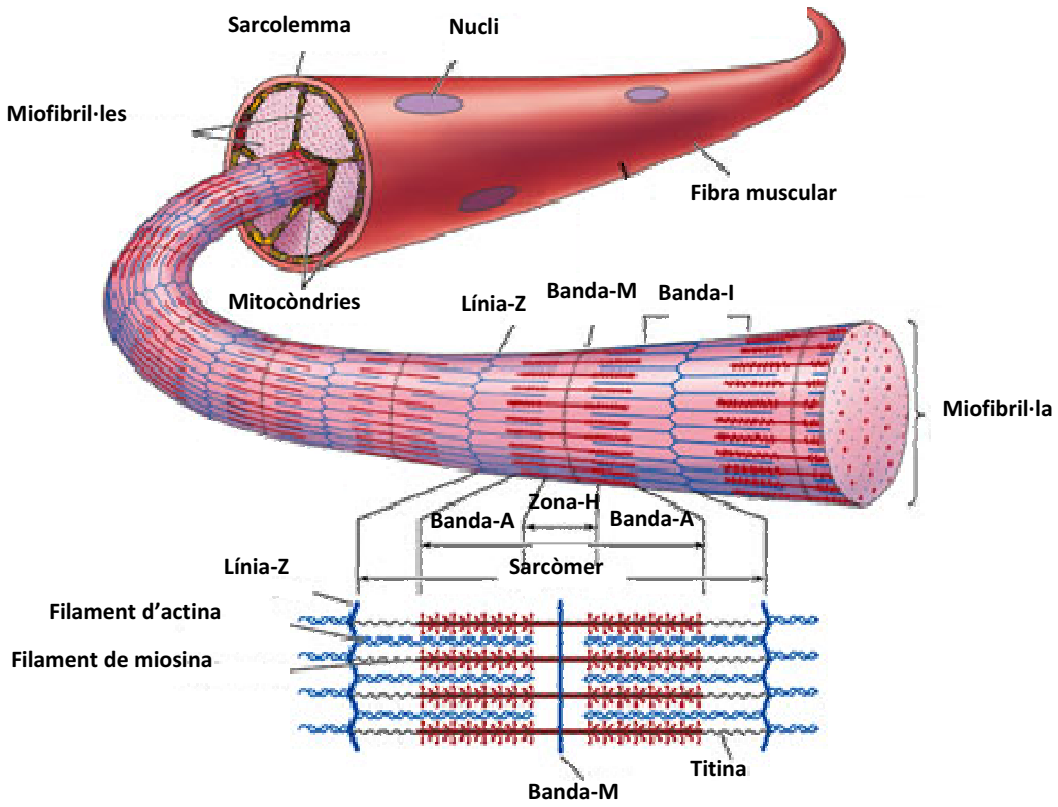


Figura 9. Estructura del múscul esquelètic: fibra muscular, miofibril·les, miofilaments i sarcòmer. Adaptada de Sadava et al., 2008.

A més de miosina i actina, moltes altres proteïnes completen l'estructura del sarcòmer (Figura 10). D'entre elles, per exemple els agregats de miosina estan connectats a la línia-Z a través de filaments elàstics de titina, que van des de la línia-Z fins a la banda o línia-M. A més a més de la interconnexió del disc-Z, els sarcòmers estan units entre ells a través dels filaments intermedis formats per desmina. També hi ha presents estructures del citoesquelet que connecten les línies-Z i -M amb el sarcolemma (membrana de la fibra muscular). Aquestes estructures s'anomenen costàmers, i es componen de proteïnes com espectrina, vinculina, α-actinina, distrofina, talina i filamina, que s'uneixen a l'actina i integrines associades a la membrana (García de la serrana et al., 2012). Les integrines estan unides al seu torn, a les proteïnes de la matriu extracel·lular, com el col·lagen i la fibronectina.

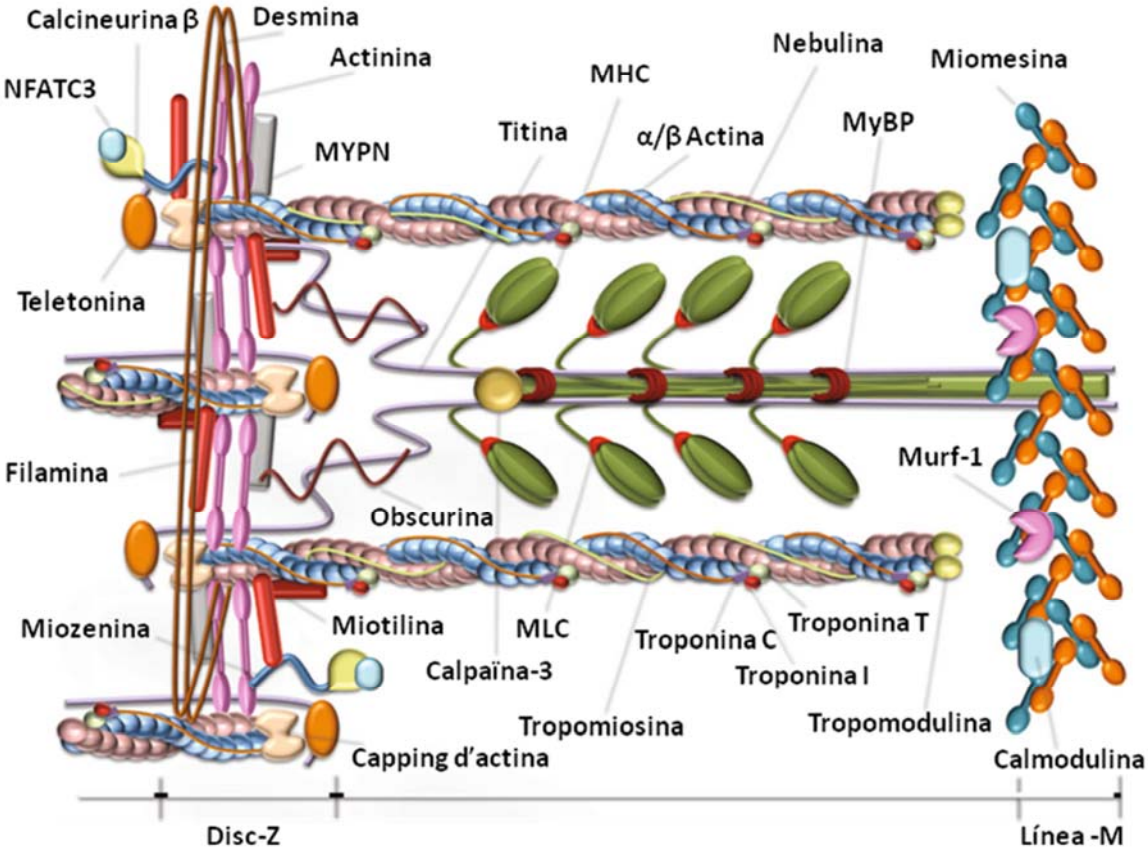


Figura 10. Reconstrucció de mig sarcòmer identificant les diverses proteïnes miofibril·lars. Adaptada de García de la serrana et al., 2012.

La quantitat de proteïna en el múscul del peix està entre el 15-20%. Les proteïnes miofibril·lars són les més abundants, més del 50%, on la miosina representa el 27% i l'actina l'11%. La miosina és la proteïna motora que interacciona amb l'actina, hidrolitza l'ATP i produeix el moviment. És un hexàmer, amb una massa molecular d'aproximadament 520 kDa, i consisteix en un parell de cadenes pesants (MHC) de 220 kDa i dos parells de cadenes lleugeres (MLC1 i

MLC2) de 20 kDa, i en un dels seus extrems té dos caps globulars units formats pels extrems N-terminals de les MHC i MLCs (Figura 10).

Molts estudis han demostrat que el debilitament de les miofibres és l'esdeveniment clau en l'estovament de la carn *post-mortem* i que el canvi estructural més consistentment reportat en aquest procés està associat amb els trencaments en la banda-I i el disc-Z (Dutaud et al., 2006; Ho et al., 1996; Koohmaraie and Geesink, 2006; Taylor et al., 1995). Les proteïnes que es degraden durant la proteòlisi de les miofibres són principalment proteïnes miofibril·lars i del citoesquelet. D'entre les proteïnes miofibril·lars que s'ha descrit que pateixen degradació *post-mortem* en el múscul de diferents espècies de peixos trobem: titina (Du et al., 2010; Seki and Watanabe, 1984), nebulina (Astier et al., 1991; Du et al., 2010), distrofina (Caballero et al., 2009; Papa et al., 1997), α-actinina (Jasra et al., 2001; Papa et al., 1996; Tsuchiya and Seki, 1991), miosina (Wu et al., 2010) i tropomiosina (Astier et al., 1991; Delbarre-Ladrat et al., 2004). La degradació de la cadena pesant de la miosina (MHC) condueix a la desestructuració de les miofibril·les, la qual influirà en la integritat del filet (Astier et al., 1991; Busconi et al., 1989).

A més de proteïnes, al voltant del 70-80% de la carn del peix està formada per aigua, però aquest percentatge pot variar en funció de l'espècie o a nivell individual; i també hi trobem lípids, tot i que la seva quantitat varia molt també en funció de l'espècie i pot variar a més segons l'època de l'any (FAO, 2012). Altres components menors del múscul són: els carbohidrats, els minerals, les vitamines i els extractes, com ara sucres, aminoàcids lliures, i substàncies volàtils, les quals canvien en funció del temps d'emmagatzematge *post-mortem* i poden servir com a indicadors de la qualitat del peix.

2.2. Proteòlisi

La taxa de creixement del múscul esquelètic es determina, en gran mesura, per l'equilibri entre la taxa de síntesi i la taxa de degradació (proteòlisi) i renovació de les seves proteïnes (Goll et al., 2008). Les proteases són els enzims que catalitzen la hidròlisi dels enllaços peptídics dins de les proteïnes. Gairebé el 2% de les proteïnes codificades pel genoma humà són proteases, constituint una de les classes d'enzims més grans (Puente et al., 2005). Les proteases exerceixen papers crítics en molts processos biològics com ara el desenvolupament, la diferenciació, la migració cel·lular, la immunitat, la cicatrització de ferides i la mort cel·lular.

Els quatre sistemes proteolítics principals coneguts són (Figura 11): el sistema dependent de calci (calpaïnes) (A), el sistema de proteases lisosomals (catepsines) (B), el sistema ATP-dependent d'ubiquitina-proteasoma (C), i el sistema de caspases (no mostrat a la figura).

Evidències recents apunten cap a la participació interactiva d'aquests sistemes de proteòlisi en la degradació muscular (Jackman and Kandarian, 2004). Les calpaïnes s'encarreguen principalment del processament proteolític, en lloc de la degradació, per modular o modificar l'activitat del substrat, especificitat, longevitat, localització i/o estructura. El sistema d'autofagosoma-lisosoma degrada principalment components cel·lulars no específics, incloent pèptids i microorganismes, continguts per membranes d'aïllament. En contrast, el sistema d'ubiquitina-proteasoma degrada i elimina proteïnes substrat-específiques marcades amb un sistema d'etiquetatge d'ubiquitina (Ub) (proteïnes poli-ubiquitinades). El sistema de les caspases és un important sistema proteolític intracel·lular amb funcions principalment apoptòtiques (Cowdhury et al., 2008).

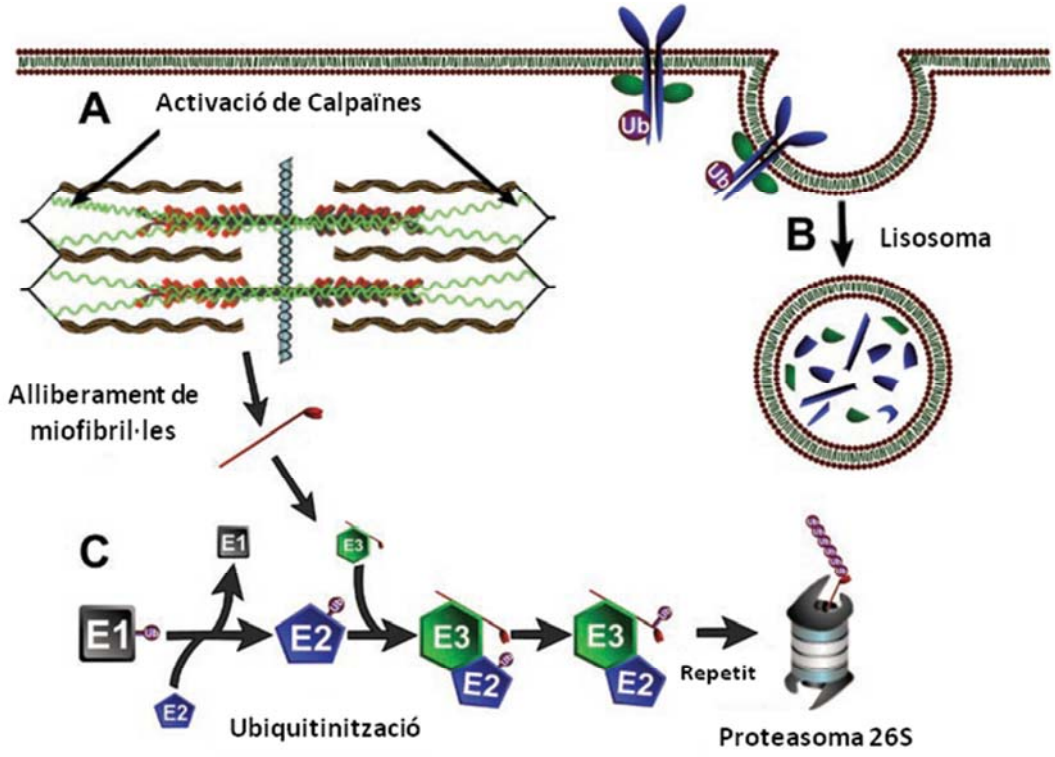


Figura 11. Principals sistemes proteolítics intracel·lulars. Adaptada de Jackman and Kandarian, 2004.

Calpaïnes

Les calpaïnes són proteases de cisteïna activades per calci que juguen un paper important en una àmplia gamma de processos cel·lulars com: l'apoptosi, la migració, la regulació del cicle cel·lular, el recanvi proteic, la remodelació, la miogènesi i el metabolisme entre d'altres (Campbell and Davies, 2012; Goll et al., 2003; Kar et al., 2010; Patel and Lane, 1999; Richard et al., 1995; Zhivotovsky and Orrenius, 2011). En mamífers s'han identificat 15 isoformes diferents de calpaïnes (Figura 12), que es poden dividir en: subunitats grans o catalítiques,

subunitats petites o reguladores i calpastatina, que és l'inhibidor específic de les calpaïnes. A més, les calpaïnes es poden agrupar en base a la presència o absència de dominis "EF-hand" d'unió a calci. Així doncs, trobem calpaïnes amb "EF-hand", les convencionals o típiques (calpaïnes 1, 2, 3, 8, 9, 11 al 14), i les atípiques (calpaïnes 5, 6, 7, 10, 15 i 16). Finalment, les calpaïnes també es poden classificar segons la seva localització, distingint-se calpaïnes ubiqües (calpaïnes 1, 2, 5, 7, 10, 13 al 16), i calpaïnes teixit-específiques (calpaïnes 3, 6, 8, 9, 11 i 12), que només s'expressen en determinats teixits, com per exemple el múscul esquelètic (calpaïna 3), el tracte gastrointestinal (calpaïnes 8 i 9), el testicle (calpaïna 11) i el fol·licle pilós (calpaïna 12) (Ono and Sorimachi, 2012).

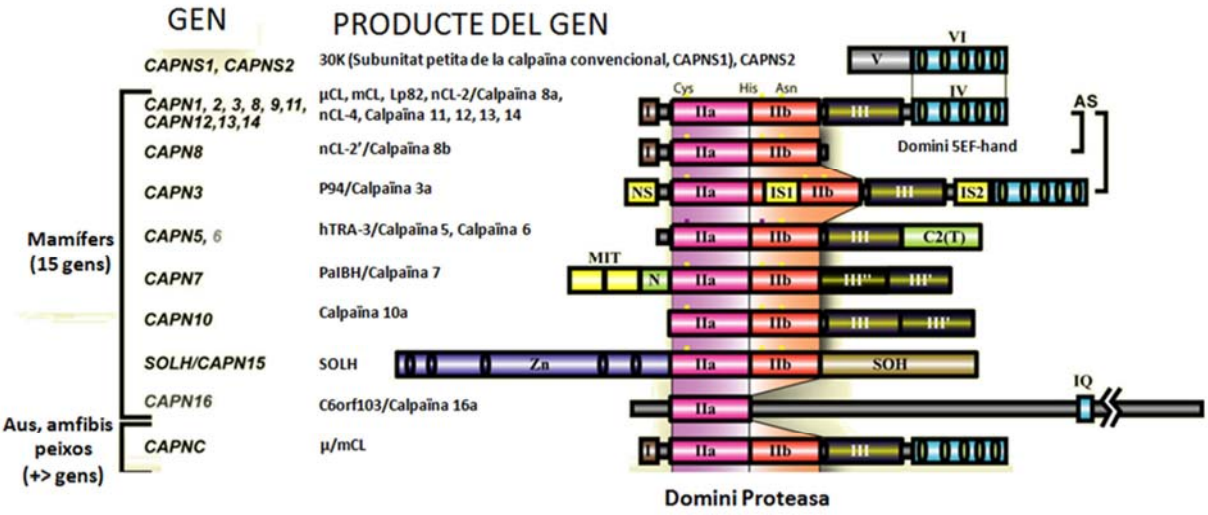


Figura 12. Estructura esquemàtica dels membres de la família de les calpaïnes identificats en vertebrats. Els símbols utilitzats són: I: domini N-terminal; IIa i IIb: sub-dominis de la proteasa que contenen els llocs actius de cisteïna i histidina, respectivament; III: domini semblant a C2 d'unió de Ca^{2+}; III' i III'': dominis moderadament i molt feblement similars al domini III, respectivament; IV i VI: domini 5-EF-hand d'unió de Ca^{2+}; V: domini hidrofòbic ric en glicina; NS, IS1 i IS2: seqüències específiques. Dades de calpain.org.

L'estructura de les calpaïnes 1 i 2, les més estudiades, revela 6 dominis: una seqüència N-terminal (I), dos dominis que constitueixen el lloc actiu (IIa i IIb), el domini III, la seqüència d'unió dels dominis III i IV, i el domini IV que conté 5 "EF-hand" (Figura 12) (Goll et al., 2003). La subunitat petita té 2 dominis: el domini V en la regió N-terminal, hidrofòbic i ric en glicines, i el domini VI amb 5 "EF-hand" (Figura 12) (Goll et al., 2003). Finalment, la calpastatina es caracteritza per tenir un domini N-terminal únic (domini XL i/o L), on el L determina la localització de l'enzim en la cèl·lula (De Tullio et al., 2009) i reprimeix parcialment l'entrada de Ca^{2+} (Minobe et al., 2006) i l'XL, que conté tres llocs potencials de fosforilació per quinases (Cong et al., 1998) té funció reguladora. A més, la calpastatina té fins a 4 dominis repetits cada

un dels quals pot inhibir una molècula heterodímer de calpaïna, encara que les seves activitats inhibidores varien (Figura 13A) (Goll et al., 2003). Cada domini inhibidor té tres regions o subdominis (A, B, i C), que s'uneixen al domini IV i al lloc actiu del domini II de la subunitat catalítica, i al domini VI de la subunitat reguladora, respectivament (Figura 13A i 13B). La regió B és l'única regió amb activitat inhibidora, ja que bloqueja l'accés dels substrats al lloc actiu de les calpaïnes (Figura 13B) (Kiss et al., 2008; Motter et al., 2009; Pfizer et al., 2008).

Les calpaïnes funcionen com un heterodímer format per la unió de dues subunitats: una subunitat gran o catalítica de 80 kDa formada generalment per les calpaïnes 1 o 2, anomenades també μ- o m-calpaïna, respectivament segons les concentracions de Ca^{2+} requerides per a la seva activació (3-50 μM i 400-800 μM, respectivament), i una subunitat petita o reguladora de 28 kDa comuna, la calpaïna s1 (Figures 12 i 13B) (Goll et al., 2003). Es creu que la subunitat petita actua com una xaperona, que possiblement ajuda a la subunitat gran en el seu correcte plegament conformacional per a que sigui activa. En ratolí s'ha demostrat que la manca de m-calpaïna o de subunitat petita és letal en embrions, mentre que els ratolins nuls per μ-calpaïna o calpastatina són sans (Arthur et al., 2000; Azam et al., 2001; Dutt et al., 2006; Takano et al., 2005; Zimmerman et al., 2000).

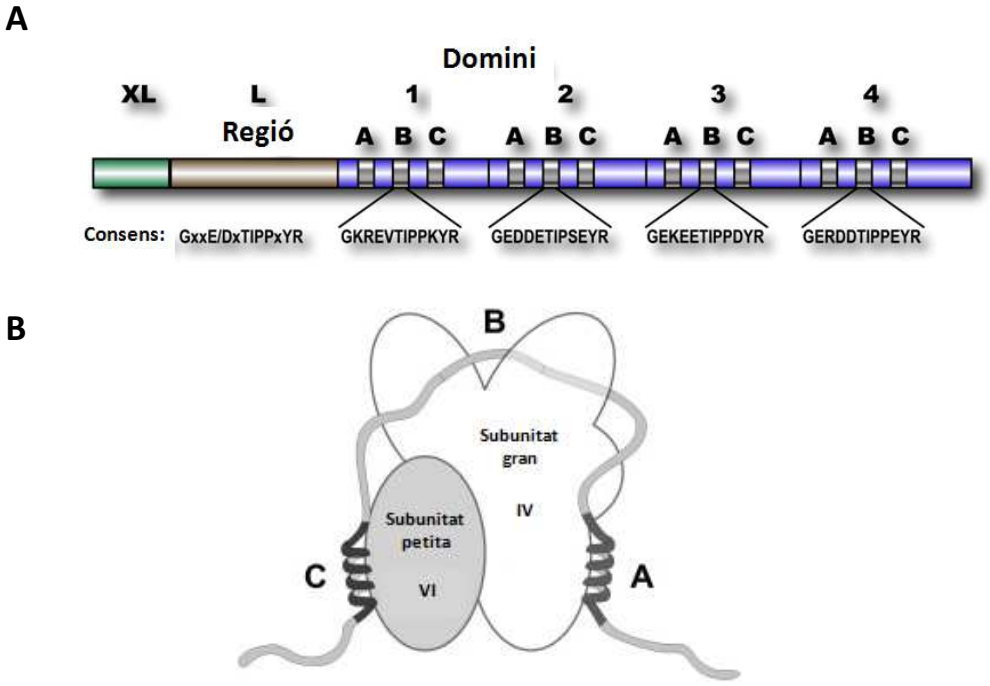


Figura 13. (A) Estructura esquemàtica de la calpastatina humana. Dades de calpain.org.
(B) Interacció entre calpastatina i calpaïna. Adaptada de Motter et al., 2009.

La μ-calpaïna i la calpastatina tenen una gran importància en mamífers per a la producció, composició i/o qualitat de la carn, ja que s´han descrit variants al·lèliques (SNP) relacionades amb aquestes característiques (Casas et al., 2006; Page et al., 2002; Page et al., 2004; White et

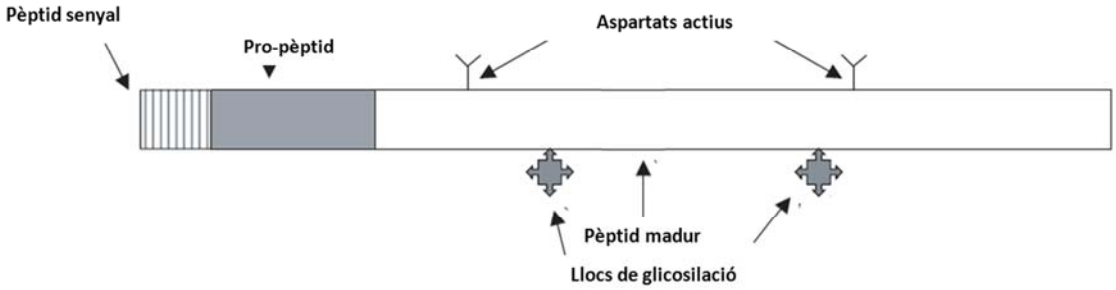
al., 2005). Un SNP (polimorfisme d'un únic nucleòtid) és un marcador molecular bi-al·lèlic i co-dominant. En la μ-calpaïna i la calpastatina s'han associat SNP específics amb diferències en la tendresa de la carn en vaques, que actualment s'utilitzen per a selecció gènica (GeneSTAR, Pfizer Animal Genetics). D'altra banda, tot i que la calpaïna 3 s'ha descrit com una calpaïna específica de múscul, s'ha trobat correlació entre els seus nivells d'expressió i la tendresa de la carn en alguns grups animals (vaques i ovelles), però no en altres (porc) (Ilian et al., 2001; Parr et al., 1999). No obstant, pel fet que també pot degradar la calpastatina i les calpaïnes ubiqües, s'ha suggerit que la calpaïna 3 pot tenir un paper regulador de l'expressió i l'activitat proteolítica de les pròpies calpaïnes a múscul (Ono et al., 2004), pel que més estudis es fan necessaris.

En peixos, també s'han descrit diversos membres de la família de les calpaïnes, així com també diverses calpastatines generades per "splicing" diferencial en diferents espècies, incloent la truita irisada (Salem et al., 2005a; Salem et al., 2005b), i s'ha descrit que la seva expressió pot estar modulada per l'estat nutricional. A més a més, l'expressió de calpastatina o la relació calpaïna/calpastatina s'ha vist que és major en truites amb creixement més ràpid i fillet més ferm que en soques de creixement més lent i carn més tova (Salem et al., 2005b). No obstant, a diferència dels mamífers, on la major part de l'estovament de la carn s'associa amb l'activitat de les calpaïnes (Koohmaraie, 1996), en peixos s'ha suggerit que les calpaïnes i les catepsines probablement actuen de forma complementària i sinèrgica en els diferents nivells de degradació de les proteïnes miofibril·lars (Delbarre-Ladrat et al., 2006).

Catepsines

Les catepsines són enzims proteolítics presents en els lisosomes de diversos tipus de cèl·lules i exhibeixen una àmplia gamma de funcions (Brix et al., 2008; Turk et al., 2001). Estan involucrades en la resposta immune i la presentació d'antígens (Zavasnik-Bergant and Turk, 2006), així com en l'activació d'hormones i enzims de vies metabòliques (Dunn et al., 1991; Hook et al., 2004). *In vivo*, la majoria de les catepsines s'activen a pH baix (de 3.5 fins a 6.5) que és el que es troba en els lisosomes (Goll et al., 2008). Les catepsines es classifiquen segons els residus nucleòfils presents en els llocs actius responsables de l'acció proteolítica (Rawlings et al., 2006; Turk et al., 2001) en: serina proteases (catepsines A i G), aspàrtic proteases (catepsines D i E), i cisteïna proteases (catepsines B, C, F, H, K, L, O, S, V, X i W). Les catepsines es sintetitzen com a zimògens, formats pel pèptid senyal, un pro-pèptid i la proteïna madura (Figura 14) (Rawlings et al., 2006). El pèptid senyal s'escindeix en el reticle endoplasmàtic i la pro-proteïna s'activa per eliminació proteolítica de l'extrem N-terminal, ja sigui per autocatàlisi

en ambients àcids, o per altres proteases. Finalment, la cadena simple amb activitat enzimàtica pateix una escissió addicional dividint-se en una cadena lleugera i una cadena pesada (Erikson et al., 1981). L'àrea de pro-pèptid de la catepsina exerceix múltiples funcions, podent actuar com a inhibidor per bloquejar l'accés al lloc actiu, regulant l'activitat de la pròpia catepsina. A més, el pro-pèptid pot actuar com una xaperona intramolecular que ajuda en el plegament, o en el tràfic intracel·lular, dirigint la proteïna al seu destí (Turk et al., 2002). Algunes de les



catepsines s'expressen ubiquament i d'altres són específiques de teixit.

Figura 14. Representació esquemàtica d'una catepsina aspàrtic peptidasa. Adaptada de Fusek and Vetvicka, 2005.

Després de la mort, quan el pH en les cèl·lules del múscul disminueix i la membrana lisosomal es veu alterada, les catepsines són alliberades al citosol, guanyant accés a les proteïnes miofibril·lars que aleshores lisen (Kemp et al., 2010). Existeix una proteïna que inhibeix l'activitat de les catepsines codificada pel gen *CTSB*. Es troba en el citosol i inhibeix les proteases alliberades dels lisosomes (Turk and Bode, 1991). També es coneix com inhibidor de catepsina B, perquè es sospita que bloqueja només l'activitat d'aquesta catepsina. Tenint en compte que les catepsines i el seu inhibidor juguen un paper important en molts processos metabòlics, han estat molts d'ells seleccionats com a gens candidats associats amb trets de la qualitat de la carn en mamífers. En porc, polimorfismes SNP dels gens de les catepsines D, F i Z, s'han associat amb el gruix del greix dorsal, el pes del pernil i el guany diari de pes (Fontanesi et al., 2011; Russo et al., 2008); i altres investigacions, també amb porc, van trobar una relació entre el genotip de catepsina B i el rendiment en el pes del pernil i el llom (Piórkowska et al., 2012).

En peixos, la degradació de les proteïnes miofibril·lars té també greus conseqüències pel teixit muscular, i s'ha demostrat que diverses proteïnes musculars són susceptibles a l'acció de les catepsines. Les catepsines B, D i L són capaces de degradar α-actinina i MHC (Delbarre-Ladrat et al., 2003). El mateix estudi va demostrar que la tropomiosina i l'actina només eren susceptibles a l'acció de la catepsina L, mentre que la troponina T i la desmina podien ser

degradades per les catepsines B i L. La degradació de l'α-actinina condueix a un debilitament del disc-Z, mentre que la proteòlisi de miosina, tropomiosina, troponina T i desmina desestructura les miofibril·les (Delbarre-Ladrat et al., 2006). Bahuaud et al., (2010) van demostrar en el salmó Atlàntic de piscifactoria que l'estrès abans del sacrifici augmenta significativament l'expressió del gen de la catepsina L muscular, i que també tendeix a augmentar l'expressió gènica de la catepsina B i la seva activitat. Aquests augments d'expressió i activitat de catepsina B i L es van correlacionar amb un augment de proteòlisi muscular i menor textura, de manera que l'activació d'aquestes catepsines induïdes per l'estrès accelera la degradació muscular. La mateixa relació entre catepsines, especialment catepsina D, i fermesa del filet també s'ha trobat en truita irisada (Godiksen et al., 2009).

Via ubiquitina-proteasoma

La via ubiquitina-proteasoma és un important mecanisme cel·lular que regula els nivells de proteïna intracel·lular, degradant proteïnes de vida curta, controlant la qualitat de les proteïnes del citoplasma i el nucli tot eliminant les proteïnes danyades, mal plegades, i mutants, i està implicada en la regulació de la transcripció de gens, i en el funcionament del sistema immunològic, entre d'altres (Glickman and Ciechanover, 2002; Lecker et al., 2006). A més, és la maquinària cel·lular bàsica en l'atròfia muscular en mamífers (Ciechanover, 1998). Els substrats de la via ubiquitina-proteasoma estan covalentment conjugats a l'Ub, una proteïna de 76-residus altament conservada. Tres components enzimàtics estan involucrats en aquest procés, E1 (enzim d'activació d'Ub), E2 (enzim de conjugació d'Ub) i E3 (lligasa d'Ub), que confereix especificitat pel sistema presentant llocs de reconeixement de substrat (Figura 11). Les proteïnes poli-ubiquitinades estan dirigides principalment als proteasomes 26S per a la seva proteòlisi en petits pèptids de 3-24 aminoàcids (Kisselev et al., 1999; Nussbaum et al., 1998; Pickart and Cohen, 2004). A més, Bodine et al., (2001) van identificar un petit subconjunt de gens que sempre es trobaven incrementats en diversos models d'atròfia, dos dels quals codifiquen Ub lligases: MuRF1 i MAFbx, també conegut com atrogina-1.

En peixos, el sistema ubiquitina-proteasoma s'ha estudiat principalment en relació amb el dejuni prolongat que aquests pateixen en la natura degut a canvis estacionals o migracions. En la truita irisada s'ha vist que diversos enzims com l'atrogina-1 i subunitats del proteasoma veuen augmentada la seva expressió, juntament amb un increment en el contingut de proteïnes poli-ubiquitinades durant el dejuni, suggerint un augment de la proteòlisi muscular en aquestes condicions; i s'ha descrit també una posterior reducció en resposta a la realimentació (Rescan et al., 2007; Seliez et al., 2008).

3. Teixit adipós

El teixit adipós és un tipus de teixit connectiu especialitzat format principalment per cèl·lules plenes de lípids, anomenades adipòcits, immerses en una matriu extracel·lular formada per una estructura majoritàriament de fibres de col·lagen. Els adipòcits generalment es descriuen com derivats de la capa mesodèrmica de l'embrió, a partir de cèl·lules mare mesenquimàtiques (MSC) (Hassan et al., 2012). A més dels adipòcits, el teixit adipós conté cèl·lules de la fracció estroma-vascular, incloent cèl·lules fibroblàstiques, leucòcits, macròfags i pre-adipòcits, que contribueixen a la integritat estructural i constitueixen al voltant del 50% del contingut cel·lular total (Hassan et al., 2012). El teixit adipós és vital pel manteniment de l'homeòstasi de l'energia del cos. Tradicionalment s'ha considerat com un simple dipòsit d'energia, sintetitzant i emmagatzemat triglicèrids (TG) en els períodes d'excés de calories, i alliberant àcids grassos (FA) i glicerol durant els períodes de privació nutricional. No obstant això, ara es reconeix que el teixit adipós juga un paper més actiu, amb múltiples funcions com a òrgan endocrí, secretant molècules biològicament actives, anomenades adipoquines, les quals actuen sobre el sistema nerviós central i els teixits perifèrics regulant molts processos, com per exemple la homeòstasi de la glucosa i el metabolisme dels lípids (Saltiel, 2001).

3.1. Adipogènesi

El teixit adipós creix per augment dels adipòcits pel que fa a la seva mida (hipertròfia), al seu nombre (hiperplàsia) o ambdós. La hipertròfia és el resultat de l'acumulació de TG en els adipòcits existents, i la hiperplàsia es basa en la formació de nous adipòcits a partir de cèl·lules precursores presents en el teixit adipós, i implica la proliferació i la diferenciació d'aquestes (Hausman et al., 2001).

La diferenciació dels adipòcits és un procés dividit en dos passos (Figura 15). En primer lloc, les MSC pluripotents se sotmeten a un procés conegut com a determinació (Boone et al., 2000; Gregoire et al., 1998). Aquest procés resulta en cèl·lules similars a fibroblasts que apareixen morfològicament idèntiques a MSC pluripotents, però que només són capaces ja de diferenciar-se en adipòcits. Com a resultat, les MSC en aquest estat de post-determinació s'anomenen pre-adipòcits o adipoblasts. La segona etapa, pròpiament de diferenciació, és la formació dels adipòcits estructuralment madurs a partir dels pre-adipòcits, i es coneix comunament com adipogènesi (Medina-Gómez, 2012).

El procés d'adipogènesi es caracteritza per l'expressió d'una sèrie de factors de transcripció en una cascada de regulació que resulta en l'expressió de diferents gens associats amb el

metabolisme lipídic del teixit adipós, els quals incrementen la capacitat lipogènica de la cèl·lula (Boone et al., 2000; Gregoire et al., 1998; Hausman et al., 2001). Primer s'activen les proteïnes d'unió al potenciador CCAAT β i δ (C/EBPβ i C/EBPδ, respectivament), les quals regulen l'expressió de C/EBPα i el receptor activat per proliferadors de peroxisomes γ (PPARγ) (Figura 15). PPARγ, membre de la superfamília de receptors nuclears, és considerat com el regulador clau de l'adipogènesi, ja que és necessari i suficient per a que es doni i es mantingui aquest procés (Rosen et al., 2000). C/EBPα i PPARγ, coordinen llavors l'expressió de gens específics de l'adipòcit com ara la proteïna d'unió a àcids grassos-2 (aP2), l'àcid gras sintasa (FAS), l'acetil-CoA carboxilasa (ACC), la lipoproteïna lipasa (LPL), l'ATP-citrat liasa (ACL) i la translocasa de FA o clúster de diferenciació 36 (CD36), molts d'ells característics dels últims estadis de diferenciació de l'adipòcit (Fajas, 2003; Farmer, 2006; Lowe et al., 2011; Rosen and MacDougald, 2006).

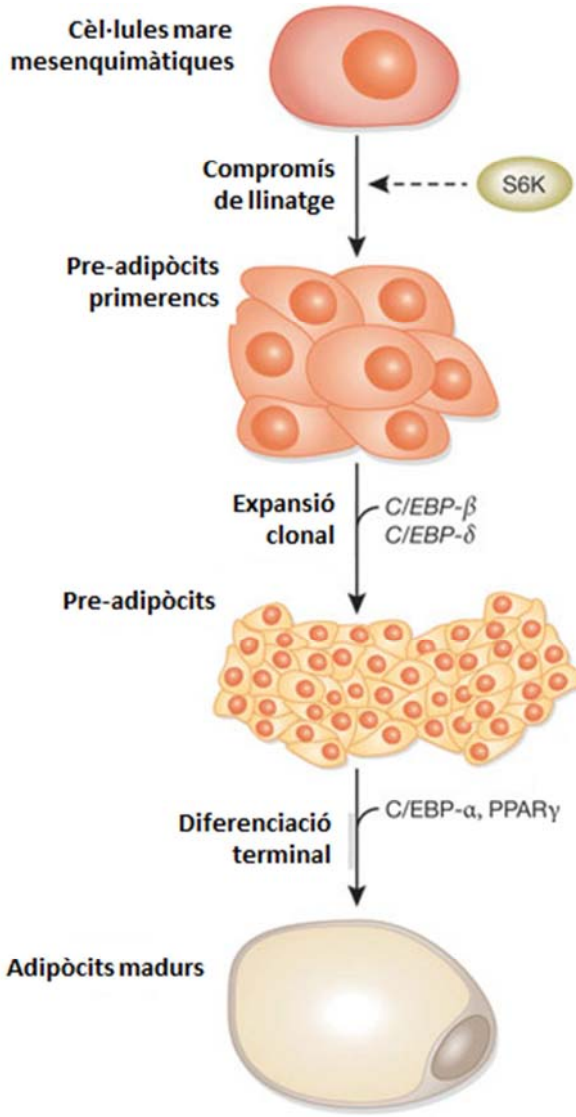


Figura 15. Representació esquemàtica del procés d'adipogènesi. Adaptada de Ricoult and Manning, 2013.

Quan es treballa amb models de pre-adipòcits *in vitro*, per induir la seva diferenciació en adipòcits madurs, és necessària l'addició al medi de compostos que activin aquests gens implicats en l'adipogènesi. Generalment 3 components s'utilitzen en el còctel de diferenciació en cèl·lules de mamífers: la insulina, la dexametasona, i el 3-Isobutil-1-metilxantina (IBMX) (Scott et al., 2011). La insulina és àmpliament utilitzada per a induir la proliferació i diferenciació dels pre-adipòcits. En altes dosis mimetitza els efectes del factor de creixement similar a la insulina (IGF-I) activant la via de la proteïna quinasa activada per mitògens (MAPK), que al seu torn activa la proteïna quinasa ribosomal S6 (S6K). La proteïna S6K estimula l'activitat del factor de transcripció dependent de determinació i diferenciació d'adipòcit-1 (ADD1)/proteïna d'unió a elements regulatoris d'esterol-1 (SREBP1), i l'expressió dels gens diana de SREBP1 (Ailhaud, 1982; Janderova et al., 2003; Ricoult and Manning, 2013). La dexametasona, un esteroide antiinflamatori, i l'IBMX, un inhibidor competitiu, no selectiu de la fosfodiesterasa, són inductors de C/EBPβ i C/EBPδ. L'IBMX, a més eleva els nivells d'adenosina monofosfat cíclica (AMPc) intracel·lular activant la proteïna quinasa A (PKA), proteïna que es requereix per a l'activació transcripcional de PPARγ. Per tant, l'IBMX en combinació amb la dexametasona regula PPARγ, promovent l'adipogènesi.

En l'última dècada, s'han establert cultius primaris de pre-adipòcits de diferents espècies de peixos, com ara en el salmó de l'Atlàntic (Vegusdal et al., 2003), l'orada del Japó (*Pagrus major*) (Oku et al., 2006), la truita irisada (Bouraoui et al., 2008), la carpa herbívora (*Ctenopharyngodon idella*) (Li, 2012) i el reig groc (*Pseudosciaena crocea*) (Wang et al., 2012). En aquests cultius primaris de pre-adipòcits de peixos, a més a més d'insulina, dexametasona i IBMX, s'ha vist que també és necessari incorporar en el còctel de diferenciació, lípids, generalment una barreja (formada principalment per oli de fetge de bacallà i colesterol), els quals s'ha demostrat que són requerits per induir la maduració completa dels adipòcits en aquestes espècies (Bouraoui et al., 2008; Oku and Umino, 2008; Oku et al., 2006).

3.2. Metabolisme lipídic

En els peixos, els lípids són importants fonts d'energia metabòlica (Sargent et al., 2002), i tant la seva biosíntesi com el seu catabolisme impliquen vies bioquímiques equivalents a les dels mamífers (Sheridan, 1988). Els peixos, així com altres vertebrats poiquiloterms, generalment emmagatzemen els lípids en diversos teixits, incloent el greix mesentèric, el múscul i el fetge (Sheridan and Kao, 1998). Les espècies de peixos classificades com a "magres", com ara el bacallà, tenen un menor contingut de greix en el múscul, però major en el fetge (Holdway and Beamish, 1984; Jørgensen et al., 2011); mentre que en les espècies anomenades "grasses", com les dels salmònids, el greix visceral, el greix de la panxa ("belly flap") i el múscul són els

principals teixits d'emmagatzematge de lípids (Jobling et al., 2002; Nanton et al., 2007; Polvi and Ackman, 1992;). Finalment, així com en els salmònids, en els espàrids, classificats com a peixos "semi-grassos" el teixit adipós i el múscul són també els principals reservoris d'energia, mentre que el fetge té un paper secundari (Takama et al., 1994). En aquestes espècies, el teixit adipós juga per tant un paper important en la regulació del metabolisme lipídic (Figura 16). Els adipòcits madurs serveixen en primer lloc com a reserva energètica, ja que agafen els FA o altres substrats procedents de la dieta (glucosa o aminoàcids) i els converteixen en TG (lipogènesi) per a l'emmagatzematge a llarg termini. En segon lloc, els adipòcits descomponen els TG en FA i glicerol mitjançant la lipòlisi pel seu alliberament a la sang durant els períodes de necessitat energètica. Un tercer destí dels lípids en el teixit adipós, tot i que minoritari, consisteix en la seva oxidació per a l'obtenció d'energia. Aquests processos de lipogènesi, lipòlisi i β-oxidació estan regulats per diverses hormones i factors de creixement, i hi ha implicats molts enzims i diversos factors de transcripció.

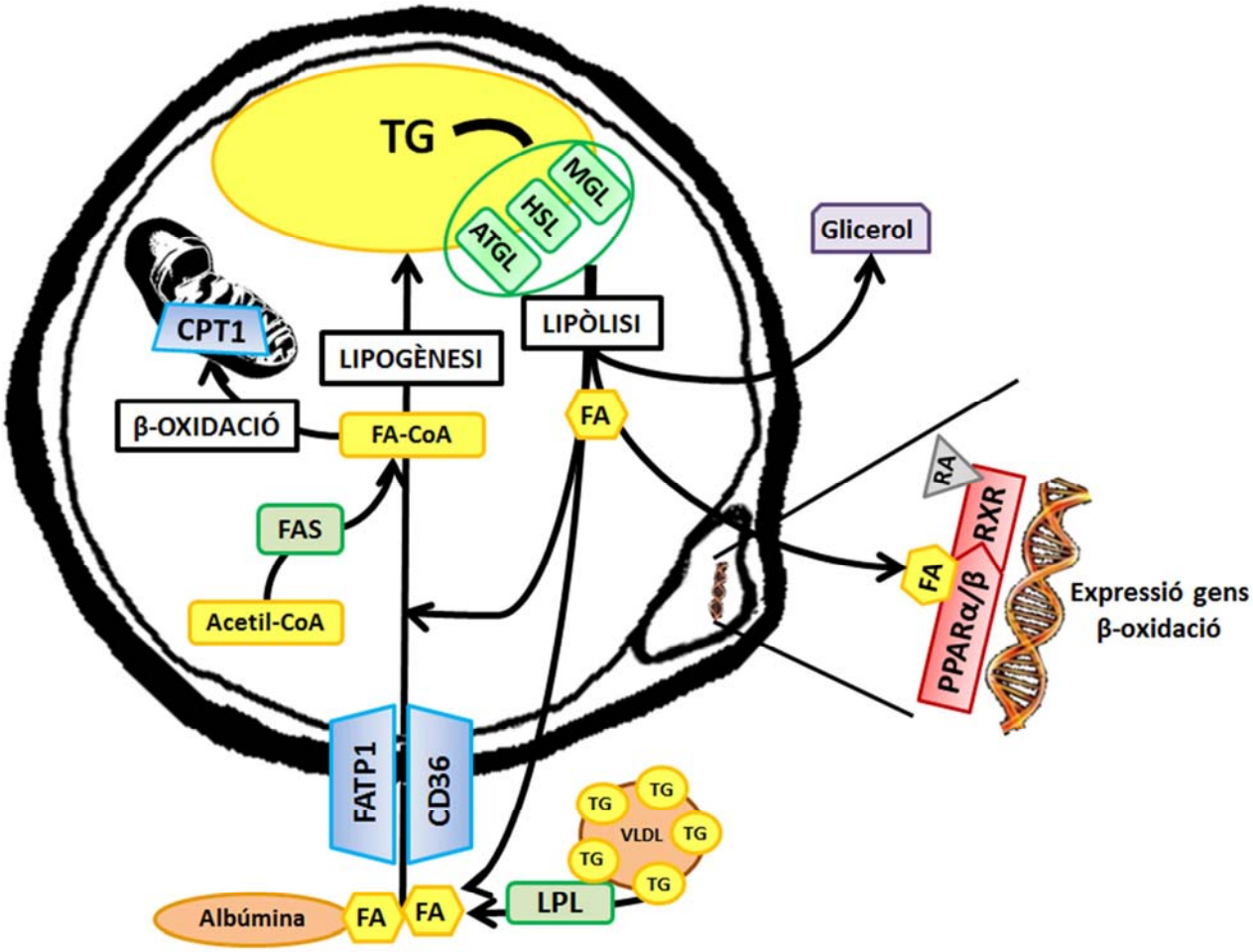


Figura 16. Metabolisme lipídic en un adipòcit: lipogènesi, lipòlisi i β-oxidació. Adaptada de Zechner et al., 2011; Zhang et al., 2009.

Lipogènesi

La lipogènesi majoritàriament es produeix en hepatòcits i adipòcits, i el transport de lípids entre aquests teixits es porta a terme a través de lipoproteïnes plasmàtiques (Figura 16). Entre els diversos enzims que influeixen en aquest procés hi ha la LPL, que hidrolitza els TG presents en aquestes lipoproteïnes, com la lipoproteïna de molt baixa densitat (VLDL) o els quilomicrons, en FA, els quals entraran al teixit adipós on principalment es re-esterificaran per a ser emmagatzemats com a reserva energètica, o s'utilitzaran per a l'obtenció d'energia mitjançant l'oxidació (Leaver et al., 2008; Mead et al., 2002). Per aquest motiu, la LPL és considerada un enzim clau en el metabolisme dels lípids, crític per regular el balanç entre deposició i catabolisme, tant en mamífers, com en peixos. En aquest sentit, l'activitat i l'expressió de la LPL en el teixit adipós de truita s'ha demostrat que disminueix amb el dejuni i augmenta amb l'alimentació (Albalat et al., 2006; Black and Skinner, 1986; Sánchez-Gurmaches et al., 2012) i s'ha descrit, tant *in vivo* com *in vitro*, que està regulada positivament per la insulina (Albalat et al., 2006; Bouraoui et al., 2012). En espàrids també s'ha descrit que la LPL es veu afectada pel dejuni i l'època de l'any *in vivo*, en relació amb els nivells d'insulina en plasma (Albalat et al., 2007; Saera-Vila et al., 2005), mentre que *in vitro* la insulina augmenta també la seva expressió (Oku et al., 2006).

L'entrada de FA, procedents de lipoproteïnes o complexes circulants FA-albúmina, en els adipòcits es dóna per difusió simple o a través de transportadors com la proteïna transportadora de FA-1 (FATP1) o el CD36 (Figura 16), l'existència dels quals també s'ha descrit en peixos. En el salmó de l'Atlàntic, l'expressió d'aquests transportadors s'ha estudiat en pre-adipòcits durant el procés de diferenciació, i en teixits procedents de peixos alimentats amb diferents dietes o sota diferents tractaments (Todorcevic et al., 2008; Torstensen et al., 2009; Sánchez-Gurmaches et al., 2011). En truita irisada, el dejuni va incrementar l'expressió d'ARNm de FATP1 i CD36, mentre que l'administració d'insulina va revertir aquests efectes (Sánchez-Gurmaches et al., 2012) i el contrari s'ha observat en el salmó de l'Atlàntic (Sánchez-Gurmaches et al., 2011), indicant diferències específiques d'espècie pel que fa a la resposta del teixit adipós davant la situació catabòlica causada pel dejuni. A més, s'ha demostrat *in vitro* que FATP1 incrementa durant el procés d'adipogènesi, mentre que CD36 augmenta durant la miogènesi en la truita irisada (Sánchez-Gurmaches et al., 2012).

En relació a la síntesis *de novo* dels FA per part de l'adipòcit, un enzim lipogènic important, limitant de la velocitat en la síntesi de FA de cadena llarga és l'ACC, que catalitza la carboxilació irreversible de l'acetil-CoA a malonil-CoA. A més, la 6-fosfogluconat deshidrogenasa (6PGD) i la

glucosa 6-fosfat deshidrogenasa (G6PD) són enzims clau que participen en la producció de NADPH, essencial per a la biosíntesi de FA (Chen et al., 2013). Finalment, en el procés de lipogènesi, la FAS catalitza en presència d'aquest NADPH, la síntesi *de novo* de FA com el palmitat donant lloc a un FA-CoA, a partir d'acetil-CoA i malonil-CoA (Figura 16) (Cowey and Walton, 1989). Aquest enzim ha estat estudiat en truita irisada principalment en fetge, on s'ha descrit un increment de la seva expressió en resposta a la realimentació i la ingesta de dietes amb alts nivells de proteïna (Lansard et al., 2009; Seiliez et al., 2011); així com també en hepatòcits aïllats, on s'ha vist que la FAS està regulada positivament per la insulina (Lansard et al., 2011; Plagnes-Juan et al., 2008).

Lipòlisi

Moltes espècies de peixos, al llarg de la seva vida, viuen períodes de manca d'aliments i estadis de la vida amb una alta demanda energètica (com per exemple durant l'esmoltificació), que impliquen un augment dels processos catabòlics, on cessa el creixement i es mobilitzen les reserves energètiques com el glicogen, els lípids i les proteïnes. En mamífers, la lipòlisi o hidròlisi neutra dels TG que donarà glicerol i tres FA requereix de tres passos consecutius que poden implicar fins a tres enzims diferents: la lipasa de TG del teixit adipós (ATGL) que catalitza l'etapa inicial de la lipòlisi, la conversió de TG a diacilglicerols (DG); la lipasa sensible a hormones (HSL) que és responsable principalment de la hidròlisi de DG a monoacilglicerols (MG), i la monoacilglicerol lipasa (MGL) que hidrolitza finalment els MG (Figura 16). En el teixit adipós de mamífers, on la lipòlisi està principalment estimulada per catecolamines i inhibida per la insulina, ATGL i HSL són responsables de més del 90% de la hidròlisi de TG (Schweiger et al., 2006).

En truita irisada i orada, diversos estudis han demostrat que la lipòlisi al teixit adipós incrementa durant el dejuni, amb una dieta formulada amb proteïna d'origen vegetal i en resposta a una injecció intraperitoneal de glucagó (Albalat et al., 2005a; Albalat et al., 2005b). A més, en adipòcits aïllats d'aquestes espècies, tant el glucagó com la hormona de creixement (GH) s'ha vist que estimulen la lipòlisi, mentre que la insulina s'ha confirmat que és anti-lipolítica com en mamífers (Albalat et al., 2005a; Albalat et al., 2005b). En orada, s'ha vist que l'activitat d'HSL en el teixit adipós, i els nivells basals de lipòlisi en els seus adipòcits aïllats, incrementen amb la substitució d'oli de peix per un 66% d'oli vegetal en la dieta (Cruz-Garcia et al., 2011a). A més en aquesta espècie, el dejú estimula l'activitat catalítica i l'expressió d'ARNm d'HSL en el teixit adipós, mentre la realimentació reverteix aquests efectes (Denver et al., 2012). Finalment, un estudi amb reig groc ha demostrat per primera vegada en peixos la

presència d'ARNm d'ATGL en pre-adipòcits, l'expressió de la qual es veu disminuïda per la insulina i el factor de necrosi tumoral α (TNFα) (Wang et al., 2012).

β-oxidació

Finalment, a través de l'oxidació de FA, les cèl·lules poden obtenir energia en forma d'ATP, acetil-coA i poder reductor (NADH i $FADH_2$). La carnitina palmitoil transferasa (CPT) és considerat el principal enzim regulador de l'oxidació de FA de cadena llarga, ja que catalitza la conversió de FA-CoA en FA-carnitina per a la seva entrada a la matriu mitocondrial (Figura 16) (Kerner and Hoppel, 2000). En truita irisada, s'ha vist que un tractament crònic d'insulina amb bomba osmòtica va reduir l'expressió de les isoformes CPT-1c i CPT-1d en el teixit adipós (Polakof et al., 2011), i que un tractament agut d'insulina amb injeccions intraperitoneals va disminuir l'expressió de CPT-1b en múscul (Polakof et al., 2010), indicant una inhibició de l'oxidació lipídica en aquests teixits en condicions anabòliques. També en truita, la incubació de miòcits en cultiu amb insulina va reduir l'expressió d'ARNm de CPT-1b, corroborant el seu paper anabòlic, mentre que l'àcid oleic la va incrementar (Sánchez-Gurmaches et al., 2010). D'altra banda, l'expressió dels gens CPT1 i CPT2 en teixit adipós de salmó de l'Atlàntic no es va veure afectada per dues setmanes de dejuni (Sánchez-Gurmaches et al., 2011), el que va indicar una baixa regulació transcripcional sota situacions catabòliques.

Diversos factors de transcripció tenen un paper intermediari en l'homeòstasi dels lípids i el procés de β-oxidació, a través de la regulació gènica d'enzims que participen en aquestes vies. Entre ells trobem els PPARs, factors de transcripció dependents de lligand, on dos subtipus, denominats PPARα i δ/β, juguen un paper fonamental en mamífers (Leone et al., 1999; Li et al., 2004; Wang et al., 2003). Tots dos PPARs estan altament expressats en teixits oxidatius, però també en el teixit adipós, i regulen gens relacionats amb l'alliberació de substrat, l'oxidació de substrat, i la fosforilació oxidativa (Figura 16) (Zechner et al., 2011). El paper dels PPARs en el teixit adipós de peixos es poc conegut; no obstant, truites irisades alimentades amb una dieta rica en FA poliinsaturats van mostrar major expressió dels gens CPT-1 i PPARβ en el teixit adipós, suggerint que aquests FA, a través de l'activació de PPARs, promouen la β-oxidació en aquest teixit (Morash et al., 2009). A més, un estudi recent ha descrit en orades amb fenotip "gras", que l'estimulació dels seus adipòcits aïllats amb TNFα incrementa la lipòlisi, a la vegada que disminueix l'expressió de PPARβ (Cruz-Garcia et al., 2009), suggerint una disminució de l'oxidació dels FA en el teixit adipós en resposta a aquesta citoquina. D'altra banda, en miòcits de truita, l'addició d'agonistes del receptor X de fetge (LXR) va incrementar

l'expressió de mRNA de PPARα, mentre va disminuir la de PPARβ (Cruz-Garcia et al., 2011b), suggerint una complexa modulació de l'oxidació de FA en aquest teixit.

3.3. Rol endocrí del teixit adipós

Després del descobriment de la leptina en el ratolí el 1994 (Zhang et al., 1994), el teixit adipós blanc va ser reconegut com a òrgan endocrí, secretor d'una important font de substàncies biològicament actives amb acció local (paracrina/autocrina) i/o sistèmica (endocrina) anomenades adipoquines (Figura 17) (Hassan et al., 2012). Es poden distingir dos grans grups d'adipoquines, que inclouen les anti-diabètiques o sensibilitzadores de la insulina on trobaríem leptina i adiponectina, i les pro-diabètiques, a on també trobaríem les citoquines pro-inflamatòries com el TNFα, les interleuquines 6 i 1β (IL-6 i IL1β, respectivament) i la resistina (Rosen and Spiegelman, 2006). A més, els adipòcits estan activament involucrats en altres processos metabòlics com l'angiogènesi, la resposta immune i l'hemostàsia. Per tant, la funcionalitat del teixit adipós és clau per l'homeòstasi de l'organisme i una secreció inadequada d'adipoquines a causa d'una quantitat excessiva de teixit adipós blanc sembla participar en el desenvolupament de processos patològics relacionats amb l'obesitat com: resistència a la insulina i diabetis tipus 2, dislipèmia, hipertensió i síndrome metabòlica, incloent un augment en el risc de patir malaltia cardíaca coronària i infart (Kwon and Pessin, 2013; Qatanani and Lazar, 2007; Zimmet et al., 2001).

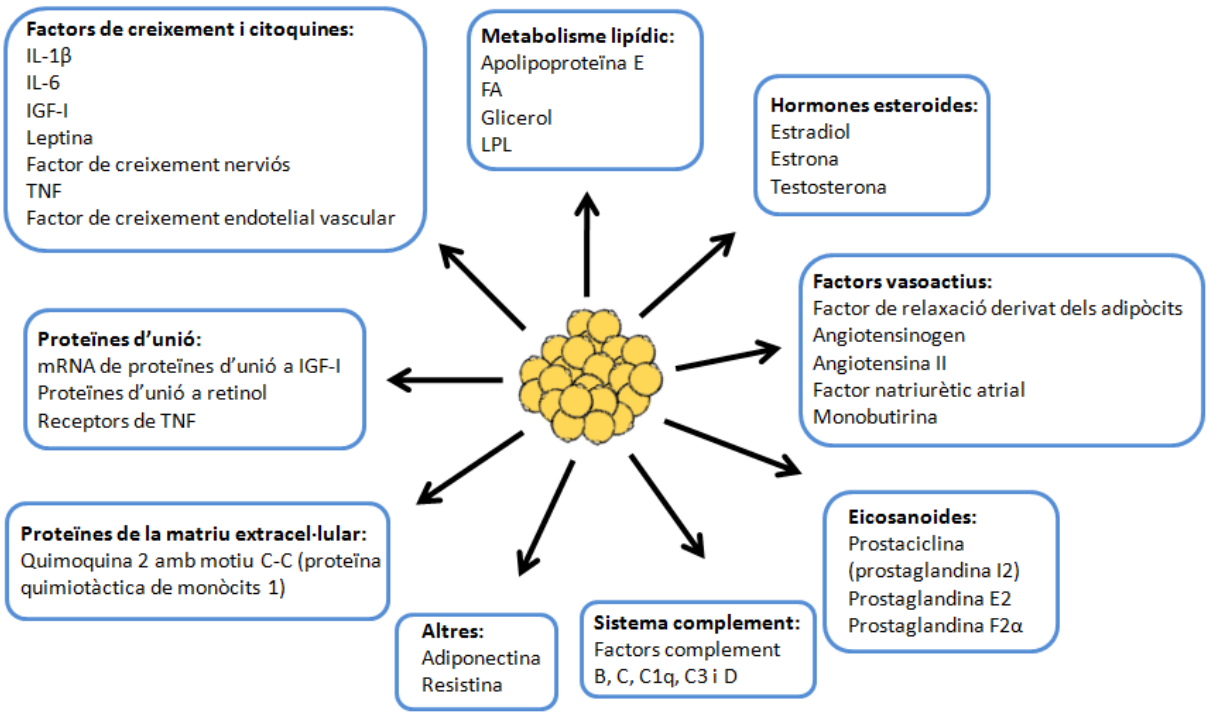


Figura 17. Factors secretats pel teixit adipós. Adaptada de Hassan et al., 2012.

3.4. Leptina i grelina

La regulació del balanç energètic és fonamental pel correcte creixement i desenvolupament al llarg de la vida. El creixement del múscul esquelètic depèn de la ingesta d'aliment, però també de l'extracció i ús/assignació d'aquesta energia. El metabolisme i el balanç energètic també inclouen la mobilització dels dipòsits energètics del fetge i del teixit adipós en etapes de la vida amb alta demanda energètica com la metamorfosi, l'esmoltificació i la pubertat. Encara que és de gran importància en l'aqüicultura, la comprensió fonamental de com es regulen aquests processos en peixos encara està molt fragmentada.

El pes corporal està regulat per un sistema complex, que inclou tant factors perifèrics com centrals. Dues de les hormones que semblen tenir un paper més important en la regulació de la ingesta d'aliments, el pes corporal i l'homeòstasi de l'energia en mamífers són la leptina i la grelina (López et al., 2007). Totes dues s'originen a la perifèria i senyalitzen a través de diferents vies al cervell, particularment a l'hipotàlem (Figura 18) (Klok et al., 2007). En l'hipotàlem, l'activació dels corresponents receptors inicia diferents cascades de senyalització que condueixen a canvis en la ingesta d'aliments (Klok et al., 2007).

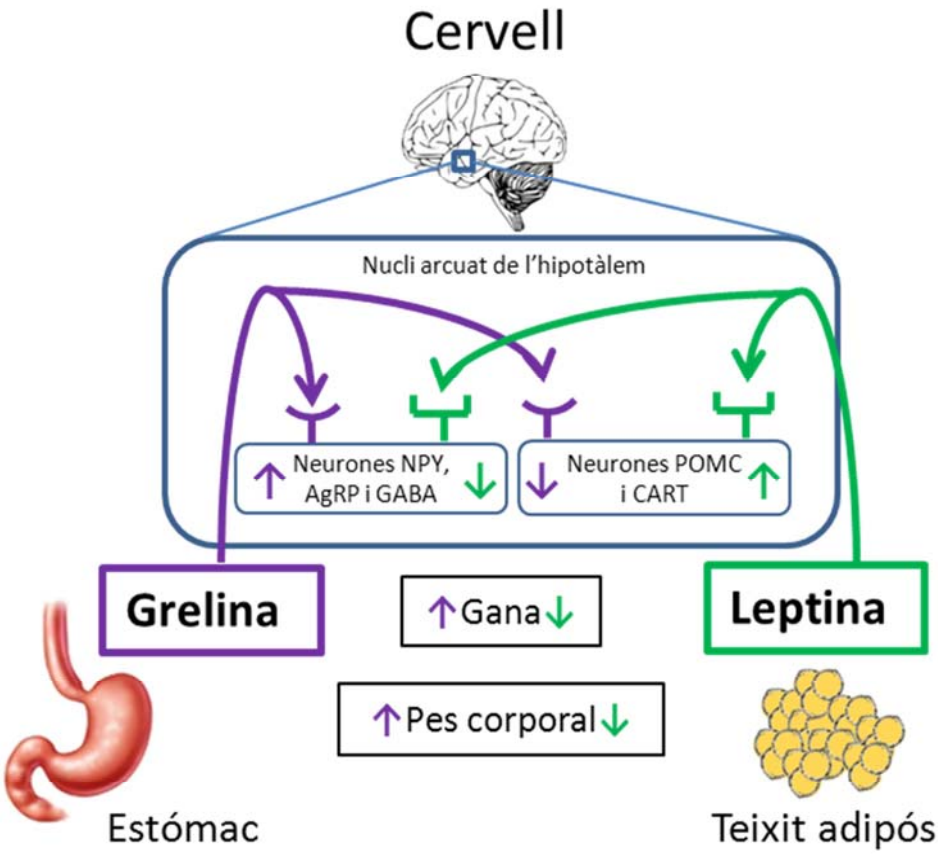


Figura 18. Accions de la leptina i la grelina en l'hipotàlem sobre neurones orexigèniques i anorexigèniques per regular la ingesta. Adaptada de www.precisionnutrition.com.

En l'estudi del balanç energètic, el nucli arcuat (ARC) ha estat molt investigat, en gran part degut a la identificació de neurones orexigèniques, neuropèptid Y/proteïna relacionada amb agouti (NPY/AgRP) i neurones anorexigèniques, pro-opiomelanocortina/trànscrit regulat per cocaïna i amfetamina (POMC/CART) en aquesta ubicació. L'activació de neurones que expressen POMC/CART suprimeix la ingesta, mentre que l'activació de neurones que expressen NPY/AgRP estimula l'alimentació. Els dos grups neuronals responen tant als senyals procedents de la leptina, alliberada pel teixit adipós, com de la grelina, que és secretada per l'estómac. Mitjançant l'alteració de l'activitat neuronal de l'ARC, la leptina inhibeix fortament la ingesta d'aliments i estimula la despesa energètica. Per contra, les concentracions de grelina en plasma augmenten en condicions de balanç energètic negatiu per estimular l'alimentació i reduir la despesa d'energia (Figura 18) (Hill, 2012).

Leptina

La leptina és una hormona de 16 kDa codificada pel gen *ob* implicada en l'homeòstasi de l'energia, l'obesitat, la reproducció, l'homeòstasi de la glucosa, la formació d'os, la cicatrització de ferides i el sistema immunològic (Peelmanet al., 2006). En els mamífers, la leptina és produïda i secretada principalment pel teixit adipós blanc (Zhang et al., 1994), tot i que també es produeix en concentracions més petites en altres teixits, com el teixit adipós marró, l'estómac, la placenta, el múscul esquelètic i els ovaris (Margetic et al., 2002). S'ha observat que la leptina en plasma està altament correlacionada amb l'índex de massa corporal (BMI) en els rosegadors i els éssers humans, i també s'ha trobat que es correlaciona amb el percentatge de greix corporal (Considine et al., 1996; Maffei et al., 1995). Així, la concentració de leptina en el plasma incrementa en proporció a la massa de teixit adipós, i regula la ingesta d'aliments i la despesa energètica per mantenir els dipòsits de greix corporal (Campfield et al., 1995; Elias et al., 1999; Halaas et al., 1995; Kamohara et al., 1997; Pelleymounter et al., 1995; Zhang et al., 1994).

En mamífers, la leptina exerceix el seu efecte anti-obesitat a través dels seus receptors (OB-R o LepR), situats en diferents àrees hipotalàmiques, principalment en el ARC (Bouret and Simerly, 2004; Fei et al., 1997; Gautron and Elmquist, 2011). Hi ha descrites sis isoformes: LepRa, LepRb, LepRc, LepRd, LepRe i LepRf (Fruhbeck, 2006). La isoforma llarga (LepRb) codifica per una proteïna amb un domini citoplasmàtic llarg, és membre de la família de receptors de citoquines de classe 1 i és considerada la principal isoforma implicada en la transducció de senyals intracel·lulars (Mantzoros et al., 2011; Marroquí et al., 2012). La leptina senyalitza a través de la via Janus quinasa (JAK)-transductor de senyal i activador de la

transcripció (STAT) (JAK/STAT). Quan la leptina s'uneix al receptor LepRb activa el receptor associat a JAK2 a través de la transfosforilació, i fosforila el receptor en tres residus de tirosina (Figura 19).

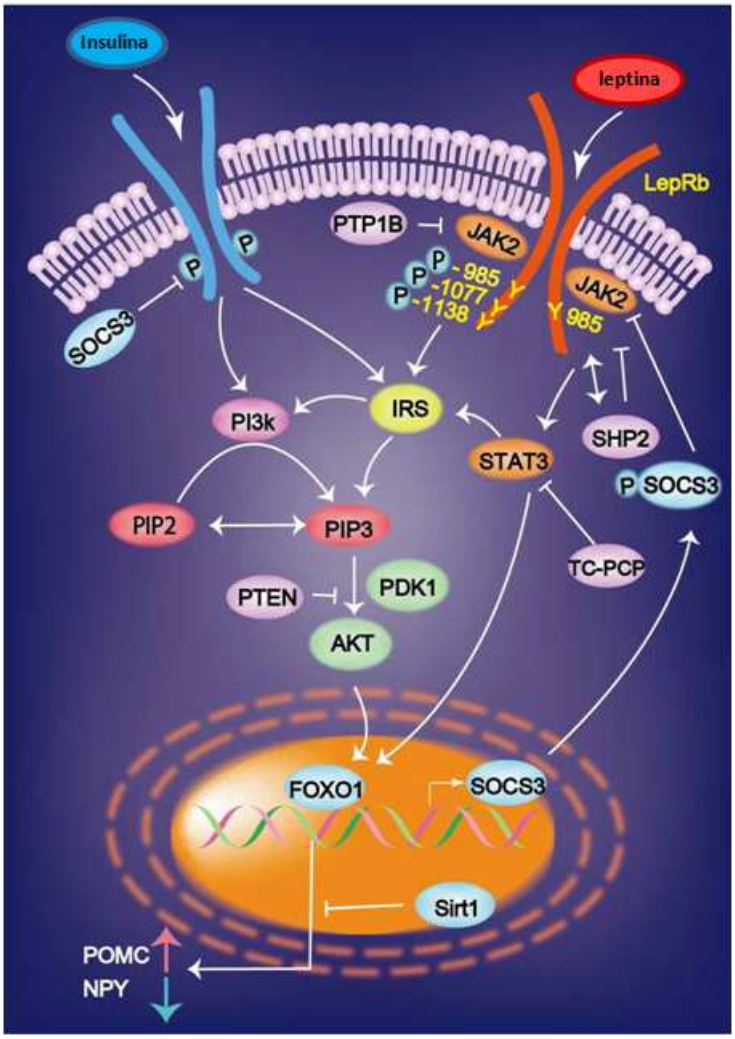


Figura 19. Vies de transducció del senyal de la leptina i la insulina en l'hipotàlem. Amitani et al., 2013.

A més de fosforilar JAK2, la leptina també fosforila STAT3 i STAT5, estimulant la seva translocació al nucli. Dins del nucli, STAT3 fosforilat incrementa l'expressió de POMC i inhibeix la de NPY (St-Pierre and Tremblay, 2012). D'altra banda, a través de JAK2, la leptina també actua mitjançant la via de la fosfatidilinositol-3-quinasa (PI3K), via de senyalització que comparteix amb la insulina (Figura 19). JAK2 estimula la fosforilació del substrat del receptor d'insulina (IRS) 1 o 2, que activa PI3K, el qual a través de la formació de fosfatidilinositol 3,4,5-trifosfat (PIP3) activa la proteïna quinasa B (PKB), també coneguda com Akt. Akt subseqüentment prevé la inhibició de l'expressió de POMC i l'expressió de NPY i AgRP per part del factor de transcripció FoxO1. Per tant, la insulina a través de la via PI3K promou l'efecte de

la leptina inhibint FoxO1 (St-Pierre and Tremblay, 2012; Wauman and Tavernier, 2011). A més a més, a partir de l'Akt i a través de la molècula diana de rapamicina (TOR), la leptina regula l'activitat de la proteïna quinasa activada per AMP (AMPK). L'AMPK és un sensor intracel·lular d'energia i un regulador de la ingesta en resposta a senyals hormonals i nutricionals en el hipotàlem (Minokoshi et al., 2004). L'AMPK regula els nivells d'energia inhibint les vies que consumeixen ATP i activant les vies que produeixen ATP, com la captació de glucosa i l'oxidació d'àcids grassos. La leptina per a restringir la ingesta, inhibeix l'activitat de l'AMPK hipotalàmica.

Recentment, el gen de la leptina ha estat identificat en diverses espècies de teleostis; el peix globus (*Takifugu rubripes*) (Kurokawa et al., 2005), la carpa comuna (*Cyprinus carpio*) (Huising et al., 2006), la truita irisada (Angotzi et al., 2013; Murashita et al., 2008), el peix zebra (*Danio rerio*) (Gorissen et al., 2009), medaka del Japó (*Oryzias latipes*) (Kurokawa and Murashita, 2009), el salmó de l'Atlàntic (Rønnestad et al., 2010), la truita alpina (*Salmo trutta*) (Frøiland et al., 2010) i el carpí daurat (*Carassius auratus*) (Tinoco et al., 2012), entre d'altres. L'estructura primària de la leptina del peix globus, la primera leptina descrita en peixos, només és un 13% idèntica a la leptina humana (Kurokawa et al., 2005); no obstant això, l'estructura terciària de la leptina de peixos és molt similar a la leptina de mamífers (Figura 20) (Gorissen et al., 2009; Huising et al., 2006; Kurokawa et al., 2005). En peixos s'han descrit dues isoformes del gen de la leptina, la leptina A (LepA) i la B (LepB) resultat de la duplicació completa del genoma (3R) en l'ancestre dels teleostis (Taylor et al., 2003; Volff, 2004). Degut al procés de tetraploidització del genoma dels salmònids fa uns 25-100 milions d'anys (4R) (Allendorf and Thorgaard, 1984; Ohno, 1970), aquests posseeixen duplicats de cada gen de LepA (LepA1 i LepA2) i LepB (LepB1 i LepB2) (Figura 20).

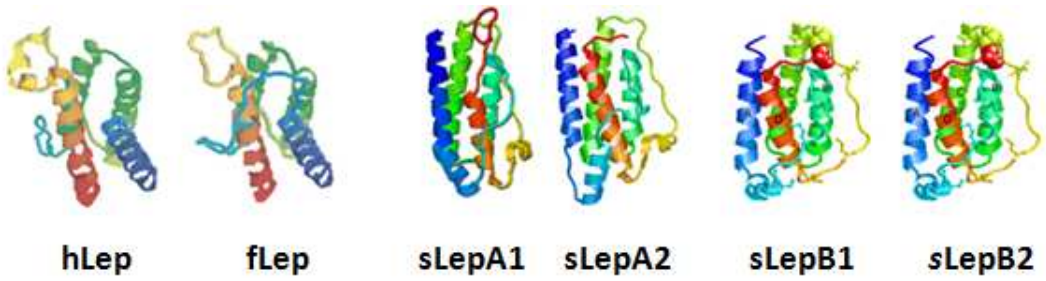


Figura 20. Estructura terciària de diferents ortòlegs de la leptina. hLep: leptina de *Homo sapiens*, fLep: leptina de *Takifugu rubripes*, sLep: leptines de *Salmo salar*. Angotzi et al., 2013; Kurokawa et al., 2005; Rønnestad et al., 2010.

En els teleostis, el gen del receptor de la leptina també ha estat identificat (Kurokawa et al., 2008; Rønnestad et al., 2010; Wong et al., 2007), i presenta una gran similitud amb la

seqüència corresponent en mamífers en els dominis que es consideren importants per a la senyalització.

En els peixos, el paper de la leptina com a senyal de sacietat no és tan clar com en mamífers. S'ha vist que quan s'injecta leptina recombinant en truita irisada, es dóna un fort efecte anorexigènic, juntament amb una disminució i un augment, respectivament, en l'expressió hipotalàmica de l'ARNm de NPY i POMC (Aguilar et al., 2010; Aguilar et al., 2011; Murashita et al., 2008). D'altra banda, el tractament a llarg termini de la carpa herbívora amb leptina homòloga no va afectar la ingesta ni el pes corporal, mentre que un tractament a curt termini sí que en va disminuir la ingesta (Li et al., 2010a). En general, les troballes són similars a l'observat anteriorment en mamífers, donant suport al paper de la leptina en la regulació de la gana en peixos, inhibint aquesta.

En els mamífers, els adipòcits són l'única font coneguda de leptina, ja que els pre-adipòcits no presenten aquesta capacitat (Matson et al., 1996). A diferència dels mamífers, en peixos, com per exemple en la truita irisada, la leptina s'expressa principalment en el fetge, i en menor mesura en ull, cor, múscul i pell (Murashita et al., 2008), i també s'ha descrit tot i que amb gran variabilitat individual, expressió de leptina al teixit adipós perivisceral (Pfundt et al., 2009). A més, en el cultiu primari de pre-adipòcits de salmó de l'Atlàntic, només els adipòcits diferenciats van expressar nivells detectables del pèptid de leptina (Vegusdal et al., 2003).

La quantitat de leptina produïda pels adipòcits està regulada a nivell transcripcional, però també a nivell de la traducció, l'emmagatzematge, la taxa de renovació i la secreció (Lee and Fried, 2006). En mamífers, s'han descrit diverses senyals hormonals i nutricionals que poden modular l'alliberació de leptina pels adipòcits. Els factors que estimulen la secreció de leptina són: el consum d'aliments (Lynch et al., 2006), la leucina (Lynch et al., 2006; Roh et al., 2003), l'àcid eicosapentaenoic (EPA) (Pérez-Matute et al., 2005), la glucosa (Levy et al., 2000), la insulina (Cheng et al., 2000; Moreno-Aliaga et al., 2003; O'Rourke et al., 2001; Ricci et al., 2005), la grelina (Giovambattista et al., 2008), el TNFα (Margetic et al., 2002), els estròGens (Margetic et al., 2002) i els glucocorticoides (Ricci et al., 2005), entre altres. Els factors que inhibeixen la secreció de leptina són aquells que augmenten els nivells d'AMPc en l'adipòcit, com el dejuni (Szkudelski et al., 2004), l'exposició al fred (Korhonen and Saarela, 2005; Rayner and Trayhurn, 2001), l'estimulació β-adrenèrgica (Margetic et al., 2002; Ricci et al., 2005; Szkudelski et al., 2005;), els andròGens (Margetic et al., 2002), els FA (Margetic et al., 2002), la GH (Margetic et al., 2002), els agonistes de PPARγ (Margetic et al., 2002) i l'exercici (Bramlett et al., 1999; Zheng et al., 1996). En peixos, no obstant, no hi ha estudis al respecte.

Quan a la regulació del metabolisme a nivell del teixit adipós, la leptina incrementa la despesa energètica, estimulant l'oxidació lipídica a la vegada que inhibeix la lipogènesi i promou la lipòlisi (Cohen et al., 2002; Hwa et al., 1997; Reidy and Weber, 2000).

Grelina

La grelina es va descobrir per primera vegada en l'estómac de rata el 1999 (Kojima et al., 1999), on es va descriure com un secretagog de GH (GHS), encarregat de l'estimulació de l'alliberament de GH a través del receptor secretagog de GH (GHS-R), descrit 3 anys abans en humans i en porcs (Howard et al., 1996). La grelina és un membre de la família dels pèptids reguladors relacionats amb la motilina. A més de la seva capacitat per estimular la secreció de GH i la motilitat gàstrica, la grelina estimula la gana, indueix un balanç energètic positiu que condueix a l'augment de pes, i està implicada en processos fisiològics tan importants com la digestió, la immunitat i la reproducció (Castañeda et al., 2010; Kaiya et al., 2008).

Des del seu descobriment, la grelina i el seu receptor s'han identificat en nombroses espècies d'animals no mamífers, incloent moltes espècies de peixos (Kaiya et al., 2013). La grelina és produïda principalment per l'estómac, però en menor mesura per altres teixits com l'intestí, el pàncrees i el cervell (Castañeda et al., 2010; Jönsson, 2013), i inclús pel teixit adipós (Murashita et al., 2009). La grelina té una modificació post-traduccional única, una acilació, generalment un àcid gras octanoic (C8:0), però també pot ser algun altre àcid gras de cadena mitja, el qual està unit al tercer residu aminoacídic de la grelina formant la grelina acilada (AG). Aquesta modificació és necessària per a que la grelina activi el seu receptor, mentre que les funcions de la grelina no acilada (UAG) no estan tant clares (Kaiya et al., 2011).

El GHS-R1a és un receptor acoblat a proteïna G (GPCR) amb set dominis transmembrana, i quan s'hi uneix la grelina, senyalitza provocant un augment en la concentració de Ca^{2+} intracel·lular, tot i que també s'ha trobat una variant sense capacitat d'incrementar el Ca^{2+} en resposta a grelina, el GHS-R1b. El receptor funcional de la grelina (GHS-R1a), està altament expressat en el sistema nerviós central, i es troba principalment en la part medial de l'ARC de l'hipotàlem (Perello et al., 2012). L'activació de GHS-R1a estimula AMPK en les neurones NPY/AgRP de l'ARC i en el nucli ventromedial de l'hipotàlem regulant el metabolisme, mentre que en el fetge i el teixit adipós resulta en la inhibició d'AMPK (Lim et al., 2010).

En mamífers, la grelina activa les neurones NPY/AgRP en el nucli paraventricular i suprimeix l'activitat neuronal de POMC regulant la ingesta (Cowley et al., 2003). Ara bé, en peixos s'han vist efectes diferents en resposta a la grelina en funció de l'espècie (Figura 21). En el carpí

daurat, els aferents del nervi vague intervenen en la senyal de la grelina derivada de l'intestí al cervell. En l'hipotàlem, les dades actuals suggereixen que la grelina estimula poblacions orexigèniques de neurones de NPY i d'orexina (Jönsson et al., 2013), a la vegada que aquesta orexina també pot estimular les neurones que contenen grelina. Aquesta acció de la grelina en l'hipotàlem de carpí daurat condueix a un augment de la ingesta d'aliments com succeeix en mamífers. En canvi, en la truita irisada, la grelina té un paper contrari, suprimint la ingesta d'aliments en actuar sobre les neurones anorexigèniques de l'hormona secretora de corticotropina (CRH) (Figura 21) (Jönsson et al., 2013).

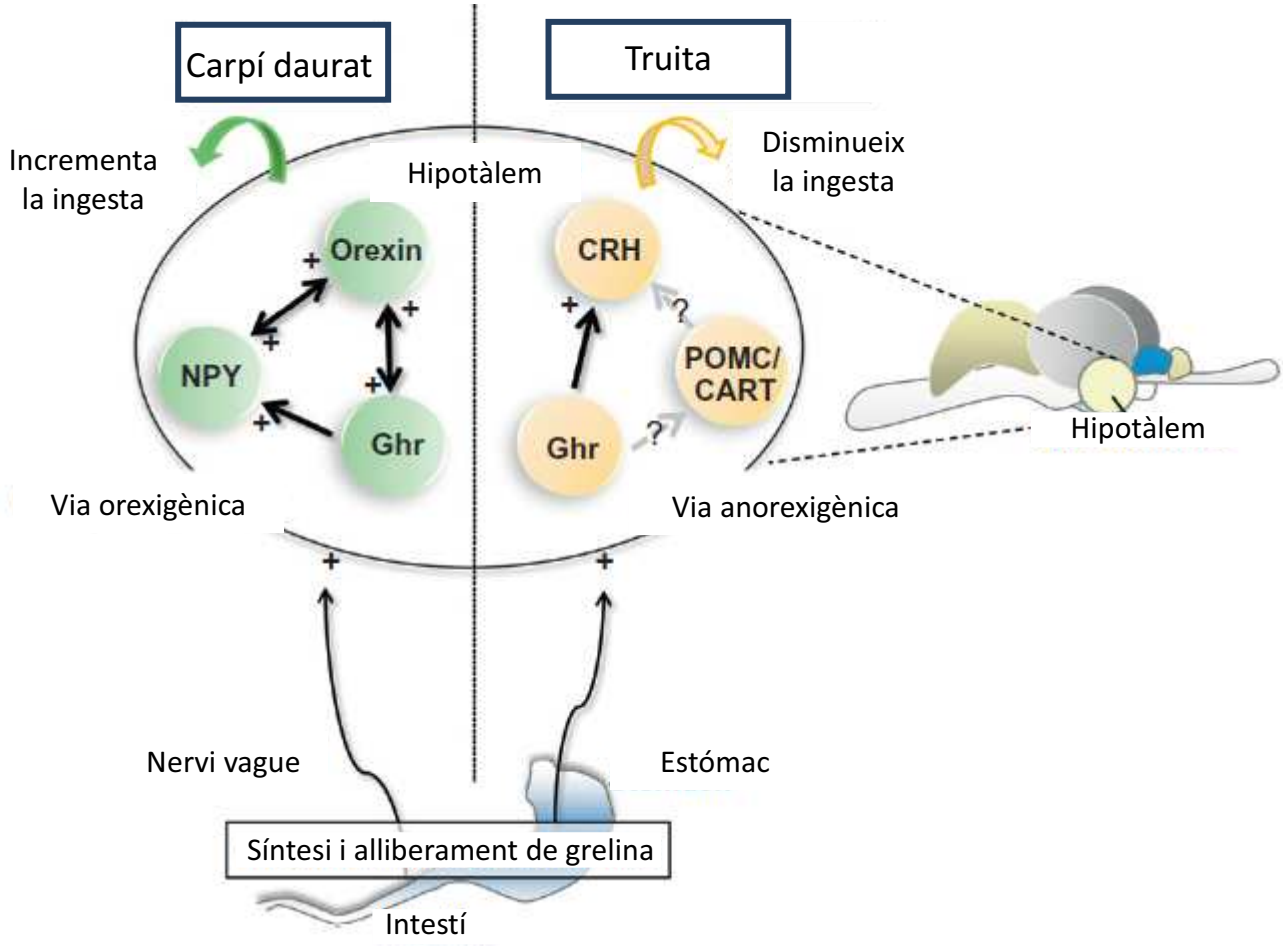


Figura 21. Model esquemàtic de l'acció de la grelina sobre la ingesta en carpí daurat i en truita irisada. Jönsson, 2013.

En el teixit adipós perivisceral, la grelina estimula l'acumulació de lípids mitjançant l'expressió de gens adipogènics com SREBP1, FAS i LPL, així com també s'ha demostrat que estimula l'adipogènesi mitjançant l'activació de gens com PPARγ (Giovambattista et al., 2008; Pulkkinen et al., 2010).

Objectius/Objectives

Objectius

Els estudis que constitueixen la present tesi es van plantejar emmarcats dins dels projectes d'investigació titulats: "Construcció d'una base de coneixements biològics sobre els cicles vitals dels peixos per a una aqüicultura europea competitiva i sostenible (LIFECYCLE)" del setè programa marc de la Unió Europea (EU-FP7 222719), "La qualitat del múscul en orada i la seva resposta a les condicions de cultiu: nous marcadors i la seva relació amb el metabolisme proteic" del Ministeri de Ciència i Innovació MICINN (AGL2009-12427), "Caracterització de les molècules clau que participen en l'osteoblastogènesi, el desenvolupament ossi i l'aparició de deformitats en l'orada (*Sparus aurata*)" MICINN (AGL2010-17324), "Caracterització i regulació de marcadors d'adipogènesi i deposició lipídica; digestió, absorció de greixos i metabolisme d'àcids grassos poliinsaturats en l'engreix de truita i orada" MICINN (AGL2008-00783) i "Grup de regulació i optimització del creixement en espècies de peixos d'interès en aqüicultura" de la Generalitat de Catalunya (2009SGR-00402).

L'objectiu global d'aquesta tesi ha estat conèixer millor els sistemes proteolítics involucrats en la degradació muscular que poden afectar la textura del filet, així com els factors reguladors de la formació del teixit adipós i el seu metabolisme lipídic, els quals poden influir en la deposició de greix perivisceral, per determinar com poder millorar la qualitat de dues espècies importants en aqüicultura, l'orada (*Sparus aurata*) i la truita irisada (*Oncorhynchus mykiss*).

Objectius concrets:

1) Identificar i caracteritzar membres dels diferents sistemes proteolítics presents en el múscul esquelètic d'orada, i estudiar la seva regulació transcripcional en diferents etapes de la vida, sota condicions de dejuni i realimentació i en resposta a dietes amb diferent contingut de proteïna i carbohidrat **(Capítols I i II)**.
2) Analitzar la relació existent entre l'expressió gènica de les proteases identificades i la textura del filet en orada, i avaluar la possibilitat d'utilitzar-les com a potencials marcadors de la qualitat del producte **(Capítol I)**.
3) Estudiar els efectes de l'estat nutricional o adiposita sobre els nivells de leptina en plasma de truita irisada, així com la regulació hormonal i nutricional de l'expressió i secreció de leptina en adipòcits **(Capítol III)**.
4) Analitzar els efectes de les hormones leptina i grelina sobre l'adipogènesi i el metabolisme lipídic dels adipòcits en la truita irisada **(Capítol IV)**.
5) Establir i caracteritzar un cultiu primari de pre-adipòcits d'orada, així com estudiar la regulació endocrina de la proliferació i la diferenciació cel·lular **(Capítol V)**.

Pla de treball

Per tal d'abordar els diferents objectius es van realitzar diversos experiments donant lloc a les publicacions científiques que constitueixen la base de la present tesi doctoral.

- Per assolir el primer objectiu es van clonar, identificar i caracteritzar diferents membres dels principals sistemes proteolítics cel·lulars en múscul esquelètic d'orada. Un cop identificats, es van realitzar 3 experiments independents per veure'n la seva regulació transcripcional segons: a) les diferents etapes de la vida (alevins, juvenils i adults), b) l'estat nutricional (dejuni i realimentació) i c) el tipus de dieta (diferent nivell de proteïna/carbohidrat) **(Capítols I i II)**.

- Pel segon objectiu, els filets de les orades alimentades amb les diferents dietes experimentals van ser analitzats amb un texturòmetre, i a nivell transcripcional es va determinar l'expressió de les diferents proteases identificades **(Capítol I)**.

- Pel tercer objectiu es va realitzar un experiment *in vivo* en que es van alimentar truites irisades amb una dieta amb un alt contingut energètic procedent de lípids. Un grup es va alimentar a sacietat (AL) i l'altre amb un nivell que es corresponia amb el 25% d'ingesta del grup AL (RE). A continuació *in vitro*, es van aïllar adipòcits de cada grup experimental (AL i RE) als que es van aplicar diferents tractaments hormonals i nutricionals per determinar els seus efectes sobre els nivells d'expressió i secreció de leptina **(Capítol III)**.

- Pel quart objectiu es van realitzar cultius de pre-adipòcits i aïllaments d'adipòcits madurs a partir de truites alimentades amb una dieta regular. A continuació, es va determinar l'efecte de les hormones leptina i grelina sobre l'adipogènesi i el metabolisme lipídic dels adipòcits, així com els nivells d'expressió i secreció de leptina. També es va analitzar la presència dels pèptids grelina i leptina i dels seus receptors, en adipòcits diferenciats **(Capítol IV)**.

- Pel cinquè objectiu es van establir les condicions òptimes pel cultiu de pre-adipòcits d'orada tenint en compte diferents factors fins a obtenir un bon creixement i desenvolupament de les cèl·lules. A continuació es van fer tractaments amb hormones i factors de creixement per estudiar el seu paper durant el procés d'adipogènesi **(Capítol V)**.

Cada capítol ha donat o donarà lloc a una publicació (veure llistat al final d'aquest apartat).

Objectives

The studies that constitute the present thesis were proposed within the research projects entitled "Building a biological knowledge-base on fish lifecycles for competitive, sustainable European aquaculture (LIFECYCLE)" from the Seventh Framework Program of the European Union (EU-FP7 222719), "Quality in gilthead sea bream muscle and its response to growing conditions: new markers and its relationship with protein metabolism" from the Spanish Ministry of Science and Innovation MICINN (AGL2009-12427), "Characterization of key molecules involved in osteoblastogenesis, bone development and the appearance of deformities in gilthead sea bream (*Sparus aurata*)" MICINN (AGL2010-17324), "Characterization and regulation of adipogenesis and lipid deposition markers; digestion, absorption of fats and polyunsaturated fatty acid metabolism in fattening trout and sea bream" MICINN (AGL2008-00783) and "Group of growth regulation and optimization in fish species with interest in aquaculture" of the Catalonian Government (2009SGR-00402).

The overall objective of this thesis was to better understand the proteolytic systems involved in muscle degradation that can affect flesh texture, as well as the factors that regulate the formation of adipose tissue and its lipid metabolism, which may influence the deposition of perivisceral fat to determine how to improve the quality of two important species for aquaculture, gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*).

Specific objectives:

1) To identify and characterize different members of the proteolytic systems present in the skeletal muscle of gilthead sea bream and, to study its transcriptional regulation at different life stages, under conditions of fasting and re-feeding and, in response to diets with different protein and carbohydrate content **(Chapters I and II)**.
2) To analyze the relationship between the gene expression of the proteases identified and the texture of the fillet in gilthead sea bream and, to evaluate the possibility of using them as potential markers of product quality **(Chapter I)**.
3) To study the effects of nutritional status or adiposity on plasma leptin levels of rainbow trout, as well as the nutritional and hormonal regulation of leptin expression and secretion in adipocytes **(Chapter III)**.
4) To analyze the effects of the hormones leptin and ghrelin on adipogenesis and adipocyte lipid metabolism in rainbow trout **(Chapter IV)**.
5) To establish and characterize a primary culture of preadipocytes in sea bream and, to study the endocrine regulation of cell proliferation and differentiation **(Chapter V)**.

Work plan

In order to address the different objectives several experiments were conducted leading to the scientific publications that form the basis of this thesis.

- To achieve the first objective we cloned, identified and characterized different members of the major cellular proteolytic systems in skeletal muscle of gilthead sea bream. Once identified, three independent experiments were conducted to see its transcriptional regulation by: a) different life stages (fingerlings, juveniles and adults), b) nutritional status (fasting and re-feeding) and, c) the type of diet (different protein/carbohydrate ratio) **(Chapters I and II)**.

- For the second objective, the fillets of the gilthead sea bream fed the different experimental diets were analyzed with a texturometer and, the expression of the different proteases identified was determined at a transcriptional level **(Chapter I)**.

- For the third objective, an experiment was performed *in vivo*, where rainbow trout were fed a diet with a high-energy content coming from lipids. One group was fed to satiation (AL) and the other one with a level that corresponded to 25% of that fed by the AL group (RE). Then *in vitro*, adipocytes were isolated from each experimental group (AL and RE) and, different nutritional and hormonal treatments were applied to determine its effects on the levels of expression and secretion of leptin **(Chapter III)**.

- For the fourth objective, preadipocytes cultures and isolations of mature adipocytes from rainbow trout fed with a regular diet were conducted. Subsequently, the effect of the hormones leptin and ghrelin on adipogenesis and adipocyte lipid metabolism was determined, as well as the levels of expression and secretion of leptin. Then, also the presence of the peptides ghrelin and leptin and their receptors in differentiated adipocytes was evaluated **(Chapter IV)**.

- For the fifth objective, the optimal conditions for the cultivation of primary preadipocytes from gilthead sea bream were established considering different factors to obtain good growth and development of the cells. Next, treatments were made with hormones and growth factors to study their role during the process of adipogenesis **(Chapter V)**.

Each chapter has or will result in a publication (see the list below).

Llista de publicacions derivada de la present tesi doctoral corresponent als diferents capítols:

List of publicatons derived from the present PhD thesis corresponding to the diferent chapters:

I- Salmerón, C., García de la serrana, D., Jiménez-Amilburu, V., Fontanillas R., Navarro, I., Johnston, I.A., Gutiérrez, J. and Capilla, E. (2013). Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata). PloS one* **8**, e75349.

II- Salmerón, C., Navarro, I., Johnston, I.A., Gutiérrez, J. and Capilla, E. Characterisation of cathepsins and ubiquitin-proteasome members in gilthead sea bream (*Sparus aurata*) skeletal muscle: changes in expression during life stages and re-feeding. In preparation.

III- Salmerón, C., Johansson, M., Angotzi, A.R., Rønnestad, I., Jönsson, E., Björnsson, B.T., Gutiérrez, J., Navarro, I. and Capilla, E. Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion in rainbow trout. In preparation.

IV- Salmerón, C., Johansson, M., Asaad, M., Angotzi, A.R., Rønnestad, I., Jönsson, E., Björnsson, B.T., Gutiérrez, J., Capilla, E., Navarro, I. Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes *in vitro*. In preparation.

V- Salmerón, C., Acerete, L., Gutiérrez, J., Navarro, I. and Capilla, E. (2013). Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*). *Domestic Animal Endocrinology* **45**, 1-10.

Llista de publicacions derivada de les col·laboracions realitzades durant la present tesi doctoral:

List of publicatons derived from the collaborations carried out during the present PhD thesis:

VI- Seiliez, I., Gutiérrez, J., Salmerón, C., Skiba-Cassy, S., Chauvin, C., Dias, K., Kaushik, S., Tesseraud, S. and Panserat, S. (2010). An *in vivo* and *in vitro* assessment of autophagy-related gene expression in muscle of rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology, Part B* **157**, 258–266.

VII- Enes, P., Pousão-Ferreira, P., Salmerón, C., Capilla, E., Navarro, I., Gutiérrez, J. and Oliva-Teles, A. (2013). Effect of guar gum on glucose and lipid metabolism in white sea bream *Diplodus sargus*. *Fish Physiology and Biochemistry* **39**, 159-169.

VIII- Jiménez-Amilburu, V., Salmerón, C., Codina, M., Navarro, I., Capilla, E. and Gutiérrez, J. (2013). Insulin-like growth factors effects on the expression of myogenic regulatory factors in gilthead sea bream muscle cells. *General and Comparative Endocrinology* **18**, 151-158.

Factor d’impacte

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La Dra. Encarnación Capilla Campos com a directora de la tesi doctoral presentada per Cristina Salmerón Salvador titulada "Paper de leptina i grelina sobre el teixit adipós i estudi de la proteòlisi muscular en peixos" manifesta la veracitat del factor d'impacte i la implicació de la doctoranda en cada article científic publicat presentat en aquesta tesi. La Dra. Capilla fa constar que la doctoranda ha participat de manera molt activa en l'elaboració de cadascun dels articles en tots els aspectes, tal i com queda palès amb el fet que la Cristina Salmerón és primera autora en tots ells. La doctoranda ha participat de forma molt notable en tots els articles des de l'inici del disseny experimental, com en la realització dels experiments i la obtenció de dades fins a l'analítica dels resultats, així com ha contribuït en la redacció dels manuscrits. Els articles 1 i 2 es van iniciar durant l'estada de la Cristina al laboratori del Prof. Ian A. Johnston del "Scottish Oceans Institute" a la Universitat de St. Andrews (Escòcia), i dades dels articles 3 i 4 es deriven també de l'estada de la doctoranda al laboratori del Prof. Björn Thrandur Björsson i la Dra. Elisabeth Jönsson a la Universitat de Göteborg (Suècia).

Article 1: Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*).

Autors: Cristina Salmerón, Daniel García de la serrana, Vanesa Jiménez-Amilburu, Ramón Fontanillas, Isabel Navarro, Ian A. Johnston, Joaquim Gutiérrez and Encarnación Capilla.

Revista: PLoS ONE

Factor d'impacte: 3.730 (Quartil 1)

Estat: Publicat

Article 2: Characterisation of cathepsins and ubiquitin-proteasome members in gilthead sea bream (*Sparus aurata*) skeletal muscle: changes in expression during life stages and re-feeding.

Autors: Cristina Salmerón, Isabel Navarro, Ian A. Johnston, Joaquim Gutiérrez and Encarnación Capilla.

Revista: -

Factor d'impacte: -

Estat: En preparació

Article 3: Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion in rainbow trout.

Autors: Cristina Salmerón, Marcus Johansson, Anna R. Angotzi, Ivar Rønnestad, Elisabeth Jönsson, Björn Thrandur Björnsson, Joaquim Gutiérrez, Isabel Navarro, Encarnación Capilla.

Revista: -

Factor d'impacte: -

Estat: En preparació

Article 4: Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes *in vitro*.

Autors: Cristina Salmerón, Marcus Johansson, Maryam Asaad, Anna R. Angotzi, Ivar Rønnestad, Elisabeth Jönsson, Björn Thrandur Björnsson, Joaquim Gutiérrez, Encarnación Capilla and Isabel Navarro.

Revista: -

Factor d'impacte: -

Estat: En preparació

Article 5: Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*).

Autors: Cristina Salmerón, Laura Acerete, Joaquim Gutiérrez, Isabel Navarro and Encarnación Capilla.

Revista: Domestic Animal Endocrinology

Factor d'impacte: 2.377 (Quartil 1)

Estat: Publicat

Dra. Encarnación Capilla

Directora de la tesi doctoral

Discussió general

The works that comprise this thesis aim to gain knowledge on the processes related with skeletal muscle protein turnover, as well as adipose tissue development and metabolism regulation in aquaculture fish. More specifically, the studies aim to determine the factors that regulate muscle proteolysis (Bloc I: chapters I and II) and those that are involved in the control of adipogenesis and lipid metabolism in adipocytes (Bloc II: chapters III, IV and V) in gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*), two species with great commercial interest in Spain and Europe. To achieve this, some key members of the different proteolytic systems (calpains, cathepsins and ubiquitin-proteasome (UbP) pathway) related with protein turnover and degradation were identified in gilthead sea bream fast skeletal muscle and characterized (Chapters I and II). Then, its gene transcription was studied at three different life stages and in fish under different nutritional status (fasting and re-feeding) and fed diets with different composition (Chapters I and II); and their potential use as genetic markers of flesh quality was also analyzed (Chapter I). Next, experiments were conducted *in vivo* in rainbow trout to explore the modulatory effect of adiposity in plasma leptin (Chapter III), and *in vitro,* to study the role of certain hormones (leptin, ghrelin and insulin) and nutrients (leucine and EPA) on adipogenesis and lipid metabolism regulation (Chapters III and IV). Finally, in order to study gilthead sea bream adipogenesis, it was established for the first time in this species, a preadipocyte primary cell culture from visceral adipose tissue; and the effects of insulin, the insulin-like growth factor (IGF-I) and growth hormone (GH) on cell proliferation and differentiation were analyzed (Chapter V). Overall, these studies have contributed to better understand the mechanisms that regulate muscle proteolysis and adipose tissue metabolism in these species, which may help to optimize muscle growth and to reduce fat accumulation improving quality and production in aquaculture.

Characterization and transcriptional analysis of genes related to muscle proteolysis and flesh texture in gilthead sea bream

One of the most important parameters of flesh quality in both mammals and fish is texture. The underlying mechanisms responsible for changes in texture *in vivo* and during *post-mortem* are not well known, but endogenous proteolytic enzymes seem to play an important role in these processes (Bond and Warner, 2007; Huff-Lonergan et al., 2010; Kemp et al., 2010). A muscle cell contains several proteolytic systems, which include primarily the cytosolic calpains, the lysosomal cathepsins and the UbP system (Jackman and Kandarian, 2004); as well as the caspases, that are mainly involved in apoptosis (Cowdhury et al., 2008).

In the present thesis, we have cloned the full-length cDNA sequences of: a) calpains1-3, (sacapn1, sacapn2, sacapn3) and two paralogues of the calpain small subunit1, (sacapns1a and sacapns1b), b) cathepsin B (SaCTSB) and a new paralog of cathepsin D (SaCTSDb); and c) ubiquitin (SaUb), and partially the proteasome subunit beta type-4 known as N3 or PSMB4 (SaN3), from gilthead sea bream fast skeletal muscle. The deduced amino acid sequences obtained shared high levels of overall identity (58–100%) with its homologous counterparts in other teleosts and vertebrate species. The typical calpain, cathepsin, Ub and N3 domain architecture was identified in all gilthead sea bream amino acid sequences. The catalytic triad (Goll et al., 2003), the cysteine or aspartyl (Turk et al., 2012; Zaidi et al., 2008) and the threonine (Nothwang et al., 1994) residues common to all calpains, cysteine or aspartyl cathepsins, and certain proteasome subunits were in each case highly conserved in comparison to vertebrates. Interestingly, the third putative N-glycosylation site present in SaCTSDa, the first SaCTSD paralog described in ovary of gilthead sea bream (Carnevali et al., 1999) is not present in SaCTSDb, as it occurs in other vertebrate sequences (Mommsen, 2004), suggesting regulatory differences between paralogues.

To further characterize the gilthead sea bream calpains and cathepsins, two separate phylogenetic trees were constructed. Each putative gilthead sea bream sequence was related to its corresponding teleost and tetrapod ortholog, with the exception of sacapn2 and SaCTSDa. On one side, sacapn2 clustered with the other teleosts' Calpain2 sequences analyzed, but this whole group formed a monophyletic clade external to the tetrapod Calpain2/8 sister group, supporting the hypothesis that they are a close related group (i.e. Calpain2-like) and the common ancestor of vertebrate putative Calpains2 and 8 (Lepage and Bruce, 2008; Macqueen et al., 2010a). On the other side, the phylogenetic analysis of cathepsin D genes provided strong evidence that both paralogues are present in most teleosts; however, the fact that SaCTSDa clustered on a branch with only fish members, suggested that SaCTSDa orthologues may have been lost in the higher vertebrate lineages (Feng et al., 2011; Mommsen et al., 2004), whereas SaCTSDb was maintained. This, together with the structural differences observed between both cathepsin D paralogues in gilthead sea bream, suggests that functional divergences may also exist.

Differential tissue expression

Tissue expression analysis of the different proteolytic members identified in gilthead sea bream showed that transcripts of sacapn1, sacapn2, sacapns1a, sacapns1b, SaCTSDa and SaCTSDb were ubiquitously expressed as previously observed in rainbow trout (Brooks et al.,

1997; Salem et al., 2004; Salem et al., 2005), Atlantic halibut (Macqueen et al., 2010), grass carp (Dong et al., 2012), channel catfish (Feng et al., 2011), zebrafish (Riggio et al., 2000), rabbits (Emori et al., 1986a; Emori et al., 1986b) or humans (Ono and Sorimachi, 2012), for instance; including also for SaCTSDa previous reports in gilthead sea bream (Acerete et al., 2007; Carnevali et al., 1999a). On the other hand, the sacapn3 transcripts were mostly detected in fast- and slow-twitch skeletal muscle as in mammals, where calpain3 is principally expressed (Sorimachi et al., 1989); but also, in the heart and to a lesser extent in other tissue types as previously reported in Atlantic halibut (Macqueen et al., 2010), suggesting a broader physiological role for Calpain3 in teleosts in comparison to mammals, deserving thus further investigation. Moreover, the SaCTSDb gene was highly expressed in muscle, especially in comparison to SaCTSDa, suggesting that the former might have a function regulating muscle protein turnover and degradation, being then more involved in fillet quality than the latter, which has been previously implicated in the follicle maturation process that occurs during oogenesis (Carnevali et al., 1999a; Carnevali et al., 1999b; Carnevali et al., 2008).

Expression during life stages

The mRNA expression of SaCTSB, SaCTSDb, SaCTSL and SaN3 was evaluated in gilthead sea bream fast skeletal muscle at three different life stages (fingerlings, juveniles and adults), and all of them decreased during lifetime. The down-regulation observed in the expression of these proteolytic enzymes suggests a decrease in protein degradation with age, which would reduce at the same time protein turnover, as younger fish (fingerlings) have also higher protein synthesis rates than older fish (Peragón et al., 1998; Peragón et al., 2001). On the other hand, the expression of SaCTSDa remained stable among life stages in muscle; thus, supporting evolutionary physiological divergences between both SaCTSD paralogues. Moreover, SaUb was the only transcript which expression decreased from fingerlings to juvenile fish and increased again in adults, in agreement with previous studies in mice and humans were Ub increased with age (Cai et al., 2004). Supporting this idea, in our study the transcript abundance of the proliferation marker, the proliferating cell nuclear antigen (PCNA) as well as that of the well-known negative regulator of muscle growth, myostatin (MSTN) also decreased in adult fish. Although gilthead sea bream presents indeterminate growth, these observations overall support in line with our results that SaUb is important during the whole life of the fish; with a role in the adult reducing the ability of muscle regeneration or being involved in some kind of muscle aging process as in mammals.

Effects on expression of nutritional status

In response to long-term fasting, fish skeletal muscle proteins can be mobilized resulting in muscle atrophy (Navarro and Gutiérrez, 1995). Besides, in anabolic situations food intake stimulates protein synthesis, but to a lesser extent, also its degradation, inducing protein turnover and growth. After 15 and 30 days of fasting, in gilthead sea bream only sacapns1b increased its expression, while the other calpains remained unaffected; although cathepsins' expression was not measured. This result is contrary to previous studies in fish were fasting stimulated the expression in fast skeletal muscle of calpain catalytic subunits (Macqueen et al., 2010; Preziosa et al., 2013; Salem et al., 2005a), but not that of the calpain regulatory subunit (Salem et al., 2005a). Contrarywise after re-feeding, only sacapn3 remained unchanged, whereas the relative expression of the other calpains, cathepsins and UbP members analyzed was significantly reduced, suggesting a generalized decrease in muscle proteolysis under these conditions. These results were in agreement with a study in Atlantic halibut, where calpain1 transcript levels significantly decreased after 7 days of re-feeding in a 60 days fasted fish (Macqueen et al., 2010). In another study in rainbow trout fasted for one month, changes on the calpain genes were not found; however, up-regulation of cathepsins B, D and S and UbP pathway genes expression was observed (Rescan et al., 2007). Moreover in the same study, a significant down-regulation on the expression of the different proteases was reported during re-feeding in agreement with the present data. Similarly, the mRNA expression of the Ub-ligase atrogin1 and the level of poly-ubiquitinated proteins were significantly increased with fasting and decreased after re-feeding in the muscle of rainbow trout (Seiliez et al., 2008). All in all, the majority of the data indicate that in fish the proteolytic systems, especially the calpains, are more sensitive to the modulation by anabolic rather than catabolic signals.

Effects on expression of diet composition

Gilthead sea bream is mainly carnivorous, but also accessorily herbivorous (Bauchot and Hureau, 1990). In the present thesis, four isolipidic diets with different protein/carbohydrate (CH) ratios were used to determine if it is possible to increase CH over the established limit of 20% without affecting growth in this species; but more interestingly, to see if the relative expression of calpain members could be modulated by the diet and/or related to muscle texture. Although no differences were observed in feed intake or plasma glucose between groups, gilthead sea bream showed decreased growth parallel to the increased level of exchange of protein by CH in the diet, supporting the limited value of 20% CH dietary inclusion to achieve good growth rates in this species (Enes et al., 2011).

Texture analysis showed that a lower protein/CH ratio in the diet significantly improved the flesh maximal strength and elasticity in the gilthead sea bream of our study, similarly as in another sparid, *Dentex dentex,* where the decrease in dietary protein content improved also textural parameters (Suárez et al., 2009). Nevertheless in that study, and contrary to our observations, diets with high CH and low lipid content resulted in lower values of muscle firmness than diets with low CH, overall indicating that an adequate balance of nutrients is required for optimal fillet texture. In terms of calpain expression, in our study sacapn1 and sacapns1a were transcriptionally affected by the diet, while the other calpains remained unaffected. Both, sacapn1 and sacapns1a relative expression decreased with dietary CH increase and protein decrease, suggesting a reduction in muscle proteolysis and an increase in muscle texture in these fish. In agreement with this observation, calpain1 mRNA levels were significantly lower in pigs fed a protein-free diet in comparison to control fed pigs (van den Hemel-Grooten et al., 1997). Conversely in rainbow trout, the substitution of fishmeal by a mix of plant protein sources in the diet, or strains with distinct growth rates and fillet firmness fed two diets with different energy content, did not show modified expression of any of the calpains analyzed (Alami-Durante et al., 2010; Salem et al., 2005b), indicating that the effects of diet on calpain expression show significant variations between fish species. Nevertheless in that study, differences were observed in calpastatin expression, indicating that the calpain inhibitor has a role in meat tenderization too (Salem et al., 2005b). Therefore, it will be interesting to identify the calpastatin gene in gilthead sea bream to study its function and regulation, as well as its level of expression in proportion to that of calpains in different situations.

To further investigate the potential use of calpains as flesh quality molecular markers, correlation analysis between textural parameters and calpains expression was performed with the data obtained from this dietary experiment. Interestingly, the relative expression levels of sacapn1 and sacapns1a were significantly negatively correlated with maximal strength in our study. Similarly in mice, calpain1 knock-out animals have a significant reduced rate of proteolysis in comparison to wild-type mice (Geesink et al., 2006). Besides, other studies in beef and lamb have supported that calpain1 is primarily responsible for meat tenderisation (Koohmaraie et al., 1987; Ilian et al., 2001); and in cattle, single nucleotide polymorphisms (SNP) for the calpain1 gene have been clearly associated with tenderness (Page et al., 2002). This has currently led to the use of markers within the calpain1 as well as the calpastatin gene to identify the genetic potential of beef cattle to produce tender meat (Casas et al., 2006), a tool that is commercially available as a genetic test (GeneSTAR, Pfizer Genetics). In this sense,

it would be very interesting to develop a similar tool for aquaculture; therefore, the present results have revealed at the moment the potential use of calpains, specifically sacapn1 and sacapns1a, as candidate genes to monitor muscle growth and fillet firmness in gilthead sea bream.

In conclusion, this thesis provides new evidences at a transcriptional level, about the regulation of important enzymes of the main endogenous proteolytic systems implicated in protein turnover and fillet tenderisation. The present results contribute to understand the role of different members of calpain, cathepsin and UbP systems in important physiological processes, under different conditions such as fasting, re-feeding, dietary regime or during life stages. Finally, for the first time it has been shown that the mRNA expression of some calpains is related to muscle texture in gilthead sea bream. This discovery opens new avenues of research on calpains as potential genetic markers of fillet quality in this species.

In vivo effects of adiposity on plasma leptin and, hormonal and nutritional control of adipogenesis and adipocyte lipid metabolism *in vitro*

Nutrition in fish is essential to produce a healthy and high quality product, but also, it is important economically because the feed represents 40-50% of the production costs in aquaculture (Craig, 2002). The current trend in aquafeeds is to use lipids, a high-energy nutrient, to partially substitute dietary protein (the most expensive component). The use of these diets can help reduce the cost; however, can have at the same time some negative effects such as that it may cause excessive fat deposition, mainly in the visceral adipose tissue and the liver, which can decrease welfare, as well as, the quality of the fish product (Craig, 2002). Therefore, the study of adipogenesis and lipid metabolism in fish can help to better understand how to reduce fat accumulation.

In vivo effects of adiposity on plasma leptin

The aim of the present study was to investigate the response of plasma leptin levels to middle-term (8 weeks) feeding with a high-energy diet at two ration levels, *ad libitum* (AL) or 25% satiation (RE). In one hand, we focused on leptin, since this cytokine has been implicated in the regulation of energy homeostasis in mammals (Grill, 2010) and teleosts (de Pedro et al., 2006; Huising et al., 2006; Murashita et al., 2008). On the other hand, we studied plasma leptin levels

in relation to adiposity, since leptin concentration and body mass index or adipose tissue mass are positively correlated in mammals (Benoit et al., 2004; Chakrabarti, 2013), where leptin is considered to act as an adiposity signal.

First, we observed higher values of adipose tissue and liver weights, mesenteric fat index (MFI) and hepatosomatic index (HSI) in AL respect to RE fish at the end of the experimental trial. Then, we found that middle-term feed restriction in rainbow trout increased plasma leptin levels in comparison to fully fed fish, which is in agreement with previous studies in rainbow trout and other teleost species, where plasma leptin levels increased with fasting or feed restriction (Frøiland et al., 2012; Fuentes et al., 2012; Johnsen et al., 2011; Kullgren et al., 2013; Trombley et al., 2012). In this sense, plasma leptin levels in the burbot (*Lota lota*) also increase in the wild after spawning, when the energy stores of the fish are small, and correlate inversely with body mass (Mustonen et al., 2002). On the contrary in mammals, fasting acutely decreases plasma leptin concentrations (Friedman and Haalas, 1998); with the exception of hibernating mammals such as the mink (*Mustela vison*), the raccoon dog (*Nyctereutes procyonoides*), the woodchuck (*Marmota monax*) or the common shrew (*Sorex araneus*), where the highest leptin levels have been observed in the animals with the lowest body adiposity (Concannon et al., 2001; Nieminen and Hyvärinen, 2000; Nieminen et al., 2000; Nieminen et al., 2002).

Previous works *in vivo* and *in vitro* have demonstrated that human or homologous leptin treatments have anorectic actions in rainbow trout (Aguilar et al., 2010; Aguilar et al., 2011; Murashita et al., 2008) and Atlantic salmon (Murashita et al., 2011). Therefore, although leptin is differently secreted in fish compared to mammals under fasting condition, the peptide activates similar pathways in the brain suppressing appetite. In agreement with all these results, Fuentes and collaborators (2012) hypothesized that the response of elevating plasma leptin levels in fasted fish may contribute to a passive survival strategy, in species that commonly experience natural food shortage periods, to limit physical foraging activity (that will require extra energy), by lowering appetite. Interestingly, this hypothesis is in agreement with the leptin response observed in mammalian hibernators.

Furthermore, plasma leptin levels in our study negatively correlated with MFI and adipose tissue mass, according to recent findings on Atlantic salmon where plasma leptin levels also negatively correlated with body lipid content in fully fed controls, whereas not in feed-restricted fish (40% satiation) (Trombley et al., 2012). Moreover in our study, plasma leptin levels were also negatively correlated with liver weight, as previously described in the burbot

during the spawning season (Mustonen et al., 2002). In rainbow trout (Murashita et al., 2008) and in other fish species, leptin mRNA is expressed primarily in liver (Gorissen et al., 2009; Huising et al., 2006; Kurokawa and Murashita, 2009; Kurokawa et al., 2005); consequently, the liver is thought to be the principal source of leptin and, to a lesser extent, the adipose tissue. A recent study in Atlantic salmon fully fed or feed-restricted (60% satiation) found in agreement with this observation that LepA1 expression in the liver negatively correlated with visceral lipid content (Moen and Finn, 2013). Although the relationship between circulating leptin and adiposity is different in fish that in mammals, all these findings suggest that leptin in fish might still convey information to the brain about reserves to regulate energy metabolism.

Nutritional and hormonal control of leptin and lipid metabolism in isolated adipocytes

Adipocytes isolated from visceral adipose tissue of AL and RE fish, plus adipocytes isolated from fish fed a regular diet, were used to assess the effects of several nutrients and hormones on a) leptin secretion and expression and, b) lipolysis and expression of lipid metabolism-related genes.

In teleosts, leptin mRNA expression is low in adipose tissue, relative to its expression in the liver (Huising et al., 2006; Pfundt et al., 2009; Rønnestad et al., 2010). Notwithstanding, in the present thesis we have demonstrated for the first time that freshly isolated rainbow trout adipocytes can express and secrete leptin; thus indicating that plasma leptin levels in fish may become in part from adipose tissue, as in mammals (Harris, 2014). Furthermore, leptin secretion was significantly higher in control and insulin-treated adipocytes from RE than AL fish, in agreement with the *in vivo* observations on plasma leptin, suggesting that the adipocytes from RE fish retain some kind of metabolic memory. Similarly in rainbow trout, the rate of lipolysis in isolated adipocytes from fasted fish was higher than in those from fed fish, accordingly to the higher levels of free fatty acids (FA) in the plasma of fasted fish echoing what is observed *in vivo* (Albalat et al., 2005b). Besides, it appeared that isolated adipocytes from AL fish show a certain degree of resistance to hormonal or nutritional stimuli, as similar LepA1 mRNA and secreted leptin levels were detected in all treatments. These results agree with the idea that animals fed *ad libitum* with a high-energy diet may develop metabolic disorders, and may have therefore lost the ability to respond to external stimuli to regulate leptin, as observed in rodent models (Ceddia, 2005). On the other hand, in adipocytes from RE fish incubated with nutrients such as leucine, a decrease in leptin secretion occurred, contrary to what it is known in mammals (Lynch et al., 2006; Roh et al., 2003). In agreement with this observation, a recent study with golden-mantled ground squirrels (*Spermophilus lateralis*) has

shown that leptin levels decrease in hibernators in association with the initiation of food intake (Healy et al., 2008). Thus, it can be speculated from our study that this increase in leucine in the medium could be interpreted at the cellular level as increased food availability, causing a decrease in leptin secretion, which in turn, *in vivo* would increase appetite, stimulating thereby the feeding/foraging behavior of the fish, as it seems to occur in hibernating mammals. Furthermore, although ghrelin or insulin did not modify leptin neither in AL nor in RE adipocytes, treatment of freshly isolated adipocytes from regular diet-fed rainbow trout with both hormones increased leptin secretion in a dose-response manner, as previously reported in mammals (Giovambattista et al., 2008; Moreno-Aliaga et al., 2003; Ricci et al., 2005). Overall, these results indicate that the nutritional status of the fish modulates the basal level of leptin secretion, as well as, it affects the ability of the tissue to respond to stimuli in order to modify this secretion.

In relation with lipid metabolism, leptin and ghrelin treatments significantly increased lipolysis, with an unaccompanied release of free FA in rainbow trout freshly isolated adipocytes. This effect was previously reported for leptin in mammalian studies, *in vivo* and *in vitro*, supporting the idea that FA are oxidized or re-esterified inside the adipocyte rather than exported (Li et al., 2010b; Tajima et al., 2005; Wang et al., 1999). However ghrelin, contrary to our results, generally inhibits lipolysis in rat adipocytes (Choi et al., 2003; Muccioli et al., 2004). On the other hand *in vivo*, ghrelin is known to stimulate the release of GH in the pituitary, a well-known lipolytic factor in vertebrates (Kojima et al., 2001; Lucidi et al., 2005). Consequently, it appears that in rainbow trout, ghrelin may be stimulating lipolysis directly and indirectly via GH, since GH has been demonstrated to increase lipolysis also in fish adipocytes (Albalat et al., 2005a; Bergan et al., 2013). Furthermore, the mRNA levels of two important lipolytic enzymes, hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Kolditz and Langin, 2010; Lass et al., 2011; Yang et al., 2011) were analyzed. The treatments had no effect on the expression of these genes, although HSL mRNA showed a tendency to increase dose-dependently after incubation with ghrelin, suggesting that ghrelin may stimulate lipolysis in part directly through HSL activation, whereas leptin may act activating other lipases.

Next, fatty acid synthase (FAS) and lipoprotein lipase (LPL), two enzymes important for adipose tissue triglyceride (TG) accumulation (Gonzales and Orlando, 2007; Notarnicola et al., 2012; Ranganathan et al., 2006; Weinstock et al., 1997), were used as lipogenic markers. FAS and LPL mRNA expression showed a significant increase in response to ghrelin, suggesting a lipogenic role for ghrelin also in rainbow trout adipocytes. This result agrees with a previous study *in vivo* in rats where the mRNA expression of various fat storage-promoting enzymes was

markedly increased in adipose tissue after central ghrelin infusion (Theander-Carrillo et al., 2006) or subcutaneous injections of ghrelin (Barazzoni et al., 2005); and with a recent study in mice, where ghrelin stimulated the expression of lipogenic genes in adipose tissue and liver through the transcription factor p53 (Porteiro et al., 2013). Moreover, we analyzed the expression of the fatty acid transporter protein-1 (FATP1). A transporter involved in the translocation of FA across the plasma membrane, playing a pivotal role with fatty acyl-CoA synthase up-regulating TG synthesis *de novo* (Hatch et al., 2002). FATP1 mRNA expression showed a significant dose-dependent decrease upon leptin treatment, suggesting that leptin decreases the transport of FA into the adipocyte as described in mammals (Ceddia, 2005). Interestingly, in a recent study in Atlantic salmon, two weeks of fasting reduced FATP1 mRNA expression in adipose tissue (Sánchez-Gurmaches, et al., 2011); therefore, since feed restriction increases plasma leptin in salmonids, we suggest that leptin may be act *in vivo*, similarly as we observed *in vitro*, reducing FA transportation.

Finally, the rate-controlling enzyme of the long-chain FA oxidation pathway, the carnitine palmitoyl transferase (CPT1b) and, the transcription factors from the peroxisome proliferator-activated receptors (PPARs) family, PPARα and PPARβ, both implicated in FA uptake and oxidation (Huss and Kelly, 2004; Leaver et al., 2008; Li and Glass, 2004; Wang et al., 2003; Leone et al., 1999), were selected as β-oxidation markers. Whereas neither leptin nor ghrelin had any effect on CPT1b or PPARα transcript levels, ghrelin significantly up-regulated the mRNA expression of PPARβ, suggesting that it increases FA oxidation. In rats, ghrelin reduced hepatic mitochondrial β-oxidation (Rigault et al., 2007) and CPT1 mRNA expression (Barazzoni et al., 2005); however, in abdominal white adipose tissue ghrelin did not change the expression of CPT1 in agreement with the present results.

Overall, these data supports an anti-lipogenic role for leptin in rainbow trout, whereas ghrelin seems to cause a general activation of lipid metabolism turnover by stimulating anabolic and catabolic pathways in adipose tissue. These results indicate that both, leptin and ghrelin regulate adipocyte energy homeostasis in fish, although nothing is known about the pathways by which they exert their actions in adipose tissue, thus deserving further attention. In mammals, the signaling pathways of both hormones convey in the regulation by phosphorylation of AMP-activated protein kinase (AMPK). Recent studies in mammals have shown that leptin exerts its effects in a tissue-dependent manner through the activation or inhibition of AMPK (reviewed by Lim et al., 2010). In adipose tissue and liver, leptin activates AMPK, thus inhibiting TG synthesis through acetyl-CoA carboxylase (ACC1) inactivation, whereas in muscle and the hypothalamus, leptin inhibits AMPK enhancing FA β-oxidation by

inhibiting ACC2 (Daval et al., 2006; Viollet et al., 2010). On the other hand in mammals, ghrelin stimulates AMPK phosphorylation in the hypothalamus activating it, while the contrary situation is observed in the adipose tissue, enhancing thus lipolysis and lipogenesis but decreasing β-oxidation (Lim et al., 2010).

Endocrine regulation of preadipocyte primary cell culture development

A thorough understanding of adipocyte proliferation and differentiation, plus a detailed study of the endocrine regulation of these processes, could importantly support the potential manipulation of adipogenesis in order to prevent excessive fat deposition in fish.

Two kinds of cell lines are currently available as models to study adipocyte development in mammals: a) preadipocyte cell lines, and b) multipotent stem cell lines (Armani et al., 2010). However, equivalent cell systems are not available in fish; therefore, to study adipogenesis primary cell cultures have been established in several fish species including red sea bream (Oku et al., 2006), rainbow trout (Bouraoui et al., 2008), Atlantic salmon (Vegusdal et al., 2003), large yellow croaker (Wang et al., 2012) and grass carp (Li, 2012), but not in gilthead sea bream.

Then, in order to study gilthead sea bream adipogenesis, it was established, for the first time in this species, a preadipocyte primary cell culture from the stromal-vascular cell fraction of visceral adipose tissue. The culture was established as previously done in our laboratory for rainbow trout (Bouraoui et al., 2008), but higher content of NaCl and incubation temperature were needed for optimal development of the cells, being the values more similar to the conditions reported to culture red sea bream preadipocytes (Oku et al., 2006). Regarding cell progression, in gilthead sea bream, cells in growth medium achieved maximum proliferation at day 16, similarly to that observed in red sea bream (Oku et al., 2006). Moreover, increased proliferation of cells incubated in adipogenic medium indicated that some cells may continue proliferating during the differentiation phase, contrary to what it occurs in mammalian cells (Gregoire et al., 1998; Otto and Lane, 2005). Morphologically, the gilthead sea bream preadipocytes in culture first adopted a fibroblast appearance and after the addition of the differentiation medium, accumulated lipid droplets and enlarged its cytoplasm, as previously described in other preadipocyte cultures (Bouraoui et al., 2008; Li, 2012; Oku et al., 2006; Vegusdal et al., 2003; Wang et al., 2012). Furthermore, as previously observed in other fish preadipocyte cultures, besides the regular components of the differentiation cocktail (insulin, dexamethasone and IBMX) used in mammals (Scott et al., 2011), the use of a lipid mixture plays an essential role in gilthead sea bream adipocyte differentiation measured.

Next, we studied the hormonal regulation of proliferation and differentiation, in both, gilthead sea bream and rainbow trout primary preadipocyte cultures, since it is well established that changes in circulating hormone concentrations can result in marked differences in adipose tissue growth (Hausman et al., 2009; Poulos et al., 2010).

In relation with proliferation, all factors tested in gilthead sea bream preadipocytes (GH, IGF-I and insulin) significantly stimulated proliferation, whereas only insulin, but not leptin or ghrelin, stimulated proliferation in rainbow trout preadipocytes. Stimulatory effects of GH on preadipocyte proliferation have been described in mammals, but depending on cell model and species it appears to be an indirect action, since GH markedly stimulates IGF-I production also in adipocytes (Gregoire et al., 1998). Notwithstanding, the mitogenic effects of insulin and IGF-I observed in our gilthead sea bream and rainbow trout preadipoctes are in agreement with the literature. Both, insulin and IGF-I have been shown to promote proliferation in cell lines from mammals (Blüher et al., 2005; Géloën et al., 1989; Grimaldi et al., 1983; Nelson et al., 2002; Scavo et al., 2004), as well as mammalian and piscine preadipocyte primary cultures (Bouraoui et al., 2008; Li, 2012; Louveau and Gondret, 2004; Oku et al., 2006; Wang et al., 2012). On the other hand, in the present study, neither leptin nor ghrelin modulated adipocyte proliferation. The effects of leptin reported in mammals are controversial, with leptin increasing or decreasing preadipocyte proliferation (Wagoner et al., 2006; Zwirska-Korczala et al., 2007) or showing no effects (Kim et al., 2008); however, conversely to our results, ghrelin in mammals increases preadipocyte proliferation (Kim et al., 2004; Zhang et al., 2004; Zwirska-Korczala et al., 2007).

Regarding adipocyte maturation, IGF-I was more potent than insulin enhancing differentiation in gilthead sea bream, whereas leptin or ghrelin had no effects in rainbow trout adipognesis. The positive effects observed for IGF-I on preadipocyte differentiation in gilthead sea bream, corroborated the evidence previously shown that IGF-I enhances the maturation of preadipocytes into adipocytes in many mammalian cell models and species (Hausman, 1989; Ramsay and Rosebrough, 2003; Ramsay et al., 1989; Schmidt et al., 1990; Smith et al., 1988). Furthermore, the lipid mixture was the major stimulator of differentiation, both in gilthead sea bream and rainbow trout adipocytes. The addition of lipid mixture enhanced lipid accumulation 10 days after the induction of differentiation in gilthead sea bream preadipocytes, and increased lipid accumulation and mRNA expression of lipogenic markers (LPL and FATP1) in rainbow trout preadipocytes. As previously mentioned, the dependence on high concentration of FA for differentiation appears to be crucial in fish and other species such as chicken (Matsubara et al., 2005), but not so much in mammals (Gregoire et al., 1998). The

molecular bases for those differences are unknown; but among others, different sensibility of PPARs to FA or distinct rates of lipogenesis have been speculated to partially explain these variations. Interestingly, leptin significantly decreased the mRNA expression in rainbow trout preadipocytes of an early differentiation marker, LPL (Bouraoui et al., 2012; Gregoire et al., 1998; MacDougal et al., 1995; Todorcević et al., 2008), suggesting a possible anti-adipogenic role for leptin in this species.

Finally, we have demonstrated in this thesis the presence of ghrelin and its functional receptor (GHS-R1a) by immunofluorescence in differentiated adipocytes of rainbow trout in culture. Ghrelin is primarily produced by the stomach in vertebrates (Castañeda et al., 2010; Jönsson, 2013), and in fish, one report in Atlantic salmon detected weak expression of ghrelin in visceral adipose tissue (Murashita et al., 2009). On the other hand, GHS-R1a is mainly present in the pituitary in mammals, but also, one study in Mozambique tilapia detected GHS-R1a transcripts in adipose tissue (Kaiya et al., 2009). Furthermore, as observed in isolated adipocytes, we have also demonstrated LepA1 mRNA expression, as well as leptin secretion in rainbow trout primary adipocytes in culture. These results are in agreement with observations in primary cultured adipocytes of Atlantic salmon (Vegusdal et al., 2003), and with previous works on mammalian cultured adipocytes and 3T3-L1 cells (Matson et al., 1996; Sheng et al., 2013; van Harmelen et al., 2002). Notwithstanding, this is the first study that shows leptin expression and secretion also in preadipocytes, since in mammals, undifferentiated adipocytes have been shown to not produce leptin (Matson et al., 1996). Moreover, LepA1 expression and leptin production increased in our culture during adipocyte differentiation, suggesting a more important role for leptin as an endocrine signal coming from mature adipocytes.

In conclusion, this thesis provides new evidences at metabolic and transcriptional levels, about the effects of adiposity in plasma leptin *in vivo* and, the role of metabolic hormones (leptin, ghrelin and insulin), growth factors (IGF-I and GH) and nutrients (EPA and leucine) on lipid metabolism and adipogenesis regulation in rainbow trout and gilthead sea bream. This is the first time that a primary culture of preadipocytes from gilthead sea bream has been established and characterized, being a useful tool for future adipose tissue *in vitro* studies. Furthermore, we have shown that plasma leptin levels correlate negatively with adiposity, contrary to what it occurs in mammals, and that leptin secretion from adipocytes is regulated by hormones and nutrients. Moreover, we have demonstrated that ghrelin and the GHS-R1a receptor are expressed in differentiated adipocytes, suggesting new roles for ghrelin as a

potential adipokine with endocrine or autocrine modes of action in rainbow trout. All in all, the present results contribute to better understand the role of leptin and ghrelin in important processes, such as adipogenesis and the regulaton of lipid metabolism in fish adipocytes.

Epilogue

Although this thesis is divided *a priori* into two different parts, the subject matter of study in each part is not totally independent, but related, instead.

Regarding leptin, some studies have found in cows that this adipokine is implicated in the regulation of different characteristics related to production and quality, such as food consumption, quality grade and fat deposition among others, and that these traits are closely related to molecular markers, including a single nucleotide polymorphism (SNP) and two microsatellites (Buchanan et al., 2002; Cerón-Muñoz et al., 2009; Fitzsimmons et al., 1998; Hale et al., 1999; Kononoff et al., 2005; Lagonigro et al., 2003; Liefers et al., 2002).

As for the calpains, it has been shown that calpains are required for the differentiation of 3T3-L1 preadipocytes into mature adipocytes, since the inhibition of calpain activity blocks the expression of the CAAT/enhancer binding protein-α (C/EBPα), a transcriptional activator of many adipogenic genes that plays a key role in adipogenesis (Patel and Lane, 1999). The authors suggested that calpains appear to be required for C/EBPβ to acquire the capacity to bind the promoter of C/EBPα, to thereby activate the transcription of this gene to continue the transcriptional cascade that takes place during the process of adipogenesis.

Therefore, this thesis opens the door to future research to better understand the role of leptin in fish muscle to determine its potential use as a molecular marker of filet quality, as well as, to study the role of calpains in fish adipogenesis to unravel their ability to regulate lipid accumulation.

Conclusions

Conclusions

1. En el múscul esquelètic d'orada (*Sparus aurata*) s'han identificat i caracteritzat diversos membres dels sistemes proteolítics de calpaïnes (capn), catepsines (CTS) i ubiquitina-proteasoma (UbP) (sacapn1, sacapn2, sacapn3, sacapns1a, sacapns1b, SaCTSB, SaCTSDb, SaUb i SaN3) que mostren els dominis estructurals i els motius catalítics específics per ser funcionals, com els seus ortòlegs en mamífers.

2. L'expressió dels gens de les catepsines (SaCTSB, SaCTSDb i SaCTSL) i dels membres de la via UbP (SaN3 i SaUb) disminueixen al llarg de la vida juntament amb PCNA i MHC, el que indica que el recanvi proteic muscular es redueix amb l'edat. No obstant, la SaUb incrementa de nou en peixos adults, suggerint un augment de la ubiquitinització en aquesta etapa de la vida.

3. El dejú incrementa només els nivells de mRNA de la sacapns1b, mentre que la realimentació redueix l'expressió de gairebé totes les proteases analitzades (calpaïnes, catepsines i membres de la via UbP), el que indica una clara regulació negativa de la proteòlisi a nivell transcripcional en condicions anabòliques.

4. El nou paràleg SaCTSDb identificat en l'orada mostra major expressió en múscul i un patró de regulació transcripcional diferent de la SaCTSDa, recolzant un paper més important de la SaCTSDb en la proteòlisi muscular.

5. L'expressió de sacapn1 i de sacapns1a és veu modulada per la composició de la dieta i es correlaciona negativament amb els paràmetres de textura del filet, assenyalant a aquests gens com a possibles marcadors moleculars de la qualitat de la carn en l'orada.

6. S'ha demostrat per primera vegada en peixos que els adipòcits, així com pels pre-adipòcits, expressen i secreten leptina, contribuint d'aquesta manera als nivells de leptina en plasma. D'altra banda, s'ha demostrat que la insulina i la grelina augmenten la secreció de leptina dependent de la dosi en adipòcits aïllats de truites irisades (*Oncorhynchus mykiss*) alimentades amb una dieta estàndard.

7. La restricció nutricional (25% de l'alimentació *ad libitum*) amb una dieta alta en energia en la truita irisada redueix la massa del teixit adipós i del fetge respecte el grup *ad libitum*, però augmenta els nivells de leptina en plasma, el que resulta en correlacions negatives entre la leptina i els pesos dels teixits. Aquestes dades suggereixen que la leptina circulant pot actuar com un senyal nutricional en el peix, transmetent informació al cervell per regular la gana.

8. L'estat nutricional afecta la producció de leptina dels adipòcits i la seva regulació, ja que els adipòcits de peixos amb restricció alimentària secreten més leptina que els dels peixos alimentats *ad libitum*. A més, només els adipòcits dels peixos amb alimentació restringida responen a la leucina (interpretat com un augment de la disponibilitat d'aliments) reduint la secreció de leptina, la qual al seu torn estimularia la gana.

9. La grelina activa el metabolisme lipídic de l'adipòcit a diferents nivells; augmentant la síntesi de triglicèrids (lipogènesi) i la seva mobilització (lipòlisi), així com l'oxidació d'àcids grassos. Per contra, la leptina té un paper anti-lipogènic en els adipòcits, ja que augmenta l'alliberament de glicerol i inhibeix l'expressió de transportadors d'àcids grassos.

10. Ni la leptina ni la grelina tenen influència en l'adipogènesi en el cultiu primari d'adipòcits de truita irisada; però, la grelina i el seu receptor s'han identificat en aquestes cèl·lules, el que indica que la grelina pot tenir altres efectes endocrins o locals en aquesta espècie.

11. Per primera vegada en orada s'ha establert un cultiu primari de pre-adipòcits, a partir de cèl·lules de la fracció vascular i de l'estroma del teixit adipós visceral, i s'ha demostrat la capacitat d'aquestes cèl·lules per diferenciar-se en adipòcits madurs en presència d'un medi adipogènic complementat amb lípids. A més, s'ha demostrat que la GH, l'IGF-I i la insulina estimulen la proliferació cel·lular, mentre que l'IGF-I també incrementa l'acumulació de lípids.

12. En resum, aquesta tesi obre noves línies d'investigació en fisiologia de peixos (per exemple, la regulació dels paràlegs de calpaïna o la grelina com a nova adipoquina), i proporciona eines útils per investigar més a fons el múscul esquelètic i el desenvolupament del teixit adipós en peixos, per tal d'ajudar a la millora d'alguns aspectes de la qualitat de l'actual producte d'aqüicultura.

Conclusions

1. In gilthead sea bream (*Sparus aurata*) skeletal muscle have been identified and characterized several members of the calpain (capn), cathepsin (CTS) and ubiquitin-proteasome (UbP) proteolytic systems (sacapn1, sacapn2, sacapn3, sacapns1a, sacapns1b, SaCTSB, SaCTSDb, SaUb and SaN3) that show the structural domains and specific catalytic motifs to be functional, as their orthologues in mammals.

2. The gene expression of cathepsins (SaCTSB, SaCTSDb and SaCTSL) and UbP members (SaN3 and SaUb) decrease during lifetime together with PCNA and MHC, indicating that muscle protein turnover is reduced with age. However, SaUb rises again in adult fish, suggesting an increase in ubiquitination in this life stage.

3. Fasting increases only sacapns1b mRNA levels, whereas re-feeding reduces the expression of almost all the proteases analyzed (calpains, cathepsins and UbP members), indicating a clear negative regulation of proteolysis at a transcriptional level under anabolic conditions.

4. The newly identified SaCTSDb paralogue in gilthead sea bream shows higher expression in muscle and a different pattern of transcriptional regulation compared to SaCTSDa, supporting a major role for SaCTSDb in muscle proteolysis.

5. The expression of sacapn1 and sacapns1a is modulated by dietary composition and correlates negatively with the textural parameters of the fillet, pointing out to these genes as potential molecular markers of flesh quality in gilthead sea bream.

6. It has been demonstrated for the first time in fish that leptin is expressed and secreted by adipocytes, as well as preadipocytes; thus contributing to the plasma leptin levels. Moreover, it has been shown that insulin and ghrelin increase dose-dependently leptin secretion from isolated adipocytes of rainbow trout (*Oncorhynchus mykiss*) fed a regular diet.

7. Nutritional restriction (25% of *ad libitum* feeding) with a high-energy diet in rainbow trout decreases adipose tissue and liver mass but increases plasma leptin levels in comparison with the *ad libitum* group, which results in negative correlations between leptin and tissue weights. These data suggest that circulating leptin may act as a nutritional signal in fish conveying information to the brain to regulate appetite.

8. The nutritional condition affects leptin production from adipocytes and its regulation, since adipocytes from feed restricted fish secrete more leptin than those of fish fed *ad libitum*. Besides, only adipocytes from feed restricted fish respond to leucine (interpreted as increased food availability) reducing leptin secretion, which in turn would stimulate appetite.

9. Ghrelin activates lipid metabolism in the adipocyte at different levels, by increasing synthesis of triglycerides (lipogenesis) and its mobilization (lipolysis), as well as fatty acid oxidation. Conversely, leptin has an anti-lipogenic role in adipocytes, as it increases glycerol release and inhibits the expression of fatty acid transporters.

10. Neither leptin nor ghrelin influence adipogenesis in rainbow trout primary cultured adipocytes; however, ghrelin and its receptor have been identified in these cells, indicating that ghrelin may have other endocrine or local effects in this species.

11. A preadipocyte primary culture from the stromal-vascular cell fraction of visceral adipose tissue has been established for the first time in gilthead sea bream. Furthermore, the ability of the cells to differentiate into mature adipocytes in the presence of an adipogenic medium supplemented with lipids has been demonstrated. It has been shown that GH, IGF-I and insulin stimulate cell proliferation, while IGF-I enhances also lipid accumulation.

12. In summary, the present thesis opens new lines of research on fish physiology (e.g. Calpains paralogues regulation, ghrelin as a new adipokine) and; provides useful tools to further investigate skeletal muscle and adipose tissue development in fish, in order to help the improvement of some of the quality traits in the current product of aquaculture.

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Publicacions

BLOC I

Estudi de la proteòlisi muscular

Study of muscle proteolysis

CAPÍTOL I

Caracterització i expressió de membres de la familia de les calpaïnes en relació a l'estat nutricional, composició de la dieta i textura del filet en orada (*Sparus aurata*)

Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*)

Publicat a la revista PLoS One el 2013

Caracterització i expressió de membres de la familia de les calpaïnes en relació a l'estat nutricional, composició de la dieta i textura del filet en orada (*Sparus aurata*)

Les calpaïnes són proteases neutres no lisosomals activades per calci que participen en una àmplia gamma de processos cel·lulars incloent la proteòlisi muscular lligada a l'estovament *post-mortem* de la carn. Els objectius d'aquest estudi van ser: (a) caracteritzar diversos membres del sistema de les calpaïnes en orada i (b) examinar la seva expressió en relació amb l'estat nutricional i l'estovament del múscul. Es va identificar el marc obert de lectura de les calpaïnes1-3, *sacapn1*, *sacapn2*, *sacapn3*, i de dos paràlegs de la subunitat petita de la calpaïna1, *sacapns1a* i *sacapns1b* en l'orada. Les seves seqüències proteiques van mostrar una identitat d'un 63-90% en comparació amb les seqüències de mamífers i altres peixos teleostis, i l'estructura característica dels dominis de les calpaïnes de vertebrats. Els trànscrits de *sacapn1*, *sacapn2*, *sacapns1a* i *sacapns1b* van mostrar una àmplia distribució tissular, mentre que *sacapn3* es va detectar gairebé exclusivament en el múscul esquelètic. A continuació, es va avaluar l'expressió dels trànscrits de les calpaïnes en el múscul esquelètic després de l'alteració de l'estat nutricional mitjançant (a) el dejuni i la realimentació o (b) l'alimentació amb quatre dietes experimentals amb diferents proporcions d'hidrats de carboni i proteïna. El dejuni va reduir significativament la glucosa en plasma i va augmentar els àcids grassos lliures i els triglicèrids, juntament amb un augment significatiu en l'expressió de la *sacapns1b*. Després de 7 dies de realimentació, els paràmetres plasmàtics van tornar als valors dels peixos alimentats i l'expressió de la *sacapn1*, la *sacapn2*, la *sacapns1a* i la *sacapns1b* va reduir-se significativament. D'altra banda, un augment en el contingut de carbohidrats de la dieta (11-39%) va disminuir el creixement però va augmentar la textura muscular, que es va correlacionar significativament amb la disminució de l'expressió de la *sacapn1* i de la *sacapns1a*, mentre que les altres calpaïnes no es van veure afectades. Aquest estudi ha demostrat que l'expressió de les calpaïnes és modulada per l'estat nutricional i la composició de la dieta en l'orada, i que l'expressió de diversos membres del sistema de les calpaïnes es correlacionen amb la textura muscular, el que indica el seu potencial ús com a marcadors moleculars de la qualitat del filet produït en aqüicultura.

Paraules clau: Textura muscular, degradació de proteïnes, creixement, dejuni i re-alimentació, qualitat del producte.

Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*)

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Short title: Gilthead sea bream calpains characterisation.

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Abstract

Calpains are non-lysosomal calcium-activated neutral proteases involved in a wide range of cellular processes including muscle proteolysis linked to *post-mortem* flesh softening. The aims of this study were (a) to characterise several members of the calpain system in gilthead sea bream and (b) to examine their expression in relation to nutritional status and muscle tenderisation. We identified the complete open reading frame of gilthead sea bream calpains1-3, *sacapn1*, *sacapn2*, *sacapn3*, and two paralogs of the calpain small subunit1, *sacapns1a* and *sacapns1b*. Proteins showed 63-90% sequence identity compared with sequences from mammals and other teleost fishes, and the characteristic domain structure of vertebrate calpains. Transcripts of *sacapn1*, *sacapn2*, *sacapns1a* and *sacapns1b* had a wide tissue distribution, whereas *sacapn3* was almost exclusively detected in skeletal muscle. Next, we assessed transcript expression in skeletal muscle following alteration of nutritional status by (a) fasting and re-feeding or (b) feeding four experimental diets with different carbohydrate-to-protein ratios. Fasting significantly reduced plasma glucose and increased free fatty acids and triglycerides, together with a significant increase in *sacapns1b* expression. Following 7 days of re-feeding, plasma parameters returned to fed values and *sacapn1*, *sacapn2*, *sacapns1a* and *sacapns1b* expression was significantly reduced. Furthermore, an increase in dietary carbohydrate content (11 to 39%) diminished growth but increased muscle texture, which showed a significant correlation with decreased *sacapn1* and *sacapns1a* expression, whilst the other calpains remained unaffected. This study has demonstrated that calpain expression is modulated by nutritional status and diet composition in gilthead sea bream, and that the expression of several calpain members is correlated with muscle texture, indicating their potential use as molecular markers for flesh quality in aquaculture production.

Keywords: Muscle texture, protein degradation, growth, fasting and re-feeding, product quality.

1. Introduction

Gilthead sea bream (*Sparus aurata*) is an important marine species reared in the Mediterranean area. In 2011, 94,4% of its production came from farms (151.346Tn), whereas only 5,6% (8.330Tn) came from extractive fishing [1]. The axial musculature or fillet is the main product of aquaculture and in gilthead sea bream represents approximately 65% of body mass.

While in terrestrial farmed animals meat tenderisation is a desirable process, in farmed fish freshness and firm texture are considered among the most important quality attributes of the flesh. Texture is influenced by various physical, chemical, biochemical and microbiological changes that may occur *post-mortem*, finally resulting in a loss of quality. An important determinant of flesh texture is muscle cellularity i.e. the number and size distribution of the fibres [2], [3]. These parameters can be affected by a number of factors such as diet [4], [5], [6], photoperiod [7], temperature [8], [9], [10] and exercise training [11]-[14].

Muscle texture also depends on the ratio between protein synthesis and degradation. During the last decades, the activity of muscle endogenous proteinases has received a great deal of attention due to their role in muscle protein turnover and growth, and *post-mortem* proteolysis. The major intracellular proteolytic systems include the proteasome, calpains, cathepsins and caspases. Currently, calpains and cathepsins (lysosomal proteases) are known to hydrolyse myofibrillar proteins, and all available evidence indicate that the structural changes that take place during *post-mortem* storage of meat are caused by muscle proteases, especially members of these two families [15].

Calpains are Ca^{2+}-dependent intracellular proteases that belong to the papain superfamily of cysteine proteases and are found in almost all eukaryotes and a few bacteria, but not in archaebacteria [16]. The human genome contains 15 genes that encode calpains. Nine of them encode the classical calpains, Calpain1 to Calpain3, Calpain8, Calpain9, and Calpain11 to Calpain14. All of them contain a C2-like (CL2) and a penta EF-hand (PEF) domain plus the calpain-like protease (CysPc) domain. The remaining non-classical calpains (Calpain5 to Calpain7, Calpain10, Calpain15 and Calpain16) lack both, the CL2 and PEF domains [16]. Depending on their expression across tissues, classical calpains are classified in humans as ubiquitous (*calpain1*, *calpain2*, *calpain13* and *calpain14*) or tissue-specific (*calpain3* in skeletal muscle, *calpain8* and *calpain9* in gastrointestinal tract, *calpain11* in testis and *calpain12* in hair follicle).

Defects in ubiquitous calpains may be lethal, as seen in *calpain2*-/- mice (*Mus musculus*) [17], whereas defects in tissue-specific calpains may cause tissue-specific phenotypes such as the

muscular dystrophy caused by mutations in *calpain3* [18]. Calpain1 and Calpain2 have been widely studied in vertebrates; both are 80 kDa catalytic subunits that independently bind a common 30 kDa regulatory subunit (Calpain4 or calpain small subunit) to respectively form a heterodimer, which is named μ-calpain or m-calpain for its activation by micro or millimolar concentrations of Ca^{2+}, respectively. A large number of proteins including cytoskeletal proteins, kinases, phosphatases, membrane-associated proteins, such as receptors or ion channels, and some transcription factors have been reported to be cleaved by calpains in *in vitro* assays [19]. Nevertheless, experimental evidence has clearly suggested that μ-calpain, but not m-calpain, has the most significant role in *post-mortem* proteolysis and meat tenderisation [20].

Previous studies in rainbow trout (*Oncorhynchus mykiss*) [21], Atlantic halibut (*Hippoglossus hippoglossus*) [22], zebrafish (*Danio rerio*) [23] and more recently in channel catfish (*Ictalurus punctatus*) [24] have shown that fish calpains have high amino acid identity and the characteristic domains of their orthologs in mammals. Retained paralogs of several calpain family members have been identified from the Whole Genome Duplication that occurred early in the adaptive radiation of the bony fishes [22], [25]. Teleosts also contain a ubiquitously expressed μ/m calpain which has one-to-one orthology with the testis-restricted *calpain11* in placental mammals [25]. It has also been reported that teleost calpains may be differentially regulated according to nutritional status. For example, in Atlantic halibut with 60 days of feed restriction, it was shown that *calpain1* transcript levels were significantly decreased after 7 days of re-feeding; at the same time that *calpain3* and *calpain11* expression significantly increased, whereas *calpain2-like* showed little response [22]. In channel catfish, 35 days of fasting increased the expression of *calpain2*, while decreasing that of *calpain1* and *calpain3* [24]. In another study in rainbow trout, fasting also for 35 days stimulated the expression of *calpain1*, *calpain2* and *calpastatin* (the endogenous specific inhibitor of ubiquitous calpains), suggesting a potential role for calpains in protein mobilization as a source of energy under catabolic conditions [21]. The same authors also observed that rainbow trout strains with reduced growth rate and softest fillet had significantly lower levels of *calpastatin* expression, but this softness effect related to the strain disappeared when fish were fed a high energy diet, indicating that diet also modulates calpain expression and texture [26]. Another study in sea bass (*Dicentrarchus labrax*) fed diets with three different levels of fat reported that a high lipid content in muscle could be responsible for faster *post-mortem* proteolysis, and suggested a possible activation of calpains related to lipid accumulation [27]. Further research is needed to elucidate the potential importance of diet, calpain expression and/or activity on texture in key aquaculture species.

The first objective of the present study was to identify and characterise different members of the calpain proteolytic system in gilthead sea bream. Secondly, in order to better understand the physiological situations that may regulate calpains expression in this species, transcript abundance was studied in fast-twitch skeletal muscle in response to: fasting/re-feeding conditions and various experimental diets with different ratios of protein and carbohydrate. Finally, the relationship between calpains expression and fillet firmness was also examined to determine their potential use as molecular markers of flesh quality.

2. Materials and methods

2.1. Ethics statement

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona (CEEA 239/09) and the Departament de Medi Ambient i Habitatge (DMAH permit number 5420, Generalitat de Catalunya, Spain) following the European Union, Spanish and Catalan Government-established norms and procedures.

2.2. Animals and experimental trials

For the tissue screening experiment 10 juvenile gilthead sea bream (67.14±9.89g, 15.86±0.88cm fork length) from Tinamenor S.L. (Pesués, Spain) were maintained at the facilities of the University of Barcelona (Barcelona, Spain), fed *ad libitum* twice a day with commercial pellets (Excel, Skretting, Burgos, Spain) and held at 21±1ºC, pH of 7.5-8 in a recirculating seawater tank (400L) with 12 h light: 12 h dark photoperiod.

For the fasting/re-feeding experiment 120 juvenile gilthead sea bream (49.52±5.91g) from the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Sant Carles de la Ràpita, Spain) were maintained at the facilities of the University of Barcelona (Barcelona, Spain) homogenously distributed in 8 recirculating seawater tanks (200L) and held at 21±1ºC, pH 7.5-8 with 12L:12D photoperiod. Fish were acclimated for a month and fed at 3% body weight twice a day (the ration was given 70% in the morning and 30% in the afternoon) with commercial pellets (Excel, Skretting, Burgos, Spain). First, 1 fish from each tank was sampled for time 0 (D0C). Then, fish were divided into two conditions: Control fed group (C) and Fasted group (F). The F group was fasted during 30 days while the C group was fed at 3% body weight for the duration of the whole experiment. Samples of 8 fish from each condition were collected at days 15 and 30 (D15C/F and D30C/F). Then, for the re-feeding experiment, fasted animals for 30 days (D0F) were re-fed at 2% body weight (lower than the control normal ration to facilitate correct

adaptation of the digestive system) during 7 and 14 days and sampled (8 fish per condition at each time, D7R and D14R).

Finally, for the dietary experiment, 204 adult gilthead sea bream with an initial average weight of 115g were maintained at IRTA facilities (Sant Carles de la Ràpita, Spain) and held at 22-24ºC and natural photoperiod. Animals were homogenously distributed in 12 seawater tanks (17 fish/tank and 3 tanks per condition) connected to a closed recirculation system with feed collectors to measure the food wasted to calculate feed intake. Fish were fed *ad libitum* twice a day for 107 days, using automatic fish feeders, with four experimental diets containing different percentages in protein/carbohydrate (CH) (46/11, 46/19, 42/35 and 40/39, respectively) and 17% lipid (Table 1). At the end of the experiment, 9 fish of each group (3 fish per tank) were sampled for plasma constituents, biometrics, colour, texture and expression analysis. Together with the sampled fish, the remaining fish were also weighted to obtain the specific growth rate (SGR) and the feed conversion rate (FCR) values of all fish.

Table 1. Ingredients and chemical composition of the experimental diets.

Diet	**46/11**	**46/19**	**42/35**	**40/39**
Raw materials (%)				
Cellulose	17,07	7,70	0,00	0,00
Fish meal	20,00	20,00	20,00	20,00
Corn gluten	13,10	13,10	14,71	9,77
Wheat gluten	20,00	20,00	20,00	21,02
Fish oil	11,27	11,27	11,29	11,52
Soya concentrate	11,53	11,53	2,67	2,86
Mineral vitamin	2,00	2,00	2,00	2,00
Yttrium premix	0,10	0,10	0,10	0,10
Wheat Starch	5,00	14,18	29,00	33,00
Total	100	100	100	100
Calculated (% dry matter)				
Protein	46,85	47,32	43,33	41,12
Fat	17,03	17,21	17,33	17,31
Starch	7,56	16,31	30,29	36,22
Analysed (% dry matter)				
Protein	45,73	46,17	42,14	39,57
Fat	16,44	17,34	17,03	17,19
Starch	10,85	18,51	35,15	38,70

Skretting designed the diets and performed the nutrient analysis. Nomenclature of the diets corresponds to the percentage of protein/carbohydrate analysed.

Before sampling, all animals were fasted 24 h to avoid regurgitation of food and to obtain basal values of plasma metabolites and also to closely mimic the market situation, since this is a common practice before sacrificing commercial products for aquaculture. The fish were then anesthetised with tricaine methane sulphonate (MS-222 0.1g/L, Sigma, Tres Cantos, Spain) and sacrificed with a blow on the head and medullar section. Blood from all fish of the dietary and fasting/re-feeding experiments was taken (1mL/fish) from the caudal vein using 23G syringes with EDTA-Na and quickly centrifuged at 5000 rpm for 10 min to separate the plasma. Biometrics including body weight, total length, hepatosomatic index (HSI), mesenteric fat index (MFI) and condition factor (CF) were measured. Flesh colour was analysed and samples of fast skeletal muscle were either taken for texture measurement and kept on ice, or immediately snap-frozen in liquid nitrogen and stored at -80ºC for gene expression analyses. The same procedure was used for 15 distinct tissue-types (fast muscle, slow muscle, fat, bone, head kidney, spleen, eye, brain, stomach, proximal intestine, distal intestine, pyloric caeca, skin, liver and heart) from 10 fish.

2.3. Plasma parameters

Plasma glucose concentration was determined by a glucose oxidase colorimetric method (Spinreact, Sant Esteve d'en Bas, Spain). Plasma free fatty acids (FFAs) concentration was measured using a commercially available kit (NEFA-HR2, Wako Chemicals GmbH, Neuss, Germany). Plasma triglycerides (TGs) were hydrolysed by a lipase, and the released glycerol was measured by a peroxidase-coupled colorimetric assay (Spinreact, Sant Esteve d'en Bas, Spain).

2.4. Flesh colour and texture

Muscle colour was measured at the time of sampling using a portable CR400 Chroma Meter (Konica Minolta, Madrid, Spain). The colorimeter was calibrated using the white standard provided. The colour system L*, a* and b* was used for analysis. L* represents lightness (L*=0 for black, L*=100 for white), a* indicates red/green (+a* intensity in red and –a* intensity in green) and b* represents yellow/blue (+b* intensity in yellow and –b* intensity in blue) [28]. Values of Chroma (colour intensity) and Hue angle (composed colour) were also obtained in the measurement. It is difficult to present standard colour values for a healthy/desirable muscle due to the great variability observed between studies; nevertheless, muscle colour in gilthead sea bream seems to be strongly related to its fat content [29], [30].

For texture analysis, a slab of ~2cm^2 of skinless flesh, containing fast- and slow-twitch skeletal muscle, was dissected from the anterior-dorsal side of the fish to the dorsal spines and immediately kept on ice until analysis, 24 h later. Thickness of the muscle fillet was taken into consideration for normalisation of the texture data. Texture analysis was done using a TA.XT2i texturometer and a Mini Kramer/Ottawa cell blade in the Escola Superior d'Agricultura de Barcelona (ESAB) facilities, (Castelldefels, Spain). Total work, maximal strength and elasticity of flesh fragments from 4-8 fish per diet were analysed.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from ~100mg of fast muscle and between 40 to 500mg of the other tissues from the tissue screening experiment, following the guanidiniumthiocyanate-phenol-chloroform method [31] using TRIreagent (Applied Biosystems, Alcobendas, Spain). Total RNA was quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain), quality was verified as 260/280 and 260/230 ratios were over 1.8 in both cases and RNA integrity was analysed by 1% (m/v) agarose gel electrophoresis. To eliminate any residual genomic DNA, total RNA was treated with DNase I (Invitrogen, Alcobendas, Spain) following the manufacturer's recommendations before cDNA synthesis. One µg of total RNA per sample was used to synthesise first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain) following the manufacturer's recommendations. cDNA samples were diluted 1:5 in milliQ H_2O for conventional polymerase chain reaction (RT-PCR) and diluted 1:100 in milliQ H_2O for real-time quantitative PCR (qPCR).

2.6. Calpains cloning and sequencing

To obtain the complete sequences of *Sparus aurata* calpain1 (*sacapn1*), calpain2 (*sacapn2*), calpain3 (*sacapn3*), calpain small subunit1a (*sacapns1a*), primers for 5' rapid amplification of cDNA ends (RACE)-PCR and specific primers were designed from gilthead sea bream ESTs (Expressed Sequence Tag) NCBI database (*sacapn1*: AM951595.1; *sacapn2*: FM155301.1, FG591123.1, FM152855.1 and FG265085.1; *sacapn3*: FG262721.1; *sacapns1a*: AM962179.1 and FM145762) and from 5'RACE-PCR amplicon products for *sacapn1* and *sacapn2* (Table S1). Also, *sacapn3* and calpain small subunit1b (*sacapns1b*) sequences were retrieved from the gilthead sea bream muscle transcriptome performed using 454 pyrosequencing (accession number: ERP000874) previously described by Garcia de la serrana *et* al., [32].

PCR products were separated by gel electrophoresis and purified using a PureLink Quick Gel Extraction Kit, ligated into T/A pCR4-TOPO vector and transformed into chemically competent

TOP10 *Escherichia coli* cells by thermal shock (all from Invitrogen, Alcobendas, Spain). At least 1-3 clones of each PCR product were sequenced in both T3/T7 orientations using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Alcobendas, Spain) and analysed at the Serveis Cientificotècnics of the University of Barcelona (Barcelona, Spain). Sequenced products were joined *in silico* using DNAMAN (Lynnon Corporation, Quebec, Canada) to produce contigs with a single open reading frame (ORF). 5'RACE-PCR reactions were performed using a 5'RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Alcobendas, Spain) following the manufacturer's recommendations.

Table S1. Calpains primer sequences used for cloning by RT-PCR and 5' RACE-PCR.

Gene	Assay	Sense strand primer (5'-3')	Antisense strand primer (5'-3')	Anneal temp (°C)	Product size (bp)
sacapn1	5' RACE GSP1a		GTCCTTATCTGTGTCC		
	5' RACE nested GSP2a	5'RACE Abridged Anchor Primer	GTCTGACCAGGCAGGTGACAAAGTT	55	
	5' RACE nested GSPa	Abridged Universal Amplification Primer	GGTGCCCGATTTGTCCAGGT	55	1006
	5' RACE GSP1b		CTTTGGAACCTCATAG		
	5' RACE nested GSP2b	5'RACE Abridged Anchor Primer	CCTCCACTCCCCCTGATACA	55	
	5' RACE nested GSPb	Abridged Universal Amplification Primer	ATCCACACTGTCCCACTCTCTG	55	425
	5' RACE GSP1c		CATCTCTGGAGTTGTC		
	5' RACE nested GSP2c	5'RACE Abridged Anchor Primer	TGTTGCTGATGTCGATGGAG	55	
	5' RACE nested GSPc	Abridged Universal Amplification Primer	CTCGTAGCAGCCGTTCAACT	55	467
	5' RACE GSP1d		ATGTCTGTGCGAGTAG		
	5' RACE nested GSP2d	5'RACE Abridged Anchor Primer	CTTTGAACCCCAGTGACGAC	55	
	5' RACE nested GSPd	Abridged Universal Amplification Primer	CGCAGGGGAACAAGCTATC	55	719
sacapn2	RT-PCR	GCTCTGGGTGACTGCTGGCTG	GACATCCAGAACTCTCCGTCCTC	64	671
	RT-PCR	GAGGACGGAGAGTTCTGGATGTC	CCACTGGTTGAAGTCGAGCTC	61	1121
	5' RACE GSP1		ACTGGAAGTGGAAGAT		
	5' RACE nested GSP2	5'RACE Abridged Anchor Primer	CTGGTCAGTGGGAACGACTC	55	
	5' RACE nested GSP	Abridged Universal Amplification Primer	ACAGCCAGCAGTCACCCAGA	55	395
sacapn3	RT-PCR	AGGGTTTCAGCCTTGAGACG	CTGGAGCCACTCCAGGACATT	56	379
sacapns1a	RT-PCR	TCCTCAACCTCAACAAAGTGC	GGAAGTGGTAGAGATGGTTGAGA	56	783

2.7. Tissue screening

Qualitative RT-PCR was used to analyse calpains transcripts expression in different tissues. Elongation factor 1-alpha (*ef1α*) was used as a control gene. Reactions were performed in a final volume of 50µL, containing 1µL of first-strand cDNA (equivalent to 4ng of reverse transcribed total RNA), 1.5U of Taq polymerase (Sigma, Tres Cantos, Spain) and 200nM (final concentration) of sense and antisense primers (Table S2). Reactions proceeded in a C1000 Thermal Cycler (Bio-Rad, El Prat de Llobregat, Spain) with the following protocol: 1 cycle at 95ºC for 5 min, 35 cycles at 95ºC for 30 s, 53-61ºC (primer dependent, see Table S2) for 30 s, 72ºC for 0.5-1.5 min and 1 cycle at 72ºC for 7 min. Each reaction product was separated by agarose gel electrophoresis and visualised using SYBR Safe DNA gel stain (Life Technologies, Alcobendas, Spain) in a LAS-3000 (Fujifilm, Madrid, Spain) to confirm that a single product was amplified, and then sequenced to confirm the specificity of each assay.

Table S2. Calpains primer sequences used for tissue screening by RT-PCR.

Gene	Assay	Sense strand primer (5'-3')	Antisense strand primer (5'-3')	Anneal temp (°C)	Product size (bp)
sacapn1	RT-PCR	GAAGGACCGCAGGAAGAAAC	GTGCTACCGCTGGATTGACT	58	1111
sacapn2	RT-PCR	GAGGACGGAGAGTTCTGGATGTC	CCACTGGTTGAAGTCGAGCTC	61	1122
sacapn3	RT-PCR	TCCTCGCTCTTCTACAGTCAC	CGTAACACCACCCGTGAAAT	58	455
sacapns1a	RT-PCR	TTCCTCAACCTCAACAAAGTGC	CAAGGCAGCCAATGTAGTT	56	590
sacapns1b	RT-PCR	ATGTTTATGGCGAAGGCG	AGAGGTATTTGAACTCGTGGAAG	53	346
ef1α	RT-PCR	CTTCAACGCTCAGGTCATCAT	GCACAGCGAAACGACCAAGGGGA	60	264

2.8. Quantitative real-time PCR

The mRNA transcript levels of gilthead sea bream calpain genes (*sacapn1*, *sacapn2*, *sacapn3*, *sacapns1a* and *sacapns1b*), the β proteasome subunit N3 (*N3*) plus three reference genes (*ef1α*, beta-actin (*β-actin*) and ribosomal protein L27a (*rpl27a*)) were assessed using qPCR across the fasting/re-feeding and diet experiments. Each qPCR reaction contained 5µL of first-strand cDNA (equivalent to 2.5ng of reverse transcribed total RNA), 10µL of iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain) and 250nM (final concentration) of sense and antisense primers (Table S3) in a final volume of 20µL. Reactions were performed in triplicate using a MyiQ thermocycler (Bio-Rad, El Prat de Llobregat, Spain) with 1 cycle of 3 min at 95ºC and 40 cycles of 10 s at 95ºC and 30 s at 56-68 ºC (primer dependent, see Table S3), followed by an amplicon dissociation analysis from 55 to 95ºC at 0.5ºC increase each 30 s, where a single peak was observed confirming the specifity of the reaction and the absence of primer-dimers formation. Also, prior to the analyses, a dilution curve with a pool of samples was run to confirm primer efficiency and to determine the appropriate cDNA dilution. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Negative controls (NTC: No Template Control; RTC: no Reverse Transcriptase Control and PCR: water) were included and ran in duplicate. Raw data were normalized to *β-actin*, the most stable of the three reference genes analysed, by the delta-delta method [33].

Table S3. Calpains primer sequences used for qPCR.

Gene	Assay	Sense strand primer (5'-3')	Antisense strand primer (5'-3')	Anneal temp (°C)	Product size (bp)
sacapn1	qPCR	CCTACGAGATGAGGATGGCT	AGTTGTCAAAGTCGGCGGT	56	114
sacapn2	qPCR	ACCCACGCTCAGACGGCAAA	CGTTCCCGCTGTCATCCATCA	61	405
sacapn3	qPCR	AGAGGGTTTCAGCCTTGAGA	CGCTTTGATCTTTCTCCACA	56	113
sacapns1a	qPCR	CGCAGATACAGCGATGAAAA	GTTTTGAAGGAACGGCACAT	56	92
sacapns1b	qPCR	ATGGACAGCGACAGCACA	AGAGGTATTTGAACTCGTGGAAG	56	51
N3	qPCR	AGACACACACTGAACCCGA	TTCCTGAAGCGAACCAGA	54	119
ef1α	qPCR	CTTCAACGCTCAGGTCATCAT	GCACAGCGAAACGACCAAGGGGA	60	263
β-actin	qPCR	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT	60	50
rpl27a	qPCR	AAGAGGAACACAACTCACTGCCCCA	GCTTGCCTTTGCCCAGAACTTTGTAG	68	159

2.9. Statistical analyses

Statistical analyses of all parameters were performed in PASW Statistics 17.0 (IBM, Chicago, USA). Normality was analysed according to the Shapiro-Wilk test and homogeneity in variance according to Levene's test. Therefore, statistical differences were assessed by one-way ANOVA, followed by Tukey's test, or t-test. Non-parametric tests, Kruskal-Wallis and U de Mann-Whitney, were used when after data transformation normality was not found. A significance of $p<0.05$ was applied to all statistical tests performed. Data are presented as mean±standard error of the mean (SEM). Correlation analyses were carried out on the dietary experiment data in order to determine whether flesh texture was related to calpains expression, or whether any of the other variables also had an effect. Non-homoscedasticity was found; therefore the Spearman's rank correlation coefficient (ρ) was performed. Correlation was considered significant at the bilateral levels of 0.05(*) or 0.01(**).

2.10. Bioinformatic resources

Sequences used in the study other than those from gilthead sea bream were obtained from either NCBI (http://www.ncbi.nlm.nih.gov/) or ENSEMBL (http://www.ensembl.org) databases. Human tissue expression patterns of calpains were obtained from the GeneNote database ([34], http://genecards.weizmann.ac.il/genenote/). BLAST searches were performed against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/blast). PSORTII [35] was used to predict nuclear localisation signals (NLSs) and Reinhardt's method for Cytoplasmic/Nuclear discrimination ([36], http://psort.hgc.jp/form2.html). Compute pI/Mw tool (ExPASy, Switzerland, http://www.expasy.org/tools/pi_tool.html) was used to estimate the molecular weight (Mw) of the predicted proteins. In addition, polypeptide sequences rich in Proline (P), Glutamic acid (E), Serine (S) and Threonine (T) (PEST) that mark proteins as targets for rapid destruction were identified using the PEST finding program (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::epestfind).

A phylogenetic tree of 48 complete amino acid sequences of calpain large subunits (Calpains 1, 2, 3, 8, 9 and 11) and small subunits (Calpains 1a and 1b), from different vertebrates was performed. Calpain8, Calpain9 and Calpain11 were included, since these family members form a sister group to Calpain2, Calpain3 and Calpain1, respectively [23], [25], [37]. Calpain sequences were initially aligned using the Mafft v.6 (http://mafft.cbrc.jp/alignment/server/index.html) and G-INS-i (recommended for <200 sequences with global homology) strategy. Evolutionary analyses were conducted in MEGA5 [38]. The evolutionary history was inferred by using the Maximum Likelihood method based on

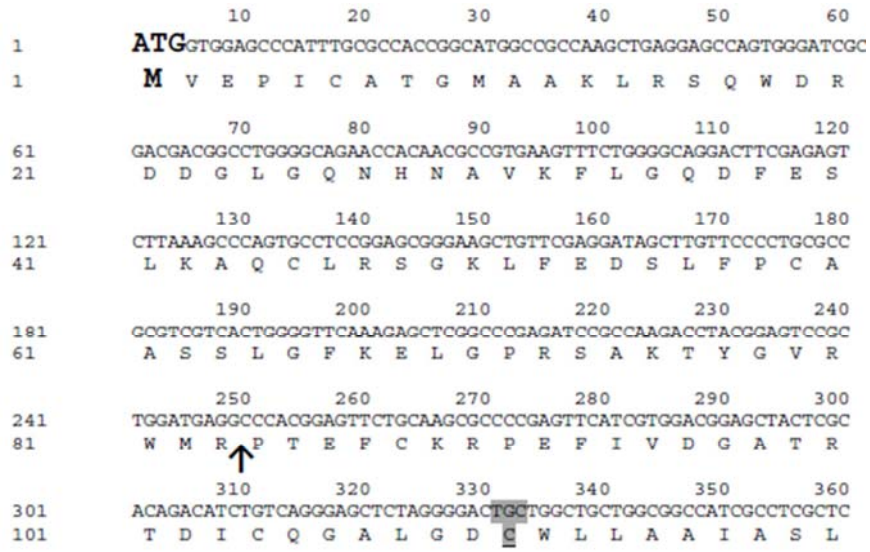
the JTT matrix-based model [39]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed [40], and a discrete Gamma distribution was used to model evolutionary rate differences among invariant sites (G+I). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The Atlantic salmon, *Salmo salar* cathepsin L (NM_001146546), a lysosomal cysteine protease, was used to root the phylogenetic tree.

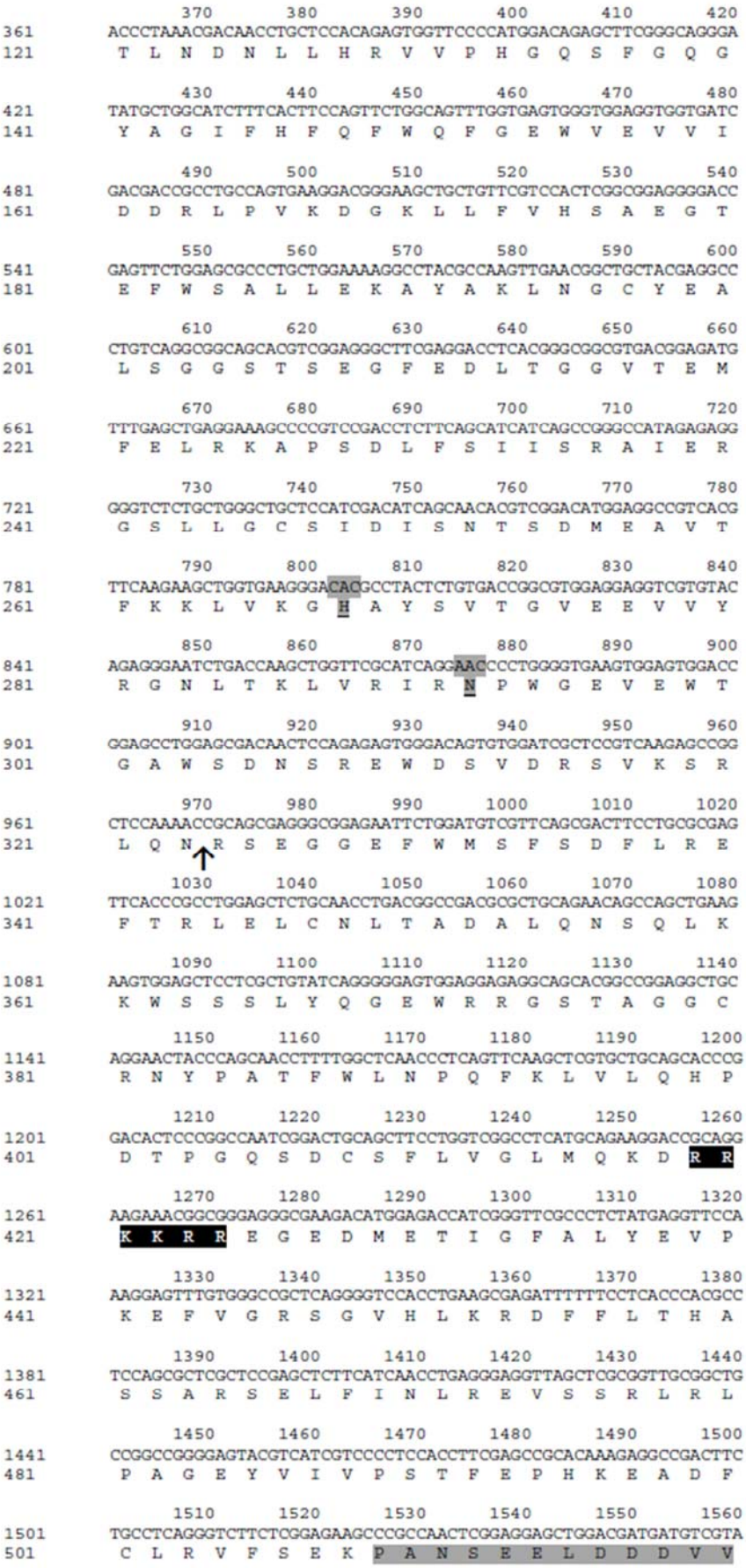
3. Results

3.1. Calpains characterisation

The cDNA sequences of five distinct gilthead sea bream calpains were obtained using PCR, 5'RACE-PCR and 454 pyrosequencing and deposited in GenBank. BLAST searches were used to examine the identity of these new sequences.

The first complete coding region of 2118 base-pairs (bp) corresponding to a single ORF of 705 amino acids (aa) and a theoretical Mw of 79.9 kDa, returned highest BLAST scores to calpain1 sequences, showing 68% and 86% identity with human (*Homo sapiens*) and Atlantic salmon capn1, respectively; therefore, it was named *sacapn1* (accession number: KF444899) (Figure S1). A 2094 bp contig, coding a 697 aa protein with a Mw 78.2 kDa, showed 63% and 90% identity with mouse and Atlantic halibut capn2, respectively, and it was named *sacapn2* (accession number: KF444900) (Figure S2). Next, a 2316 bp contig, with a single ORF of 771 aa and 89.1 kDa Mw, showed 66% identity with mouse and 86% identity with halibut capn3 and was named *sacapn3* (accession number: ERP000874) (Figure S3).




370 380 390 400 410 420
361 ACCCTAAACGACAACCTGCTCCACAGAGTGGTTCCCCATGGACAGAGCTTCGGGCAGGGA
121 T L N D N L L H R V V P H G Q S F G Q G
430 440 450 460 470 480
421 TATGCTGGCATCTTTCACTTCCAGTTCTGGCAGTTTGGTGAGTGGGTGGAGGTGGTGATC
141 Y A G I F H F Q F W Q F G E W V E V V I
490 500 510 520 530 540
481 GACGACCGCCTGCCAGTGAAGGACGGGAAGCTGCTGTTCGTCCACTCGGCGGAGGGGACC
161 D D R L P V K D G K L L F V H S A E G T
550 560 570 580 590 600
541 GAGTTCTGGAGCGCCCTGCTGGAAAAGGCCTACGCCAAGTTGAACGGCTGCTACGAGGCC
181 E F W S A L L E K A Y A K L N G C Y E A
610 620 630 640 650 660
601 CTGTCAGGCGGCAGCACGTCGGAGGGCTTCGAGGACCTCACGGGCGGCGTGACGGAGATG
201 L S G G S T S E G F E D L T G G V T E M
670 680 690 700 710 720
661 TTTGAGCTGAGGAAAGCCCCGTCCGACCTCTTCAGCATCATCAGCCGGGCCATAGAGAGG
221 F E L R K A P S D L F S I I S R A I E R
730 740 750 760 770 780
721 GGGTCTCTGCTGGGCTGCTCCATCGACATCAGCAACACGTCGGACATGGAGGCCGTCACG
241 G S L L G C S I D I S N T S D M E A V T
790 800 810 820 830 840
781 TTCAAGAAGCTGGTGAAGGGACACGCCTACTCTGTGACCGGCGTGGAGGAGGTCGTGTAC
261 F K K L V K G H A Y S V T G V E E V V Y
850 860 870 880 890 900
841 AGAGGGAATCTGACCAAGCTGGTTCGCATCAGGAACCCCTGGGGTGAAGTGGAGTGGACC
281 R G N L T K L V R I R N P W G E V E W T
910 920 930 940 950 960
901 GGAGCCTGGAGCGACAACTCCAGAGAGTGGGACAGTGTGGATCGCTCCGTCAAGAGCCGG
301 G A W S D N S R E W D S V D R S V K S R
970 980 990 1000 1010 1020
961 CTCCAAAACCGCAGCGAGGGCGGAGAATTCTGGATGTCGTTCAGCGACTTCCTGCGCGAG
321 L Q N R S E G G E F W M S F S D F L R E
1030 1040 1050 1060 1070 1080
1021 TTCACCCGCCTGGAGCTCTGCAACCTGACGGCCGACGCGCTGCAGAACAGCCAGCTGAAG
341 F T R L E L C N L T A D A L Q N S Q L K
1090 1100 1110 1120 1130 1140
1081 AAGTGGAGCTCCTCGCTGTATCAGGGGGAGTGGAGGAGAGGCAGCACGGCCGGAGGCTGC
361 K W S S S L Y Q G E W R R G S T A G G C
1150 1160 1170 1180 1190 1200
1141 AGGAACTACCCAGCAACCTTTTGGCTCAACCCTCAGTTCAAGCTCGTGCTGCAGCACCCG
381 R N Y P A T F W L N P Q F K L V L Q H P
1210 1220 1230 1240 1250 1260
1201 GACACTCCCGGCCAATCGGACTGCAGCTTCCTGGTCGGCCTCATGCAGAAGGACCGCAGG
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1270 1280 1290 1300 1310 1320
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1510 1520 1530 1540 1550 1560
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501 C L R V F S E K P A N S E E L D D D V V

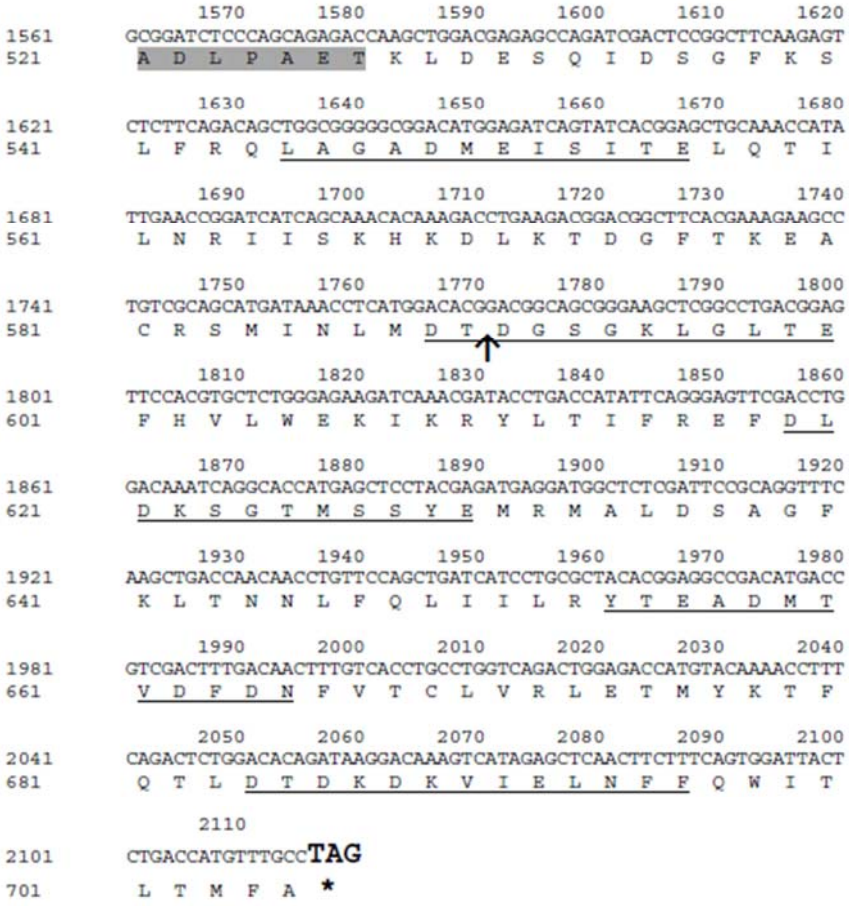
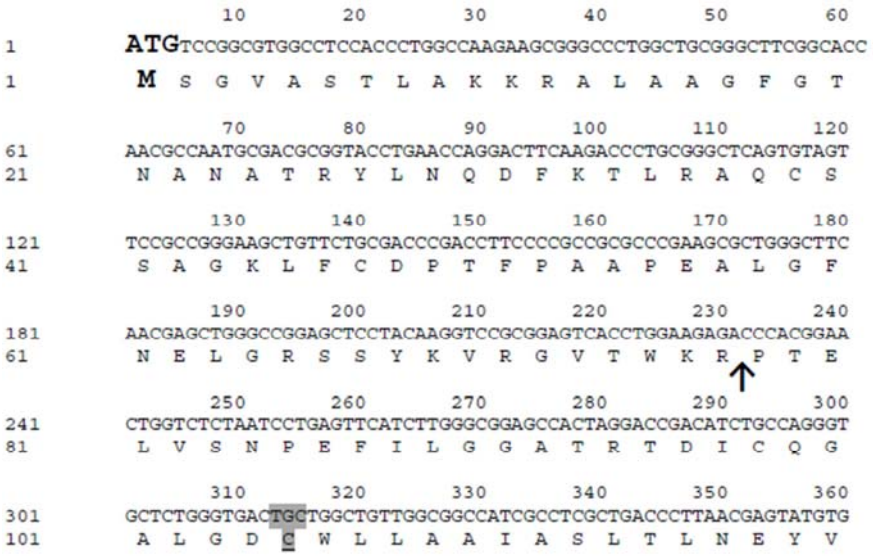
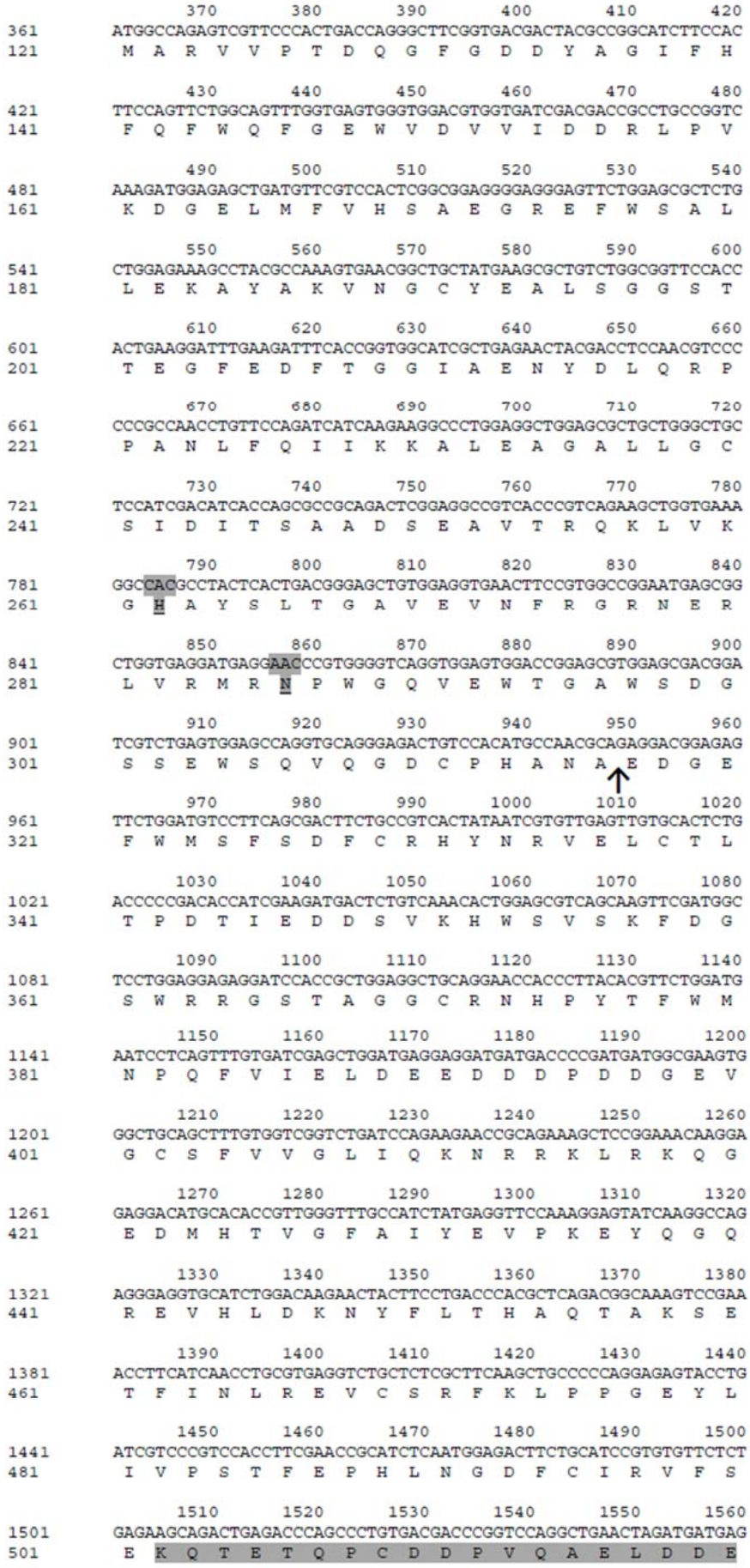


Figure S1. Complete ORF and deduced amino acid sequence of gilthead sea bream calpain1 (*sacapn1*). The initiation and stop codons are shown in bold. The conserved catalytic residues are boxed and underlined. ↑ Indicates the boundaries of domains. The nuclear localization signal (NLS) is boxed in black. PEST proteolytic signal is boxed in grey. The penta-EF-hand (PEF) sequences are underlined.

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421 TTCCAGTTCTGGCAGTTTGGTGAGTGGGTGGACGTGGTGATCGACGACCGCCTGCCGGTC
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490 500 510 520 530 540
481 AAAGATGGAGAGCTGATGTTCGTCCACTCGGCGGAGGGGAGGGAGTTCTGGAGCGCTCTG
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550 560 570 580 590 600
541 CTGGAGAAAGCCTACGCCAAAGTGAACGGCTGCTATGAAGCGCTGTCTGGCGGTTCCACC
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610 620 630 640 650 660
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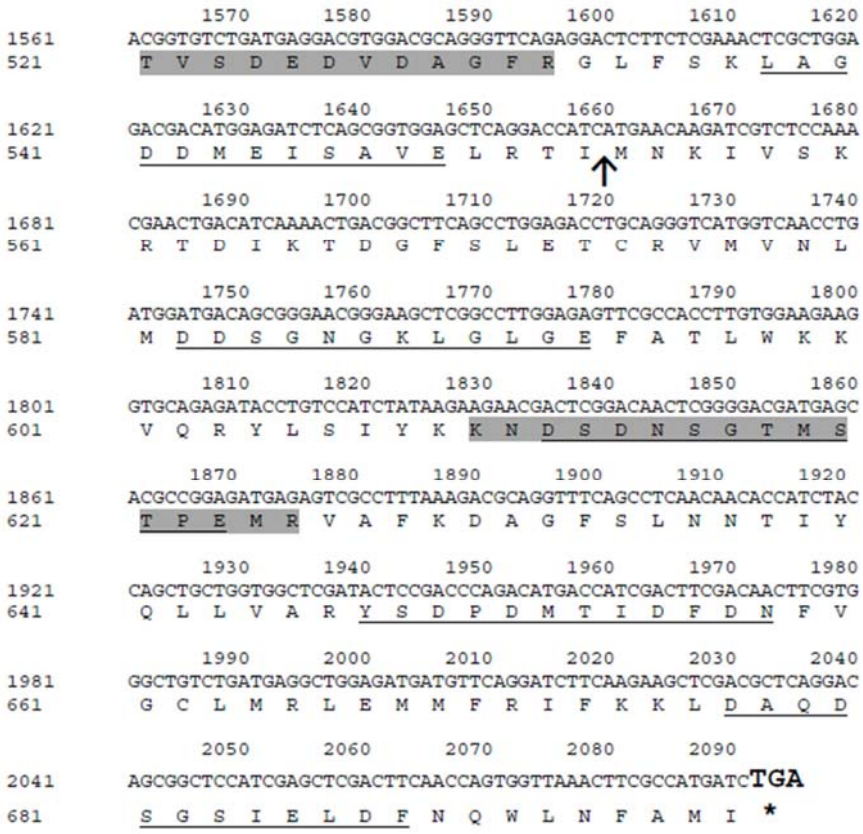
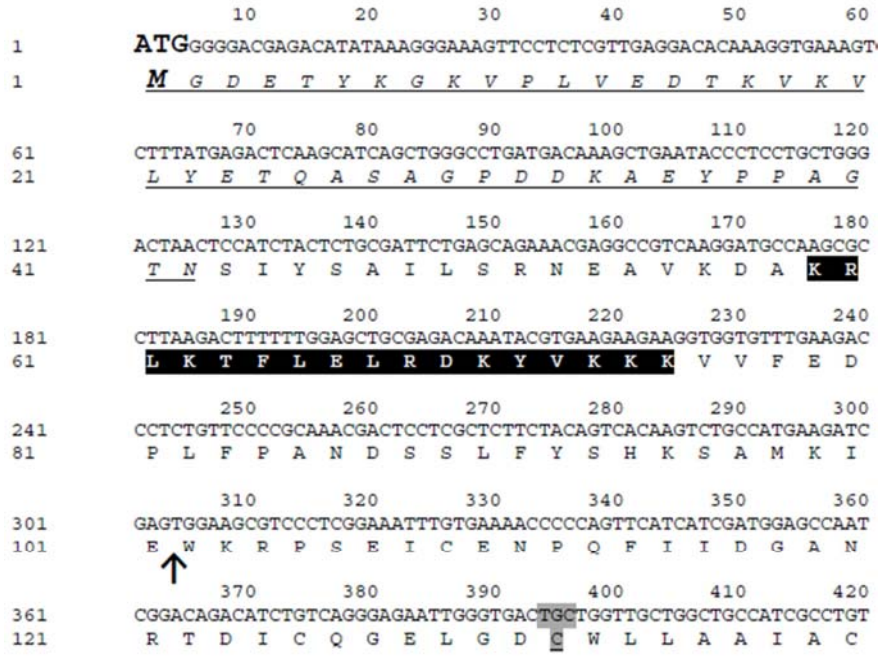
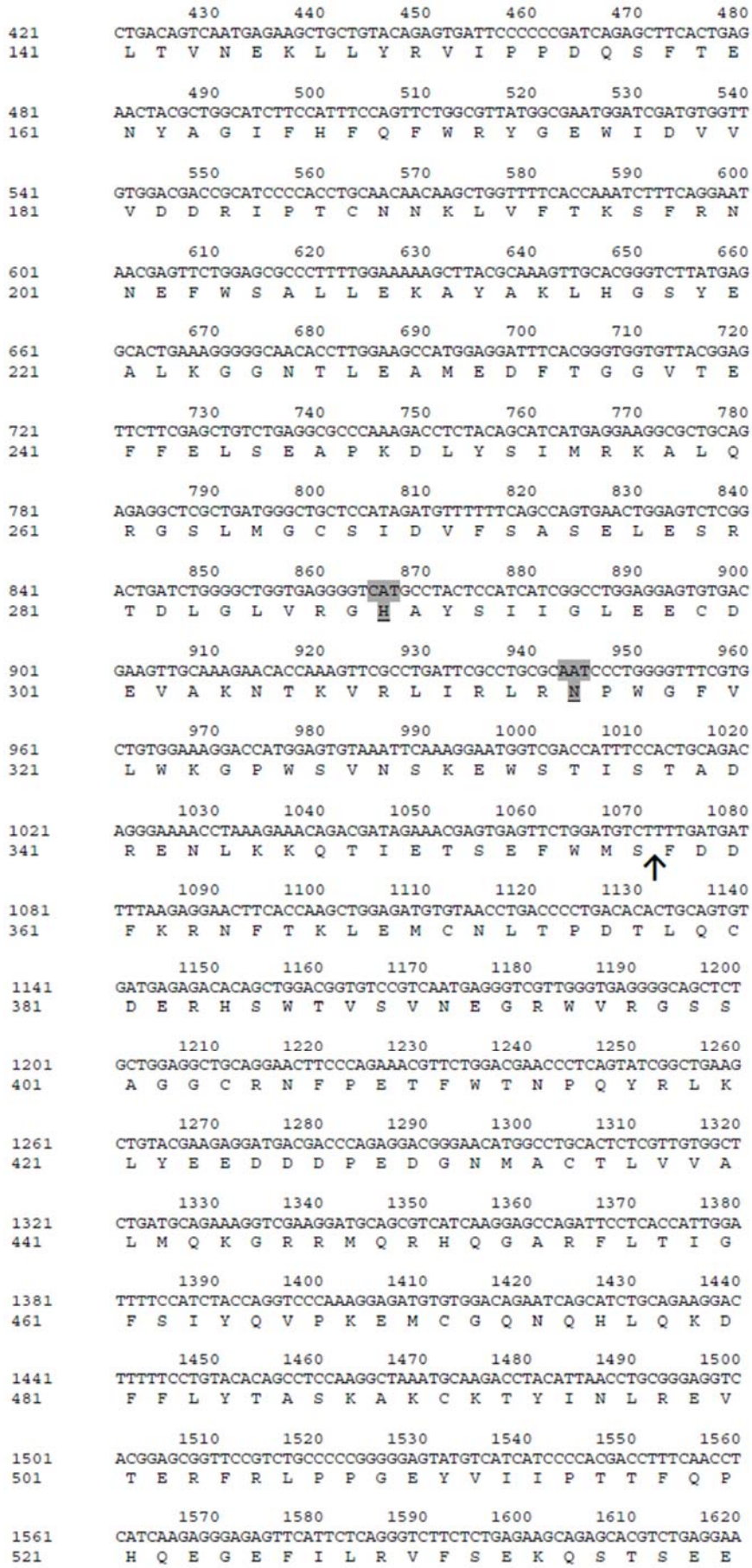


Figure S2. Complete ORF and deduced amino acid sequence of gilthead sea bream calpain2 (*sacapn2*). The initiation and stop codons are shown in bold. The conserved catalytic residues are boxed and underlined. ↑ Indicates the boundaries of domains. PEST proteolytic signals are boxed in grey. The penta-EF-hand (PEF) sequences are underlined.

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670 680 690 700 710 720
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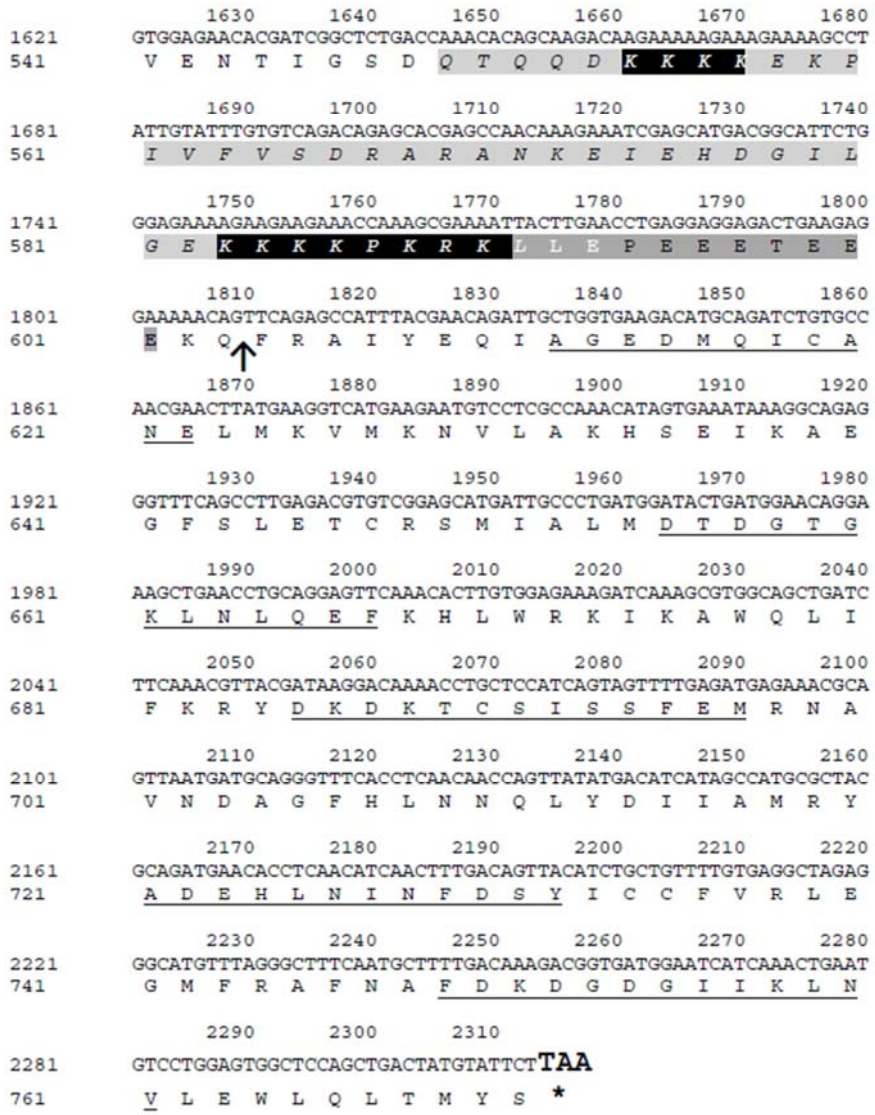


Figure S3. Complete ORF and deduced amino acid sequence of gilthead sea bream calpain3 (*sacapn3*). The initiation and stop codons are shown in bold. The teleost N-terminal sequence (NS) is shown in italics and underlined. Inserted sequence IS2 is shown in italics and boxed in pale grey. The conserved catalytic residues are boxed and underlined. ↑ Indicates the boundaries of domains. The nuclear localization signals (NLS) are boxed in black. PEST proteolytic signal is boxed in dark grey. The penta-EF-hand (PEF) sequences are underlined.

Regarding the regulatory calpains, two 651 bp contigs, with single ORFs of 216 aa and theoretical Mw of 24.6 and 24.7 kDa, respectively, returned highest BLAST scores to calpain small subunit1 sequences. The first one showed 82% and 88% identity with zebrafish (NM_001017899.2) and Atlantic salmon (BT043754.1) capns1a and capns1, respectively and it was named *sacapns1a* (accession number: KF444901) (Figure S4). The second sequence showed 72% and 75% identity with Atlantic salmon (BT047225.1) and zebrafish (BC162479.1)

capns1 and capns1b, respectively, indicating it was a sacapns1 paralog in gilthead sea bream; and thus it was named *sacapns1b* (accession number: ERP000874) (Figure S5).

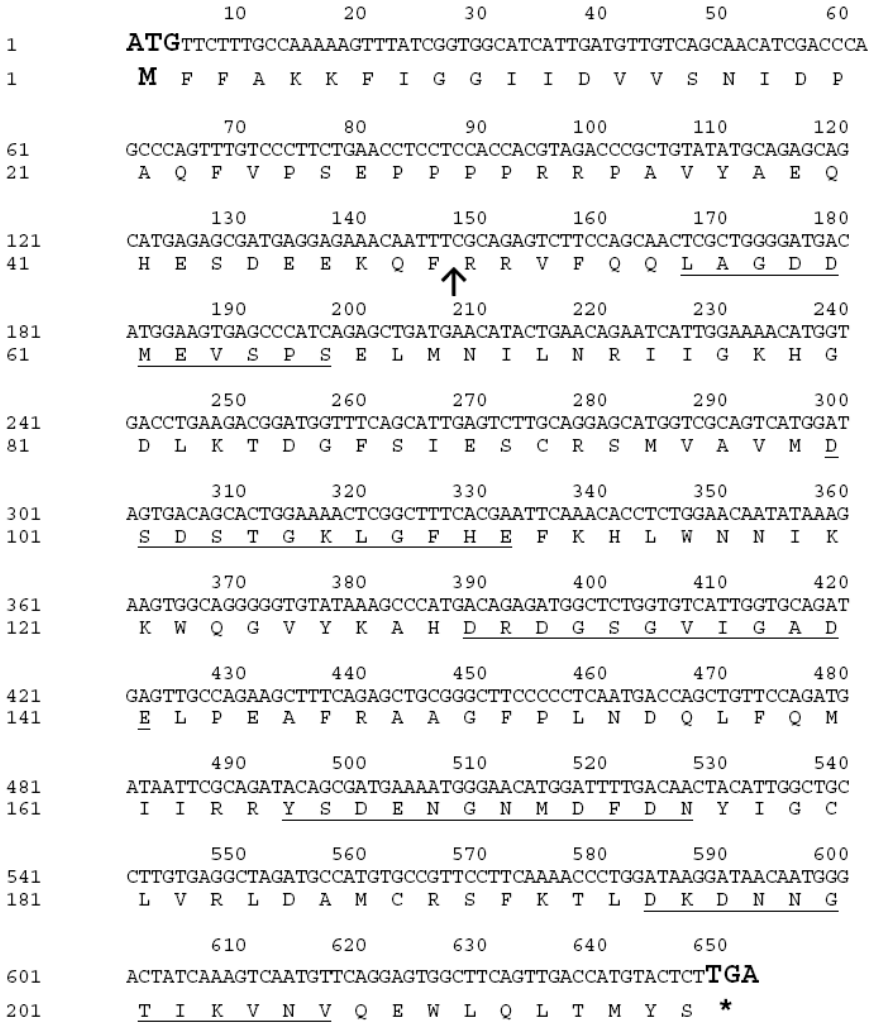
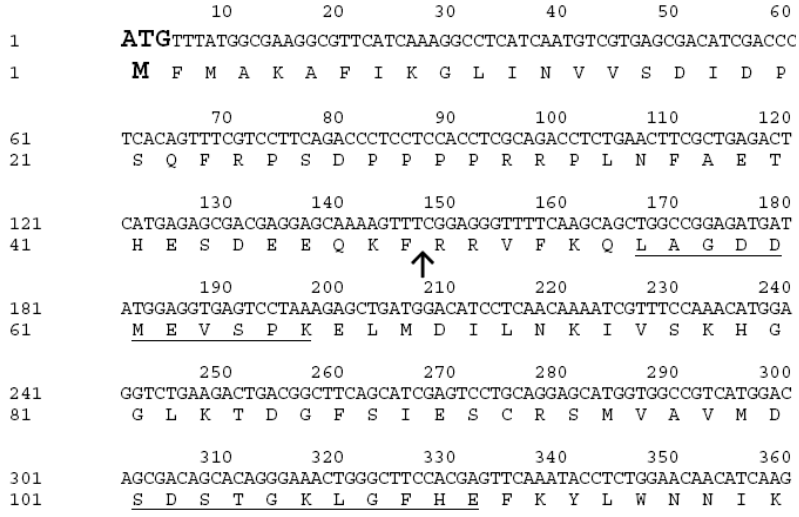


Figure S4. Complete ORF and deduced amino acid sequence of gilthead sea bream calpain small subunit1a (*sacapns1a*). The initiation and stop codons are shown in bold. ↑ Indicates the boundaries of domains. The penta-EF-hand (PEF) sequences are underlined.



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121      R  W  Q  G  I  Y  M  S  H  D  A  D  G  S  G  V  I  C  D  Q

                430        440        450        460        470        480
421     GAGCTGCCGAAAGCCTTCAAGGCTGCAGGCTTCCCTCTGAACGACCAGCTCTTCAAGCTG
141      E  L  P  K  A  F  K  A  A  G  F  P  L  N  D  Q  L  F  K  L

                490        500        510        520        530        540
481     ATTATCCGTCGCTATAGCGATGAGCACGGCAACATGGACTTTGACAACTTCGTCGGCTGC
161      I  I  R  R  Y  S  D  E  H  G  N  M  D  F  D  N  F  V  G  C

                550        560        570        580        590        600
541     CTGGTGCGACTGGACGCCATGTGCAGAGCCTTTAAGACCCTGGACAAGGACAACAGTGGC
181      L  V  R  L  D  A  M  C  R  A  F  K  T  L  D  K  D  N  S  G

                610        620        630        640        650
601     ACCATAGACTTGGACATCAAGGAGTGGCTTCAGCTGACGATGTATTCATGA
201      T  I  D  L  D  I  K  E  W  L  Q  L  T  M  Y  S  *
```

Figure S5. Complete ORF and deduced amino acid sequence of gilthead sea bream calpain small subunit1b (*sacapns1b*). The initiation and stop codons are shown in bold. ↑Indicates the boundaries of domains. The penta-EF-hand (PEF) sequences are underlined.

The calpain domain architecture was identified in all five gilthead sea bream calpain peptides (Figure 1). Sacapn1 and Sacapn2 contained four domains (D): DI or the N-terminal anchor helix region, DII or the CysPc protease domain, DIII or the C2-like domain (C2L), and DIV or the penta-EF-hand domain (PEF) (Figures S1 and S2). Also, two additional regions were present in Sacapn3: the teleost N-terminal sequence (NS) and an insertion sequence (IS2) (Figure S3). The characteristic triad of catalytic residues, the potential PEST proteolytic signals that target proteins for rapid destruction and the nuclear localization signal (NLS) were also identified.

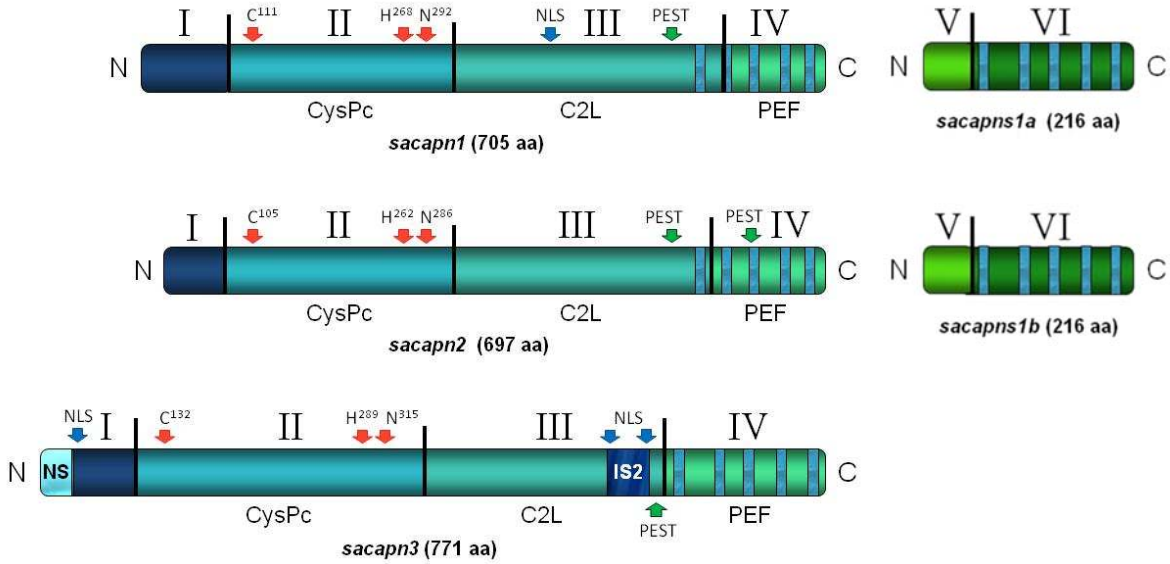
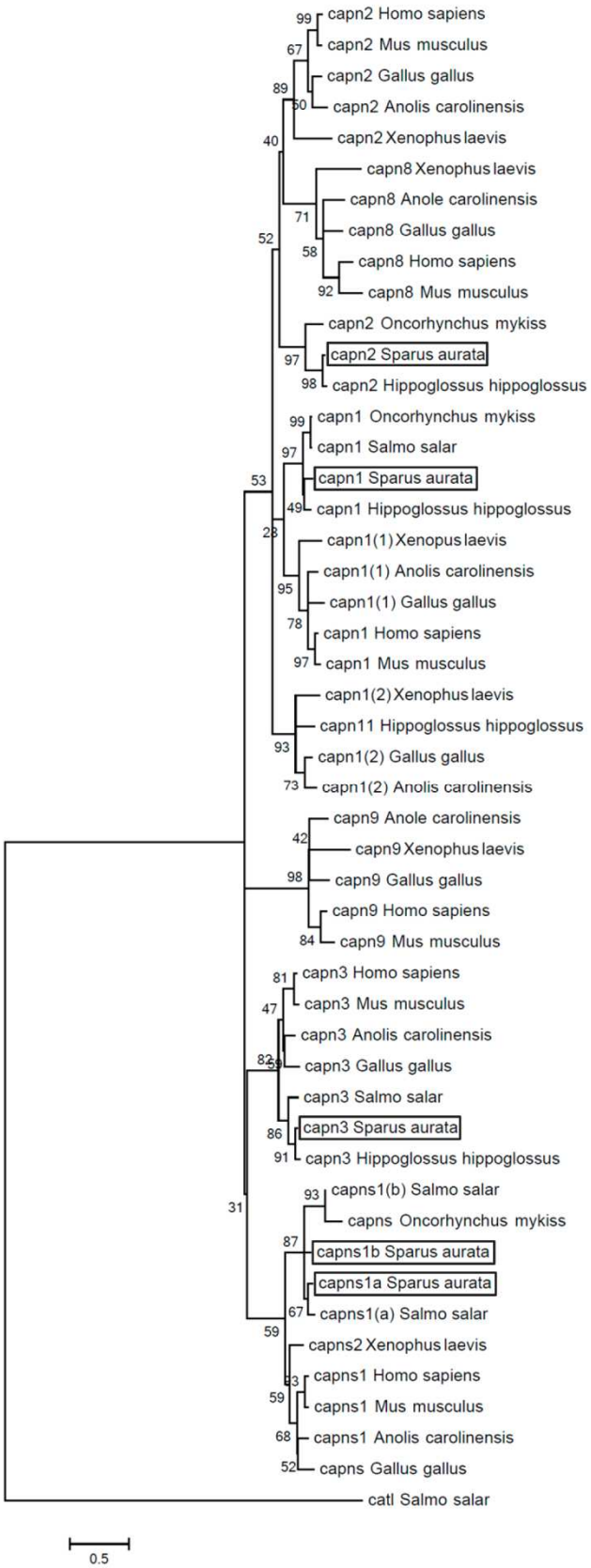


Figure 1. Schematic representation of the gilthead sea bream Calpain peptides' structural architecture. Domains are identified with roman numbers. CysPc: proteolytic domain, C2L: C2-like domain, and PEF: penta-EF-hand domain. The conserved catalytic residues, nuclear localization signals (NLS) and PEST proteolytic signals are indicated with arrows.

Both paralogs of the regulatory calpain, Sacapns1a and Sacapns1b, contained two domains: DV and DVI or PEF domain (Figures S4 and S5). Finally, analysis of the calpain amino acid sequences using the PSORTII program predicted that all were cytoplasmic proteins.



3.2 Phylogenetic analysis

A phylogenetic tree including 48 calpain sequences from different vertebrates, and an Atlantic salmon Cathepsin L sequence, was performed (Figure 2). The calpain cluster was divided into two main groups, one including the calpain large subunits (Capn1, Capn2, Capn8 and Capn11) and the second containing Capn3 and the calpain small regulatory subunits (Capns1a and Capns1b), whereas Capn9 formed a separated clade. Each putative gilthead sea bream calpain sequence was related to the corresponding calpain teleost ortholog, with both Capns paralogs forming two different sister clades.

Figure 2. Molecular phylogenetic analysis of the gilthead sea bream Calpain amino acid sequences. A rooted phylogenetic tree of gilthead sea bream (*Sparus aurata*), Anole lizard (*Anolis carolinensis*), chicken (*Gallus gallus*), Atlantic halibut (*Hippoglossus hippoglossus*), human (*Homo sapiens*), mouse (*Mus musculus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and African clawed frog (*Xenopus laevis*) Calpain1 (capn1), Calpain2 (capn2), Calpain3 (capn3),

Calpain8 (capn8), Calpain9 (capn9), Calpain11 (capn11), and Calpain small subunits 1a and 1b (capns1a and capns1b) orthologs was performed. The Atlantic salmon Cathepsin L, a lysosomal cysteine protease, was used to root the phylogenetic tree. The tree was created by UPGMA method using ClustalW multiple alignment and bootstrapped 1000 times. The scale of the given branch length indicates 0,5 amino acid substitutions per site. Accession numbers were retrieved from public databases: *Anolis carolinensis* capn1(1) XM_003229570; capn1(2) XM_003215899; capn2 XM_003216038; capn3 XM_003214560; capn8 ENSACAT00000003160; capn9 ENSACAT00000002035 and capns1 XM_003228375; *Gallus gallus* capn1(1) NM_001044672; capn1(2) NM_205303; capn2 FJ232590; capn3 FJ232591; capn8 ENSGALT00000015288; capn9 ENSGALT00000018152 and capns AB007824; *Hippoglossus hippoglossus* capn1 GQ327965; capn2 GQ327966; capn3 GQ327967 and capn11 GQ327964; *Homo sapiens* capn1 BC075862; capn2 NM_001748; capn3 BC146649; capn8 NM_001143962; capn9 ENST00000271971 and capns1 ENST00000246533; *Mus musculus* capn1 AF021847; capn2 AF015038; AF127766; capn8 ENSMUST00000048941; capn9 ENSMUST00000093033 and capns1 ENSMUST00000001845; *Oncorhynchus mykiss* capn1 AY573919; capn2 NM_001124491 and capns NM_001124331; *Salmo salar* capn1 BT059271; capn3 NM_001165408; capns1(a) BT043754; capns1(b) BT047225 and cathepsin L1 (catl1) NM_001146546; *Sparus aurata* capn1 (KF444899); capn2 (KF444900); capn3 (ERP000874); capns1a (KF444901) and capns1b (ERP000874); *Xenopus laevis* capn1(1) NM_001087016; capn1(2) NM_001013613; capn2 NM_001090244; capn8 NM_001088543; capn9 NM_001092528 and capns2 BC078469.

3.3 Tissue expression

Conventional RT-PCR was used to determine the mRNA expression of each gilthead sea bream calpain identified in 15 different tissues (Figure 3). Transcripts of *sacapn1*, *sacapn2*, *sacapns1a* and *sacapns1b*, were detected to a greater or lesser extent in each one of the 15 tissues examined. On the other hand, transcripts for *sacapn3* were detected preferentially in tissues containing striated muscle fibres, including fast and slow skeletal muscle and heart (Figure 3).

3.4 Fasting/re-feeding experiment

Calpain gene expression was analysed in fast skeletal muscle of gilthead sea bream subjected to a fasting/re-feeding experiment. Fasting for 15 days caused a minor reduction in body mass and MFI, but decreased significantly HSI. During the same period, a significant increase was observed in body weight and MFI in the control group (Table 2). Again after 7 days of re-feeding the increase in body mass and MFI was not significant, although it was for HSI (Table 2).

As expected, plasmatic glucose was significantly reduced, while FFAs and TGs were significantly increased after 15 and 30 days of fasting (Table 3). In re-fed fish significantly elevated plasma

glucose and reduced FFAs and TGs were observed (Table 3). In relation to muscle colour, no clear changes were observed during fasting for any of the parameters analysed; however, re-feeding significantly increased lightness (L*) and decreased a* and b* components towards green and blue intensities, respectively (Table S4).

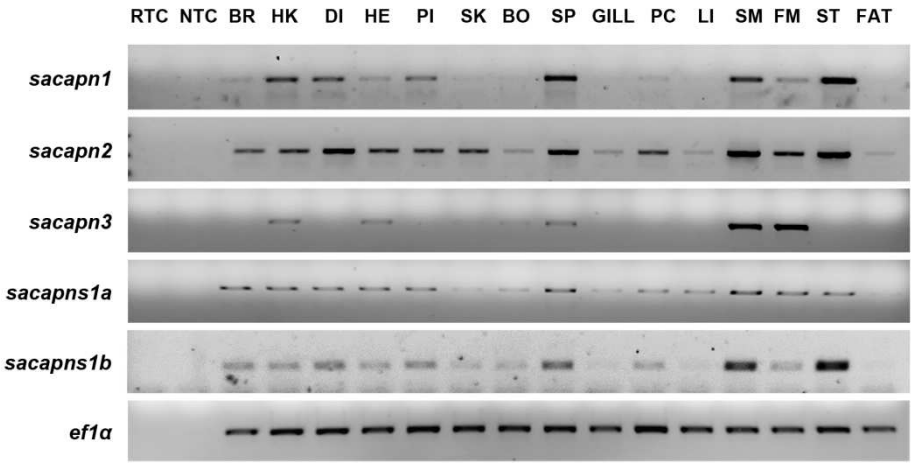


Figure 3. Tissue distribution of gilthead sea bream calpains. Qualitative transcript expression profiles of *sacapn1*, *sacapn2*, *sacapn3*, *sacapns1a*, *sacapns1b* and *ef1α*. RTC: No Reverse Transcriptase Control, NTC: No Template Control, BR: Brain, HK: Head kidney, DI: Distal intestine, HE: Heart, PI: Proximal intestine, SK: Skin, BO: Bone, SP: Spleen, GILL, PC: Pyloric caeca, LI: Liver, SM: Slow skeletal muscle, FM: Fast skeletal muscle, ST: Stomach, FAT: adipose tissue. A representative experiment from n=3 independent fish analysed is shown.

Table 2. Biometrics of gilthead sea bream from the fasting and re-feeding experiment.

Condition[4]	Body weight (g)	Total length (cm)	HSI[1]	MFI[2]	CF[3]
D0C	50,79 ± 2,13[a]	15,40 ± 0,22	1,19 ± 0,08[a]	0,35 ± 0,05[a]	1,39 ± 0,05[a]
D15C	57,28 ± 4,26[ab]	16,00 ± 0,39	1,25 ± 0,09[a]	0,43 ± 0,06[ab]	1,38 ± 0,02[a]
D15F	46,32 ± 1,70[a]	15,21 ± 0,18	0,63 ± 0,03[b]	0,32 ± 0,04[a]	1,31 ± 0,01[a]
D30C	61,77 ± 2,18[b]	15,84 ± 0,17	1,24 ± 0,08[a]	0,60 ± 0,10[b]	1,55 ± 0,04[b]
D30F	46,91 ± 1,98[a]	15,20 ± 0,18	0,58 ± 0,06[b]	0,29 ± 0,03[a]	1,34 ± 0,03[a]
D0F	46,91 ± 1,98	15,20 ± 0,18	0,58 ± 0,06[a]	0,29 ± 0,03	1,34 ± 0,03
D7R	47,07 ± 2,43	15,18 ± 0,32	0,94 ± 0,10[b]	0,30 ± 0,04	1,34 ± 0,03
D14R	47,09 ± 1,52	15,44 ± 0,25	0,89 ± 0,06[b]	0,25 ± 0,06	1,29 ± 0,05

Body weight, total length, [1]hepatosomatic index [HSI=(Liver weight/Body weight)*100], [2]mesenteric fat index [MFI=(Adipose weight/Body weight)*100], and [3]condition factor [CF=(Body weight/Total length3)*100]. Results are shown as mean ± SEM (n=7-8). Different letters ([a,b]) indicate significant

differences at p<0,05 with fasting and re-feeding periods analysed separately. [4]Condition: D: day, C: control fed fish, F: fasted fish, R: re-fed fish.

Table 3. Plasma parameters of gilthead sea bream from the fasting and re-feeding experiment.

Condition[1]	Glucose (mg/dL)	FFAs (mEq/L)	TGs (mg/dL)
D0C	58,89 ± 3,12^{a}	0,35 ± 0,04^{a}	228,30 ± 12,08^{a}
D15C	56,65 ± 4,18^{ab}	0,25 ± 0,03^{a}	233,36 ± 17,06^{a}
D15F	43,89 ± 1,39^{c}	0,55 ± 0,03^{b}	441,28 ± 67,95^{b}
D30C	58,90 ± 2,72^{a}	0,23 ± 0,03^{a}	235,73 ± 13,05^{a}
D30F	46,52 ± 1,54^{bc}	0,55 ± 0,03^{b}	906,87 ± 98,09^{c}
D0F	46,52 ± 1,54^{a}	0,55 ± 0,03^{a}	906,87 ± 98,09^{a}
D7R	61,73 ± 3,29^{b}	0,39 ± 0,04^{b}	500,15 ± 80,93^{b}
D14R	64,51 ± 2,93^{b}	0,33 ± 0,03^{b}	262,26 ± 41,54^{b}

Results are shown as mean ± SEM (n=7-8). Different letters (^{a,b,c}) indicate significant differences at p<0,05 with fasting and re-feeding periods analysed separately. [1]Condition: D: day, C: control fed fish, F: fasted fish, R: re-fed fish.

Table S4. Colour of gilthead sea bream muscle from the fasting and re-feeding experiment. Colour measurements were performed on fast skeletal muscle from the antero-dorsal region. Colour is expressed using the L* (lightness), a* (red/green) and b* (yellow/blue) system. Results are shown as mean ± SEM (n=7-8). Different letters indicate significant differences at p<0,05 with fasting and re-feeding periods analysed separately. C: control fed fish, F: fasted fish, R: re-fed fish.

Condition	L*	a*	b*	Chroma	Hue
D0C	44,77 ± 0,72^{a}	-1,36 ± 0,10	-2,84 ± 0,30^{a}	3,16 ± 0,30^{a}	243,47 ± 1,90^{a}
D15C	51,07 ± 0,62^{c}	-1,40 ± 0,07	-2,47 ± 0,23^{ab}	2,85 ± 0,22^{ab}	239,67 ± 1,87^{a}
D15F	49,82 ± 0,38^{c}	-1,46 ± 0,04	-2,72 ± 0,31^{ab}	3,11 ± 0,27^{ab}	240,10 ± 2,97^{ab}
D30C	48,69 ± 0,65^{bc}	-1,66 ± 0,08	-1,14 ± 0,28^{c}	2,11 ± 0,16^{b}	211,57 ± 6,81^{c}
D30F	46,80 ± 1,10^{ab}	-1,44 ± 0,09	-1,63 ± 0,34^{bc}	2,33 ± 0,26^{ab}	231,50 ± 2,99^{b}
D0F	46,80 ± 1,10^{a}	-1,44 ± 0,09^{a}	-1,63 ± 0,34^{a}	2,33 ± 0,26^{a}	231,50 ± 2,99
D7R	49,45 ± 1,23^{ab}	-1,72 ± 0,07^{ab}	-2,55 ± 0,14^{ab}	3,10 ± 0,13^{ab}	235,06 ± 1,51
D14R	50,75 ± 0,80^{b}	-1,77 ± 0,08^{b}	-2,90 ± 0,35^{b}	3,42 ± 0,32^{b}	237,04 ± 2,70

Interestingly, fish fasted for 15 and 30 days presented a significant increase in *sacapns1b* expression, whereas the other calpains remained unchanged (Figure 4). Moreover, re-fed fish after 14 days had significantly decreased expression of *sacapn1*, *sacapn2*, *sacapns1a* and *sacapns1b*; and also a significant decrease was observed already at 7 days after re-feeding in the expression of the proteolysis marker of the proteasome, *N3* (Figure 5).

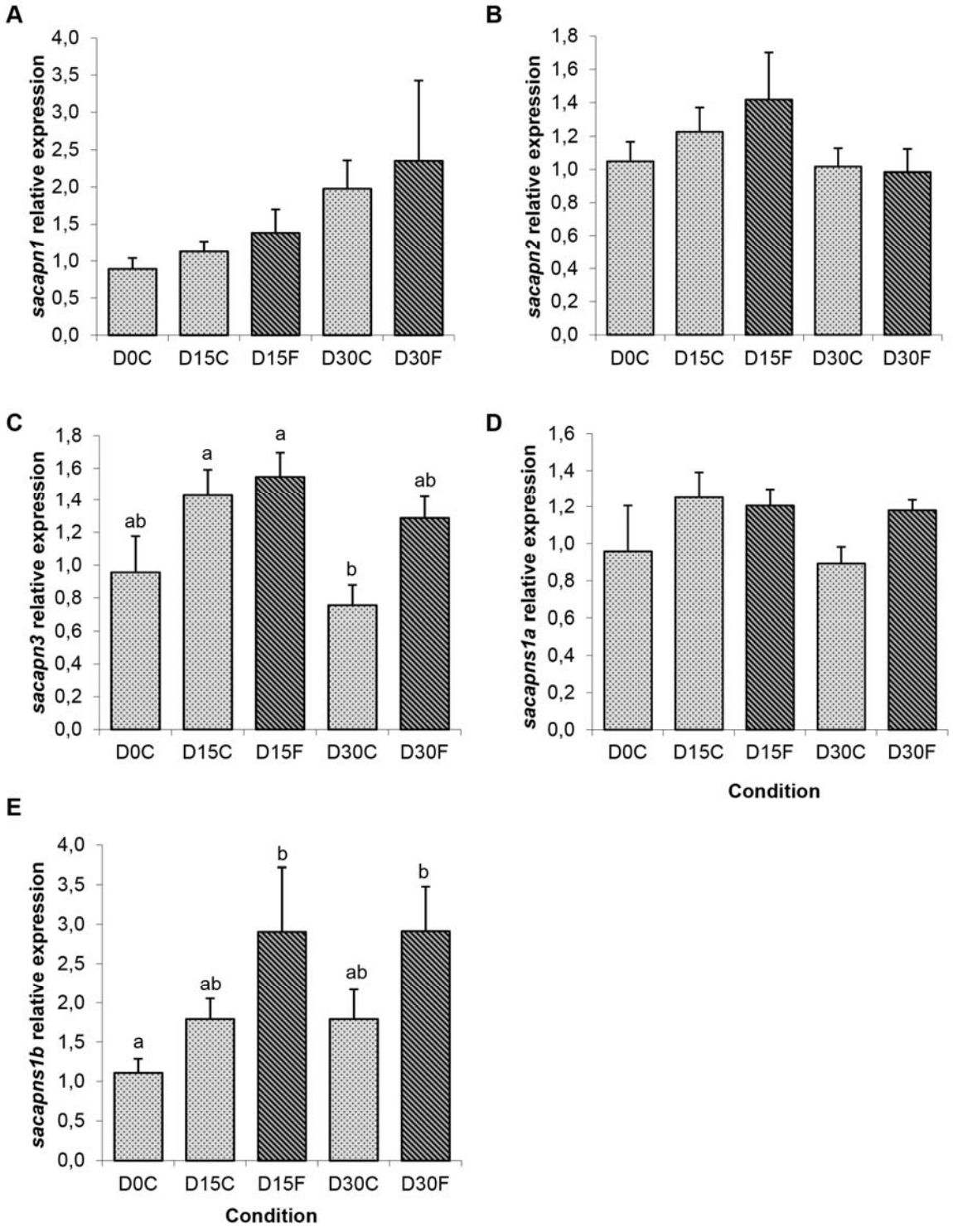


Figure 4. Calpains relative expression in gilthead sea bream from the fasting experiment. Quantitative expression relative to *β-actin* of **(A)** *sacapn1*, **(B)** s*acapn2*, **(C)** *sacapn3*, **(D)** *sacapns1a* and **(E)** *sacapns1b*. Results are shown as mean ± SEM (n=5-8). Different letters indicate significant differences at p<0,05. C: control fed fish, F: fasted fish.

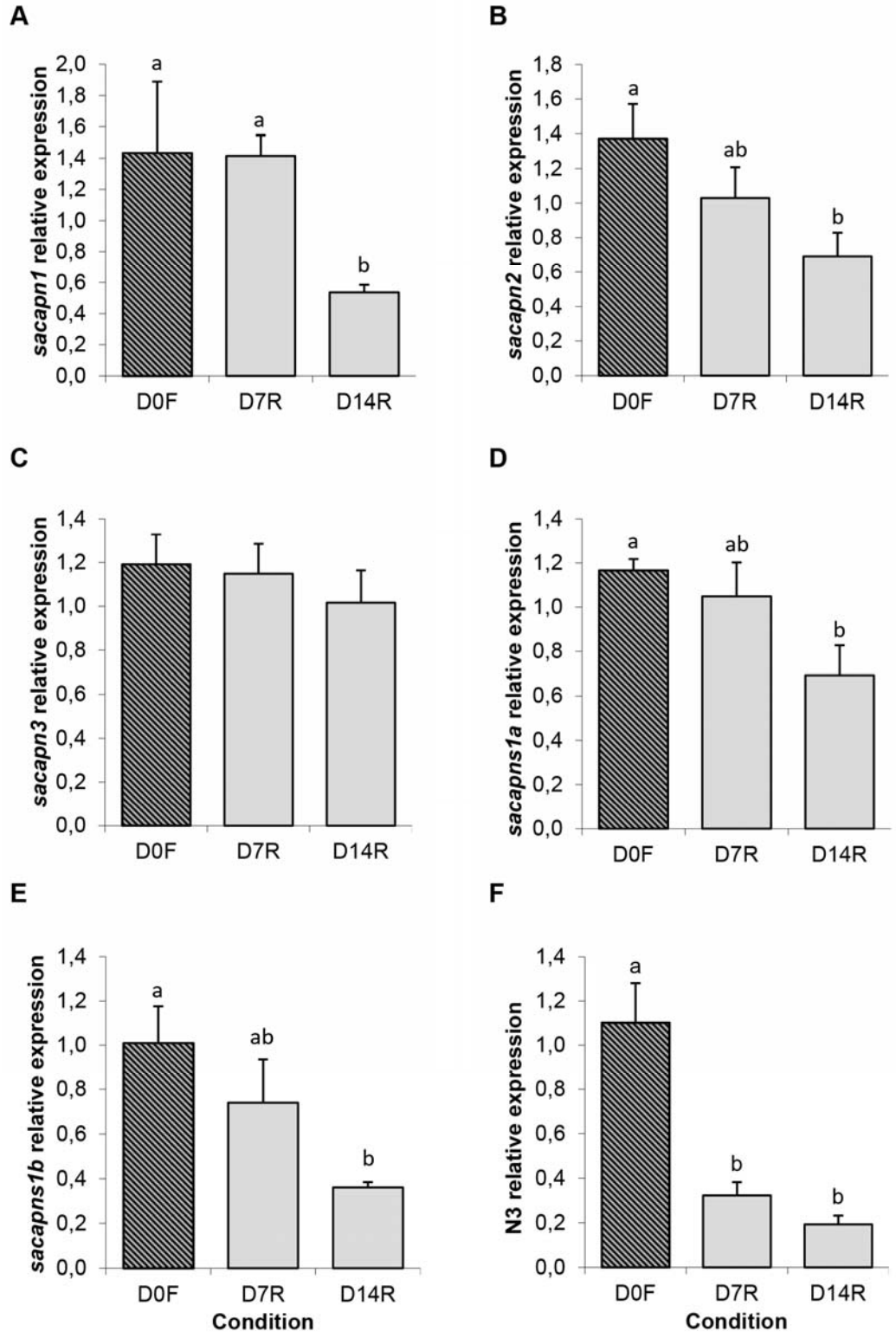


Figure 5. Calpains relative expression in gilthead sea bream from the re-feeding experiment. Quantitative expression relative to *β-actin* of **(A)** *sacapn1*, **(B)** *sacapn2*, **(C)** *sacapn3*, **(D)** *sacapns1a*, **(E)** *sacapns1b* and **(F)** N3. Results are shown as mean ± SEM (n=5-8). Different letters indicate significant differences at p<0,05. F: fasted fish, R: re-fed fish.

3.5 Diet experiment

Then, we analysed whether the gilthead sea bream calpain genes identified were transcriptionally regulated according to the different percentages of dietary protein and CH (Table 1) on fast-twitch skeletal muscle. Finally, we evaluated if the expression of any of the calpains correlated with muscle texture. At the end of the experiment, no significant differences were found in SGR between the 46% protein groups (46/11 and 46/19). Nevertheless, differences were found respect fish fed the diets with 46% protein and those fed the other diets (Table 4). Also, significant differences were observed between fish fed the 42/35 and 40/39 diets, with the fish fed the 40/39 diet showing the smallest SGR. In addition, no significant differences in FCR between diets 46/11 and 42/35 were found; however, besides all groups had equal feed intake, differences were found between those two and the other groups (Table 4). Interestingly, the fish fed the diet 46/19 had the best FCR values, and the fish fed the 40/39 diet, the worst. Moreover, the fish fed with the diet 46/11 (with the lowest amount of CH), showed the highest final body weight and total length, which was significantly different with respect to fish fed with 42/35 and 40/39 diets, and showed significantly the lowest HSI value in comparison to the diets with higher amounts of CH (Table 5). The fish fed with the diet 40/39, showed the lowest MFI followed by those fed the diets 46/19 and 42/35, and showed the lowest CF, significantly different with respect to the fish fed the diet 46/19.

Table 4. Standard growth rate (SGR), feed intake and feed conversion rate (FCR) of gilthead sea bream fed the four experimental diets.

Diet	SGR[1]	Feed intake (g)	FCR[2]
46/11	1,14 ± 0,01[a]	10961 ± 593	1,61 ± 0,07[a]
46/19	1,13 ± 0,03[a]	9099 ± 553	1,38 ± 0,14[b]
42/35	1,05 ± 0,02[b]	10157 ± 356	1,73 ± 0,06[a]
40/39	0,95 ± 0,01[c]	10697 ± 273	2,15 ± 0,02[c]

[1]Standard growth rate [SGR=(ln final weight (lnW_f)-ln initial weight (lnW_i))*100/time], [feed intake=feed offered-feed refused] and [2]feed conversion rate [FCR=dry feed intake/wet weight gain]. Results are shown as mean ± SEM (n=51). Different letters ([a,b,c]) indicate significant differences at p<0,05.

Plasma parameters, glucose and TGs were not significantly affected by diet composition. On the other hand, FFAs were significantly lower in the fish fed the 46/19 diet compared to the 46/11 and 40/39 groups (Table 6). Also, colour measurement of the dorsal muscle did not show any significant differences between groups (Table S5).

Table 5. Biometrics of gilthead sea bream fed the four experimental diets.

Diet	Body weight (g)	Total length (cm)	HSI[1]	MFI[2]	CF[3]
46/11	419,44 ± 12,65[a]	24,01 ± 0,23[a]	1,24 ± 0,03[a]	1,06 ± 0,12[ab]	3,03 ± 0,07[ab]
46/19	396,78 ± 15,69[ab]	23,16 ± 0,19[b]	1,73 ± 0,12[b]	1,32 ± 0,20[a]	3,19 ± 0,11[a]
42/35	363,10 ± 8,64[b]	23,06 ± 0,12[b]	2,12 ± 0,12[b]	1,27 ± 0,12[a]	2,96 ± 0,07[ab]
40/39	271,70 ± 7,11[c]	21,14 ± 0,21[c]	1,93 ± 0,18[b]	0,70 ± 0,09[b]	2,83 ± 0,04[b]

Body weight, total length, [1]hepatosomatic index [HSI=(Liver weight/Body weight)*100], [2]mesenteric fat index [MFI=(Adipose weight/Body weight)*100], and [3]condition factor [CF=(Body weight/Total length^3)*100]. Results are shown as mean ± SEM (n=8-9). Different letters (^{a,b,c}) indicate significant differences at p<0,05.

Table 6. Plasma parameters of gilthead sea bream fed the four experimental diets.

Diet	Glucose (mg/dL)	FFAs (mEq/L)	TGs (mg/dL)
46/11	74,41 ± 6,30	0,28 ± 0,02[a]	329,26 ± 22,62
46/19	79,27 ± 6,86	0,19 ± 0,02[b]	290,80 ± 30,10
42/35	74,46 ± 3,53	0,23 ± 0,01[ab]	380,23 ± 54,07
40/39	91,50 ± 12,45	0,28 ± 0,02[a]	340,69 ± 53,73

Results are shown as mean ± SEM (n=8-9). Different letters (^{a,b}) indicate significant differences at p<0,05.

Table S5. **Colour of gilthead sea bream muscle fed the four experimental diets.** Colour measurements were performed on fast skeletal muscle from the antero-dorsal region. Colour is expressed using the L* (lightness), a* (red/green) and b* (yellow/blue) system. Results are shown as mean ± SEM (n=7-9). No significant differences were observed at p<0,05.

Diet	L*	a*	b*	Chroma	Hue
46/11	45,82 ± 1,99	2,05 ± 0,27	-3,35 ± 0,45	4,09 ± 0,33	298,78 ± 4,45
46/19	51,96 ± 2,12	2,65 ± 0,32	-2,80 ± 0,56	4,17 ± 0,32	315,98 ± 7,77
42/35	47,85 ± 2,77	3,07 ± 0,48	-2,38 ± 0,44	4,11 ± 0,31	320,63 ± 8,47
40/39	50,34 ± 1,89	2,48 ± 0,20	-2,82 ± 0,22	3,84 ± 0,29	317,65 ± 5,31

The fish fed the 46/11 diet, showed significantly lower values in maximal strength and elasticity, while the diet 40/39 was associated with maximal values of both parameters and a firmer flesh. Total work showed a trend to increase with reduced dietary protein content, but the differences were not statistically significant (Figure 6).

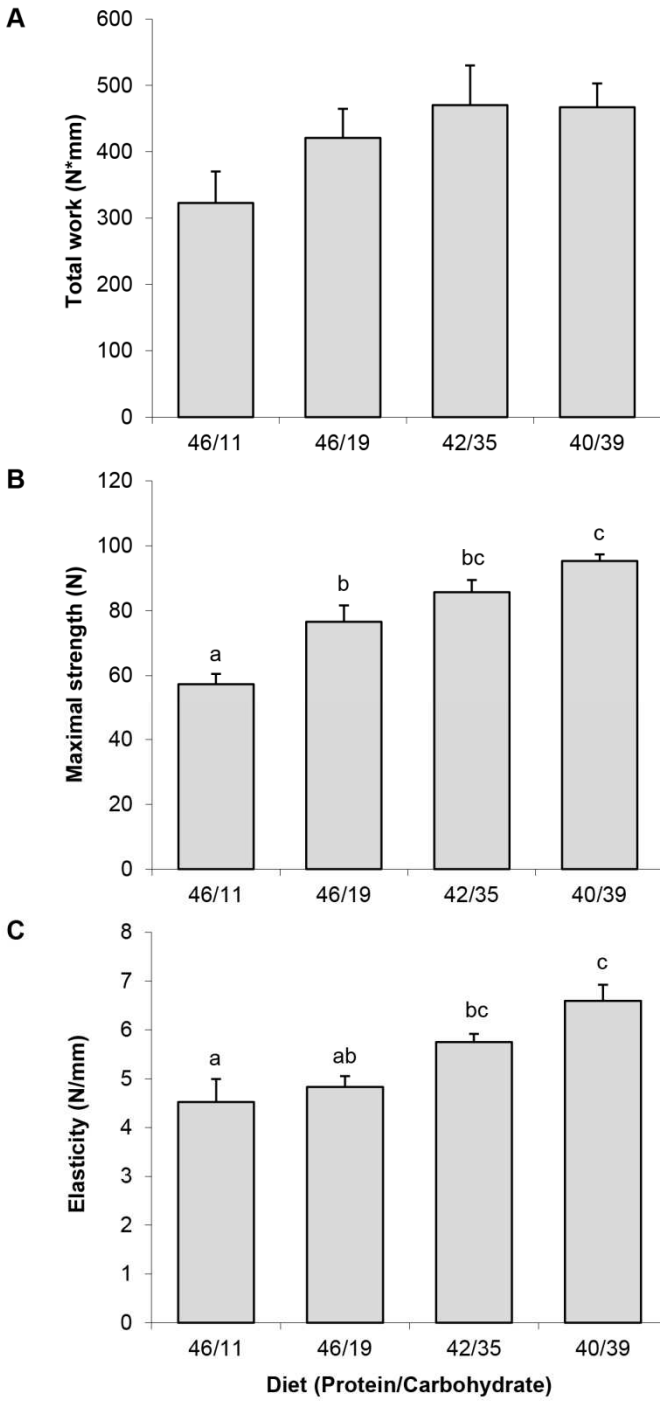


Figure 6. Fast skeletal muscle texture analysis. **(A)** total work, **(B)** maximal strength and **(C)** elasticity of gilthead sea bream fed the four experimental diets. Results are shown as mean ± SEM (n=5-8). Different letters indicate significant differences at p<0,05.

Fish fed the 46/11 diet had the highest relative expression of *sacapn1* and *sacapns1a*, while the diets 40/39 and 42/35 showed significantly lower values (Figure 7). The same decreasing trend was observed for *sacapn2* expression concomitantly with the increase of dietary CH, although no significant differences were found.

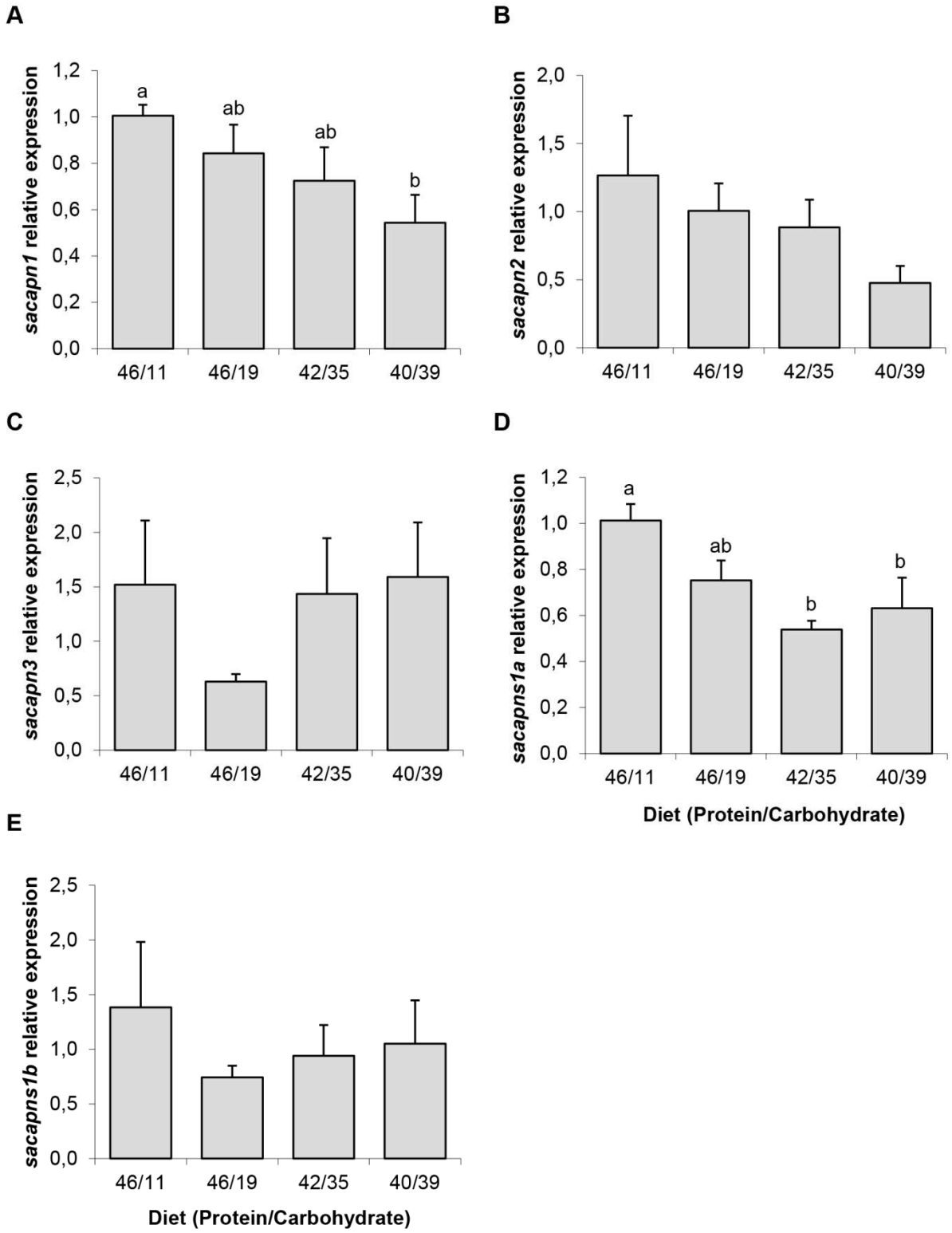


Figure 7. Calpains relative expression in gilthead sea bream fed the four experimental diets. Quantitative expression relative to β-*actin* of **(A)** *sacapn1*, **(B)** *sacapn2*, **(C)** *sacapn3*, **(D)** *sacapns1a* and **(E)** *sacapns1b*. Results are shown as mean ± SEM (n=6-8). Different letters indicate significant differences at p<0,05.

To better understand the possible relationship between calpains gene expression and flesh texture, correlations between these different parameters were performed. Significant Spearman ρ values of negative correlation were found between maximal strength and *sacapn1* as well as *sacapns1a* expression (ρ=-0.409* and ρ=-0.449*, respectively). Regarding calpain expression, significant positive correlations were observed between: *sacapn1* and *sacapn2* (ρ=0.414*), *sacapn1* and *sacapns1a* (ρ=0.814**), *sacapn2* and *sacapns1b* (ρ=0.592**), *sacapn3* and *sacapns1a* (ρ=0.455*) and *sacapns1a* and *sacapns1b* (ρ=0.489*).

4. Discussion

4.1 Calpains characterisation

In the present study, several members of the calpain system have been characterised for the first time in gilthead sea bream (*Sparus aurata*) fast-twitch skeletal muscle. The typical calpain domain architecture was identified in all five gilthead sea bream calpains, in which the triad of catalytic residues common to all calpains was in each case highly conserved in comparison to vertebrates [19]. In agreement with Atlantic halibut [22], Sacapn3 lacks the IS1 domain present in tetrapods at the C-terminal region of DII [41], which altogether with NS and IS2 has to be autocatalytically removed for Calpain3 to become proteolitically active [42]. Regarding the regulatory subunits, in the N-terminal region of DV there is missing the Gly-rich and hydrophobic region characteristic of mammalian calpain small subunits that plays a role in membrane targeting, which was previously described absent in rainbow trout calpain small subunit [43] and in zebrafish calpain small 2-like [23], suggesting divergent function and activation mechanisms of the fish calpain system compared to mammals.

4.2 Phylogenetic analysis

To further characterise the gilthead sea bream calpains, a phylogenetic tree was constructed. Importantly, each putative gilthead sea bream calpain sequence was related to the corresponding teleost ortholog as expected, as well as with its tetrapod ortholog with the exception of Calpain2. The teleost Calpain2 group formed a monophyletic clade external to the tetrapod Calpain2/8 sister group; thus, supporting the hypothesis that they are a close related group (i.e. Calpain2-like) and the common ancestor of vertebrate Calpains2/8 [23], [25]. These phylogenetic data together with conserved protein structure support a similar role for these proteinases in muscle tenderisation.

4.3 Tissue expression

Next, qualitative RT-PCR was used to identify the distinct tissue expression of each gilthead sea bream calpain. Transcripts of *sacapn1*, *sacapn2*, *sacapns1a* and *sacapns1b*, were ubiquitously expressed as observed in rainbow trout [21], [43], Atlantic halibut [22], rabbits [44] or humans [16], for instance. Furthermore as in mammals, where *calpain3* is principally expressed in skeletal muscle [41], regulating protein turnover and maintaining the sarcomere integrity [45], the *sacapn3* transcripts were mostly detected in fast-and slow-twitch skeletal muscle, but also in the heart. Moreover, *sacapn3* was also expressed to a lesser extent in other tissue types as previously reported in Atlantic halibut [25]; thus, suggesting a possible broader physiological role for Calpain3 in teleosts in comparison to mammals.

4.4 Effects of nutritional status

In the wild, many fish species including gilthead sea bream are adapted to long-term food deprivation. In response to fasting, fish mobilize energy materials stored in their tissues, and after long periods, when more readily available energy sources have been exhausted, skeletal muscle proteins can be also mobilized resulting in muscle atrophy [46]. On the other hand, in anabolic situations, food intake stimulates the synthesis of new proteins, and to a lesser extent, also its degradation to induce protein turnover and growth.

Morphological and plasma parameters in fasted fish confirmed the catabolic state. *Sacapns1b* expression significantly increased after 15 days of fasting, while *sacapn1*, *sacapn2*, *sacapn3* and *sacapns1a* expression remained unaffected. In a previous study in rainbow trout, fasting for 35 days significantly stimulated the expression of *calpain1* and *calpain2*, but not that of the calpain small subunit [21]. In Atlantic halibut, *calpain1* but not *calpain2* was up-regulated after 60 days of fasting [22], and the contrary occurred in channel catfish, where 35 days of fasting increased *calpain2*, while decreased *calpain1* expression [24]. After re-feeding, significant increases were observed in HSI and plasma parameters returned to normal values. Changes in muscle colour indicated an increase in lightness during re-feeding, which may be attributed to an increase in lipid content as previously reported [29]. Furthermore, *sacapn3* was again unchanged but the relative expression of *sacapn1*, *sacapn2*, *sacapns1a* and *sacapns1b* was significantly reduced, suggesting a decrease in muscle proteolysis under these conditions, a result supported by the significant decrease observed during re-feeding of the subunit β of the proteasome *N3*, previously used in other studies as a proteolysis marker [47], [48]. These results were in agreement with the study in Atlantic halibut, where it was also observed that after 7 days of re-feeding, fish that were fasted for 60 days had *calpain1* transcript levels

significantly decreased, but no differences were observed in *calpain2* expression, whereas a significant increase in *calpain3* was found [22]. In addition, in a recent study in gilthead sea bream fasted for 4 days, *calpain3* expression also increased 5-6 fold 24 h after re-feeding and was maintained until 6 days later [49].

Overall, these data suggests that the regulation of calpain expression with fasting and re-feeding is species-specific. In our study, *sacapns1b* expression was sensitive to fasting and re-feeding increasing and decreasing respectively, suggesting this calpain could be a potential marker to identify nutritional status in gilthead sea bream.

4.5 Effects of diet composition

In their natural diet, gilthead sea bream feed mainly on molluscs and crustaceans, but the presence of algae is common in its intestinal contents. This indicates that gilthead sea bream can use vegetables, rich in CH and fibre, as an energy source. Previous studies on gilthead sea bream have shown that it is not advisable to exceed 20% of CH in their diet, due to a persistent postprandial hyperglycemia, that can finally decrease growth [50]. In the present study, isolipidic diets with different percentages of protein and CH were used to determine if it is possible to increase CH over the limit of 20% without affecting growth, but more interestingly, to see if calpain relative expression could be related to muscle texture, a parameter that can be modulated according to dietary treatment. Interestingly, although no differences were observed in feed intake or plasma glucose between groups, gilthead sea bream showed decreased growth parallel to the amount of protein in the diet. Fish fed diets 42/35 and 40/39 obtained significantly lower SGR and final body weight values compared to the fish fed the other diets (46/11 and 46/19). These results arein agreement with a previous study [50] and support the limited value of 20% CH dietary inclusion to achieve good growth rates in this species.

Texture analysis showed that in diets with 46% of protein, increasing CH levels up to 19% improved significantly flesh maximal strength; and an increase up to 35%, with a reduction of protein from 46 to 40%, elevated also significantly flesh elasticity. In previous studies in *Dentex dentex* it was also observed that a decrease in dietary protein content from 43 to 38% improved textural parameters as firmness and water holding; however, it was observed that within each dietary protein level, diets with high CH and low lipid content resulted in lower values of muscle firmness than diets with low CH [51]. Therefore, this is the first study in *Sparids* where an increase in dietary CH seems to improve textural parameters.

Sacapn1 and *sacapns1a* were transcriptionally affected by the diet, while *sacapn2*, *sacapns1b* and *sacapn3* remained unaffected. Both, *sacapn1* and *sacapns1a* relative expression decreased with dietary CH increase and protein decrease, suggesting a reduction in muscle proteolysis and an increase in muscle texture in these fish. In agreement with this observation, *calpain1* mRNA levels were significantly lower in pigs fed a protein-free diet in comparison to control pigs [52]. In rainbow trout, it was observed that the level of *cathepsin D* expression in fast muscle increased by substitution of dietary fishmeal by a mix of plant protein sources, but *calpain2* was not modified [6]. Using different rainbow trout strains with distinct growth rates and fillet firmness and fed with two different energy diets, Salem *et* al., [26] reported that strain or diet did not affect the level of mRNAs expression for any of the calpain members analysed; however, significantly lower *calpastatin* expression was observed in the strain with softest fillet. These results suggest that the effects of diet on calpain expression show significant variation between fish species.

To further investigate the importance of the different gilthead sea bream calpains in flesh firmness, correlation analysis between texture parameters and calpains expression was performed. Interestingly, *sacapn1* and *sacapns1a* relative expression levels were significantly negatively correlated with maximal strength in our study. In mice, *calpain1* knockout animals had significantly reduced proteolysis in comparison to control mice [53]. Also, other studies in mammals have supported that calpain1, but not calpain2, is primarily responsible for meat tenderisation in beef and lamb [54], [55]; and in cattle, single nucleotide polymorphisms (SNPs) for the *calpain1* gene have been clearly associated with tenderness [56]. This has currently led to the use of markers within the *calpain1* as well as the *calpastatin* gene to identify the genetic potential of beef cattle to produce tender meat [57], a tool that is commercially available as a genetic test (GeneSTAR, Pfizer Genetics). The present results have revealed the potential use of calpains, *sacapn1* and *sacapns1a*, as candidate genes to monitor muscle growth and fillet firmness in gilthead sea bream.

In addition, *sacapn1* and *sacapn2* relative expression was significantly positively correlated, as both genes followed the same trend and decreased expression with an increased CH:protein ratio in the diet. A significant positive correlation between both *sacapns* paralogs was also found. Furthermore, the expression of small subunit paralogs, *sacapns1a* and *sacapns1b*, revealed also that each paralog was significantly correlated with each one of the catalytic calpains (*sacapns1a* with *sacapn1* and *sacapns1b* with *sacapn2*). Contrary to what it is observed in mammals, where the different catalytic calpains bind a common regulatory subunit to be fully active [19], the present data suggests that the genome duplication that

occurred in the teleost lineage resulted in each fish catalytic calpain binding to a specific calpain regulatory subunit paralog. In order to confirm this hypothesis, further studies at the protein level will be required.

Finally, a significant positive correlation was found between the expression of paralog *sacapns1b* and *sacapn3*, suggesting a different regulation for calpain3 activity in fish, because in mammals, the recombinant PEF domain of Calpain3 is known to form a stable homodimer, but it is believed not to form a heterodimer with the calpain small subunit [58]. Moreover, the muscle-specific *calpain3* did not show differences in response to dietary treatment and did not correlate with muscle texture. In mammals, a strong correlation has been shown between *calpain3* mRNA levels and tenderness in cattle and sheep, whereas no correlation was reported in pigs [59], [60]. Also, it has been shown that Calpain3 can cleave calpastatin and the ubiquitous calpains, suggesting a role for calpain3 as an endogenous regulator of calpain expression and proteolytic activity [61]; thus indicating calpain3 deserves further attention in future studies in fish.

In summary, the present data has shown that several gilthead sea bream calpains are expressed in tissues with a distribution similar to that of calpains already described in other fish species as well as in mammals. We have also shown for the first time in teleosts the presence of two paralogs of the calpain small subunit (*sacapns1a* and *sacapns1b*) and the data has suggested that they are differently activated; *sacapns1b* with fasting and *sacapns1a* with changes in diet composition. Furthermore, the present results suggested that the expression of each paralog may be related to the expression of a corresponding catalytic subunit (*sacapns1a* with *sacapn1* and *sacapns1b* with *sacapn2*). Finally, we can conclude that the expression of some gilthead sea bream calpain genes, such as *sacapn1* and *sacapns1a*, may serve as potential genetic markers of flesh quality in this species.

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CAPÍTOL II

Caracterització de catepsines i members del sistema ubiquitina-proteasoma en múscul esquelètic d'orada (*Sparus aurata*): canvis d'expressió durant les etapes de la vida i la realimentació

Characterisation of cathepsins and ubiquitin-proteasome members in gilthead sea bream (*Sparus aurata*) skeletal muscle: changes in expression during life stages and re-feeding

Caracterització de catepsines i members del sistema ubiquitina-proteasoma en múscul esquelètic d'orada (*Sparus aurata*): canvis d'expressió durant les etapes de la vida i la realimentació

El múscul esquelètic dels peixos o filet, és el producte més valuós de l'aqüicultura, per la qual cosa totes les mesures encaminades a millorar-ne el creixement i la qualitat, com ara la caracterització dels gens implicats en l'acreció de proteïna en el múscul i la seva degradació són de gran importància. Els principals sistemes que participen en la proteòlisi muscular són a) calpaïnes, b) catepsines (CTSs), i c) ubiquitina-proteasoma (UbP). En el present estudi es van identificar en el múscul esquelètic d'orada (Sa), les CTSs (SaCTSB i SaCTSDb) i membres del sistema UbP (SaN3 i SaUb), les seqüències aminoacídiques dels quals van compartir un 66-100% d'identitat global amb els seus homòlegs d'altres espècies de vertebrats, i van mostrar totes les característiques de domini i els residus catalítics per ser funcionals. El nou SaCTSD clonat en el múscul, SaCTSDb, va mostrar diferències de seqüència, filogenètiques i de distribució tissular respecte el seu paràleg, SaCTSDa, identificat prèviament en ovari. A continuació es va avaluar la seva regulació transcripcional en el múscul a) en tres etapes diferents de la vida, i b) en resposta a la realimentació. La quantificació dels nivells d'ARNm va revelar que la majoria dels gens proteolítics analitzats van ser inhibits significativament a mesura que creixien els peixos i durant la realimentació, el que indica que hi ha una disminució en la proteòlisi muscular en ambdós casos. Com a excepció, l'expressió de SaCTSDa es va mantenir estable entre les diferents etapes de la vida, el que dóna suport a les divergències evolutives a nivell funcional amb el seu paràleg, SaCTSDb. D'altra banda, l'expressió del gen SaUb va ser l'única que es va reduir d'alevins a peixos juvenils i va tornar a augmentar en els adults, el que suggereix un possible paper, limitant el creixement muscular o la capacitat de regeneració en els animals adults. Aquestes dades indiquen que les SaCTSs i els membres del sistema SaUbP són reguladors del recanvi de les proteïnes musculars, l'expressió dels quals canvia durant la vida i en relació amb l'estat nutricional dels peixos. Per tant, aquests sistemes proteolítics podrien ser modulats nutricionalment per disminuir el catabolisme proteic i l'estovament del filet *post-mortem* en orada.

Paraules clau: Múscul blanc, estadis de la vida, proteòlisi lisosomal, via ubiquitina-proteasoma, realimentació.

Characterisation of cathepsins and ubiquitin-proteasome members in gilthead sea bream (*Sparus aurata*) skeletal muscle: changes in expression during life stages and re-feeding

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Running title: Gilthead sea bream muscle proteases

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Abstract

Fish skeletal muscle or fillet, is the most valuable aquaculture product; therefore, all measures aimed to improve its growth and quality such as the characterisation of genes implicated in muscle protein accretion and degradation are of great importance. The main systems involved in muscle proteolysis are a) calpains, b) cathepsins (CTSs), and c) the ubiquitin-proteasome (UbP). In the present study we have identified in gilthead sea bream (Sa) fast skeletal muscle, the CTSs (SaCTSB and SaCTSDb) and UbP members (SaN3 and SaUb), which amino acid sequences share 66-100% overall identity with its counterparts in other vertebrate species, and possess all the domain features and catalytic residues to be functional. The new SaCTSD cloned in muscle, SaCTSDb, showed phylogenetic, sequence and tissue distribution differences respect to its paralogue, SaCTSDa, previously identified in the ovary. We evaluated next their transcriptional regulation in muscle a) at three different life stages and, b) in response to re-feeding. Quantitative mRNA analysis revealed that the majority of the proteolytic genes analysed were significantly down-regulated as the fish grow and during re-feeding, indicating that there is a decrease in muscle proteolysis in both cases. As an exception, SaCTSDa expression remained stable among life stages, supporting evolutionary functional divergences with its paralogue, SaCTSDb. Moreover, SaUb was the only gene whose expression decreased from fingerlings to juvenile fish and increased again in adults, suggesting a possible role limiting muscle growth or regeneration capability in the older. These data indicate that SaCTSs and SaUbP members are regulators of muscle protein turnover, whose expression changes during lifetime and in relation with the nutritional condition of the fish. Thus, these proteolytic systems might be nutritionally modulated to decrease protein catabolism and *post-mortem* fillet tenderisation in gilthead sea bream.

Keywords: White muscle, life stages, lisosomal proteolysis, ubiquitin-proteasome pathway, re-feeding.

1. Introduction

The 60-70% of the fish body is axial musculature or fillet, the most valuable product in aquaculture at nutritional and economic levels. Gilthead sea bream (*Sparus aurata* L.) is widely farmed around the Mediterranean area (APROMAR, 2013) and because of its commercial interest, muscle growth regulation in this species has received a great deal of attention during the last years (Ibarz et al., 2011; Jiménez-Amilburu et al., 2013; Rius-Francino et al., 2011; Sánchez-Gurmaches et al., 2013). As most teleosts, gilthead sea bream exhibits indeterminate growth, with muscle mass increasing by hypertrophy (increase of fibres size) and/or hyperplasia (production of new fibres), until mortality or senescence occur (Johnston, 2006; Mommsen, 2001). In the process of myogenesis, proliferation of precursor cells is triggered primarily upon up-regulation of proliferating cell nuclear antigen (PCNA) (García de la Serrana et al., 2014; Johnston, 2006), whereas myostatin (MSTN) has been reported as an important negative regulator of muscle growth, inhibiting proliferation and differentiation of satellite cells (Medeiros et al., 2009; Seiliez et al., 2012). Once initiated the myogenic programme, increased expression of major structural proteins such as myosin heavy chain (MHC), myosin light chain-2 (MLC2) and desmin is observed, following muscle development (García de la Serrana et al., 2013; Moutou et al., unpublished data). During the life of an animal, the skeletal muscle is in continuous protein turnover, which includes protein synthesis as well as degradation, with positive balance allowing muscle growth. Contrary to synthesis, muscle proteolysis, implicated in protein mobilization for energy production or remodelling, and also, *post-mortem* fillet softening, has been little studied in gilthead sea bream (Caballero et al., 2009; Salmerón et al., 2013).

In vertebrates, four catabolic systems are known to be involved in muscle proteolysis: a) the Ca^{2+}-dependent proteinases (calpains), b) the autophagy-lysosome system (cathepsins), c) the ATP-dependent ubiquitin-proteasome (UbP) pathway and, d) the apoptosis protease system (caspases) (Argilés et al., 2008; Jackman and Kandarian, 2004; Kachaeva and Shenkman, 2012; Salem et al., 2006a). Calpains are considered a system of primary protein degradation, with a regulatory or signalling function, since calpains do not cleave proteins to amino acids or small peptides (Goll et al., 2003); being the cathepsins and the UbP pathway the systems that finally degrade the protein substrates completely (Ono and Sorimachi, 2012).

The UbP pathway operates through a multisubunit proteolytic complex, the proteasome, and is characterised by specific targeting the proteins for destruction through a three-step process that covalently links a polyubiquitin (Ub) chain to the protein substrate to be degraded. This proteolytic system in mammals is especially important during muscle atrophy either caused by

starvation or wasting diseases (Lecker and Goldberg, 2002; Lecker et al., 2004; Medina et al., 1995), and it is also involved in the age-related loss of muscle mass (sarcopenia) (Cai et al., 2004; Altun et al., 2012). In fish, starvation has been clearly shown to induce muscle atrophy (Beaulieu and Guderley, 1998; Guderley et al., 2003), whereas signs of sarcopenia as the fish age have been reported only for species with determinate growth such as the zebrafish (Froehlich et al., 2013). Previous studies in rainbow trout have shown that fasting increased the 20S proteasome activity (Salem et al., 2007) and the mRNA expression of several UbP pathway members, which expression decreased with re-feeding (Rescan et al., 2007; Seiliez et al., 2008). Nevertheless in the same species, the UbP system was not modified in muscle of fertile fish during the proteolytic process induced during spawning (Salem et al., 2006a; Salem et al., 2006b).

Cathepsins are lysosomal proteases optimally active in a slightly acidic environment, divided according to the amino acid found in their active site in three groups as aspartic, serine or cysteine proteases (Turk et al., 2012). The role of cathepsins in muscle proteolysis and *post-mortem* degradation has been investigated in several fish species. The enzymatic degradation of key sarcoplasmatic proteins, including structural and extracellular matrix proteins during post-mortem tenderisation seem to be conducted by calpains as well as cathepsins (Delbarre-Ladrat et al., 2006). In salmon, in the muscle protein degradation that occurs at the last stages of maturation (during migration or natural starvation), the lysosomal cathepsins, D and sometimes L are especially involved (Mommsen et al 2004). In rainbow trout, fasting and re-feeding increased and decreased, respectively, the mRNA expression of cathepsins B, D and S (Rescan et al., 2007), and the spawning-induced muscle deterioration in this species was associated also with greater mRNA accumulation of cathepsins L and D (Salem et al., 2006a; Salem et al., 2006b). In gilthead sea bream, Carnevali and co-workers (Carnevali et al., 1999a; Carnevali et al., 1999b; Carnevali et al., 2008) identified in the reproductive ovary the cathepsins B, D and L and characterised them during the process of oogenesis, showing that its gene expression and enzymatic activity changed significantly during gonadal development, confirming a specific function for these enzymes during the follicle maturation process. However, the role of cathepsins in gilthead sea bream skeletal muscle and its transcriptional regulation remain unknown.

We have recently characterised in gilthead sea bream muscle several members of the calpain system, and have demonstrated that its expression is modulated by nutritional status and diet composition (Salmerón et al., 2013). Thus, the main objectives of the present study were using gilthead sea bream as a model: a) to identify cathepsins and UbP pathway members expressed

in muscle and, b) to characterise its expression patterns together with those of muscle growth markers at different life stages and, c) to analyse its expression during re-feeding after a 30-days fasting period in juveniles.

2. Materials and methods

2.1 Ethics statement

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona (CEEA 239/09) and the Departament de Medi Ambient i Habitatge (DMAH permit number 5420, Generalitat de Catalunya, Spain) following the European Union, Spanish and Catalan Government established norms and procedures.

2.2 Animals and experimental trials

Animals used for the cloning, screening and life stages experiment were obtained from a fish farm in Northern Spain and animals for the re-feeding experiment were obtained from the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) facilities (Sant Carles de la Ràpita, Spain). All animals were acclimatized to the facilities at the University of Barcelona (Barcelona, Spain) for two weeks minimum before sampling or experimental manipulations, fed *ad libitum* twice daily with commercial pellets (Excel, Skretting, Burgos, Spain) and held at 21ºC in 200 or 400 L recirculating seawater tanks with 12 h dark:12 h light photoperiod. For cloning we used 10 juvenile gilthead sea bream of 43±3 g and for the tissue screening 6 juveniles of 151±12 g. For the life stages experiment, we used groups of 5 fish each of 15±1 g (fingerlings, FL), 47±5 g (juveniles, JV) and 503±37 g (adults, AD). For the re-feeding experiment 24 juvenile gilthead sea bream (50±6g) were homogenously distributed in 4 tanks (200L), acclimated as indicated above and then fasted during 30 days. At that time, 2 fish per tank were sampled as day 0 and the remaining animals were re-fed at 2% body weight with the same diet as in the acclimatization period. Then, 2 fish per tank were sampled at days 7 and 14 of re-feeding. Before sampling all fish were fasted 24 h, anesthetized with tricaine methane sulphonate (MS-222 0.1g/L, Sigma, Tres Cantos, Spain) and sacrificed with a blow on the head and medullar section. Samples of fast skeletal muscle (FM) for the cloning and all experiments and also, samples of slow skeletal muscle (SM), heart (HE), brain (BR), liver (LI), spleen (SP) and immature gonad (GO) for the tissue screening were taken with sterile dissection material and immediately snap-frozen in liquid nitrogen and stored at -80ºC for cloning or gene expression analyses.

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from 20-130 mg of tissue following the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 2006) using TRIreagent (Applied Biosystems, Alcobendas, Spain), was quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and, RNA quality was analysed by 1% (m/v) agarose gel electrophoresis. One µg of total RNA per sample was used to synthesise first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain) or the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Las Rozas, Spain) in the case of the tissue screening, following the manufacturer's instructions. cDNA samples were diluted 1:5 in milliQ H2O for conventional polymerase chain reaction (PCR) and diluted 1:40 to 1:100 in milliQ H2O for real-time quantitative PCR (qPCR).

2.4 Cloning and sequencing

To obtain the complete sequences of gilthead sea bream (Sa) cathepsin B (SaCTSB), the new paralogue of cathepsin D (SaCTSDb), the proteasome beta type-4 subunit (SaN3, also known as PSMB4) and the ubiquitin (SaUb) from fast skeletal muscle, specific primers for PCR were designed using the gilthead sea bream ESTs (Expressed Sequence Tag) NCBI database (SaCTSB: HS985610 and FG26781; SaCTSDb: FM146030 and FG26194; SaN3: HS988518; SaUb: AM955423) (Table 1). The cloning was done as previously described (Salmerón et al., 2013). Briefly, PCR products were separated by gel electrophoresis and purified from the agarose gel using the PureLink Quick Gel Extraction Kit (Invitrogen, Alcobendas, Spain). The purified PCR product was ligated into T/A pCR4-TOPO vector and transformed into chemically competent TOP10 *Escherichia coli* cells (all from Invitrogen, Alcobendas, Spain). One to three clones of each PCR product were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Alcobendas, Spain) and analysed at the Serveis Cientificotècnics of the University of Barcelona (Barcelona, Spain). Sequenced products were joined in silico using the sequence alignment editor and sequence analysis program BioEdit (Hall, 1999) to produce contigs with a single open reading frame (ORF). Sequences generated were analysed for similarity with other known sequences using the BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 Sequence and phylogenetic analyses

The putative protein architecture (domains, active sites and other important motifs) from the sequences generated was determined according to the literature and the conserved domain search program of NCBI (Marchler-Bauer et al., 2013) and the simple modular architecture

research tool (SMART) version 4.0 (http://smart.embl-heidelberg.de, Letunic et al., 2004). Compute pI/Mw tool (ExPASy, Switzerland, http://www.expasy.org/tools/pi_tool.html) was used to estimate the molecular weight (Mw) of the predicted proteins andNetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict the N-Glycosylation sites. All the alignments were created with MAFFT version 7 (http://mafft.cbrc.jp/alignment/software/) and G-INS-i (recommended for <200 sequences with global homology) strategy. Sequences used in the present study other than those cloned from gilthead sea bream fast skeletal muscle were obtained from NCBI. Phylogenetic analyses, including multiple sequence alignment and Maximum Parsimony (MP) tree prediction of 27 vertebrate cathepsin orthologs of CTSB, CTSD and CTSL were conducted using MEGA version 5.0 (Tamura et al., 2011). MP trees were obtained using the Close-Neighbor-Interchange algorithm and bootstrap values were inferred from 1000 replicates. The gilthead sea bream calpain 1 (accession number: KF444899), a cytosolic cysteine protease, was used to root the phylogenetic tree.

Table 1. Primer sequences used to clone in gilthead sea bream (Sa) fast muscle the cathepsins (SaCTSDb and SaCTSB) and ubiquitin-proteasome members (SaUb and SaN3). FW: forward, RV: reverse.

Primers ID	Primer sequences (5'-3')	Annealing temperature (ºC)	Product size (bp)
SaCTSDb_FW	TCGGACTGTTACGATGAGGA	56	1240
SaCTSDb_RV	CTTTGCACTTGGACGAGTTG		
SaCTSB_FW	CCCGAAGATTATAACCAAGTTGAC	59	1249
SaCTSB_RV	GTGACTTGTGCTCAGAAACGTAGT		
SaUb_FW	CGGAAGTAAGAGGAACCAACAC	56	1132
SaUb_RV	AAGCAGTCAGAATGCAAAGTCA		
SaN3_FW	CAGGTTTGAAGCTGAGTTTCTG	58	759
SaN3_RV	CTGACCATGTGAGCGATGTC		

2.6 Conventional PCR

The mRNA levels of both SaCTSD paralogues under normal physiological conditions were measured in gilthead sea bream tissues using qualitative PCR and elongation factor 1-α (EF1α) was used as a loading control gene. Reactions were performed in a final volume of 50µL, containing 1µL of first-strand cDNA (equivalent to 4ng of reverse transcribed total RNA), 1.5U of Taq polymerase (Sigma, Tres Cantos, Spain) and 200nM (final concentration) of sense and antisense primers (Table 2). Reactions proceeded in a MyiQ Thermal Cycler (Bio-Rad, El Prat de Llobregat, Spain) with the following protocol: 1 cycle at 95ºC for 5 min, 35 cycles at 95ºC for 30 s, 56-60ºC (primer dependent, see Table 2) for 30 s, 72ºC for 30s and 1 cycle at 72ºC for 7 min.

Each reaction product was separated by agarose gel electrophoresis and visualised using SYBR Safe DNA gel stain (Life Technologies, Alcobendas, Spain) in a LAS-3000 (Fujifilm, Madrid, Spain) to confirm that a single product was amplified, and then sequenced to confirm the specificity of each assay as explained above in 2.4.

Table 2. Primer sequences against gilthead sea bream (Sa) cathepsins (SaCTSDa, SaCTSDb, SaCTSL and SaCTSB), ubiquitin-proteasome members (SaUb and SaN3) and muscle growth markers (SaPCNA, SaMSTN, SaMHC and SaMLC2) and reference genes (Saβ-actin, Sa18S and SaEF1α) used for conventional PCR and qPCR analyses. qFW: forward, qRV: reverse.

Primers ID	Primer sequences (5'-3')	Annealing temperature (ºC)	Product size (bp)
SaCTSDa_qFW	CCTCCATTCACTGCTCCTTC	56	107
SaCTSDa_qRV	ACCGGATGGAAAACTCTGTG		
SaCTSDb_qFW	AAATTCCGTTCCATCAGACG	56	131
SaCTSDb_qRV	CTTCAGGGTTTCTGGAGTGG		
SaCTSL_qFW	ACTCCTTGGGCAAACACA	54	116
SaCTSL_qRV	CCTTGAACTTCCTCTCCGT		
SaCTSB_qFW	GCAGCCTTCCTGTTATTGG	57	185
SaCTSB_qRV	AGGTCCCTTCAGCATCGTA		
SaUb_qFW	ACTGGCAAGACCATTACCTT	54	160
SaUb_qRV	TGGATGTTGTAGTCGGAAAG		
SaN3_qFW	AGACACACACTGAACCCGA	54	118
SaN3_qRV	TTCCTGAAGCGAACCAGA		
SaPCNA_qFW	TGTTTGAGGCACGTCTGGTT	58	201
SaPCNA_qRV	TGGCTAGGTTTCTGTCGC		
SaMSTN_qFW	GTACGACGTGCTGGGAGACG	60	201
SaMSTN_qRV	CGTACGATTCGATTCGCTTG		
SaMHC_qFW	AGCAGATCAAGAGGAACAGCC	58	166
SaMHC_qRV	GACTCAGAAGCCTGGCGATT		
SaMLC2_qFW	GCTGGCAATGTGGACTACAA	58	208
SaMLC2_qRV	GAGCTGCAAAGCGACAGAG		
Saβ-actin_qFW	TCCTGCGGAATCCATGAGA	60	50
Saβ-actin_qRV	GACGTCGCACTTCATGATGCT		
Sa18S_qFW	CAGACAAATCGCTCCACCAACTA	56	119
Sa18S_qRV	CTCAACACGGGAAACCTCACC		
SaEF1α_qFW	CTTCAACGCTCAGGTCATCAT	60	262
SaEF1α_qRV	GCACAGCGAAACGACCAAGGGGA		

2.7 Quantitative real-time PCR (qPCR)

To characterise the two cathepsin D paralogues in gilthead sea bream, mRNA abundance in fast muscle and immature gonad from the tissue screening samples was evaluated using qPCR.

Then, the transcriptional levels of the different cathepsins and UbP genes in fast skeletal muscle from the life stages and re-feeding experiments were evaluated. In the life stages experiment, the expression of PCNA (AY550963), MSTN (AF258448), MHC (NM131404) and MLC2 (AF150904) as muscle growth markers was also determined. In addition, β-actin, 18S and EF1α were tested as reference genes. The qPCR assay was conducted as previously described (Salmerón et al., 2013). Reactions contained first-strand cDNA (equivalent to 2.5ng of reverse transcribed total RNA), iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain) and 250nM (final concentration) of sense and antisense primers (Table 2), and were performed in triplicate using a MyiQ or a CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain). The protocol consisted on 1 cycle of 3 min at 95ºC and 40 cycles of 10 s at 95ºC and 30 s at 54-60 ºC (primer dependent, see Table 2), followed by an amplicon dissociation analysis from 55 to 95ºC at 0.5ºC increase each 30 s, where a single peak was observed confirming the specifity of the reaction. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Expression results were normalized to β-actin, the most stable of the three genes tested, and analysed by the delta-delta method (Livak and Schmittgen, 2001).

2.8 Statistical analyses

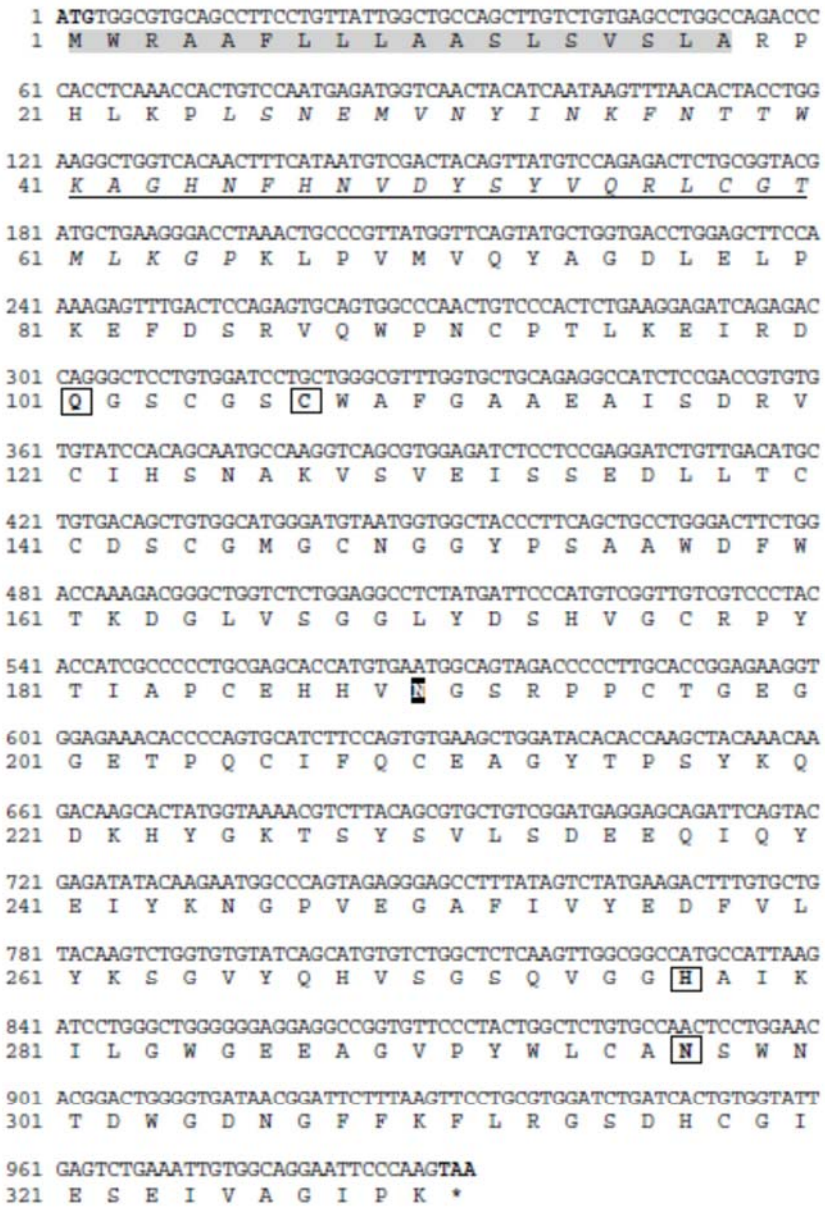
Statistical analyses of all parameters were performed in PASW Statistics 17.0 (IBM, Chicago, USA). Normality was analysed according to the Shapiro-Wilk test and homogeneity in variance according to Levene's test. Then, statistical differences were assessed by one-way ANOVA, followed by Tukey's *post hoc* test, or t-test. A significance of $p<0.05$ was applied to all statistical tests performed. Data are presented as mean ± standard error of the mean (SEM).

3. Results

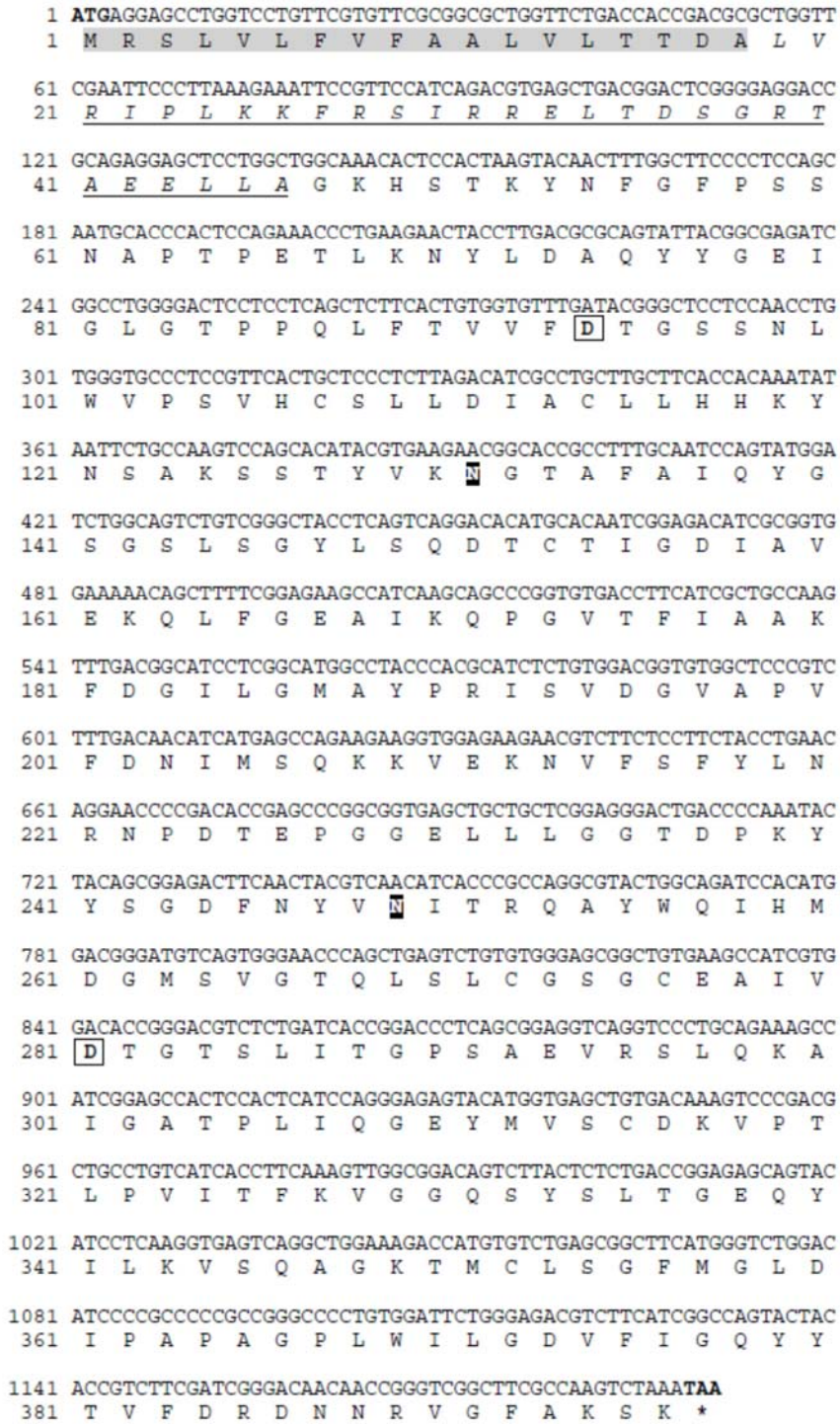
3.1. Sequence analysis of SaCTSB, SaCTSDb, SaN3 and SaUb

The ORF of SaCTSB consisted of 993 base-pairs (bp) that encode a protein of 330 amino acids (aa) with a molecular mass (Mw) of 36.41kDa (Accession number: to be submitted). BLAST analysis showed that SaCTSB shares 70-82% overall sequence identity with the cathepsin B proteins of a number of fish, amphibian and mammalian species (Table 3). *In silico* analysis identified in SaCTSB an N-terminal signal peptide (I29/Propeptide C1), a cathepsin B propeptide region, and a papain family cysteine protease domain (Supp. Fig. 1). The protease domain contains the four essential residues for catalysis, i.e. Q101, C107, H277 and N297, which are highly conserved among vertebrates plus a predicted N-glycosylation site located at position 190. The ORF of SaCTSDb has 1191 bp encoding a 396 aa protein with a Mw of 42.98 kDa (Accession number: to be submitted). SaCTSDb consists of a putative N-terminal signal

peptide, a cathepsin D propeptide region (A1_Propeptide), and an aspartyl protease domain (Supp. Fig. 2), with the two aspartyl, D94 and D281 catalytic residues, conserved. A comparison of the SaCTSDb with other vertebrate cathepsin D protein sequences revealed a high degree of sequence similarity (66-88%; Table 3). Moreover, the sequence analysis of SaCTSDb predicted two possible N-glycosylation sites, located at positions131 and 249 (Fig. 1 and Suppl. Fig. 2).



Supp. Fig. 1. Nucleotide and deduced amino acid sequences of gilthead sea bream cathepsin B (SaCTSB, accession number: to be submitted). In the nucleotide sequence, the translation start and stop codons are in bold. In the amino acid sequence, the putative signal peptide sequence is highlighted in grey, and the propeptide region (I29/Propeptide C1) is underlined and in italics. The predicted N-glycosylation site is in bold and black shaded. The two conserved catalytic residues (C and H), the glutamine (Q) of the oxyanion hole and the asparagine (N) that orients the imidazolium ring are in bold and boxed.



Supp. Fig. 2. Nucleotide and deduced amino acid sequences of a new paralogue of gilthead sea bream cathepsin D (SaCTSDb, accession number: to be submitted). In the nucleotide sequence, the translation start and stop codons are in bold. In the amino acid sequence, the putative signal peptide sequence is highlighted in grey, the propeptide region (A1_Propeptide) is underlined and in italics. The predicted N-glycosylation sites are in bold and black shaded, and the two aspartyl (D) conserved catalytic residues are in bold and boxed.

Table 3. Percentages of amino acid sequence identity between the gilthead sea bream (Sa) and the *Mus musculus*, *Xenopus laevis*, *Takifugu rubripes*, *Danio rerio* and *Salmo salar* cathepsins (B, L, Da and Db) and the ubiquitin-proteasome members (N3 and Ub).

	M. musculus	*X. laevis*	*T. rubripes*	*D. rerio*	*S. salar*
SaCTSB	71	72	70	81	82
SaCTSL	63	76	88	83	84
SaCTSDa	58	58	58	66	73
SaCTSDb	66	77	88	83	87
SaN3	74	80	93	90	90
SaUb	100	100	100	100	100

Compared to the previously reported cathepsin D in gilthead sea bream ovary (SaCTSDa), the newly identified SaCTSDb has 57% aa identity, but lacks the third residue of N-glycosylation at position 337 (Fig. 1).

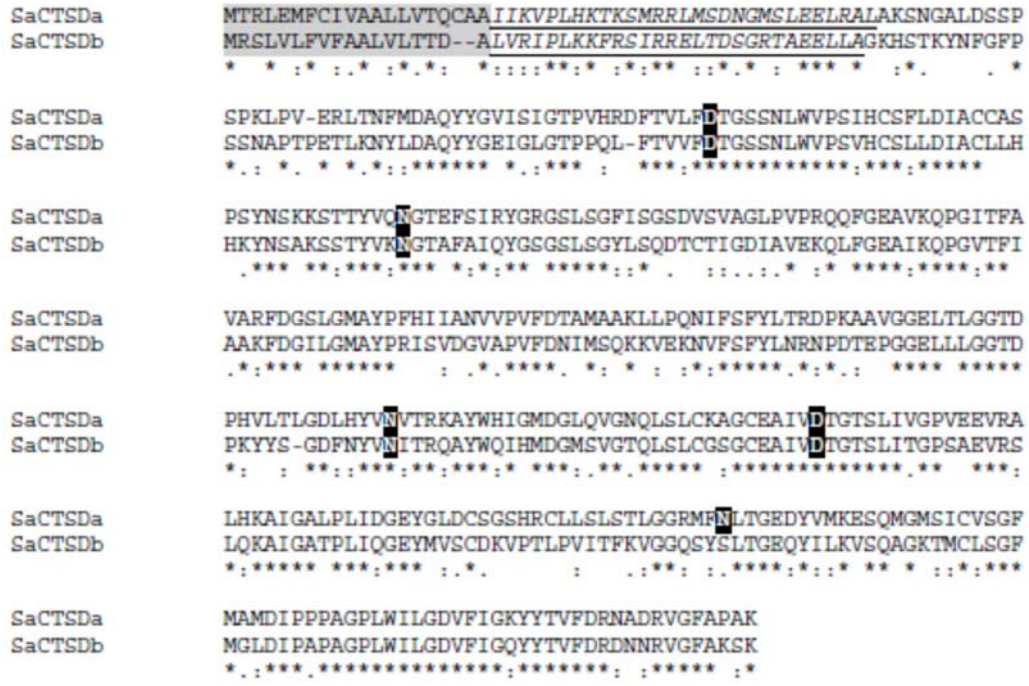
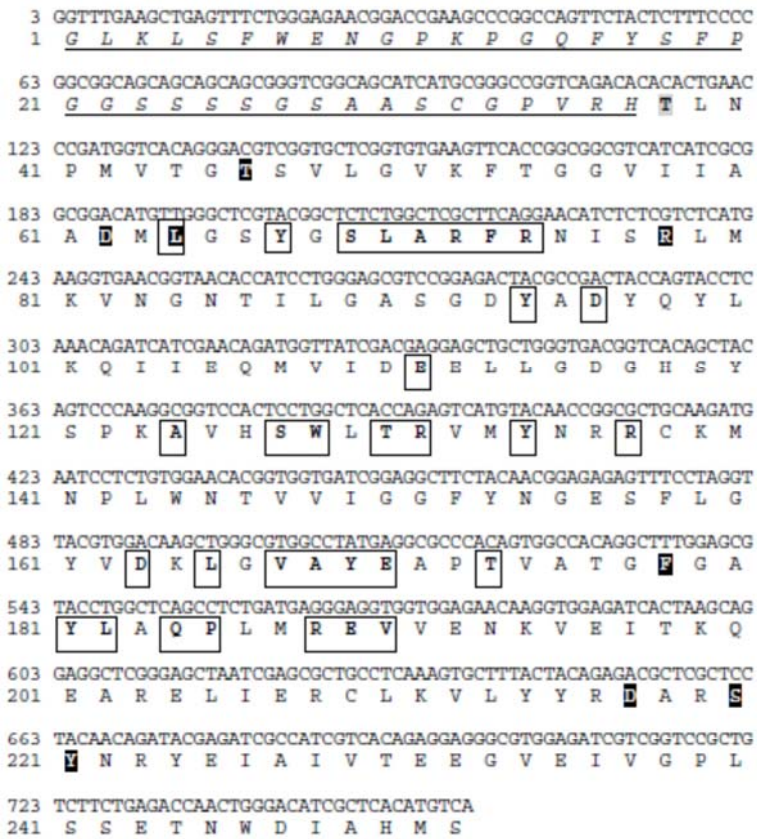
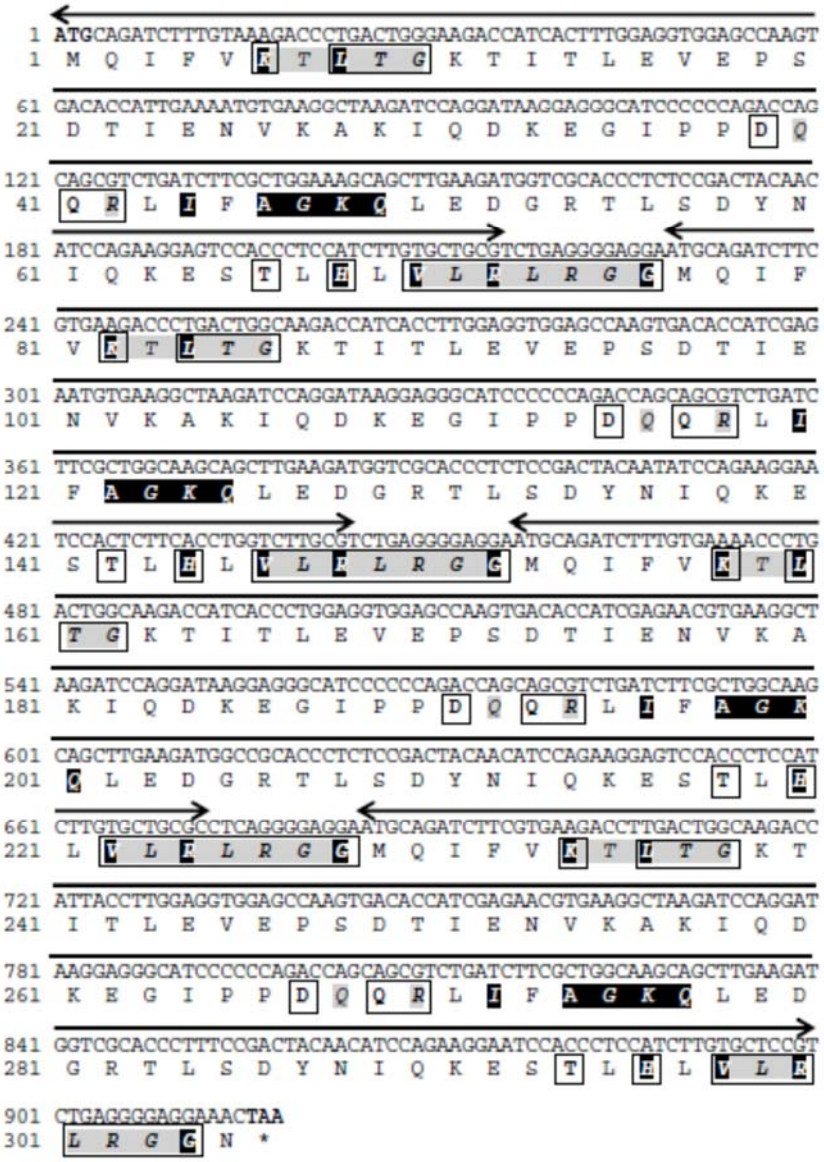


Figure 1. Sequence comparison of gilthead sea bream cathepsin D paralogues. Alignment of deduced amino acid sequence of both gilthead sea bream (Sa) cathepsin D paralogues; SaCTSDa (GenBank: AF036319) and SaCTSDb (Accession number: to be submitted), via MAFFT (v7.058b) and L-INS-I method. Symbols: (*) identical residues in both sequences; (:) conservative substitutions and (.) semiconservative substitutions. The putative signal peptide sequence is highlighted in grey and the propeptide region (A1_Propeptide) is underlined and in italics. The predicted N-glycosylation sites (N) and the two aspartyl (D) conserved catalytic residues are shaded in black.

The partially sequenced SaN3 (97% of the molecule) contained 756 bp and encoded 252 aa (Accession number: to be submitted). SaN3 showed high levels of identity (74-93%) with other vertebrate proteasome N3/PSMB4 sequences (Table 3). The partial SaN3 protein sequenced contains the proteasome domain with threonine endopeptidase activity and shows the characteristic aa in the active site and interaction site typical of proteasome beta type-4 subunits (Supp. Fig. 3). Finally, we identified the SaUb with an ORF of 918 bp, encoding a 305 aa protein with a Mw of 34.32 kDa (Accession number: to be submitted). Four identical aa repeat units, termed R1 to R4, were present in the SaUb sequence each consisting of 216 bp coding for a 72 aa Ub monomer (Supp. Fig. 4). Each Ub monomer showed an interaction site with the Ub-conjugating enzyme (E2) and also an interaction site with the C-terminal hydrolase (UCH) as well as binding sites to the CUE domain of the Cue2 protein (Supp. Fig. 4). The protein sequence of SaUb is 100% identical to all vertebrate Ub protein sequences analysed (Table 3).



Supp. Fig. 3. Partial nucleotide and deduced amino acid sequences of gilthead sea bream proteasome subunit beta type-4 (SaN3, also known as PSMB4, accession number: to be submitted). The predicted leader sequence that is cleaved to release the mature peptide is underlined and the key threonine essential for autocatalytic and proteolytic function is in bold and grey shaded. The amino acids that form the active site are shaded in black, and the ones corresponding to the beta subunit interaction site are in bold and boxed.



Supp. Fig.4. Nucleotide and deduced amino acid sequences of gilthead sea bream ubiquitin (SaUb, accession number: to be submitted). In the nucleotide sequence, the translation start and stop codons are in bold. The four conserved protein domains, identified using the SMART program are showed on top with bold arrows. In the amino acid sequence, the binding sites to the catalytic domain of the E2 ligase are shaded in grey and in italics. The C-terminal hydrolase (UCH) interaction sites are in bold and boxed. The interaction sites to the CUE domain of the Cue2 protein are black shaded.

3.2. Phylogenetic analysis of SaCTSs

Phylogenetic analysis of cathepsins B, D and L from representative mammals, amphibians and fish, including the SaCTSB, SaCTSDa, SaCTSDb and SaCTSL, produced an MP-phylogenetic tree that contained two distinct branches. The cysteine proteases, cathepsins B and L clustered together separated from the aspartic proteases, the cathepsins D (Fig. 2). The SaCTSB, SaCTSL, SaCTSDa and SaCTSDb were clustered together with their homologous vertebrate cathepsins

(Fig. 2). The relationships revealed in the phylogenetic tree were in agreement with the concept of traditional taxonomy. In addition, the new cathepsin D paralogue cloned from gilthead sea bream fast muscle, the SaCTSDb, appeared phylogenetically related to its CTSD orthologues from other teleost and tetrapod species, whereas the other paralogue previously cloned in ovary, SaCTSDa, formed a clade with only CTSD sequences of teleost species (Fig. 2).

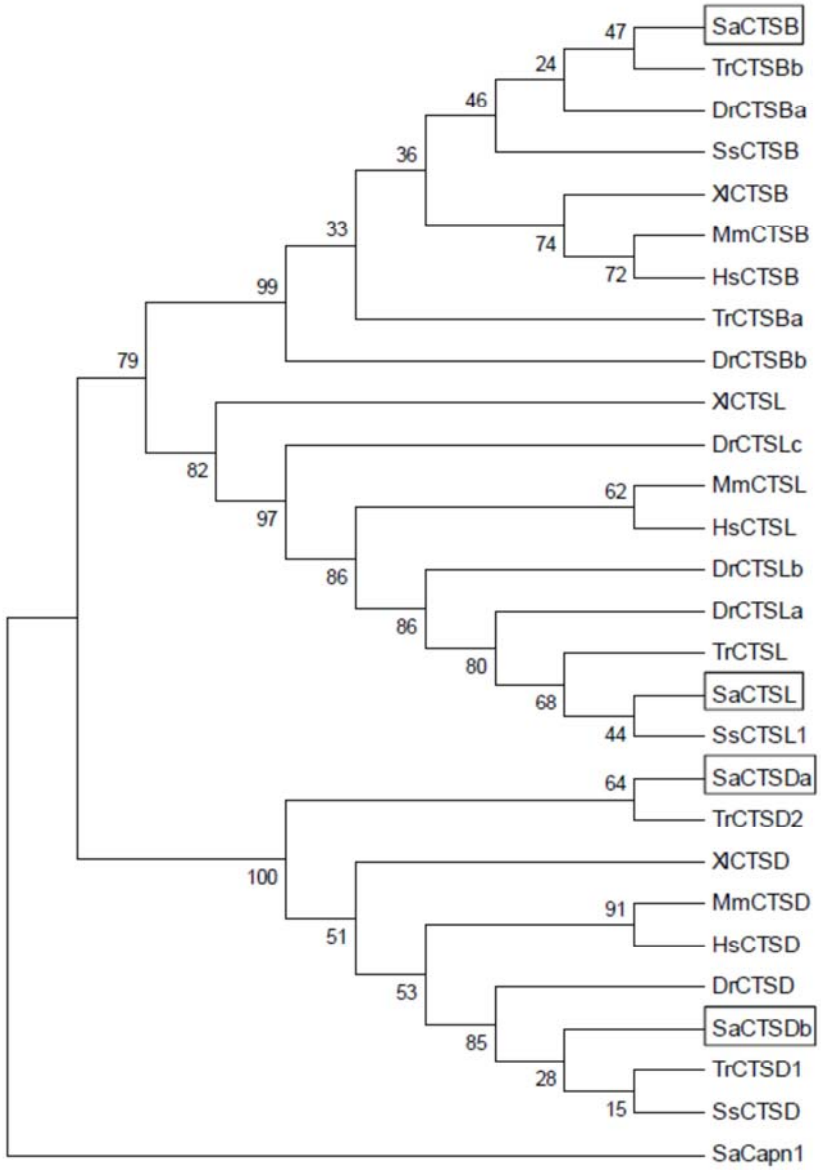


Figure 2. Phylogenetic analysis of gilthead sea bream cathepsins D, B and L. Unrooted Maximum Parsimony tree predicting the evolutionary relationship between *Sparus aurata* (Sa), *Danio rerio* (Dr), *Homo sapiens* (Hs), *Mus musculus* (Mm), *Salmo salar* (Ss), *Takifugu rubripes* (Tr) and *Xenopus laevis* (Xt) CTSB, CTSD and CTSL orthologues. The calpain 1 sequence of gilthead sea bream (SaCapn1) (accession number: KF444899) was used as outgroup. Bootstrap values, calculated from 1000 replicates, are indicated at the nodes. Gilthead sea bream cathepsins are boxed. The NCBI GenBank accession numbers for the analysed sequences are: *D. rerio* (DrCTSBa: NM_213336; DrCTSBb: NM_001110478; CTSD:

NM_131710; DrCTSLa: BC066490; DrCTSLb: NM_131198), *H. sapiens* (HsCTSB: L16510; HsCTSD: NM_001909; HsCTSL: NM_001912), *M. musculus* (MmCTSB: NM_007798; MmCTSD: NM_009983; MmCTSL: NM_009984), *S. salar* (SsCTSB; NM_001140522; SsCTSD: BT043515; SsCTSL1: NM_001146546), *S. aurata* (SaCTSB: to be submitted; SaCTSDa: AF036319; SaCTSDb: to be submitted and; SaCTSL: DQ875329), *T. rubripes* (TrCTSBa: XM_003971718; TrCTSBb: XM_003969499; TrCTSD1: AB179548; TrCTSD2: AB179549; TrCTSL: XM_003975074) and *X. laevis* (XlCTSB: NM_001086101; XlCTSD: AB103479; XlCTSL: NM_001092267).

3.3. Tissue Expression of SaCTSDa and SaCTSDb

Qualitative PCR analysis showed that under normal physiological conditions, both SaCTSDa and SaCTSDb mRNA levels were ubiquitously expressed in all tissues analysed, although SaCTSDa mRNA was detected in higher levels in gonad, while SaCTSDb mRNA was more abundant in fast and slow skeletal muscle (Fig. 3A).

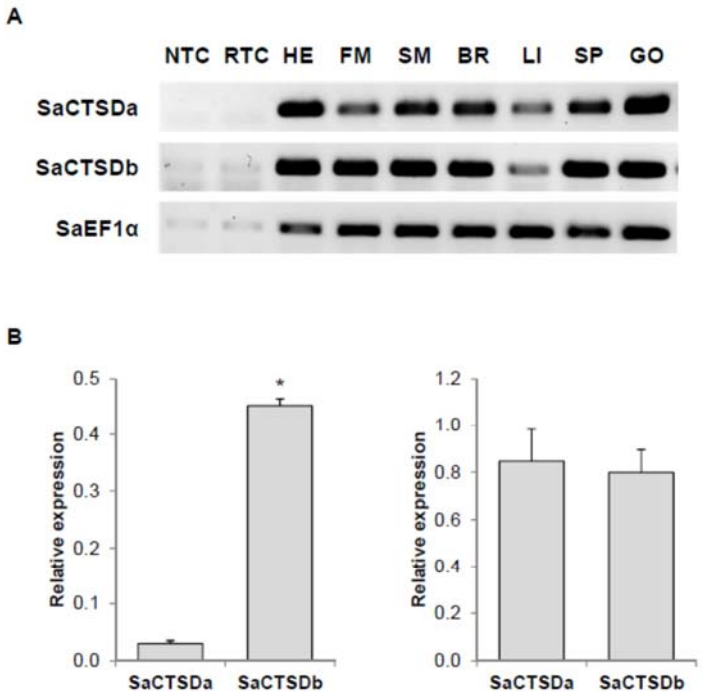


Figure 3. Tissue expression of gilthead sea bream cathepsin D paralogues. (A) Qualitative transcript expression profiles of SaCTSDa and SaCTSDb across adult gilthead sea bream tissue types: fast skeletal muscle (FM), slow skeletal muscle (SM), heart (HE), brain (BR), liver (LI) spleen (SP) and immature gonad (GO). SaEF1α was also amplified as a reference gene to confirm a steady-state level of expression among tissues. No template (NTC) and no reverse transcriptase (RTC) negative controls were also included to confirm primer specificity and the absence of genomic DNA. Images are representative from n=3 fish. (B) Quantitative relative expression normalized to β-actin of SaCTSDa and SaCTSDb from gilthead sea bream (Sa) fast skeletal muscle (left) and ovary (right). Results are shown as mean ± SEM (n=4-6). Different letters indicate significant differences at $p<0.05$.

Furthermore, quantitative analysis of the expression of both paralogues in gilthead sea bream muscle and gonad corroborated that SaCTSDb has significantly higher expression in muscle than SaCTSDa, whereas similar levels of expression were observed in the gonad (Fig. 3B).

3.4. Expression of SaCTSs, SaN3 and SaUb during life stages

To study the transcriptional regulation of the different proteolytic members present in the fast muscle of gilthead sea bream, we first analysed the differential expression of the SaCTSs and UbP members at three different life stages. qPCR analysis showed that the mRNA expression of SaCTSB, SaCTSL, and SaCTSDb decreased significantly as fish grow, being greater in the muscle of fingerlings than in juveniles or adult fish (Figs. 4A, 4B and 4D), whereas the expression of SaCTSDa remained unchanged (Fig. 4C). Regarding the UbP members, SaN3 expression also was down-regulated with time, being significantly higher in fingerlings than in adult fish (Fig. 4E). Nevertheless, SaUb expression was significantly reduced in juvenile fish compared to fingerlings, but contrary the other genes analysed, its expression increased again significantly in the muscle of adult respect to juvenile fish (Fig. 4F).

To better understand the changes that may be occurring in the muscle as the fish grow, the expression of several genes related to muscle growth were also analysed. The expression of the proliferation marker PCNA was also reduced with time, being significantly different between fingerlings and adult fish (Fig. 5A). Similarly, the expression of the negative regulator of muscle growth MSTN also showed a tendency to decrease during the life cycle of the fish (Fig. 5B). Furthermore, the expression of two important structural proteins, MHC and MLC2 revealed that MHC was significantly down-regulated in juveniles and adult fish in comparison with fingerlings (Fig. 5C), whereas MLC2 showed a tendency to increase as the fish grow (Fig. 5D).

3.5. Expression of SaCTSs, SaN3 and SaUb in response to re-feeding

Finally, the transcriptional regulation of CTSs and UbP members was studied in fish muscle at two time points (7 and 14 days) during re-feeding after 30 days of fasting. The mRNA levels of all genes analysed was significantly decreased in response to re-feeding. The decrease in SaCTSL (Fig. 6B), SaCTSDb (Fig. 6D), SaN3 (Fig. 6E) and SaUb (Fig. 6F) expression showed significant differences already after 7 days of re-feeding, while the expression of SaCTSB (Fig. 6A) and SaCTSDa (Fig. 6C) decreased significantly after re-feeding the fish for 14 days.

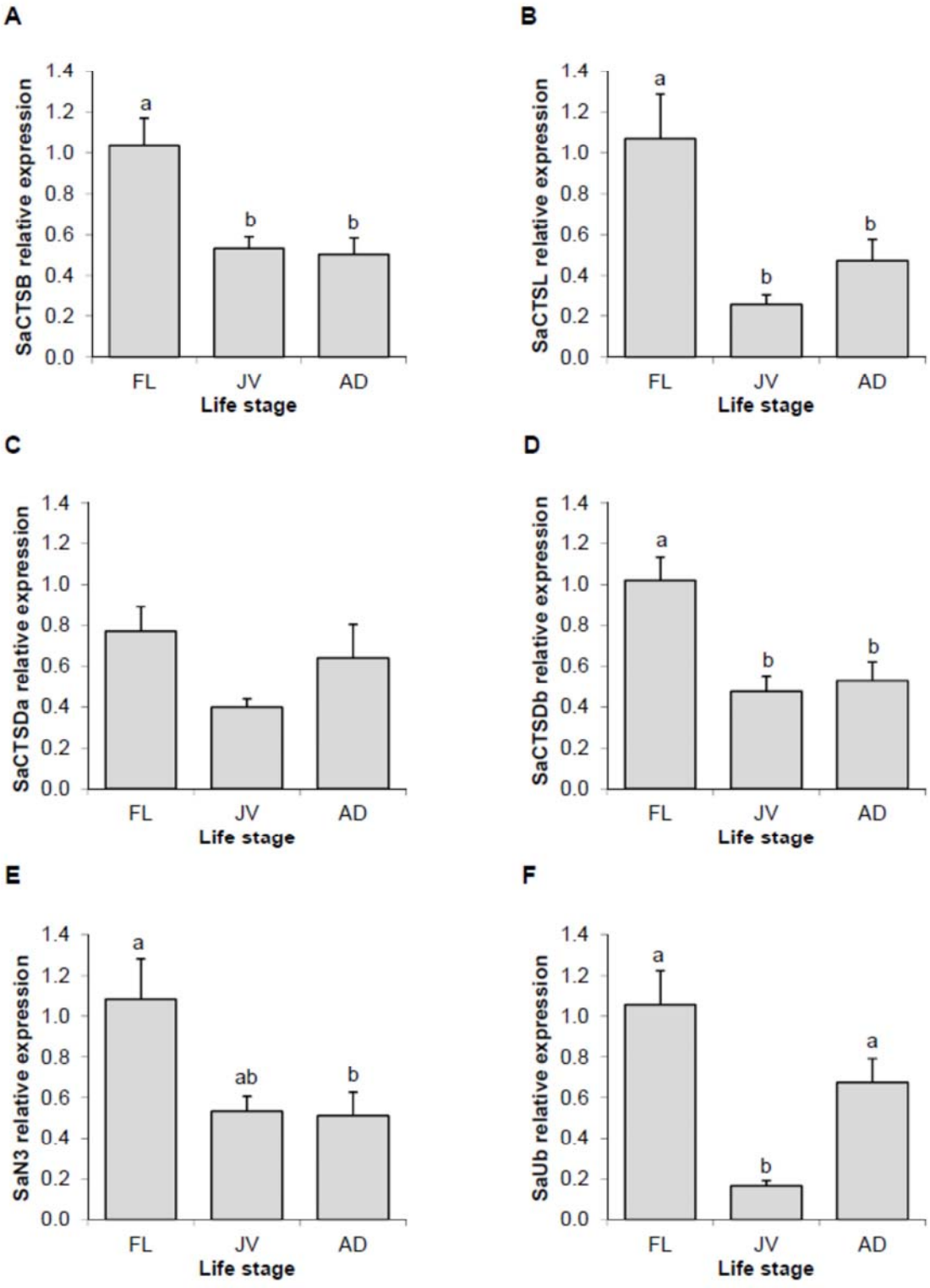


Figure 4. Proteolytic markers expression in gilthead sea bream muscle at different life stages. Quantitative relative expression normalized to β-actin of (A) SaCTSB, (B) SaCTSL, (C) SaCTSDa, (D) SaCTSDb, (E) SaN3 and (F) SaUb from gilthead sea bream (Sa) fast skeletal muscle from fish at three different life stages: fingerlings (FL), juveniles (JV) and adults (AD). Results are shown as mean ± SEM (n=4-5). Different letters indicate significant differences at $p<0.05$.

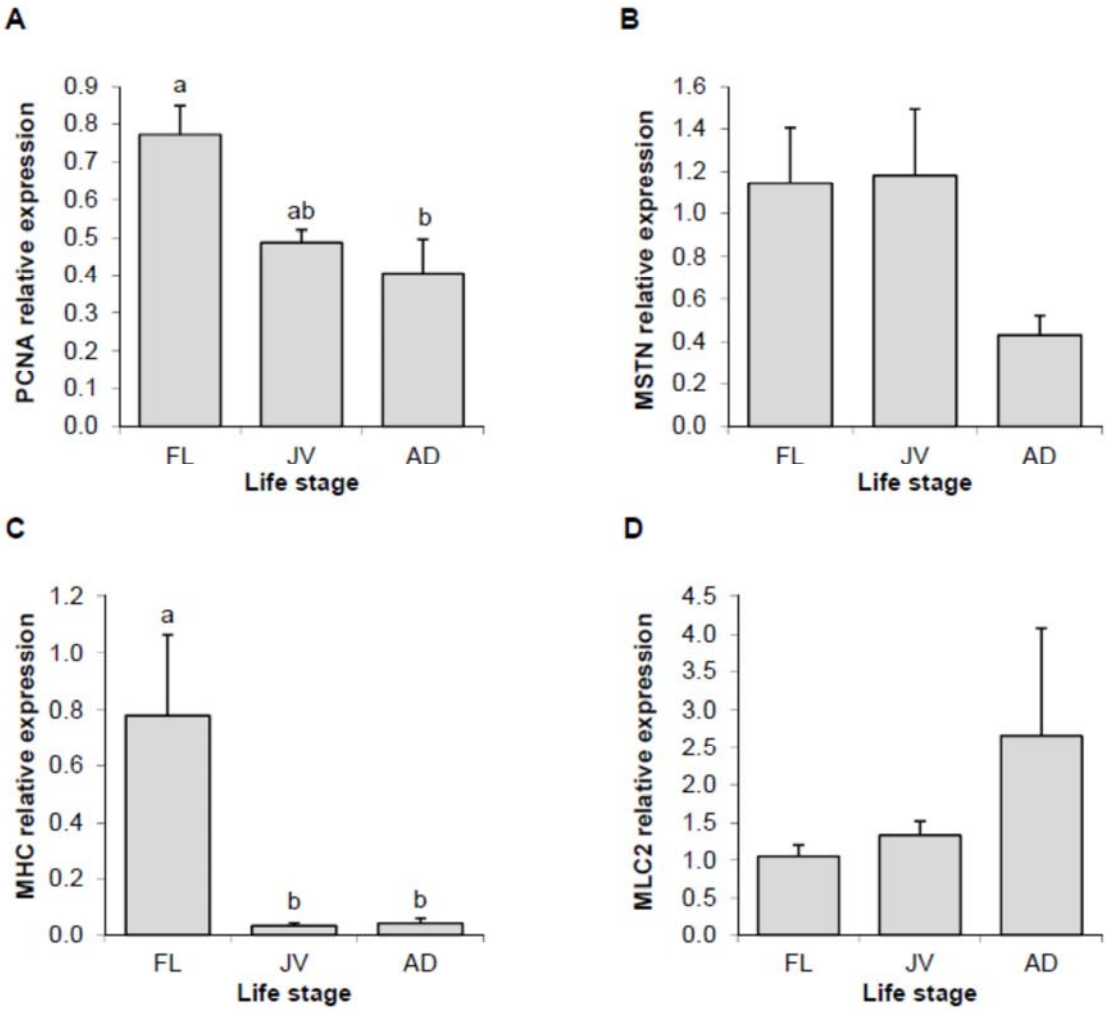


Figure 5. Growth markers expression in gilthead sea bream muscle at different life stages. Quantitative relative expression normalized to β-actin of (A) PCNA, (B) MSTN, (C) MHC and (D) MLC2 from gilthead sea bream (Sa) fast skeletal muscle from animals at three different life stages: fingerlings (FL), juveniles (JV) and adults (AD). Results are shown as mean ± SEM (n=4-5). Different letters indicate significant differences at $p<0.05$.

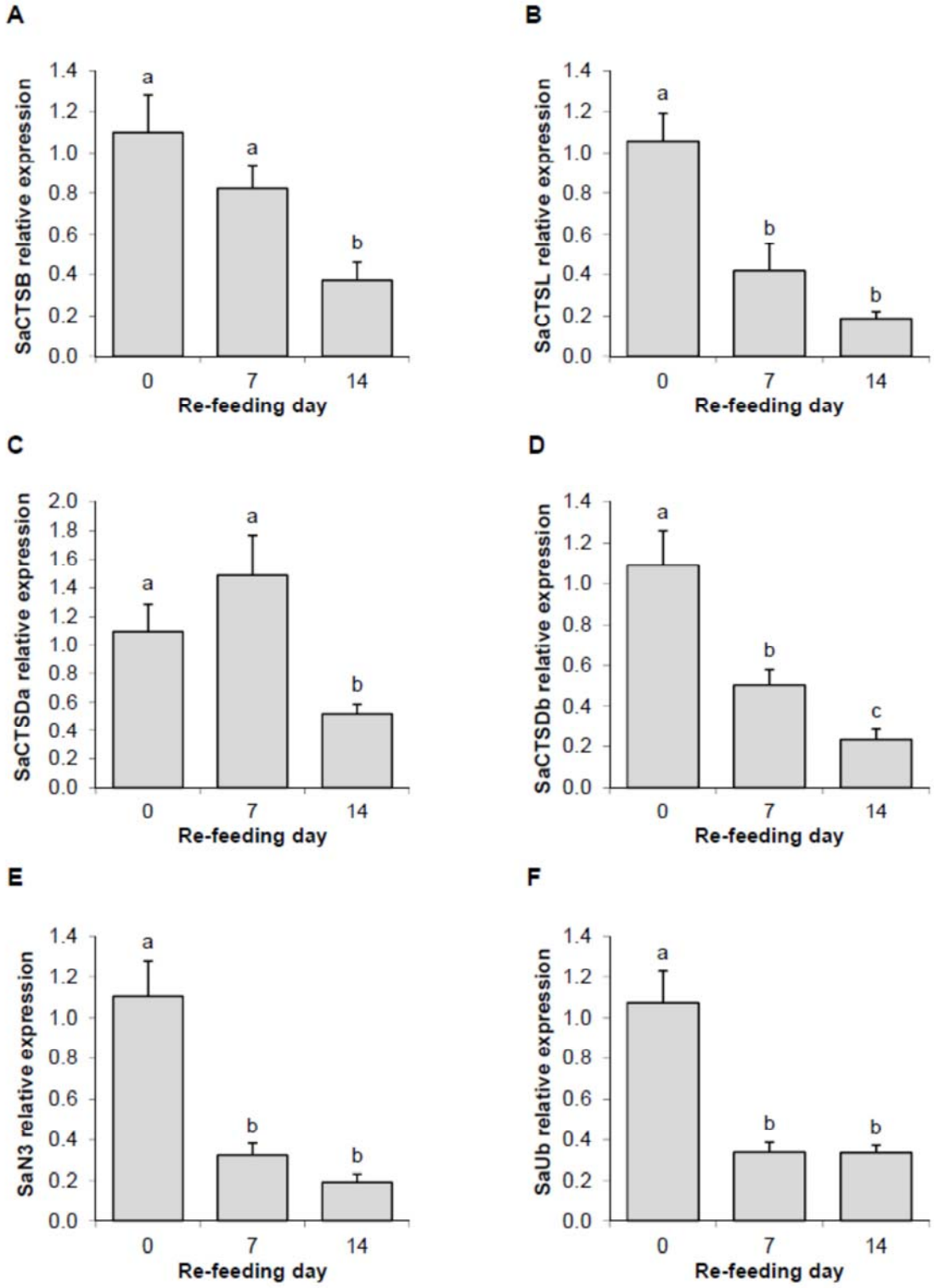


Figure 6. Proteolytic markers expression in gilthead sea bream muscle during re-feeding. Quantitative relative expression normalized to β-actin of (A) SaCTSB, (B) SaCTSL, (C) SaCTSDa, (D) SaCTSDb, (E) SaN3 and (F) SaUb from gilthead sea bream (Sa) fast skeletal muscle from fish at 0, 7 and 14 days of re-feeding after a period of 30 days fasting. Results are shown as mean ± SEM (n=5-7). Different letters indicate significant differences at $p<0.05$.

4. Discussion

In the present study, we have cloned the full-length cDNA sequences of cathepsin B (SaCTSB), a new paralogue of cathepsin D (SaCTSDb), ubiquitin (SaUb), and partially the proteasome subunit beta type-4 known as N3 or PSMB4 (SaN3), from gilthead sea bream fast skeletal muscle. The deduced amino acid sequences obtained shared high levels of overall identity with the correspondents CTSB, CTSD, Ub and N3/PSMB4 of teleosts and other vertebrate species. Like their orthologues in other vertebrates, SaCTSB and SaCTSDb, possess an N-terminal signal peptide, a propeptide region, and a cysteine or aspartyl catalytic protease domain, respectively, with complete conservation at the catalytic residues (Turk et al., 2012; Zaidi et al., 2008), indicating they are the structural homologues of cathepsins B and D in gilthead sea bream. The SaN3 presented a histidine residue replacing the mammalian arginine before the key threonine essential for proteolytic function (Nothwang et al., 1994) as previously reported in the N3 sequence of rainbow trout (Martin et al., 2002). The beta type subunits are synthesized as inactive precursors and activated after this autocatalytic internal cleavage (Heinemeyer et al., 2004), which proposed function, as in other proteasome 20S beta subunits, is to maintain the subunit in an inactive form until the proteasome is assembled (Heinemeyer et al., 2004). Analysis of the SaN3 sequence showed also the active site conserved, and the beta subunit interaction site as in other vertebrate N3 sequences (Unno et al., 2002). Finally, the SaUb cDNA identified has four 228 bp repeats encoding each a 72 aa Ub monomer, which has 100% identity with other vertebrate Ub sequences. The final repeat has an additional asparagine, contrary to the usual mammalian tyrosine (Hayashi et al., 1994), as has been shown to be the case also in Ub from rainbow trout (Martin et al., 2002).The structural features and the high levels of identity with the respective vertebrate sequences indicate that SaN3 and SaUb are the structural and functional homologues of N3 and Ub, respectively in gilthead sea bream.

The phylogenetic analysis of gilthead sea bream cathepsins B, Da, Db and L, suggests the existence of a cathepsin-like ancestral gene in all vertebrate superfamily members. The tree showed two main branches; one with the cysteine CTSB and CTSL proteases and the other one with the aspartyl CTSDs. The cysteine proteases arose early during evolution and the high percent identity among residues across taxa suggests that both cathepsin subfamilies evolved from gene duplication events (Berti and Storer, 1995). Interestingly, SaCTSDb seems to be the more common and less derived SaCTSD paralogue from the ancient teleostean genome duplication (Taylor et al., 2003) as predicted by Mommsen (2004). Moreover, this new paralogue SaCTSDb, clustered with other fish and mammalian cathepsins D, whereas SaCTSDa

clustered on a separate branch with only fish members, suggesting functional divergences between both paralogues.

SaCTSDb lacks the third putative N-glycosylation site present in SaCTSDa, as it occurs in other vertebrate sequences, which possess only one or two, as previously reported (Mommsen, 2004). A previous study in human cells with mono-glycosylated and non-glycosylated cathepsin D mutants has shown that glycosylation is not necessary for folding or enzyme activity but it is required for targeting the enzyme to lysosomes (Fortenberry et al., 1995). Both SaCTSD are ubiquitously expressed in all tissues analysed as previously reported in other fish, avian and mammalian species (Bourin et al., 2012; Mei et al., 2008; Riggio et al., 2000; Sakai et al., 1989). The SaCTSDa gene is predominantly expressed in gonad as previously observed for its orthologue in rainbow trout (Brooks et al., 1997), in agreement with its role during the cleavage of vitellogenin to yield yolk proteins for the fish (Brooks et al., 1997; Carnevali et al., 1999a; Hiramatsu et al., 2002; Kwon et al., 2001; Sire et al., 1994; Yamamura et al., 1995). On the other hand, the SaCTSDb gene is highly expressed in skeletal muscle tissue, suggesting that this isoform might have a function regulating muscle protein turnover, being thus more involved in fillet quality.

In the present study, most cathepsins and UbP members' expression decreased in gilthead sea bream muscle during lifetime. Dietary protein requirements decrease as the fish grow, e.g. the protein and energy content in diets are higher in smaller (<100 g) than in larger fish to fulfil the needs to grow (Lupatsch, 2005). Therefore, we propose that the decrease in the proteolytic systems parallels protein turnover, where younger fish (fingerlings) have higher proteolysis rates than older fish (juveniles and adults), as well as higher rates of protein synthesis, as previously shown for example in rainbow trout (Peragón et al., 2001; Peragón et al., 1998). Supporting this, the transcript abundance of the proliferation marker PCNA as well as that of the negative regulator of muscle growth, MSTN also decreased in adult fish. Moreover, one of the main structural proteins of muscle, MHC, showed an almost complete suppression of expression in juvenile and adult fish in comparison to fingerlings; thus, suggesting that synthesis of new basic structural proteins in those life stages is limited in this species. On the other hand, although SaCTSDb was reduced as the other SaCTSs, SaCTSDa remained unchanged at all times, showing that both paralogues are differentially regulated at the transcriptional level during the life of the fish, supporting the functional differences previously suggested. In line with these observations, SaCTSDb appears to be linked to muscle proteolysis in gilthead sea bream, whereas SaCTSDa seems to be of less importance. Furthermore, in the present study SaUb mRNA expression decreased between fingerlings and juveniles, but

increased again in adult fish. In mammals, a relationship exists between aging and loss of skeletal muscle mass, and members of the UbP pathway have been demonstrated to play a key role in this process, because of increased demand of degradation of aberrant proteins to limit the risk of entering a state of proteotoxicity (Altun et al., 2012). Furthermore, at advanced age, poor capacity to regenerate muscle due to impaired signalling, exhaustion of the satellite cell pool or changes in the extracellular matrix limiting the regeneration of myofibers can occur (Altun et al., 2012). In agreement with this observation, a previous study found that Ub protein expression was also up-regulated in rat and human fast muscle fibres during aging, and showed using C2C12 muscle cells that Ub suppresses proliferation, which may be associated with the poor healing potential in the older (Cai et al., 2004). Although gilthead sea bream presents indeterminate growth, these observations support in line with our results that SaUb is important not only for basal proteolysis during the whole life of the fish, but also during the last life stages where it may reduce the ability of muscle regeneration or be involved in some kind of muscle aging process also in fish.

Finally in our study, qPCR analyses revealed a significant decrease in the expression of all the studied SaCTSs and SaUbP genes with re-feeding. In a recent study under the same conditions, we also demonstrated the down-regulation of several catalytic and regulatory members of the calpain system (Salmerón et al., 2013), overall suggesting a generalised decrease in proteolytic systems' activity and thus, in muscle proteolysis in response to re-feeding in this species. Although in our studies, we have analysed only the variations at a transcriptional level, and changes in the protein content or the activity of the different proteases may also occur. In line with our results, Rescan and coworkers (2007) found in rainbow trout, an up-regulation of cathepsins B, D and S and genes from the UbP pathway during fasting, as well as a significantly reduced expression during re-feeding (Rescan et al., 2007). Similarly, the mRNA expression of the Ub-ligase atrogin1 and the level of poly-ubiquitinated proteins in the muscle of the same species was significantly increased with fasting and decreased after re-feeding, although major changes were not observed in the activity of the main proteasomal peptidases (trypsin and chymotrypsin-like) (Seiliez et al., 2008).

In conclusion, this is the first study that has examined the changes during life stages and the effect of the nutritional status (fasting/re-feeding), on fast muscle proteolytic markers expression (cathepsins and UbP members) in vivo in gilthead sea bream. Our results suggest that SaCTSs and SaUbP pathway genes might be implicated in muscle growth regulation throughout life and muscle protein mobilization during fasting and re-feeding in fish. This information will be important to better understand muscle protein turnover to improve

growth performance, enhancing protein synthesis and decreasing muscle degradation in cultured fish.

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BLOC II

Paper de leptina i grelina sobre el teixit adipós

Role of leptin and ghrelin on adipose tissue

CAPÍTOL III

Efectes de l'estat nutricional sobre els nivells de leptina en plasma i regulació de l'expressió i la secreció de leptina *in vitro* en adipòcits de truita irisada

Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion in rainbow trout

Efectes de l'estat nutricional sobre els nivells de leptina en plasma i regulació de l'expressió i la secreció de leptina *in vitro* en adipòcits de truita irisada

L'objectiu del present estudi va ser explorar l'efecte modulador de l'adipositat en la leptina plasmàtica, i el paper d'una selecció de factors en la regulació de l'expressió de l'ARNm i la secreció de leptina en els adipòcits de truita irisada. Els peixos van ser alimentats amb una dieta alta en energia a dos nivells de racionament, *ad libitum* (grup AL) o 25% de sacietat (grup RE) durant 8 setmanes. Els peixos RE presentaven significativament un menor creixement (-47%, p<0,01) i pes del teixit adipós (52%, p<0,01) en comparació amb els peixos AL, però els seus nivells de leptina en plasma van ser més alts (36%, p=0,022). A diferència dels mamífers, la leptina plasmàtica es va correlacionar negativament amb la massa de teixit adipós i l'índex de greix mesentèric. Els adipòcits aïllats de peixos alimentats amb la dieta alta en energia i amb una dieta regular van ser tractats amb insulina, grelina, leucina, àcid eicosapentaenoic, o es van deixar sense tractament (control). En els adipòcits dels peixos alimentats amb una dieta estàndard, la insulina i la grelina van augmentar la secreció de leptina de manera dosi dependent (p=0,002 i p=0,033, respectivament). D'altra banda, la secreció de leptina en els adipòcits control va ser significativament major en els peixos RE que en els AL (29%, p=0,022), recolzant el resultats observats *in vivo* de leptina en plasma. No es van observar efectes de cap tractament en els adipòcits dels grups de la dieta alta en energia (AL i RE), ni en l'expressió ni en la secreció de leptina, llevat que la secreció de leptina es va reduir significativament en resposta a la leucina en els peixos del grup RE (p=0,025). Aquestes dades mostren, per primera vegada en la truita irisada, que els nivells circulants de leptina poden transmetre informació sobre l'estat energètic del cos, i que la regulació de la secreció de leptina dels adipòcits és modulada per la història nutricional dels peixos i per la influència d'hormones metabòliques i nutrients.

Paraules clau: Teleostis, teixit adipós, grelina, insulina, leucina.

Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion in rainbow trout

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Running title: Rainbow trout adipocyte leptin regulation

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Abstract

The aim of the present study was to explore the modulatory effect of adiposity on plasma leptin, and the role of selected potential factors on the regulation of leptin mRNA expression and secretion in rainbow trout adipocytes. Fish were fed a high-energy diet at two ration levels, *ad libitum* (AL group) or 25% satiation (RE group) for 8 weeks. RE fish had significantly reduced growth (-47%; $p<0.01$) and adipose tissue weight (-52%; $p<0.01$) in comparison to AL fish, but higher plasma leptin levels (+36%; $p=0.022$). Contrary to mammals, plasma leptin was negatively correlated with adipose tissue mass and mesenteric fat index. Isolated adipocytes from fish fed the high-energy and a regular diet were treated with insulin, ghrelin, leucine, eicosapentaenoic acid or left untreated (Control). In adipocytes from fish fed a regular diet, insulin and ghrelin increased leptin secretion dose-dependently ($p=0.002$ and $p=0.033$, respectively). Furthermore, leptin secretion in control adipocytes was significantly higher in RE than in AL fish (+29%; $p=0.022$), supporting the *in vivo* findings on plasma leptin. No treatment effects were observed in adipocytes from the high-energy diet groups (AL and RE), neither in leptin expression nor secretion, except that leptin secretion was significantly reduced by leucine in RE fish ($p=0.025$). These data show for the first time in rainbow trout that circulating leptin levels may convey information on the energy status of the body, and that regulation of leptin secretion from adipocytes is modulated by the nutritional history of the fish and influenced by metabolic hormones and nutrients.

Keywords: Teleost, adipose tissue, ghrelin, insulin, leucine.

1. Introduction

Leptin, a 16-kDa hormone coded by the *ob* gene (*obese*), was discovered in mouse in 1994 (72), and a year later its receptor was identified as the product of the *db* gene (*diabetes*) (65). Leptin is a member of the cytokine superfamily implicated in mammals in energy homeostasis, obesity, reproduction, bone formation, wound healing and immunity among other biological functions (57). Also in mammals, leptin is produced and secreted primarily by white adipose tissue (72), and then only by mature adipocytes (49). Leptin acts on hypothalamic orexigenic and anorexigenic neurons via transmembrane receptors to suppress food intake and increase energy expenditure (15). A strong, positive correlation between plasma leptin levels, body mass index and adipose tissue mass exist in rodents and humans (17, 47). When fat mass decreases, circulating leptin decreases leading to stimulated appetite and suppressed energy expenditure, while an opposite trend is observed when fat mass increases (20). This hormone is therefore considered to act as a lipostatic signal, communicating the body's lipid stores to the brain (27). It should be noted, however, that mammalian data also indicate rapid regulatory mechanisms of leptin linked to short-term nutritional status, rather than long-term changes in adiposity (14, 29).

The amount of leptin produced in mammalian adipocytes appears to be regulated at transcriptional and translational levels, but also through storage, turnover, and secretion (41). To date, several nutritional and hormonal signals have been well-described to modulate leptin release in isolated adipocytes. Among these, food consumption (45), leucine (45, 62), eicosapentaenoic fatty acid (EPA) (58), glucose (42), insulin (50, 61) and ghrelin (24) appear to stimulate leptin secretion; while fasting (64), peroxisome proliferator-activated receptor-γ (PPARγ) agonists (48), free fatty acids (FFA) (48), growth hormone (GH) (48) and exercise (10, 74) have been reported as leptin inhibitors.

The leptin gene in fish was first discovered in pufferfish (40), and since, leptin A and B isoforms resulting from an ancient fish whole genome duplication event (WGD; 3R) (66, 70) have been identified in zebrafish (25), Japanese medaka (39) and orange-spotted grouper (71). Salmonids possess duplicates for both the LepA and LepB genes (7, 63), most probably generated by the lineage specific WGD (4R) resulting in the tetraploidization of salmonids about 25-100 Mya (6, 54). Leptin teleost primary structure is only 13 to 25% identical to human leptin. However, predicted tertiary structures of fish leptins are very similar to that of their mammalian counterparts (25, 28, 40, 43, 51, 63), thus supporting functional homology.

In fish, the role of leptin as a satiety or lipostatic signal is not as clear as in mammals. In rainbow trout, short-term injections with homologous leptin decreased food intake (51). Similarly, in grass carp, a short-term treatment decreased feeding, whereas long-term leptin treatment did not affect food intake or body weight (43). Besides, plasma leptin levels are influenced by energy status in rainbow trout (37) and Atlantic salmon (36), where long-term fasting or restricted feeding increased plasma leptin levels. These observations have been confirmed in other studies and species (21, 23, 31, 63, 68), supporting the hypothesis of a different mode of action for this hormone between fish and mammals.

At mRNA level, LepA1 in rainbow trout is mainly expressed in liver, and to a lesser extent in eye, heart, muscle and ventral skin (51). Hepatic LepA1 expression is modified by the nutritional status in several fish species (22, 28, 67). Nevertheless, little is known about leptin expression and secretion by adipose tissue and its regulation in fish. A specific signal for LepA1 mRNA was detected in visceral adipose tissue of rainbow trout, albeit not in all individuals tested (59). Also, the peptide has been detected in primary cultured mature adipocytes of Atlantic salmon (69). However, there are very few reports indicating a possible relationship between leptin and adiposity in fish. Only one study has shown a positive correlation between plasma leptin levels and lipid content of adipose tissue in juvenile GH-treated rainbow trout (36). In Arctic charr, no correlation was found between liver leptin mRNA expression and the amount of whole body or liver lipid stores (21, 22) and similar results have been obtained in other fish species such as common carp (28), zebrafish (25) and goldfish (67).

A lipostatic model for food intake regulation has been proposed also in fish, suggesting that the central nervous system (CNS) senses the amount of lipid stores and modulate/adjust feeding behavior accordingly (30). In order to evaluate leptin as a potential endocrine signal of adiposity in fish, rainbow trout at two different states of adiposity were established. To further shed light on the hormonal and/or nutritional regulation of adipose tissue transcription and secretion of leptin, isolated adipocytes from rainbow trout under different feeding regimes were used as an *in vitro* model (5, 19).

2. Materials and methods

2.1 Animals and in vivo experimental trial

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the European Union, Spanish and Catalan Governments established legislation (reference numbers CEEA 237/12 and DAAM 6755).

2.1.1 Regular diet fish

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) for the regular diet experiment were obtained from Truites del Segre (Oliana, Lleida, Spain) and were held at the facilities of the University of Barcelona (Barcelona, Spain) until sampling. A total of 48 adult fish were equally distributed in 6 fiberglass tanks of 400L each and, maintained in a recirculation system at 15±1ºC with 12 h light: 12 h dark photoperiod. The fish were acclimated for 2 weeks and fed twice daily *ad libitum* with a commercial diet (38% protein, 24% fat and 19.8 MJ/Kg digestible energy; Trout evolution, Dibaq Diproteg S.A., Segovia, Spain) before performing the adipocyte isolations for the *in vitro* experiments.

2.1.2 High-energy diet experiment: *ad libitum* and feed restricted fish

Rainbow trout from the fish farm Viveros de los Pirineos S.A. (El Grado, Huesca, Spain) were held at the facilities of the University of Barcelona (Barcelona, Spain) where the experimental trial was carried out. A total of 98 adult fish were equally distributed in 6 fiberglass tanks of 400L each (16-17 trout/tank), maintained in a recirculation system kept at 15±1ºC with 12:12 L:D photoperiod. The fish were allowed to acclimate for 2 weeks and during this period they were fed to satiation with a commercial feed once a day (Trout evolution, Dibaq Diproteg S.A., Segovia, Spain). After this period, fish were fasted 24h whereupon a total of 8 animals were rapidly netted at time 0 (t=0). Fish were anesthetized with MS-222 (0.1g/L) and once opercular frequency decreased and the fish began to float, they were sampled. Blood was obtained by a heparinized syringe (sodium heparin 5000 IU/mL) drawn from the caudal vein. The blood obtained was quickly centrifuged (5000rpm, 10min), and plasma stored at -80ºC until analysis. Then, animals were quickly sacrificed by a blow to the head and body weight and length were recorded. Fish were then externally sterilized with 70% ethanol and tissue samples were collected. Whole liver and visceral adipose tissue without that surrounding the pyloric caeca were collected and weighed to calculate hepatosomatic index (HSI) and mesenteric fat index (MFI), respectively. The remaining 90 fish were also anesthetized with MS-222 (0.1g/L), weighed, measured, tagged intramuscularly below the dorsal fin with passive integrated transponder tags (PIT-tags, ID100-B T.CRISTAL, Trovan, Madrid, Spain) after disinfecting the area with diluted povidone iodine (Betadine), and returned to their corresponding tanks for recovery.

At the start of the experiments, the fish were divided into two equally-sized groups (three tanks each), which were fed different rations of the same feed; one group was fed to satiation (AL group) while the ration of the other group was restricted (RE group) to 25% of the feed

given to the AL group. Fish were fed once daily in the morning with a commercial feed with high energy content (40% protein, 30% fat and 22.1 MJ/Kg digestible energy; Aquatex 30 HMD, Dibaq Diproteg S.A., Segovia, Spain). During the first experimental week, the feed eaten by the AL group was weighed daily, and the corresponding 25% given to the RE group. The daily average ration obtained during week 1 was used to feed the RE fish during weeks 2 and 3. On week 4 (t=4), in order to monitor the proper operation of the experiment, all fish were fasted 24h, sedated with MS-222 (0.03-0.04g/L), weighed and measured, individually identified with a chip reader, and finally recovered in their tanks. Again, during week 4, the same procedure used during the first week was repeated to recalculate the RE ration for weeks 5 and 6. During the last 2 weeks (weeks 7 and 8), the feed ration was recalculated daily based on the animals remaining in each tank.

Samplings were carried out on 7 different days over weeks 7 and 8 (t=8) in order to minimize the stress associated with handling, and to carry out the *in vitro* isolation of adipocytes. On each sampling day, 24h-fasted fish from the AL and RE groups (n=4-9) from a single tank per treatment were sampled as described above for t=0. Besides being taken to determine the MFI and to perform gene expression analysis, the remaining adipose tissue was used for the isolation of adipocytes.

2.2 Adipocyte isolation and in vitro studies

2.2.1 Regular diet experiment

Adipocytes were isolated following the method described by Albalat et al., (5) with minor modifications. For each isolation, a pool of adipose tissue of ~42g was obtained from 7-10 fish (average weight 234.6±5.5g), which had been fasted for 24 h. The adipose tissue was kept in Krebs-Hepes buffer pre-gassed with CO_2 (pH 7.3-7.4), and then minced into small pieces and incubated for 60min in Krebs-Hepes buffer containing collagenase type II (130U/mL) plus 1% bovine serum albumin (BSA) at 18ºC in a shaking water bath (Unitronic OR, JP Selecta, Abrera, Spain). The cell suspension was then filtered through a 100µm cell strainer and washed by flotation 1-2 times with Krebs-Hepes buffer with 1% BSA and then 1-2 times with Krebs-Hepes buffer supplemented with 2% BSA depending on the blood remaining. Finally, cells were carefully resuspended at the desired concentration in Krebs-Hepes buffer containing 2% BSA after counting using a Fuchs-Rosenthal chamber.

Approximately 2 million isolated adipocytes (~0.5mL of cell cake and ~0.5mL of Krebs-Hepes buffer with 2% BSA) were incubated in triplicate during 3h in polypropylene tubes in the

shaking water bath at 18ºC, in the absence (Control, C) or presence of different concentrations of insulin at 10nM (I10) or 100nM (I100); or ghrelin at 0.1nM (G0.1), 1nM (G1) or 10nM (G10). Porcine insulin was supplied by Sigma-Aldrich (Tres Cantos, Spain), while synthetic 20 amino-acid octanoylated rainbow trout ghrelin (MW: 2206Da) was obtained from the Peptide Institute Inc., Osaka. Japan. Porcine insulin was used as piscine insulin was not available and also because its effects in fish adipose cells have been demonstrated previously (5, 8, 9). After incubation, cells and media were quickly transferred to a sterile 1.5mL tube, and following a short centrifugation (2700g for 2min at 4ºC) cell-free aliquots of each triplicate were immediately transferred to a new sterile 1.5mL tube and kept at -80ºC until analysis of leptin secretion. After removing all the media, the remaining cells from each triplicate were recovered together with 1mL of TRI Reagent solution (Ambion, Alcobendas, Spain) and transferred to a single RNase/DNase free 1.5mL tube and kept at -80ºC until leptin gene expression analysis. Results are the average of n=6 independent adipocyte isolations.

2.2.2 High-energy diet experiment

Adipocytes were isolated on each sampling day as described above in section 2.2.1. For each experiment a pool of adipose tissue of 20-30g from 4-9 fish from each group (AL and RE) was obtained. Approximately 2 million isolated adipocytes (~0.5mL of cell cake and ~0.5mL of Krebs-Hepes buffer 2% BSA) were incubated in triplicate during 3h in polypropylene tubes in the shaking bath at 18ºC, in absence (Control, Ctrl) or presence of different treatments: insulin 100nM (Ins), ghrelin 10nM (Ghrel), leucine 5mM (Leu) and EPA 100µM (EPA). The dose used for insulin and ghrelin was the dose found to be most effective in the regular diet experiment, and for leucine and EPA, the concentrations used were based on previous studies (11, 52, 58, 62). Porcine insulin, leucine and EPA were supplied by Sigma-Aldrich (Tres Cantos, Spain), while ghrelin was obtained from the Peptide Institute Inc., Osaka. Japan. After incubation, cell and media were collected for leptin secretion and leptin mRNA expression analysis as explained above in section 2.2.1. Results are the average of n=6-7 independent adipocyte isolations, with each treatment performed 2-4 times.

2.3 Plasma glucose, triglycerides (TG) and FFA

Plasma glucose, TG and FFA were analyzed with commercially available kits according to the manufacturer's instructions. Plasma glucose concentration was determined by a glucose oxidase colorimetric method (Spinreact, Sant Esteve d'en Bas, Spain). TG and FFA concentrations were measured enzymatically with the Serum Triglyceride Determination Kit

(Sigma-Aldrich, Tres Cantos, Spain) and the Non-Esterified Fatty Acid kit (NEFA-HR2, Wako Chemicals GmbH, Neuss, Germany), respectively.

2.4 Leptin radioimmunoassay (RIA)

Leptin was measured in plasma and media using the homologous RIA protocol established to perform plasma analyses by Kling et al., (37). The salmonid RIA was developed using a sequence of 14 amino acids identical between *Salmo* and *Oncorhynchus* species as antigen, and a polyclonal antibody was raised in rabbit against that same peptide. The RIA was validated for leptin detection in adipocyte media samples determining parallelism between leptin standard curves and media dilutions. Plasma samples were used undiluted and media samples from isolated adipocytes were diluted 5.7 times.

2.5 Quantitative real-time PCR (qPCR)

Total RNA from isolated adipocytes was extracted with TRI Reagent solution (Ambion, Alcobendas, Spain) according to the manufacturer's protocol, and cDNA reversely transcribed using 2µg of total RNA in conjunction with oligo(dT)12-18 primer and Superscript III (Invitrogen, Carlsbad, CA, USA) following standard procedures. Relative expression of leptin was quantified on the CFX-96 Real-Time PCR detection system platform (Bio-Rad, Hercules, CA, USA) using SYBR Green (QuantiTec SYBR Green PCR kit, Qiagen, Hilden, Germany). Each qPCR reaction comprised 12.5µl 2xSYBR Green PCR Master Mix, 300nM of forward and reverse primers, 100ng cDNA template and nuclease-free water up to a final volume of 25µl. To visualize potential genomic amplification in qPCR samples, the primer assay was designed in regions spanning exon-exon boundaries (LepA1 F: TTGCTCAAACCATGGTGATTAGGA and LepA1 R: GTCCATGCCCTCGATCAGGTTA; LepA2 F: TGGAAACCAAAAAGCTCCCTTCCTCTT and LepA2 R: GCCTTCTATAGGCTGGTCTCCTGCA; elongation factor (Ef1α) F: ATTAACATTGTGGTCATTGGCCATGTC and Ef1α R: ATCTCAGCTGCTTCCTTCTCGAACTTTT). The qPCR conditions were as follows: 3min at 95ºC, amplification for 45 cycles at 95ºC for 15s and 60ºC for 1min. A serial dilution in nuclease-free water of cDNA derived from a RNA pool of experimental samples was amplified to construct standard curves (2-fold dilutions) for both target and reference genes. Standard curves were included in each run to determine amplification efficiency (E) calculated as the slope from the plot of log RNA concentration versus threshold cycle (Ct) values using the following formula: E=10(−1/slope). However, only LepA1, but not LepA2 was sufficiently expressed in adipocytes to be analyzed; therefore, we only measured LepA1. Fold change in target gene expression was determined using the "2-ddCT" method (44), using Ef1α as endogenous reference gene (55). Melting curves were

recorded to evaluate specificity of amplification and lack of primer-dimers. Product specificity was also confirmed by agarose gel analysis.

2.6 Calculations and statistical analysis

Specific growth rate for body weight (SGR_W) and length (SGR_L) were determined according to the formulas $(\ln(BW_f\text{-}BW_i)/D)100$ and $(\ln(BL_f\text{-}BL_i)/D)100$, where BW_f and BL_f are the final body weight and length, respectively, BW_i and BL_i represent the initial body weight and length respectively, while D is the number of days between measurements. Three different sampling periods were studied for SGR_W and SGR_L: P0-4, P4-8 and P0-8, where the number indicates the sampling week. Condition factor (CF) was calculated as $(BW_f x BL_f^{-3})100$. HSI and MFI were calculated as $(X x BW_f^{-1})100$, where X represents liver or adipose weight, respectively.

Differences in body weight and SGR_W were analyzed with a two-way ANOVA using group (AL and RE) and sampling time (t=0, t=4 and t=8) or period (P0-4, P4-8 and P0-8) respectively, as fixed factors. This was followed by a one-way ANOVA and Tukey'spost hoc test for each group through sampling time or period, and finally a Student's t-test between groups to analyze the effect of sampling time or period. Body length, SGR_L and CF did not adhere to the ANOVA assumption for boral distribution. Therefore these variables were analyzed using non-parametric tests, as Friedman and Wilcoxon test within each group through sampling time or period, and a Mann-Whitney U test between groups at each sampling time or period. Adipose tissue and liver weight, MFI and HSI, and plasma parameters between t=0 or t=8 and between AL and RE groups at t=8 were analyzed by Student's t-test. Linear correlation analyses were carried out at the individual level for body, liver and adipose tissue weight, HSI and MFI versus plasma leptin using a Pearson Correlation (P). Differences in leptin secretion into the media and LepA1 relative expression by treatments from isolated adipocytes within each group were analyzed by one-way ANOVA followed by a Tukey'spost hoc test, and a Student's t-test between groups for each treatment. In all cases, normality and homoscedasticity were confirmed by Shapiro-Wilk and Levene's tests, respectively. Numerical values are expressed as mean ± standard error of the mean (s.e.m.), and differences were accepted as statistically different at the level of $p<0.05$. Statistical analyses were performed using the SPSS Statistics 20 software (IBM, Chicago, USA).

3. Results

3.1 Biometrics

There was a significant main effect of time/period and group, and a significant interaction between these factors on body weight, length, CF and SGR_W ($p<0.01$), where the values observed were always higher in AL compared to RE fish (Table 1). Neither main effect of period, nor interaction between period and group for SGR_L were observed ($p=0.177$ and $p=0.986$, respectively). However, there was a significant main effect of group with higher values for AL fish compared to RE fish ($p<0.01$, Table 1). SGR_W, but not SGR_L, showed that AL and RE fish grew significantly faster during the first experimental period (P0-4) than during the second (P4-8) (Table 1). Furthermore, SGR_W and SGR_L were significantly higher in AL fish compared to RE fish for all periods studied (P0-4, P4-8 and P0-8) (Table 1).

Table 1. Biometric parameters of *ad libitum* and feed restricted rainbow trout.

Sampling time (week)	t=0		t=4		t=8	
Group	**AL**	**RE**	**AL**	**RE**	**AL**	**RE**
Body weight (g)	205.84 ± 5.42^{A}	204.60 ± 6.06^{a}	362.83 ± 7.45^{B}	235.13 ± 5.81^{b}*	480.39 ± 10.95^{C}	256.10 ± 6.79^{c}*
Body length (cm)	26.90 ± 0.21^{A}	26.83 ± 0.27^{a}	30.00 ± 0.25^{B}	28.00 ± 0.27^{b}*	32.49 ± 0.27^{C}	28.68 ± 0.25^{c}*
CF	1.05 ± 0.02^{A}	1.05±0.02	1.34 ± 0.02^{B}	1.07±0.02*	1.40 ± 0.02^{B}	1.08±0.02*
Sampling period (weeks)	P 0-4		P 4-8		P 0-8	
Group	**AL**	**RE**	**AL**	**RE**	**AL**	**RE**
SGR_W	1.97 ± 0.06^{A}	0.49 ± 0.05^{a}*	1.15 ± 0.12^{B}	0.36 ± 0.17^{b}*	1.60±0.06	0.44±0.07*
SGR_L	0.38±0.01	0.15±0.01*	0.33±0.05	0.10±0.04*	0.36±0.02	0.13±0.02*

Body weight, length and condition factor (CF) at different sampling times (week t=0, t=4 and t=8), and specific growth rate weight (SGR_W) and length (SGR_L) during three different sampling periods (P0-4, P4-8 and P0-8 in weeks). Data are shown as mean ± s.e.m. (n=42-44). Different capital or lower case letters indicate significant differences between sampling times within the AL or RE group, respectively, and asterisks indicate significant differences between groups for comparisons within sampling times or sampling periods ($p<0.05$).

After 8 weeks of feeding the high-energy diet, AL fish had significantly higher adipose tissue and liver weights, MFI and HSI, compared with the RE group at t=8, as well as in comparison to t=0 except for the MFI ($p<0.01$, Fig. 1A, 1B, 1C and 1D). Furthermore, RE fish had a significantly larger liver, and higher HSI values at t=8 than at t=0 ($p<0.01$, Fig. 1B and 1D), although their adipose tissue weight did not change with time ($p=0.200$, Fig. 1A) and the MFI was significantly reduced ($p<0.01$, Fig. 1C).

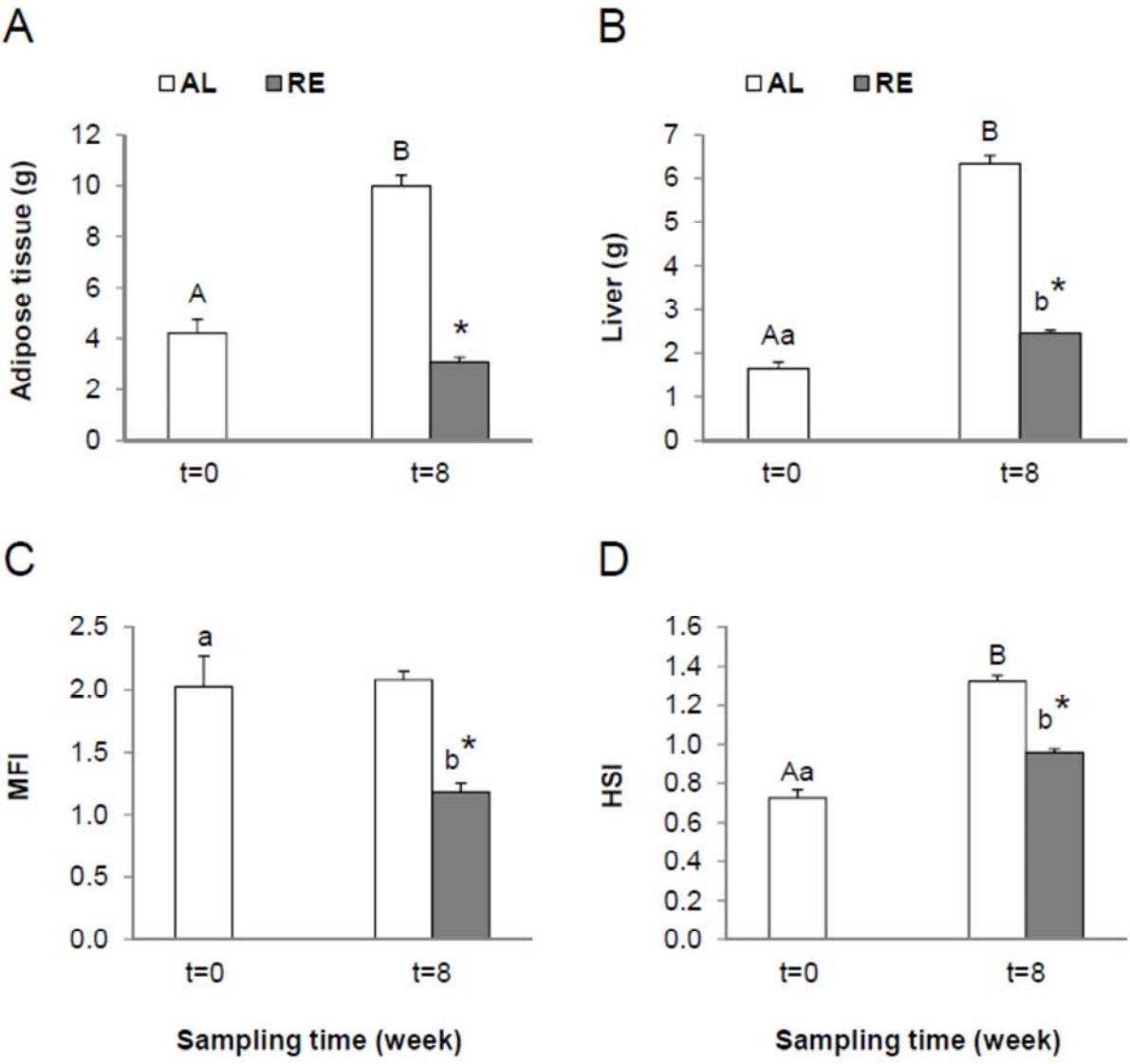

Figure 1. Biometric parameters of *ad libitum* and feed restricted rainbow trout. **(A)** Adipose tissue and **(B)** liver weight, **(C)** mesenteric fat index (MFI) and **(D)** hepatosomatic index (HSI) at two different sampling times (week t=0 and t=8). Data are shown as mean ± s.e.m. (n=7-8 for t=0 and n=41-44 for t=8). Different capital or lower case letters indicate significant differences between sampling times within the AL or RE group, respectively, and asterisks indicate significant differences between groups at t=8 ($p<0.05$).

3.2 Plasma leptin, glucose, TG and FFA

Plasma leptin concentration did not change with time in AL fish, while an increase was observed with time in RE fish, thus showing significant differences between the two groups at t=8 (p=0.022, Fig. 2).

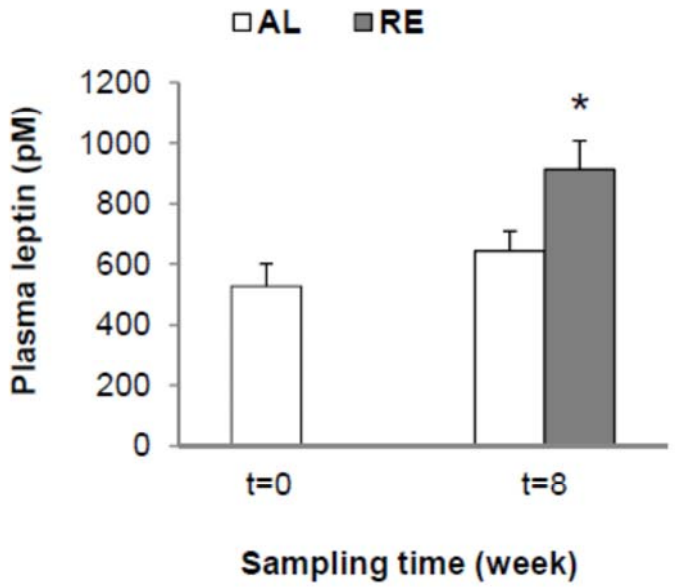

Figure 2. Plasma leptin levels in *ad libitum* and feed restricted rainbow trout. Data at different sampling times (week t=0 and t=8) are shown as mean ± s.e.m. (n=7-8 for t=0 and n=42-43 for t=8). Different capital or lower case letters indicate significant differences between sampling times within the AL or RE group, respectively and asterisks indicate significant differences between groups at t=8 ($p<0.05$).

After 8 weeks of feeding the high-energy diet, plasma glucose levels remained unaffected by time and nutritional treatment (Table 2). Plasma TG concentrations significantly increased with the high-energy diet for both groups at t=8 in comparison to t=0; however, no-significant differences were found between the AL and RE groups (Table 2). Furthermore, plasma FFA levels significantly increased during the experimental period in AL fish, and were significantly higher at t=8 when compared to RE fish (Table 2).

Table 2. Plasma parameters of *ad libitum* and feed restricted rainbow trout.

Sampling time (week)	t=0	t=8	
Group		**AL**	**RE**
Glucose (mM)	5.44±0.94	4.74±0.11	4.98±0.15
TG (mM)	1.66 ± 0.07^{Aa}	4.15 ± 0.49^{B}	2.55 ± 0.14^{b}
FFA (mg/dL)	9.83 ± 0.48^{A}	16.05 ± 0.92^{B}	11.88±0.48*

Glucose, triglycerides (TG) and free fatty acids (FFA) at two different sampling times (week t=0 and t=8). Data are shown as mean ± s.e.m. (n=7-8 for t=0 and n=41-44 for t=8). Different capital or lower case letters indicate significant differences between sampling times within the AL or RE group, respectively, and asterisks indicate significant differences between groups at t=8 ($p<0.05$).

3.3 Plasma leptin correlations

To further evaluate the possible role of leptin as an adiposity signal, correlation analyses were carried out between plasma leptin concentration and factors related to energy and nutritional status (weight of adipose tissue, liver and body, as well as MFI and HSI). There was a significant negative correlation between plasma leptin concentrations and adipose tissue (P=-0.258, p=0.017, Fig. 3A), liver weight (P=-0.217, p=0.047, Fig. 3B) and MFI (P=-0.282, p=0.009, Fig. 3C). On the other hand, plasma leptin levels correlated negatively, but not significantly, with HSI (P=-0.153, p=0.165, Fig. 3D) and body weight (P=-0.197, p=0.070, figure not shown).

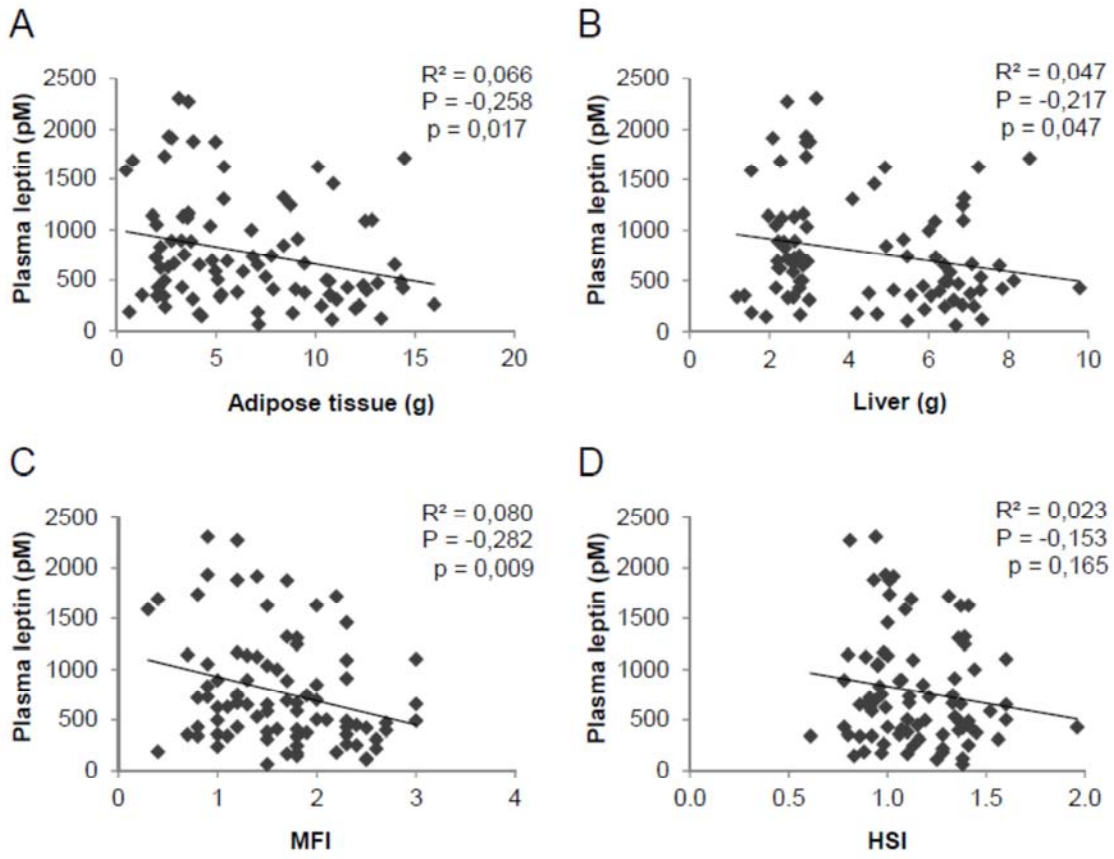

Figure 3. Correlations between plasma leptin and biometric parameters of *ad libitum* and feed restricted rainbow trout. Plasma leptin and **(A)** adipose tissue and **(B)** liver weight, **(C)** mesenteric fat index (MFI) and **(D)** hepatosomatic index (HSI) at week t=8. R^2 of the linear regression and correlation coefficient (P) and p value (p) from the Pearson correlation are indicated.

3.4 Leptin secretion and expression in isolated adipocytes

3.4.1 Regular diet experiment

Leptin secretion of isolated adipocytes from fish fed a regular diet increased in a dose-dependent manner after incubation with insulin or ghrelin (Fig. 4A and 4B). The effects were significantly different in comparison to untreated control adipocytes (incubated in the absence of peptides) at 100nM insulin concentration (p=0.001, Fig. 4A) or ghrelin at 10nM (p=0.033, Fig. 4B). On the other hand, LepA1 expression remained unaffected in all conditions tested either with insulin or ghrelin (Fig. 4C and 4D), although a trend towards a reduced expression was observed in the presence of insulin (p=0.062) (Fig. 4C). Moreover, secreted leptin concentration into the media showed a trend towards negative correlation to LepA1 expression levels after both, insulin and ghrelin incubation (P=-0.396, p=0.093 and P=-0.048, p=0.811, respectively).

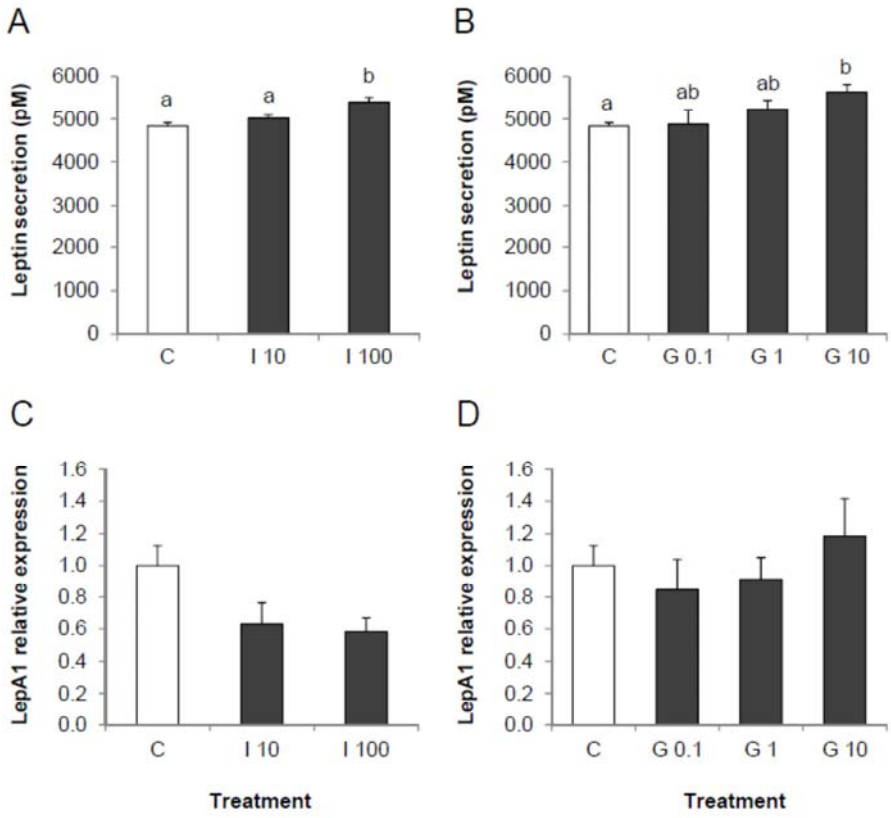

Figure 4. Leptin secretion and expression in isolated adipocytes from regular fed rainbow trout. **(A,B)** Leptin concentration in the media and **(C,D)** quantitative relative expression of LepA1 normalized to EF1α. Adipocytes were isolated as explained in Materials and Methods and left untreated (C) or treated either with insulin (I) at 10 or 100nM or ghrelin (G) at 0.1, 1 or 10nM for 3h. Data are shown as mean ± s.e.m. (n=5-6). Different letters indicate significant differences between treatments (p<0.05).

3.4.2 High-energy diet experiment

Leptin secretion from the adipocytes of the high-energy diet fish in the *in vitro* experiment, supported the findings on circulating plasma leptin, with significantly higher leptin levels in the media of control adipocytes from RE than AL fish (p=0.022), as well as in the media of insulin-treated adipocytes from RE fish (p=0.005, Fig. 5A). There was a significant main effect of group (p=0.002) and treatment (p=0.001) on leptin secretion into the media, but no interaction between these factors was found (p=0.069, Fig. 5A). In AL fish, adipocyte leptin secretion into the media remained unaffected by the different treatments applied (p=0.556, Fig. 5A). In RE fish, leptin secretion was significantly reduced by leucine in comparison to the control treatment (p=0.025, Fig. 5A). A similar but non-significant effect was observed after EPA treatment (p=0.690, Fig 5A). Insulin treatment tended to increase the secretion of leptin (p=0.226, Fig. 5A). Regarding the regulation of LepA1 expression, there was a close to significant main effect of treatment (p=0.051), no main effect of group (p=0.509), and no treatment-group interaction (p=0.725) (Fig. 5B). Moreover, one-way ANOVA results showed that LepA1 expression did not change in response to treatment neither in AL nor RE isolated adipocytes (p=0.434 and p=0.171, respectively) (Fig. 5B). However, in isolated adipocytes from AL fish, LepA1 expression showed a tendency to increase after EPA treatment in comparison to control adipocytes (p=0.094, Fig. 5B). A final correlation analysis was carried out between secreted leptin levels and LepA1 expression levels using the data obtained from both groups together, AL and RE, where media leptin concentration correlated positively but not significantly to LepA1 mRNA expression (P=0.005, p=0.965).

4. Discussion

In the present study, groups of rainbow trout developed significantly different levels of visceral adiposity after being fed with a high-energy diet at two ration levels, *ad libitum* (AL) or 25% satiation (RE), for 8 weeks, with the AL group being fatter than the RE group. Other factors, including biometric and plasma parameters, were also modified by the diet and were higher in AL in comparison to RE fish. This corroborates that the experimental design used was effective in generating two groups of fish with clearly differentiated nutritional status and fat reserves.

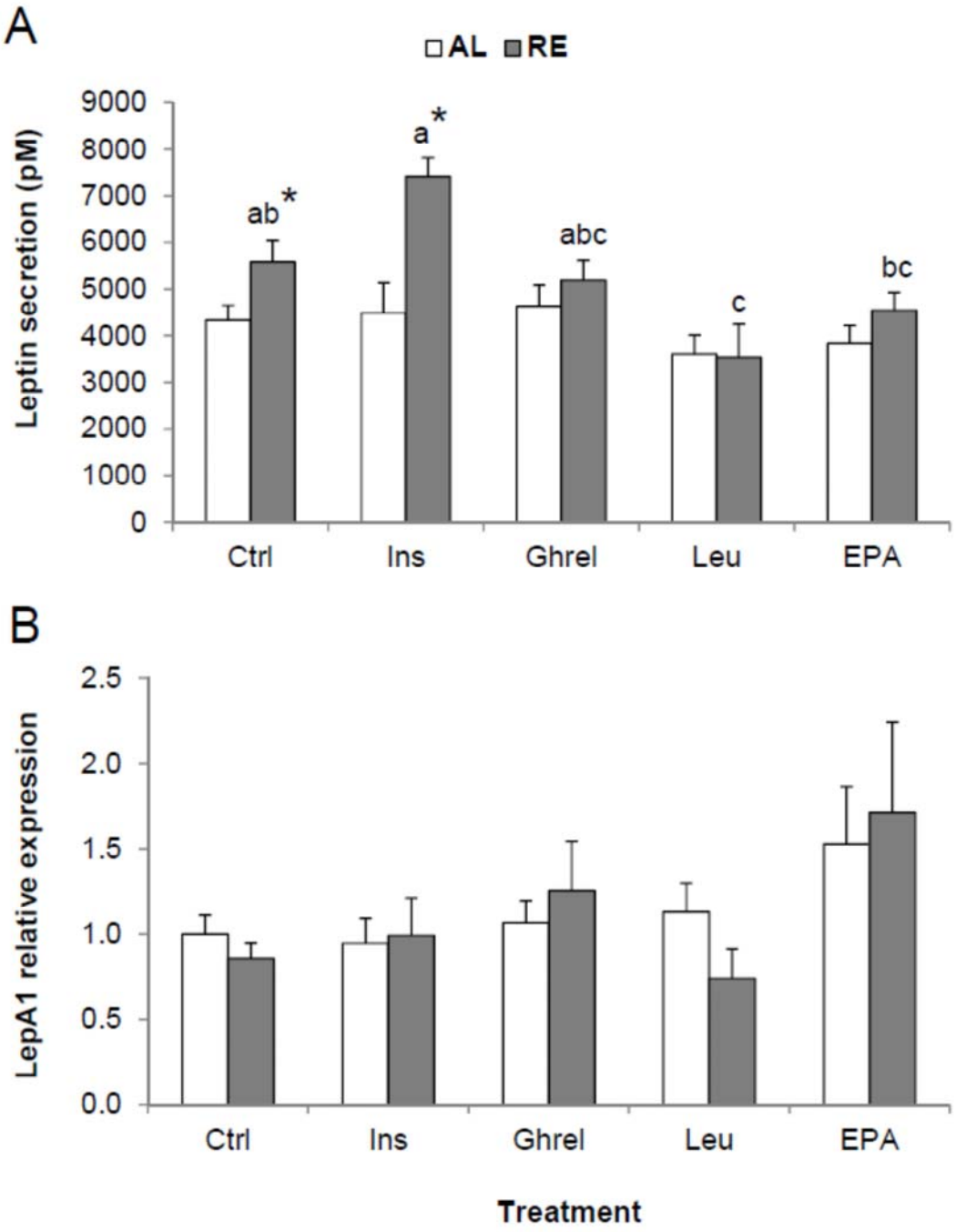

Figure 5. Leptin secretion and expression in *ad libitum* and feed restricted rainbow trout isolated adipocytes. **(A)** Leptin concentration in the media and **(B)** quantitative relative expression of LepA1 normalized to EF1α of isolated adipocytes from AL and RE fed rainbow trout at week t=8. Adipocytes were isolated as explained in Materials and Methods and left untreated (Ctrl) or treated either with insulin 100nM (Ins), ghrelin 10nM (Ghrel), leucine 5mM (Leu) or EPA 100µM (EPA) for 3h. Data are shown as mean ± s.e.m. (n=6-7 with 2-4 treatment replicates per group). Different capital or lower case letters indicate significant differences between treatments within the AL or RE group, respectively, and asterisks indicate significant differences between groups within treatments ($p<0.05$).

4.1 Nutritional modulatory effects on plasma leptin

In the current study, plasma leptin in the RE group was significantly higher than in the AL group, which is in agreement with previous studies in rainbow trout and other teleost species, where plasma leptin levels increased with fasting or feed restriction (21, 23, 31, 38, 68). An exception is the absence of effect of a reduced ration (60% of fully fed controls) in plasma leptin reported in one study performed in Atlantic salmon (63). The increase in the levels of plasma leptin in fish in response to situations where food is limited or absent is opposite to that known in mammals, where leptin levels decrease during fasting. This is often interpreted to be a consequence of decreased adipose tissue mass, as this is the principal leptin-secreting tissue in mammals (2, 18). This may reflect fundamental differences in the site and regulation of leptin production between fish and mammals. In fish, other tissues than adipocytes, e.g. liver, produce significant amounts of leptin. As an explanation for this observation in a wider perspective, Fuentes and colleagues (23) suggested that increased leptin levels may be linked to a survival behavior in fish, which experience naturally prolonged periods of food shortage, lowering appetite and limiting energy-wasting foraging activity.

Moreover in the present study, leptin levels in fish from the AL group remained stable after 2 months of feeding the high-energy diet, during which time these fish showed marked appetite and increased their CF from 1.05 to 1.4. Similarly in rats, a study showed high-fat diet feeding associated with reduced leptin secretion, which promoted hyperphagia and increased adiposity (3), suggesting that the effects of dietary lipids on leptin may be dependent on the type and length of the dietary fat treatment. In this sense, a previous study on Atlantic salmon showed that fish fed a high-energy diet for 68 days had significantly lower plasma leptin levels than fish fed with a low-energy diet (31); however, that study reported also equal leptin levels between groups at initial sampling, which took place after 215 days of preconditioning under the same diets.

Furthermore, the present study shows that plasma leptin levels negatively correlate with adipose tissue and MFI. These results agree with recent findings on Atlantic salmon where plasma leptin levels negatively correlated with body lipid content in *ad libitum* fed fish (68). Contrary to these observations, a positive correlation between plasma leptin levels and visceral adipose tissue and belly flap lipid content has been observed in juvenile rainbow trout treated with a sustained-release GH implant, although the GH-treated fish had lower CF than the sham-treated controls (36); since as in other vertebrates, GH has a lipolytic function in fish (4, 53). It may therefore be the nutritional status and/or the interplay of metabolic hormones

which determines the relationship between leptin levels and adiposity, rather than adiposity *per se*. Thus, it may be of relevance if the fat mass changes are e.g. due to feed restriction or GH-induced energy mobilization (36).

The current study also found a negative correlation between plasma leptin and liver weight. This is contrary to mammals (26) and interesting as the liver is not the main lipid/energy storing tissue in salmonids (1), but agrees with the fact demonstrated in several studies where the liver is the major tissue expressing leptin in fish species including salmonids (25, 28, 36, 39, 40, 51, 59, 63, 68). The negative correlation between plasma leptin and adipose tissue or liver weights observed in the present study, suggests that when the energy stores in these organs are small, the body responds by increasing leptin secretion into the blood in agreement with recent hypothesis (23). Conversely, when more fat is stored, less leptin is secreted, with consequent reduction of anorectic signals to the CNS, as an indication that the feed restriction time has ended. Overall, these data demonstrate that the present experimental trial can be a good model to study the metabolic and/orendocrine responsesof leptin in fish with different degrees of adiposity.

4.2 Regulation of leptin mRNA expression and secretion in isolated adipocytes

In teleosts, leptin mRNA expression is low in adipose tissue, relative to the leptin mRNA expression in the liver, which appears to be a highly active site for leptin production (25, 28, 39, 40, 51). On the other hand, the liver is only about 1% of salmonid body mass. Notwithstanding, the present study demonstrates for the first time that isolated rainbow trout adipocytes can express and secrete leptin as well as to respond to nutritional and hormonal stimuli. Incubation of isolated adipocytes from regular diet-fed rainbow trout with insulin increased leptin secretion, a similar stimulatory effect as that observed in mammals (24, 50, 61). Changes in plasma insulin levels affect adipose tissue mRNA and plasma levels of leptin in mammals, e.g. in rats treated with streptozotocin, where reduction in leptin mRNA expression was quickly reversed after insulin administration (46, 56). Furthermore, an acute increase in plasma leptin levels significantly inhibits glucose-stimulated insulin secretion *in vivo* in rats (12), thus suggesting a cross-regulation between these two hormones. Studies in common carp have shown that hepatic LepA1 and LepA2 expression increases transiently following the postprandial plasma glucose peak, suggesting that leptin expression is at least in part regulated indirectly by hormones such as insulin and cholecystokinin (28). In the present study, although insulin stimulated leptin secretion, LepA1 mRNA expression had a tendency to decrease in a dose-response manner, contrary to what has been observed in mammals, and carp (16, 28).

The current findings suggest that in rainbow trout, insulin could act in a dual-mode fashion through both a feedback mechanism down-regulating LepA1 expression and post-transcriptionally by increasing adipocyte leptin secretion.

In the present study, isolated adipocytes from regular diet-fed rainbow trout incubated with ghrelin increase their leptin secretion in parallel. Nonetheless, previous studies in rainbow trout, have demonstrated that ghrelin appears to act through the anorexigenic CRH neurons to also suppress food intake (33). In contrast, fasting or feed restriction in rainbow trout increases plasma leptin levels (37), whereas it decreases ghrelin levels (32), suggesting that ghrelin and leptin may interact to maintain energy homeostasis. In mammals leptin levels fall during fasting (2), while ghrelin levels increase (34). The same occurs at a transcriptional level (73) and the opposite pattern of expression is observed after food intake (34). Moreover, leptin inhibits *in vivo* both the secretion of gastric ghrelin and the stimulation of feeding by ghrelin (35). On the other hand the stimulation of rat adipocytes with ghrelin causes an increase in leptin gene expression and secretion (24). Altogether, these results suggest that insulin, ghrelin and leptin regulate each other through negative feedback mechanisms in rainbow trout, in a similar but distinct interrelationship to that found in mammals, but directed also to control food intake and energy homeostasis.

The *in vitro* adipocytes from the fish fed the high-energy diet at different rations seem to retain a “metabolic memory", as the adipocytes from RE fish secreted more leptin into the media than adipocytes from the AL fish, both under control conditions and after insulin treatments, echoing the different plasma leptin levels of these fish groups. This observation reinforces the idea that the *in vitro* results reflect what occurs *in vivo*, and validates the use of the present experimental protocol to study the regulation of leptin mRNA expression and secretion. In the present study, it also appears that isolated adipocytes from the AL fish show acertain degree of resistance to the hormonal or nutritional stimuli, as similar LepA1 mRNA and secreted leptin levels were detected in all treatments. This agrees with the hypothesis that animals fed with a high-energy diet *ad libitum* may develop metabolic disorders, and may have therefore lost the ability to respond to hormonal or nutritional external stimuli to regulate leptin, as observed in rodent models (13). On the other hand, isolated adipocytes from RE fish responded to hormones and nutrients, and leptin was differently secreted upon treatment. When adipocytes were incubated with anabolic hormones such as insulin, an increase in leptin secretion occurred, as observed in insulin-treated regular diet adipocytes. Intriguingly, when the adipocytes were incubated with ghrelin, the stimulatory effects observed in regular diet adipocytes were reduced. These results suggest that ghrelin may lose its secretory effects

when the animal is in a period of feed restriction, which may reflect what occurs *in vivo*, as ghrelin decreases in plasma in response to fasting in rainbow trout (32). Moreover, in adipocytes from RE fed animals incubated with nutrients such as leucine, a decrease in leptin secretion occurred, contrary to what is known in mammals (45, 62), and a similar tendency was also found following EPA treatment. It can be speculated that increases in leucine or EPA levels could be interpreted at the cellular level as increased food availability, causing a decrease in leptin secretion. In turn, this would increase appetite and thereby stimulate the feeding/foraging behavior of the fish.

At a transcriptional level, although there were no statistically significant differences between groups or treatments, interesting tendencies were observed. Insulin did not modify LepA1 mRNA in any of the two groups, contrary to the results from regular diet adipocytes. In cultured rat adipocytes, cycloheximide abolished the effects of insulin stimulating leptin secretion, which indicated that *de novo* synthesis and perhaps transcription is required for insulin-mediated increase in leptin secretion (50). Moreover, contrary to the observed effects on leptin secretion, EPA had a tendency to elevate LepA1 mRNA expression in both groups. In primary cultured rat adipocytes and 3T3-L1 cells, EPA stimulates leptin gene expression and secretion (58), although other studies have reported that dietary n-3 fatty acids decrease leptin mRNA expression, maybe acting through the nuclear receptor PPARγ (60).Therefore, the regulatory role of fatty acids such as EPA on leptin expression and secretion in fish deserves to be further explored.

Altogether, the current findings suggest that the physiological role of leptin is linked to the degree of adiposity in rainbow trout, and support the view that leptin has a role in regulating food intake and energy expenditure in salmonid fish such as the rainbow trout. Furthermore, leptin seems to be under the influence of other important metabolic/appetite regulating hormones such as insulin and ghrelin, which modulate leptin secretion. Overall, the data suggest that interactions between these three hormones are crucial for the regulation of metabolism and energy balance. Also, the present data reveal several aspects of the underlying mechanisms regulating leptin secretion from adipocytes in rainbow trout, which can be related to the nutritional status. Finally, the present work shows for the first time a negative correlation between plasma leptin and adipose tissue mass in rainbow trout, and provides further evidence that to feed trout *ad libitum* with high-energy diets may eventually affect the health of the animal and quality of the aquaculture product.

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CAPÍTOL IV

Rols de la leptina i la grelina en l'adipogènesi i el metabolisme lipídic en adipòcits de truita irisada *in vitro*

Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes *in vitro*

Rols de la leptina i la grelina en l'adipogènesi i el metabolisme lipídic en adipòcits de truita irisada *in vitro*

La leptina i la grelina són reguladors ben coneguts de l'homeòstasi energètica, però el seu paper en els peixos encara no està totalment clar. En el present estudi, vem examinar els efectes d'aquestes dues hormones en l'adipogènesi i l'expressió de gens relacionats amb el metabolisme lipídic en adipòcits de truita irisada. Tot i que la grelina no va influir ni en la proliferació ni en la diferenciació dels pre-adipòcits, tant el pèptid com el seu receptor es van identificar en els adipòcits en cultiu, el que indica que la grelina podria tenir altres efectes endocrins o locals en la truita irisada. D'altra banda, també es va detectar expressió i secreció de leptina, augmentant de pre-adipòcits a adipòcits madurs, tot i que la leptina tampoc no va tenir efectes sobre el procés d'adipogènesi en aquesta espècie. La leptina i la grelina van augmentar la lipòlisi en els adipòcits madurs aïllats, però l'expressió d'ARNm dels marcadors de lipòlisi no es va veure modificada significativament. La leptina no va afectar l'expressió dels gens de la lipogènesi o de la β-oxidació estudiats, però va suprimir de forma significativa l'expressió de la proteïna transportadora d'àcids grassos-1, el que suggereix una disminució de l'adquisició i l'emmagatzematge d'àcids grassos. D'altra banda, la grelina va incrementar significativament l'expressió d'ARNm de la lipoproteïna lipasa, de la sintasa d'àcids grassos i del receptor activat per proliferadors de peroxisomes-β; per tant, sembla estimular la síntesi de triglicèrids, així com la seva mobilització. En general, aquestes dades indiquen que la grelina sembla ser una potenciadora de la renovació del metabolisme lipídic en el teixit adipós de la truita irisada, mentre que la leptina sembla regular-lo negativament, i que aquests efectes podrien ocórrer, en part, d'una manera autocrina o paracrina. No obstant això, els seus mecanismes d'acció s'han d'estudiar més a fons per tal de comprendre millor la regulació de l'adipositat en les espècies cultivades.

Paraules clau: Teixit adipós, cultiu primari, proliferació, diferenciació, insulina, lipòlisi.

Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes *in vitro*

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Running title: Leptin and ghrelin effects in trout adipocytes

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Abstract

Leptin and ghrelin are well-known regulators of energy homeostasis; however, their roles in fish are still not fully elucidated. In the present study, we have examined the effects of leptin and ghrelin on adipogenesis and the expression of lipid metabolism-related genes in rainbow trout adipocytes. Although ghrelin did not influence preadipocyte proliferation or differentiation, both peptide and receptor were identified in cultured adipocytes, indicating that ghrelin may have other endocrine or local effects in rainbow trout. Moreover, leptin expression and secretion was also detected, and increased from preadipocytes to mature adipocytes, despite leptin did not affect either the process of adipogenesis in this species. Leptin and ghrelin increased lipolysis in mature freshly isolated adipocytes; nevertheless, the mRNA expression of lipolysis markers was not significantly modified. Leptin did not affect the expression of any of the lipogenesis or β-oxidation genes studied, besides it significantly suppressed the fatty acid transporter-1 expression, suggesting a decrease in fatty acid uptake and storage. Furthermore, ghrelin significantly up-regulated the mRNA expression of lipoprotein lipase, fatty acid synthase and peroxisome proliferator-activated receptor-β; thus, it appears to stimulate synthesis of triglycerides as well as mobilization. Overall, these data indicate that ghrelin seems to be an enhancer of adipose tissue lipid metabolism turnover in rainbow trout, whereas leptin seems to regulate it negatively, and that those effects could occur in part in an autocrine or paracrine manner. However, the mechanisms of action need to be further explored in order to better understand the regulation of adiposity in cultured fish species.

Keywords: Adipose tissue, primary culture, proliferation, differentiation, insulin, lipolysis.

1. Introduction

Obesity is a worldwide burden for human health, whereas in fish, excessive adiposity not only affects animal's welfare, but it is also critical for the quality of the final product for aquaculture. Consequently, it is of great interest to gain knowledge on the molecular mechanisms involved in adipose tissue (AT) growth and development in fish, as well as the regulation of lipid metabolism in adipocytes in order to prevent excessive fat accumulation. AT is not a static tissue, during the life of an animal may be increased or reduced according to external and internal stimuli. AT grows by hyperplasia (cell number increase), hypertrophy (cell size increase) or both (Otto & Lane, 2005). The classical functions exerted by the AT are the synthesis (lipogenesis) and storage of energy in the form of triglycerides (TG), and its break down (lipolysis) into free fatty acids (FFAs) and glycerol, for their release as fuels for peripheral tissues (Sheridan, 1988; Steinberg, 1963). Notwithstanding, the AT has been also increasingly evidenced as an important endocrine organ through the secretion of a wide range of factors that among other functions control feeding behavior, metabolism, reproduction and immunity (Fischer-Posovszky et al., 2007; Rondinone, 2006).

Evidence in mammals has demonstrated that among others, leptin and ghrelin are two of the main factors involved in the control of food intake and energy balance, in particular regulating central and peripheral lipid metabolism, which has led to an increase in the research focused on their potential use as therapeutic targets to treat obesity. A decade ago, both peptide hormones were also identified in fish, and since then, their effects on appetite have been studied showing similar and dissimilar responses depending on the species, in comparison to mammals (Copeland et al., 2011; Jönsson, 2013; Kang et al., 2011). Nevertheless, nothing is known about leptin and ghrelin regulating adipogenesis and fat metabolism in fish AT.

Leptin is primarily secreted by the AT in mammals and acts as an adiposity signal promoting anorexia (Saladin et al., 1995; Zigman and Elmquist, 2003). In fish, leptin is predominantly expressed in the liver (Gorissen et al., 2009; Huising et al., 2006; Murashita et al., 2008), besides recent studies in rainbow trout have demonstrated that mature adipocytes also express and secrete leptin (Pfundt et al., 2009). More recently, duplicated leptin genes (A and B) with two paralogues each (1 and 2) have been described in salmonids, due to the whole genome duplication events occurred during evolution (Angotzi et al., 2013; Rønnestad et al., 2010). Several studies in mammals have investigated the effects of leptin on proliferation and differentiation of adipocytes, with contradictory results among them. Leptin has been shown, using primary preadipocytes as well as 3T3-L1 cells, to either have no effects on preadipocyte proliferation (Kim et al., 2008), increase (Machinal-Quélin et al., 2002; Ramsay, 2005), cause a

dose-dependent reduction (Zwirska-Korczala et al., 2007); or even to have a biphasic effect depending on the concentrations of leptin used (Ambati et al., 2007; Wagoner et al., 2006). Regarding differentiation in response to leptin, inhibition (Thomas et al., 1999), stimulation (Machinal-Quélin et al., 2002), and no effects (Ramsay, 2005; Wagoner et al., 2006) have been also reported. In relation to lipid metabolism, the roles of leptin on the other hand are very consistent. Leptin in mammals increases energy expenditure *in vivo* enhancing lipid oxidation at the same time that inhibits insulin-induced lipogenesis and promotes lipolysis in adipocytes, hepatocytes and other cell types (Cohen et al., 2002; Hwa et al., 1997; Reidy and Weber, 2000). Importantly, some of these effects have been shown to be modulated also through autocrine or paracrine signaling using *in vitro* cultured adipocytes (Frühbeck et al., 1997; Kim et al., 2008). Besides nothing is known in fish regarding the effects of leptin on adipogenesis, few studies on lipid metabolism have been reported using heterologous leptin injections *in vivo*. In goldfish for example, leptin treatment has been shown to cause a decrease in liver lipid content (de Pedro et al., 2006). In terms of its modulatory effect on the expression of lipid-related genes, a decrease in hepatic stearoyl CoA desaturase and lipoprotein lipase (LPL), as well as an increase in the fatty acid binding protein and the carnitine palmitoyl transferase-1 (CPT1) have been reported in grass carp and green sunfish, respectively (Li et al., 2010a; Londraville and Duvall, 2002); thus supporting an anti-adipogenic role for leptin in fish too.

Ghrelin is mainly produced by the stomach in fish as in mammals, but to a lesser extent, also by other tissues such as intestine, pancreas and brain (Castañeda et al., 2010; Jönsson, 2013), and a study in Atlantic salmon found that it is also weakly expressed in AT (Murashita et al., 2009). First described in rat as a growth hormone (GH)-secretagogue (Kojima et al., 1999), ghrelin has been shown to promote growth and adiposity (Castañeda et al., 2010; Choi et al., 2003; Kamegai et al., 2001; Tschöp et al., 2000). The effects of ghrelin stimulating preadipocyte proliferation have been well described in mammalian models (Kim et al., 2004; Liu et al., 2009; Zhang et al., 2004; Zwirska-Korczala et al., 2007). Furthermore, it has been shown *in vitro* that ghrelin also enhances adipocyte differentiation, with a concomitant increase in the mRNA expression of two key adipogenic transcription factors, the peroxisome proliferator-activated receptor-γ (PPARγ) and the CAAT/enhancer binding protein α (C/EBPα) (Giovambattista et al., 2008; Liu et al., 2009; Pulkkinen et al., 2010). Moreover, ghrelin treatment in rodents induced abdominal obesity and hepatic steatosis increasing the number of lipid droplets and their lipid content as well as decreasing fat utilization (Davies et al., 2009; Tschöp et al., 2000). In visceral AT, ghrelin has been shown also to stimulate lipid accumulation by enhancing the expression of several genes including fatty acid synthase (FAS), LPL and perilipin among others (Pulkkinen

et al., 2010). In fish, *in vivo* ghrelin treatment has been reported to increase lipid deposition in the liver and muscles of Mozambique tilapia and goldfish (Kang et al., 2011; Riley et al., 2005), but not in rainbow trout (Jönsson et al., 2010). Nevertheless, as with leptin, no data is available about ghrelin effects on adipocyte proliferation and differentiation or adipocyte metabolism in piscine models.

In this study, we used rainbow trout cultured preadipocytes and freshly isolated mature adipocytes, both well established *in vitro* adipocyte model systems (Albalat et al., 2005b; Bouraoui et al., 2008; Cruz-Garcia et al., 2009; Salmerón et al., 2013) to explore the effects of leptin and ghrelin regulating the development and lipid metabolism in fish adipocytes.

2. Materials and methods

2.1. Fish

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the European Union, Spanish and Catalan Governments established legislation (reference numbers CEEA 237/12 and DAAM 6755).

Three different batches of rainbow trout (*Oncorhynchus mykiss*) from the fish farm Viveros de los Pirineos S.A. (El Grado, Huesca, Spain) were held at the facilities of the University of Barcelona (Barcelona, Spain) in 6 fiberglass tanks of 400L (8-9 trout/tank) in a recirculation system. Animals were maintained at 15±1ºC with 12h light: 12h dark photoperiod and fed to satiation twice daily with a commercial feed (38% protein, 24% fat and 19.8 MJ/Kg digestible energy; Trout evolution, Dibaq Diproteg S.A., Segovia, Spain). For all experiments fish were fasted before sampling for 24h to avoid regurgitation of food and to ensure clean intestines, anesthetized with MS-222 (0.1g/L) and sacrificed by a blow to the head and medullar section. Then, fish were weighed, externally sterilized with 70% ethanol and visceral AT was taken with sterile dissection material. We used for the leptin expression and secretion experiment, a total of 45 adult fish with a mean body weight of 430.5±18g; for the immunofluorescence, proliferation and differentiation experiments, a total of 50 adult fish with a body weight of 181.1±10g; and finally, for the isolated adipocytes experiment, a total of 49 adult fish with an average body weight of 264.5±25g.

2.2. Peptides

Recombinant rainbow trout leptin was produced in the Department of Biology, University of Bergen (Bergen, Norway) following the procedure described in (Murashita et al., 2008) and, synthetic 20 amino-acid octanoylated rainbow trout ghrelin was obtained from the Peptide

Institute Inc., Osaka. Japan. Porcine insulin was obtained from Sigma (Tres Cantos, Spain) and used as piscine insulin is not available and because its effects in fish cells have been shown previously (Albalat et al., 2005b; Albalat et al., 2006; Bouraoui et al., 2010; Bouraoui et al., 2012a).

2.3. Preadipocyte culture experiments

Preadipocytes were obtained following the protocol described previously by Bouraoui et al., (2008) with minor modifications. For each experiment a pool of AT of approximately 28g obtained from 4-9 fish was kept in Krebs-Hepes buffer pre-gassed with CO2 (pH 7.3-7.4) and supplemented with 1% (v/v) antibiotic/antimycotic solution (A/A). AT was minced into small pieces and incubated for 60min in Krebs-Hepes buffer containing collagenase type II (130U/mL) and 1% bovine serum albumin (BSA) at 18ºC with gentle agitation. Cell suspension was filtered through a 100µm cell strainer and centrifuged at 2.000xg for 10min. Then, the pellet was treated with erythrocyte lysing buffer for 10min at room temperature (RT). Cells were centrifuged again and the resulting pellet was resuspended in "growth medium" (GM) containing Leibovitz's L-15 (L-15), 10% fetal bovine serum (FBS) and 1% A/A. Finally, cells were counted, diluted, and plated into 6- or 12-well plates treated with 1% gelatin (those for immunofluorescence containing glass cover slips) at a density of 1.2 to 3.0 x 105 cells per well and incubated at 18°C in GM.

2.3.1. Immunofluorescence

To detect the presence of leptin and ghrelin peptides and receptors in mature adipocytes, cells were plated on glass cover slips and incubated in GM up to day 7. Then, when confluence was reached, differentiation into adipocytes was induced by changing to a "differentiation medium" (DM) composed of GM plus 10mg/mL insulin, 0.5mM 1-methyl-3-isobutylxanthine and 0.25mM dexamethasone; and, supplemented also with lipid mixture (5µL/mL) since it has been demonstrated that lipids are required to induce complete maturation of fish adipocytes (Bouraoui et al., 2008; Oku and Umino, 2008; Oku et al., 2006; Salmerón et al., 2013). This medium was maintained for 3 days and then, it was changed again to an "adipocyte medium" (AM) that contained GM plus lipid mixture only (5µL/mL) to maintain the cells already differentiated until day 13, when the cells were fixed.

Immunostaining was performed as described by Capilla et al., (2007) using 3T3-L1 adipocytes. Briefly, cells at day 13 were washed with PBS and fixed with 4% paraformaldehyde for 30min at RT. After washing with phosphate buffered saline (PBS), cells were permeabilized with 0.2%

Triton X-100 for 15min at RT and blocked with 1% BSA and 5% donkey serum in PBS (blocking buffer, BB) for 60min at RT. Cells were then incubated over night at 4ºC in the dark with BB alone as a negative control or the primary antibodies against either ghrelin (1:10000), ghrelin receptor (GHS-R1a) (1:20000), leptin (1:500) or the long isoform of leptin receptor (LEPRB) (1:10000) diluted in BB. The anti-rat ghrelin antibody was a kind gift of Dr. Hiroshi Hosoda (Japan) and all other antibodies were developed against rainbow trout in rabbits by Agrisera (Vännäs, Sweden) and previously described (Einarsdóttir et al., 2011; Jönsson et al., 2010). Next, cells were washed again with PBS, rinsed with BB and incubated with BB containing a secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody (A21069 Life Technologies, Alcobendas, Spain; 1:1000) in combination with a Hoechst 342 stain (Life Technologies, Alcobendas, Spain; 1:2000) for 1h at RT in the dark. Finally, cells were mounted with Prolong (Invitrogen, Alcobendas, Spain) and immunofluorescence was captured with a confocal microscope (Leica TCS SP2). The images were analyzed with the imaging software ImageJ. All images showed autofluorescence, due to the presence of lipid droplets characteristic of mature adipocytes. To eliminate the autofluorescence and to obtain the corresponding specific signal, the images acquired using the green channel (unspecific signal) were subtracted to the images acquired using the red channel with the ImageJ software. Four independent experiments were performed.

2.3.2. Expression and secretion of leptin

To study leptin mRNA expression and secretion during culture development plated cells were incubated in GM up to day 7. At that time, once confluence was reached, the GM was changed to DM including 5µL/mL of lipid mixture to induce differentiation. At day 10, and every 2 days the medium was changed again to AM to maintain differentiation. Samples of media were obtained from individual wells of a 6-well plate, each one with only 0.5mL medium to concentrate leptin, from preadipocytes at day 7 and, from differentiated cells at day 16. Leptin secretion into the media was measured using the homologous RIA protocol established by Kling et al. (2009) and previously validated for adipocyte media samples (Salmerón et al., unpublished data). For the analyses, media samples from preadipocytes and mature adipocytes were used undiluted. After removing all the media, the remaining cells were recovered from 3 wells together with 1mL of TRI Reagent solution (Ambion, Alcobendas, Spain), transferred to a single tube and kept at -80ºC until leptin gene expression analysis. Results are the average of n=6-8 independent experiments.

2.3.3. Effects of leptin and ghrelin on preadipocyte proliferation

The effects of leptin, ghrelin and insulin on cell proliferation were studied using preadipocytes at day 3. Cells were starved for 5h by changing the GM to a medium consisting of L-15 with 1% A/A and only 0.02% FBS. After that, the medium was changed again to a stimulation medium consisting of L-15 containing 1% A/A, 2% FBS, and the corresponding dose of peptide. Leptin was tested at 100nM (L100); ghrelin at 10nM (G10); and insulin at 1000nM (I1000) final concentrations in duplicate wells in a 12-well plate. Proliferation was analyzed after 48h of incubation (day 5) by an MTT assay consisting on the reduction by mitochondrial reductases of methylthiazolyldiphenyl-tetrazolium bromide (MTT) into formazan, as previously described for fish and mammalian adipocytes (Bai et al., 2007; Salmerón et al., 2013; Yang et al., 2009). The MTT solution (0.5mg/mL) was added to the cells 18h before the end of the experiment. Then, cells were washed with PBS, and the blue crystals resuspended in 150µL of dimethyl sulfoxide per well and incubated for 3h before spectrophotometric evaluation. Proliferation values were obtained by subtracting the background read at 650nm from the absorbance measured at 570nm. Cells with PBS instead of MTT were used as nonspecific, and the value from this reading was also subtracted from all the other data. Results are the average of n=6 independent experiments. Data are presented as fold change with the absorbance values normalized to the control condition.

2.3.4. Effects of leptin and ghrelin on preadipocyte differentiation

To analyze the actions of leptin, ghrelin and insulin on cell differentiation, confluent and undifferentiated cells were used at day 7 after plating, when differentiation is usually induced (Bouraoui et al., 2008). At this time, cells were incubated during 6 days at 18ºC with DM as a control condition (C), or with DM containing the different treatments: 5µL/mL lipid mixture (LIP) as a positive control, leptin 100nM (L100), ghrelin 10nM (G10) or insulin 1000nM (I1000), or were left with GM as a negative condition (non-treated cells, NT). At day 13, cells from 2 wells from a 6-well plate were recovered together as described above in 2.3.2 for gene expression analysis and stored at -80ºC. Furthermore, in a parallel set of 12-well plates, cell differentiation was evaluated in duplicate by measuring lipid accumulation using the protocol of oil red O (ORO) staining (Koopman et al., 2001) previously adapted for fish adipocytes (Capilla et al., 2011; Salmerón et al., 2013). Quantification of cell lipid content was measured at 490nm and was divided by the cell protein content reading at 630nm (Capilla et al., 2011). Results are the average of n=6 independent experiments. Data are presented in fold change with the values normalized to the control condition.

2.4. Isolated adipocytes experiment

Adipocytes were isolated following the protocol established by Albalat et al., (2005a). For each experiment a pool of visceral AT of 20-28g obtained from 3-10 fish was minced and incubated 60min in collagenase type II (130U/mL) prepared in Krebs-Hepes buffer containing 1% BSA at 18ºC in a shaking water bath. Cell suspension was filtered through a 100µm cell strainer and washed by flotation 1-2 times each with Krebs-Hepes buffer with 1% BSA and 2% BSA. Approximately 2 million isolated adipocytes (~0.5mL cell cake and ~0.5mL Krebs-Hepes buffer 2% BSA) were incubated in triplicate tubes during 3h in the shaking bath at 18ºC, in the absence (Control, C) or presence of different doses of leptin 10 and 100nM (L10 and L100, respectively) or ghrelin 0.1, 1 and 10nM (G0.1, G1 and G10, respectively). After incubation samples were centrifuged at 2700xg for 2 min at 4ºC. Cell-free aliquots of each triplicate were immediately transferred to a new tube and kept at -80ºC until measurement of glycerol and FFAs; and, the cells from each triplicate recovered together with 1mL of TRI Reagent solution (Ambion, Alcobendas, Spain), transferred to a single tube and kept at -80ºC until gene expression analysis. Glycerol and FFAs released from adipocytes into the media were determined enzymatically using commercial kits (Free glycerol determination kit, Sigma, Tres Cantos, Spain for glycerol and NEFA-HR2, Wako Chemicals GmbH, Neuss, Germany for FFAs) following the manufacturer's instructions with minor modifications to adapt the protocol to a 96-well microtiter plate. Results are the average of n=8 independent experiments.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted following the guanidiniumthiocyanate-phenol-chloroform method using TRI Reagent (Ambion, Alcobendas, Spain). Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and RNA integrity was analyzed by 1% (m/v) agarose gel electrophoresis. To eliminate any residual genomic DNA, total RNA was treated with DNase I (Invitrogen, Alcobendas, Spain) before cDNA synthesis following the manufacturer's instructions. An amount of 0.25 or 1.45µg of total RNA from the isolated adipocytes or preadipocytes from the differentiation experiment, respectively, were used to synthesize first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain) following the manufacturer's recommendations. To analyze the expression of leptin in cultured adipocytes, 2µg of total RNA were reversely transcribed using Oligo(dT)12-18 Primer and Superscript III (Invitrogen, Carlsbad, CA, USA) following standard procedures.

2.6. Quantitative real-time PCR (qPCR)

The relative expression of leptin during preadipocyte culture development was quantified on the CFX-96 Real-Time PCR detection system platform (Bio-Rad, Hercules, CA, USA) using SYBR Green (QuantiTect SYBR Green PCR kit, Qiagen, Hilden, Germany). Each qPCR reaction comprised 12.5µl SYBR Green PCR Master Mix, 300nM of forward and reverse primers (Table 1), 100ng cDNA and nuclease-free water up to a final volume of 25µl. The qPCR conditions were as follows: 3min at 95ºC, amplification for 45 cycles at 95ºC for 15s and 60ºC for 1 min. Then, the mRNA transcript levels of different genes related with lipid metabolism and preadipocyte differentiation (Table 1) in isolated and cultured adipocytes were analyzed. Each qPCR reaction contained 5µL of diluted first-strand cDNA, 10µL of iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain) and 250nM (final concentration) of sense and antisense primers (Table 1) in a final volume of 20µL. Reactions were performed in triplicate using a MyiQ thermocycler (Bio-Rad, El Prat de Llobregat, Spain) with 1 cycle of 3min at 95ºC and 40 cycles of 10s at 95ºC and 30s at 54-61ºC (primer dependent, see Table 1), followed by an amplicon dissociation analysis from 55 to 95ºC at 0.5ºC increase each 30s, where a single peak was observed confirming the specificity of the reaction and the absence of primer-dimers formation. Also, a dilution curve with a pool of samples was run to confirm primer efficiency. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Negative controls (NTC: No Template Control; RTC: no Reverse Transcriptase Control and PCR: water) were included and ran in duplicate. Raw data were normalized to elongation factor 1α (EF1α), which was used as a reference gene in all cases, and analyzed by the delta-delta method (Livak and Schmittgen, 2001).

2.7. Statistical analyses

Statistical analyses of all parameters were performed in IBM SPSS Statistics 20.0 (IBM, Chicago, USA). Normality was analyzed according to the Shapiro-Wilk test and homogeneity in variance according to Levene's test. Statistical differences were assessed by one-way ANOVA followed by the post hoc Tukey's test or Dunnett's T3, or t-test. Non-parametric tests, Kruskal-Wallis and Mann-Whitney U test were used if after data transformation normality was not found. A significance of $p<0.05$ was applied to all statistical tests performed. Data are presented as mean±standard error of the mean (SEM). Pearson correlation analyses were carried out between leptin expression and secretion data from cultured adipocytes.

Table 1. Nucleotide sequences of the primers used to evaluate mRNA abundance by quantitative real time PCR (qPCR) in rainbow trout adipocytes.

Gene	Primer Sequence (5'-3')	Anneal temp (ºC)	Product size (bp)	Database	Accession number	References
HSL1	FW: AGGGTCATGGTCATCGTCTC RV: CTTGACGGAGGGACAGCTAC	58	175	DFCI	TC172767	Sánchez-Gurmaches et al., 2012
ATGL	FW: CGTGTCCGAGTTCAAGTC RV: GGAGAGATGCTGATGGTG	56	174	GenBank	BX318925	Present study
LPL	FW: TAATTGGCTGCAGAAAACAC RV: CGTCAGCAAACTCAAAGGT	59	164	GenBank	AJ224693	Sánchez-Gurmaches et al., 2012
FAS	FW: GAGACCTAGTGGAGGCTGTC RV: TCTTGTTGATGGTGAGCTGT	54	186	Sigenae	tcaa0001c.m.06_5.1.om.4	Cruz-Garcia et al., 2011
FATP1	FW: AGGAGAGAACGTCTCCACCA RV: CGCATCACAGTCAAATGTCC	60	157	DFCI	CA373015	Sánchez-Gurmaches et al., 2012
CPT1b	FW: CCCTAAGCAAAAAGGGTCTTCA RV: CATGATGTCACTCCCGACAG	60	149	GenBank	AF606076	Polakof et al., 2012
PPARα	FW: CTGGAGCTGGATGACAGTGA RV: GGCAAGTTTTTGCAGCAGAT	54	195	GenBank	AY494835	Cruz-Garcia et al., 2011
PPARβ	FW: CTGGAGCTGGATGACAGTGA RV: GTCAGCCATCTTGTTGAGCA	60	195	GenBank	AY356399.1	Cruz-Garcia et al., 2011
PPARγ	FW: GACGGCGGGTCAGTACTTTA RV: ATGCTCTTGGCGAACTCTGT	60	171	GenBank	HM536192.1	Cruz-Garcia et al., 2011
GAPDH	FW: TCTGGAAAGCTGTGGAGGGATGGA RV: AACCTTCTTGATGGCATCATAGC	61	210	GenBank	NM_001123561	Cruz-Garcia et al., 2009
LepA1 (1)	FW: TTGCTCAAACCATGGTGATTAGGA RV: GTCCATGCCCTCGATCAGGTTA	60	68	GI	NM_225543246	King et al., 2012
LepA1 (2)	FW: GGTGATTAGGATCAAAAAGCTGGA RV: GACGAGCAGTAGGTCCTGGTAGAA	54	148	GenBank	NM_225543246	Angotzi et al., 2013
LepA2	FW: TGGGAATCAAAAAGCTCCCTTCCTCTT RV: GCCTCCTATAGGCTGGTCTCCTGCA	60	95	GenBank	GU584004	Rønnestad et al., 2010
EF1α (1)	FW: ATTAACATTGTGGTCATTGGCCATGTC RV: ATCTCAGCTGCTTCCTTCTCGAACTTTT	60	125	GenBank	AF498320	Angotzi et al. unpublished
EF1α (2)	FW: TCCTCTTGGTCGTTTCGCTG RV: ACCCGAGGGACATCCTGTG	59	159	GenBank	AF498320	Bouraoui et al., 2011

HSL: hormone sensitive lipase; ATGL: adipose triglyceride lipase; LPL: lipoprotein lipase; FAS: fatty acid synthase; FATP1: fatty acid transporter 1; CPT1b: carnitine palmitoyltransferase 1b; PPARα: peroxisome proliferator-activated receptor α; PPARβ: peroxisome proliferator-activated receptor β; PPARγ: peroxisome proliferator-activated receptor γ; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LepA1: leptin A1; LepA2: leptin A2; and EF1α: elongation factor 1α. (1) LepA1 and EF1α primers used in the leptin expression analyses in cultured adipocytes and (2) LepA1 and EF1α primers used in the remaining experiments. FW: forward primer; RV: reverse primer; bp: base pair; DFCI: Dana Farber Gene Indices, GI: GenInfo Identifier.

3. Results

3.1. Presence of leptin and ghrelin in cultured preadipocytes

To identify the presence of leptin, ghrelin and their receptors in differentiated adipocytes we performed immunofluorescence staining at day 13 of culture. Control cells incubated with secondary antibody only did not present any specific signal and showed only autofluorescence, as exact coincidence was observed in both, the images acquired with the green and the red channels (Figure 1, panels a-c). Nonetheless, cells incubated with ghrelin primary antibody, showed a clear specific signal in the cytoplasm, in the structures surrounding the nuclei and inside the nuclei, as revealed by Hoechst staining co-localization (Figure 1, panel f). Furthermore, cells incubated with GHS-R1a primary antibody, showed a clear specific signal in the cytoplasm, and especially in the plasma membrane of the adipocytes, mainly in the junction points between cells (Figure 1, panel i). Finally, cells incubated either with leptin or

LEPRB antibodies did not show specific signals (Supplementary Figure 1) independently of all the dilutions and conditions tested, suggesting that these antibodies are unsuitable for immunofluorescence assays.

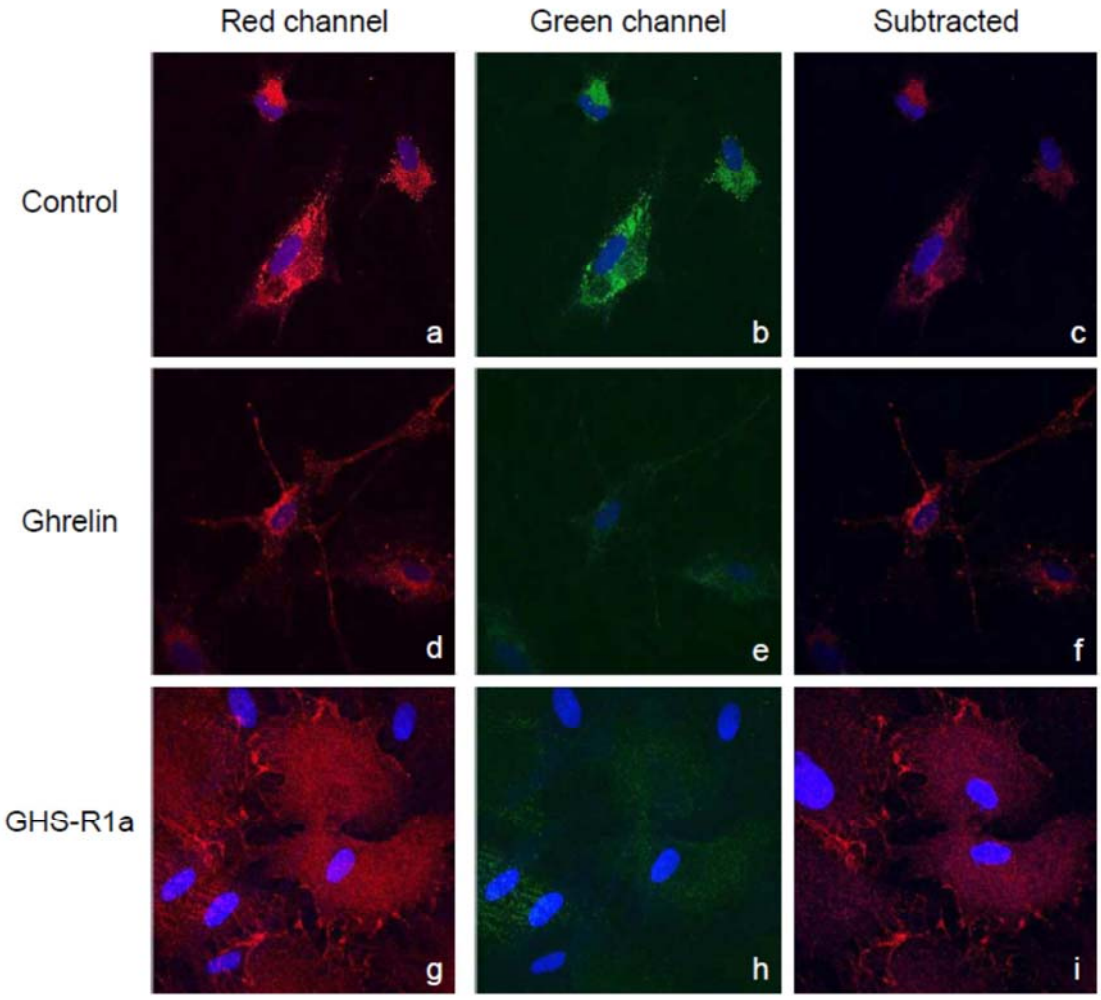
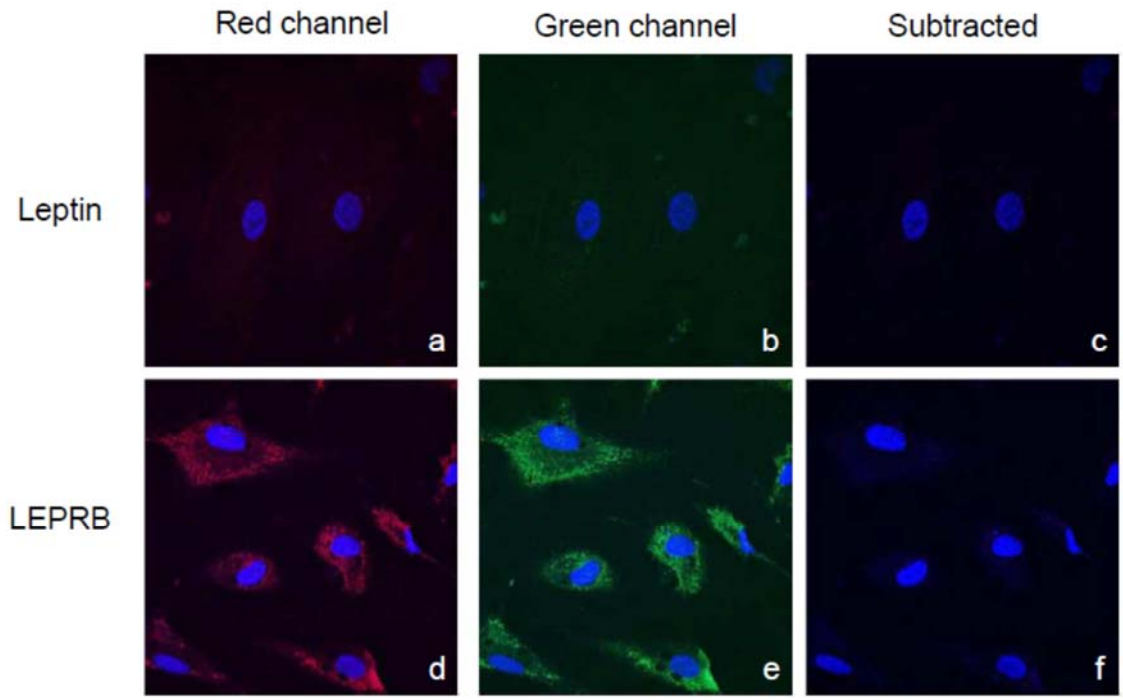


Figure 1. Immunofluorescence of ghrelin and ghrelin receptor 1a (GHS-R1a) in rainbow trout primary cultured adipocytes. Primary and secondary goat anti-rabbit Alexa Fluor 568 antibodies incubations and nuclei counterstain with Hoechst were performed as described in Materials and Methods. Hoechst staining is visualized in blue. In the first column (left), images acquired in the red channel (target signal) are shown; in the middle column are the images acquired in the green channel (unspecific signal); and in the last column (right), the final images, product of the subtraction of the signal from the green channel (autofluorescence) to the image from the red channel to obtain the specific target signal are shown. Cells incubated with secondary antibody only (negative control) are in panels a-c, and cells incubated with ghrelin or GHS-R1a primary antibodies are in panels d-f and g-i, respectively. All images were captured with the objective of 63x in immersion oil. Representative images from n=4 independent experiments are shown.



Sup. Fig.1. Immunofluorescence of leptin and leptin receptor long isoform (LEPRB) in rainbow trout primary cultured adipocytes. Primary and secondary goat anti-rabbit Alexa Fluor 568 antibodies incubations and nuclei counterstain with Hoechst were performed as described in Materials and Methods. Hoechst staining is visualized in blue. In the first column (left), images acquired in the red channel (target signal) are shown; in the middle column are the images acquired in the green channel (unspecific signal); and in the last column (right), the final images are shown, product of the subtraction of the signal from the green channel (autofluorescence) to the image from the red channel to obtain the specific target signal. Cells incubated with leptin and LEPRB primary antibodies are in panels a-c and d-f, respectively. All images were captured with the objective of 63x in immersion oil.

Next, we examined whether leptin is expressed and secreted differentially during the differentiation of preadipocytes into adipocytes in primary cells in culture. Figure 2 shows the expression and the amount of leptin secreted into the media at two different time points during culture development. LepA1 expression was detected both, in preadipocytes and mature adipocytes, with higher levels of expression observed in the latter (p=0.069) (Figure 2A). LepA2 mRNA expression was very low, almost undetectable. In addition, mature adipocytes at day 16, had significantly increased leptin secreted into the media in comparison with day 7 cells (p= 0.018) (Figure 2B). Although LepA1 expression and secretion increased in parallel during adipocyte differentiation, no significant correlation was observed (P=-0.037, p=0.880).

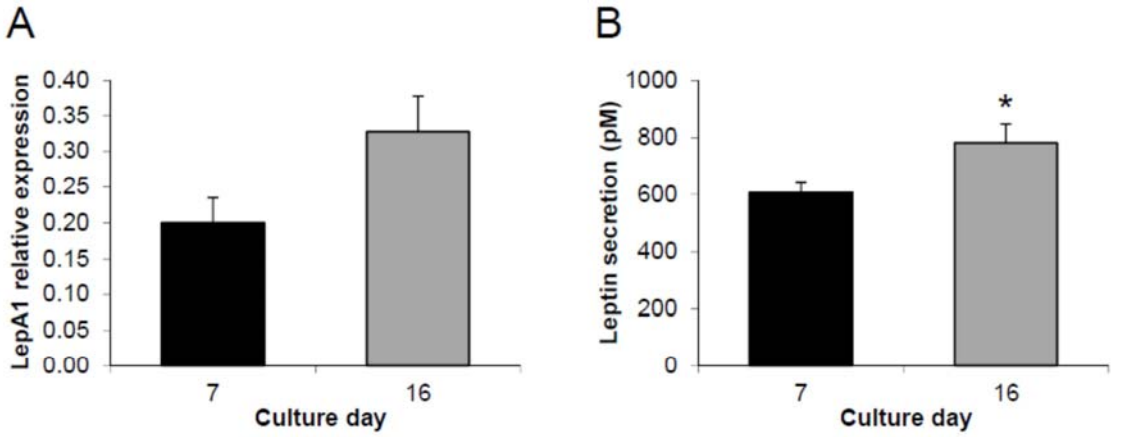


Figure 2. Leptin expression and secretion in rainbow trout primary cultured cells during adipocyte differentiation. (A) Quantitative relative expression of LepA1 normalized to EF1α and **(B)** leptin concentration in the media of primary cultured preadipocytes at day 7 and mature adipocytes at day 16. Data are shown as mean ± SEM (n=6-8). Asterisks indicate significant differences between culture days at $p<0.05$. LepA1: leptin A1; EF1α: elongation factor 1α.

3.2. Effects of leptin and ghrelin on preadipocyte proliferation and differentiation

Then, we investigated the role of leptin and ghrelin on rainbow trout preadipocyte development. Proliferation was significantly higher in cells growing with insulin in comparison to the control (positive control, p=0.016), but remained unchanged with either leptin or ghrelin treatments (Figure 3A). Next, as an indicator of differentiation, accumulated intracellular lipids were measured in day 13 adipocytes after 6 days of treatment. As expected (Figure 3B), lipid mixture (used as a positive control condition) stimulated differentiation compared with cells in GM (NT, non-treated cells) (p=0.017), DM (C, control) (p=0.019), or cells under leptin, ghrelin or insulin treatments (p=0.018, p=0.016 and p=0.012, respectively). In contrast, leptin, ghrelin or insulin alone were not able to increase lipid accumulation in these cells in comparison with the control cells incubated in DM (Figure 3B).

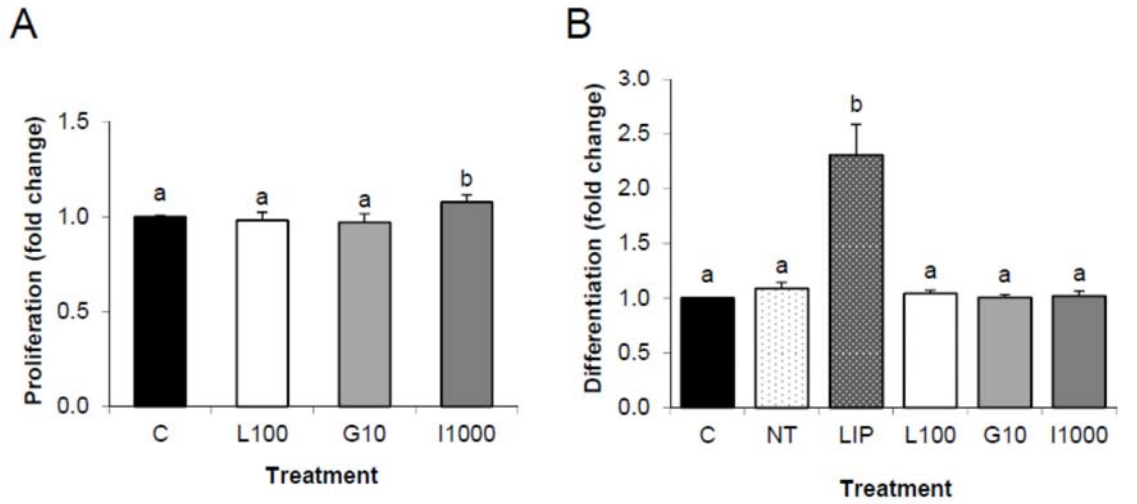


Figure 3. Proliferation and differentiation profiles of rainbow trout primary cultured adipocytes under different treatments. (A) Proliferation of cells at day 5 growing in medium alone (C) or with leptin 100nM (L100), ghrelin 10nM (G10) or insulin 1000nM (I1000) was measured with the MTTassay. Data are shown as mean ± SEM (n=6). Asterisks indicate significant differences between treatments and control condition at $p<0.05$. **(B)** Differentiation of cells at day 15 growing in DM (C), GM (NT), or DM with lipid mixture (LIP), leptin 100nM (L100), ghrelin 10nM (G10) or insulin 1000nM (I1000) was evaluated by measuring lipid accumulation using the protocol of ORO staining and extraction. Data are shown as mean ± SEM (n=5-6). Different letters indicate significant differences between treatments at $p<0.05$. MTT: methylthiazolyldiphenyl-tetrazolium bromide; DM: differentiation media; GM: growth media; ORO: oil red O.

To further explore a possible impact of leptin and ghrelin at the transcriptional level on adipocyte differentiation, we assessed gene expression levels in adipocytes under the same treatments. The mRNA levels of a master regulator of adipogenesis (PPARγ), as well as metabolism-related genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), LPL and fatty acid transporter-1 (FATP1) were determined. The mRNA levels of the adipogenesis-associated transcription factor PPARγ and the marker of late adipocyte differentiation GAPDH were not significantly affected by any treatment (Figure 4A and 4B). Conversely, treatment with lipid mixture significantly increased the expression of LPL ($p=0.020$) (Figure 4C) and FATP1 ($p=0.001$) (Figure 4D) in comparison with control cells in DM, whereas treatment with leptin or GM only (NT), significantly decreased the expression of LPL ($p=0.048$ and $p=0.032$, respectively) (Figure 4C).

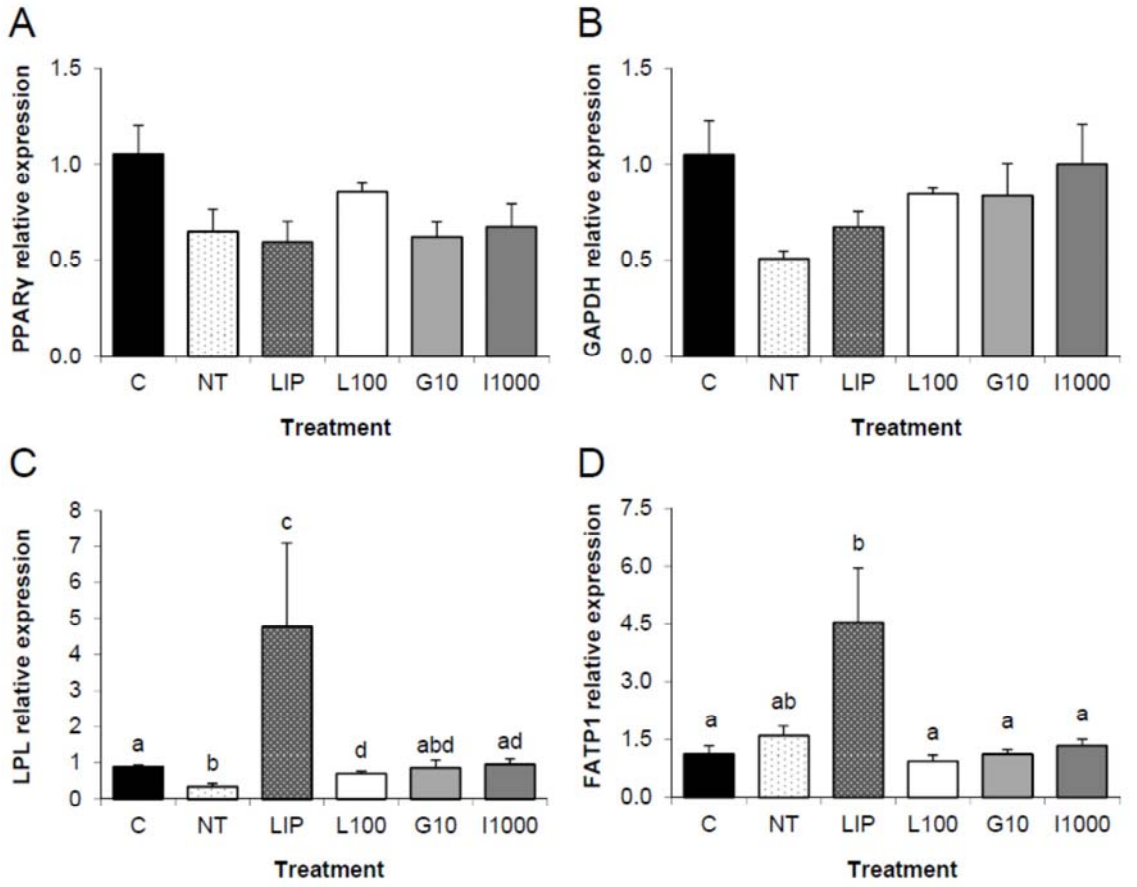


Figure 4. Expression of genes related to adipocyte differentiation in rainbow trout primary cultured adipocytes under different treatments. Quantitative relative expression of PPARγ **(A)**, GAPDH **(B)**, LPL **(C)** and FATP1 **(D)** normalized to EF1α in adipocytes at day 15 growing in DM (C), GM (NT), or DM with lipid mixture (LIP), leptin 100nM (L100), ghrelin 10nM (G10) or insulin 1000nM (I1000). Data are shown as mean ± SEM (n=5-6). Different letters indicate significant differences between treatments at $p<0.05$. DM: differentiation media; GM: growth media; PPARγ: peroxisome proliferator-activated receptor γ; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LPL: lipoprotein lipase; FATP1: fatty acid transporter 1; EF1α: elongation factor 1α.

3.3. Effects of leptin and ghrelin on lipid metabolism in isolated adipocytes

To determine the actions of leptin and ghrelin on lipolysis, we compared the release of glycerol and FFAs from rainbow trout adipocytes incubated with different concentrations of leptin or ghrelin with untreated adipocytes. Leptin (Figure 5A) or ghrelin (Figure 5B) treatment significantly increased glycerol release from isolated adipocytes in a dose-response manner ($p=0.008$ and $p=0.001$, respectively), whereas neither leptin (Figure 5C) nor ghrelin (Figure 5D) modified the FFAs released into the media.

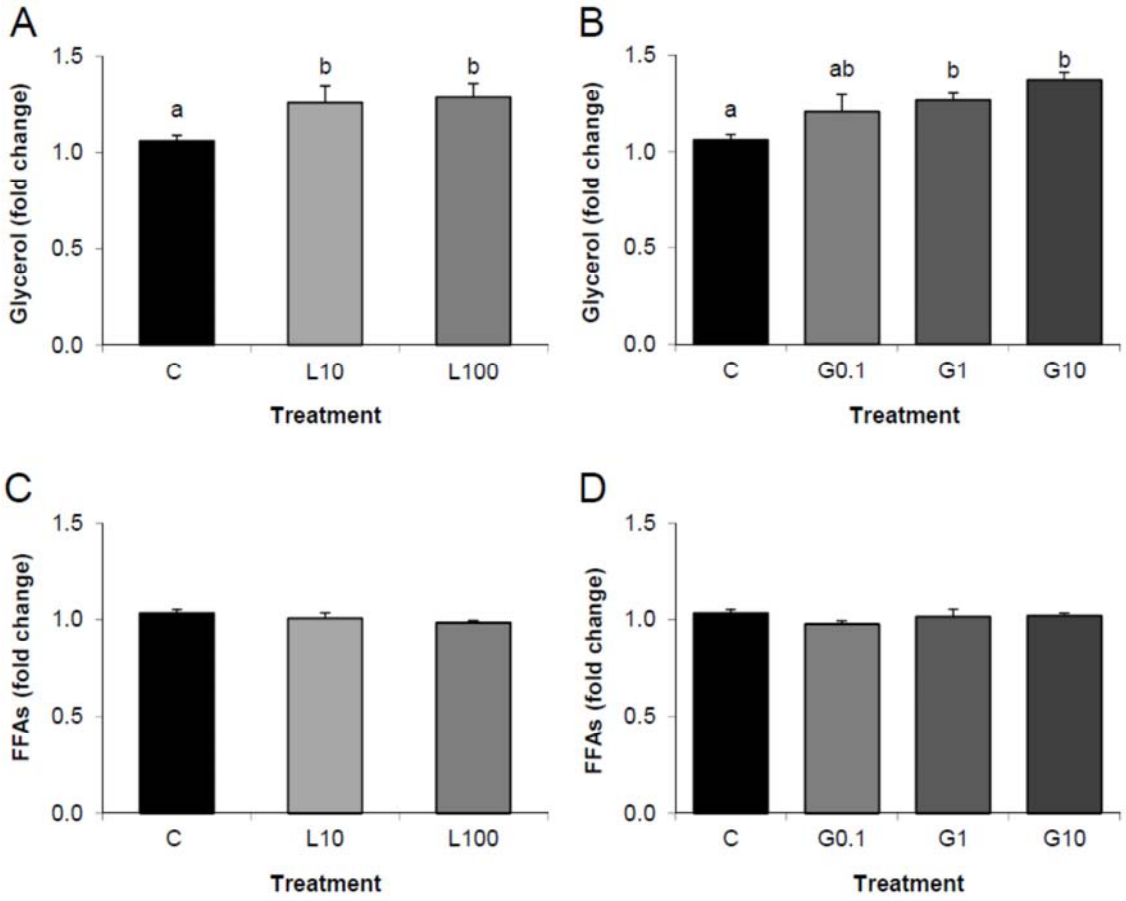
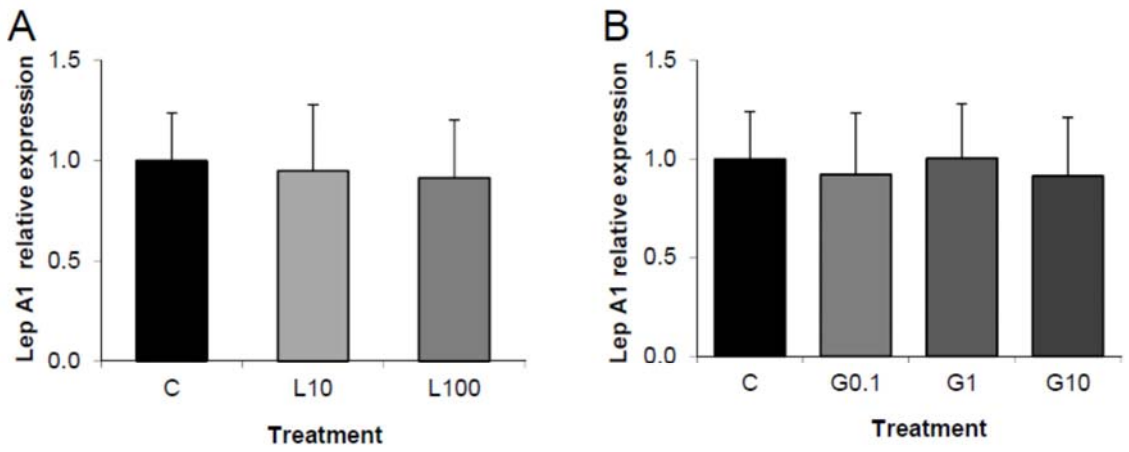


Figure 5. Glycerol and FFAs released into the media in rainbow trout isolated adipocytes treated with leptin or ghrelin. Glycerol **(A and B)** and FFAs **(C and D)** secreted into the media of isolated adipocytes untreated (control, C) or treated during 3h with leptin at 10 and 100nM (L10 and L100) or ghrelin at 0.1, 1 and 10nM (G0.1, G1 and G10), respectively. Data are shown normalized to control adipocytes as mean ± SEM (n=8). Different letters indicate significant differences between treatments at $p<0.05$. FFAs: Free Fatty Acids.

Then, we analyzed in isolated adipocytes under the same treatments, the transcriptional effects caused by leptin or ghrelin. First, the mRNA expression levels of leptin were determined. As occurred in adipocytes in culture, LepA2 expression was almost undetectable in mature adipocytes and, the mRNA expression of LepA1 was not significantly affected neither with leptin nor ghrelin (Supplementary Figure 2).



Sup. Fig. 2. Leptin expression in rainbow trout isolated adipocytes treated with leptin (A) or ghrelin (B). Quantitative relative expression normalized to EF1α of isolated adipocytes untreated (control, C) or treated during 3h with leptin 10 and 100nM (L10 and L100) or ghrelin 0.1, 1 and 10nM (G0.1, G1 and G10), respectively. Data are shown normalized to control adipocytes as mean ± SEM (n=5-13). Different letters indicate significant differences between treatments at $p<0.05$. LepA1: leptin A1; EF1α: elongation factor 1α.

Next, the mRNA levels of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), LPL and FAS, genes involved in fatty acid metabolism, were determined. The mRNA expression of HSL, ATGL, LPL and FAS in adipocytes treated with leptin was not significantly affected; however, LPL and FAS expression showed a significant increase in a dose-dependent manner after incubation with ghrelin (Figures 6 and 7). Then, the mRNA levels of FATP1 and CPT1b, genes involved in fatty acid transport into the cell and the mitochondria, respectively were determined. The mRNA expression of FATP1 and CPT1b in isolated adipocytes was not significantly affected upon ghrelin treatment; nevertheless, FATP1 expression showed a significant decrease in response to leptin (Figure 8). Finally, we determined the mRNA levels of PPARα and PPARβ, transcription factors involved in the activation of several genes and pathways related to lipid metabolism, in particular β-oxidation. As shown in Figure 9, adipocyte mRNA expression of PPARα (Figure 9A and 9B), was not significantly affected by any treatment. However, PPARβ expression was significantly increased after incubation with ghrelin at 1nM concentration compared to the control condition ($p=0.012$) (Figure 9D), while it remained unchanged after leptin treatment (Figure 9C).

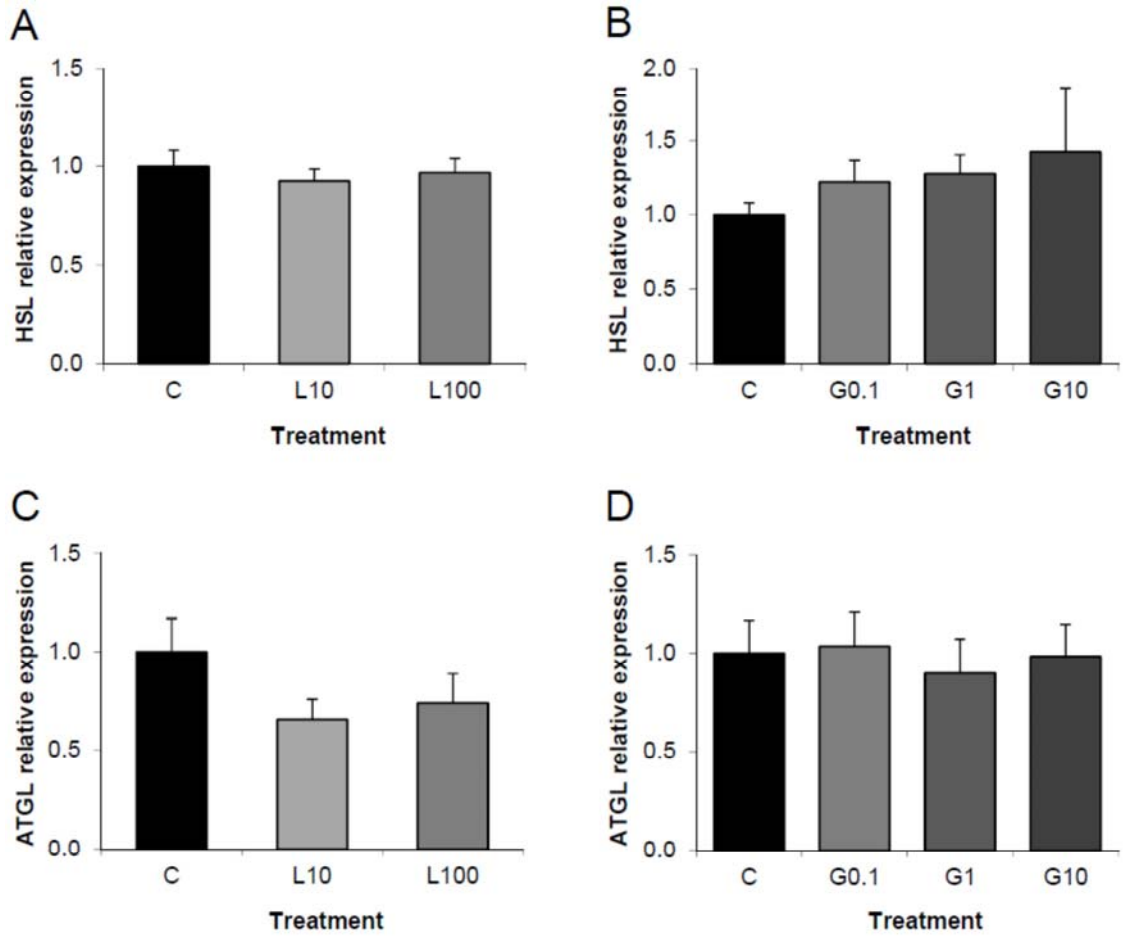


Figure 6. Expression of genes related to lipolysis in rainbow trout isolated adipocytes treated with leptin or ghrelin. HSL **(A and B)** and ATGL **(C and D)** quantitative relative expression normalized to EF1α of isolated adipocytes untreated (control, C) or treated during 3h with leptin 10 and 100nM (L10 and L100) or ghrelin 0.1, 1 and 10nM (G0.1, G1 and G10), respectively. Data are shown normalized to control adipocytes as mean ± SEM (n=5-10). Different letters indicate significant differences between treatments at $p<0.05$. HSL: hormone sensitive lipase; ATGL: adipose triglyceride lipase; EF1α: elongation factor 1α.

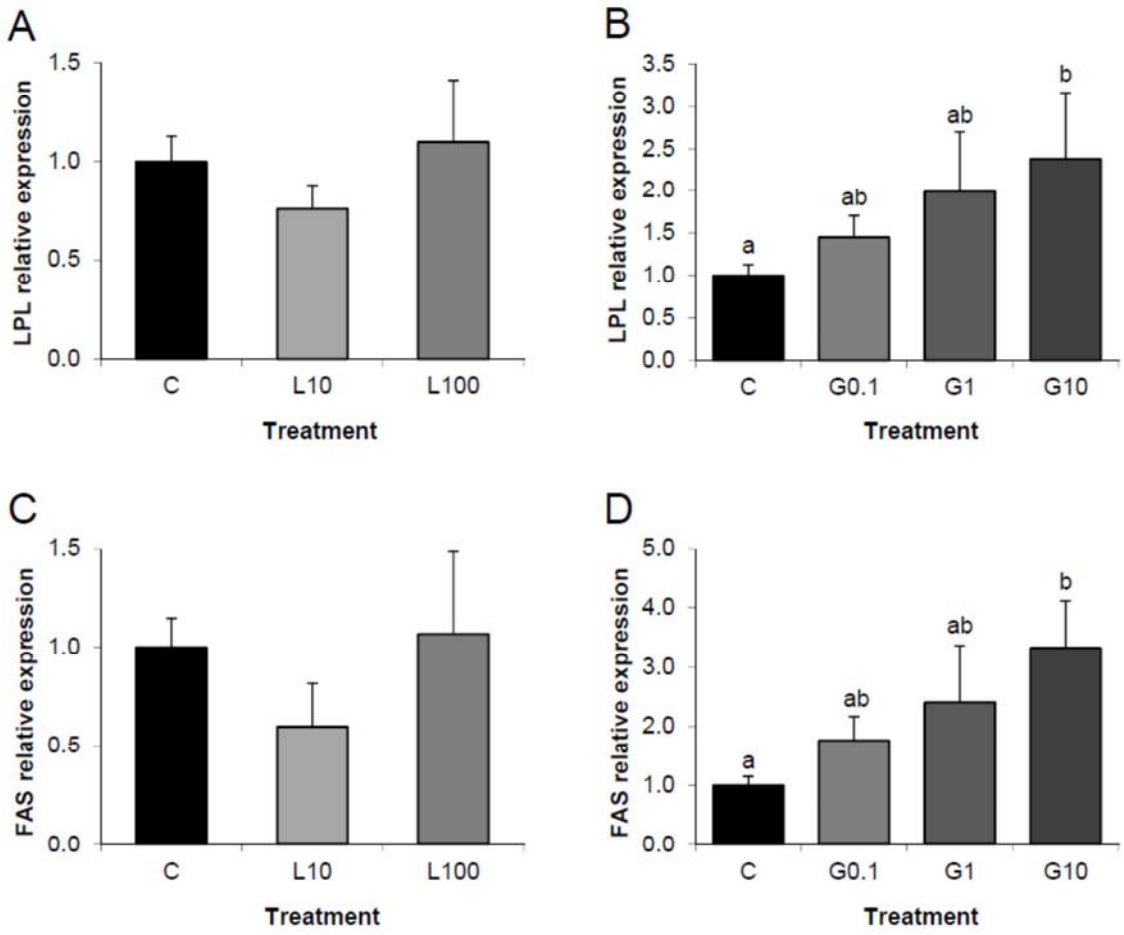


Figure 7. Expression of genes related to lipogenesis in rainbow trout isolated adipocytes treated with leptin or ghrelin. LPL **(A and B)** and FAS **(C and D)** quantitative relative expression normalized to EF1α of isolated adipocytes untreated (control, C) or treated during 3h with leptin 10 and 100nM (L10 and L100) or ghrelin 0.1, 1 and 10nM (G0.1, G1 and G10), respectively. Data are shown normalized to control adipocytes as mean ± SEM (n=5-11). Different letters indicate significant differences between treatments at $p<0.05$. LPL: lipoprotein lipase; FAS: fatty acid synthase; EF1α: elongation factor 1α.

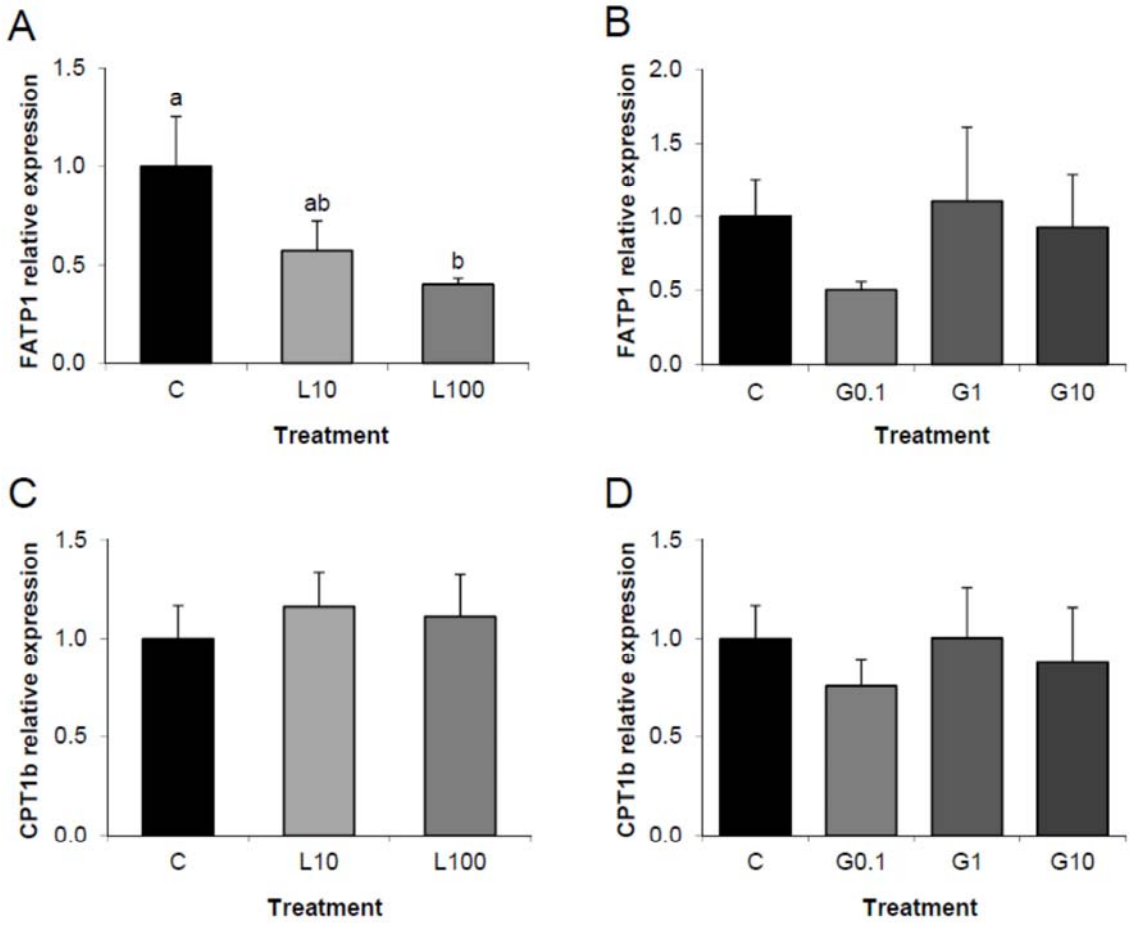


Figure 8. Expression of genes related to fatty acid transport and β-oxidation in rainbow trout isolated adipocytes treated with leptin or ghrelin. FATP1 **(A and B)** and CPT1b **(C and D)** quantitative relative expression normalized to EF1α of isolated adipocytes untreated (control, C) or treated during 3h with leptin 10 and 100nM (L10 and L100) or ghrelin 0.1, 1 and 10nM (G0.1, G1 and G10), respectively. Data are shown normalized to control adipocytes as mean ± SEM (n=4-11). Different letters indicate significant differences between treatments at $p<0.05$. FATP1: fatty acid transporter 1; CPT1b: carnitine palmitoyltransferase 1b; EF1α: elongation factor 1α.

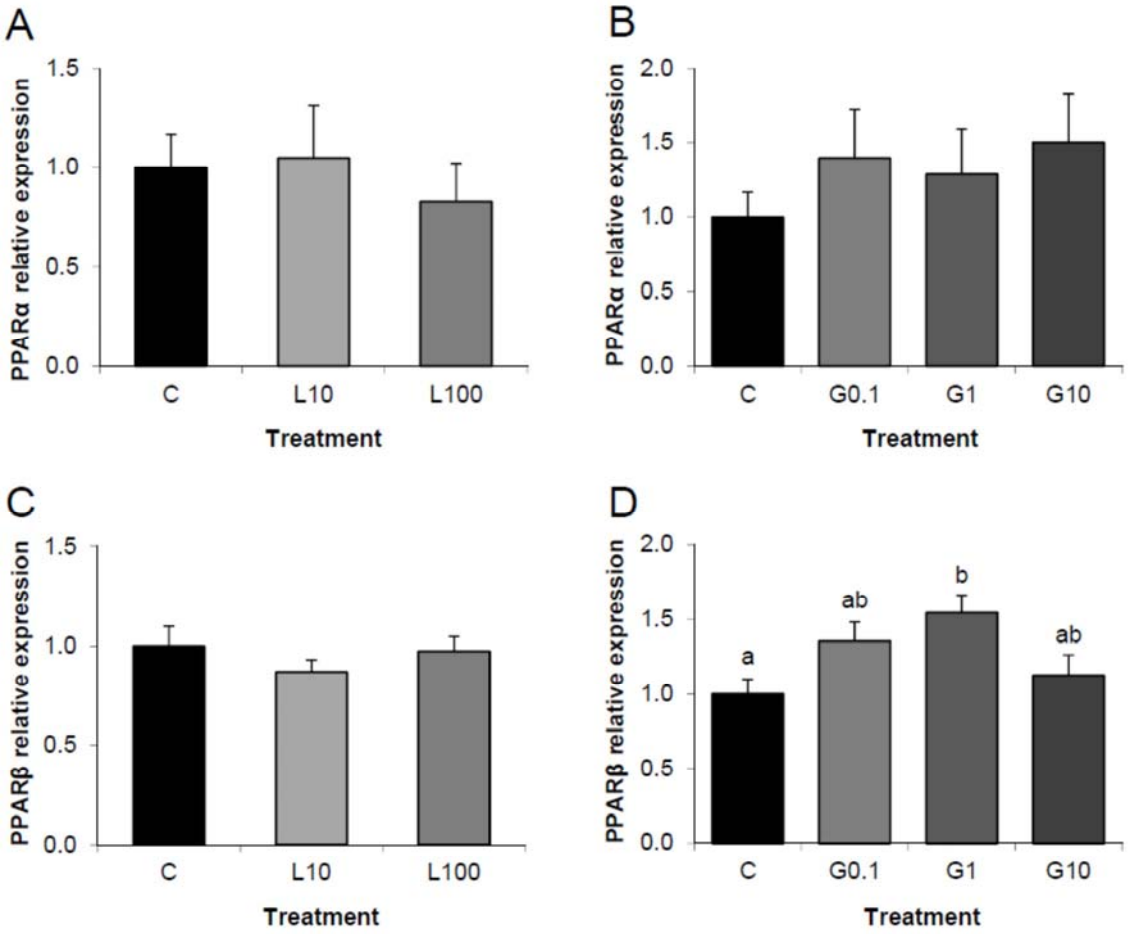


Figure 9. Expression of transcription factors in rainbow trout isolated adipocytes treated with leptin or ghrelin. PPARα **(A and B)** and PPARβ **(C and D)** quantitative relative expression normalized to EF1α of isolated adipocytes untreated (control, C) or treated during 3h with leptin 10 and 100nM (L10 and L100) or ghrelin 0.1, 1 and 10nM (G0.1, G1 and G10), respectively. Data are shown normalized to control adipocytes as mean ± SEM (n=5-11). Different letters indicate significant differences between treatments at $p<0.05$. PPARα: peroxisome proliferator-activated receptor α; PPARβ: peroxisome proliferator-activated receptor β; EF1α: elongation factor 1α.

4. Discussion

In the present study we have demonstrated the presence of ghrelin and its receptor, as well as the expression and secretion of leptin in rainbow trout cultured adipocytes. We have shown for the first time in a fish species that although leptin and ghrelin do not seem to control adipogenesis, both have direct effects on different parameters related with adipocyte lipid metabolism, and have suggested that some of these actions can occur in an autocrine or paracrine manner.

4.1. Presence of leptin and ghrelin in cultured preadipocytes

We have demonstrated in this study the presence of ghrelin and the GHS-R1a receptor by immunofluorescence in cultured differentiated adipocytes of rainbow trout, while preadipocytes were not tested. Ghrelin is mainly produced by the stomach in vertebrates (Castañeda et al., 2010; Jönsson, 2013), and in fish, one report in Atlantic salmon detected weak gene expression of ghrelin in visceral AT (Murashita et al., 2009). On the other hand, GHS-R1a is mainly detected in the pituitary in mammals, but in fish, one study in Mozambique tilapia detected also GHS-R1a transcripts in AT (Kaiya et al., 2009). Moreover, besides we were not able to detect the presence of leptin and its receptor with the antibodies tested by immunofluorescence; previous studies demonstrated their presence in AT by means of western blot analysis (Gong et al., 2013; Pfundt et al., 2009). All these data confirm that leptin and ghrelin are able to interact with the AT, and suggest that apart from the endocrine effects, an autocrine role is possible for both hormones.

In line with previous observations, the present study has also demonstrated that LepA1, but not LepA2 mRNA is expressed in rainbow trout adipocytes, as well as that leptin is produced and secreted from these cells. These results are in agreement with previous works on mammalian cultured adipocytes and 3T3-L1 cells (Matson et al., 1996; Sheng et al., 2013; van Harmelen et al., 2002), and with observations in primary cultured adipocytes of Atlantic salmon (Vegusdal et al., 2003). Notwithstanding, this is the first study that shows leptin expression and secretion in preadipocytes, since undifferentiated adipocytes in mammals do not present this competence (Matson et al., 1996). Furthermore, LepA1 expression as well as the amount of leptin secreted into the media increased in our culture during adipocyte differentiation, suggesting a more important role for leptin as an endocrine signal originated by mature adipocytes.

4.2. Effects of leptin and ghrelin on preadipocyte proliferation and differentiation

Regarding the endocrine regulation of preadipocyte proliferation, we observed a significant stimulation with insulin, our positive control, according to the facilitatory actions of insulin reported previously in preadipocytes from mammals (Géloën et al., 1989; Grimaldi et al., 1983) and other fish species (Bouraoui et al., 2008; Li, 2012; Oku et al., 2006; Salmerón et al., 2013; Wang et al., 2012), which confirms that the effects of insulin as a growth factor are maintained among vertebrates. However, proliferation of rainbow trout preadipocytes in our study remained unchanged in response to the treatments by leptin or ghrelin at 100 and 10 nM concentrations, respectively. In mammals, several studies have shown that ghrelin stimulates

preadipocyte proliferation at concentrations that range from 0.1 pM to 1 μM (Kim et al., 2004; Zhang et al., 2004; Zwirska-Korczala et al., 2007). On the other hand, the effects of leptin are controversial. In rat preadipocyte primary culture, leptin increased proliferation at 50 ng/ml concentration, whereas decreased it at 250 and 500 ng/ml (Wagoner et al., 2006). Other studies in 3T3-L1 cells, showed that leptin at concentrations from 10 pM to 1 μM caused a significant reduction in proliferation (Zwirska-Korczala et al., 2007), whereas another study showed no effects either with 5, 50 or 500 ng/ml leptin (Kim et al., 2008). Therefore, leptin in fish might show a wide array of effects depending on time and dosage as in mammalian preadipocytes, whereas it seems that the role of ghrelin stimulating proliferation of preadipocyte cells has not been conserved between fish and mammals.

In our study, neither leptin nor ghrelin were able to induce differentiation of preadipocytes measured as cell lipid accumulation. In terms of gene expression regulation, leptin and ghrelin showed a tendency to decrease PPARγ expression in comparison to DM alone. In agreement with this, some studies in rat primary preadipocytes and undifferentiated 3T3-L1 cells have demonstrated that leptin had no effect on TG accumulation or GAPDH activity (Kim et al., 2008; Wagoner et al., 2006); and in murine primary adipocytes, a study showed also that leptin inhibits the rosiglitazone-induced PPARγ expression (Rhee et al., 2008). Regarding ghrelin effects, the majority of studies have shown stimulation of adipocyte differentiation by ORO staining and PPARγ mRNA expression in rodent preadipocytes (Choi et al., 2003; Liu et al., 2009), although Zhang et al., (2004) have reported in 3T3-L1 cells that ghrelin inhibits differentiation, suppressing PPARγ mRNA and protein expression, and stimulating on the other hand, cell proliferation.

Furthermore in our study, only the addition of lipids to the DM significantly induced adipocyte lipid accumulation. These results are in agreement with previous studies in primary cultured preadipocytes in fish where the presence of lipids has a much more pronounced effect stimulating adipocyte differentiation than hormones, suggesting that one or more components of the lipid mixture may act as adipogenic factors (Bouraoui et al., 2008; Li, 2012; Oku et al., 2006; Salmerón et al., 2013; Vegusdal et al., 2003). It is well known that FFAs bind to PPARγ, the master regulator of adipogenesis, to stimulate transcription of target genes (Krey et al., 1997; Spiegelman, 1998; Yu et al., 2011); however in our study, the mRNA expression of PPARγ was not affected neither by lipids nor insulin. Thus, one possibility is that PPARγ is already highly activated and constantly expressed without responding to further stimuli, as previously shown in primary cultured adipocytes from red sea bream or Atlantic salmon (Oku and Umino, 2008; Vegusdal et al., 2003).

On the other hand, the mRNA expression of genes involved in fatty acid metabolism such as FATP1 and LPL (Gregoire et al., 1998) was up-regulated with lipid mixture incubation, while the addition of leptin significantly decreased LPL expression. We have previously reported that the expression of both genes increase during culture development in rainbow trout, confirming that they could be good markers of adipocyte differentiation also in fish (Bouraoui et al., 2012b; Sánchez-Gurmaches et al., 2012). Thus, our results suggest a possible inhibitory role for leptin in rainbow trout adipocyte differentiation. Moreover in our study, ghrelin did not modify the expression of neither FATP1 nor LPL, contrary to previous studies in human adipocytes that showed that ghrelin increases the expression of fat storage-related genes such as FAS and LPL (Rodríguez et al., 2009). In fish, a previous study showed that troglitazone (a PPARγ activator) required the presence of insulin to modulate adipogenesis in rainbow trout, increasing significantly the expression of LPL (Bouraoui et al., 2012a); and another study in red sea bream, showed also increased LPL mRNA expression using a combination of insulin with cortisol (Oku et al., 2006). Thus, the fact that ghrelin did not affect LPL expression in fish, might suggest that its role on adipocyte development needs of the interaction with other factors.

4.3. Effects of leptin and ghrelin on lipid metabolism in isolated adipocytes

Leptin and ghrelin treatments significantly increased glycerol release unaccompanied with a proportional release of FFAs in rainbow trout isolated adipocytes. These data are in agreement with previous studies in mammals, where leptin stimulated lipolysis increasing glycerol without a proportional release of FFAs both, *in vivo* and *in vitro*, supporting the idea that FFAs are oxidized or re-esterified inside the adipocyte rather than exported (Li et al., 2010b; Tajima et al., 2005; Wang et al., 1999). Moreover, our results showed that ghrelin also stimulated lipolysis in isolated adipocytes, contrary to that generally observed in rat adipocytes, where ghrelin has been shown to have anti-lipolytic effects by inhibiting isoproterenol-induced lipolysis (Choi et al., 2003; Muccioli et al., 2004). Nevertheless *in vivo*, it is well-known that ghrelin stimulates the release of GH in the pituitary, which then increases lipolysis in vertebrates AT (Kojima et al., 2001; Lucidi et al., 2005). Thus, this suggests that in rainbow trout, ghrelin may stimulate lipolysis directly and indirectly via GH, since GH has been demonstrated to be lipolytic also in fish (Albalat et al., 2005a; Bergan et al., 2013). In the present study, HSL mRNA expression in isolated adipocytes showed a tendency to increase dose-dependently after incubation with ghrelin, but not after leptin treatment, suggesting that ghrelin may stimulate lipolysis directly through HSL, whereas leptin may act activating other lipases. For this reason, we also tested the expression of ATGL, a novel lipase that collaborates with HSL in the lipolytic process, and is modulated by leptin through different signaling

pathways in mammalian adipocytes (Lampidonis et al., 2011; Li et al., 2010b). This is the first time that the expression of ATGL has been reported in trout AT, although no regulation by either leptin or ghrelin was observed. Nevertheless, nothing is known about the mechanisms of action of leptin or ghrelin in fish AT, including the activation of key signaling pathways by modulating the phosphorylation of AMP-activated protein kinase (AMPK), a matter of great interest for future studies.

The expression of FAS and LPL showed a significant increase in response to ghrelin, but not with leptin, suggesting a lipogenic role for ghrelin in rainbow trout adipocytes. In agreement with this observation, a previous study *in vivo* in rats found that the mRNA expression of various fat storage-promoting enzymes such as LPL, acetyl-CoA carboxylase, FAS, and stearoyl-CoA desaturase was markedly increased in AT with central ghrelin infusion (Theander-Carrillo et al., 2006). On the other hand, FATP1 mRNA expression showed a significant dose-dependent decrease with leptin treatment, but did not change after ghrelin incubation. FATP1 has been reported to be hormonally and nutritionally regulated in the AT of rainbow trout, although its regulation appears to be very complex and species-specific (Sánchez-Gurmaches et al., 2011; Sánchez-Gurmaches et al., 2012). Thus our results, suggest that leptin decreases the transport of FFAs into the adipocyte, whereas both hormones would have no effects on the fatty acid oxidation mediated by CPT1b.

In this line, PPARα and PPARβ activate transcription of genes involved in lipid utilization pathways, including fatty acid uptake and oxidation, and thus, have been characterized as central regulators of mitochondrial lipid catabolism (Huss and Kelly, 2004; Leaver et al., 2008). In the present study, PPARα mRNA remained unchanged after leptin or ghrelin treatments, while PPARβ mRNA expression significantly increased with ghrelin, suggesting that ghrelin might regulate fat metabolism and oxidation through the activation of this transcription factor. Regarding other possible actions of PPARβ, it has been demonstrated using liposarcoma cell lines, that PPARβ directly represses leptin expression through the PPAR/RxR binding element in the leptin promoter (Wagner et al., 2011); however, LepA1 mRNA expression remained unchanged in our study independently of the treatment, suggesting that the increase of PPARβ mRNA expression observed with ghrelin incubation does not seem to exert a direct effect on LepA1 expression in this species.

In conclusion, the present study has shown for the first time the presence of ghrelin and its receptor in the AT of rainbow trout, and a direct effect of ghrelin enhancing lipolysis and the expression of lipogenesis and fatty acid oxidation genes, suggesting that ghrelin causes a

general activation of lipid metabolism by stimulating anabolic and catabolic pathways. On the other hand, leptin has been shown to stimulate lipolysis, and to decrease fatty acid uptake in adipocytes, supporting an anti-adipogenic role for leptin in rainbow trout. Therefore, this study indicates that both, leptin and ghrelin, regulate adipocyte energy homeostasis in fish, although more studies are required to better understand the pathways by which they exert their actions in AT. Overall, this will contribute to better understand lipid metabolism in fish, in order to prevent excessive fat accumulation to help improve the quality of the final product for aquaculture.

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CAPÍTOL V

Caracterització i regulació endocrina de la proliferació i la diferenciació del cultiu primari de preadipocits d'orada (*Sparus aurata*)

Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*)

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Caracterització i regulació endocrina de la proliferació i la diferenciació del cultiu primari de pre-adipòcits d'orada (*Sparus aurata*)

Es va establir el cultiu primari de pre-adipòcits d'orada (*Sparus aurata*) per tal d'adquirir coneixements sobre el desenvolupament del seu teixit adipós, ja que l'orada és una de les espècies marines més àmpliament produïdes en l'aqüicultura mediterrània. Els pre-adipòcits obtinguts a partir de cèl·lules de la fracció vascular i de l'estroma del teixit adipós van proliferar en el cultiu fins aconseguir la confluència al voltant de dia 8. En aquest moment, l'addició d'un medi adipogènic va promoure la diferenciació de les cèl·lules en adipòcits madurs, que van mostrar un citoplasma engrandit ple de gotes lipídiques. En primer lloc, la proliferació i la diferenciació cel·lular es van analitzar sota condicions control i adipogèniques. A continuació, es van avaluar els efectes de la insulina, de l'hormona del creixement (GH) i del factor de creixement semblant a la insulina tipus-I (IGF-I) sobre la proliferació cel·lular a dia 8. Tots els pèptids van estimular significativament la proliferació cel·lular després de 48 h d'incubació ($P<0.002$ per a la GH i l'IGF -I i $P<0.05$ per a la insulina), tot i que no hi va haver diferències entre les diferents dosis provades. Posteriorment, es van estudiar els efectes de la insulina i l'IGF-I en el manteniment de la diferenciació afegint-los al medi de creixement a dia 11, després de 3 dies de la inducció amb medi adipogènic. Els resultats van mostrar que l'IGF-I incrementa més la diferenciació que la insulina ($P<0.01$ per l'IGF-I en comparació amb el control). En resum, s'ha caracteritzat el cultiu primari de pre-adipòcits d'orada i s'han avaluat els efectes de diversos reguladors del creixement i desenvolupament. Aquest sistema cel·lular pot ser un bon model per estudiar el procés de l'adipogènesi en peixos, el qual pot ajudar a millorar la qualitat del producte d'aqüicultura.

Paraules clau: Teixit adipós, cultiu de cèl·lules de peix, adipogènesi, insulina, IGF-I.

Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*)

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Abstract

A preadipocyte primary cell culture was established to gain knowledge regarding adipose tissue development in gilthead sea bream (*Sparus aurata*), one of the most extensively produced marine aquaculture species in the Mediterranean. The preadipocytes obtained from the stromal-vascular cell fraction of adipose tissue proliferated in culture reaching confluence around day 8. At that time, the addition of an adipogenic medium promoted differentiation of the cells into mature adipocytes, which showed an enlarged cytoplasm filled with lipid droplets. First, cell proliferation and differentiation were analyzed under control and adipogenic conditions during culture development. Next, the effects of insulin, growth hormone (GH) and insulin-like growth factor-I (IGF-I) on cell proliferation were evaluated at day 8. All peptides significantly stimulated proliferation of the cells after 48 h of incubation ($P<0.002$ for GH and IGF-I and $P<0.05$ for insulin), despite no differences were observed between the different doses tested. Subsequently, the effects of insulin and IGF-I maintaining differentiation when added to growth medium were studied at day 11, after 3 d of induction with adipogenic medium. The results showed that IGF-I is more potent than insulin enhancing differentiation ($P<0.01$ for IGF-I compared to the control). In summary, a primary culture of gilthead sea bream preadipocytes has been characterized and the effects of several regulators of growth and development have been evaluated. This cellular system can be a good model to study the process of adipogenesis in fish, which may help improve the quality of the product in aquaculture.

Keywords: Adipose tissue, fish cell culture, adipogenesis, insulin, IGF-I.

1. Introduction

Global aquaculture production has increased in recent decades to meet an increasing human population's demand for fish and fishery products worldwide [1]. Some aquaculture practices, such as the use of hyperlipidic diets or new raw dietary materials, together with the low swimming activity of farmed fish, may have negative effects, such as reduced fish growth and animal welfare, and increased visceral adiposity [2,3]. Fat increment can induce precocious sexual maturation, alterations in lipid metabolism, as well as a change in the fatty acid profile of the flesh [4,5].

Fat mass expansion can occur either by hypertrophy (increase in size of existing adipocytes) or by hyperplasia (increase in cell number by proliferation of precursor cells). At least in mammals, it is well known that mature adipocytes arise from multipotentmesenchymal stem cells (MSCs) after commitment and differentiation [6]. In fish, primary cultures of preadipocytes have been developed in the last years from Atlantic salmon [7], red sea bream [8], rainbow trout [9], grass carp [10] and the large yellow croaker [11]. Since in the Mediterranean area, gilthead sea bream (*Sparus aurata*) is one of the most important commercialized species, we were interested in the development of an *in vitro* system to study adipogenesis in this marine species to help improve the quality of the aquaculture product.

In fish, as in mammals, growth hormone (GH) is the main factor regulating growth, as it has been clearly demonstrated using GH acute injections or long term treatments with implants, as well as with transgenic approaches [12-15]. However, most of the GH growth-related functions are mediated through the insulin-like growth factor-I (IGF-I), which is produced primarily in the liver, but also in other tissues in response to GH stimulation. *In vivo*, IGF-I plasma levels have been clearly correlated with body weight and length in chinook and coho salmon [16,17]. IGF-I is structurally and functionally related to insulin, a hormone also involved in many regulatory functions [18,19], which plasma levels had been also correlated with body mass in salmonids [20,21].

At the cellular level, in mammals IGF-I is considered to be the major regulator of proliferation and differentiation of preadipocytes [22]. In fish, several *in vitro* studies have also demonstrated the effects of IGF-I stimulating cell proliferation, including gilthead sea bream myocytes [23] and osteoblasts [24], and rainbow trout adipocytes [9]. On the other hand, the mitogenic effects of insulin are contradictory, since positive effects were observed in eel cartilage [25] and large yellow croaker preadipocytes [11], but no significant effects were observed in zebrafish cells [26] or rainbow trout myocytes [27]. Regarding differentiation, the

positive role of IGF-I has been well reported in mammals; both in cell lines, such as the mouse 3T3L1 cells [28,29], as well as in primary cultures of preadipocytes of human, rat and porcine origin [30,31].

The objective of the present study was to establish a primary culture of preadipocytes from gilthead sea bream; and to analyze the roles of insulin, GH and IGF-I in cell proliferation and differentiation, in order to better understand the process of adipogenesis in this marine species, which is of high commercial value.

2. Materials and methods

2.1 Establishment of the preadipocyte primary culture

All plasticware for tissue culture was obtained from Nunc (Barcelona, Spain); and all reagents were purchased from Sigma-Aldrich (Tres Cantos, Spain) unless stated otherwise.

Gilthead sea bream (*Sparus aurata*) were obtained from the fish farm Tinamenor S.L. (Pesués, Cantabria, Spain) and maintained in the animal facility of the Faculty of Biology at the University of Barcelona. Fish were kept in 200 L fiberglass tanks under 12 h light/12 h dark photoperiod at 21±1ºC and fed *ad libitum* twice daily with a commercial diet (Skretting España S.A., Burgos, Spain). All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the European Union, Spanish and Catalan Government-established norms and procedures.

Following the protocol previously described for rainbow trout by Bouraoui et al. [9], we set up the conditions for culturing gilthead sea bream preadipocytes. We first tested multiple factors that could affect culture performance including fish size (50 or 500 g), base medium (Dulbecco's Modified Eagle Medium, DMEM or Leibovitz's, L15), NaCl concentration (30 or 60 mM), collagenase type (I or II), lysing of erythrocytes or not during the isolation procedure, cell density at the time of plating (2 to $5x10^4$ cells/cm2) and incubation temperature (18 or 23ºC) among others. Cells were routinely observed under the microscope and some proliferation assays were run. For example, the number of cells recovered from 500 g fish was low, and they did not proliferate as well as those from 50 g fish. Also, proliferation was higher in cells growing in DMEM in comparison to L15, as well as at a temperature of 23ºC in comparison to 18ºC. Results are not shown except for the NaCl concentration effects as an example (see Figure 2 in section 3.2).

The final protocol consisted of extracting the visceral adipose tissue of fish that had a body weight of approximately 50 g. Ten fish on average were used for each culture. Anesthetized

fish with ethyl 3-aminobenzoate methanesulfonate (MS-222, 0.1 g/L, Sigma A5040) were killed by a blow to the head and externally sterilized with 70% ethanol. Then, visceral adipose tissue was removed using autoclaved dissection material and collected in Krebs-Hepes buffer (pH 7.4) supplemented with 1% (vol/vol) antibiotic/antimycotic solution (A/A, Sigma A5955). Tissue was minced to small pieces by mechanical disruption with the use of two scalpels in sterile conditions inside a laminar air flow hood. Then, enzymatic digestion was performed by incubating the tissue with collagenase type II at 18ºC with gentle agitation (250 rpm) for 1 h in a Movil-Rod agitator (JP Selecta, Abrera, Spain). Collagenase at 130 U/mL was prepared in Krebs-Hepes buffer supplemented with 1% bovine serum albumin (BSA). After that, the stromal-vascular cell fraction was recovered, which includes in addition to preadipocytes other cell types such as MSCs, endothelial cells and macrophages. All these cells mostly disappear as the adipocytes progress in culture. Cells were then filtered through a 100 µm cell strainer to remove the large undigested tissue, washed with Krebs-Hepes buffer and centrifuged 10 min at 2000 X g in an oscillating rotor to get rid of mature adipocytes. Differently from Bouraoui et al. [9] cells at this point were not treated with an erythrocyte lysing buffer, since they proliferated better without this treatment, and the erythrocytes were easily removed from the culture with the first medium change because they did not attach to the plate. Finally, the cells were resuspended in growth medium (GM) and plated in gelatin-treated 12-wells plates at a density of $4.3x10^4$ cells/cm2 and incubated at 23°C with 2.5% CO2. Gelatin was used according to Bouraoui et al. [9] as it was established as a better substrate in terms of attachment and proliferation of the cells. The GM was composed of DMEM with 10% fetal bovine serum (FBS), 1% A/A and 60 mMNaCl, since the cells proliferated better with this NaCl concentration (see Figure 2 in section 3.2), in addition to present better appearance when observed under the microscope (data not shown).

2.2 Cell culture development characterization

Cells were maintained up to 21 d with fresh media replacement every 2-4 d. At day 8 after plating, once confluence was reached, the GM was either maintained or changed to an adipogenic medium (AM) containing GM plus 10 µg/mL insulin, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 0.25 µM dexamethasone and 5 µL/mL lipid mixture (that contained cholesterol and fatty acids from cod liver oil, Sigma L5146) to induce differentiation and test the ability of the preadipocytes to become mature adipocytes. Development of the cells under both growing conditions (GM and AM) was followed, and images were taken at different times using an Axiovert 40C inverted microscope (Zeiss, Germany) coupled to a Canon digital

camera. Specific assays to measure the degree of proliferation and differentiation of the cells were performed at different culture times as explained below.

2.2.1 Measurement of preadipocyte proliferation

Cell proliferation during culture development was analyzed using the assay based on the reduction by mitochondrial reductases of methylthiazolyldiphenyl-tetrazolium bromide (MTT) into formazan, as it was previously done for sea bream cells and pig primary adipocytes and mouse 3T3L1 cells [24,32,33] with minor modifications. The MTT solution (0.5 mg/mL final concentration, Sigma M5655) was added to the cells 18 h before the end of the experiment. Then, cells were washed with phosphate buffered saline (PBS) and the blue formazan crystals formed were resuspended in 150 µL of dimethyl sulfoxide (DMSO) per well and incubated for 3 h before spectrophotometric evaluation. Proliferation values were obtained subtracting the background read at 650 nm from the absorbance measured at 570 nm. Cells with PBS instead of MTT were used as nonspecific, and the value from this reading was also subtracted from all the other data. Results are presented as fold change with the absorbance values normalized to day 4.

2.2.2 Measurement of adipocyte differentiation

To study the ability of the preadipocytes to differentiate into mature adipocytes, cells were induced to differentiate by the addition of AM. To evaluate differentiation, accumulation of neutral lipids into the cells was observed by Oil Red O (ORO) staining following the protocol of Koopman et al. [34] previously adapted for gilthead sea bream adipocytes by Capilla et al. [24]. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm corresponding to cell protein content, which was obtained after Comassie blue staining for 1 h and dye extraction by incubation of the cells with 85% propylene glycol during 3 h at 60ºC [24]. Data are presented in fold change with the values normalized to day 12 cells growing in GM.

2.3 Endocrine regulation of preadipocyte proliferation

The effects of insulin, GH and IGF-I on cell proliferation were analyzed. On day 8, the cells were starved by changing the GM to a medium consisting on DMEM with 1% A/A and only 0.02% FBS for 5 h. After that, the medium was changed again to a DMEM containing 1% A/A, 2% FBS and the corresponding dose of peptide. GH was tested at 1 and 10 nM, IGF-I at 10 and 100 nM and insulin at 10, 100 and 1000 nM final concentrations. Porcine insulin was obtained from Sigma-Aldrich (Tres Cantos, Spain), recombinant human IGF-I was purchased from Bachem

(Weil am Rhein, Germany) and recombinant human GH from Genway Biotech Inc. (San Diego, USA). We used mammalian peptides due to availability, and because their mitogenic effects in fish cells have been demonstrated to be similar than those caused by their homologous counterparts [23,35]. Proliferation was measured by the MTT assay as explained above by adding the MTT solution to the medium 30 h later, to end the experiments exactly after 48 h of peptide stimulation (counting the last 18 h in the presence of MTT). Data were obtained from the absorbance readings as described above. Values are presented in fold change respect to control condition without peptide.

2.4 Endocrine regulation of adipocyte differentiation

To analyze the endocrine regulation of cell differentiation, cells at day 8 were first induced to differentiate with AM for 3 d. At day 11, the medium was changed again to GM containing either insulin (1000 nM), IGF-I (100 nM) or lipid mixture (5 µL/mL), which was used as a positive control condition, or a combination of lipid mixture with insulin or IGF-I. Lipid accumulation in the cells was assayed 5 or 10 d later using the protocol of ORO staining and extraction. Data were obtained from the absorbance readings as described above and presented in fold change respect to control cells with only GM.

2.5 Statistical analysis

Results are presented as mean ± SEM. To perform the statistical analyses, first the data were log-transformed. Then, it was confirmed that the data were normally distributed according to the Shapiro-Wilk test and that presented homogeneity in the variance according to Levene's test. Finally, statistical differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. When the data did not follow the ANOVA presumptions, the non-parametric Kruskal-Wallis followed by Mann-Whitney tests were performed. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1 Morphological characterization of cell culture development

In the present study we have established a primary culture of preadipocytes from gilthead sea bream as a model of study. Once the culture conditions were set up, we proceeded to characterize cell growth and development. First, we observed the cells routinely under a microscope to determine morphology.

Figure 1 shows representative images of the cells at different culture days growing in GM or AM. One day after plating, the cells attached corresponding to preadipocytes presented the characteristic small triangular appearance of MSCs (Figure 1, panel a).

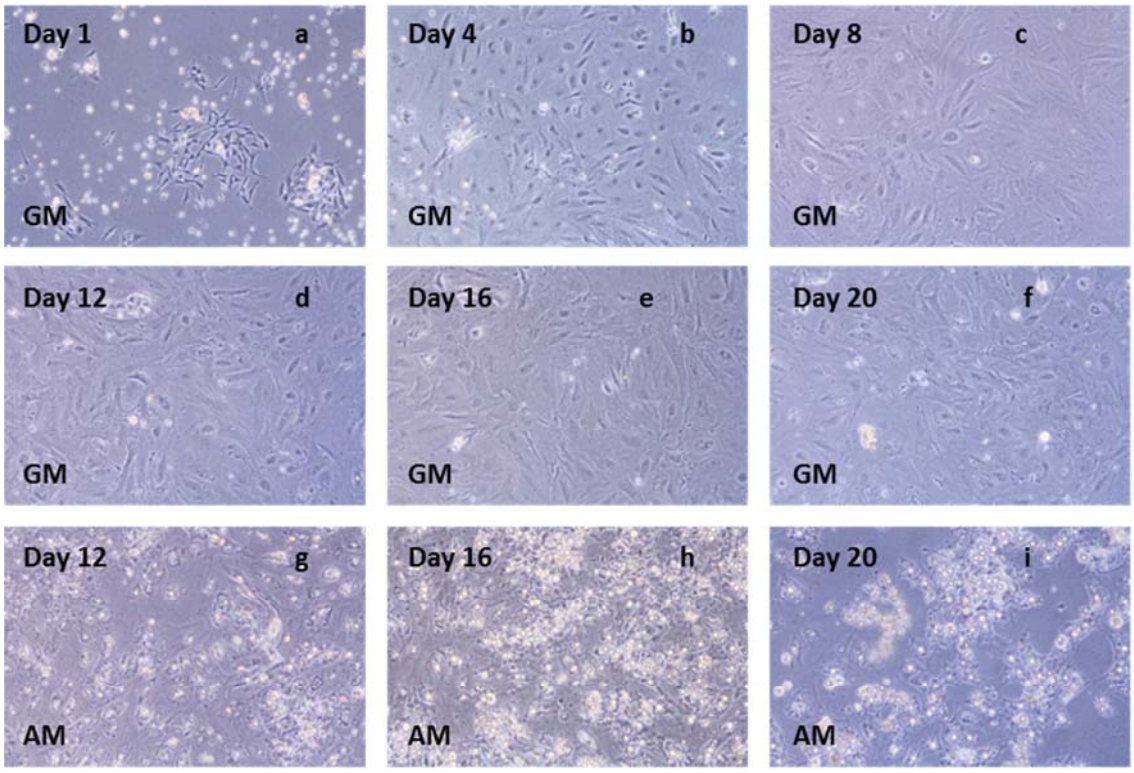

Figure 1. Representative phase-contrast images of gilthead sea bream preadipocyte cells at different days of culture (1, 4, 8, 12, 16 and 20). The cells were growing in control (GM, panels a to f) or adipogenic conditions (AM, panels g to i) as described in section 2. Magnification: 10x.

The majority of other cell types present in the stromal-vascular cell fraction including erythrocytes, macrophages or endothelial cells did not attach and were washed with the medium change. From there, preadipocytes in GM proliferated showing an elongated appearance already observed at day 4 (Figure 1, panel b). This shape was maintained in the cells growing in GM up to day 20 (Figure 1, panels c to f). At day 8, when AM was added to some cells to induce the formation of adipocytes, these cells changed to a more rounded shape with an enlarged cytoplasm that became filled with lipid droplets (Figure 1, panels g to i), characteristic of mature cultured adipocytes.

3.2 Characterization of preadipocyte proliferation

Next, to investigate proliferation of the cells during the culture, MTT assays were performed in parallel to morphological studies. Previously, during the set up process, several culture conditions were tested to obtain the best performing results. Figure 2 shows the proliferation profiles obtained with cells growing at two different NaCl concentrations (30 or 60 mM). The cells presented similar profiles, but proliferation was higher in cells growing with 60 mM NaCl. In comparison to their own day 4 (corresponding to each NaCl concentration), significant differences were observed at days 12 ($P<0.001$) and 16 ($P<0.001$) for the cells in 60 mM NaCl, whereas with 30 mM NaCl these differences were only significant at day 16 ($P<0.004$) (Figure 2).

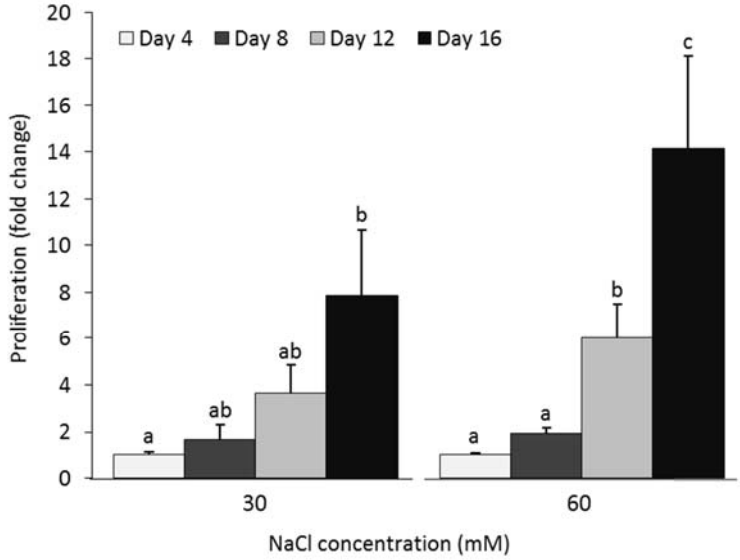

Figure 2. Proliferation profile of gilthead sea bream preadipocyte cells at different days of culture in media with two different NaCl concentrations (30 and 60 mM). Proliferation of cells growing in control (GM, days 4 and 8) or adipogenic conditions (AM, days 12 and 16) was measured using the MTT assay as described in section 2. Data are means ± SEM of 3-4 independent experiments with wells run in duplicate. Means with different superscripts differ ($P<0.05$), with each NaCl concentration analyzed separately.

Once the culture conditions were established, and following the characterization of the cells, MTT assays were run for cells growing in GM or AM up to day 20. Cells in GM showed increased proliferation from day 4 to day 12, with significant differences respect to day 4, both at day 8 ($P<0.02$) and at day 12 ($P<0.001$), and then reached a plateau (Figure 3). From days 12 to 20, there were no significant differences with time within the incubation media (GM or AM); however, at day 20 there was a significantly higher number of cells in AM in comparison to GM

(P<0.01) (Figure 3), suggesting that some component in this medium may be stimulating cell proliferation.

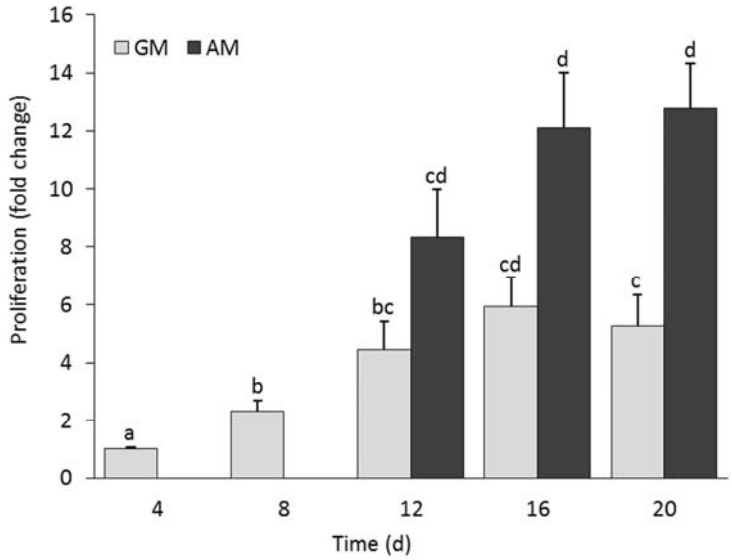

Figure 3. Proliferation profile of gilthead sea bream preadipocyte cells at different days of culture (4, 8, 12, 16 and 20). Proliferation of cells growing in control (GM) or adipogenic conditions (AM) was measured using the MTT assay as described in section 2. Data are means ± SEM of 3-4 independent experiments with wells run in duplicate. Means with different superscripts differ (P<0.05).

3.3 Characterization of adipocyte differentiation

To further characterize the cell culture, the ability of the cells to differentiate into mature adipocytes was studied using ORO to stain accumulated intracellular lipids at different days during the culture. A representative image of day 20 cells growing in GM or AM, and stained with ORO is shown in Figure 4A. The difference in shape between cells growing on each media can be observed, and specially, the presence of several lipid droplets stained in red, in the cytoplasm of cells growing in AM, which have differentiated into adipocytes. The ORO quantification showed as expected, significantly higher lipid content in the cells growing in AM at all times respect to GM (P<0.001), with significant differences within control cells growing in GM at day 20 of culture (P<0.02) (Figure 4B).

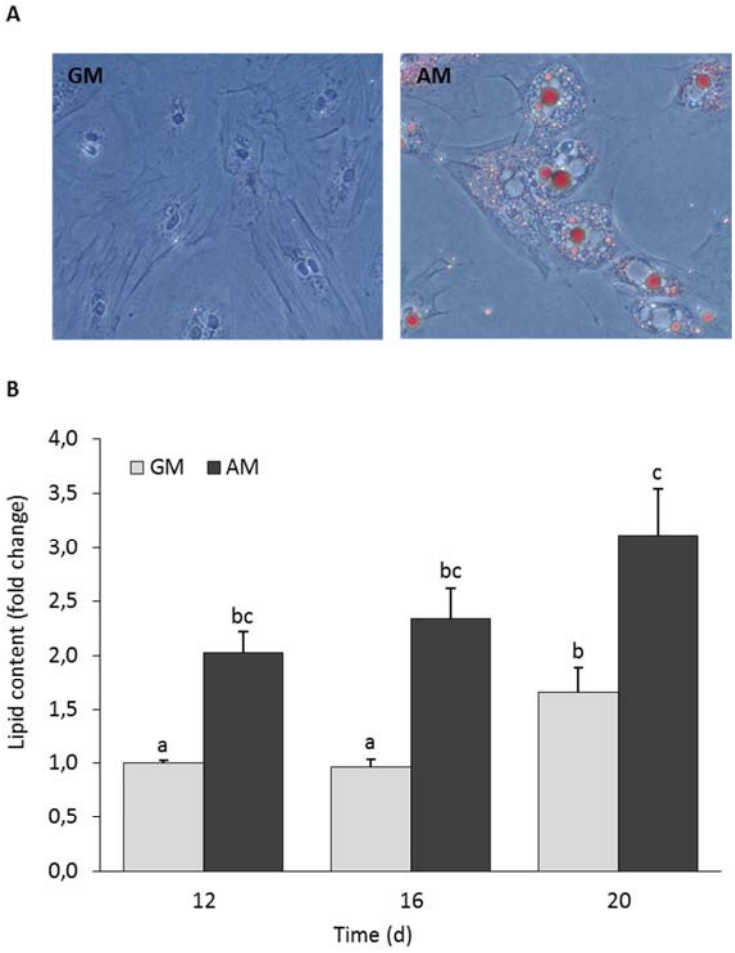

Figure 4. (**A**) Representative phase-contrast images of gilthead sea bream preadipocyte cells stained with Oil Red O at day 20 of culture growing in control (GM) or adipogenic conditions (AM). Magnification 20x. (**B**) Differentiation profile of gilthead sea bream preadipocyte cells at different days of culture (12, 16 and 20). Adipogenic differentiation of the cells growing under control (GM) or adipogenic conditions (AM) was measured by extraction of the Oil Red O stain as described in section 2. Data are means ± SEM of 6-7 independent experiments with wells run in duplicate. Means with different superscripts differ ($P<0.05$).

3.4 Insulin, GH and IGF-I effects on preadipocyte proliferation

The endocrine regulation of proliferation in the newly established gilthead sea bream preadipocyte cell culture was also studied. We tested the effects of different doses of insulin, GH and IGF-I after 48 h of incubation in day 8 cells. All three peptides significantly increased proliferation (measured with the MTT assay) compared to the control situation ($P<0.002$ for GH and IGF-I and $P<0.05$ for insulin), but no concentration-dependence was observed for any of the hormones (Figure 5).

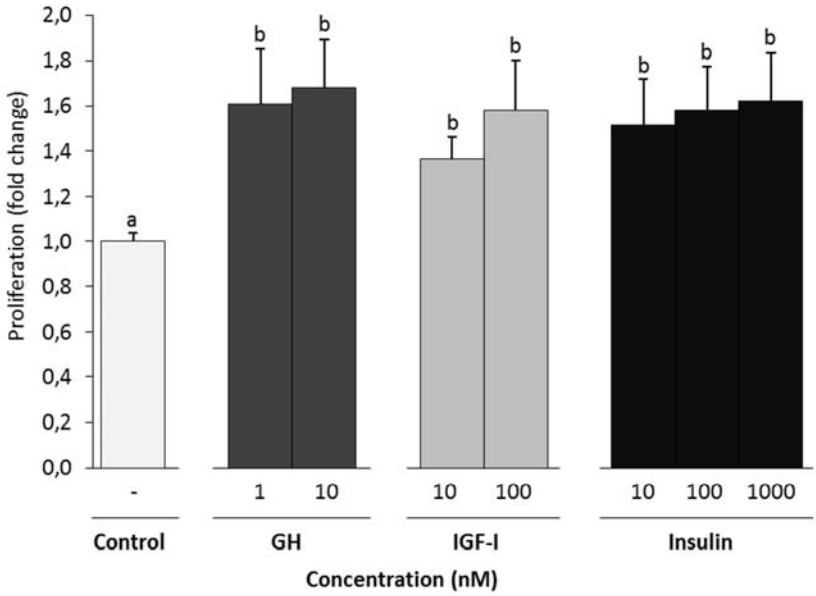

Figure 5. Effects of GH (1 and 10 nM), IGF-I (10 and 100 nM) and insulin (10, 100 and 1000 nM) on the proliferation of gilthead sea bream preadipocyte cells. On day 8, after 5 h of starvation (DMEM+0.02% FBS), stimulation with the different doses of peptides was performed in DMEM with 2% FBS for 48 h. Proliferation of the cells was measured using the MTT assay as described in section 2. Data are means ± SEM of 4-5 independent experiments with wells run in duplicate. Means with different superscripts differ (P<0.05), with each peptide analyzed separately but with the control in all cases.

3.5 Insulin and IGF-I effects on adipocyte differentiation

Finally, we studied the endocrine regulation of cell differentiation. The effects of insulin and IGF-I alone or in combination with lipid mixture increasing the differentiation of day 11 cells, previously induced to mature by the addition of AM for 3 d, were analyzed by means of ORO staining at two different time points. As expected, at day 16 the lipid mixture (used as a positive control condition) stimulated differentiation with respect to control cells (with GM only, P<0.001), and in a similar degree also it did IGF-I alone (P<0.01) (Figure 6A). On the contrary, insulin by itself was not able to increase lipid accumulation in the cells in comparison to the control cells with only GM at this time. The combination of lipid mixture with insulin or with IGF-I gave results similar to those obtained with lipid mixture alone (P<0.001 and P<0.01, respectively compared to the control), thus no additive effects of lipids and peptides were observed. In day 21 cells, the effects of lipid, alone or combined with either peptide, were similar to those observed at day 16 (Figure 6B). Regarding the peptides, no significant differences were observed after 10 d of incubation neither with insulin or IGF-I.

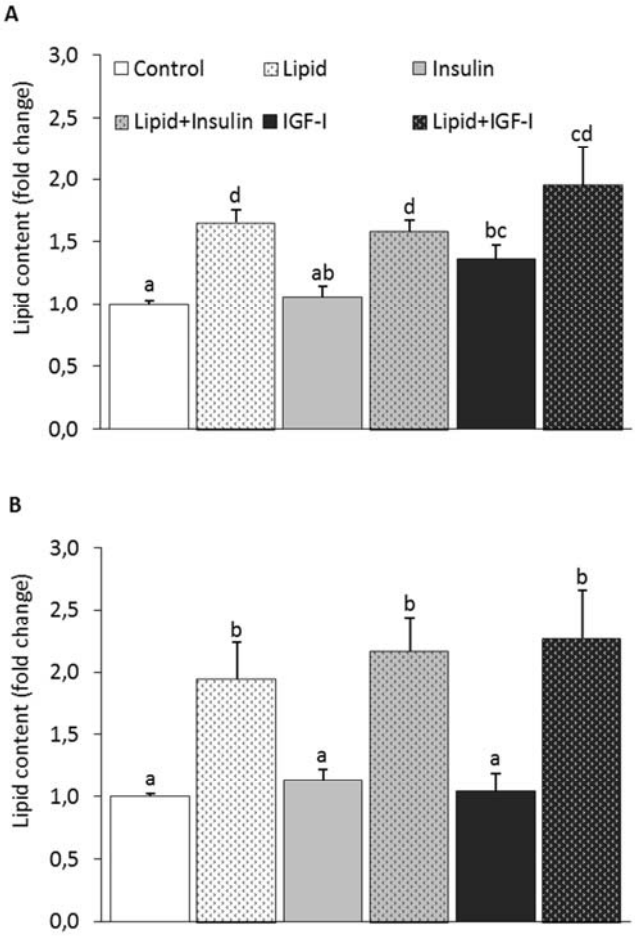

Figure 6. Effects of insulin (1000 nM), IGF-I (100 nM), lipid mixture (5 µL/mL) and combinations of lipid with insulin or IGF-I on the differentiation of gilthead sea bream preadipocyte cells. On day 8, the cells were induced to differentiate with complete AM medium (10 µL/mL lipid mixture) for 3 d. Then, stimulation of the differentiation with the lipid mixture or the peptides was performed in GM medium for 5 d (A) or 10 d (B). Adipogenic differentiation was measured by extraction of the Oil Red O stain as described in section 2. Data are means ± SEM of 4 independent experiments with wells run in duplicate. Means with different superscripts differ ($P<0.05$).

4. Discussion

Adipose tissue accumulation in fish can induce some problems in aquaculture including a decrease in efficiency production due to an increase in the proportion of tissue that is finally discarded, reducing the quality of the final product. In farmed gilthead sea bream, the quantity of visceral adipose tissue is notably higher in comparison with the content found in wild fish [36]. Visceral fat in gilthead sea bream is positively correlated with whole body fat content, according to a preferential perivisceral fat deposition [37]. Thus, increases in visceral fat depots can even result in metabolic alterations that potentially may affect fish welfare [2-4].

Therefore, a better knowledge on the regulatory factors that affect adipose tissue growth and development in fish is of great importance nowadays, especially in species such as gilthead sea bream with notable accumulation of visceral fat [36].

For the first time in gilthead sea bream, we have set up cell culture conditions to obtain differentiated adipocytes *in vitro* from this Mediterranean species. The difficulties to achieve the optimal conditions of incubation temperature, medium salinity, cell density or fish size to obtain good growth and development of preadipocytes revealed that species-specific culture characteristics are crucial. Specifically in gilthead sea bream, higher content of NaCl (60 mM) and higher temperature of culture (23ºC) than in rainbow trout (no NaCl added to the DMEM and 18ºC) [9] were needed; whereas more similar NaCl concentration and incubation temperature values (65 mM and 25ºC) were used to culture red sea bream preadipocytes [8]. These adjustments are in agreement with the natural living conditions of these species; besides as a euryhaline species, gilthead sea bream has been shown to adequately adapt to live in variable environmental salinities [38,39].

In a similar way, differences in culture conditions and medium composition to induce cells to differentiate have been observed in different mammalian species, chicken and in the few fish species where primary adipocyte cultures have been established. In mammalian preadipose cells, including cell lines, it is well known the use of a cocktail composed of insulin, dexamethasone and IBMX to induce mature adipocyte differentiation [40-42]. A common trait in fish is that lipids, especially high concentrations of fatty acids, play an essential role in adipocyte differentiation [7-11], while the presence of lipids is not so crucial for the differentiation of preadipocyte cell lines, such as the 3T3L1 cells [40,41], or primary preadipocytes derived from distinct fat depots of humans, rodents, rabbits, or pigs [42-44]. However, activated PPARγ by fatty acids and other specific ligands has been described to be involved in the differentiation-linked adipogenic gene expression in mammals [42]. In fish, the mechanism involved in the activation of cell differentiation by fatty acids has not been elucidated, and contradictory results have been found. PPARγ gene expression was activated by 2-bromopalmitate (a PPAR agonist) in red sea bream, but its expression level was not linked to adipocyte differentiation during cell culture [45], whereas PPARγ gene or protein expression was increased with adipocyte differentiation in trout and salmon [7,8,46]. Further studies are necessary to better understand the role of fatty acids in piscine adipocyte differentiation.

The observed profile of proliferation during the culture again was similar to that of red sea bream [8] but appeared to be slower than in rainbow trout [9], since the plateau for maximum

proliferation was reached after 7-9 d in rainbow trout whereas in gilthead sea bream 16 d were needed to achieve maximum levels of proliferation. Moreover, the increased level of proliferation observed after the addition of differentiation medium indicated that some cells may continue proliferating during the differentiation phase in gilthead sea bream adipocyte culture contrary to what has been well described for mammalian cells [6,42]. Nevertheless, morphological changes throughout gilthead sea bream culture were very similar to those previously described for other cultured fish preadipocytes [7-11], changing from a fibroblast appearance to the more rounded shape with an enlarged cytoplasm with lipid droplets accumulation, characteristic of differentiated cells.

Furthermore, despite a certain percentage of cells was able to differentiate spontaneously into adipocytes, evidenced by the increase in lipid content in cells maintained in only growth medium by day 20, the adipogenic medium used clearly enhanced lipid accumulation in the cells and induced the morphological change to mature adipocytes. This is a characteristic of this type of primary culture, and also of adipocyte cell lines such as the 3T3L1 cells [40,41], whereas other cell models differentiate spontaneously *in vitro*, such as the cultured myocytes derived from muscle isolated enriched satellite cells [23,27,35,47]. Regardless the species analyzed, proliferated cells from stromal-vascular fraction need the addition of a cocktail or medium of differentiation to completely differentiate in a relatively short time in culture. This makes the preadipocyte culture an interesting model to study the effects of hormones on proliferation and differentiation into mature adipocytes. Besides, this is not only interesting from a point of view of *in vitro* studies, since new adipocytes form constantly to replace lost adipocytes such that approximately 10% of total human adipocytes are renewed every year [48]. And also, it is well established that changes in circulating hormone concentrations can result in marked differences in adipose tissue growth [49,50], which is also very important in fish production. Therefore, in vitro studies like the present one can be the base to study the effects of other factors such as nutrients in adipose tissue development.

In the present study, stimulation of cell proliferation with GH incubation was similar to the increase in proliferating cells observed after treatment with insulin or IGF-I. Stimulatory effects of GH on preadipocyte proliferation have been described in mammals [42], but the effects were contradictory depending on cell model and species. In primary culture, GH has been shown to stimulate or to not affect preadipocyte proliferation in human and rat, but to reduce proliferation in pigs [51]. In addition, GH has been recently reported to be mitogenic in gilthead sea bream myocytes [23]. This hormone has been commonly shown species-specificity effects, and in the study of Rius-Francino et al. [23] sea bream GH resulted more potent than

human GH stimulating proliferation. Nevertheless, in our study even using human GH the effects on proliferation were very clear.

Besides the well-known direct effects of GH in lipid metabolism [52], the action of GH in proliferation appeared to be indirect, since GH markedly stimulated IGF-I production in rat preadipocytes, which in turn promoted cell proliferation [42]. Furthermore, additional studies have revealed that adipocytes recently differentiated from precursor cells in response to incubation with GH are much more sensitive to the mitogenic effect of IGF-I than precursor cells, resulting mainly in proliferation of young differentiated cells rather than in proliferation of preadipocytes [22]. In the present study we have not measured the production of IGF-I in cultured cells or its possible contribution to GH effects, neither in precursor cells or early differentiated adipocytes, but we have clearly observed a mitogenic effect with the addition of IGF-I in gilthead sea bream preadipocytes. This finding is in agreement with the literature, where IGF-I has been shown to promote proliferation in primary culture of pig preadipocytes [51], and rainbow trout cells [9]. In addition, IGF-I has been reported to stimulate proliferation of 3T3L1 preadipocytes [22] and bone marrow-derived human mesenchymal stem cells during differentiation into adipocytes [53]. Moreover, the same effect has been reported in other piscine cell models such as zebrafish embryonic cells [26], rainbow trout [27] and gilthead sea bream [23] myoblasts, and gilthead sea bream osteoblasts [24]. Moreover, human or trout IGF-I have been previously demonstrated to be equally effective in fish cells [35].

In addition to GH and IGF-I, insulin has been reported to be mitogenic on mammalian [54,55] and piscine cultured preadipocytes [8,11]; and this mitogenic effect has now been also confirmed in our gilthead sea bream preadipoctes. We have previously demonstrated the presence of both insulin and IGF-I receptors in trout adipose tissue [56], in trout preadipocytes in culture [57] and in sea bream muscle [58]. Although it has been suggested that insulin receptors are more important in metabolism, whereas IGF-I receptors mediate growth, this dichotomy is no so clear in fish [18,19,58]. Nevertheless, insulin at high concentrations could mimic the effects of IGF-I in gilthead sea bream preadipocyte proliferation as reported in mammals [22]. And a possible synergic effect between insulin and dexamethasone on cell proliferation cannot be discarded, as described in rat preadipocytes [59]. From the two main signaling transduction pathways shared by insulin and IGF-I receptors, the mitogen-activated protein kinase, MAPK pathway is preferentially stimulated by both peptides at early stages of cell culture in rainbow trout and gilthead sea bream myocytes [47,60] and rainbow trout adipocytes [57], which has been associated with increases in cell proliferation. Further studies

should be done in order to understand the mechanisms of action that mediate the mitogenic effects observed in gilthead sea bream cultured preadipocytes.

Regarding the endocrine regulation of differentiation, the observed positive effects of IGF-I on gilthead sea bream preadipocyte maturation, estimated as lipid accumulation, corroborated the evidence previously shown that IGF-I enhances the maturation of preadipocytes into adipocytes in many mammalian adipocyte models and species [28,29,31,61,62]. The fact that IGF-I was more effective than insulin enhancing gilthead sea bream preadipocyte differentiation at early stages (5 d of stimulation) does not mean that insulin is not necessary for the differentiation of these cells, since insulin was present in the adipogenic medium first used to induce differentiation. It has been previously demonstrated that the presence of insulin plus troglitazone (a peroxisome proliferated-activated receptor, PPAR gamma agonist of the drug class of thiazolidinediones known as an anti-diabetic agent) significantly increased lipid content in rainbow trout adipocytes maintained in growth medium; and insulin alone was able to stimulate lipoprotein lipase (LPL) expression, which is associated with the adipocyte phenotype, already at early stages of differentiation [8,63]. In red sea bream and the large yellow croaker, insulin was also able to increase lipid accumulation and glycerol-3-phosphate dehydrogenase, GPDH activity, and to stimulate LPL expression [8,11]. Therefore, it is possible that insulin stimulates gilthead sea bream adipocyte differentiation potentiating the effects of other adipogenic compounds present in the differentiation medium. On the other hand, only the addition of lipid mixture and not the presence of hormones enhanced lipid accumulation after 10 d, which suggested that hormones can trigger the process of differentiation, but once it is already switched on, the presence of hormones does not further induce more lipid synthesis and accumulation. Nevertheless, as mentioned before, the dependence on the presence of lipids or high concentration of fatty acids for differentiation appears to be crucial in fish and other species such as chicken, but not so much in mammals. The molecular bases for those species differences are unknown; among other, different sensibility of fatty acids to PPARs or distinct rates of lipogenesis could partially explain variations in the dependency of exogenous lipid supply for differentiation.

In conclusion, we have established a cell culture system to study the processes of proliferation and differentiation in gilthead sea bream adipocytes, and we have investigated the possible endocrine regulators of adipogenesis in this species. These results contribute to the knowledge on the hormonal control of adipose tissue growth and development in gilthead sea bream, and potentially in the improvement of aquaculture products' quality.

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Anexos

Articles publicats en el format de la revista:

Peer-reviewed publications in the journal format:

Article 1: Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*).

Autors: Cristina Salmerón, Daniel García de la serrana, Vanesa Jiménez-Amilburu, Ramón Fontanillas, Isabel Navarro, Ian A. Johnston, Joaquim Gutiérrez and Encarnación Capilla.

Revista: PLoS ONE

Factor d'impacte: 3.730 (Quartil 1)

Estat: Publicat

Article 5: Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*)

Autors: Cristina Salmerón, Laura Acerete, Joaquim Gutiérrez, Isabel Navarro and Encarnación Capilla.

Revista: Domestic Animal Endocrinology

Factor d'impacte: 2.377 (Quartil 1)

Estat: Publicat