Universitat Autònoma de Barcelona

ANÁLISIS MOLECULAR Y FUNCIONAL DE LAS SIRTUÍNAS COMO MARCADORES METABÓLICOS EN DORADA (SPARUS AURATA)

PAULA SIMÓ MIRABET

UNIVERSIDAD AUTÓNOMA DE BARCELONA

Departamento de Biología Animal, Biología Vegetal y Ecología

Tesis doctoral

ANÁLISIS MOLECULAR Y FUNCIONAL DE LAS SIRTUÍNAS COMO MARCADORES METABÓLICOS EN DORADA (*SPARUS AURATA*)

MEMORIA PRESENTADA POR
PAULA SIMÓ MIRABET

PARA OPTAR AL TÍTULO DE
Doctora por la Universidad Autónoma de Barcelona

PROGRAMA EN ACUICULTURA

Esta tesis se ha realizado en el Grupo de Nutrigenómica y Endocrinología del Crecimiento de Peces del Instituto de Acuicultura Torre de la Sal (IATS-CSIC) bajo la dirección del Dr. **Jaume Pérez Sánchez** y bajo la tutorización del Dr. **Francesc Padrós Bover** del Departamento de Biología Animal, Biología Vegetal y Ecología de la Universidad Áutonoma de Barcelona.

Enero 2020

Director
Jaume Pérez Sánchez
Instituto de Acuicultura
Torre de la Sal

Tutor
Francesc Padrós Bover
Universidad Autónoma
de Barcelona

Doctoranda
Paula Simó Mirabet

“Que tot està per fer

i tot és possible”

Miquel Martí i Pol

Agradecimientos

Mientras escribo el resumen de mis últimos 4 años no puedo creer que esto sea el final de una etapa y el comienzo de otra nueva. Durante este tiempo, han pasado muchas cosas que me han hecho aprender en todos los sentidos. Por este motivo, creo que este apartado es necesario para agradecer a todas aquellas personas el granito de arena que han aportado durante este camino.

En primer lugar, quiero agradecer a mi director Jaume la oportunidad que me ha dado de formar parte de Nutrigroup, por el aprendizaje y por las horas invertidas. A Josep por su apoyo y su paciencia. A mi tutor Sito por su ayuda durante todo este camino.

También quiero agradecer la oportunidad de conocer otro laboratorio y otra ciudad a la gente que me acogió durante mi estancia en Cádiz, gracias Juanmi, Gonzalo, Neda y Juan Antonio por hacerme sentir como en casa.

Por supuesto, a mis compañeros de despacho, que estuvieron y que están, por las charlas absolutamente necesarias tanto superficiales como profundas que hemos tenido en estos años. Necesito dedicarle unas líneas en especial a:

Juan Antonio y Vero, habéis sido mi apoyo, mis amigos y mi familia y ya para siempre. Os quiero y le doy gracias al universo por haberos conocido. Cinta y Lucia ha sido un placer conoceros, compartir con vosotras cada comida y cada conversación. Atuneros, habéis sido una pieza esencial en este camino. Erick, gracias por las charlas, las explicaciones y por tu apoyo. Nando, gràcies per ser com ets, has sigut

un recolzament molt important per a mi. Et trobaré a faltar perquè sempre tens un somriure a la cara que és tan necessari, no el perdis mai. Per cert, perquè vegis que sóc una noia de paraula: gràcies pels teus arbres filogenètics! Kike, has arribat l' últim però t' he d' agrair la comprensió y el recolzament que m'has donat. No m'oblido, tenim una cosa pendent.

Als meus amics de Barcelona, a la meva Tati, Humbi, Sergi i Laura per aquells retrobaments com si no hagués passat el temps.

A la meva segona família de Castellterçol, per totes aquelles estones compartides que m'han ensenyat que cal gaudir del petits moments que et dóna la vida. En especial a tu Sari, perquè el futur ens regali molts més moments juntes.

I ara ve l'agraïment més difícil de tots perquè no hi ha prou pàgines que el puguin contenir, intentaré ser breu. Als meus pares, els hi dec tot, l'educació, la confiança i l'amor que m'han donat sempre, perquè no han fallat mai. Sóc el que sóc per vosaltres i mai podré estar prou agraïda. Us estimo fins a l' infinit. Als meus avis, per haver-me cuidat, consentit i ensenyat uns valors. Als meus tiets, per estar sempre allà i sobretot per donar-me uns cosins increïbles. Als meus sogres, per haver estat allà sempre que els he necessitat. Finalment, al meu company de viatge, gràcies Xavi per cada minut viscut en aquests últims anys, per les converses i pels silencis tan necessaris. Gràcies per la paciència i per estar allà sempre. T'estimo molt! Per cert, gràcies Gaia per rebrem al tornar a casa com si no m' haguessis vist en anys i treure'm sempre un somriure.

Tabla de contenido

ABREVIATURAS

AADPR	2'- O-acetil-ADP-ribosa
AAE	aminoácido esencial
ADN	ácido desoxiribonucleico
ADP	adenosín difosfato
AG	ácido graso
AGCM	ácido graso de cadena media
AGE	ácido graso esencial
AGM	ácido graso monoinsaturado
AGP	ácido graso poliinsaturado
AKT	proteína quinasa B
ALA	ácido α-linolénico
AMPK	proteína quinasa activada por AMP
APE	endonucleasa apurínica/apirimidínica
ARN	ácido ribonucleico
AROS	regulador activo de SIRT1
ATP	adenosín trifosfato
BMAL1	receptor nuclear translocador de aril hidrocarburos en cerebro y músculo
C	citosina
CGI	isla CpG
ChREBP	elemento de respuesta a carbohidratos
CLOCK	circadian locomotor output cycles kaput
CMYB	proteína proto-oncogénica c-myb
CpG	dinucleótido citosina y guanina
CPS1	carbamoil-fosfato sintasa 1
CPT-1	carnitina palmitoiltransferasa-1
CRE	secuencia de unión a elementos de respuesta a AMPc
CREB	proteína de union a elementos de respuesta a AMPc
CRTC2	coactivador transcripcional regulado por CREB 2
C/EBP-α/β	proteína de unión al amplificador CCAAT alpha/beta
DBC1	deleted in breast cancer
DHA	ácido docosahexaenoico

DNMT	metiltransferasa de ADN
EGR1	proteína de respuesta temprana al crecimiento 1
ELOVL2/4	elongasa 2/4
EPA	ácido eicosapentaenoico
ERR-α	receptor nuclear relacionado con estrógenos
FABP	proteína de unión a ácidos grasos
FADS2	desaturasa de ácidos grasos 2
FOXO	forkhead Box O
G	guanina
GCN5	histona acetiltransferasa GCN5
GH	hormona del crecimiento
GOAT	grelina-o-acilransferasa
HAT	acetiltransferasa de histonas
HDAC	deacetilasa de histonas
HIF-1α/2α	subunidad alfa del factor 1/2 inducible por hipoxia
HSI	índice hepatosomático
HTM	metiltransferasas de histonas
IATS	Instituto de Acuicultura Torre de la Sal
Ig	inmunoglobulina
IGF-I	factor de crecimiento insulínico tipo I
KDAC	deacilasa de lisina
KLF2	factor 2 kruppel-like
LSD1	demetilasa de histonas 1 específica de lisina
mC	citosina metilada
mTOR	diana de rapamicina en células de mamífero
miARN	micro ARN
MRL	nivel máximo de residuo establecido y/o recomendado
MSI	índice mesentérico
MYC	MYC proto-oncogen
NA	ácido nicotínico
NAD^+	dinucleótido de nicotinamida adenina forma oxidadada
NADH	dinucleótido de nicotinamida adenina forma reducida
NAM	nicotinamida
NAMPT	fosforibosiltransferasa de nicotinamida

NCoR1	correpresor 1 de receptor nuclear
NF-kB	factor nuclear Kappa B
NMN	mononucleótido nicotinamida
NMNAT	mononucleótido nicotinamida adenilil transferasa
NRF1	factor respiratorio nuclear 1
OCP	organoclorado
PAH	hidrocarburo policíclico aromático
PARP	poli ADP ribosa polimerasa
PAX6	paired box 6
PCR	reacción en cadena de la polimerasa
PGC-1α	peroxisoma proliferador activado del receptor-γ co-activador 1α
PIT-1	factor de transcripción específico de la hipófisis anterior
POP	contaminante orgánico persistente
PPAR	receptor activado por el proliferador de peroxisomas
P300	histona acetiltransferasa p300
P53	proteína tumoral P53
QTL	locus de un carácter cuantitativo
ROS	especies reactivas de oxígeno
SCD1A	estearoil-coA desaturasa 1
SIRT	sirtuína
SMRT	mediador del silenciamiento de receptores de hormonas retinoideas y tiroideas
SP1	proteína de especificidad 1
STAT5	transductor de señal y activador transcripcional 5A
t	toneladas
T	timina
TSS	lugar de inicio de la transcripción
U	uracil
UCP	proteína mitocondrial desacoplante
USP7	proteasa específica de ubiquitina 7
XBP1	proteína de unión a la caja X 1

CAPÍTULO 1- Introducción

1.1. Acuicultura

1.1.1. Situación actual de la acuicultura

Los sistemas de producción primaria tienen como gran desafío proporcionar alimentos a una población que superará los 9.000 millones de personas a mediados del siglo XXI, al mismo tiempo que abordar los problemas derivados del cambio climático y de un uso más sostenible de los recursos disponibles. Por tanto, la pesca y en mayor medida la acuicultura, jugarán un papel esencial para cubrir la demanda creciente de alimentos a nivel mundial.

Las pesquerías se han estabilizado en los últimos 20 años en torno a los 90 millones de toneladas (t) anuales, habiéndose alcanzado los niveles máximos de explotación sostenible de los recursos pesqueros. Por tanto, la estabilización de las capturas, junto con el aumento de la demanda de productos acuáticos, deberá impulsar más si cabe el desarrollo de la acuicultura. De hecho, con el estancamiento de la actividad pesquera se ha disparado la producción de la acuicultura, alcanzándose un máximo histórico de 111,9 millones de t en 2017 (un 3,5% más que el año anterior), lo que supera en más de un 15% la producción derivada de la pesca. Es más, como muestra la **Fig. 1.1**, a partir de los años sesenta, la producción mundial de la acuicultura ha crecido de manera constante, aunque en los últimos años se ha producido un ligero decaimiento en el ritmo de crecimiento. Sin embargo, el sector sigue manteniendo una vigorosa tasa de crecimiento del 6-8% anual, aunque cabe destacar que la mayor parte de la producción está concentrada en Asia (91,9%) y el resto en América (3,2%), Europa (2,7%), África (2,0%) y Oceanía (0,2%).

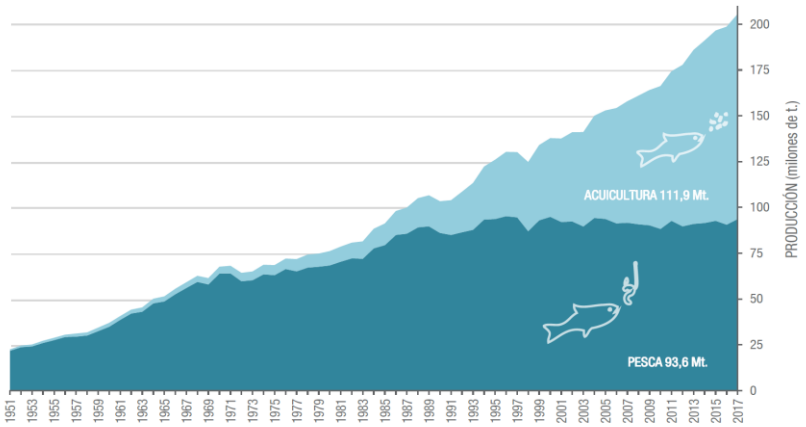


Figura 1.1 Evolución de la producción acuícola mundial (acuicultura más pesca) desde 1951 hasta 2017. Modificada de Informe APROMAR, 2019.

1.1.2. Demanda actual de productos de origen acuático a nivel mundial

Los océanos aportan casi el 50% de la biomasa animal y vegetal que se produce en el planeta. Sin embargo, los alimentos procedentes del mar solo constituyen el 2% de las calorías y el 15% de las proteínas consumidas (Informe "Alimentos procedentes de los océanos"; disponible en http://bit.ly/2oWMzGP).

Entre 1961 y 2016, el consumo de pescado aumentó anualmente en un 3,2% superando el crecimiento de la población mundial (1,6%) y el consumo de proteína animal terrestre (2,8%) (FAO, 2018). Por consiguiente, el consumo *per cápita* de pescado aumentó de 9 kg en 1961 a 20,5 kg en 2017, con una tasa media de crecimiento anual del 1,5%. Este incremento no solo se debe al aumento de la producción, sino también a otros factores como la mejora de las técnicas de conservación del pescado, la reducción de los desechos y a una distribución y comercialización más eficiente de un producto de elevado valor

nutricional por su alto contenido en proteínas digestibles con un buen aporte en aminoácidos esenciales (AAE), además de vitaminas, minerales y ácidos grasos esenciales (AGE) (omega-3; ácido docosahexaenoico (DHA) y eicosapentaenoico (EPA)). A pesar de ello, el margen de mejora sigue siendo alto ya que, según la FAO, en 2015 el consumo de pescado tan solo representó el 17% de la proteína animal consumida. En la medida que esta situación se vaya revirtiendo, se conseguirá una mejora de los hábitos alimentarios a la vez que una producción de proteína animal más ética y sostenible con el medio ambiente. Como prueba de ello, el consumo de agua para producir un kg de cerdo o ternera varía entre 5-10 m^3, disminuyendo hasta 3-4 m^3 para los huevos y las aves y 1-2 m^3 en el caso de los productos lácteos y el pescado (**Fig. 1.2**).

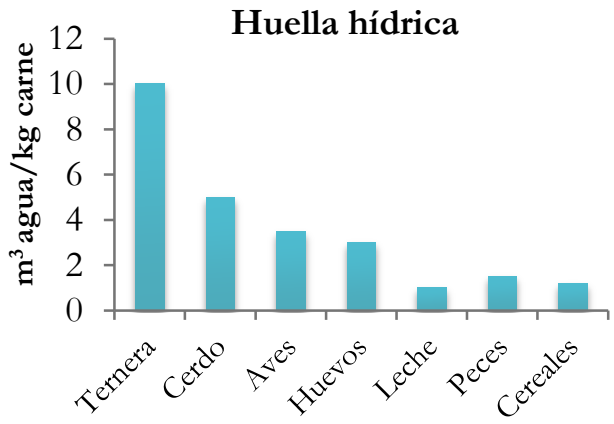


Figura 1.2 Consumo de agua para la producción de carne y derivados en m^3 de agua por kg de carne. Datos de Pahlow et al., 2015 y Water Footprint Network disponible en https://waterfootprint.org.

1.1.3. Estado de la acuicultura en España

La acuicultura europea representa una importante fuente de productos acuícolas. Aun así, solo constituye el 19,2% de la producción, frente al 80,8% de la pesca extractiva. Sin embargo, en algunos países de la Unión Europea, la relevancia económica y social de la acuicultura ya supera la de la pesca, tal y como ocurre en algunas Comunidades Autónomas españolas. De hecho, España es el estado miembro de la Unión Europea con una mayor producción en acuicultura (23% del total en 2017) seguido a cierta distancia por Reino Unido (16,4%) y Francia (12,3%). Sin embargo, cuando se normaliza por el valor de la producción, el Reino Unido ocupa el primer lugar y España el cuarto (Informe APROMAR, 2019).

En concreto, la cosecha de la acuicultura española en 2018 fue de 348.395 t de acuerdo con el siguiente desglose: mejillón (273.600 t), lubina (22.460 t), trucha arcoíris (18.856 t), dorada (14.930 t), rodaballo (7.450 t) y otras (11.099 t). En el caso de los peces marinos, el incremento de la producción española fue sostenido desde los años 80 hasta el 2009, donde sufrió un estancamiento que comenzó a revertirse en 2015 con la recuperación tras la última crisis económica (**Fig. 1.3**). A pesar de todo, el consumo doméstico de pescado se ha reducido progresivamente desde el 2010 en un 15,8%, siendo la merluza, la sardina, el salmón, el lenguado, el bacalao y el atún los pescados más demandados por los españoles. Sin embargo, hay cada vez una mayor aceptación de las especies típicamente cultivadas, con un incremento del consumo de lubina, dorada y rodaballo del 13,5%, 5,0% y 2,3% durante el 2018 (Panel de Consumo del MAPA).

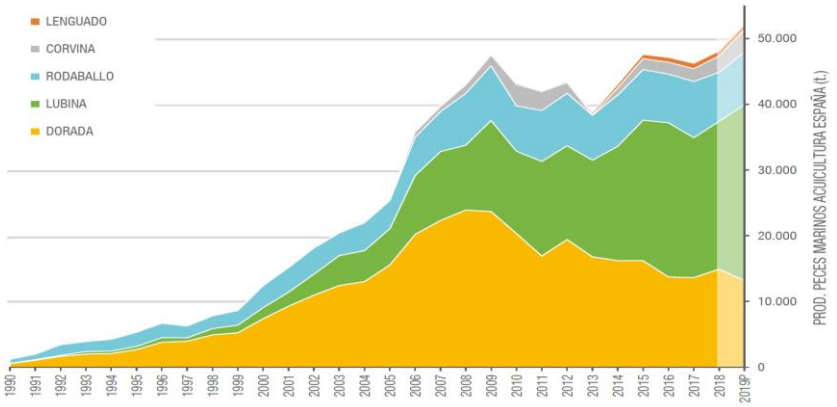


Figura 1.3 Evolución del cultivo de peces marinos (en toneladas) de acuicultura en España desde 1990 hasta 2019. Modificada del Informe APROMAR, 2019.

1.1.4. Retos de la acuicultura

En un contexto de limitada disponibilidad de recursos naturales, el progreso científico y la innovación son herramientas imprescindibles para poder aumentar la producción de proteína animal y satisfacer las necesidades de la población en un mundo globalizado con grandes desigualdades económicas. La acuicultura es posiblemente la mejor apuesta de futuro, aunque sigue siendo necesario incrementar el rendimiento de la producción de una manera sostenible y respetuosa con el medio ambiente. Por tanto, las investigaciones en curso deben hacer especial hincapié en la mejora de los conocimientos sobre el estado energético y la salud de los peces, así como en la optimización de las dietas y los métodos de selección genética y programación nutricional.

1.1.5. Evolución de las dietas de peces en cultivo

1.1.5.1. Materias primas y disponibilidad. Posibles efectos negativos

La acuicultura es actualmente el sector con una mayor demanda de harinas y aceites de pescado (Tacon y Metian, 2008; Naylor et al., 2009) con un consumo en 2012 del 68% y 74% del total de harinas y aceites disponibles (Tacon y Metian, 2015). Ello es consecuencia de los altos niveles de inclusión de ingredientes de origen marino en los piensos de acuicultura para cubrir los requerimientos nutricionales tanto de macronutrientes como de micronutrientes establecidos para las especies cultivadas (NRC, 2011). No obstante, los requerimientos de proteína son menores para los peces cultivados de agua dulce, que suelen ser omnívoros o herbívoros, por lo que el contenido en proteínas de sus dietas no supera el 25-35%. Por el contrario, los peces marinos cultivados suelen ser carnívoros y tienen mayores requerimientos proteicos (40-55%). Por tanto, teniendo en cuenta los datos de producción y los requerimientos propios de cada especie, los mayores consumidores de harinas de pescado son los camarones, el salmón y las especies de peces típicamente marinas (Tacon et al., 2011; Hasan, 2017). Las mismas consideraciones son válidas para los aceites, ya que las especies marinas poseen una capacidad limitada para sintetizar EPA (20:5n-3) y DHA (22:6n-3) a partir de ácido linolénico (ALA, 18:3n-3) (Bell y Tocher, 2009), al carecer de la Δ5 desaturasa y de la elongasa 2 (*elovl2*) (**Fig. 1.4**). Algunos autores han sugerido que la carencia de *elovl2* puede paliarse en parte por la acción de la elongasa 4 (*elovl4*) (Monroig et al., 2010; 2011), aunque los resultados en dorada muestran una expresión

relativamente baja de esta enzima a nivel hepático (Benedito-Palos et al., 2014).

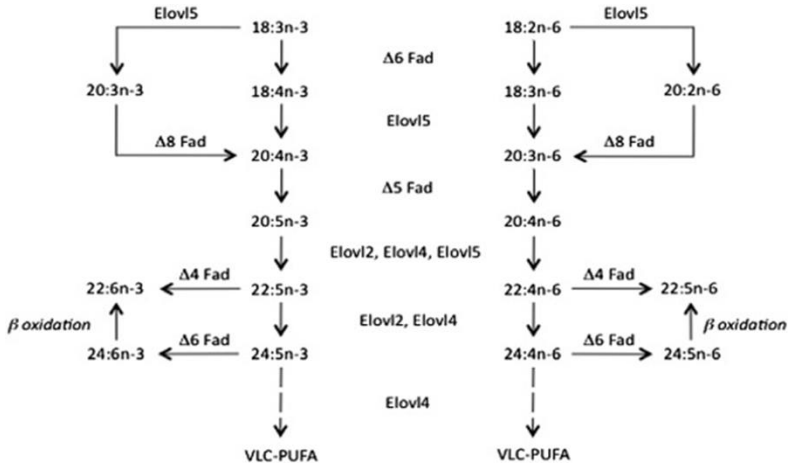


Figura 1.4 Ruta de biosíntesis de los ácidos grasos poliinsaturados de cadena larga a partir de los precursores de 18 carbonos, ácido α-linolénico (ALA; 18:3n-3) y ácido linoleico (LA; 18:2n-6). Modificada de Tocher, 2015.

De acuerdo con lo expuesto anteriormente, los peces de agua dulce (pero no los marinos) son productores netos de EPA y DHA, aunque su capacidad de síntesis y elongación a partir de ácidos grasos (AGs) de 18 átomos de carbono es igualmente muy limitada una vez cubiertos los requerimientos en AGEs de la especie. En otras palabras, para que los peces de cultivo sigan siendo la fuente más importante de EPA y DHA en la dieta humana, tanto las especies marinas como dulceacuícolas siguen requiriendo un aporte alto de EPA y DHA en la fase finalizadora (Trushenski y Bowzer, 2013). En esta fase, los cambios producidos en la composición en AGs del filete siguen un modelo de dilución simple, que se ha evidenciado tanto en salmónidos como en dorada y rodaballo (Robin et al., 2003; Jobling 2004a; 2004b; Benedito-

Palos et al., 2009). Es más, la composición del filete es altamente predecible por un modelo de regresión "dummy", con la composición en AGs de la dieta y el contenido graso del filete como variables independientes (Ballester-Lozano et al., 2014). Este modelo predictivo está disponible para dorada, lubina y lenguado a través del siguiente enlace (http://nutrigroup-iats.org/aquafat/index.php) (**Fig. 1.5**).

Figura 1.5 Vista de la página web que incluye la herramienta interactiva de predicción de la composición en ácidos grasos de peces.

En base a lo expuesto anteriormente, es necesario encontrar el equilibrio adecuado que permita cubrir las necesidades del consumidor y de la especie en cultivo, a la vez que un desarrollo sostenible del sector basado en el respeto por el medio ambiente. Ello forma parte de la estrategia para disminuir las emisiones de gases de efecto invernadero y la dependencia de las materias primas finitas de origen marino (Tacon y Metian, 2015; Hasan y Soto, 2017). En esta línea, las proteínas alternativas más usadas en los últimos años son las de origen vegetal, especialmente las procedentes de semillas oleaginosas (Tacon et al., 2011; Hasan y New, 2013; Little et al., 2016; FAO, 2018). Sin embargo,

formulaciones inadecuadas o lotes con altos niveles de factores antinutricionales pueden desencadenar efectos no deseados sobre la ingesta, la acreción proteica, la salud intestinal o la respuesta inmune en sentido amplio (Francis et al., 2001; Krogdahl et al., 2010; Hajra et al., 2013). Todo ello, junto con la competencia con otros sectores (cosmética, alimentación humana, ganadería, producción de biodiesel, etc.) (Gasco et al., 2018) está promoviendo la búsqueda de otras fuentes alternativas basadas en hidrolizados proteicos (Martínez-Álvarez et al., 2015), microalgas (Byreddy et al., 2019), macroalgas (Wan et al., 2019) o proteínas de insectos (Henry et al., 2015) de acuerdo con los principios de la economía circular tal y como se está abordando en el proyecto europeo GAIN "*Green aquaculture intensification in Europe*".

La buena noticia es que la industria del salmón noruego ha conseguido disminuir el uso de ingredientes marinos en piensos de engorde hasta niveles de inclusión próximos al 30% (Ytrestøyl et al., 2015; Shepherd et al., 2017), a pesar de la alta susceptibilidad de esta especie a la enteritis de la soja (Booman et al., 2018). La trucha es menos susceptible a este tipo de enteritis y peces alimentados con dietas vegetales desde la primera ingesta presentan buenos datos de crecimiento, en especial aquellas familias seleccionadas previamente para este carácter productivo (Callet et al., 2017; Lazzarotto et al., 2018). También se han obtenido resultados satisfactorios en lubina y dorada con niveles de inclusión de ingredientes marinos del orden del 10-15% (Benedito-Palos et al., 2016; Torrecillas et al., 2017). En el caso de la dorada, estas nuevas formulaciones son capaces de soportar un alto crecimiento -con valores récord para la especie- hasta completar la madurez sexual en peces de 3-4 años (Simó-Mirabet et al., 2018). Es más,

estudios recientes demuestran que formulaciones con bajos contenidos en harinas y aceites de pescado siguen siendo válidas en un modelo de "common garden" con familias seleccionadas por bajo o alto crecimiento, lo que va acompañado de diferentes trayectorias de crecimiento a lo largo del ciclo de producción de carácter marcadamente estacional (Perera et al., 2019). En cualquier caso, es interesante tener presente que las harinas de pescado por sí solas cubren los requerimientos en aminoácidos, fosfolípidos, AGEs y otros micronutrientes cuando sus niveles de inclusión están por encima del 50%, por lo que la sustitución total de aceites de pescado por aceites vegetales es factible sin afectar aparentemente al crecimiento y la salud del animal (Regost et al., 2003; Piedecausa et al., 2007; Benedito-Palos et al., 2007; 2008; 2009; Bouraoui et al., 2011). Otros tipos de respuestas, como es el caso del estrés, también están reguladas nutricionalmente, aunque los resultados existentes en la bibliografía suelen ser contradictorios. Por ejemplo, el uso de dietas basadas en aceites vegetales suele ir asociado a niveles altos de cortisol en la dorada (Montero et al., 2003). Sin embargo, al disminuir el nivel de instauración de la dieta con aceites vegetales, también disminuye el riesgo de estrés oxidativo. Como prueba de ello, la respuesta de marcadores mitocondriales tras un estrés por confinamiento se ve mejorada en juveniles de dorada alimentados con dietas vegetales que cubren los requerimientos en AGEs (Pérez-Sánchez et al., 2013a).

Otro de los efectos negativos asociados al uso de dietas vegetales es un estado proinflamatorio que se manifiesta especialmente a nivel intestinal. Como se indica más adelante este efecto negativo puede paliarse mediante el empleo de prebióticos o probióticos que favorezcan

la reversión, al menos en parte, al patrón salvaje (Estensoro et al., 2011; 2016; Piazzon et al., 2016; 2017).

1.1.5.2. Seguridad y calidad alimentaria del producto final

El uso de piensos con harinas y aceites de pescado lleva asociado la presencia de contaminantes orgánicos persistentes no polares (POPs) que son fácilmente acumulables debido a sus características lipofílicas (Berntssen et al., 2005). Estos compuestos se depositan en el filete a una mayor tasa que los contaminantes polares como los pesticidas no organoclorados (OCPs) y las micotoxinas (Nácher-Mestre et al., 2013; 2014; 2015), que suelen encontrarse en los ingredientes vegetales terrestres. Por tanto, la sustitución de ingredientes de origen marino por ingredientes de origen vegetal ha reducido los niveles de POPs tanto en el pienso como en los filetes (Berntssen et al., 2005; Nácher-Mestre et al., 2009; Berntssen et al., 2010; Bell et al., 2012), aunque en contraposición el riesgo de bioacumulación es mayor para las micotoxinas y los pesticidas. Sin embargo, las concentraciones de estos compuestos suelen mantenerse por debajo del nivel máximo de residuo establecido y/o recomendado (MRL). Lo mismo ocurre con los hidrocarburos policíclicos aromáticos (PAHs), aunque esta familia de compuestos, también presente en los ingredientes de origen marino, es la que muestra unos factores de transferencia más altos (Nácher-Mestre et al., 2018).

En todo caso, el uso de materias primas de origen vegetal en las formulaciones de piensos tiene importantes efectos sobre la calidad del producto final (Benedito-Palos et al., 2009; Rosenlund et al., 2011; Ballester-Lozano et al., 2016). De hecho, como ya se ha comentado anteriormente, el valor nutricional del filete se ve comprometido por el

uso de aceites pobres en EPA y DHA (Izquierdo et al., 2005; Benedito-Palos et al., 2008). Todo ello, junto con el creciente interés por el consumo de AGs poliinsaturados (AGPs) de cadena larga por sus beneficios en la salud humana, comporta una alta demanda de aceite de pescado, lo que se traduce en presiones directas sobre los productores para que los productos derivados del mar sigan presentando niveles altos de EPA y DHA. En todo caso, otras características como la textura del filete, la vida útil del producto y sus propiedades organolépticas no se ven afectadas en la carpa común, la trucha o la dorada (Grigorakis et al., 2018; Turchini et al., 2018) por los actuales niveles de sustitución de las dietas comerciales.

1.1.5.3. Aditivos, selección genética y programación nutricional

Para conseguir formular piensos con bajos niveles de inclusión de harinas de pescado, éstos deben estar suplementados con nutrientes esenciales como aminoácidos, AGEs, vitaminas o minerales. Asimismo, con la finalidad de mejorar la salud y la resistencia a enfermedades, los antibióticos se han usado de forma indiscriminada durante muchos años para prevenir, controlar y tratar enfermedades, al mismo tiempo que como promotores del crecimiento. Con la prohibición del uso indiscriminado de los mismos en la Unión Europea y otros países debido a la aparición de resistencia cruzada, ha incrementado el interés por los aditivos alimentarios naturales que presentan unos efectos negativos mínimos tanto en el pez como en los consumidores y el medio ambiente (Meena et al., 2013). De acuerdo con ello, algunos prebióticos y probióticos han demostrado tener efectos beneficiosos sobre el estado inmunitario, la tolerancia al estrés, la eficiencia alimentaria y el

crecimiento en varias especies de peces. Concretamente, el butirato, un AG de cadena corta, presenta efectos positivos sobre la salud intestinal de varios modelos de animales de cultivo (Guilloteau et al., 2010). También, en la dorada, la suplementación con butirato ayuda a prevenir la inflamación del epitelio intestinal causado por las dietas vegetales, preservando la integridad de la barrera epitelial del intestino (Estensoro et al., 2016). Otro efecto beneficioso está asociado a cambios en el proteoma del mucus y el microbioma del intestino de doradas alimentadas con dietas vegetales, mejorando de esta manera la progresión de la enfermedad en animales retados con bacterias o parásitos intestinales (Piazzon et al., 2017). Además, la suplementación con butirato revierte, al menos en parte, los cambios asociados a la proporción de sexos en un hermafrodita protándrico como la dorada (Simó-Mirabet et al., 2018).

Por lo que se refiere a la selección genética, ésta se centra en aspectos como la mejora del crecimiento y la conversión del alimento entre otras características de interés productivo. Así, por ejemplo, se ha demostrado que la habilidad para crecer con dietas basadas en vegetales es genéticamente variable en trucha y lubina (Le Boucher et al., 2011; 2013), aunque doradas seleccionadas por distintas tasas de crecimiento son capaces de crecer de manera eficiente con dietas vegetales con un alto grado de sustitución (Perera et al., 2019) en base a las formulaciones del proyecto europeo ARRAINA "*Advanced research initiatives for Nutrition & Aquaculture, 7FP European Project*".

Otra estrategia para mejorar el uso de dietas con formulaciones más sostenibles es la programación nutricional. Por un lado, los estudios llevados a cabo en peces de agua dulce, como la trucha arcoíris, han

permitido mejorar la aceptación de las dietas vegetales tras la programación durante estadios tempranos (alevines) con este tipo de dietas (Geurden et al., 2013; Balasubramanian et al., 2016). En especies marinas, los estudios de programación nutricional se han centrado en aspectos relacionados con el metabolismo lipídico, actuando en los primeros estadios larvarios o indirectamente sobre los reproductores. Por ejemplo, en lubina, se ha observado un aumento persistente de la expresión de la *fads2* en juveniles alimentados durante el estado larvario con dietas deficientes en AGPs omega-3 (Vagner et al., 2007; 2009; Geay et al., 2010). En dorada se han observado resultados similares, pero con una alta mortalidad, por lo que un protocolo de actuación es difícilmente estandarizable a nivel práctico (Turkmen et al., 2017a). Es por ello que de forma alternativa se ha prestado especial atención a la programación a nivel de progenitores mediante el uso de dietas pobres en EPA y DHA, con el objeto de inducir la expresión de la *fads2* y, en consecuencia, aumentar el nivel de instauración de los fosfolípidos de la membrana citoplasmática. No obstante, esta programación tiene sus limitaciones y dietas con un 80% de sustitución de aceite de pescado por aceites vegetales reducen la fecundidad de los progenitores, así como la calidad de la progenie. Por el contrario, un nivel de sustitución menor (60%) mejora el crecimiento de la descendencia cuando ésta es expuesta meses más tarde a dietas vegetales (Izquierdo et al., 2015; Turkmen et al., 2017b). Ahora bien, cuando la programación está basada en el aumento de AGs de 18 carbonos (ALA) sin comprometer el aporte de EPA y DHA, la expresión de la *fads2* (Δ6 desaturasa) en la progenie es altamente refractaria a dicha programación nutricional (Turkmen et al., 2019); mientras que la expresión de la *scd1a* (Δ9 desaturasa), una enzima

limitante en la formación de AGs monoinsaturados (AGMs) se muestra especialmente sensible a estos cambios (**Fig. 1.6**). Esta enzima lipogénica disminuye los signos de deficiencias en AGPs estimulando la síntesis de AGMs (16:1n-7, 18:1n-9). El riesgo asociado es un incremento desproporcionado de la cantidad de depósitos grasos principalmente a nivel hepático. Es por ello, que uno de los objetivos de la programación nutricional con dietas basadas en aceites vegetales es mantener regulada la expresión de *scd1a,* evitando una respuesta desproporcionada que a su vez limite la deposición grasa, disminuyendo el riego de esteatosis hepática. Estos cambios de expresión están negativamente correlacionados con el nivel de metilación del promotor de la *scd1a*, lo que demuestra la participación de mecanismos epigenéticos en este tipo de regulación (Perera et al., 2020).

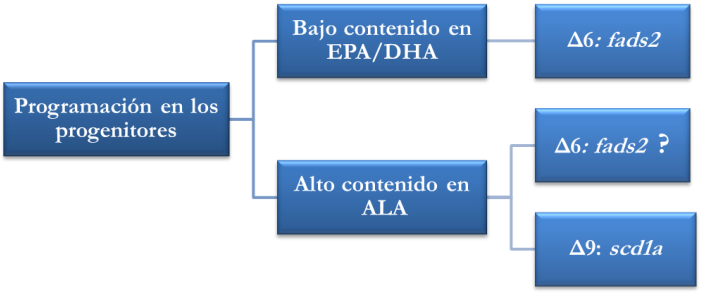


Figura 1.6 Resumen de los avances en la programación nutricional en los progenitores.

1.1.6. ¿Por qué la dorada cómo objeto de estudio?

Actualmente la acuicultura es el sector alimentario que presenta un crecimiento más rápido, siendo a la vez el más diverso en términos de número de especies, ambiente y tecnología usada (Harvey et al., 2017). Recientemente, algunos factores ambientales y sociales como la demanda

de mercado, las oportunidades de financiación, la disponibilidad de recursos o emplazamientos, la reducción de especies en el medio salvaje o el cambio climático han sido los principales impulsores de la diversificación de la acuicultura. Esto ha llevado al desarrollo proyectos (p. ej. Diversify "*Exploring the biological and socio-economic potential of new/emerging candidate fish species for expansion of the European aquaculture industry*") donde se ha explorado el potencial biológico y socioeconómico de nuevas especies de peces con la finalidad de expandir la industria acuícola europea, aumentando la oferta de productos y desarrollando nuevos mercados. La diversificación puede añadir un valor económico, social o ecológico a los sistemas de acuicultura, aunque los costes, retos y riesgos asociados a la diversificación son altos, dadas las actuales limitaciones tecnológicas. En cualquier caso, la estrategia de diversificación debe contemplar aumentar el número de especies cultivadas, así como la selección de cepas o variedades que cumplan con características específicas de interés productivo, para lo que es necesaria una visión integral de aspectos de economía de mercado, zootecnia y biología para resolver los problemas de las especies susceptibles de cultivo. Entre ellas, se encuentra sin lugar a duda la dorada, dada su fácil adaptación a las condiciones de cultivo intensivo y su elevada valorización por parte del consumidor. Como prueba de ello, en 2018 (Informe APROMAR, 2019), la producción acuícola de dorada fue de 246.839 t (un 10,7% superior a la de 2017), siendo los principales productores Turquía (33,6% de la producción total), Grecia (24,7%), Egipto (14,6%) y España (6,0%). A nivel global, esta producción se puede considerar modesta, al ocupar el puesto 62 dentro de las especies

cultivadas, pero en Europa (95.390 t) y España (14.930 t) su producción es relevante, al ocupar la tercera y cuarta posición del ranking.

La dorada (*Sparus aurata*) es un pez teleósteo del litoral marino que pertenece a la familia Sparidae del orden de los Perciformes (**Fig. 1.7**). Su hábitat natural se distribuye a lo largo del mar Mediterráneo y de las costas orientales del océano Atlántico, desde Gran Bretaña y el estrecho de Gibraltar hasta Cabo Verde y las Islas Canarias. Estudios sobre la estructura genética de la dorada, a través de distintos marcadores moleculares (aloenzimas, microsatélites o amplificación aleatoria de ADN polimórfico) han demostrado la existencia de subpoblaciones geográficas. Por ejemplo, entre el océano Atlántico y el mar Mediterráneo se ha observado una diferenciación genética significativa, aunque débil (Alarcón et al., 2004; De Innocentiis et al., 2004; Rossi et al., 2006). Sin embargo, en áreas más reducidas se han observado subdivisiones genéticas más fuertes como ocurre a lo largo de las costas de Túnez (Slimen et al., 2004) y entre Francia y Argelia (Chaoui et al., 2009). En cualquier caso, la falta de barreras geográficas disminuye la variabilidad genética, tal y como se ha puesto de relieve a lo largo de la costa italiana (Franchini et al., 2012).

La dorada es una especie eurihalina y euriterma, por lo que puede vivir en distintos ambientes, desde marinos o salobres hasta lagunas costeras y zonas estuáricas. Se distribuye en zonas poco profundas (hasta los 30 m) en el caso de los juveniles, mientras que los adultos se pueden encontrar hasta los 150 m. Es una especie hermafrodita protándrica que durante los dos primeros años es un macho funcional (Zohar et al., 1978; 1989) con espermatogénesis asincrónica, que acaba desarrollándose como hembra al alcanzar tallas superiores a los 30 cm. Sin embargo, el

cambio de sexo no solo está relacionado con el determinismo individual, sino que puede depender de las condiciones sociales y ambientales (Happe y Zohar, 1988). El desarrollo ovárico también es asincrónico y las hembras pueden poner unos 20.000-80.000 huevos al día a lo largo de los meses de octubre-diciembre, dependiendo de la latitud y las condiciones ambientales. Las puestas se realizan en mar abierto y los juveniles migran al principio de la primavera hacia aguas costeras donde hay abundantes recursos y temperaturas más templadas.

Figura 1.7 Dorada (*Sparus aurata*).

Todas estas características hacen de la dorada una especie con una gran capacidad de adaptación a un ambiente cambiante, incluyendo una alta tolerancia a cambios de salinidad del agua, concentración de oxígeno disuelto, temperatura y composición de la dieta. En este sentido, la dorada es una especie con una alta plasticidad que ha quedado demostrada en una gran cantidad de estudios de nutrición (Benedito-Palos et al., 2016; Simó-Mirabet et al., 2018; Gil-Solsona et al., 2019), cronobiología (Mata-Sotres et al., 2015; Yúfera et al., 2017), comportamiento alimentario (López-Olmeda et al., 2009; Sánchez et al., 2009), estrés (Calduch-Giner et al., 2010; Castanheira et al., 2013; Pérez-Sánchez et al., 2013b; Bermejo-Nogales et al., 2014; Magnoni et al., 2017; Martos-Sitcha et al., 2017; 2019) y resistencia a enfermedades (Cordero et al., 2016; Estensoro et al., 2016; Piazzon et al., 2018).

1.2. Metabolismo energético

1.2.1. La mitocondria y la fosforilación oxidativa

La demanda de energía cambia a lo largo del desarrollo y con el estado nutricional o las condiciones de salud y estrés. El 90% de la energía en forma de adenosín trifosfato (ATP) es producida por los organismos aeróbicos a través de la fosforilación oxidativa, por lo que la regulación de la actividad y de la biogénesis mitocondrial es clave para una adecuada función del metabolismo energético. En consecuencia, el número de mitocondrias y su nivel de actividad varia a nivel celular y tisular (Schaefer et al., 2013; Barbour y Turner, 2014), habiéndose demostrado en la dorada que la biogénesis mitocondrial y la fosforilación oxidativa están altamente reguladas a nivel transcripcional bajo diferentes condiciones de estrés ambiental y nutricional (Bermejo-Nogales et al., 2014; 2015; Silva-Marrero et al., 2017).

El estrés nutricional suele deberse a un aporte insuficiente de nutrientes y energía. Sin embargo, un exceso de energía también puede desencadenar diferentes tipos de estrés celular, como consecuencia de un aumento en la producción de especies reactivas de oxígeno (ROS) a nivel mitocondrial (Wellen y Thompson, 2010), lo que acaba inhibiendo la ingesta voluntaria de alimento (Saravanan et al., 2012) y el crecimiento de los peces en cultivo (Fernández-Díaz et al., 2006; Rise et al., 2015). Por consiguiente, se debe conseguir un equilibrio entre los mecanismos de producción y eliminación de ROS para que la célula y el organismo en su conjunto funcionen correctamente una vez alcanzado el nivel de alostasis. Para ello, las llamadas proteínas desacoplantes (UCPs) actúan como válvulas de seguridad, activando ciclos fútiles de energía (Mailloux

y Harper, 2011) que se desactivan rápidamente cuando el aporte de nutrientes no excede la demanda energética (Nabben y Hoeks, 2008; Bermejo-Nogales et al., 2011; 2014). Asimismo, el sistema de defensa antioxidante que incluye, entre otras, las enzimas superóxido dismutasa, glutatión peroxidasa, glutatión reductasa, tioredoxina, tioredoxina reductasa y catalasa (Martínez-Álvarez et al., 2005; Pacitti et al., 2014), también se activa para eliminar el exceso de ROS y disminuir el riesgo de estrés oxidativo.

1.2.2. Sistemas de regulación

Tanto en levaduras como en vertebrados superiores, los principios fundamentales del metabolismo energético son muy parecidos, estando su regulación sujeta a un estricto balance entre la energía ingerida, su utilización y su almacenamiento. Es más, en todos los organismos vivos, la energía celular es producida y usada mediante rutas altamente conservadas, siendo la energía almacenada y transferida a través del ATP y el NADH, en base a un delicado balance entre el anabolismo y el catabolismo (**Fig. 1.8**).

Las rutas clásicas de señalización de exceso de nutrientes utilizan la mTOR que es activada mediante aminoácidos ramificados (p. ej. leucina) o la vía de la insulina y del factor de crecimiento IGF-I. La mTOR promueve el metabolismo mitocondrial, la síntesis proteica y el crecimiento celular (Wellen y Thompson, 2010; Laplante y Sabatini, 2012). Otro de los mecanismos relacionados con la detección de nutrientes incluye la ChREBP que responde a niveles altos de glucosa, activando la glicólisis y la lipogénesis (Filhoulaud et al., 2013). Asimismo, la GOAT detecta AGs de cadena media (AGCM) y es una pieza clave

para la activación de la grelina que participa en la secreción de la GH, la estimulación del apetito y la adipogénesis, así como en la secreción de insulina y la homeostasis de la glucosa (Hougland, 2019). En contraposición, la restricción calórica comporta niveles bajos de ATP, con la consiguiente activación de la AMPK que inhibe la proliferación celular. En paralelo, las SIRTs, al usar como cofactor el NAD^+, están implicadas en una gran variedad de funciones biológicas desde el silenciamiento génico, pasando por el control del ciclo celular y la apoptosis, hasta la homeostasis energética.

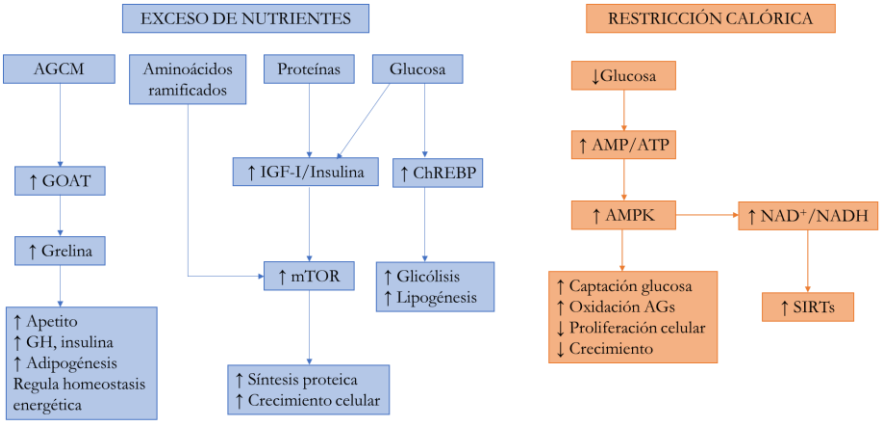


Figura 1.8 Esquema de las rutas clásicas de señalización de exceso de nutrientes (izquierda, en azul) y de restricción calórica (derecha, en naranja). *Abreviaturas: AGCM, ácidos grasos de cadena media; AMP y ATP, adenosin monofosfato y trifosfato; AMPK, proteína quinasa activada por AMP; ChREBP, elemento de respuesta a carbohidratos; GH, hormona del crecimiento; GOAT, O-acetiltransferasa grelina; IGF-I, factor de crecimiento insulínico tipo I; mTOR, diana de rapamicina en células de mamífero; NAD^+ y NADH, dinucleótido de nicotinamida adenina forma oxidadada y reducida; SIRTs, sirtuínas.*

1.2.3. Las sirtuínas

1.2.3.1. Filogenia y localización celular

Las SIRTs son una familia de proteínas altamente conservada a lo largo de la evolución, que presentan efectos pleiotrópicos sobre la regulación y la función celular. El origen de su nombre proviene del primer miembro de la familia descrito, la Sir2 de la levadura ("silent information regulator 2", homóloga a la SIRT1 de vertebrados) que actúa silenciando loci específicos del genoma deacetilando histonas (Imai et al., 2000). Desde su descubrimiento, el interés por las SIRTs aumentó exponencialmente debido a su efecto positivo sobre la esperanza de vida en diferentes modelos experimentales, habiéndose observado que la activación de las SIRTs ejerce efectos parecidos a los de la restricción calórica, que hoy por hoy es la forma más eficiente que se conoce a nivel fisiológico para aumentar la esperanza de vida. Desde entonces, se han publicado un gran número de estudios referentes a las SIRTs, destacando su importante papel en respuesta a la restricción calórica y a las enfermedades relacionadas con la edad y la homeostasis metabólica (Fiorino et al., 2014).

La familia de las SIRTs está presente en todos los seres vivos y el número de isotipos diferentes dentro de un mismo organismo va desde 1 en bacterias hasta 7 en vertebrados. El análisis filogenético de las SIRTs de organismos procariotas y eucariotas ha llegado a establecer cinco clases distintas (Frye, 2000; Vassilopoulos et al., 2011). La clase I incluye la SIRT1, 2 y 3; la clase II la SIRT4; la clase III la SIRT5 y la clase IV la SIRT6 y 7. Finalmente, también existe la clase U que está presente en bacterias y que podría ser el precursor de la clase I y la clase IV. De

acuerdo con esta clasificación, se considera que las SIRTs pertenecientes a las clases II, III y U aparecen primero en la evolución. Por eso, los procariotas contienen solo estas 3 categorías mientras que la mayoría de los eucariotas contienen las 4 clases de SIRTs (I-IV), aunque algunos organismos como los insectos, nematodos y levaduras acaban perdiendo alguna clase (**Fig. 1.9**).

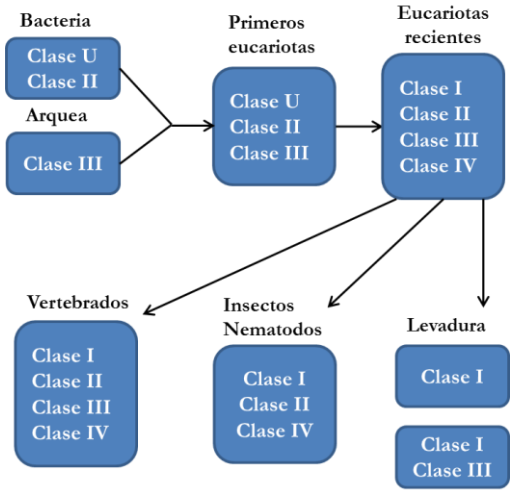


Figura 1.9 Modelo hipotético para la evolución y la distribución de las 4 clases de SIRTs presentes en eucariotas. Modificada de Frye, 2000.

La diversificación de las SIRTs está acompañada por una amplia compartimentalización subcelular. La SIRT1 y la SIRT6 son generalmente nucleares, mientras que la SIRT7 se encuentra principalmente en el nucléolo. Sin embargo, la SIRT1 (Tanno et al., 2007) y la SIRT6 también se pueden localizar en el citoplasma (asociada a gránulos de estrés) (Jedrusik-Bode et al., 2013; Simeoni et al., 2013). La SIRT2 es mayoritariamente citoplasmática, pero pasa al núcleo para participar en el ciclo celular (North y Verdin, 2007). Las SIRT3, 4 y 5 se localizan predominantemente en la mitocondria, aunque la SIRT3 parece

tener funciones en el citosol y en el núcleo (Scher et al., 2007; Sundaresan et al., 2008; Huang et al., 2010) según la longitud de sus isoformas (Schwer et al., 2002; Scher et al., 2007; Iwahara et al., 2012). La SIRT5 también se encuentra en compartimentos no mitocondriales (citosol, núcleo y peroxisomas) (Matsushita et al., 2011; Park et al., 2013; Chen et al., 2018), por lo que cada isoforma acaba presentando una preferencia distinta por los diferentes compartimentos celulares.

1.2.3.2. Estructura y reacción estequiométrica

Los distintos isotipos de SIRTs varían en longitud, pero todos ellos poseen un dominio catalítico central de unión al NAD^+ de unos 275 aminoácidos (Frye, 2000), que está rodeado por secuencias N- y C-terminales muy variables. Un análisis estructural de la región catalítica muestra que dicha región está formada por dos dominios: un dominio grande que consiste en una estructura Rossman fold α/β que es característica de las proteínas de unión al NAD^+ y un dominio más pequeño y variable que incluye un módulo de unión al Zn^{+2} (**Fig. 1.10**). Entre estos dos dominios se encuentran varios bucles que forman un bolsillo donde se une el NAD^+ y los sustratos acilados, conformando el lugar activo de la enzima (Sanders et al., 2010). La variabilidad entre los dominios de Zn^{+2} presentes en las distintas SIRTs es una de las razones por la que algunos miembros de esta familia son capaces de llevar a cabo diferentes actividades enzimáticas. Un ejemplo de ello es la SIRT5, que presenta una baja actividad deacetilasa siendo más eficiente como desuccinilasa y demalonilasa (Du et al., 2011). Además, las regiones N- y C- terminales, que difieren en longitud y en secuencia entre las distintas SIRTs, son las principales dianas de las modificaciones postraduccionales

(ver apartado 1.2.3.4.3), por lo que en gran medida condicionan la localización celular y las funciones propias de cada uno de los miembros de la familia (Flick y Luscher, 2012).

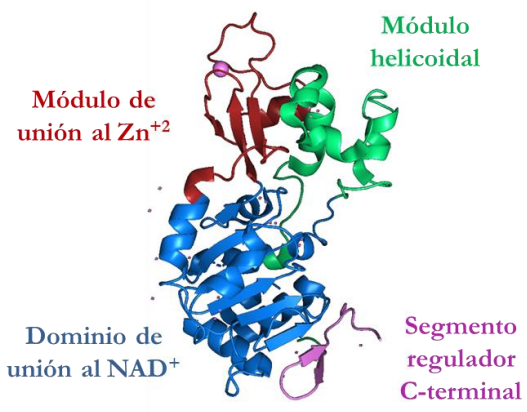


Figura 1.10 Estructura tridimensional de la SIRT1 de humanos. Modificada con PyMOL Molecular Graphics System, Version 2.2.3 Schrödinger, LLC.

La reacción estequiométrica de las SIRTs combina tres reactivos como sustratos-cosustratos: el NAD^+, el agua y un péptido o proteína acilada (concretamente un residuo de lisina). Tras la reacción se forman productos como la NAM, la proteína deacilada y un nuevo compuesto llamado AADPR (**Fig. 1.11**). Cuando la NAM se encuentra a altas concentraciones inhibe la actividad de las SIRTs mediante retroalimentación negativa. El AADPR ha sido menos estudiado, pero parece actuar como molécula de señalización (Tong y Denu, 2010), habiéndose relacionado con una disminución de los niveles de ROS, con el silenciamiento génico y con la activación de canales iónicos.

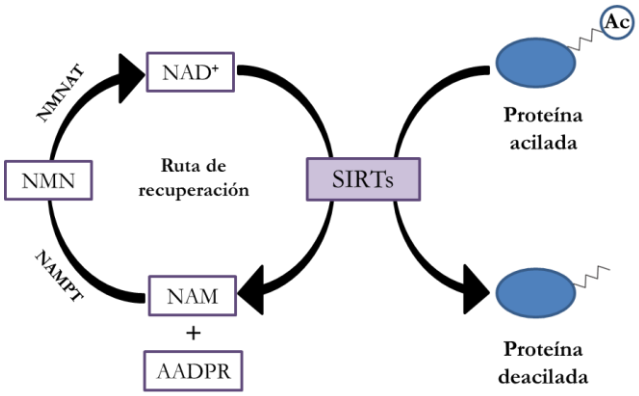


Figura 1.11 La actividad enzimática de las SIRTs y la ruta de recuperación del NAD^+. *Abreviaturas: AADPR, 2'-O-acetil-ADP-ribosa; NAM, nicotinamida; NAMPT, fosforibosiltransferasa de nicotinamida; NMN, mononucleótido nicotinamida; NMNAT, nicotinamida mononucleótido adenilil transferasa.*

1.2.3.3. Especificidad a nivel de sustrato y actividad enzimática

Las SIRTs son deacilasas de lisina dependientes de NAD^+. Los residuos de lisina están altamente presentes en los sitios activos o de unión de las proteínas y debido a sus propiedades químicas, su modificación juega un papel importante en la estructura proteica y sus interacciones. Además, la lisina presenta una gran variedad de modificaciones postraduccionales incluyendo la metilación, ubiquitinación y varios tipos de acilación, siendo la principal la acetilación. Sin embargo, varios estudios proteómicos han permitido identificar nuevos tipos de modificaciones como la formilación, propionilación, butirilación, crotonilación, malonilación, succinilación y glutarilación (Houtkooper, 2016), muchas de las cuales pueden ser eliminadas por las SIRTs. Varias de las anteriores modificaciones postraduccionales han sido detectadas tanto en histonas como en enzimas metabólicas (Choudhary et al., 2014). Por este motivo, y a pesar de su clasificación inicial como deacetilasas de histonas (HDACs), se ha demostrado que la mayoría de los sustratos de las SIRTs

no son las histonas, sino otras proteínas que mayoritariamente son enzimas del metabolismo intermediario. En definitiva, el hecho de que algunas SIRTs se encuentren en compartimentos celulares donde las histonas no están presentes y la posibilidad de llevar a cabo otro tipo de deacilaciones además de la deacetilación, ha hecho que algunos autores hayan sugerido que deberían nombrarse deacilasas de lisina (KDACs) (Yang y Sauve, 2016).

En cualquier caso, la especificad a nivel de sustrato y de localización celular, junto con las diferencias estructurales, confieren a cada isotipo una actividad enzimática específica. En este sentido, además de la acetilación, hay otras acilaciones de residuos de lisina reversibles (Lin et al., 2012) sobre las cuales cada SIRT muestra distintos niveles de preferencia, aunque el mecanismo catalítico es el mismo que el de la deacetilación (Zhou et al., 2012). De hecho, aunque todas las SIRTs, excepto la SIRT4, poseen actividad deacetilasa, la clase I (SIRT1-3) es la que posee una mayor actividad deacetilasa para una gran variedad de sustratos (Michishita et al., 2005). Estudios *in vitro* también han señalado que las SIRTs con una fuerte actividad deacetilasa son capaces de catalizar otras reacciones de deacilación como la decrotonilación (Bao et al., 2014), depropionilación, debutirilación (Smith et al., 2008) y demiristoilación (He et al., 2014). Por el contrario, las SIRTs de las clases II-IV presentan una débil o nula actividad deacetilasa. Por ejemplo, la SIRT4 y la SIRT6, actúan principalmente como ADP-ribosiltransferasas. La SIRT6 también presenta una eficiente actividad como deacilasa de AGs de cadena larga (Jiang et al., 2013), a la vez que la SIRT4 posee un amplio rango de actividades enzimáticas (Anderson et al., 2017). De modo similar, la SIRT5 actúa principalmente como demalonilasa,

desuccinilasa (Du et al., 2011; Peng et al., 2011) y deglutarilasa (Feldman et al., 2012; Tan et al., 2014). La SIRT7 también presenta otras actividades enzimáticas a parte de la deacetilación, como la desuccinilación y la deacilación de AGs (Wu et al., 2018) (**Tabla 1.1**). En consecuencia, las SIRTs no son simplemente deacetilasas, sino que son deacilasas dependientes de NAD^+ capaces de actuar sobre un gran número de sustratos acilo.

Tabla 1.1 Localizaciones celulares y actividades enzimáticas principales descritas en las SIRTs.

SIRTs	Localización	Actividad enzimática
SIRT1	**Núcleo**	
	Citoplasma	Deacetilasa
SIRT2	**Citoplasma**	Debutirilasa
	Núcleo	Decrotonilasa
SIRT3	**Mitocondria**	Demiristoilasa
	Citoplasma	Depropionilasa
	Núcleo	
SIRT4	**Mitocondria**	ADP-ribosiltransferasa
		Debiotilnilasa
		Dehidroximetilglutarilasa
		Delipoilasa
		Demetilglutaconilasa
		Demetilglutarilasa
SIRT5	**Mitocondria**	Deacetilasa (débil)
	Citoplasma	Deglutarilasa
	Núcleo	Demalonilasa
		Desuccinilasa
SIRT6	**Núcleo**	Deacetilasa (débil)
	Citoplasma	ADP-ribosiltransferasa
		Demiristoilasa
		Depalmitoilasa
SIRT7	**Nucléolo**	Deacetilasa (débil)
	Núcleo	Debutirilasa
		Demiristoilasa
		Desuccinilasa

1.2.3.4. Mecanismos de regulación

1.2.3.4.1. Disponibilidad del NAD^+ como cosustrato

Las SIRTs son capaces de llevar a cabo distintas actividades enzimáticas que requieren específicamente el NAD^+ (Feldman et al., 2012), hecho que las distingue de otras clases de deacetilasas de proteínas. En consecuencia, esta familia de proteínas ha evolucionado para que la disponibilidad de NAD^+, que a su vez informa del estado redox, sirva para desencadenar una determinada respuesta biológica, proporcionando una conexión directa entre la deacilación de las proteínas y el metabolismo intermediario. En otras palabras, el NAD^+ y su forma reducida NADH, regulan y coordinan reacciones metabólicas por su participación directa en vías metabólicas que son sensibles a señales nutricionales y ambientales (Imai, 2009a). En este sentido, debemos especificar que los niveles intracelulares de NAD^+ son dinámicos y su disponibilidad depende de su nivel de biosíntesis, que puede ser: i) *de novo*, a partir del aminoácido triptófano (quinurenina); ii) a través de la ruta Preiss-Handler, a partir del ácido nicotínico (NA) y iii) a través de la ruta de salvamiento o recuperación a partir de NAM. Los cambios en los niveles de NAD^+ también están acoplados a rutas de degradación, incluyendo las actividades de los consumidores de NAD^+ como por ejemplo las poli ADP-ribosa polimerasas (PARPs), las propias SIRTs y las NAD^+ glicohidrolasas.

Según Houtkooper et al. (2010), de todas las vías posibles de biosíntesis de NAD^+, la más importante es la de recuperación (**Fig. 1.11**). Esta ruta recicla el NAD^+ a partir de NAM, el producto derivado del consumo de NAD^+. La síntesis de NMN desde NAM es catalizada por la

enzima NAMPT. El NMN se adenila a través de NMNAT1-3 para regenerar el NAD^+ (Canto et al., 2015). La interrupción de las rutas de síntesis de recuperación del NAD^+ lleva a una disminución de sus niveles y de la actividad de las SIRTs (Araki et al., 2004). El NAMPT es la enzima limitante en esta ruta y sus niveles son cruciales para ajustar los niveles de NAD^+ (Yang et al., 2007a), mientras que una mayor expresión de otra enzima de esta ruta, NMNAT, no altera los niveles de NAD^+ (Araki et al., 2004; Revollo et al., 2004). En base a todo ello, los factores que afectan a la homeostasis del NAD^+ están estrechamente relacionados con diferentes respuestas metabólicas. Por ejemplo, la falta de nutrientes y el ejercicio modifica el balance redox aumentando los niveles de NAD^+ y potenciando la actividad de la SIRT1, al mismo tiempo que la expresión de NAMPT (Fulco et al., 2008; Costford et al., 2010). Los niveles celulares de NAD^+ también se pueden aumentar inhibiendo farmacológicamente el consumo de NAD^+ por enzimas como la PARP-1, lo cual puede tener fuertes implicaciones metabólicas (Bai et al., 2011) ya que es el principal consumidor de NAD^+ en la célula. Los efectos de los cambios en los niveles de NAD^+ sobre la actividad de las SIRTs se han demostrado especialmente en el caso de la SIRT1 y la SIRT3 (Imai, 2009b; Bai et al., 2011; Yoshino et al., 2011; Imai y Yoshino, 2013; Brown et al., 2014).

1.2.3.4.2. Actividad transcripcional

A pesar de que existen numerosos estudios sobre los sustratos que regulan las SIRTs, no existe tanta información sobre su regulación a nivel transcripcional, siendo la *SIRT1* la más estudiada. Así, por ejemplo, se sabe que determinados factores de transcripción se unen al promotor de

la *SIRT1* regulando su expresión como respuesta a la restricción calórica (CREB) o a la disponibilidad de nutrientes (ChREBP) (Noriega et al., 2011), mientras que el FOXO3a lo hace mediante un mecanismo basado en la interacción proteína-proteína donde también participa el factor de transcripción p53 (Nemoto et al., 2004). Además, la familia de los factores de transcripción PPAR también participa en la regulación transcripcional de la *SIRT1*. Otros ejemplos son: el C/EBP-α, que aumenta la expresión de la *SIRT1* durante la adipogénesis y el complejo C/EBP-β-HDAC1, que reprime su expresión con el aumento de la edad (Jin et al., 2010; 2011). También cabe destacar la regulación de la *SIRT1* por medio del factor de transcripción CLOCK/BMAL1 que media las variaciones circadianas, el metabolismo y la sensibilidad a la insulina (Zhou et al., 2014). La *SIRT1* también está implicada en rutas relacionadas con el estrés, que controlan su expresión al unirse a su promotor varios factores de transcripción como es el caso del NF-κB (Zhang et al., 2010; Katto et al., 2013), la proteína EGR1 (Pardo y Boriek, 2012) y la proteína APE (Antoniali et al., 2014). Asimismo, la SIRT1 se ha relacionado con el cáncer, pero su función es compleja, ya que se ha demostrado que puede actuar como supresor o promotor de tumores según el tipo de cáncer (Lin y Fang, 2013).

Según la reciente revisión de Buler et al. (2016), se sabe que en respuesta al ejercicio o a la falta de nutrientes el receptor relacionado con los estrógenos ERR-α se une al promotor de la *SIRT3* y estimula su transcripción mediante la coactivación con PGC-1α. La *SIRT5*, también mitocondrial, está regulada por factores de transcripción comunes como el PGC-1α y el AMPK, de manera positiva y negativa respectivamente. Asimismo, el factor de transcripción NK2 homeobox 2.2 se une al

promotor de la *SIRT2* y disminuye su expresión, mientras que CREB2 regula la expresión de la *SIRT4*. Varios factores de transcripción regulan la *SIRT6*, dependiendo del tipo celular y de la condición fisiológica. Por ejemplo, en células hepáticas, NRF1 y otros coactivadores inducen la expresión de la *SIRT6* en una situación de falta de energía, mientras que c-Jun la reprime durante un estrés genotóxico. Otros activadores de la expresión de la *SIRT6* son PAX6, KLF2 mientras que C/EBP-β, CMYB, y E2F1 son inhibidores de ésta (Hong et al., 2018). Finalmente, la *SIRT7* está regulada por varios factores de transcripción que aumentan (XBP1) o reprimen (HDAC3 y C/EBP-α) su expresión.

Otra forma de regulación de las *SIRTs* es mediante microARNs (miARNs), que son ARNs no codificantes que se unen a la región no traducida 3' del ARN mensajero diana para degradarlo o bloquear su traducción (Ambros, 2004). Hasta el momento se han descrito un gran número de miARNs que regulan la expresión y actividad de la *SIRT1* (Buler et al., 2016). Entre ellos está el miR-34a que también regula la actividad de la *SIRT6* y *SIRT7*. Otros miARNs que regulan la actividad de la *SIRT6* a nivel hepático son el miR-33a/miR-33b (Elhanati et al., 2013) y el miR-122 (Elhanati et al., 2016), que acaban controlando varios aspectos de la fisiología hepática.

1.2.3.4.3. Modificaciones postraduccionales e interacción proteína-proteína

Las actividades enzimáticas de las SIRTs pueden verse afectadas por modificaciones postraduccionales, principalmente en los extremos N- y C- terminales. De hecho, la SIRT1 presenta 15 lugares de fosforilación cuya modificación afecta a la actividad de la enzima y a su abundancia a nivel de proteína a través de procesos de degradación dependientes o

independientes del proteasoma (Hwang et al., 2013). Además, la SIRT1 está sujeta a otros tipos de modificaciones postraduccionales como la sumoilación (Yang et al., 2007b), la metilación (Liu et al., 2011) y la nitrosilación (Kornberg et al., 2010).

Se sabe menos sobre el resto de SIRTs, pero se han identificado lugares de fosforilación y de acetilación (Musgrove, 2006; Pandithage et al., 2008) en la SIRT2 que parecen estar afectando su actividad. En la SIRT3, 4 y 6 se han encontrado lugares de fosforilación que es posible que modulen su actividad, pero éste no parece ser el caso de la SIRT5 (Flick y Lücher et al., 2012). Por el contrario, la SIRT7 puede ser fosforilada por la AMPK en una situación de estrés energético, determinando su distribución celular y su degradación (Sun et al., 2016). Además, la SIRT7 puede ser deubiquitinada por la proteína USP7, disminuyendo su actividad y, en consecuencia, también la gluconeogénesis (Jiang et al., 2017).

Las SIRTs presentan otro nivel de regulación basado en la formación de complejos con otras proteínas. Por ejemplo, la proteína AROS regula positivamente a la SIRT1 (Kim et al., 2007) y la metiltranferasa de histonas LSD1 y la SIRT1 forman un complejo que reprime genes regulados por la ruta de señalización Notch relacionada con el desarrollo (Mulligan et al., 2011). Por otro lado, hay varios reguladores negativos como NCoR1 y SMRT, que forman un complejo con la SIRT1 y el PPARγ durante el ayuno para prevenir la adipogénesis (Picard et al., 2004). De la misma manera, la DBC1 forma un complejo estable con la SIRT1 e inhibe su actividad (Kim et al., 2008).

1.2.3.5. Función metabólica

La importancia de la acetilación de las lisinas en el estado de la cromatina y en la expresión génica es bien conocida. Por otra parte, la mayoría de las enzimas del metabolismo intermediario relacionadas con la glicólisis, la gluconeogénesis, el ciclo de Krebs, el ciclo de la urea, el metabolismo de AGs y del glicógeno están acetiladas (Zhao et al., 2010). Es más, la acetilación de estas enzimas cambia en respuesta a alteraciones en la disponibilidad extracelular de nutrientes y afecta directamente a su actividad o estabilidad. Más allá de la acetilación proteica, se han identificado nuevos tipos de modificaciones (acilaciones) de la lisina como se ha descrito anteriormente. Estas modificaciones postranscripcionales están conservadas evolutivamente y varían con el estado celular en respuesta a diferentes factores estresantes y mutaciones genéticas (Hirschey y Zhao, 2015). De hecho, estudios proteómicos han permitido identificar varios tipos de acilaciones en un gran número de proteínas mitocondriales que participan en diferentes rutas metabólicas (Colak et al., 2013; Park et al., 2013; Rardin et al., 2013; Weinert et al., 2013; Tan et al., 2014; Yang y Gibson, 2019).

En todo caso, estudios funcionales, especialmente centrados en la SIRT1, han demostrado que la expresión/actividad de esta enzima aumenta bajo restricción calórica o con el ejercicio (Guarente, 2013; Radaka et al., 2013), en respuesta a un incremento en los niveles de NAD^+. Además, el sensor metabólico AMPK también regula (y está regulado por) la SIRT1 para controlar la función mitocondrial a través de PGC-1α (Canto et al., 2009; Price et al., 2012). Esta acción sobre la función mitocondrial cobra especial relevancia en las SIRT3-5 que están codificadas por el ADN nuclear, pero que se localizan mayoritariamente

en la mitocondria, por lo que contribuyen activamente en los procesos de envejecimiento, la respuesta al estrés oxidativo y la estabilidad del genoma (Carafa et al., 2016).

La SIRT3 es sin duda el isotipo mitocondrial más estudiado, ya que además de participar en la regulación del ciclo de Krebs (Finley y Haigis, 2012), también regula de manera directa o indirecta todos los complejos mitocondriales y la ATP sintasa, demostrando su amplio control en la regulación de la capacidad de la fosforilación oxidativa. Contrariamente a la SIRT3, la expresión de la SIRT4, y consecuentemente su actividad, está reducida en condiciones de restricción calórica (Guarente, 2013), lo que sugiere una función y regulación diferente de cada isotipo. En particular, la SIRT4 regula el metabolismo de la glutamina, la homeostasis lipídica y el ciclo de Krebs en respuesta a daños en el ADN (Jeong et al., 2013; Laurent et al., 2013). Además, la SIRT5 participa en el ciclo de la urea deacetilando y activando la CPS1 (Nakagawa et al., 2009) y también desuccinila proteínas mitocondriales responsables de la regulación de la producción de cuerpos cetónicos y de la oxidación de AGs (Rardin et al., 2013).

Por último, la SIRT2 (mayoritariamente citoplasmática) participa en el control del ciclo celular y bajo condiciones de estrés puede jugar un papel importante en la apoptosis y en la señalización oxidativa, disminuyendo los niveles de ROS (Nie et al., 2014; Cao et al., 2016). También está implicada en la homeostasis metabólica participando en la señalización insulínica (Ramakrishnan et al., 2014) mediante el control de la AKT, la cual también está controlada por la SIRT1 (Sundaresan et al., 2011; Ramakrishnan et al., 2014), lo que sugiere un estricto control de las distintas funciones de las SIRTs con varios sustratos comunes. La SIRT2

también deacetila enzimas relacionadas con la glicólisis, la glicogénesis y la gluconeogénesis, procesos que también regula la SIRT6 (Kuang et al., 2018). Según parece, la SIRT7 también puede aumentar la expresión de genes mitocondriales (Ryu et al., 2014) y controla el metabolismo hepático (Yoshizawa et al., 2014), por lo que todo ello es un claro ejemplo de una compleja regulación y coordinación de las funciones de las SIRTs en el metabolismo energético.

1.2.3.6. Participación en mecanismos de regulación epigenética

En las células eucarióticas, la cromatina es una estructura bien organizada y dinámica susceptible a procesos epigenéticos que modifican su arquitectura, regulando la actividad génica sin implicar cambios en la secuencia de ADN. La modificación covalente del ADN, las histonas o la asociación de proteínas no histónicas regulan la transición entre la heterocromatina, altamente condensada e inhibida transcripcionalmente, y la eucromatina, más accesible a los factores de transcripción y por tanto transcripcionalmente activa (Richards y Elgin, 2002). Las modificaciones postraduccionales que ocurren en los aminoácidos del extremo N-terminal de las histonas incluyen la acetilación, la ADP-ribosilación, la metilación, la fosforilación y la ubiquitinación. Estas modificaciones regulan fuertemente el empaquetamiento de la cromatina y la expresión génica. Una de las modificaciones postraduccionales más estudiadas es la acetilación de los residuos de lisina de las histonas. Esta reacción es el resultado de un balance entre las acetiltransferasas de histonas (HATs) y las HDACs, que reducen e incrementan respectivamente el empaquetamiento de la cromatina, regulando la accesibilidad de los factores de transcripción y la ARN polimerasa II afectando así a la

transcripción génica (**Fig. 1.12**). De este modo, como se señala en la revisión de Jing y Lin (2015), las SIRT1, 2, 6 y 7 son las que han demostrado tener una importante función a nivel epigenético. La SIRT1 regula la estructura de la cromatina mediante la deacetilación de histonas, especialmente las histonas H3 y H4 (especialmente en las lisinas H3K9 y H4K16; y en menor medida sobre las H3K14, H4K8 y H4K12). Además, el papel de la SIRT1 en la represión de la transcripción incluye la regulación de la actividad de otras enzimas epigenéticas como HATs, metiltransferasas de histonas (HTMs) y de ADN (DNMTs). La SIRT1 también puede deacetilar numerosos factores de transcripción como p53, PGC-1α, FOXOs, HIF-1α, HIF-2α, NF-κB y MYC. En cuanto al resto de SIRTs, también se ha demostrado que deacetilan histonas (SIRT2: H4K16, H3K18 y H3K56; SIRT6: H3K9 y H3K56; SIRT7: H3K18) y factores de transcripción (SIRT2: p300, FOXOs, HIF-1α, PGC-1α, NF-κB; SIRT6: GCN5, FOXO1). Todo esto muestra claramente la participación de las SIRTs en procesos de regulación epigenética.

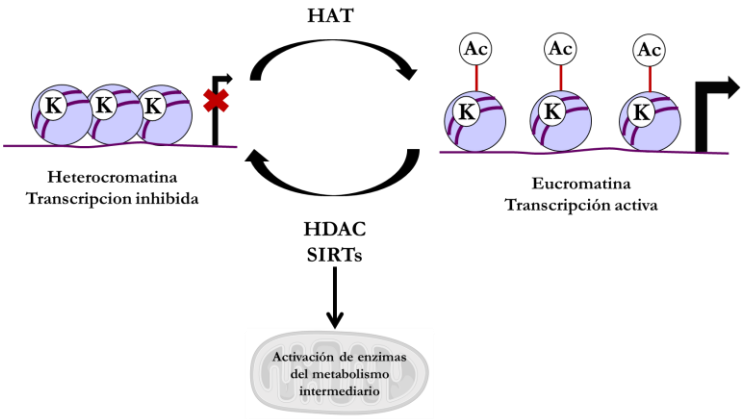


Figura 1.12 Las sirtuínas actúan sobre las histonas inhibiendo la transcripción génica y sobre varias enzimas del metabolismo energético activándolas.

1.2.3.7. Conocimiento actual en peces

Desde su descubrimiento, las SIRTs se han estudiado ampliamente en diferentes modelos experimentales, desde la levadura hasta vertebrados superiores, principalmente roedores y humanos. Estos estudios se han centrado en su habilidad para regular la esperanza de vida y la homeostasis energética, pudiéndose usar como potenciales nutracéuticos en distintas enfermedades metabólicas o disfunciones asociadas a la edad, como la obesidad, el cáncer o el Alzheimer. Sin embargo, los posibles beneficios que este tipo de estudios pueden comportar sobre la producción animal, y en concreto sobre la acuicultura y especies de peces modelo, son limitados (Ghinis-Hozumi et al., 2013). A pesar de ello, estudios recientes en pez cebra (*Danio rerio*) muestran los efectos nutracéuticos de distintos compuestos como el resveratrol, un activador de las SIRTs, para tratar con éxito los glaucomas (Sheng et al., 2018). Las disfunciones relacionadas con la angiogénesis postnatal, el crecimiento vascular (Potente et al., 2007) o la esteatosis hepática (Schneider et al., 2017) son otros procesos fisiológicos en los que se ha estudiado la acción de la SIRT1 como diana de nuevos tratamientos terapéuticos en pez cebra. Otra especie de pez modelo es el Notho de cola roja (*Nothobranchius guentheri*), que es especialmente útil para el estudio del envejecimiento y de la acción de las SIRTs en tal proceso (Kabiljo et al., 2019). Otros estudios con esta misma especie han mostrado que el resveratrol presenta efectos positivos, mediante la regulación de la actividad de las SIRTs, sobre la fertilidad de las hembras (Lee et al., 2018), la esperanza de vida y el estrés oxidativo (Liu et al., 2015). Asimismo, minimiza los efectos de la edad sobre el metabolismo hepático (Liu et al., 2017) y la salud intestinal (Liu et al., 2018).

Otros estudios recientes demuestran que la suplementación con resveratrol mejora la utilización de dietas de alto contenido en carbohidratos en la carpa (*Megalobrama amblycephala*), mediante la activación del mecanismo AMPK-SIRT1-PGC-1α y la regulación de genes relacionados (Shi et al., 2018). De hecho, algunos efectos de este tipo de dietas sobre el metabolismo glucolipídico están regulados, en parte, por el miARN-34a, que tiene como diana la *sirt1* (Miao et al., 2019). Por otro lado, la activación de la *sirt1* ha resultado tener efectos beneficiosos sobre metabolismo lipídico y glucídico de la carpa tras alimentar los peces con dietas ricas en grasas (Zhang et al., 2018).

Las SIRTs también parecen facilitar la adaptación a cambios medioambientales. La disminución de la *sirt2* en medios de baja concentración de oxígeno se ha relacionado con una estrategia adaptativa de los mugílidos que viven en estuarios con altos niveles de contaminantes (Ekambaram et al., 2017). Distintas *sirts* también responden a la aclimatación al frío en el espinosillo (*Gasterosteus aculeatus*), aumentando la expresión de *sirt1* y *sirt2* en el hígado y la de *sirt1* en el músculo pectoral, mientras que disminuye la de *sirt3* tanto en hígado como en músculo, manteniéndose invariable la expresión de *sirt4* (Teigen et al., 2015). También se ha identificado la *sirt6* como un biomarcador a la exposición al amonio en el eperlano de estanque (*Hypomesus transpacificus*) (Connon et al., 2011).

Por último, estudios en trucha han revelado cambios en la expresión de la *sirt1,* que funciona como un sensor de nutrientes, concretamente de glucosa (Otero-Rodiño et al., 2016) y han demostrado su participación en el sistema circadiano y en la respuesta al estrés (Naderi et al., 2018; 2019; Hernández-Pérez et al., 2019). Por el

contrario, no se ha observado ninguna respuesta de la *sirt1* en músculo rojo y blanco durante la natación sostenida (Magnoni et al., 2014) y tampoco a nivel hipotalámico como respuesta a un tratamiento con la preproteína grelina-obestatina (Velasco et al., 2017). Sin embargo, la *sirt1* participa en la activación de cascadas de señalización relacionadas con la capacidad cognitiva del carpín dorado (*Carassius auratus*) (Rajan et al., 2015).

1.2.3.8. Uso potencial de las sirtuínas como biomarcadores

Los biomarcadores se usan como indicadores de distintas condiciones biológicas como el crecimiento, la salud o cualquier otra característica de valor productivo. Desde un punto de vista funcional dichos marcadores se pueden clasificar en tres grandes categorías: de valor pronóstico (predicción sobre posibles problemas), diagnóstico (evaluación de los problemas en un carácter de interés) y terapéutico (indicativo de la corrección de un problema después de tratarlo). Algunos de estos marcadores están regulados nutricionalmente y uno de los objetivos del proyecto europeo ARRAINA "*Advanced research initiatives for Nutrition & Aquaculture, 7FP European Project*" ha sido la identificación y validación mediante técnicas bioquímicas, histológicas y moleculares de un complejo set de biomarcadores relacionados con el crecimiento, la capacidad reproductora, el estado nutricional y la salud y bienestar animal, accesibles a través del siguiente enlace (www.nutrigroup-iats.org/arraina-biomarkers). Las *sirts* son candidatos para formar parte de esta lista de biomarcadores por su estrecha relación con el nivel de ingesta y el estado de demanda energética del organismo, que puede desencadenar determinadas respuestas encaminadas a preservar la masa

muscular en diferentes estados de demanda energética o crecimiento a lo largo del ciclo biológico.

Las aproximaciones epigenéticas merecen especial mención al proporcionar un nexo entre el ambiente y el fenotipo. La epigenética se refiere a cualquier proceso que resulte en un cambio fenotípico sin que ello implique cambios en la secuencia de ADN (Jablonka y Lamb, 2002). Los mecanismos epigenéticos incluyen: i) cambios en la metilación del ADN; ii) modificaciones postraduccionales de las histonas (principalmente acetilación, fosforilación y metilación) y iii) cambios en la expresión de ARNs no codificantes. La metilación del ADN es la marca epigenética mejor estudiada (Gavery y Roberts, 2017), estando metiladas el 70-80% de las posiciones CpG de los genomas de vertebrados, cuyo número es limitado debido a las propiedades mutagénicas de la metilcitosina (Coulondre et al., 1978). En cualquier caso, las regiones ricas en C+G, llamadas islas CpG (CGIs), se encuentran normalmente hipometiladas y están asociadas con el 70% de los promotores de vertebrados (cercanas al lugar de inicio de la transcripción o TSS), siendo ésta una característica altamente conservada a nivel evolutivo (Saxonov et al., 2006).

En las CGIs, los nucleosomas se ensamblan de forma inestable (Ramirez-Carrozzi et al., 2009), atrayendo proteínas que están relacionadas con la modificación de la cromatina lo que permite la activación del promotor (**Fig. 1.13**). Así pues, la metilación del ADN a nivel de promotor suele estar negativamente correlacionada con la expresión génica. Es más, los patrones de metilación pueden cambiar en función de los tipos celulares, el estadio del desarrollo, la edad y la interacción del organismo con el medio. De acuerdo con ello,

prácticamente la mitad de las CGIs están localizadas en regiones promotoras de genes ampliamente expresados (genes constitutivos). La otra mitad, correspondiente a las CGIs huérfanas, se localiza tanto en regiones intragénicas como intergénicas (**Fig. 1.13**); actuando muchas de estas CGIs huérfanas como promotores alternativos, al estar localizadas en lugares de inicio de la transcripción de transcritos alternativos o de ARNs no codificantes (Mendizabal y Yi, 2016).

Figura 1.13 Representación de CGIs en dos genes hipotéticos. Una CGI metilada y la otra no metilada en el promotor del gen (cercanas al TSS) y una huérfana entre los dos genes.

La metilación de las CGIs tiene un papel especialmente importante a lo largo del desarrollo embrionario como mecanismo de silenciamiento génico a nivel de tejido (Shen et al., 2007; Ehrlich y Lacey, 2013; Lim et al., 2019). No obstante, la adquisición de modificaciones epigenéticas a lo largo de la vida es posible y algunas pueden ser potencialmente reversibles (Bishop y Ferguson, 2015). Es más, en peces hay evidencias de la existencia de herencias epigenéticas transgeneracionales (Shao et al., 2014; Knecht et al., 2017), lo que raramente ocurre en mamíferos. No obstante, son pocos los estudios que asocian modificaciones epigenéticas con características de interés comercial en acuicultura. En este sentido, cobran especial importancia la familia de las SIRTs, ya que al deacetilar histonas, factores de transcripción y enzimas epigenéticas actúan como moduladores

epigenéticos. Esta regulación que teóricamente está mediada, al menos en parte, por cambios en el nivel de metilación de los genes diana, también es extensible a las propias SIRTs, aunque este tipo de estudios queda reducido a humanos y roedores (Silva et al., 2008; Furuya et al., 2012; Sahin et al., 2014; Zullo et al., 2018).

A nivel metodológico, frente a los cambios de metilación del genoma a nivel global (**Fig. 1.14, 1 y 2**), también son factibles aproximaciones dirigidas a establecer el nivel de metilación de regiones o genes específicos (**Fig. 1.14, 3 y 4**). Dentro de los métodos destinados a detectar cambios en la metilación de genes específicos, la pirosecuenciación (**Fig. 1.14, 3.3**) es un método cuantitativo de uso rutinario para el análisis de la metilación de varios sitios CpG consecutivos. Dicho método es fácil y rápido de llevar a cabo, es económicamente asequible y ofrece una mayor capacidad de procesamiento comparado con otras tecnologías alternativas. El principio del análisis se basa en la conversión del ADN con bisulfito y su posterior amplificación por PCR. En el primer paso del proceso, el ADN genómico es tratado con bisulfito y el resultado es que la citosina (C) no metilada se convierte en uracil (U), mientras que la citosina metilada (mC) no sufre ninguna modificación. El segundo paso es la amplificación por PCR, donde el U es amplificado en timina (T) y la mC sigue siendo una C.

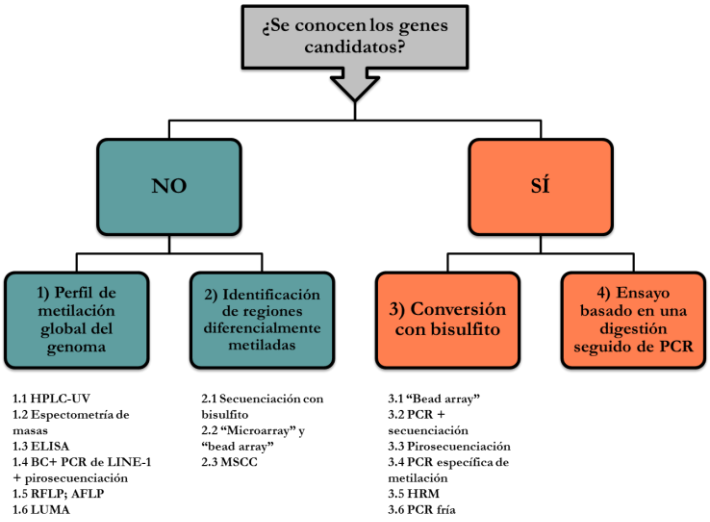


Figura 1.14 Los distintos métodos para llevar a cabo análisis de metilación del ADN. Modificada de Kurdyukov y Bullock, 2016. *Abreviaturas: AFLP, polimorfismo de longitud de los fragmentos amplificados; BC, conversión con bisulfito; ELISA, ensayo por inmunoabsorción ligado a enzimas; HPLC-UV, cromatografía líquida de alto rendimiento; HRM, fusión de alta resolución; LINE, elemento nuclear largo intercalado; LUMA, ensayo de metilación luminométrico; MSCC, contaje de cortes sensibles a la metilación; PCR, reacción en cadena de la polimerasa; RFLP, polimorfismo de longitud de los fragmentos de restricción.*

Por tanto, la medida del nivel de C es igual al grado de metilación de cada sitio CpG (**Fig. 1.15**). Después, la pirosecuenciación cuantifica la ratio C/T mediante la incorporación secuencial de nucleótidos a la cadena molde simple de ADN. Usa un sistema de cascada que consiste en 4 enzimas y sustratos específicos y cuando se forma una pareja de bases con la base complementaria de la cadena molde de ADN se emite luz. La intensidad de la luz es proporcional al número de nucleótidos incorporados. El resultado es un pirograma que es una representación gráfica donde se muestran las incorporaciones de los nucleótidos en relación con la intensidad de la luz resultante. El pirograma muestra la

secuencia de nucleótidos (como picos) y también la representación cuantitativa de la incorporación de éstos (altura de los picos). El grado de metilación se calcula a partir de la altura de los picos de las incorporaciones de C y T.

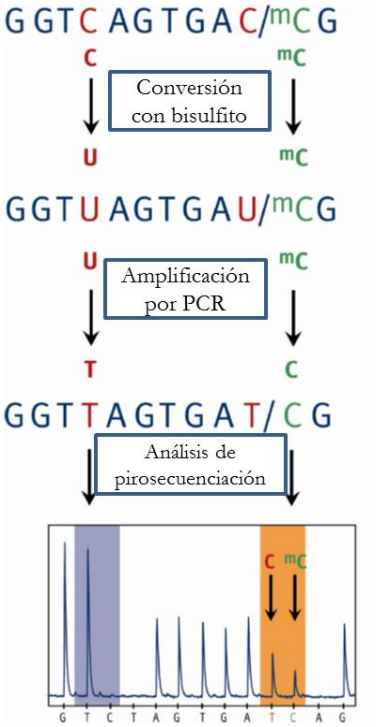


Figura 1.15 Un ejemplo de la conversión con bisulfito de una secuencia de ADN y su posterior amplificación por PCR y pirosecuenciación. Modificada de Biotage AB, Sweden.

Bibliografía

Alarcón JA, Magoulas A, Georgakopoulos T, Zouros E, Alvarez MC. 2004. Genetic comparison of wild and cultivated European populations of the gilthead sea bream (*Sparus aurata*). *Aquaculture* 230:65-80.

"Alimentos procedentes de los océanos"; Informe del mecanismo Asesoramiento Científico de la Comisión Europea (SAM). Disponible en http://bit.ly/2oWMzGP.

Ambros V. 2004. The functions of animal microRNAs. *Nature* 431:350-355.

Anderson KA, Huynh FK, Fisher-Wellman K, Stuart JD, Peterson BS, Douros JD, et al. 2017. SIRT4 is a lysine deacylase that controls leucine metabolism and insulin secretion. *Cell Metabolism* 25:838-855.

Antoniali G, Lirussi L, D'Ambrosio C, Dal Piaz F, Vascotto C, Casarano E, et al. 2014. SIRT1 gene expression upon genotoxic damage is regulated by APE1 through nCaRE-promoter elements. *Molecular Biology of the Cell* 25:532-547.

APROMAR. 2019. La acuicultura marina de peces en España.

Araki T, Sasaki Y, Milbrandt J. 2004. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 305:1010-1013.

Bai P, Canto C, Oudart H, Brunyanszki A, Cen Y, Thomas C, et al. 2011. PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. *Cell Metabolism* 13:461-468.

Balasubramanian MN, Panserat S, Dupont-Nivet M, Quillet E, Montfort J, Le Cam A, et al. 2016. Molecular pathways associated with the nutritional programming of plant-based diet acceptance in rainbow trout following an early feeding exposure. *BMC Genomics* 17(1):449.

Ballester-Lozano GF, Benedito-Palos L, Riaza A, Navarro JC, Rosel J, Pérez-Sánchez J. 2014. Dummy regression analysis for modelling the nutritionally tailored fillet fatty acid composition of turbot and sole using gilthead sea bream as a reference subgroup category. *Aquaculture Nutrition* 20(4):421-430.

Ballester-Lozano GF, Benedito-Palos L, Mingarro M, Navarro JC, Pérez-Sánchez J. 2016. Up-scaling validation of a dummy regression approach for predictive modelling the fillet fatty acid composition of cultured European sea bass (*Dicentrarchus labrax*). *Aquaculture Research* 47: 1067-1074.

Bao X, Wang Y, Li X, Li X-M, Liu Z, Yang T, et al. 2014. Identification of 'erasers' for lysine crotonylated histone marks using a chemical proteomics approach. *Elife* 3:e02999.

Barbour JA, Turner N. 2014. Mitochondrial stress signaling promotes cellular adaptations. *International Journal of Cell Biology* 214:156020.

Bell MV, Tocher DR. 2009. Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions. In Lipids in aquatic ecosystems pp. 211-236. Springer, New York, NY.

Bell JG, Dick JR, Strachan F, Guy DR, Berntssen MHG, Sprague M. 2012. Complete replacement of fish oil with a blend of vegetable oils affects dioxin, dioxin-like polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in 3 Atlantic salmon (*Salmo salar*) families differing in flesh adiposity. *Aquaculture* 324-325:118-126.

Benedito-Palos L, Saera-Vila A, Calduch-Giner JA, Kaushik S, Pérez-Sánchez J. 2007. Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis. *Aquaculture* 267(1-4):199-212.

Benedito-Palos L, Navarro JC, Sitjà-Bobadilla A, Bell JG, Kaushik S, Pérez-Sánchez J. 2008. High levels of vegetable oils in plant-protein rich diets fed to gilthead sea bream (*Sparus aurata* L.): Growth performance, muscle fatty acid profiles and histological alterations of target tissues. *British Journal of Nutrition* 100:992-1003.

Benedito-Palos L, Navarro JC, Bermejo-Nogales A, Saera-Vila A, Kaushik S, Pérez-Sánchez J. 2009. The time course of fish oil wash-out follows a simple dilution model in gilthead sea bream (*Sparus aurata* L.) fed graded levels of vegetable oils. *Aquaculture* 288:98-105.

Benedito-Palos L, Ballester-Lozano GF, Pérez-Sánchez J. 2014. Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene* 547:34-42.

Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz Á, Calduch-Giner J, et al. 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). *Aquaculture* 454:8-18.

Bermejo-Nogales A, Benedito-Palos L, Calduch-Giner JA, Pérez-Sánchez J. 2011. Feed restriction up-regulates uncoupling protein 3 (UCP3) gene expression in heart and red muscle tissues of gilthead sea bream (*Sparus aurata* L.): new insights in substrate oxidation and energy expenditure. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 159:296-302.

Bermejo-Nogales A, Nederlof M, Benedito-Palos L, Ballester-Lozano GF, Folkedal O, Olsen RE, et al. 2014. Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata*) to environmental stress: New insights in fish mitochondrial phenotyping. *General and Comparative Endocrinology* 205:305-315.

Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. 2015. Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. *PLOS ONE* 10(4):e0122889.

Berntssen MHG, Lundebye AK, Torstensen BE. 2005. Reducing the levels of dioxins and dioxin-like PCBs in farmed Atlantic salmon by substitution of fish oil with vegetable oil in the feed. *Aquaculture Nutrition* 11:219-231.

Berntssen MHG, Julshamn K, Lundebye AK. 2010. Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional versus alternative feed ingredients. *Chemosphere* 78:637-646.

Bishop KS, Ferguson LR. 2015. The interaction between epigenetics, nutrition and the development of cancer. *Nutrients* 7(2):922-947.

Booman M, Forster I, Vederas JC, Groman DB, Jones SR. 2018. Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar*) and Chinook salmon (*Oncorhynchus tshawytscha*) but not in pink salmon (*O. gorbuscha*). *Aquaculture* 483:238-243.

Bouraoui L, Sánchez-Gurmaches J, Cruz-Garcia L, Gutiérrez J, Benedito-Palos L, Pérez-Sánchez J, et al. 2011. Effect of dietary fish meal and fish oil replacement on lipogenic and lipoprotein lipase activities and plasma insulin in gilthead sea bream (*Sparus aurata*). *Aquaculture Nutrition* 17(1):54-63.

Brown KD, Maqsood S, Huang JY, Pan Y, Harkcom W, Li W, et al. 2014. Activation of SIRT3 by the NAD(+) precursor nicotinamide riboside protects from noise induced hearing loss. *Cell Metabolism* 20:1059-1068.

Buler M, Andersson U, Hakkola J. 2016. Who watches the watchmen? Regulation of the expression and activity of sirtuins. *The FASEB Journal* 30(12):3942-3960.

Byreddy AR, Yoganantharjah P, Gupta A, Gibert Y, Puri M. 2019. Suitability of novel algal biomass as fish feed: accumulation and distribution of omega-3 long-chain polyunsaturated fatty acid in zebrafish. *Applied Biochemistry and Biotechnology* 188(1):112-123.

Calduch-Giner JA, Davey G, Saera-Vila A, Houeix B, Talbot A, Prunet P, et al. 2010. Use of microarray technology to assess the time course of liver

stress response after confinement exposure in gilthead sea bream (*Sparus aurata* L.). *BMC Genomics* 11:193.

Callet T, Médale F, Larroquet L, Surget A, Aguirre P, Kerneis T, et al. 2017. Successful selection of rainbow trout (*Oncorhynchus mykiss*) on their ability to grow with a diet completely devoid of fishmeal and fish oil, and correlated changes in nutritional traits. *PLOS ONE* 12(10):e0186705.

Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al. 2009. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity *Nature* 458:1056-1060.

Canto C, Menzies KJ, Auwerx J. 2015. NAD+ metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cell Metabolism* 22(1):31-53.

Cao W, Hong Y, Chen H, Wu F, Wei X, Ying W. 2016. SIRT2 mediates NADH-induced increases in Nrf2, GCL, and glutathione by modulating Akt phosphorylation in PC12 cells. *FEBS Letters* 590:2241-2255.

Carafa V, Rotili D, Forgione M, Cuomo F, Serretiello E, Hailu GS, et al. 2016. Sirtuin functions and modulation: from chemistry to the clinic. *Clinical Epigenetics* 8:61.

Castanheira MF, Herrera M, Costas B, Conceição LEC, Martins CIM. 2013. Linking cortisol responsiveness and aggressive behaviour in gilthead seabream *Sparus aurata*: indication of divergent coping styles. *Applied Animal Behaviour Science* 143:75-78.

Chaoui L, Kara, MH, Quignard JP, Faure E, Bonhomme F. 2009. Forte différenciation génétique de la daurade *Sparus aurata* (L., 1758) entre les deux rives de la Méditerranée occidentale. *Comptes Rendus Biologies* 332:329-335.

Chen XF, Tian MX, Sun RQ, Zhang ML, Zhou LS, Jin L, et al. 2018. SIRT5 inhibits peroxisomal ACOX1 to prevent oxidative damage and is downregulated in liver cancer. *EMBO Reports* 19(5):e45124.

Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M. 2014. The growing landscape of lysine acetylation links metabolism and cell signalling. *Nature Reviews Molecular Cell Biology* 15:536-550.

Colak G, Xie Z, Zhu AY, Dai L, Lu Z, Zhang Y, et al. 2013. Identification of lysine succinylation substrates and the succinylation regulatory enzyme CobB in *Escherichia coli*. *Molecular & Cellular Proteomics* 12:3509-3520.

Connon RE, Deanovic LA, Fritsch EB, D'Abronzo LS, Werner I. 2011. Sublethal responses to ammonia exposure in the endangered delta smelt; *Hypomesus transpacificus* (Fam. Osmeridae). *Aquatic Toxicology* 105:369-377.

Cordero H, Cuesta A, Meseguer J, Esteban MA. 2016. Characterization of the gilthead seabream (*Sparus aurata* L.) immune response under a natural lymphocystis disease virus outbreak. *Journal of Fish Diseases* 39:1467-1476.

Costford SR, Bajpeyi S, Pasarica M, Albarado DC, Thomas SC, Xie H, et al. 2010. Skeletal muscle NAMPT is induced by exercise in humans. *American Journal of Physiology-Endocrinology and Metabolism* 298:E117-E126.

Coulondre C, Miller JH, Farabaugh PJ, Gilbert W. 1978. Molecular basis of base substitution hotspots in *Escherichia coli*. *Nature* 274(5673):775.

De Innocentiis S, Lesti A, Livi S, Rossi AR, Crosetti D, Sola L. 2004. Microsatellite markers reveal population structure in gilthead sea bream *Sparus auratus* from the Atlantic Ocean and Mediterranean Sea. *Fisheries Science* 70:852-859.

Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. 2011. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. *Science* 334:806-809.

Ehrlich M, Lacey M. 2013. DNA methylation and differentiation: silencing, upregulation and modulation of gene expression. *Epigenomics* 5:553-568.

Ekambaram P, Parasuraman P. 2017. Differential expression of sirtuin 2 and adipocyte maturation restriction: an adaptation process during hypoxia in fish. *Biology Open* 6(9):1375-1382.

Elhanati S, Kanfi Y, Varvak A, Roichman A, Carmel-Gross I, Barth S, et al. 2013. Multiple regulatory layers of SREBP1/2 by SIRT6. *Cell Reports* 4:905-912.

Elhanati S, Ben-Hamo R, Kanfi Y, Varvak A, Glazz R, Lerrer B, et al. 2016. Reciprocal regulation between SIRT6 and miR-122 controls liver metabolism and predicts hepatocarcinoma prognosis. *Cell Reports* 14(2):234-242.

Estensoro I, Benedito-Palos L, Palenzuela O, Kaushik S, Sitjà-Bobadilla A, Pérez-Sánchez J. 2011. The nutritional background of the host alters the disease outcome in a fish-myxosporean sytem. *Veterinary Parasitology* 175:141-150.

Estensoro I, Ballester-Lozano GF, Benedito-Palos L, Grammes F, Martos-Sitcha JA, Mydland L-T, et al. 2016. Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. *PLOS ONE* 11:e0166564.

FAO. 2018. El estado mundial de la pesca y la acuicultura 2018. Cumplir los objetivos de desarrollo sostenible. Roma. Licencia: CC BY-NC-SA 3.0 IGO.

Feldman JL, Dittenhafer-Reed KE, Denu JM. 2012. Sirtuin catalysis and regulation. The *Journal of Biological Chemistry* 287:42419-42427.

Fernández-Díaz C, Kopecka J, Cañavate JP, Sarasquete C, Solé M. 2006. Variations on development and stress defences in *Solea senegalensis* larvae fed on live and microencapsulated diets. *Aquaculture* 251:573-584.

Filhoulaud G, Guilmeau S, Dentin R, Girard J, Postic C. 2013. Novel insights into ChREBP regulation and function. *Trends in Endocrinology & Metabolism* 24(5):257-268.

Finley LW, Haigis MC. 2012. Metabolic regulation by SIRT3: implications for tumorigenesis. *Trends in Molecular Medicine* 18(9):516-523.

Fiorino E, Giudici M, Ferrari A, Mitro N, Caruso D, De Fabiani E, et al. 2014. The sirtuin class of histone deacetylases: regulation and roles in lipid metabolism. *IUBMB Life* 66(2):89-99.

Flick F, Lüscher B. 2012. Regulation of sirtuin function by posttranslational modifications *Frontiers in Pharmacology* 3:29.

Franchini E, Brito CJ, Artioli GG. 2012. Weight loss in combat sports: physiological, psychological and performance effects *Journal of the International Society of Sports Nutrition* 20129:52.

Francis G, Makkar HP, Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199(3-4):197-227.

Frye RA. 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochemical and Biophysical Research Communications* 273:793798.

Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, et al. 2008. Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Developmental Cell* 14:661-673.

Furuya TK, da Silva PN, Payão SL, Rasmussen LT, de Labio RW, Bertolucci PH, et al. 2012. SORL1 and SIRT1 mRNA expression and promoter methylation levels in aging and Alzheimer's Disease. *Neurochemistry International* 61:973e5.

Gasco L, Gai F, Maricchiolo G, Genovese L, Ragonese S, Bottari T, et al. 2018. Fishmeal alternative protein sources for aquaculture feeds. In: Feeds for the aquaculture sector Springer, Cham, pp 1-28.

Gavery MR, Roberts SB. 2017. Epigenetic considerations in aquaculture. *PeerJ* 5:e4147.

Geay F, Santigosa E, Corporeau C, Boudry P, Dreano Y, Corcos L, et al. 2010. Regulation of FADS2 expression and activity in European sea bass

(*Dicentrarchus labrax*, L.) fed a vegetable diet. *Comparative Biochemistry and Physiology B* 156:237-243.

Geurden I, Borchert P, Balasubramanian MN, Schrama JW, Dupont-Nivet M, Quillet E, et al. 2013. The positive impact of the early-feeding of a plant-based diet on its future acceptance and utilisation in rainbow trout. *PLOS ONE* 8(12):e83162.

Ghinis-Hozumi Y, Antaramian A, Villarroya F, Pina E, Mora O. 2013. Potential role of sirtuins in livestock production. *Animal* 7(1):101-108.

Gil-Solsona R, Calduch-Giner JA, Nácher-Mestre J, Lacalle-Bergeron L, Sancho JV, Hernández F, et al. 2019. Contributions of MS metabolomics to gilthead sea bream (*Sparus aurata*) nutrition. Serum fingerprinting of fish fed low fish meal and fish oil diets. *Aquaculture* 498:503-512.

Grigorakis K, Kogiannou D, Genevieve C, Pérez-Sánchez J, Agnes A, Zsuzsanna JS. 2018. Impact of diets containing plant raw materials as fish meal and fish oil replacement on rainbow trout (*Oncorhynchus mykiss*), gilthead sea bream (*Sparus aurata*), and common carp (*Cyprinus carpio*) freshness. *Journal of Food Quality* 2018:1717465.

Guarente L. 2013. Calorie restriction and sirtuins revisited. *Genes Development* 27(19):2072-2085.

Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutrition Research Reviews* 23(2):366-384.

Hajra A, Mazumder A, Verma A, Ganguly DP, Mohanty BP, Sharma AP. 2013. Antinutritional factors in plant origin fish feed ingredients: the problems and probable remedies. *Advances in Fish Research* 2013:193-202.

Happe A, Zohar Y. 1988. Self-fertilization in the protandrous hermaphrodite *Sparus aurata*: development of the technology. In: Reproduction in fish: basic and applied aspects in endocrinology and genetics: proceedings of the French-Israeli symposium Tel Aviv (Israel). INRA, Paris, France, pp 177-180.

Harvey B, Soto D, Carolsfeld J, Beveridge M, Bartley DM. 2017. Planning for aquaculture diversification: the importance of climate change and other drivers. In: FAO Technical Workshop No 47 pp 23-25.

Hasan MR, New MB, 2013. On-farm feeding and feed management in aquaculture. Documento técnico de pesca y acuicultura de la FAO No. 583. FAO, Roma.

Hasan MR. 2017. Keynote presentation: Status of world aquaculture and global aquafeed requirement with special notes on Artemia. In: Report of the FAO expert workshop on sustainable use and management of Artemia

resources in Asia, Appendix 4 pp 16-17. Tianjin, China. FAO Fisheries and Aquaculture Report No. 1198. FAO, Rome.

Hasan M, Soto S. 2017. Improving feed conversion ratio and its impact on reducing greenhouse gas emissions in aquaculture. In: FAO Non-Serial Publication. FAO, Roma, 33 pp.

He B, Hu J, Zhang X, Lin H. 2014. Thiomyristoyl peptides as cell-permeable Sirt6 inhibitors. *Organic and Biomolecular Chemistry*. 12:7498-7502.

Henry M, Gasco L, Piccolo G, Fountoulaki E. 2015. Review on the use of insects in the diet of farmed fish: past and future. *Animal Feed Science and Technology* 203:1-22.

Hernández-Pérez J, Naderi F, Chivite M, Soengas JL, Míguez JM, López-Patiño MA. 2019. Influence of stress on liver circadian physiology. A study in rainbow trout, *Oncorhynchus mykiss*, as fish model. *Frontiers in Physiology* 10.

Hirschey MD, Zhao Y. 2015. Metabolic regulation by lysine malonylation, succinylation, and glutarylation. *Molecular & Cellular Proteomics* 14(9):2308-2315.

Hong JY, Meia CG, Li SJ, Wanga HB, Zhaoa CP, Zan LS. 2018. Coordinate regulation by transcription factors and DNA methylation in the core promoter region of SIRT6 in bovine adipocytes. *Archives of Biochemistry and Biophysics* 659:1-12.

Hougland JL. 2019. Ghrelin octanoylation by ghrelin O-acyltransferase: Unique protein biochemistry underlying metabolic signaling. *Biochemical Society Transactions* 47(1):169-178.

Houtkooper RH, Canto C, Wanders RJ, Auwerx J. 2010. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocrine Reviews* 31:194-223.

Houtkooper RH. 2016. Sirtuins, proteins and cell regulation 10. Springer, Netherlands.

Huang JY, Hirschey MD, Shimazu T, Ho L, Verdin E. 2010. Mitochondrial sirtuins. *Biochimica et Biophysica Acta* 1804:1645-1651.

Hwang J-W, Yao H, Caito S, Sundar IK, Rahman I. 2013. Redox regulation of SIRT1 in inflammation and cellular senescence. *Free Radical Biology and Medicine* 61:95-110.

Imai S, Johnson FB, Marciniak RA, McVey M, Park PU, Guarente L. 2000. Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. *Cold Spring Harbor Symposia on Quantitative Biology* 65:297-302.

Imai S. 2009a. The NAD World: a new systemic regulatory network for metabolism and aging- Sirt1, systemic NAD biosynthesis, and their importance. *Cell Biochemistry and Biophysics* 53:65-74.

Imai S. 2009b. Nicotinamide phosphoribosyltransferase (Nampt): a link between NAD biology, metabolism, and diseases. *Current Pharmaceutical Design* 15:20-28.

Imai S, Yoshino J. 2013. The importance of NAMPT/NAD/SIRT1 in the systemic regulation of metabolism and ageing. *Diabetes, Obesity and Metabolism* 15:26-33.

Iwahara T, Bonasio R, Narendra V, Reinberg D. 2012. SIRT3 functions in the nucleus in the control of stress-related gene expression. *Molecular and Cellular Biology* 32:5022-5034.

Izquierdo MS, Montero D, Robaina L, Caballero MJ, Rosenlund G, Gines R. 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture* 250:431-444.

Izquierdo MS, Turkmen S, Montero D, Zamorano MJ, Afonso JM, Karalazos V, et al. 2015. Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. *Aquaculture* 449:18-26.

Jablonka E, Lamb MJ. 2002. The changing concept of epigenetics. *Annals of the New York Academy of Sciences* 981(1):82-96.

Jedrusik-Bode M, Studencka M, Smolka C, Baumann T, Schmidt H, Kampf J, et al. 2013. The sirtuin SIRT6 regulates stress granule formation in *C. elegans* and mammals. *Journal of Cell Science* 126:5166-5177.

Jeong SM, Xiao C, Finley LW, Lahusen T, Souza AL, Pierce K, et al. 2013. SIRT4 has tumor-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting mitochondrial glutamine metabolism. *Cancer Cell* 23:450-463.

Jiang H, Khan S, Wang Y, Charron G, He B, Sebastian C, et al. 2013. SIRT6 regulates TNF-alpha secretion through hydrolysis of long-chain fatty acyl lysine. *Nature* 496:110-113.

Jiang L, Xiong J, Zhan J, Yuan F, Tang M, Zhang C, et al. 2017. Ubiquitin-specific peptidase 7 (USP7)-mediated deubiquitination of the histone deacetylase SIRT7 regulates gluconeogenesis. *Journal of Biological Chemistry* 292:13296-13311.

Jin J, Iakova P, Jiang Y, Medrano EE, Timchenko NA. 2011. The reduction of SIRT1 in livers of old mice leads to impaired body homeostasis and to inhibition of liver proliferation. *Hepatology* 54:989-998.

Jin Q, Zhang F, Yan T, Liu Z, Wang C, Ge X, et al. 2010. C/EBPalpha regulates SIRT1 expression during adipogenesis. *Cell Research* 20:470-479.

Jing H, Lin H. 2015. Sirtuins in epigenetic regulation. *Chemical Reviews* 115(6):2350-2375.

Jobling M. 2004a. 'Finishing' feeds for carnivorous fish and the fatty acid dilution model. *Aquaculture Research* 35:706-709.

Jobling M. 2004b. Are modifications in tissue fatty acid profiles following a change in diet the result of dilution?: Test of a simple dilution model. *Aquaculture* 232:551-562.

Kabiljo J, Murko C, Pusch O, Zupkovitz G. 2019. Spatio-temporal expression profile of sirtuins during aging of the annual fish *Nothobranchius furzeri*. *Gene Expression Patterns* 33:11-19.

Katto J, Engel N, Abbas W, Herbein G, Mahlknecht U. 2013. Transcription factor NFkB regulates the expression of the histone deacetylase SIRT1. *Clinical Epigenetics* 5:11.

Kim EJ, Kho JH, Kang MR, Um SJ. 2007. Active regulator of SIRT1 cooperates with SIRT1 and facilitates suppression of p53 activity. *Molecular Cell* 28:277-290.

Kim JE, Chen J, Lou Z. 2008. DBC1 is a negative regulator of SIRT1. *Nature* 451:583-586.

Knecht AL, Truong L, Marvel SW, Reif DM, Garcia A, Lu C, et al. 2017. Transgenerational inheritance of neurobehavioral and physiological deficits from developmental exposure to benzo [a] pyrene in zebrafish. *Toxicology and Applied Pharmacology* 329:148-157.

Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JV, Snowman AM, et al. 2010. GAPDH mediates nitrosylation of nuclear proteins. *Nature Cell Biology* 12:1094-1100.

Krogdahl A, Penn M, Thorsen J, Refstie S, Bakke AM. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquaculture Research* 41:333e344.

Kuang J, Chen L, Tang Q, Zhang J, Li Y, He J. 2018. The role of Sirt6 in obesity and diabetes. *Frontiers in Physiology* 9:135.

Kurdyukov S, Bullock M. 2016. DNA methylation analysis: choosing the right method. *Biology* 5(1):3.

Laplante M, Sabatini DM. 2012. mTOR signaling in growth control and disease. *Cell* 149:274-293.

Laurent G, German NJ, Saha AK, de Boer VC, Davies M, Koves TR, et al. 2013. SIRT4 coordinates the balance between lipid synthesis and catabolism by repressing malonyl CoA decarboxylase. *Molecular Cell* 50:686-698.

Lazzarotto V, Médale F, Larroquet L, Corraze G. 2018. Long-term dietary replacement of fishmeal and fish oil in diets for rainbow trout (*Oncorhynchus mykiss*): effects on growth, whole body fatty acids and intestinal and hepatic gene expression. *PLOS ONE* 13:e0190730.

Le Boucher R, Quillet E, Vandeputte M, Lecalvez JM, Goardon L, Chatain B, et al. 2011. Plant-based diet in rainbow trout (*Oncorhynchus mykiss* Walbaum): are there genotype-diet interactions for main production traits when fish are fed marine vs. plant-based diets from the first meal? *Aquaculture* 321:41-48.

Le Boucher R, Vandeputte M, Dupont-Nivet M, Quillet E, Ruelle F, Vergnet A, et al. 2013. Genotype by diet interactions in European sea bass (L.): nutritional challenge with totally plant-based diets. *Journal of Animal Science* 91:44-56.

Lee Y, Drake AC, Thomas NO, Ferguson LG, Chappell PE, Shay KP. 2018. Dietary resveratrol increases mid-life fecundity of female *Nothobranchius guentheri*. Comparative *Biochemistry and Physiology Part C: Toxicology & Pharmacology* 208:71-76.

Lim WJ, Kim KH, Kim JY, Jeong S, Kim N. 2019. Identification of DNA-methylated CpG islands associated with gene silencing in the adult body tissues of the Ogye chicken using RNA-seq and reduced representation bisulfite sequencing. *Frontiers in Genetics* 10:346.

Lin H, Su X, He B. 2012. Protein lysine acylation and cysteine succination by intermediates of energy metabolism. *ACS Chemical Biology* 7(6):947-960.

Lin Z, Fang D. 2013. The roles of SIRT1 in cancer. *Genes Cancer* 4:97-104.

Little DC, Newton RW, Beveridge MCM. 2016. Aquaculture: a rapidly growing and significant source of sustainable food? Status, transitions and potential. *Proceedings of the Nutrition Society* 75(3):274-286.

Liu S, Zheng Z, Ji S, Liu T, Hou Y, Li S, et al. 2018. Resveratrol reduces senescence-associated secretory phenotype by SIRT1/NF-κB pathway in gut of the annual fish *Nothobranchius guentheri*. *Fish & Shellfish Immunology* 80:473-479.

Liu T, Qi H, Ma L, Liu Z, Fu H, Zhu W, et al. 2015. Resveratrol attenuates oxidative stress and extends life span in the annual fish *Nothobranchius guentheri*. *Rejuvenation Research* 18:225-233.

Liu T, Liu S, Ma L, Li F, Zheng Z, Chai R, et al. 2017. Oogenesis, vitellogenin-mediated ovarian degeneration and immune response in the annual fish *Nothobranchius guentheri*. *Fish & Shellfish Immunology* 66:86-92.

Liu X, Wang D, Zhao Y, Tu B, Zheng Z, Wang L, et al. 2011. Methyltransferase Set7/9 regulates p53 activity by interacting with Sirtuin 1 (SIRT1). *Proceedings of the National Academy of Sciences USA* 108:1925-1930.

López-Olmeda JF, Montoya A, Oliveira C, Sánchez-Vázquez FJ. 2009. Synchronization to light and restricted-feeding schedules of behavioral and humoral daily rhythms in gilthead seabream (*Sparus aurata*). *Chronobiology International* 26:1389-1408.

Magnoni LJ, Palstra AP, Planas JV. 2014. Fueling the engine: induction of AMP-activated protein kinase in trout skeletal muscle by swimming. *The Journal of Experimental Biology* 217:1649-1652.

Magnoni LJ, Martos-Sitcha JA, Queiroz A, Calduch-Giner JA, Gonçalves JFM, Rocha CM, et al. 2017. Dietary supplementation of heat-treated *Gracilaria* and *Ulva* seaweeds enhanced acute hypoxia tolerance in gilthead sea bream (*Sparus aurata*). *Biology Open* 6:897-908.

Mailloux RJ, Harper ME. 2011. Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radical Biology and Medicine* 51:1106-1115.

Martínez-Álvarez RM, Morales AE, Sanz A. 2005. Antioxidant defenses in fish: biotic and abiotic factors. *Reviews in Fish Biology and Fisheries* 15:75-88.

Martínez-Álvarez O, Chamorro S, Brenes A. 2015. Protein hydrolysates from animal processing by-products as a source of bioactive molecules with interest in animal feeding: a review. *Food Research International* 73:204-212.

Martos-Sitcha JA, Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. 2017. Gene expression profiling of whole blood cells supports a more efficient mitochondrial respiration in hypoxia-challenged gilthead sea bream (*Sparus aurata*). *Frontiers in Zoology* 14:34.

Martos-Sitcha JA, Simó-Mirabet P, de las Heras V, Calduch-Giner JA, Pérez-Sánchez J. 2019. Tissue-specific orchestration of gilthead sea bream resilience to hypoxia and high stocking density. *Frontiers in Physiology* 10:840.

Mata-Sotres JA, Martínez-Rodríguez G, Pérez-Sánchez J, Sánchez-Vázquez FJ, Yúfera M. 2015. Daily rhythms of clock gene expression and feeding

behavior during the larval development in gilthead seabream, *Sparus aurata*. *Chronobiology International* 32:1061-1074.

Matsushita N, Yonashiro R, Ogata Y, Sugiura A, Nagashima S, Fukuda T, et al. 2011. Distinct regulation of mitochondrial localization and stability of two human Sirt5 isoforms. *Genes Cells* 16:190-202.

Meena D, Dad P, Kumar S, Mandal S, Prusty A, et al. 2013. Beta-glucan: an ideal immunostimulants in aquaculture (a review). *Fish Physiology and Biochemistry* 39:431-457.

Mendizabal I, Yi SV. 2016. Whole-genome bisulfite sequencing maps from multiple human tissues reveal novel CpG islands associated with tissue-specific regulation. *Human Molecular Genetics* 25(1):69-82.

Miao LH, Lin Y, Pan WJ, Huang X, Ge XP, Liu B, et al. 2019. MiR-34a regulates the glucose metabolism of Blunt snout bream (*Megalobrama amblycephala*) fed high-carbohydrate diets through the mediation of the Sirt1/FoxO1 axis. *Aquaculture* 500:206-214.

Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. 2005. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Molecular Biology of the Cell* 16(10):4623-4635.

Monroig Ó, Rotllant J, Cerdá-Reverter JM, Dick JR, Figueras A, Tocher DR. 2010. Expression and role of Elovl4 elongases in biosynthesis of very long-chain fatty acids during zebrafish *Danio rerio* early embryonic development. *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids* 1801(10):1145-1154.

Monroig Ó, Webb K, Ibarra-Castro L, Holt GJ, Tocher DR. 2011. Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. *Aquaculture* 312(1-4):145-153.

Montero D, Kalinowski T, Obach A, Robaina L, Tort L, Caballero MJ, et al. 2003. Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on fish health. *Aquaculture* 225(1-4):353-370.

Mulligan P, Yang F, Di Stefano L, Ji JY, Ouyang J, Nishikawa JL, et al. 2011. A SIRT1-LSD1 corepressor complex regulates Notch target gene expression and development. *Molecular Cell* 42(5):689-699.

Musgrove EA. 2006. Cyclins: roles in mitogenic signaling and oncogenic transformation. *Growth Factors* 24:13-19.

Nabben M, Hoeks J. 2008. Mitochondrial uncoupling protein 3 and its role in cardiac-and skeletal muscle metabolism. *Physiology & Behavior* 94:259-269.

Nácher-Mestre J, Serrano R, Benedito-Palos L, Navarro JC, López FJ, Pérez-Sánchez J. 2009. Effects of fish oil replacement and re-feeding on the bioaccumulation of organochlorine compounds in gilthead sea bream (*Sparus aurata* L.) of market size. *Chemosphere* 76:811-817.

Nácher-Mestre J, Ibanez M, Serrano R, Pérez-Sánchez J, Hernández F. 2013. Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry. *Journal of Agricultural and Food Chemistry* 61:2077-2087.

Nácher-Mestre J, Serrano R, Portolés T, Berntssen MH, Pérez-Sánchez J, Hernández F. 2014. Screening of pesticides and polycyclic aromatic hydrocarbons in feeds and fish tissues by gas chromatography coupled to high-resolution mass spectrometry using atmospheric pressure chemical ionization. *Journal of Agricultural and Food Chemistry* 62(10):2165-2174.

Nácher-Mestre J, Serrano R, Beltrán E, Pérez-Sánchez J, Silva J, Karalazos V, et al. 2015. Occurrence and transfer of mycotoxins in gilthead sea bream and Atlantic salmon by use of novel alternative feed ingredients. *Chemosphere* 128:314-320.

Nácher-Mestre J, Ballester-Lozano GF, Garlito B, Portolés T, Calduch-Giner J, Serrano R, et al. 2018. Comprehensive overview of feed-to-fillet transfer of new and traditional contaminants in Atlantic salmon and gilthead sea bream fed plant-based diets. *Aquaculture Nutritrion* 24:1782-1795.

Naderi F, Hernández-Pérez J, Chivite M, Soengas JL, Míguez JM, López-Patiño MA. 2018. Involvement of cortisol and sirtuin1 during the response to stress of hypothalamic circadian system and food intake-related peptides in rainbow trout, *Oncorhynchus mykiss*. *Chronobiology International* 35(8):1122-1141.

Naderi F, Míguez JM, Soengas JL, López-Patiño MA. 2019. SIRT1 mediates the effect of stress on hypothalamic clock genes and food intake regulators in rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 235:102-111.

Nakagawa T, Lomb DJ, Haigis MC, Guarente L. 2009. SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. *Cell* 137(3):560-570.

National Research Council (NRC). 2011. Nutrient requirements of fish and shrimp. National Academy Press, Washington DC, 376 pp.

Naylor RL, Hardy RW, Bureau DP, et al. 2009. Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences USA* 106(36):15103-15110.

Nemoto S, Fergusson MM, Finkel T. 2004. Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. *Science* 306:2105-2108.

Nie H, Hong Y, Lu X, Zhang J, Chen H, Li Y, et al. 2014. SIRT2 mediates oxidative stress-induced apoptosis of differentiated PC12 cells. *Neuroreport* 25(11):838-842.

Noriega LG, Feige JN, Canto C, Yamamoto H, Yu J, Herman MA, et al. 2011. CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. *EMBO Reports* 12:1069-1076.

North BJ, Verdin E. 2007. Mitotic regulation of SIRT2 by cyclin-dependent kinase 1-dependent phosphorylation. *Journal of Biological Chemistry* 282:19546-19555.

Otero-Rodiño C, Librán-Pérez M, Velasco C, Álvarez-Otero R, López-Patiño MA, Míguez JM, et al. 2016. Glucosensing in liver and Brockmann bodies of rainbow trout through glucokinase-independent mechanisms. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 199:29-42.

Pacitti D, Wang T, Martin SAM, Sweetman J, Secombes CJ. 2014. Insights into the fish thioredoxin system: expression profile of thioredoxin and thioredoxin reductase in rainbow trout (*Oncorhynchus mykiss*) during infection and in vitro stimulation. *Developmental & Comparative Immunology* 42:261-277.

Pahlow M, van Oel PR, Mekonnen MM, Hoekstra AY. 2015. Increasing pressure on freshwater resources due to terrestrial feed ingredients for aquaculture production. *Science of the Total Environment* 536:847-857.

Pandithage R, Lilischkis R, Harting K, Wolf A, Jedamzik B, Lüscher-Firzlaff J, et al. 2008. The regulation of SIRT2 function by cyclin-dependent kinases affects cell motility. *Journal of Cell Biology* 180:915-929.

Pardo PS, Boriek AM. 2012. An autoregulatory loop reverts the mechanosensitive Sirt1 induction by EGR1 in skeletal muscle cells. *Aging* 4:456-461.

Park J, Chen Y, Tishkoff DX, Peng C, Tan M, Dai L, et al. 2013. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. *Molecular Cell* 50:919-930.

Peng C, Lu Z, Xie Z, Cheng Z, Chen Y, Tan M, et al. 2011. The first identification of lysine malonylation substrates and its regulatory enzyme. *Molecular & Cellular Proteomics* 10(12):M111-012658.

Perera E, Simó-Mirabet P, Shin HS, Rosell-Moll E, Naya-Catalá F, de las Heras V, et al. 2019. Selection for growth is associated in gilthead sea bream

(*Sparus aurata*) with diet flexibility, changes in growth patterns and higher intestine plasticity. *Aquaculture* 507:349-360.

Perera E, Turkmen S, Simó-Mirabet P, Zamorano MJ, Xu H, Naya-Català F, et al. 2020. Stearoyl-CoA desaturase (*scd1a*) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). *Epigenetics*. doi: 10.1080/15592294.2019.1699982.

Pérez-Sánchez J, Estensoro I, Redondo MJ, Calduch-Giner JA, Kaushik S, Sitjà- Bobadilla A. 2013a. Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. *PLOS ONE* 8:e65457.

Pérez-Sánchez J, Borrel M, Bermejo-Nogales A, Benedito-Palos L, Saera-Vila A, Calduch-Giner JA, et al. 2013b. Dietary oils mediate cortisol kinetics and the hepatic expression profile of stress responsive genes in juveniles of gilthead sea bream (*Sparus aurata*) exposed to crowding stress. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 8(2):123-130.

Piazzon C, Galindo-Villegas J, Pereiro P, Estensoro I, Calduch-Giner JA, Gómez-Casado E, et al. 2016. Differential modulation of IgT and IgM upon parasitic, bacterial, viral and dietary challenges in a perciform fish. *Frontiers in Immunology* 7:637.

Piazzon MC, Calduch-Giner JA, Fouz B, Estensoro I, Simó-Mirabet P, Puyalto M, et al. 2017. Under Control: how a dietary additive can restore the gut microbiome and proteomic profile, and imporve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* 5:164.

Piazzon MC, Estensoro I, Calduch-Giner JA, del Pozo R, Picard-Sánchez A, Pérez-Sánchez J, et al. 2018. Hints on T cell responses in a fish-parasite model: *Enteromyxum leei* induces differential expression of T cell signature molecules depending on the organ and the infection status. *Parasites & Vectors* 11(1):443.

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, de Oliveira RM. 2004. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. *Nature* 429(6993):771-776.

Piedecausa MA, Mazón MJ, García BG, Hernández MD. 2007. Effects of total replacement of fish oil by vegetable oils in the diets of sharpsnout seabream (*Diplodus puntazzo*). *Aquaculture* 263:211-219.

Potente M, Ghaeni L, Baldessari D, Mostoslavsky R, Rossig L, Dequiedt F, et al. 2007. SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes & Development* 21(20):2644-2658.

Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, et al. 2012. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metabolism* 15:675-690.

Radaka Z, Koltaia E, Taylora AW, Higuchi M, Kumagai S, Ohno H, et al. 2013. Redox-regulating sirtuins in aging, caloric restriction, and exercise. *Free Radical Biology and Medicine* 58:87-97.

Rajan KE, Thangaleela S, Balasundaram C. 2015. Spatial learning associated with stimulus response in goldfish *Carassius auratus*: relationship to activation of CREB signalling. *Fish Physiology and Biochemistry* 41:685-694.

Ramakrishnan G, Davaakhuu G, Kaplun L, Chung WC, Rana A, Atfi A, et al. 2014. Sirt2 deacetylase is a novel AKT binding partner critical for AKT activation by insulin. *Journal of Biological Chemistry* 289:6054-6066.

Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, et al. 2009. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* 138(1):114-128.

Rardin MJ, He W, Nishida Y, Newman JC, Carrico C, Danielson SR, et al. 2013. SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks. *Cell Metabolism* 18:920-933.

Regost C, Arzel J, Robin J, Rosenlund G, Kaushik SJ. 2003. Total replacement of fish oil by soybean or linseed oil with a return to fish oil in turbot (*Psetta maxima*): 1. Growth performance, flesh fatty acid profile, and lipid metabolism. *Aquaculture* 217:465-482.

Revollo JR, Grimm AA, Imai S. 2004. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *Journal of Biological Chemistry* 279:50754-50763.

Richards EJ, Elgin SCR. 2002. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* 108:489-500.

Rise ML, Hall JR, Nash GW, Xue X, Booman M, Katan T, et al. 2015. Transcriptome profiling reveals that feeding wild zooplankton to larval Atlantic cod (*Gadus morhua*) influences suites of genes involved in oxidation-reduction, mitosis, and selenium homeostasis. *BMC Genomics* 16:1016.

Robin JH, Regost C, Arzel J, Kaushik SJ. 2003. Fatty acid profile of fish following a change in dietary fatty acid source: model of fatty acid composition with a dilution hypothesis. *Aquaculture* 225:283-293.

Rosenlund G, Corraze G, Izquierdo M, Torstensen BE. 2011. The effects of fish oil replacement on nutritional and organoleptic qualities of farmed

fish. In: Turchini GM, Ng WK, Tocher DR (eds.) Fish oil replacement and alternative lipid sources in aquaculture feeds. CRC Press, Boca Raton, FL, pp 487-522.

Rossi AR, Perrone E, Sola L. 2006. Genetic structure of gilthead seabream *Sparus aurata* in the Central Mediterranean Sea. *Central European Journal of Biology* 1:636-647.

Ryu D, Jo YS, Lo Sasso G, Stein S, Zhang H, Perino A, et al. 2014. A SIRT7-dependent acetylation switch of GABPbeta1 controls mitochondrial function. *Cell Metabolism* 20:856-869.

Sahin K, Yilmaz S, Gozukirmizi N. 2014. Changes in human sirtuin 6 gene promoter methylation during aging. *Biomedical Reports* 2:574e8.

Sánchez JA, López-Olmeda JF, Blanco-Vives B, Sánchez-Vázquez FJ. 2009. Effects of feeding schedule on locomotor activity rhythms and stress response in sea bream. *Physiology & Behavior* 98:125-129.

Sanders BD, Jackson B, Marmorstein R. 2010. Structural basis for sirtuin function: what we know and what we don't. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* 1804(8):1604-1616.

Saravanan S, Schrama JW, Figueiredo-Silva AC, Kaushik SJ, Verreth JA, Geurden I. 2012. Constraints on energy intake in fish: the link between diet composition, energy metabolism, and energy intake in rainbow trout. *PLOS ONE* 7(4):e34743.

Saxonov S, Berg P, Brutlag DL. 2006. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proceedings of the National Academy of Sciences USA* 103(5):1412-1417.

Schaefer AM, Walker M, Turnbull DM, Taylor RW. 2013. Endocrine disorders in mitochondrial disease. *Molecular and Cellular Endocrinology* 379:2-11.

Scher MB, Vaquero A, Reinberg D. 2007. SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress. *Genes Development* 21:920-928.

Schneider ACR, Gregório C, Uribe-Cruz C, Guizzo R, Malysz T, Faccioni-Heuser MC, et al. 2017. Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver. *World Journal of Hepatology* 9(8):418.

Schwer B, North BJ, Frye RA, Ott M, Verdin E. 2002. The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. *Journal of Cell Biology* 158:647-657.

Shao C, Li Q, Chen S, Zhang P, Lian J, Hu Q, et al. 2014. Epigenetic modification and inheritance in sexual reversal of fish. *Genome Research* 24(4):604-615.

Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, et al. 2007. Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. *PLoS Genetics* 3(10):e181.

Sheng W, Lu Y, Mei F, Wang N, Liu ZZ, Han YY, et al. 2018. Effect of resveratrol on Sirtuins, OPA1, and Fis1 expression in adult zebrafish retina. *Investigative Ophthalmology & Visual Science* 59(11):4542-4551.

Shepherd CJ, Monroig O, Tocher DR. 2017. Future availability of raw materials for salmon feeds and supply chain implications: The case of Scottish farmed salmon. *Aquaculture* 467:49-62.

Shi HJ, Xu C, Liu MY, Wang BK, Liu WB, Chen DH, et al. 2018. Resveratrol improves the energy sensing and glycolipid metabolism of blunt snout bream *Megalobrama amblycephala* fed high-carbohydrate diets by activating the AMPK–SIRT1–PGC-1α network. *Frontiers in Physiology* 9:1258.

Silva PNO, Gigek CO, Leal MF, Bertolucci PHF, de Labio RW, Payao SLM, et al. 2008. Promoter methylation analysis of SIRT3, SMARCA5, HTERT and CDH1 genes in aging and Alzheimer's disease. *Journal of Alzheimer's Disease* 13(2):173-176.

Silva-Marrero JI, Sáez A, Caballero-Solares A, Viegas I, Almajano MP, Fernández F, et al. 2017. A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). *BMC Genomics* 18:768.

Simeoni F, Tasselli L, Tanaka S, Villanova L, Hayashi M, Kubota K, et al. 2013. Proteomic analysis of the SIRT6 interactome: novel links to genome maintenance and cellular stress signaling. *Scientific Reports* 3:3085.

Simó-Mirabet P, Felip A, Estensoro I, Martos-Sitcha JA, De las Heras V, Calduch-Giner JA, et al. 2018. Impact of low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle. *Aquaculture* 490:64-74.

Slimen HB, Guerbej H, Othmen AB, Brahim IO, Blel H, Chatti N, et al. 2004. Genetic differentiation between populations of gilthead seabream (*Sparus aurata*) along the Tunisian coast. *Cybium* 28:45-50.

Smith BC, Hallows WC, Denu JM. 2008. Mechanisms and molecular probes of sirtuins. *Chemistry & Biology* 15:1002-1013.

Sun L, Fan G, Shan P, Qiu X, Dong S, Liao L, et al. 2016. Regulation of energy homeostasis by the ubiquitin-independent REGγ proteasome. *Nature Communications* 7:12497.

Sundaresan NR, Samant SA, Pillai VB, Rajamohan SB, Gupta MP. 2008. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. *Molecular and Cellular Biology* 28:6384-6401.

Sundaresan NR, Pillai VB, Wolfgeher D, Samant S, Vasudevan P, Parekh V, et al. 2011. The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Science Signaling* 4.182:ra46-ra46.

Tacon AGJ, Metian M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* 285:146-158.

Tacon AGJ, Hasan MR, Metian M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects. In: FAO Fisheries and Aquaculture Technical Paper No. 564. FAO, Rome, 87 pp.

Tacon AG, Metian M. 2015. Feed matters: satisfying the feed demand of aquaculture. *Reviews in Fisheries Science & Aquaculture* 23(1):1-10.

Tan M, Peng C, Anderson KA, Chhoy P, Xie Z, Dai L, et al. 2014. Lysine glutarylation is a protein post-translational modification regulated by SIRT5. *Cell Metabolism* 19:605-617.

Tanno M, Sakamoto J, Miura T, Shimamoto K, Horio Y. 2007. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. *Journal of Biological Chemistry* 282:6823-6832.

Teigen LE, Orczewska JI, McLaughlin J, O'Brien KM. 2015. Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in threespine stickleback. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 188:139-147.

Tocher DR. 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* 449:94-107.

Tong L, Denu JM. 2010. Function and metabolism of sirtuin metabolite O-acetyl-ADP-ribose. *Biochimica et Biophysica Acta* 1804:1617-1625.

Torrecillas S, Mompel D, Caballero MJ, Montero D, Merrifield D, Rodiles A, et al. 2017. Effect of fishmeal and fish oil replacement by vegetable meals and oils on gut health of European sea bass (*Dicentrarchus labrax*). *Aquaculture* 468:386-398.

Trushenski JT, Bowzer JC. 2013. Having your omega 3 fatty acids and eating them too: strategies to ensure and improve the long-chain polyunsaturated fatty acid content of farm-raised fish. In: Omega-6/3 Fatty Acids. Humana Press, Totowa, NJ, pp 319-339.

Turchini GM, Hermon KM, Francis DS. 2018. Fatty acids and beyond: fillet nutritional characterisation of rainbow trout (*Oncorhynchus mykiss*) fed different dietary oil sources. *Aquaculture* 491:391-397.

Turkmen S, Castro PL, Caballero MJ, Hernández-Cruz CM, Saleh R, Zamorano MJ, et al. 2017a. Nutritional stimuli of gilthead seabream (*Sparus aurata*) larvae by dietary fatty acids: effects on larval performance, gene expression and neurogenesis. *Aquaculture Research* 48:202-213.

Turkmen S, Zamorano MJ, Fernández-Palacios H, Hernández-Cruz CM, Montero D, Robaina L, et al. 2017b. Parental nutritional programming and a reminder during juvenile stage affect growth, lipid metabolism and utilisation in later developmental stages of a marine teleost, the gilthead sea bream (*Sparus aurata*). *British Journal of Nutrition* 118:500-512.

Turkmen S, Perera E, Zamorano MJ, Simó-Mirabet P, Xu H, Pérez-Sanchez J, Izquierdo M. 2019. Effects of dietary lipid composition and fatty acid desaturase 2 expression in broodstock gilthead sea bream on lipid metabolism-related genes and methylation of the *fads2* gene promoter in their offspring. *International Journal of Molecular Sciences* 20:6250.

Vagner M, Zambonino-Infante JL, Robin JH, Person-Le Ruyet J. 2007. Is it possible to influence European sea bass (*Dicentrarchus labrax*) juvenile metabolism by a nutritional conditioning during larval stage? *Aquaculture* 267:165-174.

Vagner M, Robin JH, Zambonino-Infante JL, Tocher DR, Person-Le Ruyet J. 2009. Ontogenic effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. *British Journal of Nutrition* 101:1452-1462.

Vassilopoulos A, Fritz KS, Petersen DR, Gius D. 2011. The human sirtuin family: evolutionary divergences and functions. *Human Genomics* 5(5):485.

Velasco C, Moreiras G, Conde-Sieira M, Leao JM, Míguez JM, Soengas JL. 2017. Ceramide counteracts the effects of ghrelin on the metabolic control of food intake in rainbow trout. *Journal of Experimental Biology* 220(14):2563-2576.

Wan AH, Davies SJ, Soler-Vila A, Fitzgerald R, Johnson MP. 2019. Macroalgae as a sustainable aquafeed ingredient. *Reviews in Aquaculture* 11(3):458-492.

Weinert BT, Schölz C, Wagner SA, Iesmantavicius V, Su D, Daniel JA, et al. 2013. Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Reports* 4:842-851.

Wellen KE, Thompson CB. 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. *Molecular Cell* 40(2):323-332.

Wu D, Li Y, Zhu KS, Wang H, Zhu WG. 2018. Advances in cellular characterization of the sirtuin isoform, SIRT7. *Frontiers in Endocrinology* 9:652.

Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, et al. 2007a. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell* 130:1095-1107.

Yang Y, Fu W, Chen J, Olashaw N, Zhang X, Nicosia SV, et al. 2007b. SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. *Nature Cell Biology* 9:1253-1262.

Yang Y, Sauve AA. 2016. Biochemistry and enzymology of sirtuins. In: Sirtuins. Springer, Dordrecht, pp 1-27.

Yang Y, Gibson GE. 2019. Succinylation links metabolism to protein functions. *Neurochemical Research* 44(10):2346-2359.

Yoshino J, Mills KF, Yoon MJ, Imai S. 2011. Nicotinamide mononucleotide, a Key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metabolism* 14:528-536.

Yoshizawa T, Karim MF, Sato Y, Senokuchi T, Miyata K, Fukuda T, et al. 2014. SIRT7 controls hepatic lipid metabolism by regulating the ubiquitin-proteasome pathway. *Cell Metabolism* 19:712-721.

Ytrestøyl T, Aas TS, Åsgård T. 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture* 448:365-374.

Yúfera M, Perera E, Mata-Sotres JA, Calduch-Giner J, Martínez-Rodríguez G, Pérez-Sánchez J. 2017. The circadian transcriptome of marine fish (*Sparus aurata*) larvae reveals highly synchronized biological processes at the whole organism level. *Scientific Reports* 7: 12943.

Zhang D, Yan Y, Tian H, Jiang G, Li X, Liu W. 2018. Resveratrol supplementation improves lipid and glucose metabolism in high-fat diet-fed blunt snout bream. *Fish Physiology and Biochemistry* 44(1):163-173.

Zhang H-N, Li L, Gao P, Chen H-Z, Zhang R, Wei Y-S, et al. 2010. Involvement of the p65/RelA subunit of NF-kappaB in TNF-alpha-induced SIRT1 expression in vascular smooth muscle cells. *Biochemical and Biophysical Research Communications* 397:569-575.

Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, et al. 2010. Regulation of cellular metabolism by protein lysine acetylation. *Science* 327(5968):1000-1004.

Zhou B, Zhang Y, Zhang F, Xia Y, Liu J, Huang R, et al. 2014. CLOCK/BMAL1 regulates circadian change of mouse hepatic insulin sensitivity by SIRT1. *Hepatology* 59:2196-2206.

Zhou Y, Zhang H, He B, Du J, Lin H, Cerione RA, et al. 2012. The bicyclic intermediate structure provides insights into the desuccinylation mechanism of human sirtuin 5 (SIRT5). *Journal of Biological Chemistry* 287:28307-28314.

Zohar Y, Abraham M, Gordon H. 1978. The gonad cycle of the captivity-reared hermaphroditic teleost *Sparus aurata* during the first two years of life. *Annales de Biologie Animale Biochimie Biophysique* 18:877-882.

Zohar Y. 1989. Fish reproduction: its physiology and artificial manipulation. In: Shilo M and Sarig S (eds.) Fish culture in warm water systems: problem and trends. CRC Press, Boca Raton, FL, pp 65-119.

Zullo A, Simone E, Grimaldi M, Gagliardi M, Zullo L, Matarazzo MR, et al. 2018. Effect of nutrient deprivation on the expression and the epigenetic signature of sirtuin genes. *Nutrition, Metabolism & Cardiovascular Diseases* 28(4):418-42.

CAPÍTULO 2- Objetivos

El **objetivo principal** de la presente Tesis Doctoral ha sido la caracterización molecular y la validación de las *sirts* como indicadores del estado energético y del crecimiento a lo largo del ciclo biológico y de producción de la dorada, como especie modelo de la acuicultura mediterránea, haciendo especial hincapié en los últimos avances científicos encaminados a promover un desarrollo sostenible del sector.

Los **objetivos específicos** relacionados con los indicadores del estado energético y metabólico de doradas en cultivo incluyen:

i) Caracterizar molecularmente a nivel de transcrito y de estructura génica (exón-intrón) las *sirts* de la dorada.

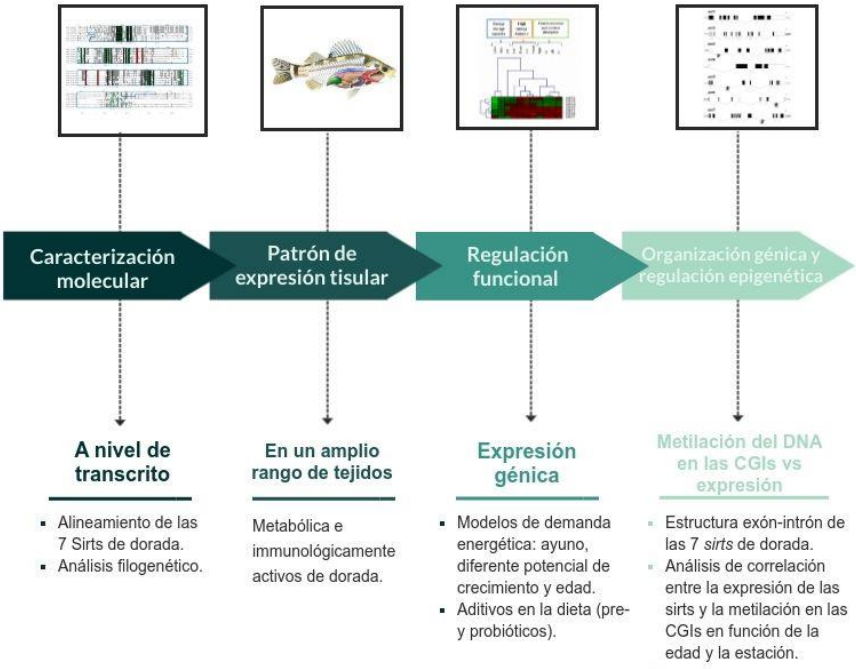
ii) Determinar el patrón de expresión génica de las *sirts* en tejidos metabólica e inmunológicamente activos.

iii) Analizar la regulación funcional de las *sirts* de la dorada en respuesta al uso de aditivos en la dieta y diferentes modelos experimentales de demanda energética (ayuno, potencial de crecimiento y edad).

iv) Mapear las islas CpG (CGIs) en las regiones promotoras de las *sirts* de dorada.

v) Analizar la correlación entre la expresión de las *sirts* y el nivel de metilación de las CGIs de sus regiones promotoras, usando como variables la edad y la estación o época del año.

Plan de trabajo



Tras los dos capítulos preliminares, los resultados obtenidos de acuerdo con el plan de trabajo se muestran en los capítulos 3-6:

Capítulo 3: Caracterización molecular y análisis funcional de la regulación de las *sirts* en peces. Se describe la caracterización molecular de las 7 de *sirts* de dorada, su patrón de expresión en un amplio rango de tejidos y su diferente regulación en un modelo de ayuno.

Capítulo 4: Regulación de la expresión génica de las *sirts* en dos cepas de dorada con diferente potencial de crecimiento. Se describe el diferente patrón de crecimiento de dos cepas de dorada genéticamente distintas, en base a parámetros de crecimiento, marcadores bioquímicos y moleculares, analizando la expresión de las *sirts* junto con otros marcadores del metabolismo energético y lipídico (en hígado, músculo, tejido adiposo) y de la función y salud intestinal (en intestino anterior y posterior).

Capítulo 5: Descripción de los efectos de determinados aditivos sobre el estado metabólico, la salud intestinal y las *sirts*. Se muestran los efectos de la suplementación de la dieta con dos aditivos de diferente naturaleza (un ácido orgánico de cadena media y un probiótico) en juveniles de dorada. Se analizan e integran datos de crecimiento, bioquímica de la sangre, histología y expresión génica de las *sirts* y otros marcadores de la función y la arquitectura intestinal en el intestino anterior y posterior.

Capítulo 6: Organización génica de las 7 *sirts* de dorada y la correlación entre los cambios de metilación y expresión de las *sirts* en función de la edad y la estación. Se describe la estructura exón-intrón de las 7 *sirts* de dorada y la presencia de CGIs en la región promotora. Se muestran los perfiles de expresión de las *sirts* y otros marcadores relacionados con el metabolismo oxidativo y el desacople de la respiración en hígado y músculo de peces de 1 y 3 años en condiciones estivales e invernales, correlacionando los patrones de metilación de las CGIs de la *sirt1* y *sirt3* con sus niveles de expresión.

Los capítulos 3-6 se corresponden con las cuatro publicaciones científicas que han constituido la base de la presente Tesis Doctoral:

Capítulo 3: Simó-Mirabet P, Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. 2017. **Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*).** *Journal of Comparative Physiology B* 187:153-163. IF: 2.517 (Physiology Q3/Zoology Q1).

Capítulo 4: Simó-Mirabet P, Perera E, Calduch-Giner JA, Afonso JM, Pérez-Sánchez J. 2018. **Co-expression analysis of sirtuins and related metabolic biomarkers in juveniles of gilthead sea bream (*Sparus aurata*) with differences in growth performance.** *Frontiers in Physiology* 9:608. IF: 3.201 (Physiology Q2).

Capítulo 5: Simó-Mirabet P, Piazzon, MC, Calduch-Giner, JA, Ortiz Á, Puyalto M, Sitjà-Bobadilla A, Pérez-Sánchez J. 2017. **Sodium salt medium-chain fatty acids and *Bacillus*-based probiotic strategies to improve growth and intestinal health of gilthead sea bream *Sparus aurata*).** *PeerJ* 5:e4001. IF: 2.118 (Multidisciplinary Sciences Q2).

Capítulo 6: Simó-Mirabet P, Perera, Calduch-Giner JA, Pérez-Sánchez J. **Local DNA methylation helps to regulate muscle sirtuin 1 gene expression across season and advancing age in gilthead sea bream (*Sparus aurata*).** En revisión en *Frontiers in Zoology*.

Nota: los capítulos 3-6 mantienen los requisitos de uniformidad de las revistas en las que se publicaron, aunque se han editado para facilitar su lectura y adaptarlos al formato de la presente Tesis Doctoral.

Finalmente, el **capítulo 7** incluye una discusión general, abordando globalmente las cuestiones planteadas a lo largo del desarrollo de la presente Tesis Doctoral y su relación con los trabajos adicionales en los que la Doctoranda ha participado activamente durante los últimos cinco años:

- Benedito-Palos L, Ballester-Lozano GF, **Simó P**, Karalazos V, Ortiz A, Calduch-Giner JA, Pérez-Sánchez J. 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). *Aquaculture* 454:8-18.
- Pérez-Sánchez J, Terova G, **Simó-Mirabet P**, Rimoldi S, Folkedal O, Calduch-Giner JA, Olsen RE, Sitjà-Bobadilla A. 2017. Skin mucus of gilthead sea bream (*Sparus aurata* L.). Protein mapping and regulation in chronically stressed fish. *Frontiers in Physiology* 8:34.
- Piazzon MC, Calduch-Giner JA, Fouz B, Estensoro I, **Simó-Mirabet P**, Puyalto M, Karalazos V, Palenzuela O, Sitjà-Bobadilla A, Pérez-Sánchez J. 2017. Under control: how a dietary

additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* 5:164.

- **Simó-Mirabet P**, Felip A, Estensoro I, Martos-Sitcha JA, De las Heras V, Calduch-Giner J, Puyalto M, Karalazos V, Sitjà-Bobadilla A, Pérez-Sánchez J. 2018. Impact of low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle. *Aquaculture* 490:64-74.
- Martos-Sitcha JA, **Simó-Mirabet P**, Piazzon MC, De las Heras V, Calduch-Giner JA, Puyalto M, Tinsley J, Makol A, Sitjà-Bobadilla A, Pérez-Sánchez J. 2018. Dietary sodium heptanoate helps to improve feed efficiency, growth hormone status and swimming performance in gilthead sea bream (*Sparus aurata*). *Aquaculture Nutrition* 24:1638-1651.
- Pérez-Sánchez J, **Simó-Mirabet P**, Naya-Català F, Martos-Sitcha JA, Perera E, Bermejo-Nogales A, Benedito-Palos L, Calduch-Giner JA. 2018. Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Frontiers in Endocrinology* 9:687.
- Perera E, **Simó-Mirabet P,** SukShin H, Rosell-Moll E, Naya-Català F, De las Heras V, Matos-Sitcha JA, Karalazos V, Armero E, Arizcun M, Chaves E, Berbel C, Manchado M, Afonso JM, Calduch-Giner JA, Pérez-Sánchez J. 2019. Selection for growth is associated in gilthead sea bream (*Sparus aurata*) with diet

flexibility, changes in growth patterns and higher intestine plasticity. *Aquaculture* 507:349-360.

- Martos-Sitcha JA, **Simó-Mirabet P**, De Las Heras V, Calduch-Giner JA, Pérez-Sánchez J. 2019. Tissue-specific orchestration of gilthead sea bream resilience to hypoxia and high stocking density. *Frontiers in Physiology* 10:840.
- Piazzon MA, Naya-Català F, **Simó-Mirabet P**, Picard-Sánchez A, Roig F, Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J. 2019. Sex, age and bacteria: How the intestinal microbiota is modulated in a protandrous hermaphrodite fish. *Frontiers in Microbiology* 10:2512.
- Turkmen S, Perera E, Zamorano MJ, **Simó-Mirabet P**, Xu H, Pérez-Sanchez J, Izquierdo M. 2019. Effects of dietary lipid composition and fatty acid desaturase 2 expression in broodstock gilthead sea bream on lipid metabolism-related genes and methylation of the *fads2* gene promoter in their offspring. *International Journal of Molecular Sciences* 20:6250.
- Perera E, Turkmen S, **Simó-Mirabet P**, Zamorano MJ, Xu H, Naya-Català F, Izquierdo M, Pérez-Sánchez J. 2020. Stearoyl-CoA desaturase (*scd1a*) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). *Epigenetics*. doi: 10.1080/15592294.2019.1699982.

Financiación

La presente Tesis Doctoral se ha desarrollado dentro del marco del proyecto nacional MI2-Fish "*Integrative phenotyping for the improvement of fish feed efficiency and production: unravelling metabolic, intestinal and immunopathological status*" (2014-2018; AGL2013-48560) que lleva asociada una beca de formación de personal investigador (BES-2014-069250).

Se ha obtenido financiación adicional de la Generalitat Valenciana (PROMETEO FASE II-2014/085) y de los proyectos europeos ARRAINA "*Advanced research initiatives for Nutrition & Aquaculture, 7FP European Project"* (2012-2016; FP7-KBBE-2011-5-288925) y PerformFISH *"Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain*" (2016-2021; H2020-SFS-2016-2017; 727610).

CAPÍTULO 3- Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*)

Paula Simó-Mirabet[1], Azucena Bermejo-Nogales[2], Josep Alvar Calduch-Giner[1], Jaume Pérez-Sánchez[1]

[1]Nutrigenomics and Fish Growth Endocrinology, Institute of Aquaculture Torre de la Sal, IATS-CSIC, Castellón, Spain

[2]Present Address: Endocrine Disruption and Toxicity of Contaminants, Department of Environment, INIA, Madrid, Spain

Journal of Comparative Physiology B (2017) 187:153-163

Abstract

The seven sirtuin (SIRT) counterparts of higher vertebrates were identified and molecularly characterized in a farmed fish of the Sparidae family, order Perciformes. These proteins are NAD^+-dependent deacetylases that couple protein deacetylation with the energy status of the cell via the cellular NAD^+/NADH ratio with a strict conservation of the characteristic catalytic domain surrounded by divergent N- and C-terminal regions. Phylogenetic analysis showed three major clades corresponding to SIRT1–3, SIRT4–5, and SIRT6–7 that reflected the present hierarchy of vertebrates and the accepted classification of SIRTs. Transcriptional studies revealed a ubiquitous SIRT gene expression that was tissue-specific for each SIRT. This was evidenced by multivariate analyses, which established two main clusters corresponding to SIRTs with relatively high (SIRT1, 2, and 5) and low (SIRT3, 4, 6, and 7) gene expression levels. A nutritional regulation was also evidenced in 10-day fasted fish, and SIRT2–4 exhibited an overall downregulated expression. SIRT1, 5, 6, and 7 were mostly upregulated, although clustering analyses evidenced a highly regulated response that was different for each SIRT according to the different tissue metabolic capabilities. These findings supported the use of SIRTs alone or in combination with other biomarkers for the metabolic phenotyping of farmed fish and gilthead sea bream in particular.

Keywords: Biomarkers; Energy sensing; Fasting; Gilthead sea bream; Oxidative phosphorylation; Sirtuins.

3.1. Introduction

Sirtuins (SIRTs) are NAD^+-dependent deacetylases/deacylases/ADP-ribosyltransferases that couple protein deacetylation of histone and non-histone substrates with the energy status of the cell via the cellular NAD^+/NADH ratio (Schwer and Verdin 2008). This yields a highly regulated proteome with more than 4000 acetylated proteins in rat tissues (Lundby et al. 2012). Thus, nearly all enzymes of glycolysis, gluconeogenesis, glycogen metabolism, fatty acid oxidation, nitrogen metabolism, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS) are abundantly acetylated (Zhao et al. 2010) with a frequent conservation of acetylation sites across diverse organisms (Choudhary et al. 2014). Indeed, SIRTs are virtually ubiquitous throughout all kingdoms of life with a number of distinct sirtuins that range from only one in bacteria to seven in vertebrates (Greiss and Gartner 2009). This feature offers the possibility of a complementary but also non-redundant tissue-specific energy sensing mechanisms, exhibiting SIRT1–3 a strong deacetylase activity, whereas the other SIRTs have weak (SIRT5–7) or undetectable (SIRT4) protein deacetylase activity (Newman et al. 2012). In this sense, other enzymatic activities have been reported for SIRT4 and SIRT6, which act as ADP-ribosyltransferases. Moreover, SIRT5 is primarily a protein with demalonylase and desuccinylase activity (Houtkooper et al. 2012). This functional diversification of SIRTs is also illustrated by their different cellular location. Thus, SIRT1, SIRT6, and SIRT7

generally reside in the nucleus. Conversely, SIRT2 is primarily cytoplasmic, whereas SIRT3–5 are mostly located in the mitochondria (Greiss and Gartner 2009).

SIRTs were first linked to aging by studies in yeast, in which the life span of yeast mother cells was proportional to the SIR2 gene dosage (Kaeberlein et al. 1999). In this line, studies in *C. elegans* and *Drosophila* indicated that SIRT2 orthologs can extend life span when overexpressed (Hoffmann et al. 2013; Schmeisser et al. 2013). Transgenic mice for SIRT1 did not display extension of life span, but showed slower aging as measured by symptoms of metabolic decline, indicative of diabetes prevalence, bone loss or neurodegenerative/pro-inflammatory/ cardiovascular diseases (Guarente 2013). SIRT1 actions on metabolism have also been evidenced to be mediated by regulating the role of multiple hormones implicated in energy balance (as reviewed in Quiñones et al. 2014). Other works also point out the interaction of SIRT1 with molecular regulators of the circadian rhythms (clock genes). In this sense, the activity of SIRT1 is regulated in a circadian manner, and SIRT1 physically associates with CLOCK and contributes to the acetylated state of CLOCK targets (Nakahata et al. 2008). Other clock regulators, such as BMAL1 and PER2, are also targets of SIRT1 action (Asher et al. 2008; Nakahata et al. 2008). These features show the ability of SIRTs to influence metabolism, circadian rhythms, and potentially life span through their function as protein deacetylases.

It is also well known that natural (resveratrol) or novel synthetic SIRT activators protect mice against metabolic decline (Camins et al. 2010) and elicit transcriptional changes that largely overlap with changes induced by caloric restriction (Barger et al. 2008). Similar results have

been reported in many other organisms, including zebrafish (Pereira et al. 2011; Schirmer et al. 2012). However, SIRTs are not yet widely studied in livestock animals (Ghinis-Hozumi et al. 2013), specifically in farmed fish, although SIRT6 was identified as a biomarker of sub-lethal responses to ammonia exposure in delta smelt (Connon et al. 2011). More recently, experimental evidence showed that cold acclimation increased the expression of SIRT1–2 in stickleback (Teigen et al. 2015), whereas SIRT1 could contribute to the activation of signaling cascades involved in synaptic plasticity and memory formation in goldfish (Rajan et al. 2015). A lack of response has also been reported, and SIRT1 expression did not change in the red or white skeletal muscles of swimming rainbow trout (Magnoni et al. 2014). However, SIRTs are, with no doubt, a family of proteins of arising interest in many research areas. Thus, the aim of this study was to underline in a successfully cultured fish, such as gilthead sea bream (*Sparus aurata*), the molecular identity, and tissue-specific gene expression pattern of each member of the SIRT family, addressing their different responsiveness under a negative energy balance induced by short-term fasting as well.

3.2. Materials and methods

3.2.1. Sequences and phylogenetic analysis

The updated transcriptomic database of gilthead sea bream (http://www.nutrigroup-iats.org/seabreamdb) was used to identify SIRT transcripts. First, this database was term-searched for automatically annotated SIRT genes. Next, SIRT-coding genes were identified by BLAST queries using SIRT-sequences and predictions from mammals

and fish model species, respectively. When multiple gilthead sea bream sequences were identified, they were manually curated for frame-shifting errors, and a PCR approach was used to confirm that the constructs belonged to the same gene transcript. The edited sequences were then blasted for searching conserved domains, and mitochondrial target peptides were identified by means of the online CBS prediction services.

Multiple sequence alignments were carried out with ClustalW version 2.1 and edited using GeneDoc software version 2.7. The phylogenetic tree was constructed on the basis of amino acid differences (*p* distance method) with the neighbor-joining algorithm (complete deletion) in MEGA version 6.0. A total of 49 SIRT sequences from seven representative species (human, chicken, alligator, turtle, frog, zebrafish, and gilthead sea bream) were used in the analysis. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

3.2.2. Experimental setup

Juveniles of gilthead sea bream of Atlantic origin were raised from early life stages with commercial standard diets (INICIO Forte 824/EFICO Forte 824; BioMar, Palencia, Spain) in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS–CSIC) under natural photoperiod and temperature conditions (40°5'N; 0°10'E). The oxygen content of water was always higher than 85 % saturation, unionized ammonia remained below toxic levels (<0.02 mg/l), and rearing density was maintained lower than 15 kg/m^3.

Tissue screening for SIRT gene expression was carried out in 2-year-old fish (approximately 300 g body weight) before the male-sex

reversal of this protandrous fish. Three randomly selected fish from the IATS stock were sampled and 14 target tissues (brain, head kidney, gills, liver, skin, adipose tissue, red and white skeletal muscle, heart, esophagus, stomach, and anterior, middle, and posterior intestine segments) were rapidly excised and deep frozen in liquid nitrogen as reported below.

Samples for analyzing the effect of fasting on SIRT gene expression came from an earlier study (Benedito-Palos et al. 2014). Briefly, fish of 86 g average body weight were allocated in summer in 500 l tanks in two groups of 30 fish each. One group of fish continued to be fed to visual satiety (CTRL group), whereas the second group remained unfed for 10 days with a loss of 6–8 % of body weight mass and a significant reduction of hepatosomatic index (0.64 vs. 2.10) and viscerosomatic index (5.41 vs. 8.52), calculated as the percentage of the organ weight to body weight. At the end of this 10-day fasting period, eight fish per experimental condition were randomly taken for tissue sampling (brain, head kidney, liver, adipose tissue, skin, white muscle, red muscle, heart, and intestine).

All lethal samplings were performed in overnight fasted fish between 10.00 and 11.00 a.m. to reduce the biologic variability due to circadian rhythms and postprandial-mediated changes. Fish were decapitated under anesthesia with 3-aminobenzoic acid ethyl ester (100 μg/ml). Target tissues were rapidly excised, frozen in liquid nitrogen in less than 10 min, and stored at −80 °C until RNA extraction and gene expression analysis. All procedures were carried out according to the national (IATS–CSIC Review Board) and present European animal

directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) on the handling of experimental animals.

3.2.3. Gene expression analyses

Total RNA was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500 ng total RNA in a final volume of 100 µl. Reverse transcriptase (RT) reactions were incubated 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without RT. Quantitative realtime PCR (qPCR) was performed using an iCycler IQ Realtime Detection System (Bio-Rad, Hercules, CA, USA). Diluted RT reactions were conveniently used for PCR reactions in 25 µl volume in combination with an SYBR Green Master Mix (Bio-Rad) and specific primers for SIRT1–7 (**Table 3.1**) at a final concentration of 0.9 µM. The 96-well PCR-array layout was designed for the simultaneous profiling under uniform cycling conditions of all SIRT genes in a given sample tissue. The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>90 %) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C) and linearity of serial dilutions of RT reactions. Reactions were performed in triplicate, and the

fluorescence data acquired during the extension phase were normalized by the delta–delta Ct method (Livak and Schmittgen 2001) using β-actin as the housekeeping gene. For multigene analysis, data on gene expression were in reference to the expression level of SIRT1, for which a value of 1 was arbitrarily assigned in the corresponding tissue (CTRL fish in the case of fasting experiment). When gene expression values in the fasting experiment were individually analyzed, fold-change calculations from each gene were in reference to the expression ratio between fasted and CTRL fish (values >1 indicate fasting upregulated genes; values <1 indicate fasting downregulated genes).

Table 3.1 Forward (F) and reverse (R) primers for real-time PCR.

Gene	Accession number		Primer sequence	Position
SIRT1	KF018666	F R	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC CCT CAG AAT GGT CCT CGG ATC GGT CTC	510-569
SIRT2	KF018667	F R	GAA CAA TCC GAC GAC AGC AGT GAA G AGG TTA CGC AGG AAG TCC ATC TCT	222-289
SIRT3	KF018668	F R	CTG CCA AGT CCT CAT CCC CTT CAC CAG ACG AGC CAC	597-649
SIRT4	KF018669	F R	GGC TGG CGG AGT CGG ATG TCC TGA ATA CAC CTG TGA CGA AGA C	812-870
SIRT5	KF018670	F R	CAG ACA TCC TAA CCC GAG CAG AG CCA CGA GGC AGA GGT CAC A	740-796
SIRT6	KF018671	F R	ACT CCA CCA CCA CCG ATG TCA A CTC CTC CTC CTT CAC CTT TCG CTT TG	995-1062
SIRT7	KF018672	F R	CTG GAG CAA CCT CTA AAC TGG AA CAC CTT CAG ACT GGA GCC TAA	799-885

3.2.4. Statistics

Multivariate analysis (principal component analysis, PCA; hierarchical clustering, HCL) to assess SIRT gene expression across different tissues at a given time or nutritional condition was carried out by means of Genesis software (Sturn et al. 2002). More specifically, the SIRT gene

expression pattern in a given tissue was analyzed by one-way analyses of variance followed by Student–Newman–Keuls test. Fasting-mediated effects upon a specific-tissue and SIRTs were analyzed by Student *t* test. The significance level was set at $P < 0.05$.

3.3. Results

3.3.1. Molecular characterization

Searches in the gilthead sea bream transcriptomic database recognized (*E*-value = 0) seven contigs (6–120 clones in depth) as complete codifying sequences of 307–697 amino acids in length (**Table 3.2**). These new gilthead sea bream sequences were uploaded to GenBank with accession numbers KF018666 (SIRT1), KF018667 (SIRT2), KF018668 (SIRT3), KF018669 (SIRT4), KF018670 (SIRT5), KF018671 (SIRT6), and KF018672 (SIRT7).

Table 3.2 Classification of identified genes according to BLAST-X searches.

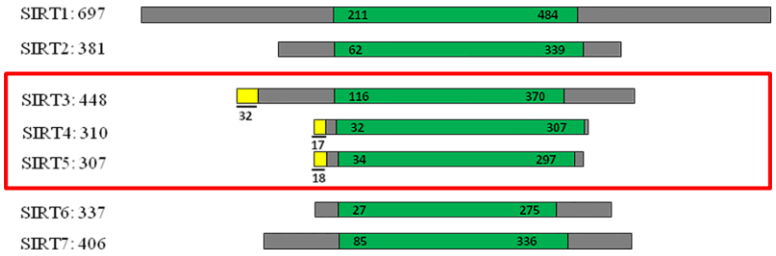
Contig	F[a]	Size (nt)	Annotation[b]	Best match[c]	E[d]	CDS[e]
C2_11708	48	3316	SIRT1	XP_010732868	0	39-2132
C2_5566	120	2356	SIRT2	XP_010754755	0	156-1307
C2_14108	26	2052	SIRT3	KKF16986	0	281-1627
C2_35254	6	1804	SIRT4	XP_003454623	0	79-1011
C2_6042	71	1660	SIRT5	ACQ58544	0	67-990
C2_43421	8	1384	SIRT6	XP_008289713	0	136-1149
C2_37007	9	1457	SIRT7	KKF28658	0	55-1275

[a]Number of reads composing the contig.
[b]Gene identity determined through BLAST-X searches.
[c]Best BLAST-X protein sequence match.
[d]Expectation value.
[e]Codifying sequence.

Schematic representation of gilthead sea bream SIRT family with data of sequence identity/similarity is shown in **Fig. 3.1.** A conserved catalytic domain of about 250 amino acids in length was identified, containing a Rossman fold with many of the hallmarks of a typical NAD^+-binding site, and a smaller conserved domain consisting of a tetrad of cysteine residues. Mitochondrial peptide targets of 32, 17, and 18 amino acids in length were recognized in SIRT3, 4, and 5, respectively. Amino acid sequence identity and similarity for all the full gilthead sea bream sequences ranged from 7 to 32 % and from 14 to 44 %, respectively. Overall, the highest degree of conservation was found between SIRT1–3; SIRT4 and 5; and SIRT6 and 7. For sequence details and multiple sequence alignments, see **Fig. 3.2**.

Phylogenetic analysis evidenced three major nodes (SIRT1–3, class I; SIRT4–5, classes II and III; and SIRT6–7, class IV), according to the present hierarchy of vertebrate species and the current SIRT classification of Frye (2000) (**Fig. 3.3**). Thus, all branches of the tree were recognized as monophyletic clusters and SIRT sequences of fish (zebrafish and gilthead sea bream) were closely grouped with the exception of SIRT3 sequences. In this case, gilthead sea bream was more related to frog rather than zebrafish, which was clustered with the other species in the analysis (human, chicken, alligator, and turtle).

A



B

	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
SIRT1	16 (25)	17 (27)	8 (16)	9 (14)	7 (16)	7 (17)
SIRT2		32 (44)	13 (27)	13 (25)	14 (25)	12 (24)
SIRT3			12 (23)	10 (18)	14 (25)	11 (23)
SIRT4				20 (35)	14 (26)	13 (24)
SIRT5					15 (27)	11 (21)
SIRT6						21 (33)

Figure 3.1 A Graphical representation of gilthead sea bream SIRTs with varying open reading frames (307–697 amino acids). The enzymatic core domain is labeled in *green*. Mitochondrial target peptides of SIRT3, 4, and 5 (*boxed* in *red*) are labeled in *yellow*. **B** Percentages of identity and similarity (in *parentheses*) between gilthead sea bream SIRTs.

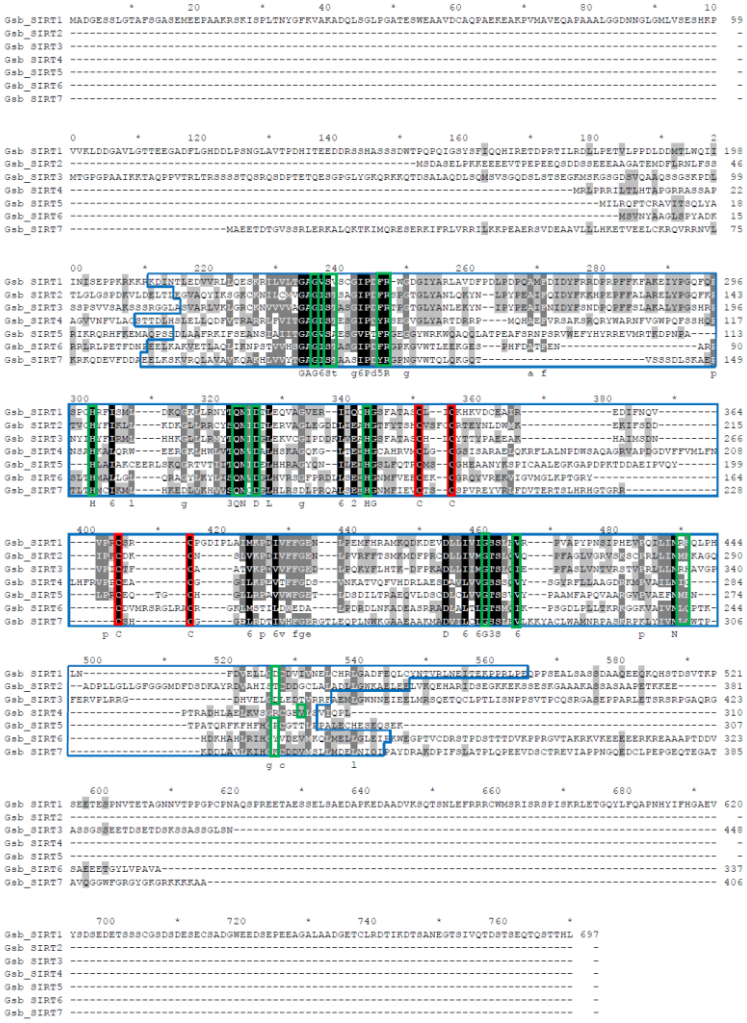


Figure 3.2 Amino acid alignment of gilthead sea bream SIRTs. Black and grey scale shading corresponds to 100%, ≥70%, ≥40% and < 40% sequence identity and similarity. Capital letters of the consensus sequence indicate 100% identity. Small letters of the consensus sequence indicates 70-85% identity. Numbers of the consensus sequence indicate the type of amino acids similarity. Boxes indicate special residues: blue for the core domain, green for the hallmarks of the NAD$^+$-binding region and red for zinc-binding cysteines.

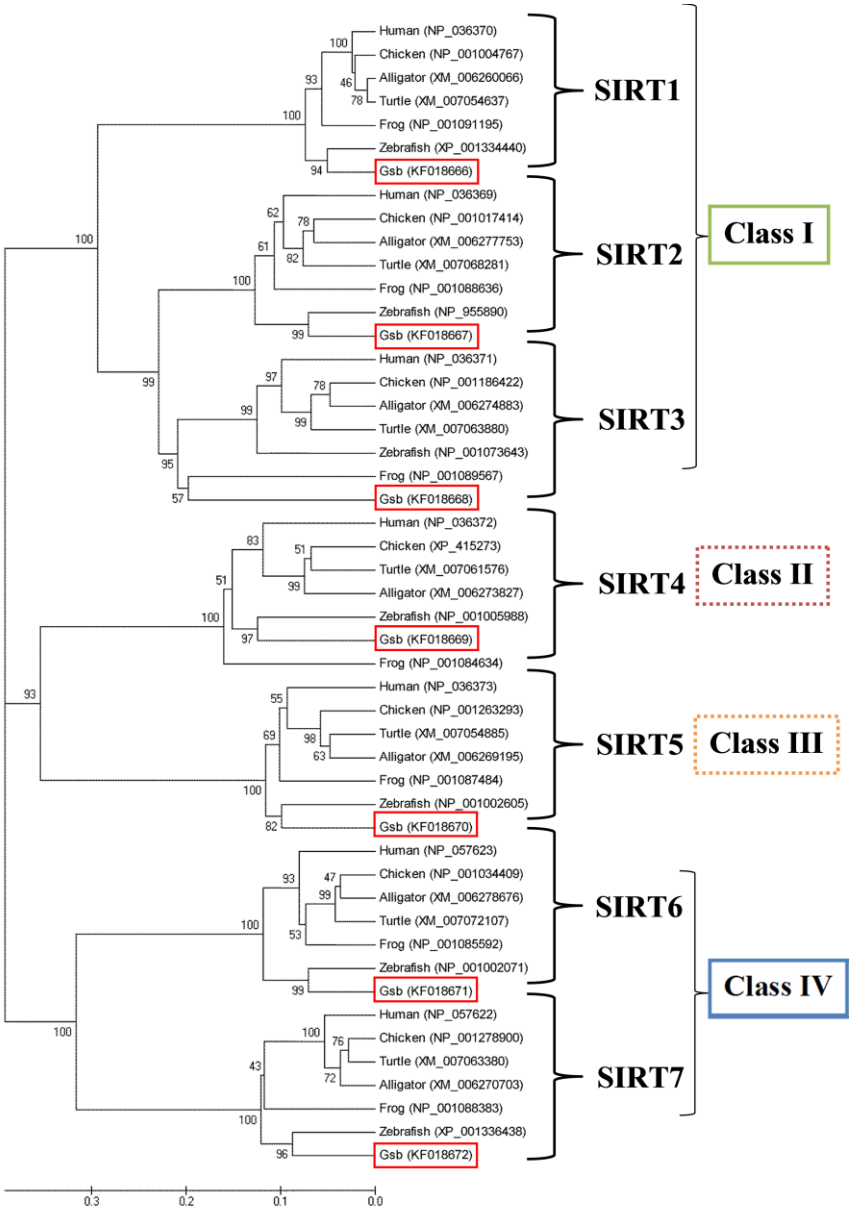


Figure 3.3 Phylogenetic analysis of SIRTs. GenBank accession numbers are provided in *parentheses* for each sequence. SIRT classes (I–IV) defined by Frye (2000) are indicated.

3.3.2. Gene expression analysis

The expression pattern of gilthead sea bream SIRTs was tissue-specific for each SIRT, and all data on relative gene expression were referred to SIRT1 in each tissue (**Table 3.3**). The two first components of PCA explained 90.50 % of total variance (**Fig. 3.4A**). The first component counted for the highest variation (76.27 %) grouping along the *X*-axis two main groups, corresponding to SIRTs with high (SIRT1, 2, and 5) and low (SIRT3, 4, 6, and 7) gene expression levels. HCL analysis highlighted deviations of this cluster association for SIRT2 and 5 in gills, head kidney, posterior intestine, and adipose tissue; and for SIRT3 in head kidney and posterior intestine (**Fig. 3.4B**).

Data on gene expression in CTRL and fasted fish are shown in **Table 3.4**. From these data, PCA of fold-changes calculated as the ratio of fasted/CTRL fish explained the 70.94 % of total variance (**Fig. 3.5A**). The first component counted for 54.47 % of variance, differentiating two main groups corresponding to upregulated (SIRT1, 5, 6, and 7) or downregulated (SIRT2, 3, and 4) SIRTs in fasted fish, although, in the case of liver, this downregulation was extensive to SIRT5–6. HCL classification revealed a graded-tissue response that ranged from a significant or weak downregulation in tissues with energy-storage capacity (liver, skin, and adipose tissue) to a consistent upregulation in tissues with a high energy demand (red muscle, brain, head kidney, and heart) (**Fig. 3.5B**). As a corollary (**Fig. 3.5C**), SIRT2–6 were significantly downregulated by fasting in the liver tissue. SIRT2 was also downregulated in adipose tissue, skin, and anterior intestine, whereas SIRT3 and 7 were downregulated in medium intestine and adipose tissue, respectively. The upregulated response was exemplified by SIRT1

in brain, adipose tissue, head kidney, intestine, and muscle tissues. SIRT5–7 were upregulated through all the intestine. The same pattern was found for SIRT5 in white muscle, whereas SIRT6 was specifically upregulated in head kidney and heart.

Table 3.3 Relative mRNA expression of gilthead sea bream sirtuins in different tissues. Expression values are the mean ± SEM of 3 fish. Different superscript letters in each row indicate significant differences ($P < 0.05$) among sirtuins in each tissue. All data values in a given tissue were in reference to the expression level of SIRT1. Values labeled with red, yellow and green circles are the higher, average and lower levels within each tissue, respectively.

	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7	P-value[1]
Brain	1.01 ± 0.18^{ab}	1.04 ± 0.15^{a}	0.23 ± 0.03^{bc}	0.15 ± 0.02^{c}	0.71 ± 0.20^{abc}	0.26 ± 0.07^{abc}	0.47 ± 0.10^{abc}	<0.001
HK	1.01 ± 0.11^{a}	0.64 ± 0.16^{ab}	1.06 ± 0.08^{a}	0.09 ± 0.02^{c}	0.86 ± 0.10^{a}	0.31 ± 0.02^{bc}	0.21 ± 0.03^{bc}	<0.001
Gills	1.00 ± 0.03^{a}	0.63 ± 0.08^{b}	0.46 ± 0.05^{b}	0.13 ± 0.01^{c}	0.65 ± 0.03^{b}	0.16 ± 0.02^{c}	0.16 ± 0.01^{c}	<0.001
Liver	1.02 ± 0.20^{a}	2.97 ± 0.08^{a}	0.52 ± 0.10^{a}	0.17 ± 0.01^{a}	2.91 ± 0.64^{a}	0.35 ± 0.05^{a}	0.49 ± 0.02^{a}	0.006
AT	1.01 ± 0.15^{a}	0.75 ± 0.05^{ab}	0.53 ± 0.04^{bc}	0.06 ± 0.01^{d}	0.61 ± 0.004^{b}	0.19 ± 0.02^{cd}	0.19 ± 0.03^{cd}	<0.001
Skin	1.01 ± 0.15^{ab}	1.26 ± 0.05^{a}	0.60 ± 0.02^{bcd}	0.14 ± 0.02^{c}	0.63 ± 0.11^{bcd}	0.22 ± 0.01^{cd}	0.32 ± 0.02^{cd}	<0.001
WM	1.00 ± 0.04^{a}	1.47 ± 0.03^{b}	0.18 ± 0.08^{c}	0.16 ± 0.02^{c}	1.87 ± 0.15^{d}	0.09 ± 0.004^{e}	0.29 ± 0.03^{c}	<0.001
RM	1.00 ± 0.03^{ab}	1.86 ± 0.20^{a}	0.42 ± 0.01^{b}	0.32 ± 0.03^{b}	7.00 ± 0.46 ^{c}	0.16 ± 0.02^{b}	0.22 ± 0.01^{b}	<0.001
Heart	1.00 ± 0.02^{ab}	1.06 ± 0.05^{a}	0.44 ± 0.09^{ab}	0.14 ± 0.02^{ab}	2.44 ± 0.39^{c}	0.17 ± 0.01 ^{b}	0.25 ± 0.003^{ab}	<0.001
Esophagus	1.00 ± 0.09^{a}	1.11 ± 0.06^{a}	0.75 ± 0.19^{a}	0.22 ± 0.002^{b}	1.00 ± 0.003^{a}	0.26 ± 0.01^{b}	0.26 ± 0.02^{b}	<0.001
Stomach	1.02 ± 0.20^{a}	1.29 ± 0.03^{ab}	0.45 ± 0.12^{c}	0.22 ± 0.004^{c}	1.64 ± 0.05^{b}	0.21 ± 0.03^{c}	0.29 ± 0.01^{c}	<0.001
AI	1.00 ± 0.07^{a}	1.48 ± 0.02^{b}	0.45 ± 0.06^{c}	0.25 ± 0.003^{c}	1.21 ± 0.14^{ab}	0.23 ± 0.04^{c}	0.20 ± 0.005^{c}	<0.001
MI	1.01 ± 0.15^{ab}	1.87 ± 0.13^{c}	0.67 ± 0.17^{ab}	0.26 ± 0.02^{a}	1.26 ± 0.27^{ac}	0.21 ± 0.02^{a}	0.24 ± 0.01^{a}	<0.001
PI	1.00 ± 0.09^{a}	0.41 ± 0.04^{a}	2.71 ± 0.45^{b}	0.32 ± 0.02^{a}	0.12 ± 0.09^{a}	0.50 ± 0.06^{a}	0.21 ± 0.02^{a}	<0.001

[1]Result values from one-way analysis of variance.

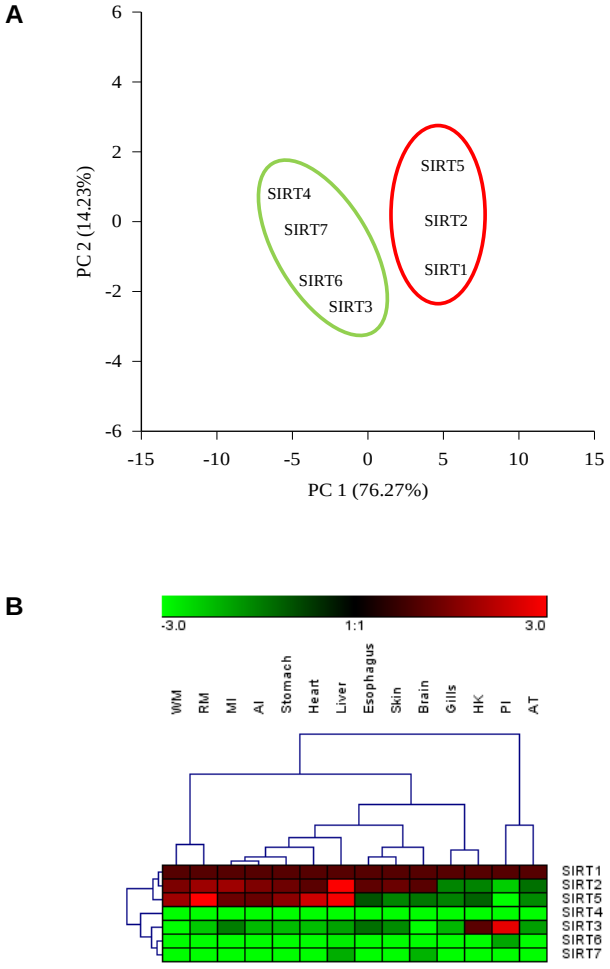
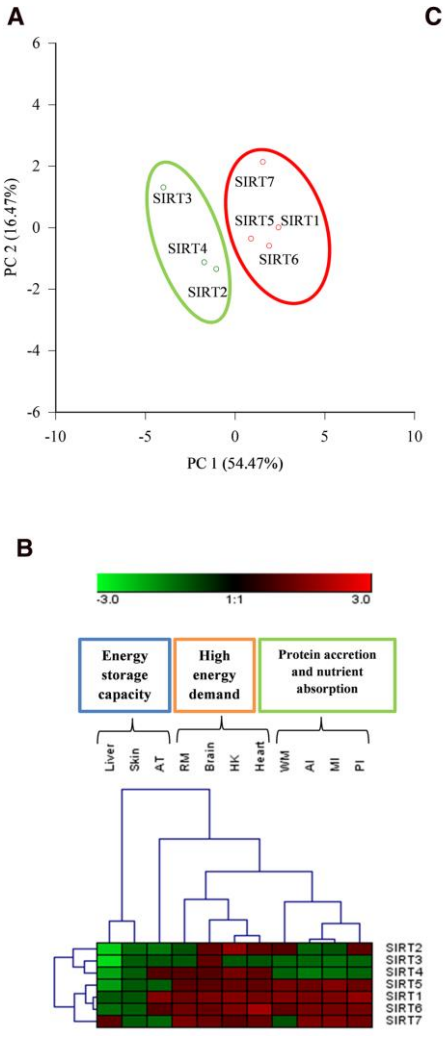


Figure 3.4 A Principal component analysis of the tissue-specific gene expression pattern of SIRTs in gilthead sea bream. SIRTs encircled in *green* were expressed at relatively low levels. SIRTs encircled in *red* were expressed at relatively high levels. **B** Hierarchical clustering of the gene expression pattern of SIRTs according their expression profile in the 14 analyzed tissues (*AI* anterior intestine, *AT* adipose tissue, *HK* head kidney, *MI* middle intestine, *PI* posterior intestine, *RM* red muscle, and *WM* white muscle).



C

SIRT2	
LIVER	0.41(0.01)***
AT	0.7(0.07)*
SKIN	0.76(0.03)***
AI	0.84(0.03)*
BRAIN	1.16(0.04)*
HK	1.74(0.3)*
SIRT3	
LIVER	0.38(0.03)***
MI	0.74(0.07)*
SIRT4	
LIVER	0.51(0.04)*
SIRT1	
PI	1.73(0.08)**
HK	1.57(0.22)*
AT	1.55(0.17)*
WM	1.52(0.07)***
MI	1.47(0.09)***
BRAIN	1.43(0.13)*
AI	1.43(0.08)**
HEART	1.36(0.07)**
RM	1.28(0.11)*
SIRT5	
MI	1.57(0.13)**
WM	1.41(0.14)*
AI	1.31(0.07)*
PI	1.29(0.12)*
LIVER	0.53(0.04)***
SIRT6	
HEART	1.94(0.12)***
HK	1.46(0.3)*
AI	1.40(0.08)**
PI	1.38(0.11)*
MI	1.37(0.08)*
LIVER	0.75(0.05)*
SIRT7	
MI	1.55(0.07)***
AI	1.50(0.10)**
PI	1.34(0.12)*
AT	0.80(0.03)*

Figure 3.5 A Principal component analysis of the nutritionally-regulated expression pattern of SIRTs in 10-day fasted fish. Data are expressed as a fold-change of fasted vs. CTRL fish. SIRTs encircled in *green* were downregulated by fasting. SIRTs encircled in *red* were upregulated by fasting. **B** Hierarchical cluster of the gene expression pattern of SIRTs according their different regulation in tissues with different metabolic capabilities (*AI* anterior intestine, *AT* adipose tissue, *HK* head kidney, *MI* middle intestine, *PI* posterior intestine, *RM* red muscle, and *WM* white muscle). **C** Fold-changes of SIRTs significantly regulated by fasting (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Table 3.4 Effect of fasting on relative mRNA expression of gilthead sea bream sirtuins. Expression values are the mean ± SEM of 8 fish of each group (CTRL and fasted). Asterisks indicate significant differences (* P< 0.05, ** P< 0.01, *** P< 0.001, Student t-test) between fasted vs CTRL groups in each sirtuin for single tissue. All data values in a given tissue were in reference to the expression level of SIRT1 of CTRL fish.

		SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
Brain	CTRL	1.02 ± 0.08	1.28 ± 0.06	0.33 ± 0.04	0.23 ± 0.02	0.78 ± 0.04	0.33 ± 0.04	0.55 ± 0.05
	Fasted	1.45 ± 0.13*	1.49 ± 0.05*	0.39 ± 0.04	0.23 ± 0.01	0.82 ± 0.05	0.40 ± 0.03	0.71 ± 0.05
HK	CTRL	1.01 ± 0.05	0.65 ± 0.04	1.11 ± 0.06	0.09 ± 0.01	0.78 ± 0.05	0.34 ± 0.03	0.25 ± 0.03
	Fasted	1.58 ± 0.21*	1.14 ± 0.20*	1.06 ± 0.06	0.13 ± 0.02	0.94 ± 0.10	0.49 ± 0.05*	0.27 ± 0.03
Liver	CTRL	1.01 ± 0.07	3.30 ± 0.28	0.62 ± 0.07	0.30 ± 0.05	2.58 ± 0.21	0.37 ± 0.03	0.37 ± 0.04
	Fasted	0.95 ± 0.05	1.36 ± 0.04***	0.24 ± 0.02***	0.15 ± 0.01*	1.36 ± 0.09***	0.28 ± 0.02*	0.41 ± 0.03
AT	CTRL	1.02 ± 0.08	0.90 ± 0.08	0.66 ± 0.08	0.09 ± 0.02	0.77 ± 0.09	0.27 ± 0.03	0.21 ± 0.02
	Fasted	1.58 ± 0.18*	0.63 ± 0.06*	0.64 ± 0.04	0.10 ± 0.01	0.67 ± 0.06	0.28 ± 0.03	0.17 ± 0.01*
Skin	CTRL	1.00 ± 0.04	1.33 ± 0.04	0.69 ± 0.03	0.22 ± 0.04	0.68 ± 0.02	0.27 ± 0.03	0.33 ± 0.02
	Fasted	0.97 ± 0.02	1.02 ± 0.04***	0.62 ± 0.02	0.20 ± 0.02	0.63 ± 0.04	0.24 ± 0.02	0.30 ± 0.01
WM	CTRL	1.01 ± 0.06	1.61 ± 0.08	0.24 ± 0.03	0.19 ± 0.03	1.96 ± 0.16	0.13 ± 0.01	0.35 ± 0.03
	Fasted	1.54 ± 0.07***	1.62 ± 0.10	0.20 ± 0.02	0.16 ± 0.01	2.76 ± 0.28*	0.15 ± 0.01	0.35 ± 0.02
RM	CTRL	1.01 ± 0.04	2.24 ± 0.10	0.50 ± 0.03	0.48 ± 0.05	8.60 ± 0.44	0.17 ± 0.02	0.24 ± 0.01
	Fasted	1.28 ± 0.11*	2.23 ± 0.23	0.48 ± 0.04	0.48 ± 0.05	9.11 ± 0.88	0.22 ± 0.04	0.27 ± 0.02
Heart	CTRL	1.01 ± 0.05	1.09 ± 0.05	0.46 ± 0.03	0.19 ± 0.03	2.60 ± 0.21	0.15 ± 0.01	0.24 ± 0.01
	Fasted	1.38 ± 0.07**	1.25 ± 0.09	0.44 ± 0.04	0.22 ± 0.03	2.99 ± 0.38	0.28 ± 0.02***	0.29 ± 0.02
AI	CTRL	1.01 ± 0.07	1.53 ± 0.07	0.59 ± 0.08	0.34 ± 0.06	1.17 ± 0.11	0.24 ± 0.02	0.22 ± 0.02
	Fasted	1.45 ± 0.09**	1.28 ± 0.05*	0.48 ± 0.05	0.23 ± 0.02	1.52 ± 0.09*	0.34 ± 0.02**	0.33 ± 0.02**
MI	CTRL	1.01 ± 0.05	1.69 ± 0.08	0.72 ± 0.06	0.36 ± 0.05	1.15 ± 0.10	0.24 ± 0.02	0.28 ± 0.03
	Fasted	1.48 ± 0.08***	1.66 ± 0.08	0.53 ± 0.05*	0.26 ± 0.03	1.81 ± 0.15**	0.33 ± 0.02*	0.44 ± 0.02***
PI	CTRL	1.02 ± 0.08	1.47 ± 0.10	0.73 ± 0.04	0.33 ± 0.06	1.05 ± 0.06	0.23 ± 0.03	0.44 ± 0.04
	Fasted	1.76 ± 0.09**	1.68 ± 0.09	0.60 ± 0.06	0.27 ± 0.02	1.34 ± 0.13*	0.31 ± 0.02*	0.59 ± 0.05*

3.4. Discussion

SIRTs are part of a regulatory and evolutionarily conserved energy sensing system, which responds to changes in nutrient intake, use and storage, contributing the results presented herein to demonstrate that they play an important role in the regulation of energy homeostasis in farmed fish. Seven SIRTs, corresponding to class I–IV of the classification of Frye (Frye 2000), have been described in higher vertebrate species, and the present study highlighted their conservation in a modern fish that belongs to family Sparidae, order Perciformes. Therefore, SIRT1–7 are ancient in animal evolution, which contrasts with the selective and extensive loss of SIRT genes in insects, nematodes, and plants (Greiss and Gartner 2009). The molecular characterization of gilthead sea bream SIRTs also highlighted a highly conserved catalytic domain with the Rossman fold domain, which is commonly found in proteins that bind NAD^+ or $NADP^+$ (Sauve et al. 2006). The cysteine tetrad within the Zn^{+2}-binding domain is also conserved in all gilthead sea bream SIRTs. Other structural features that are variable within the SIRT family are the N- and C-terminal regions surrounding the catalytic domain. In this sense, our results corroborate the fact that SIRT1 is consistently the longest SIRT through vertebrate evolution, whereas SIRT4–5 are regularly the shortest. This explains the relative low conservation of different SIRTs within a given organism, but importantly the range of amino acid similarity of gilthead sea bream SIRTs (14–44 %) is of the same order of magnitude than those calculated for the SIRT sequences of amphibians (14–53 %), reptiles (17–51 %), birds (12–51 %), and humans (12–49 %) used by us in the

phylogenetic analysis. This rendered clear monophyletic clusters, as reported elsewhere in more large-scale phylogenetic studies with more than 50 species of all kingdoms of life (Costantini et al. 2013).

Most of the changes induced by SIRT activation are related to the increase of mitochondrial metabolism and antioxidant protection. However, the specific regulation of each SIRT is far to be completed within and among different tissues and animal models. In this regard, it must be noted that all SIRTs were found at detectable levels in pig (Jin et al. 2009) or human (Michishita et al. 2005) tissues. SIRT2 expression was not detectable in kidney and spleen of zebrafish, and the same pattern was found for SIRT4 in spleen and SIRT7 in gills and skeletal muscle (Pereira et al. 2011). However, our results clearly indicated that all SIRTs were expressed at detectable levels in the 14 analyzed tissues of gilthead sea bream, evidencing the multivariate analysis higher expression levels of SIRT1, 2 and 5. Importantly, SIRT1 appeared ubiquitously expressed, whereas SIRT2 and 5 showed higher expression levels in liver, a pattern that was the same for the mitochondrial SIRT5 in aerobic muscle tissues (heart, skeletal red muscle). The emerging role of SIRTs in skeletal muscle regeneration and survival under catabolic stress has been recently reviewed by Sharples et al. (2015), and current research is underway to determine if bioenergetics of muscle tissues is specifically mediated or not by SIRT5 in gilthead sea bream.

Conversely to SIRT5, SIRT3 was categorized as an SIRT with relatively low expression levels in gilthead sea bream, but importantly SIRT3 shared high expression levels in head kidney and posterior intestine, which are now recognized as immune-relevant tissues in fish (Rombout et al. 2011; Secombes and Wang 2012). However, the

expression of SIRT3 in these tissues was not regulated by fasting, whereas both SIRT1 and 6 were upregulated by nutrient scarcity in the head kidney and posterior intestine of our experimental fish. Whether these animals benefit from the anti-inflammatory properties of caloric restriction described in rodents, and other species (Johnson et al. 2007; González et al. 2012; Youm et al. 2015) remains to be established. However, it is noteworthy that SIRT1 overexpression downregulated pro-inflammatory genes in mice (Pfluger et al. 2008; Yoshizaki et al. 2010), whereas obesity with chronic inflammation was associated with reduced levels of SIRT1 (Vachharajani et al. 2016). Likewise, other studies suggest the involvement of SIRT6 on the immune response of mammals, although its precise role in inflammation and immunity remains to be elucidated (Bruzzone et al. 2009; Lee et al. 2013). Less evident is the immunoregulatory role of SIRT5, although its desuccinylase activity seems to be an important factor of immune response (Tannahill et al. 2013).

Changes in nutrient scarcity due to caloric restriction or fasting induce other important adaptive responses that were substantiated in our 10-day fasting model by a loss of body mass and fat depots, followed by a pronounced downregulation of hepatic lipogenic enzymes, including fatty acid elongases and desaturases (Benedito-Palos et al. 2014). Experimental evidence also indicates that these fasting-mediated effects on lipid metabolism were related to a consistent downregulated expression of all the five-complex enzyme units of the mitochondrial respiratory chain (Bermejo-Nogales et al. 2015). This metabolic feature is explained by a reduced energy demand due to the fasting inhibition of hepatic lipogenesis, which is considered a major energy-demanding

process in the liver tissue (Rui 2014). This was encompassed in the present study by a reduced hepatic expression of five out of the seven SIRTs. Among them was included SIRT2, which was also downregulated in other tissues (mesenteric adipose tissue and skin submucosa) with fat storage capacity in gilthead sea bream. Indeed, it appears likely that SIRT2 is mostly under a negative control in both hepatocytes and adipocytes of mesenteric adipose tissue and skin submucosa. In the case of liver, the downregulation of SIRT2 was encompassed by the three mitochondrial SIRTs, which is indicative of a consistent response involving more than one SIRT, as previously reported in other experimental models of humans and rodents (Lai et al. 2013; Ghiraldini et al. 2013). Conversely, both SIRT1–2 were upregulated by fasting in gilthead sea bream brain, a tissue with high metabolic activity that should be protected from oxidative stress during highly energy demand process, as has been reviewed during neurodegenerative disease or metabolic decline in mammals (Calabrese et al. 2008).

Nutrient availability by itself is a major factor driving switches in muscle protein turnover and mitochondrial activity, as reported earlier in gilthead sea bream by microarray gene expression profiling of glycolytic and aerobic muscle tissues in fish fed to maintenance ration (Calduch-Giner et al. 2014). This is consistent with the upregulation of OXPHOS, although both in gilthead sea bream (Bermejo-Nogales et al. 2015) and in previous studies in pigs (da Costa et al. 2004) and mice (Suzuki et al. 2002), the response of skeletal and cardiac muscle tissues to feed deprivation or restriction is not only opposite, but also weaker than in the liver. The physiological significance of these findings is far from being fully established, although it can be viewed as a different metabolic

plasticity of glycolytic and highly oxidative muscle tissues, which was encompassed herein by the upregulation of SIRT1 in all muscle tissues, whereas the upregulation of SIRT5 and 6 was specific of white skeletal muscle and heart, respectively. In this context, SIRT1 has been shown to be necessary for the switch of glucose utilization to fatty acid oxidation during nutrient deprivation states in mice (Gerhart-Hines et al. 2007), which is largely mediated in skeletal muscle of rodents by the SIRT1-regulated deacetylation of proliferator-activated receptor gamma coactivator 1 alpha (Dominy et al. 2010). The protective role of SIRT1 against oxidative stress has been also proved during heart ischemia (Hsu et al. 2010) and muscle cardiac hypertrophy (Planavila et al. 2011). Likewise, SIRT6 protects rodent cardiomyocytes from a hypertrophic response through the suppression levels of the pro-inflammatory nuclear factor NF-kappa-B (Yu et al. 2012). In contrast, the specific involvement of SIRT5 on the regulation of muscle energy sensing is poorly documented in both humans and rodents, although its role in amino acid catabolism, TCA cycle, and fatty acid metabolism has been studied in different experimental models (Park et al. 2013; Yu et al. 2013).

In summary, seven SIRT sequences of gilthead sea bream have been unequivocally identified and molecularly characterized in gilthead sea bream. The tissue expression pattern was specific of each SIRT with relatively high levels of expression of SIRT1, 2, and 5 in comparison with SIRT3, 4, 6, and 7. Overall, SIRT1 and SIRT5–7 were upregulated by fasting, whereas SIRT2–4 were mostly downregulated, which was especially evident for SIRT1 and 2, respectively (see **Fig. 3.6** as a corollary). Current studies are underway to underline their tissue-specific

regulation by nutrient deficiencies or fish-strain differences in growth and energy demand.

SIRTs	Tissue Up-regulated	Tissue Down-regulated
SIRT1	Brain, HK, AT, WM, RM, Heart, AI, MI, PI	-
SIRT2	Brain, HK	Liver, AT, Skin, AI
SIRT3	-	Liver, MI
SIRT4	-	Liver
SIRT5	WM, AI, MI, PI	Liver
SIRT6	HK, Heart, AI, MI, PI	Liver
SIRT7	AI, MI, PI	AT

Figure 3.6 Corollary of the tissue-specific regulation by fasting of SIRT gene expression in gilthead sea bream target tissues (AI anterior intestine, AT adipose tissue, HK head kidney, MI middle intestine, PI posterior intestine, RM red muscle, and WM white muscle). For each SIRT, the degree of upregulation or downregulation in the analyzed tissues is expressed in red and green, respectively.

Acknowledgments

This study was funded by the European Union (ARRAINA, FP7-KBBE-2011-5-288925, Advanced research initiatives for nutrition and aquaculture). The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission. Additional funding was obtained from the Spanish MINECO (MI2-Fish, AGL2013-48560) and from Generalitat Valenciana (PROMETEO FASE II-2014/085).

References

Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW, Schibler U (2008) SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell 134:317–328. doi:10.1016/j.cell.2008.06.050

Barger JL, Kayo T, Vann JM, Arias EB, Wang J, Hacker TA, Wang Y, Raederstorff D, Morrow JD, Leeuwenburgh C, Allison DB, Saupe KW, Cartee GD, Weindruch R, Prolla TA (2008) A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS One 3:e2264. doi:10.1371/journal.pone.0002264

Benedito-Palos L, Ballester-Lozano G, Pérez-Sánchez J (2014) Widegene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (Sparus aurata). Gene 547:34–42. doi:10.1016/j.gene.2014.05.073

Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J (2015) Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. PLoS One 10:e0122889. doi:10.1371/journal.pone.0122889

Bruzzone S, Fruscione F, Morando S et al (2009) Catastrophic NAD+ depletion in activated T lymphocytes through Nampt inhibition reduces demyelination and disability in EAE. PLoS One 4:e7897. doi:10.1371/journal.pone.0007897

Calabrese V, Cornelius C, Mancuso C, Pennisi G, Calafato S, Bellia F, Bates TE, Giuffrida Stella AM, Schapira T, Dinkova Kostova AT, Rizzarelli E (2008) Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. Neurochem Res 33:2444–2471. doi:10.1007/s11064-008-9775-9

Calduch-Giner JA, Echasseriau Y, Crespo D, Baron D, Planas JV, Prunet P, Pérez-Sánchez J (2014) Transcriptional assessment by microarray analysis and large-scale meta-analysis of the metabolic capacity of cardiac and skeletal muscle tissues to cope with reduced nutrient availability in gilthead sea bream (Sparus aurata L.). Mar Biotechnol 16:423–435. doi:10.1007/ s10126-014-9562-3

Camins A, Sureda FX, Junyent F, Verdaguer E, Folch J, Pelegri C, Vilaplana J, Beas-Zarate C, Pallàs M (2010) Sirtuin activators: designing molecules to extend life span. Biochim Biophys Acta 1799:740–749. doi:10.1016/j.bbagrm.2010.06.005

Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M (2014) The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol 15:536–550. doi:10.1038/ nrm3841

Connon RE, Deanovic LA, Fritsch EB, D'Abronzo LS, Werner I (2011) Sublethal responses to ammonia exposure in the endangered delta smelt;

Hypomesus transpacificus (Fam. Osmeridae). Aquat Toxicol 105:369–377. doi:10.1016/j.aquatox.2011.07.002

Costantini S, Sharma A, Raucci R, Costantini M, Autiero I, Colonna G (2013) Genealogy of an ancient protein family: the Sirtuins, a family of disordered members. BMC Evol Biol 13:60. doi:10.1186/1471-2148-13-60

da Costa N, McGillivray C, Bai Q, Wood JD, Evans G, Chang KC (2004) Restriction of dietary energy and protein induces molecular changes in young porcine skeletal muscles. J Nutr 134:2191–2199

Dominy JE, Lee Y, Gerhart-Hines Z, Puigserver P (2010) Nutrientdependent regulation of PGC-1alpha's acetylation state and metabolic function through the enzymatic activities of Sirt1/ GCN5. Biochim Biophys Acta 1804:1676–1683. doi:10.1016/j. bbapap.2009.11.023

Frye RA (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem Biophys Res Commun 273:793–798. doi:10.1006/bbrc.2000.3000

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P (2007) Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. EMBO J 26:1913–1923. doi:10.1038/sj.emboj.7601633

Ghinis-Hozumi Y, Antaramian A, Villarroya F, Piña E, Mora O (2013) Potential role of sirtuins in livestock production. Animal 7:101– 108. doi:10.1017/S1751731112001115

Ghiraldini FG, Crispim ACV, Mello MLS (2013) Effects of hyperglycemia and aging on nuclear sirtuins and DNA damage of mouse hepatocytes. Mol Biol Cell 24:2467–2476. doi:10.1091/mbc. E13-04-0186

González OA, Tobia C, Ebersole JL, Novak MJ (2012) Caloric restriction and chronic inflammatory diseases. Oral Dis 18:16– 31. doi:10.1111/j.1601-0825.2011.01830.x

Greiss S, Gartner A (2009) Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. Mol Cells 28:407–415. doi:10.1007/s10059-009-0169-x

Guarente L (2013) Sirtuins and ageing—new findings. EMBO Rep 14:750. doi:10.1038/embor.2013.121

Hoffmann J, Romey R, Fink C, Yong L, Roeder T (2013) Overexpression of Sir2 in the adult fat body is sufficient to extend lifespan of male and female Drosophila. Aging 5:315–327

Houtkooper RH, Pirinen E, Auwerx J (2012) Sirtuins as regulators of metabolism and healthspan. Nat Rev Mol Cell Biol 13:225–238. doi:10.1038/nrm3293

Hsu CP, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, Shao D, Takagi H, Oka S, Sadoshima J (2010) Sirt1 protects the heart from ischemia/reperfusion. Circulation 122:2170–2182. doi:10.1161/CIRCULATIONAHA.110.958033

Jin D, Tan HJ, Lei T, Gan L, Chen XD, Long QQ, Feng B, Yang ZQ (2009) Molecular cloning and characterization of porcine sirtuin genes. Comp Biochem Physiol B 153:348–358. doi:10.1016/j. cbpb.2009.04.004

Johnson JB, Summer W, Cutler RG, Martin B, Hyun DH, Dixit VD, Pearson M, Nassar M, Maudsley S, Carlson O, John S, Laub DR, Mattson MP (2007) Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma. Free Radic Biol Med 42:665–674. doi:10.1016/j. freeradbiomed.2006.12.005

Kaeberlein M, McVey M, Guarente L (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes Dev 13:2570–2580 Lai CC, Lin PM, Lin SF, Hsu CH, Lin HC, Hu ML, Hsu CM, Yang MY (2013) Altered expression of SIRT gene family in head and neck squamous cell carcinoma. Tumour Biol 34:1847–1854. doi:10.1007/s13277-013-0726-y

Lee HS, Ka SO, Lee SM, Lee SI, Park JW, Park BH (2013) Overexpression of sirtuin 6 suppresses inflammatory responses and bone destruction in mice with collagen-induced arthritis. Arthritis Rheum 65:1776–1785. doi:10.1002/art.37963

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT. Methods 25:402–408

Lundby A, Lage K, Weinert BT, Bekker-Jensen DB, Secher A, Skovgaard T, Kelstrup CD, Dmytriyev A, Choudhary C, Lundby C, Olsen JV (2012) Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. Cell Rep 2:419–431. doi:10.1016/j. celrep.2012.07.006

Magnoni LJ, Palstra AP, Planas JV (2014) Fueling the engine: induction of AMP-activated protein kinase in trout skeletal muscle by swimming. J Exp Biol 217:1649–1652. doi:10.1242/jeb.099192

Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I (2005) Evolutionarily conserved and nonconserved cellular localizations and

functions of human SIRT proteins. Mol Biol Cell 16:4623– 4635. doi:10.1091/mbc.E05-01-0033

Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP, Sassone-Corsi P (2008) The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell 134:329–340. doi:10.1016/j. cell.2008.07.002

Newman JC, He W, Verdin E (2012) Mitochondrial protein acylation and intermediary metabolism: regulation by sirtuins and implications for metabolic disease. J Biol Chem 287:42436–42443. doi:10.1074/jbc.R112.404863

Park S, Mori R, Shimokawa I (2013) Do sirtuins promote mammalian longevity?: a critical review on its relevance to the longevity effect induced by calorie restriction. Mol Cells 35:474–480. doi:10.1007/s10059-013-0130-x

Pereira TC, Rico EP, Rosemberg DB, Schirmer H, Dias RD, Souto AA, Bonan CD, Bogo MR (2011) Zebrafish as a model organism to evaluate drugs potentially able to modulate sirtuin expression. Zebrafish 8:9–16. doi:10.1089/zeb.2010.0677

Pfluger PT, Herranz D, Velasco-Miguel S, Serrano M, Tschöp MH (2008) Sirt1 protects against high-fat diet-induced metabolic damage. Proc Natl Acad Sci 105:9793–9798. doi:10.1073/ pnas.0802917105

Planavila A, Iglesias R, Giralt M, Villarroya F (2011) Sirt1 acts in association with PPARα to protect the heart from hypertrophy, metabolic dysregulation, and inflammation. Cardiovasc Res 90:276–284. doi:10.1093/cvr/cvq376

Quiñones M, Al-Massadi O, Fernø J, Nogueiras R (2014) Crosstalk between SIRT1 and endocrine factors: effects on energy homeostasis. Mol Cell Endocrinol 397:42–50. doi:10.1016/j. mce.2014.08.00

Rajan KE, Thangaleela S, Balasundaram C (2015) Spatial learning associated with stimulus response in goldfish *Carassius auratus*: relationship to activation of CREB signalling. Fish Physiol Biochem 41:685–694. doi:10.1007/s10695-015-0038-9

Rombout JH, Abelli L, Picchietti S, Scapigliati G, Kiron V (2011) Teleost intestinal immunology. Fish Shellfish Immunol 31:616– 626. doi:10.1016/j.fsi.2010.09.001

Rui L (2014) Energy metabolism in the liver. Compr Physiol 4:177– 197. doi:10.1002/cphy.c130024

Sauve AS, Wolberger C, Schramm VL, Boeke JD (2006) The biochemistry of sirtuins. Annu Rev Biochem 75:435–465. doi:10.1146/annurev.biochem.74.082803.133500

Schirmer H, Pereira TCB, Rico EP, Rosemberg DB, Bonan CD, Bogo MR, Souto AA (2012) Modulatory effect of resveratrol on SIRT1, SIRT3, SIRT4, PGC1α and NAMPT gene expression profiles in wild-type adult zebrafish liver. Mol Biol Rep 39:3281–3289. doi:10.1007/s11033-011-1096-4

Schmeisser K, Mansfeld J, Kuhlow D et al (2013) Role of sirtuins in lifespan regulation is linked to methylation of nicotinamide. Nat Chem Biol 9:693–700. doi:10.1038/nchembio.1352

Schwer B, Verdin E (2008) Conserved metabolic regulatory functions of sirtuins. Cell Metab 7:104–112. doi:10.1016/j. cmet.2007.11.006

Secombes CJ, Wang T (2012) The innate and adaptive immune system of fish. In: Austin B (ed) Infectious disease in aquaculture: prevention and control. Woodhead Publishing, Oxford, Cambridge, Philadelphia, New Delhi, pp 3–68. doi:10.1016/ B978-0-85709-016-4.50001-5

Sharples AP, Hughes DC, Deane CS, Saini A, Selman C, Stewart CE (2015) Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging Cell 14:511–523. doi:10.1111/acel.12342

Sturn A, Quackenbush J, Trajanoski Z (2002) Genesis: cluster analysis of microarray data. Bioinformatics 18:207–208. doi:10.1093/ bioinformatics/18.1.207

Suzuki J, Shen WJ, Nelson BD, Selwood SP, Murphy GM, Kanehara H, Takahashi S, Oida K, Miyamori I, Kraemer FB (2002) Cardiac gene expression profile and lipid accumulation in response to starvation. Am J Physiol Endocrinol Metab 283:E94–E102. doi:10.1152/ajpendo.00017.2002

Tannahill GM, Curtis AM, Adamik J et al (2013) Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature 496:238–242. doi:10.1038/nature11986

Teigen LE, Orczewska JI, McLaughlin J, O'Brien KM (2015) Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in threespine stickleback. Comp Biochem Physiol A: Mol Integr Physiol 188:139–147. doi:10.1016/j.cbpa.2015.06.028

Vachharajani VT, Liu T, Wang X, Hoth JJ, Yoza BK, McCall CE (2016) Sirtuins link inflammation and metabolism. J Immunol Res. doi:10.1155/2016/8167273

Yoshizaki T, Schenk S, Imamura T, Babendure JL, Sonoda N, Bae EJ, Oh DY, Lu M, Milne JC, Westphal C, Bandyopadhyay G, Olefsky JM (2010) SIRT1 inhibits inflammatory pathways in macrophages and modulates insulin sensitivity. Am J Physiol Endocrinol Metab 298:E419–E428. doi:10.1152/ajpendo.00417.2009

Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, D'Agostino D, Planavsky N, Lupfer C, Kanneganti TD, Kang S, Horvath TL, Fahmy TM, Crawford PA, Biragyn A, Alnemri E, Dixit VD (2015) The ketone metabolite β-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. Nat Med 21:263–269. doi:10.1038/nm.3804

Yu W, Fu YC, Wang W (2012) Cellular and molecular effects of resveratrol in health and disease. J Cell Biochem 113:752–759. doi:10.1002/jcb.23431

Yu J, Sadhukhan S, Noriega LG, Moullan N, He B, Weiss RS, Lin H, Schoonjans K, Auwerx J (2013) Metabolic characterization of a Sirt5 deficient mouse model. Sci Rep 3:2806. doi:10.1038/ srep02806

Zhao S, Xu W, Jiang W et al (2010) Regulation of cellular metabolism by protein lysine acetylation. Science 327:1000–1004. doi:10.1126/science.1179689

CAPÍTULO 4- Co-expression analysis of sirtuins and related metabolic biomarkers in juveniles of gilthead sea bream (*Sparus aurata*) with differences in growth performance

Paula Simó-Mirabet[1], Erick Perera[1], Josep A. Calduch-Giner[1], Juan M. Afonso[2], Jaume Pérez-Sánchez[1]

[1]Nutrigenomics and Fish Growth Endocrinology, Institute of Aquaculture Torre de la Sal-CSIC, Castellón, Spain.

[2]Aquaculture Research Group, Institute of Sustainable Aquaculture and Marine Ecosystems (IU-ECOAQUA), University of Las Palmas de Gran Canaria (GIA), Las Palmas, Spain.

Frontiers in Physiology (2018) 9:608

Abstract

Sirtuins (SIRTs) represent a conserved protein family of deacetylases that act as master regulators of metabolism, but little is known about their roles in fish and livestock animals in general. The present study aimed to assess the value of SIRTs for the metabolic phenotyping of fish by assessing their co-expression with a wide-representation of markers of energy and lipid metabolism and intestinal function and health in two genetically different gilthead sea bream strains with differences in growth performance. Fish from the fast-growing strain exhibited higher feed intake, feed efficiency and plasma IGF-I levels, along with higher hepatosomatic index and lower mesenteric fat (lean phenotype). These observations suggest differences in tissue energy partitioning with an increased flux of fatty acids from adipose tissue toward the liver. The resulting increased risk of hepatic steatosis may be counteracted in the liver by reduced lipogenesis and enhanced triglyceride catabolism, in combination with a higher and more efficient oxidative metabolism in white skeletal muscle. These effects were supported by co-regulated changes in the expression profile of SIRTs (liver, *sirt1*; skeletal muscle, *sirt2*; adipose tissue, *sirt5-6*) and markers of oxidative metabolism (*pgc1α*, *cpt1a*, *cs*, *nd2*, *cox1*), mitochondrial respiration uncoupling (*ucp3*) and fatty acid and triglyceride metabolism (*pparα*, *pparγ* , *elovl5*, *scd1a*, *lpl*, *atgl*) that were specific to each strain and tissue. The anterior intestine of the fast-growing strain was better suited to cope with improved growth by increased expression of markers of nutrient absorption (*fabp2*), epithelial barrier integrity (*cdh1*, *cdh17*) and immunity (*il1β*, *cd8b*, *lgals1*, *lgals8*, *sIgT*, *mIgT*), which were correlated with low expression levels of *sirt4* and markers of fatty acid oxidation (*cpt1a*). In the posterior intestine, the fast-

growing strain showed a consistent up-regulation of *sirt2*, *sirt3*, *sirt5* and *sirt7* concurrently with increased expression levels of markers of cell proliferation (*pcna*), oxidative metabolism (*nd2*) and immunity (*sIgT*, *mIgT*). Together, these findings indicate that SIRTs may play different roles in the regulation of metabolism, inflammatory tone and growth in farmed fish, arising as powerful biomarkers for a reliable metabolic phenotyping of fish at the tissue-specific level.

Keywords: Fish; Feed efficiency; Lean phenotype; Elongase 5; Delta 9-desaturase; Triacylglycerol lipase; Lipoprotein lipase; Immunoglobulin T.

4.1. Introduction

The capacity of aquaculture to meet the future demand for seafood will largely depend on the use of highly efficient domesticated animal stocks. Currently, less than 10% of the aquaculture production comes from genetically improved animals (Olesen et al., 2015); however, different selective breeding programs are in progress for most farmed European fish species, including the gilthead sea bream (*Sparus aurata* L.), a highly cultured perciform fish in all the Mediterranean basin. The main trait goals for gilthead sea bream breeding companies are growth performance, morphology, disease resistance and product quality with expected improvements in growth performance of 10–15% per generation (Gjedremand Baranski, 2009; Janssen et al., 2015). However, the application of genomic tools in aquaculture is in its infancy (McAndrew and Napier, 2011), and few gilthead sea bream companies are currently using marker-assisted selection (MAS) (Janssen et al., 2015). The identification of new candidate genes for MAS, particularly for productive traits that are not easy to measure (e.g., feed efficiency, redox homeostasis, intestinal health), can be fueled using wide or targeted transcriptomic approaches (Chen et al., 2011; Cardoso et al., 2014; Choi et al., 2015). The interplay between nutrition and immune system is well recognized; however, the true integration of research on fish nutrition, growth, chronobiology, energy status, immune function and intestinal health is still far from clear despite recent and important advances in this field (Calduch-Giner et al., 2016; Estensoro et al., 2016; Martin and Król, 2017; Piazzon et al., 2017; Yúfera et al., 2017).

Fish exposed to sub-optimal rearing conditions are hampered with respect to health and growth, and genes known as master regulators of energy sensing are of special relevance for disclosing these types of metabolic disturbances. Most organisms have evolved to efficiently transition between anabolic and catabolic states, allowing them to survive in an environment in which nutrient availability is variable (Houtkooper et al., 2012; Laplante and Sabatini, 2012). Nutrient stress is generally considered from the standpoint of how cells detect and respond to an insufficient supply of nutrients (Wellen and Thompson, 2010). However, cells and organisms also experience stress with nutrient excess as a major readout of nutrient uptake is the level of reactive oxygen species (ROS) produced by mitochondria (Wellen and Thompson, 2010), which limit voluntary feed intake (Saravanan et al., 2012) and growth (Fernández-Díaz et al., 2006; Rise et al., 2015) in farmed fish. Different mechanisms operate within cells to balance ROS production and scavenging to keep ROS within physiological levels. The mitochondrial uncoupling proteins (UCPs) act as a highly conserved safety valve that activates futile cycles of energy to alleviate ROS production (Mailloux and Harper, 2011). These cycles become rapidly inactive when the oxidative capacity of the tissue is improved, or the supply of metabolic fuels does not exceed the tissue energy demand in a wide range of experimental models, including fish (Nabben and Hoeks, 2008; Bermejo-Nogales et al., 2011, 2014). Moreover, the antioxidant defense system relies mostly on superoxide dismutase, glutathione peroxidase, glutathione reductase, thioredoxin, thioredoxin reductase and catalase, operating as ROS scavengers (MartínezÁlvarez et al., 2005; Pacitti et al., 2014). As part of this complex regulatory system, nutrient

and energy availability are sensed at multiple levels. AMP-activated protein kinase (AMPK) inhibits proliferation and growth in response to ATP depletion, while the mammalian target of rapamycin (mTOR) is activated by nutrients and signaling growth factors to promote mitochondrial metabolism, protein synthesis and cell growth (Wellen and Thompson, 2010; Laplante and Sabatini, 2012). In addition, protein post-translational modifications such as O-GlcNAcylation, glycosylation and acetylation/deacetylation play key roles in the adaptation to metabolic stress produced by elevated levels of intracellular metabolites, including ROS (Wellen and Thompson, 2010).

Among protein post-translational modifications, deacetylation is particularly sensitive to metabolic states through the action of deacetylases, first represented by NAD^+-dependent sirtuin deacetylases/deacylases/ADP-ribosyltransferases (SIRTs). Most SIRTs couple protein deacetylation of histone and non-histone substrates with the energy status of the cell via the cellular NAD^+/NADH ratio (Schwer and Verdin, 2008; Houtkooper et al., 2012; Schmeisser et al., 2013; Masri, 2015). Proteomic studies following the initial discovery of histone acetylation have also revealed that thousands of proteins are abundantly acetylated (Zhao et al., 2010; Guan and Xiong, 2011; Choudhary et al., 2014), and their deacetylation commonly leads to increased stability and catalytic activity in the case of metabolic enzymes (Verdin et al., 2010; Houtkooper et al., 2012). In contrast, deacetylation of histones is an epigenetic mechanism associated with repression of gene expression (Lundby et al., 2012). These mechanisms yield a highly regulated proteome with key roles played by SIRTs, at both the transcriptional and post-translational levels, in the maintenance of energy homeostasis

(Schwer and Verdin, 2008; Zhao et al., 2010; Houtkooper et al., 2012) as well as in muscle growth through negative regulation of IGF-I and mTOR signaling (Ghosh et al., 2010; Sharples et al., 2015). Accordingly with this central role in metabolism regulation, SIRTs are virtually ubiquitous throughout all kingdoms of life, ranging in abundance from one type in bacteria to seven types in vertebrates (Greiss and Gartner, 2009). This feature offers the possibility of complementary but also non-redundant and tissue-specific energy sensing mechanisms, which is reflected by the different cellular locations of different SIRTs. SIRT1, SIRT6, and SIRT7 generally reside in the nucleus; SIRT2 is primarily cytosolic, although it is shuttled to the nucleus during the G2/M transition of the cell cycle (Gomes et al., 2015); and SIRT3-5 are mitochondrial proteins (Jing and Lin, 2015).

Most research on SIRTs has been carried out in humans and rodents, which limits our understanding of the evolution of SIRT regulation and function. However, the seven SIRT counterparts of higher vertebrates have been molecularly characterized in gilthead sea bream (Simó-Mirabet et al., 2017a). The sequence analysis of these counterparts has revealed a strict conservation of the characteristic catalytic domain, and phylogenetic analysis has revealed three major clades corresponding to SIRT1-3, SIRT4-5, and SIRT6-7 that reflect the accepted classification of SIRTs (Frye, 2000). Gene expression profiling has also demonstrated that the molecular signatures of fish SIRTs in gilthead sea bream are strongly influenced by nutrient availability and tissue-specific metabolic capabilities (Simó-Mirabet et al., 2017a). In this scenario, changes in the SIRT gene expression pattern contribute to triggering the metabolic switch from adipogenesis to lipolysis with the

increased demand of metabolic fuels by peripheral tissues during fasting or caloric restriction. Other studies in fish have related SIRTs to ammonia levels (Connon et al., 2011), cold exposure (Teigen et al., 2015), spatial learning (Rajan et al., 2015), changes in blood glucose (Otero-Rodiño et al., 2016) and adipocyte maturation during hypoxia (Ekambaram and Parasuraman, 2017). Nevertheless, SIRT function and regulation remain poorly studied in fish and in livestock animals in general (Ghinis-Hozumi et al., 2013).

The present study aimed to assess the gene expression pattern of SIRTs in fish under non-restricted feeding and the value of their gene expression profile as a new tool for metabolic phenotyping of farmed fish. This was accomplished by measuring SIRTs' co-regulated expression with markers of intermediary metabolism, immunological status, and intestine function and integrity in two gilthead sea bream strains with known differences in growth performance. The rationale of the study was that differences in key performance indicators necessarily reflect different uses of nutrients and energy, being voluntary feed intake and growth limited by the capacity of fish to preserve redox balance (Saravanan et al., 2012; Rise et al., 2015; Danzmann et al., 2016). Accordingly, the working hypothesis was that fish with higher growth rates would be able to grow efficiently in a cellular milieu with an enhanced risk of oxidative stress, contributing the differential regulation of SIRTs to readjust and preserve metabolic homeostasis at each tissue.

4.2. Material and methods

4.2.1. Fish

The gilthead sea bream is highly cultured in Europe, with several hatcheries operating mostly, but not exclusively, in the Mediterranean basin (Janssen et al., 2015). In the present study, we used fish from two geographically distant hatcheries (henceforth called strains 1 and 2), which have regularly performed differently under the same growout conditions in our experimental facilities.

For the genotyping of these two populations, thirty fish of each strain were characterized for specific microsatellite markers. Both fish populations were recognized as genetically different, with 3.5% of genetic differentiation (Fst = 0.0351), by SMsa1 multiplex PCR of 10 loci (A5, C3, C12, D4, E1, E4, F6, I9, L11, M5) (Lee-Montero et al., 2013) (**Table 4.1**). Briefly, DNA was extracted from the fin by using the BioSprint 96 DNA Blood Kit (QIAGEN®) operated by a Biosprint 96 robot. The concentration of extracted DNA was measured by using a NanoDrop 8000 spectrophotometer v.3.7 (Thermo Fisher Scientific) and normalized to 80 ng/µl prior to PCR amplification. PCR reactions were carried out by using a TECAN robot Freedom Evo (Tecan Schweiz AG, Switzerland), and Freedom Evowar® Standard v.2.5 software following the manufacturer's instructions. Genotypes were estimated by GENEMAPPER v.3.7 software using the SMsa1-kit created by Lee-Montero et al. (2013).

4.2.2. Feeding trial

Gilthead sea bream juveniles of strain 1 and strain 2 were acclimatized for 6 weeks to the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC). Then, 13-15 g fish from both strains were distributed among 90 L tanks in triplicate groups of 25 fish each. The trial was conducted under natural photoperiod and temperature conditions at the latitude of the IATS (40º5'N; 0º10'E) from May to July (8 weeks), increasing the water temperature from 20º to 25ºC. The oxygen content of water was consistently higher than 75% saturation, and unionized ammonia remained below toxic levels (<0.02 mg/L). Fish were fed with a standard diet (EFICO YM 568; BioMar, Spain) twice a day until visual satiety.

At the end of the trial, 12 fish per strain (four randomly selected fish per tank) were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 100 µg/mL) and blood was quickly taken from caudal vessels with heparinized syringes. One aliquot was used for hemoglobin measurements. The remaining blood was centrifuged at 3,000 × g for 20 min at 4ºC, and the plasma was stored at −80ºC until biochemical assays. Liver, white skeletal muscle, adipose tissue, and anterior and posterior intestine sections were rapidly excised from 12 fish per strain, frozen in liquid nitrogen and stored at −80ºC until RNA extraction.

All procedures were carried out according to present IATS-CSIC Review Board and European (2010/63/EU) animal directives and Spanish laws (Royal Decree RD53/2013) on the handling of experimental animals.

Table 4.1 Genetic diversity in strains 1 and 2 by using SMsa1 (Lee-Montero et al., 2013).

LOCUS	Locus-1		Locus-2		Locus-3		Locus-4		Locus-5		Locus-6		Locus-7		Locus-8		Locus-9		Locus-10		
Internal Code	L11		E4		A5		M5		F6		19		C12		C3		D4		E1		AVERAGE
Linkage group	10		11		20		24		3		19		18		6		1		23		
Population (Strain)	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
Heterocigocity observed	0.67	0.85	1.00	0.85	0.60	0.38	0.70	0.88	0.62	0.62	0.86	0.77	0.40	0.42	0.97	0.81	0.60	0.55	0.83	0.92	0.72	0.70
Chi^2-Pearson	12.59		74		58.28		361.67		100.11		67.38		197.29		129.78		98.89		68		1,517.26
Significance	0.083		0		0		0		0		0		0		0		0		0		**0**
Locus-NAME	Did-16-F		Bld-18-F		Bt-14-F		G27		Hd-25-F		At37		D47		Eid-39-T		P3		Bd-68-T		Strain 1	Strain 2
No. Alleles	4	5	8	8	4	3	10	13	5	6	5	6	4	5	16	17	7	8	5	5	6.8	7.6

4.2.3. Blood biochemistry

Hemoglobin (Hb) concentration was determined using a HemoCue B-Hemoglobin Analyser® (AB, Leo Diagnostic, Sweden). Plasma glucose was measured by the glucose oxidase method (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total plasma cholesterol was determined using cholesterol esterase/cholesterol dehydrogenase reagent

(Thermo Fisher Scientific). Plasma soluble proteins were measured with the Bio-Rad protein reagent (Hercules, California, USA), with bovine serum albumin as a standard. Plasma growth hormone (GH) was determined by a homologous gilthead sea bream radioimmunoassay (RIA) as previously detailed (Martínez-Barberá et al., 1995). The sensitivity and midrange (ED50) of the GH RIA assay were 0.15 and 1.8 ng/mL, respectively. Plasma insulin-like growth factors (IGFs) were extracted by acid-ethanol cryoprecipitation (Shimizu et al., 2000), and the concentration of IGF-I was measured by a generic fish IGF-I RIA validated for several Mediterranean perciform fish (de Celis et al., 2004). The sensitivity and midrange of the IGF-I RIA assay were 0.05 and 0.7−0.8 ng/mL, respectively. Plasma cortisol levels were analyzed using an EIA kit (kit RE52061, IBL, International GmbH, Germany). The detection limit of the cortisol assay was 50 pg/mL, with a midrange of 700 pg/mL. All commercial kits were used according to the manufacturers' instructions.

4.2.4. Gene expression profiling

RNA was extracted using the MagMAX-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50–100 µg, with 260:280 nm absorbance ratios (A260/280) of 1.9-2.1. RNA integrity number (RIN) values of 8-10 (Agilent 2100 Bioanalyzer) were indicative of clean and intact RNA. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Negative control reactions were run without reverse transcriptase. Two different 96-well PCR-arrays of 28–39 markers of metabolic and

intestinal health condition were designed for the simultaneous gene expression profiling of liver/white skeletal muscle/adipose tissue and intestine, respectively (**Table 4.2**). A housekeeping gene (β-Actin) and controls of PCR performance were included in each array. Briefly, 660 pg of total cDNA was used in 25 µL PCR reactions. PCR wells contained 2x SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 µM (**Table 4.3** and **4.4**). All pipetting operations for the PCR-arrays were performed by an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. Real-time quantitative PCR was carried out in an Eppendorf Mastercycler Ep Realplex (Eppendorf, Germany). The PCR amplification program consisted of an initial denaturation step at 95ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95ºC and annealing/extension for 60 s at 60ºC. The efficiency of the PCR reactions was consistently higher than 90% and similar among all the genes. The specificity of the reactions was verified by melting curve analysis (ramping rates of 0.5ºC/10 s over a temperature range of 55–95ºC). Negative controls without a template were routinely performed for each primer set. Gene expression was calculated using the delta-delta Ct method (Livak and Schmittgen, 2001). For multi-gene analysis, all values for a given tissue were referenced to the expression level of *sirt1* in strain 1 fish, for which a value of 1 was arbitrarily assigned. Fold-changes in gene expression were calculated as the expression ratio between strain 1 and strain 2. A value > 1 indicates higher expression levels in strain 1, and values < 1 indicate lower expression levels in strain 1.

Table 4.2 Genes included in the intestine (†) and liver/muscle/adipose (*) tissue pathway-focused PCR arrays.

Gene name/category	Symbol
Energy sensing	
Sirtuin 1	*sirt1* *†
Sirtuin 2	*sirt2* *†
Sirtuin 3	*sirt3* *†
Sirtuin 4	*sirt4* *†
Sirtuin 5	*sirt5* *†
Sirtuin 6	*sirt6* *†
Sirtuin 7	*sirt7* *†
Oxidative metabolism	
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1a* *†
Carnitine palmitoyltransferase 1A	*cpt1a* *†
Citrate synthase	*cs* *†
NADH-ubiquinone oxidoreductase chain 2	*nd2* *†
Cytochrome c oxidase subunit I	*cox1* *†
Mitochondrial respiration uncoupling	
Uncoupling protein 1	*ucp1* *†
Uncoupling protein 2	*ucp2* *
Uncoupling protein 3	*ucp3* *
Cell differentiation and proliferation	
Proliferating cell nuclear antigen	*pcna* †
Intestinal epithelial barrier	
Occludin	*ocln* †
Claudin-15	*cldn15*†
Cadherin-1	*cdh1* †
Cadherin-17	*cdh17* †
Enterocyte mass and nutrient absorption	
Intestinal-type alkaline phosphatase	*alpi* †
Liver type fatty acid-binding protein	*fabp1* †
Intestinal fatty acid-binding protein	*fabp2* †
Ileal fatty acid-binding protein	*fabp6* †
Mucus production and Goblet cell differentiation	
Mucin 2	*muc2* †
Mucin 13	*muc13* †
Transcription factor HES-1-B	*hes1-b* †

(Continued)

Table 4.2 Continued

Lipid metabolism		Immunological/inflammatory status	
Peroxisome proliferator-activated receptor α	*ppara* *	Tumor necrosis factor-alpha	*tnfa* †
Peroxisome proliferator-activated receptor γ	*pparγ* *	Interleukin-1 beta	*il1b* †
Elongation of very long chain fatty acids 4	*elovl4* *	Interleukin-6	*il6* †
Elongation of very long chain fatty acids 5	*elovl5* *	Interleukin-8	*il8* †
Elongation of very long chain fatty acids 6	*elovl6* *	Interleukin-10	*il10* †
Fatty acid desaturase 2	*fads2* *	CD4	*cd4* †
Stearoyl-CoA desaturase 1a	*scd1a* *	CD8 alpha	*cd8a* †
Stearoyl-CoA desaturase 1b	*scd1b* *	CD8 beta	*cd8b* †
Phosphatidylethanolamine N-methyltransferase	*pemt* *	Galectin-1	*lgals1* †
Hepatic lipase	*hl* *	Galectin-8	*lgals8* †
Lipoprotein lipase	*lpl* *	Secreted Immunoglobulin M	*sIgM* †
Hormone sensitive lipase	*hsl* *	Secreted Immunoglobulin T	*sIgT* †
Adipose triglyceride lipase	*atgl* *	Membrane Immunoglobulin M	*mIgM* †
		Membrane Immunoglobulin T	*mIgT* †

Table 4.3 Forward and reverse primers of the liver/skeletal muscle/adipose tissue pathway-focused PCR array.

Gene name	Symbol	Primer sequence	
Sirtuin1	*sirt1*	F	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC
		R	CCT CAG AAT GGT CCT CGG ATC GGT CTC
Sirtuin2	*sirt2*	F	GAA CAA TCC GAC GAC AGC AGT GAA G
		R	AGG TTA CGC AGG AAG TCC ATC TCT
Sirtuin3	*sirt3*	F	CTG CCA AGT CCT CAT CCC
		R	CTT CAC CAG ACG AGC CAC
Sirtuin4	*sirt4*	F	GGC TGG CGG AGT CGG ATG
		R	TCC TGA ATA CAC CTG TGA CGA AGA C
Sirtuin5	*sirt5*	F	CAG ACA TCC TAA CCC GAG CAG AG
		R	CCA CGA GGC AGA GGT CAC A
Sirtuin6	*sirt6*	F	ACT CCA CCA CCA CCG ATG TCA A
		R	CTC CTC CTC CTT CAC CTT TCG CTT TG
Sirtuin7	*sirt7*	F	CTG GAG CAA CCT CTA AAC TGG AA
		R	CAC CTT CAG ACT GGA GCC TAA
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1a*	F	CGT GGG ACA GGT GTA ACC AGG ACT C
		R	ACC AAC CAA GGC AGC ACA CTC TAA TTC T
Citrate synthase	*cs*	F	TCC AGG AGG TGA CGA GCC
		R	GTG ACC AGC AGC CAG AAG AG
NADH-ubiquinone oxidoreductase chain 2	*nd2*	F	TAG GTT GAA TGA CCA TCG TA
		R	GGC TAA GGA GTT GAG GTT

(Continued)

Table 4.3 Continued

Cytochrome c oxidase subunit I	*cox1*	F	GTC CTA CTT CTT CTG TCC CTT CCT GTT CT
		R	AGG TTT CGG TCT GTA AGG AGC ATT GTA ATC
Uncoupling protein1	*ucp1*	F	GCA CAC TAC CCA ACA TCA CAA G
		R	CGC CGA ACG CAG AAA CAA AG
Uncoupling protein2	*ucp2*	F	CGG CGG CGT CCT CAG TTG
		R	AAG CAA GTG GTC CCT CTT TGG TCA T
Uncoupling protein3	*ucp3*	F	AGG TGC GAC TGG CTG ACG
		R	TTC GGC ATA CAA CCT CTC CAA AG
Peroxisome proliferator-activated receptor α	*pparα*	F	TCT CTT CAG CCC ACC ATC CC
		R	ATC CCA GCG TGT CGT CTC C
Peroxisome proliferator-activated receptor γ	*pparγ*	F	CGC CGT GGA CCT GTC AGA GC
		R	GGA ATG GAT GGA GGA GGA GGA GAT GG
Elongation of very long chain fatty acids 4	*elovl4*	F	CGG TGG CAA TCA TCT TCC
		R	TCA ACT GGC TGT CTG TGT
Elongation of very long chain fatty acids 5	*elovl5*	F	CCT CCT GGT GCT CT ACA AT
		R	GTG AGT GTC CTG GCA GTA
Elongation of very long chain fatty acids 6	*elovl6*	F	GTG CTG CTC TAC TCC TGG TA
		R	ACG GCA TGG ACC AAG TAG T
Fatty acid desaturase 2	*fads2*	F	GCA GGC GGA GAG CGA CGG TCT GTT CC
		R	AGC AGG ATG TGA CCC AGG TGG AGG CAG AAG
Stearoyl-CoA desaturase 1a	*scd1a*	F	CGG AGG CGG AGG CGT TGG AGA AGA AG
		R	AGG GAG ACG GCG TAC AGG GCA CCT ATA TG

(Continued)

Table 4.3 Continued

Stearoyl-CoA desaturase 1b	*scd1b*	F	GCT CAA TCT CAC CAC CGC CTT CAT AG
		R	GCT GCC GTC GCC CGT TCT CTG
Phosphatidylethanolamine N-methyltransferase	*pemt*	F	TTG GTG CCA GTC CTG TTG GTC TC
		R	TGA TAG ATC AGT CCA GTG AAT GGT CCT TC
Hepatic lipase	*hl*	F	TTG TAG AAG GTG AGG AAA ACT G
		R	GCT CTC CAT CAG ACC ATC C
Lipoprotein lipase	*lpl*	F	CGT TGC CAA GTT TGT GAC CTG
		R	AGG GTG TTC TGG TTG TCT GC
Hormone sensitive lipase	*hsl*	F	GCT TTG CTT CAG TTT ACC ACC ATT TC
		R	GAT GTA GCG ACC CTT CTG GAT GAT GTG
Adipose triglyceride lipase	*atgl*	F	GTG CTT CAG TCC TGG ATG TCT TC
		R	AGC CTT GCA GGT CCA TGT TGA
β-Actin	*actb*	F	TCCTGCGGAATCCATGAGA
		R	GACGTCGCACTTCATGATGCT

Table 4.4 Forward and reverse primers of the intestine tissue pathway-focused PCR array.

Gene name	Symbol		Primer sequence
Sirtuin1	*sirt1*	F	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC
		R	CCT CAG AAT GGT CCT CGG ATC GGT CTC
Sirtuin2	*sirt2*	F	GAA CAA TCC GAC GAC AGC AGT GAA G
		R	AGG TTA CGC AGG AAG TCC ATC TCT

(Continued)

Table 4.4 Continued

Sirtuin3	*sirt3*	F	CTG CCA AGT CCT CAT CCC
		R	CTT CAC CAG ACG AGC CAC
Sirtuin4	*sirt4*	F	GGC TGG CGG AGT CGG ATG
		R	TCC TGA ATA CAC CTG TGA CGA AGA C
Sirtuin5	*sirt5*	F	CAG ACA TCC TAA CCC GAG CAG AG
		R	CCA CGA GGC AGA GGT CAC A
Sirtuin6	*sirt6*	F	ACT CCA CCA CCA CCG ATG TCA A
		R	CTC CTC CTC CTT CAC CTT TCG CTT TG
Sirtuin7	*sirt7*	F	CTG GAG CAA CCT CTA AAC TGG AA
		R	CAC CTT CAG ACT GGA GCC TAA
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1a*	F	CGT GGG ACA GGT GTA ACC AGG ACT C
		R	ACC AAC CAA GGC AGC ACA CTC TAA TTC T
Carnitine palmitoyltransferase 1A	*cpt1a*	F	GTG CCT TCG TTC GTT CCA TGA TC
		R	TGA TGC TTA TCT GCT GCC TGT TTG
Citrate synthase	*cs*	F	TCC AGG AGG TGA CGA GCC
		R	GTG ACC AGC AGC CAG AAG AG
NADH-ubiquinone oxidoreductase chain 2	*nd2*	F	TAG GTT GAA TGA CCA TCG TA
		R	GGC TAA GGA GTT GAG GTT
Cytochrome c oxidase subunit I	*cox1*	F	GTC CTA CTT CTT CTG TCC CTT CCT GTT CT
		R	AGG TTT CGG TCT GTA AGG AGC ATT GTA ATC
Uncoupling protein1	*ucp1*	F	GCA CAC TAC CCA ACA TCA CAA G
		R	CGC CGA ACG CAG AAA CAA AG
Proliferating cell nuclear antigen	*pcna*	F	CGT ATC TGC CGT GAC CTG T
		R	AGA ACT TGA CTC CGT CCT TGG

(Continued)

Table 4.4 Continued

Occludin	*ocln*	F	GTG TCA GAA CCT CTA CCA GAC CAG CTA CTC
		R	GAA AGC CTC CCA CTC CTC CCA TCT
Cadherin-1	*cdh1*	F	TGC TCC ATA CAG CGT CAC CTT ACA
		R	CTC GTT CAT CCT AGC CGT CCA GTT
Cadherin-17	*cdh17*	F	GAT GCC CGC AAC CCA GAG
		R	CCG TTG ATT CAC TGC CGT AGA C
Intestinal-type alkaline phosphatase	*alpi*	F	CCG CTA TGA GTT GGA CCG TGA T
		R	GCT TTC TCC ACC ATC TCA GTA AGG G
Intestinal fatty acid-binding protein	*fabp2*	F	CGA GCA CAT TCC GCA CCA AAG
		R	CCC ACG CAC CCG AGA CTT C
Ileal fatty acid-binding protein	*fabp6*	F	ACC CAG GAC GGC AAT ACC
		R	CGA CGG TGA AGT TGT TGG T
Mucin 2	*muc2*	F	ACG CTT CAG CAA TCG CAC CAT
		R	CCA CAA CCA CAC TCC TCC ACA T
Mucin 13	*muc13*	F	TTC AAA CCC GTG TGG TCC AG
		R	GCA CAA GCA GAC ATA GTT CGG ATA T
Transcription factor HES-1-B	*hes1-b*	F	GCC TGC CGA TAT GAT GGA A
		R	GGA GTT GTG TTC ATG CTT GC
Tumor necrosis factor-alpha	*tnfa*	F	CAG GCG TCG TTC AGA GTC TC
		R	CTG TGG CTG AGA GCT GTG AG
Interleukin-1 beta	*il1β*	F	GCG ACC TAC CTG CCA CCT ACA CC
		R	TCG TCC ACC GCC TCC AGA TGC
Interleukin-6	*il6*	F	TCT TGA AGG TGG TGC TGG AAG TG
		R	AAG GAC AAT CTG CTG GAA GTG AGG

(Continued)

Table 4.4 Continued

Interleukin-10	*il10*	F	AAC ATC CTG GGC TTC TAT CTG
		R	GTG TCC TCC GTC TCA TCT G
CD4	*cd4*	F	TCC TCC TCC TCG TCC TCG TT
		R	GGTGTCTCATCTTCCGCTGTCT
CD8 alpha	*cd8a*	F	GCA GCA ACG GTA ACA CGA ACG
		R	CCAGTATGAGCGGAGTACAGAACA
CD8 beta	*cd8b*	F	CCG AAA TGT GGA AGA CTG GAA CTC
		R	CTTTGGAGGTAAGGTTGGAGGGAT
Galectin-1	*lgals1*	F	GTG TGA GGA GGT CCG TGA TG
		R	ACT GTA GAG CCG TCC GAT AGG
Galectin-8	*lgals8*	F	GGC GGT GAA CGG CGG TCA
		R	GCT CCA GCT CCA GTC TGT GTT GAT AC
Secreted Immunoglobulin M	*sIgM*	F	ACC TCA GCG TCC TTC AGT GTT TAT GAT GCC
		R	CAG CGT CGT CGT CAA CAA GCC AAG C
Secreted Immunoglobulin T	*sIgT*	F	GCT GTC AAG GTG GCC CCA AAA G
		R	CAA CAT TCA TGC GAG TTA CCC TTG GC
Membrane Immunoglobulin M	*mIgM*	F	GCTATGGAGGCGGAGGAAGATAACA
		R	GCAGAGTGATGAGGAAGAGAAGGATGAA
Membrane Immunoglobulin T	*mIgT*	F	AGA CGA TGC CAG TGA AGA GGA TGA GT
		R	CGA AGG AGG AGG CTG TGG ACC A
β-Actin	*actb*	F	TCCTGCGGAATCCATGAGA
		R	GACGTCGCACTTCATGATGCT

4.2.5. Statistical analyses

Data pertaining to growth performance, blood biochemistry and gene expression of the two fish strains in liver, white skeletal muscle and adipose tissue were analyzed by Student's t-test. Two way analysis of variance (ANOVA) was carried out to analyze intestinal gene expression, with both the intestine segment and the fish strain as sources of variation. The significance level was set to $P < 0.05$ in all tests performed. These analyses were conducted using SigmaPlot version 13.0 (Systat Software, San Jose, CA).

To confirm the genetic differentiation between both fish strain used, the following log-linear model was used in SPSS software (IBM Corp., Armonk, N.Y., USA) for statistical analysis when both populations were compared for different microsatellite markers (referred as factors A and B):

$\ln f_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij}$, where

f_{ij} = is the expected frequency in row *i*, column *j* of the two-way contingency table

μ = is the mean of the logarithms of the expected frequencies

α_i = is the effect of category *i* of factor A

β_j = is the effect of category *j* of factor B

$\alpha\beta_{ij}$ = is the interaction term indicating the dependence of category *i* of factor A on category *j* of factor B.

The genetic flow between populations was estimated through Fst (Nei, 1973), by using the GENEPOP software (Raymond and Rousset, 1995; Rousset, 2008).

4.3. Results

4.3.1. Growth performance and blood biochemistry

Data pertaining to growth performance and blood biochemistry of the two genetically different (3.5% of genetic differentiation) strains are shown in **Table 4.5**. Fish of strain 1 showed higher feed intake and grew faster than fish of strain 2 with specific growth rates of 2.1 and 1.6, respectively. Feed efficiency (FE) was also significantly improved (1.2-fold higher) in fish of strain 1. Organosomatic indexes were determined for viscera, liver and mesenteric fat as tissue to body weight ratios. The resulting viscerosomatic (VSI) and mesenteric fat (MFI) indexes were significantly lower in fish of strain 1, whereas the opposite was observed for the hepatosomatic index (HSI). Regarding blood biochemistry, significant effects of fish strain on circulating levels of hemoglobin, glucose, GH and cortisol were not found. However, plasma levels of cholesterol, proteins and IGF-I were higher in strain 1 fish than in strain 2 fish.

Table 4.5 Growth performance and blood biochemistry of two different gilthead sea bream strains fed to satiety over the course of 8-weeks (May-July) under natural light and temperature conditions. Data on body weight, feed intake, and growth indices are the mean ± SEM of triplicate tanks (25 fish/tank). Data on blood biochemistry, viscera, liver and mesenteric fat weights are the mean ± SEM of 12 fish (4 fish/tank; 12 fish/strain).

	STRAIN 1	STRAIN 2	*P*[1]
Initial body weight (g)	15.1 ± 0.04	13.2 ± 0.03	*<0.001*
Final body weight (g)	50.5 ± 0.60	32.3 ± 0.03	*<0.001*
Feed intake (g DM/fish)	42.1 ± 0.20	27.5 ± 0.70	*<0.001*
SGR (%)[2]	2.11 ± 0.02	1.57 ± 0.01	*<0.001*
FE (%)[3]	0.84 ± 0.01	0.69 ± 0.02	*0.006*
Viscera (g)	4.95 ± 0.17	3.97 ± 0.22	*0.002*
Liver (g)	1.06 ± 0.15	0.68 ± 0.03	*<0.001*
Mesenteric fat (g)	0.74 ± 0.07	0.91 ± 0.10	*0.181*
VSI (%)[4]	9.28 ± 0.18	10.1 ± 0.31	*0.037*
HSI (%)[5]	1.98 ± 0.07	1.76 ± 0.07	*0.05*
MFI (%)[6]	1.38 ± 0.12	2.29 ± 0.22	*0.002*
Blood biochemistry			
Haemoglobin (g/dL)	6.19 ± 0.11	5.90 ± 0.24	*0.281*
Glucose (mg/dL)	43.8 ± 1.47	44.2 ± 2.13	*0.431*
Total cholesterol (mg/dL)	148.2 ± 8.46	100.2 ± 20.5	*0.001*
Total proteins (g/L)	45.4 ± 1.41	39.0 ± 0.86	*<0.001*
GH (ng/mL)	4.90 ± 2.10	6.85 ± 2.06	*0.130*
IGF-I (ng/mL)	57.5 ± 4.12	28.8 ± 3.88	*<0.001*
Cortisol (ng/mL)	12.2 ± 3.01	14.1 ± 7.40	*0.788*

[1]Result values from t-test.
[2]Specific growth rate = 100 x (ln final body weight - ln initial body weight/days).
[3]Feed efficiency = weight gain/dry feed intake.
[4]Viscerosomatix index = (100 x viscera weight)/fish weight.
[5]Hepatosomatic index = (100 x liver weight)/fish weight.
[6]Mesenteric fat index = (100 x mesenteric fat weight)/fish weight.

4.3.2. Transcriptional profiling of liver, adipose tissue, and skeletal muscle

Data regarding relative gene expression in liver, adipose and white skeletal muscle tissue are shown in **Table 4.6**. To simplify the visualization of the results, only fold-changes (calculated as the ratio strain 1/strain 2) of differentially expressed genes are represented in **Fig. 4.1**. The exception is *atgl*, which is included in the graphical representation of all tissues, although it's overall increased expression in fish of strain 1 was only statistically significant in skeletal muscle.

In liver (**Fig. 4.1A**), *sirt1* and mitochondrial genes related to oxidative metabolism, including markers of mitochondrial biogenesis and glucose/fatty acid (FA) metabolism (*pgc1α*), tricarboxylic acid (TCA) cycle (*cs*), oxidative phosphorylation (OXPHOS; *nd2*, *cox1*), mitochondrial FA transport and β-oxidation (*cpt1a*), were expressed at a lower rate in fish of strain 1. The expression of the lipogenic *pparγ* was also lower in fish of strain 1. Conversely, the FA elongase *elovl5* and the delta 9-desaturase *scd1a* exhibited higher expression levels in the liver of fish of strain 1. The same trend was observed for the intracellular triacylglycerol lipase *atgl*, although the observed changes were not statistically significant. In adipose tissue (**Fig. 4.1B**), *sirt5*, *sirt6*, and *pparα* exhibited higher expression rates in strain 1 fish than in strain 2 fish. The same trend was observed for *atgl*, although it was not statistically significant. In the white skeletal muscle (**Fig. 4.1C**), higher *sirt2* transcript abundance in fish of strain 1 occurred along with higher gene expression levels of key genes of mitochondrial biogenesis (*pgc1α*), tissue FA uptake (*lpl*), lipid catabolism (*atgl*, *pparα*) and metabolism of

monounsaturated FAs (*scd1a*). Conversely, the expression rate of the mitochondrial respiration uncoupling protein of skeletal muscle tissues (*ucp3*) was lower in fish of strain 1.

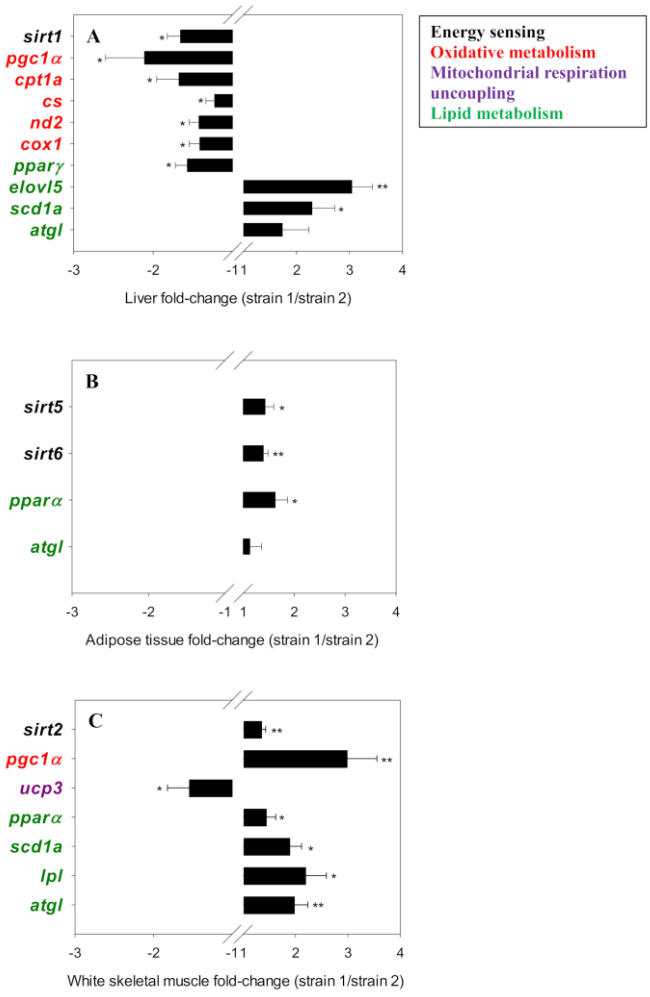


Figure 4.1 Fold-changes (strain 1/strain 2) of differentially expressed genes in liver tissue **(A)**, adipose tissue **(B)** and white skeletal muscle **(C)**. The asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01) between strains. Values >1 indicate up-regulated genes in fish of strain 1; values <1 indicate down-regulated genes in fish strain 1.

Table 4.6 Relative mRNA expression in liver, adipose tissue (AT) and white skeletal muscle (WSM) of selected markers of intermediary metabolism in two different gilthead sea bream strains. Values are the mean ± SEM of 6 fish. P-values are the result of Student t-test between strains in a given tissue. Bold values indicate statistically significant differences (P<0.05). All data values for each tissue were in reference to the expression level of *sirt1* of STRAIN 1 with an arbitrary assigned value of 1.

	LIVER			**AT**			**WSM**		
	STRAIN 1	STRAIN 2	P-value	STRAIN 1	STRAIN 2	P-value	STRAIN 1	STRAIN 2	P-value
sirt1	1.02 ± 0.11	1.69 ± 0.17	**0.011**	1.02 ± 0.09	0.86 ± 0.03	0.150	1.03 ± 0.11	1.12 ± 0.10	0.566
sirt2	2.52 ± 0.25	2.71 ± 0.11	0.508	0.82 ± 0.16	0.60 ± 0.07	0.246	1.90 ± 0.11	1.41 ± 0.05	**0.002**
sirt3	0.37 ± 0.05	0.36 ± 0.03	0.835	0.44 ± 0.04	0.42 ± 0.02	0.661	0.16 ± 0.02	0.17 ± 0.01	0.641
sirt4	0.20 ± 0.05	0.21 ± 0.03	0.983	0.08 ± 0.02	0.07 ± 0.01	0.530	0.15 ± 0.02	0.14 ± 0.01	0.764
sirt5	2.36 ± 0.12	2.39 ± 0.16	0.886	0.76 ± 0.09	0.53 ± 0.02	**0.048**	2.22 ± 0.27	2.18 ± 0.12	0.884
sirt6	0.25 ± 0.04	0.28 ± 0.02	0.214	0.28 ± 0.02	0.20 ± 0.01	**0.002**	0.13 ± 0.02	0.14 ± 0.01	0.660
sirt7	0.63 ± 0.07	0.63 ± 0.03	0.469	0.32 ± 0.05	0.24 ± 0.02	0.177	0.43 ± 0.05	0.42 ± 0.03	0.911
pgc1α	0.27 ± 0.06	0.56 ± 0.09	**0.034**	0.02 ± 0.01	0.01 ± 0.002	0.356	1.00 ± 0.19	0.34 ± 0.06	**0.006**
cpt1a	4.52 ± 0.72	7.57 ± 0.76	**0.016**	2.08 ± 0.21	2.14 ± 0.07	0.818	9.43 ± 1.23	10.2 ± 1.18	0.666
cs	7.32 ± 0.63	8.98 ± 0.25	**0.028**	6.21 ± 0.75	5.28 ± 0.45	0.358	59.9 ± 3.10	60.3 ± 2.69	0.918
nd2	306.9 ± 25.8	436.9 ± 47.7	**0.038**	75.8 ± 8.96	63.2 ± 4.19	0.266	248.8 ± 33.9	233.3 ± 14.4	0.682
cox1	593.4 ± 57.8	838.4 ± 57.9	**0.016**	225.1 ± 27.2	167.1 ± 9.3	0.133	1028.3 ± 198.9	918.5 ± 53.8	0.606
ucp1	100.6 ± 7.8	113.2 ± 13.0	0.410	-	-	-	-	-	-
ucp2	0.02 ± 0.003	0.02 ± 0.003	0.871	0.05 ± 0.01	0.06 ± 0.01	0.566	0.80 ± 0.14	0.81 ± 0.13	0.980
ucp3	-	-	-	-	-	-	13.4 ± 2.20	20.8 ± 1.59	**0.026**
pparα	29.1 ± 3.73	31.3 ± 3.27	0.661	1.23 ± 0.18	0.75 ± 0.08	**0.045**	3.52 ± 0.34	2.45 ± 0.19	**0.044**
pparγ	4.14 ± 0.42	6.51 ± 0.92	**0.047**	11.98 ± 0.76	15.2 ± 3.72	0.422	0.86 ± 0.14	0.80 ± 0.06	0.699
elovl4	4.21 ± 0.92	5.56 ± 0.26	0.190	0.13 ± 0.03	0.10 ± 0.02	0.423	0.28 ± 0.02	0.38 ± 0.06	0.161
elovl5	37.7 ± 4.85	12.4 ± 3.48	**0.002**	0.64 ± 0.11	0.49 ± 0.06	0.225	0.69 ± 0.30	0.55 ± 0.06	0.635
elovl6	26.1 ± 5.05	21.2 ± 5.71	0.527	0.73 ± 0.13	0.54 ± 0.11	0.273	0.32 ± 0.04	0.35 ± 0.04	0.545
fads2	92.8 ± 17.7	88.1 ± 4.98	0.799	0.57 ± 0.18	0.39 ± 0.19	0.491	0.11 ± 0.01	0.15 ± 0.03	0.282
scd1a	12.8 ± 2.40	5.58 ± 1.13	**0.027**	1.31 ± 0.25	1.04 ± 0.32	0.512	1.91 ± 0.24	1.01 ± 0.26	**0.032**
scd1b	32.6 ± 9.2	36.5 ± 19.5	0.863	50.12 ± 10.17	50.3 ± 15.7	0.991	4.22 ± 1.02	2.30 ± 0.38	0.110
pemt	6.50 ± 0.49	6.98 ± 0.61	0.545	0.17 ± 0.05	0.15 ± 0.01	0.724	0.41 ± 0.05	0.36 ± 0.03	0.371
hl	178.9 ± 44.7	184.9 ± 12.1	0.900	-	-	-	-	-	-
lpl	49.2 ± 19.2	51.0 ± 6.80	0.932	99.42 ± 14.8	90.9 ± 27.6	0.345	5.04 ± 1.30	2.31 ± 0.29	**0.020**
hsl	2.94 ± 0.64	3.64 ± 0.31	0.348	10.54 ± 1.23	13.9 ± 2.17	0.205	1.19 ± 0.34	0.89 ± 0.04	0.358
atgl	2.38 ± 0.68	1.38 ± 0.25	0.150	1.21 ± 0.25	1.07 ± 0.18	0.647	0.73 ± 0.10	0.37 ± 0.03	**0.004**

4.3.3. Transcriptional profiling of intestine

Results regarding the intestinal expression of genes related to intermediary metabolism and intestine function and integrity are shown in **Table 4.7**. The two-way ANOVA indicated that most genes included in the intestine PCR-array were spatially regulated, with 30 genes out of 39 being differentially expressed along the intestine. A fish strain effect was also observed for 19 genes in at least one intestine segment. In the anterior intestine (**Fig. 4.2A**), the expression of *sirt4* and *cpt1a* was lower in strain 1 fish, but the opposite trend was found for the other differentially expressed genes, including markers of enterocyte mass and intracellular FA transport (*fabp2*), epithelial barrier integrity (*cdh1*, *cdh17*), mucus production and Goblet cell differentiation (*muc2*, *hes1-b*) and immunological/inflammatory status (*il1β*, *cd8b*, *lgals1*, *lgals8*, *sIgT*, *mIgT*). In the posterior intestine (**Fig. 4.2B**), the transcript abundance of different SIRTs (*sirt2*, *sirt3*, *sirt5*, *sirt7*) was significantly higher in fish of strain 1. This higher abundance occurred in combination with increased expression of markers of OXPHOS (*nd2*), cell proliferation (*pcna*) and immunity (*sIgT*, *mIgT*).

Table 4.7 Relative mRNA expression of selected markers of intermediary metabolism and intestine function and integrity in two different gilthead sea bream strains. Values are the mean ± SEM of 6-9 fish. P-values are the result of two-way analysis of variance. The asterisks indicate statistically significant differences (* P<0.05, ** P<0.01, *** P<0.001) between strains in a given intestine segment. All data values for each tissue were in reference to the expression level of *sirt1* of STRAIN 1 in the anterior intestine segment with an arbitrary assigned value of 1.

	Anterior intestine		Posterior intestine		P-value		
	STRAIN 1	STRAIN 2	STRAIN 1	STRAIN 2	Int. Section	Strain	Interaction
sirt1	1.02 ± 0.09	0.97 ± 0.08	1.30 ± 0.09	1.12 ± 0.06	**0.019**	0.174	0.432
sirt2	1.17 ± 0.06	1.01 ± 0.04	1.31 ± 0.07*	1.02 ± 0.07	0.233	**0.003**	0.301
sirt3	0.28 ± 0.02	0.28 ± 0.04	0.37 ± 0.03*	0.24 ± 0.03	0.453	**0.046**	0.061
sirt4	0.19 ± 0.01***	0.30 ± 0.03	0.14 ± 0.02	0.14 ± 0.01	**<0.001**	**<0.001**	**<0.001**
sirt5	0.87 ± 0.07	0.79 ± 0.07	1.24 ± 0.14*	0.77 ± 0.06	0.070	**0.008**	**0.047**
sirt6	0.17 ± 0.01	0.16 ± 0.01	0.21 ± 0.02	0.18 ± 0.01	**0.047**	0.148	0.635
sirt7	0.26 ± 0.01	0.26 ± 0.02	0.41 ± 0.03*	0.31 ± 0.01	**<0.001**	**0.013**	**0.018**
pgc1α	3.18 ± 0.19	3.24 ± 0.47	2.66 ± 0.17	2.23 ± 0.20	**0.007**	0.442	0.333
cpt1a	6.36 ± 0.40**	8.43 ± 0.32	4.82 ± 0.19	4.31 ± 0.45	**<0.001**	**0.037**	**0.002**
cs	33.11 ± 2.23	37.7 ± 1.65	15.3 ± 1.75	13.3 ± 0.90	**<0.001**	0.475	0.088
nd2	118.1 ± 6.11	116.7 ± 9.02	112.5 ± 11.7*	73.8 ± 5.35	**0.012**	**0.031**	**0.042**
cox1	279.5 ± 24.2	281.7 ± 22.6	463.9 ± 37.2	383.1 ± 51.6	**0.001**	0.277	0.253
ucp1	6.41 ± 0.78	4.37 ± 1.03	2.76 ± 1.19	2.95 ± 0.91	**0.024**	0.375	0.288
pcna	5.63 ± 0.39	5.23 ± 0.30	5.08 ± 0.58*	2.64 ± 0.46	**0.003**	**0.006**	**0.038**
ocln	4.73 ± 0.63	4.34 ± 0.48	9.08 ± 1.73	11.4 ± 1.33	**<0.001**	0.409	0.247
cldn15	29.0 ± 3.70	26.0 ± 2.72	54.8 ± 10.8	60.7 ± 9.81	**<0.001**	0.843	0.562
cdh1	16.2 ± 1.52*	12.5 ± 0.99	12.1 ± 2.22	15.0 ± 1.41	0.606	0.786	**0.043**
cdh17	57.5 ± 6.76*	39.4 ± 3.16	28.1 ± 9.55	27.8 ± 1.68	**0.001**	0.124	0.136
alpi	119.6 ± 13.2	106.3 ± 17.5	19.4 ± 6.24	23.4 ± 1.89	**<0.001**	0.699	0.473
fabp1	67.4 ± 5.48	75.0 ± 5.98	20.2 ± 12.8	19.5 ± 6.25	**<0.001**	0.674	0.609
fabp2	306.4 ± 49.2*	159.2 ± 32.7	83.8 ± 47.4	97.6 ± 29.0	**0.001**	0.103	0.051
fabp6	-	-	2491.5 ± 995.1	1744.5 ± 331.7	**<0.001**	0.460	0.460
muc2	39.1 ± 4.97*	26.8 ± 2.66	39.6 ± 12.8	32.3 ± 3.23	0.661	0.162	0.716
muc13	82.6 ± 7.89	95.0 ± 24.3	92.5 ± 27.4	76.6 ± 5.89	0.822	0.925	0.460
hes1-b	2.94 ± 0.34*	1.88 ± 0.17	3.11 ± 0.61	3.93 ± 0.64	**0.029**	0.802	0.061
tnfα	0.12 ± 0.02	0.08 ± 0.01	0.18 ± 0.05	0.16 ± 0.02	**0.014**	0.355	0.619
il1β	0.03 ± 0.01*	0.02 ± 0.0	0.05 ± 0.01	0.06 ± 0.01	**<0.001**	0.672	0.070
il6	0.01 ± 0.0	0.02 ± 0.0	0.02 ± 0.01	0.02 ± 0.00	0.134	0.486	0.725
il8	0.21 ± 0.03	0.22 ± 0.05	0.37 ± 0.09	0.31 ± 0.03	**0.046**	0.679	0.563
il10	0.12 ± 0.01	0.11 ± 0.01	0.36 ± 0.12	0.22 ± 0.03	**0.004**	0.192	0.242
cd4	0.26 ± 0.04	0.22 ± 0.03	0.71 ± 0.15	0.60 ± 0.05	**<0.001**	0.363	0.653
cd8a	0.54 ± 0.09	0.36 ± 0.04	0.95 ± 0.23	0.79 ± 0.13	**0.004**	0.233	0.956
cd8b	0.07 ± 0.01*	0.04 ± 0.01	0.13 ± 0.04	0.11 ± 0.03	**0.006**	0.275	0.915
lgals1	5.68 ± 0.44***	2.49 ± 0.49	12.0 ± 2.44	12.8 ± 1.64	**<0.001**	0.428	0.210
lgals8	3.97 ± 0.34**	2.81 ± 0.21	5.70 ± 0.95	6.38 ± 0.81	**<0.001**	0.721	0.168
sIgM	1.71 ± 0.67	1.60 ± 0.53	3.69 ± 1.53	3.27 ± 0.84	0.069	0.786	0.872
sIgT	0.04 ± 0.01*	0.02 ± 0.0	0.07 ± 0.03*	0.01 ± 0.0	0.335	**0.004**	0.152
mIgM	0.13 ± 0.03	0.11 ± 0.02	0.52 ± 0.13	0.37 ± 0.05	**<0.001**	0.235	0.412
mIgT	0.20 ± 0.02*	0.12 ± 0.03	0.90 ± 0.20*	0.32 ± 0.05	**<0.001**	**0.002**	**0.013**
sIgM/ mIgT	10.2 ± 3.71	12.9 ± 2.89	3.51 ± 1.93	8.32 ± 2.18	**0.050**	0.186	0.706

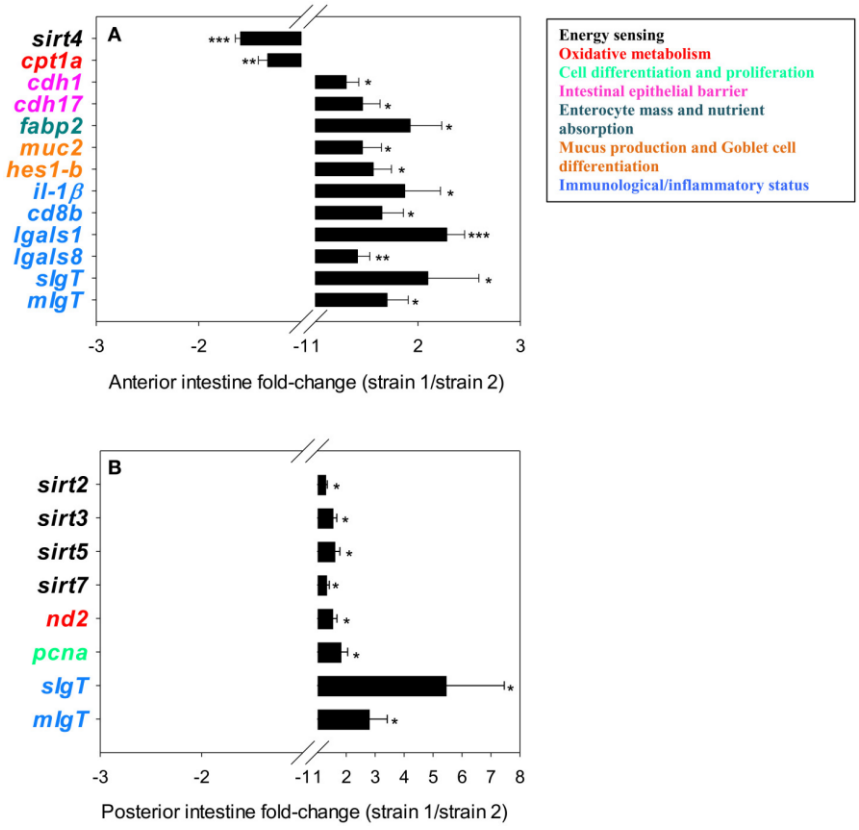


Figure 4.2 Fold-changes (strain 1/strain 2) of differentially expressed genes in the anterior **(A)** and posterior **(B)** intestinal segments. The asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01, ***P < 0.001) between strains. Values >1 indicate up-regulated genes in fish of strain 1; values <1 indicate down-regulated genes in fish strain 1.

4.4. Discussion

It is now recognized that SIRTs protect cells from ROS-induced damage across a wide range of biological systems, although the fine regulation of their expression and activity in maintaining cellular homeostasis is not fully understood (Santos et al., 2016). The ultimate mechanisms driving these processes at the cellular level were not addressed in this fish study. However, the integration of transcriptomic profiles from two genetically different gilthead sea bream strains with differences in growth performance contributes to linking the molecular signature of SIRTs to downstream markers of energy and lipid metabolism and immunological/inflammatory status, which allows for improvement in intestinal health and more efficient nutrient utilization. Certainly, in our experimental model, the highest feed intake and FE of fish from strain 1 was related to changes in blood-biochemical indicators of nutritional condition, such as total plasma protein and cholesterol levels, as previously reported for this (Sala-Rabanal et al., 2003; Peres et al., 2013) and other fish species (Congleton and Wagner, 2006; Chatzifotis et al., 2010). The same relationship held true for markers of the GH/IGF axis, and we found that plasma level of IGF-I closely reflected differences in growth potentiality between fish strains, as previously observed when comparing the growth performance of gilthead sea bream with that of the stress sensitive common dentex (Bermejo-Nogales et al., 2007). Experimental evidence regarding the gilthead sea bream also indicates that IGF-I is highly responsive to changes in growth performance due to biotic and abiotic factors, including season and developmental stage (Mingarro et al., 2002; Saera-Vila et al., 2007), ration size (Pérez-Sánchez

et al., 1995), crowding and handling stress (Rotllant et al., 2001), physical activity (Vélez et al., 2016), hypoxia (Martos-Sitcha et al., 2017) and dietary protein and lipid source (Gómez-Requeni et al., 2004; Benedito-Palos et al., 2007; Ballester-Lozano et al., 2015; Simó-Mirabet et al., 2017b). Most of these changes in circulating levels of IGF-I are inversely correlated with plasma levels of GH due to the IGF-I feedback inhibition of pituitary GH synthesis and secretion (Pérez-Sánchez, 2000). The same trend was observed herein, although it was not statistically significant. However, it is noteworthy that the trend occurred along with changes in organosomatic indexes, which suggests an enhanced flux of lipids from mesenteric adipose tissue toward the liver and perhaps skeletal muscle. This flux would be mediated, at least in part, by the lipolytic action of GH, which protects tissues from excessive lipid deposition when energy is largely available (Pérez-Sánchez, 2000).

The liver is a key metabolic organ with a remarkable capacity for regeneration based on the assumption that hepatocytes sense changes in metabolic loads and react to buffer them, counteracting, for instance, the risk of hepatic steatosis (Hohmann et al., 2014). Certainly, in our experimental model, different anti-steatosic mechanisms could be triggered with the increase in HSI and feed intake in the fast-growing fish strain. First, the FA elongase ELOVL5 is known to control hepatic triglyceride (TG) storage in higher vertebrates, and a modest increase in hepatic ELOVL5 activity in obese mice dramatically reduced hepatic TGs (Tripathy et al., 2014), whereas knockouts of this gene promoted fatty livers (Moon et al., 2009). In obese mice, the effects of this enzyme on TG metabolism are linked to the increased activity of adipocyte TG lipase without affecting FA β-oxidation (Tripathy et al., 2014). This

metabolic situation is similar to that emerging from our gene expression profiling in livers of fish from strain 1, with increased *elovl5* and *atgl* expression in combination with low expression of *cpt1a*, a key step in the mitochondrial uptake of FAs for β-oxidation. Concurrently, these fish showed a reduced expression of the lipogenic transcription factor *pparγ* (Schadinger et al., 2005). Because lipogenesis is considered the most energy-demanding process in liver tissue (Rui, 2014), the probable inhibition of this metabolic pathway was also substantiated by a reduced expression of i) master regulators of mitochondrial biogenesis and activity (*pgc1α*), ii) key enzymes (*cs*) of the TCA cycle and iii) enzyme subunits of Complexes I (*nd2*) and IV (*cox1*) of the mitochondrial respiratory chain. In previous gilthead sea bream studies, *pgc1α* has been targeted as a gene showing a high hepatic response to thermal and husbandry stressors (Bermejo-Nogales et al., 2014). Moreover, the down-regulation of *pgc1α* during the fasting inhibition of hepatic lipogenesis has been related to a marked down-regulation of nearly all the components of the OXPHOS pathway (Bermejo-Nogales et al., 2015), several FA elongases (*elovl4*, *elovl5*, *elovl6*), and FA desaturases with Δ6 (*fasd2*) and Δ9 (*scd1a* and *scd1b*) activities (Benedito-Palos et al., 2014).

In the present study, we also observed that the expression of hepatic *scd1a* was higher in fish of strain 1, which would prevent the lipotoxic effect of saturated FAs by favoring their conversion to more safely stored mono-unsaturated FAs (Li et al., 2009; Silbernagel et al., 2012). Therefore, at the liver tissue level, different adaptive mechanisms might act in concert to mitigate the detrimental metabolic effects of enhanced feed intake and tissue lipid storage. How this mechanisms are coupled to SIRT regulation remains unclear; however, the co-regulated

down-regulation of *sirt1* and *pgc1α* in the liver tissue of fish of strain 1 is noteworthy, as it could be indicative of a reduced energy demand, oxidative metabolism and oxidative stress, as widely demonstrated in rodents (Gerhart-Hines et al., 2007; Austin and St-Pierre, 2012; Santos et al., 2016). The hepatic SIRT profile in response to the enhanced growth of fish of strain 1 (low *sirt1* expression with no changes in the expression of the other *sirts*) was clearly opposite to that found during short-term fasting (no changes in *sirt1* expression in combination with an overall down-regulation of *sirt2* to *sirt6*) (Simó-Mirabet et al., 2017a). This effect might reflect the complementarity rather than the redundancy of SIRT actions when organisms are facing different types of increased energy demand (i.e., fasting vs. fast growth).

Adipose tissue plays a central role in regulating whole body lipid and energy homeostasis, and it undergoes continuous lipid trafficking to different metabolically active tissues, mostly liver and muscle (Hodson and Fielding, 2010). In our experimental model, the number of differentially expressed genes at the adipose tissue level was relatively low. However, the low MFI of fish of strain 1 suggests an increased flux of FAs from adipose tissue toward liver and muscle rather than low lipid deposition rates. Certainly, ATGL encodes for an intracellular TG lipase that is a key enzyme for both lipid storage and mobilization (Schweiger et al., 2006; Hodson and Fielding, 2010), and its increased expression at the adipose tissue level is typical of a lean phenotype in mice (Shimizu et al., 2015). In the present study, we only found a modest up-regulation of *atgl* in the adipose tissue of fish of strain 1, but this up-regulation occurred in association with the up-regulation of *sirt6*. Fat-specific *Sirt6* KO mice promoted high fat-diet induced obesity by impairing ATGL

expression inhibiting the lipolytic activity. In addition, adipose SIRT6 level is decreased in obese human patients (Kuang et al., 2017). Our fast-growing fish also exhibited other lipolytic features such as a high *pparα* expression, which prevents obesity in mice (Guerre-Millo et al., 2000) and chickens (Ji et al., 2014). Then, the lipolytic state of fish of strain 1 could be mainly orchestrated by the up-regulation of *sirt5* and *sirt6*, whereas short-term fasting up-regulated *sirt1* and down-regulated *sirt2* and *sirt7* (Simó-Mirabet et al., 2017a). Because most lipolytic factors, including PPARα (Delerive et al., 2001; Wahli and Michalik, 2012) and SIRT5-6 (Kuang et al., 2017; Wang et al., 2017) have anti-inflammatory effects, the lean phenotype is largely recognized as a healthy condition in a wide range of animals. Certainly, measures of lean fish based on gross measurements of body fat are currently used in breeding selection programs to produce more efficient fish (Kause et al., 2016); we consider that such approaches can be refined and improved by the gene expression profiling of SIRTs and other metabolic biomarkers of adipose tissue.

White skeletal muscle accounts for up to 60% of the body weight of fish (Johnston et al., 2011) and is a high energy consumer during growth. In the present study, the differences observed in gene expression pattern of white skeletal muscle between strains suggest that the fast-growing strain was metabolically more active and efficient than fish of strain 2. Notably, the muscle of fast-growing fish exhibited high expression levels of *pgc1α*, a well-recognized marker of increased mitochondrial activity and thereby aerobic oxidative capacity (Austin and St-Pierre, 2012; Wenz, 2013). This finding is in contrast to the observations of Robledo et al. (2017) in turbot indicating the up-

regulation of the glycolytic pathway in the muscle of selected fast-growing fish, which could reflect changes in energy demand as well as in swimming and feeding behavior. While the increased *pgc1α* expression in fast-growing fish did not occur along with changes in OXPHOS gene expression, it is known that PGC1α-mediated enhance of oxidative capacity may result from an increase in the number of mitochondria (Srivastava et al., 2009) or from the effects of PGC1α on the activity of the enzymes (Austin and St-Pierre, 2012) without altering OXPHOS gene expression. In accordance with the suggested increased oxidative capacity of strain 1, we observed indication of enhanced FA oxidation, such as higher expression of genes coding for enzymes (*lpl* and *atgl*) and transcription factors (*pparα*) involved in lipoprotein metabolism, tissue FA uptake and TG catabolism. The up-regulation of this lipolytic machinery is a well-known process in both gilthead sea bream and European sea bass during fasting (Benedito-Palos et al., 2014; Rimoldi et al., 2016), which supports the notion that both fasting and enhanced growth are highly demanding energy processes for skeletal muscle. Moreover, the better performance of fish of strain 1 was associated to a down-regulation of the muscle-specific uncoupling protein 3 (*ucp3*). Both in fish and other vertebrates, nutrient and energy overflow activates UCP for protecting mitochondria against oxidative stress (Bermejo-Nogales et al., 2011). Our results may indicate higher metabolic efficiency through a more coupled respiration in this strain, and agree with a higher oxidative capacity. Improved oxidative capacity in higher vertebrates (e.g., through endurance training) down-regulates UCP3 (Schrauwen-Hinderling et al., 2003). Experimental evidence in humans and rodents indicates that SIRT2 integrates changes in energy demand, lipid oxidation and redox

homeostasis by increasing FAs oxidation via activation of PGC1α (Krishnan et al., 2012) and activating ROS-scavenging enzymes (Austin and St-Pierre, 2012). Physiological studies in humans also reveal a regulatory role of SIRT2 in muscle stem cell proliferation and differentiation (Dryden et al., 2003; Wu et al., 2014; Stanton et al., 2017). Single nucleotide polymorphism of SIRT2 has also been associated with different body size traits in Quinchuan cattle (Gui et al., 2015). Importantly, we herein found that the expression of muscle *sirt2* was markedly up-regulated in the fast-growing fish strain, whereas it remains mostly unaltered during short-term fasting (Simó-Mirabet et al., 2017a). All of these findings provide further evidence of a differential regulation of cell energy sensors depending on the intensity and type of the energy-demanding stimuli.

The intestinal tract is involved not only in digestion and feed absorption but also in water and electrolyte balance, nutrient sensing and immunity (Cain and Swan, 2010). This diversity is now starting to be elucidated, and microarray gene expression profiling of European sea bass intestine revealed pronounced spatial transcriptional changes with an over-representation of nutrient transporters and mucosal chemosensors of intestinal motility and secretion in anterior-medium intestine segments, whereas immunity markers are highly over-expressed in the posterior intestine segment (Calduch-Giner et al., 2016). This expression pattern has also been inferred for gilthead sea bream in both this and previous studies (Pérez-Sánchez et al., 2015; Estensoro et al., 2016; Simó-Mirabet et al., 2017b) using intestinal PCR-arrays of selected markers of intestinal architecture and function. Moreover, the expression pattern of the fast-growing strain appears to be better suited to cope

with enhanced feed intake and growth rates, as inferred by the up-regulated expression in the anterior intestine segment of genes involved in cell adhesion and epithelial integrity (*cdh1* and *cdh17*), mucus production (*muc2*), Goblet cell differentiation (*hes1-b*) and FA transport (*fabp2*). Intriguingly, this molecular feature is concurrent with the down-regulation of *sirt4*. Unlike other SIRT family members, SIRT4 exhibits no deacetylation activity, and this novel regulator of lipid homeostasis is active in nutrient replete conditions for repressing FA oxidation while activating lipogenesis (Laurent et al., 2013). Accordingly, SIRT4 knockdown leads to increased FA oxidation in liver and muscle tissues (Nasrin et al., 2010), and low circulating levels of SIRT4 mirror attempts to increase FA oxidation in obese humans (Tarantino et al., 2014). To our knowledge, few studies have addressed the regulation of SIRT4 at the intestine level. However, the intestinal down-regulation of *sirt4* in fast-growing fish with enhanced feed intake might indicate a protective mechanism for avoiding the damaging effects of excessive accumulation of lipid droplets in enterocytes, which would be counter-regulated by the reduced expression of *cpt1a*, a key limiting enzyme of mitochondrial FA uptake and β-oxidation. Together, these results highlight the potential use of intestinal SIRT4 as a biomarker of diagnostic as well as predictor of growth potentiality and nutritional condition, particularly when used in combination with other nutritionally regulated biomarkers of blood biochemistry and tissue histopathological data scoring (Ballester-Lozano et al., 2015).

Regarding markers of cell proliferation and immunity, we also observed different gene expression patterns across the intestine of fish with differences in growth performance. A significant increase in the

expression of both secreted and membrane *IgT* was observed in fast-growing fish. *IgT* is the key mucosal immunoglobulin in teleost fish (Zhang et al., 2010), and the importance of its fine regulation upon infection has been recently described in fish with different nutritional backgrounds (Piazzon et al., 2016). There is also evidence of enhanced expression of *IgT* in the intestine of fish fed with the probiotic *Bacillus amyloliquefaciens* CET 5940 (Simó-Mirabet et al., 2017b), leading to better disease outcomes in fish challenged with the intestinal parasite *Enteromyxum leei* (Piazzon et al., 2016). Because anti-inflammatory action has been reported in rodents and humans for most SIRT isotypes, including SIRT2 (Wang et al., 2016), SIRT3 (Liu et al., 2012, 2015), SIRT5 (Tannahill, 2013; Qin et al., 2017) and SIRT7 (Vakhrusheva et al., 2008), their enhanced expression in the posterior intestine of gilthead sea bream can be considered a preventive response to keep regulated the immune system of fish with a pre-stimulatory condition, as determined by the enhanced expression of markers of OXPHOS pathway (*nd2*) and cell proliferation (*pcna*). Likewise, in the intestine of gilthead sea bream, the anti-inflammatory action of the *Bacillus* probiotic was related to an overall decrease in SIRT gene expression for *sirt1*, *sirt2*, *sirt3*, and *sirt7* (Simó-Mirabet et al., 2017b).

In summary, as shown in **Fig. 4.3**, this study illustrates the metabolic crosstalk among different tissues, identifying metabolic features that led to a lean, fast-growing and feed-efficient fish phenotype. These metabolic features are accompanied by tissue-specific mechanisms that would protect the organisms against possible lipotoxicity, oxidative stress and inflammation, processes that are related to a particular tissue-specific SIRTs expression pattern. Accordingly, in our model of fast-

growing fish, several SIRT isotypes may play anti-inflammatory roles in the intestine (*sirt2*, *3*, *5*, and *7*) and adipose tissue (*sirt5* and *6*), also favoring an increased flux of lipids from adipose tissue toward the liver and perhaps skeletal muscle. At the same time, high expression levels of *sirt2* may play a role in accelerated muscle growth in combination with an enhanced FA oxidative capacity and reduced hepatic lipogenesis, which might be sensed by reduced hepatic *sirt1* expression. As is typical in terrestrial livestock animals, lean farmed fish appear to be highly efficient and visceral fat content is currently used for indirect selection of improved feed conversion ratio in salmonids (Kause et al., 2016). The advantage of using SIRTs and SIRT-related biomarkers has been discussed to improve and refine the genetic selection programs of farmed fish to finely discriminate among low fat measurements that may arise from reduced feed intake, nutritional imbalances or any other metabolic dysfunction. However, further research is still needed to clarify whether differences in the SIRT profile between fish strains results from genetic or epigenetic sources of variation affecting the regulation of SIRTs at the transcriptional or protein level, or from the action of other genes leading to different pathways upstream of SIRTs.

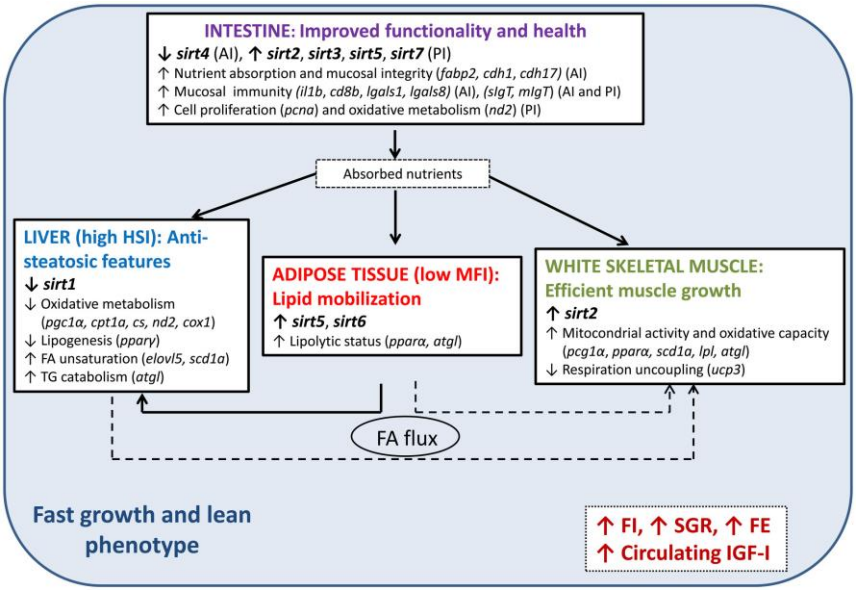


Figure 4.3 Tissue-specific expression patterns of SIRTs and the inferred metabolic features leading to a fast-growing and lean phenotype in gilthead sea bream. Arrows indicate the direction of change in metabolic processes and expression of the indicated genes. Only the genes that are the most informative about these metabolic features are shown in parentheses. HSI, hepatosomatic index; MFI, mesenteric fat index; FI, feed intake; SGR, specific growth rate; FE, feed efficiency; IGF-I, plasma insulin growth factor-1; FA, fatty acid; AI, anterior intestine; PI, posterior intestine; TG, triglyceride. For gene names, refer to **Table 4.1.**

Funding

This work was funded by the Spanish MINECO (MI2-Fish, AGL2013-48560) and from Generalitat Valenciana (PROMETEO FASE II-2014/085). Additional funding was obtained from PerformFISH (Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain) EU Project (H2020-SFS-2016-2017; 727610). This publication reflects the views only of the authors and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

Acknowledgments

The authors thank M. A. González for technical assistance with gene expression analyses, and I Vicente for technical assistance with fish husbandry and samplings. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

References

Austin, S., and St-Pierre, J. (2012). PGC1a and mitochondrial metabolism–emerging concepts and relevance in ageing and neurodegenerative disorders. J. Cell. Sci. 125, 4963–4971. doi: 10.1242/jcs.113662

Ballester-Lozano, G. F., Benedito-Palos, L., Estensoro, I., Sitjà-Bobadilla, A., Kaushik, S., and Pérez-Sánchez, J. (2015). Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets. Br. J. Nutr. 114, 713–726. doi: 10.1017/S0007114515002354

Benedito-Palos, L., Ballester-Lozano, G., and Pérez-Sánchez, J. (2014). Wide gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). Gene 547, 34–42. doi: 10.1016/j.gene.2014.05.073

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J., Sadasivam, K., and Pérez-Sánchez, J. (2007). Combined replacement of fish meal and oil in

practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis. Aquaculture 267, 199–212. doi: 10.1016/j.aquaculture.2007.01.011

Bermejo-Nogales, A., Benedito-Palos, L., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2011). Feed restriction up-regulates uncoupling protein 3 (UCP3) gene expression in heart and red muscle tissues of gilthead sea bream (*Sparus aurata* L.): new insights in substrate oxidation and energy expenditure. Comp. Biochem. Phys. A 159, 296–302. doi: 10.1016/j.cbpa.2011.03.024

Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2015). Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. PLoS ONE 10:e0122889. doi: 10.1371/journal.pone.0122889

Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G. F., Folkedal, O., Olsen, R. E., et al. (2014).Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: new insights in fish mitochondrial phenotyping. Gen. Comp. Endocrinol. 205, 305–315. doi: 10.1016/j.ygcen.2014.04.016

Bermejo-Nogales, A., Saera-Vila, A., Calduch-Giner, J. A., Navarro, J. C., Sitjà-Bobadilla, A., and Pérez-Sánchez, J. (2007). Differential metabolic and gene expression profile of juvenile common dentex (*Dentex dentex* L.) and gilthead sea bream (*Sparus aurata* L.) in relation to redox homeostasis. Aquaculture 267, 213–224. doi: 10.1016/j.aquaculture.2007.01.024

Cain, K., and Swan, C. (2010). "Barrier function and immunology," in Fish Physiology, Vol. 30, eds M. Grosell, A. P. Farrell, and C. J. Brauner (San Diego, CA: Academic Press), 111–134.

Calduch-Giner, J. A., Sitjà-Bobadilla, A., and Pérez-Sánchez, J. (2016).Gene expression profiling reveals functional specialization along the intestinal tract of a carnivorous teleostean fish (*Dicentrarchus labrax*). Front. Physiol. 7:359. doi: 10.3389/fphys.2016.00359

Cardoso, D. C., Martinati, J. C., Giachetto, P. F., Vidal, R. O., Carazzolle, M. F., Padilha, L., et al. (2014). Large-scale analysis of differential gene expression in coffee genotypes resistant and susceptible to leaf miner-toward the identification of candidate genes for marker assisted-selection. BMC Genomics 15:66. doi: 10.1186/1471-2164-15-66

Chatzifotis, S., Panagiotidou, M., Papaioannou, N., Pavlidis, M., Nengas, I., and Mylonas, C. C. (2010). Effect of dietary lipid levels on growth, feed utilization, body composition and serum metabolites of meagre

(*Argyrosomus regius*) juveniles. Aquaculture 307, 65–70. doi: 10.1016/j.aquaculture.2010.07.002

Chen, Y., Gondro, C., Quinn, K., Herd, R. M., Parnell, P. F., and Vanselow, B. (2011). Global gene expression profiling reveals genes expressed differentially in cattle with high and low residual feed intake. Anim. Genet. 42, 475–490. doi: 10.1111/j.1365-2052.2011.02182.x

Choi, M.-J., Kim, G.-D., Kim, J.-M., and Lim, H. K. (2015).Differentially-expressed genes associated with faster growth of the Pacific Abalone, *Haliotis discus hannai*. Int. J. Mol. Sci. 16, 27520–27534. doi: 10.3390/ijms161126042

Choudhary, C., Weinert, B. T., Nishida, Y., Verdin, E., and Mann, M. (2014). The growing landscape of lysine acetylation links metabolism and cell signalling. Nat. Rev. Mol. Cell Biol. 15, 536–550. doi: 10.1038/nrm3841

Congleton, J. L., and Wagner, T. (2006). Blood-chemistry indicators of nutritional status in juvenile salmonids. J. Fish Biol. 69, 473–490. doi: 10.1111/j.1095-8649.2006.01114.x

Connon, R. E., Deanovic, L. A., Fritsch, E. B., D'Abronzo, L. S., and Werner, I. (2011). Sublethal responses to ammonia exposure in the endangered delta smelt; *Hypomesus transpacificus* (Fam. Osmeridae). Aquat. Toxicol. 105, 369–377. doi: 10.1016/j.aquatox.2011.07.002

Danzmann, R. G., Kocmarek, A. L., Norman, J. D., Rexroad, C. E., and Palti, Y. (2016). Transcriptome profiling in fast versus slowgrowing rainbow trout across seasonal gradients. BMC Genomics 17:60. doi: 10.1186/s12864-016-2363-5

Delerive, P., Fruchart, J. C., and Staels, B. (2001). Peroxisome proliferator-activated receptors in inflammation control. Int. J. Endocrinol. 169, 453–459. doi: 10.1677/joe.0.1690453 Dryden, S. C.,

Nahhas, F. A., Nowak, J. E., Goustin, A. S., and Tainsky, M. A. (2003). Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol. Cell. Biol. 23, 3173–3185. doi: 10.1128/MCB.23.9.3173-3185.2003

Ekambaram, P., and Parasuraman, P. (2017). Differential expression of sirtuin 2 and adipocyte maturation restriction: an adaptation process during hypoxia in fish. Biol. Open 6, 1375–1382. doi: 10.1242/bio.027334

Estensoro, I., Ballester-Lozano, G., Benedito-Palos, L., Grammes, F., Martos-Sitcha, J. A., Mydland, L.-T., et al. (2016). Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. PLoS ONE 11:e0166564. doi: 10.1371/journal.pone.01 66564

Fernández-Díaz, C., Kopecka, J., Cañavate, J. P., Sarasquete, C., and Solé, M. (2006). Variations on development and stress defences in *Solea senegalensis* larvae fed on live and microencapsulated diets. Aquaculture 251, 573–584. doi: 10.1016/j.aquaculture.2005.06.014

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. 273, 793–798. doi: 10.1006/bbrc.2000.3000

Gerhart-Hines, Z., Rodgers, J., Bare, O., Lerin, C., Kim, S.-H., Mostoslavsky, R., et al. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1 alpha. EMBO J. 26, 1913–1923. doi: 10.1038/sj.emboj.7601633

Ghinis-Hozumi, Y., Antaramian, A., Villarroya, F., Piña, E., and Mora, O. (2013). Potential role of sirtuins in livestock production. Animal 7, 101–108. doi: 10.1017/S1751731112001115

Ghosh, H. S., McBurney, M., and Robbins, P. D. (2010). SIRT1 negatively regulates the mammalian target of rapamycin. PLoS ONE 5:e9199. doi: 10.1371/journal.pone.0009199

Gjedrem, T., and Baranski, M. (2009). Selective Breeding in Aquaculture: An Introduction. Dordrecht: Springer Science & Business Media. Gomes, P., Outeiro, T. F., and Cavadas, C. (2015). Emerging role of Sirtuin 2 in the regulation of mammalian metabolism. Trends Pharmacol. Sci. 36, 756–768. doi: 10.1016/j.tips.2015.08.001

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J. A., Médale, F., Martin, S. A. M., Houlihan, D. F., et al. (2004). Protein growth performance, amino acid utilization and somatotropic axis responsiveness to fishmeal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232, 493–510. doi: 10.1016/S0044-8486(03)00532-5

Greiss, S., and Gartner, A. (2009). Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. Mol. Cells 28, 407–415. doi: 10.1007/s10059-009-0169-x

Guan, K. L., and Xiong, Y. (2011). Regulation of intermediary metabolism by protein acetylation. Trends Biochem. Sci. 36, 108–116. doi: 10.1016/j.tibs.2010.09.003

Guerre-Millo, M., Gervois, P., Raspe, E., Madsen, L., Poulain, P., Derudas, B., et al. (2000). Peroxisome proliferator-activated receptor a activators improve insulin sensitivity and reduce adiposity. J. Biol. Chem. 275, 16638–16642. doi: 10.1074/jbc.275.22.16638

Gui, L., Hao, R., Zhang, Y., Zhao, X., and Zan, L. (2015). Haplotype distribution in the class I sirtuin genes and their associations with

ultrasound carcass traits in Qinchuan cattle (*Bos taurus*). Molec. Cell. Probes 29, 102–107. doi: 10.1016/j.mcp.2015.03.007

Hodson, L., and Fielding, B. A. (2010). Trafficking and partitioning of fatty acids: the transition from fasted to fed state. Clin. Lipidol. 5, 131–144. doi: 10.2217/clp.09.72

Hohmann, N., Weiwei, W., Dahmen, U., Dirsch, O., Deutsch, A., and Voss-Böhme, A. (2014). How does a single cell know when the liver has reached its correct size? PLoS ONE 9:e93207. doi: 10.1371/journal.pone.0093207

Houtkooper, R. H., Pirinen, E., and Auwerx, J. (2012). Sirtuins as regulators of metabolism and healthspan. Nat. Rev. Mol. Cell. Biol. 13, 225–238. doi: 10.1038/nrm3293

Janssen, K., Chavanne, H., Berentsen, P., and Komen, H. (2015). Gilthead Seabream (*Sparus aurata*)–Current status of selective breeding in Europe. Available online at: http://www.fishboost.eu/reports-on-current-status-of-selective-breedingin-europe.html (Accessed on January 2018)

Ji, B.,Middleton, J. L., Ernest, B., Saxton, A.M., Lamont, S. J., Campagna, S. R., et al. (2014).Molecular and metabolic profiles suggest that increased lipid catabolism in adipose tissue contributes to leanness in domestic chicken. Physiol. Genomics 46, 315–327. doi: 10.1152/physiolgenomics.00163.2013

Jing, H., and Lin, H. (2015). Sirtuins in epigenetic regulation. Chem. Rev. 115, 2350–2375. doi: 10.1021/cr500457h

Johnston, I. A., Bower, N. I., and Macqueen, D. J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. J. Exp. Biol. 214, 1617–1628. doi: 10.1242/jeb.038620

Kause, A., Kiessling, A., Martin, S. A. M., Houlihan, D., and Ruohonen, K. (2016). Genetic improvement of feed conversion ratio via indirect selection against lipid deposition in farmed rainbow trout (*Oncorhynchus mykiss* Walbaum). Br. J. Nutr. 116, 1656–1665. doi: 10.1017/S00071145160 03603

Krishnan, J., Danzer, C., Simka, T., Ukropec, J., Walter, K. M., Kumpf, S., et al. (2012). Dietary obesity-associated Hif1a activation in adipocytes restricts fatty acid oxidation and energy expenditure via suppression of the Sirt2-NAD+ system. Genes Dev. 26, 259–270. doi: 10.1101/gad.180406.111

Kuang, J., Zhang, Y., Liu, Q., Shen, J., Pu, S., Cheng, S., et al. (2017). Fat-specific Sirt6 ablation sensitizes mice to high-fat diet-induced obesity and insulin resistance by inhibiting lipolysis. Diabetes 66, 1159–1171. doi: 10.2337/db16-1225

Laplante, M., and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. Cell 149, 274–293. doi: 10.1016/j.cell.2012.03.017 Laurent, G., de Boer, V. C., Finley, L. W., Sweeney, M., Lu, H., Schug, T. T., et al. (2013). SIRT4 represses peroxisome proliferator-activated receptor α activity to suppress hepatic fat oxidation. Mol. Cell. Biol. 33, 4552–4561. doi: 10.1128/MCB.00087-13

Lee-Montero, I., Navarro, A., Borrell, Y., García-Celdrán, M., Martín, N., Negrín-Báez, D., et al. (2013). Development of the first standardised panel of two new microsatellite multiplex PCRs for gilthead seabream (*Sparus aurata* L.). Anim. Genet. 44, 533–546. doi: 10.1111/age.12037

Li, Z. Z., Berk, M., McIntyre, T. M., and Feldstein, A. E. (2009). Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase. J. Biol. Chem. 284, 5637–5644. doi: 10.1074/jbc.M807616200

Liu, T. F., Brown, C.M., El Gazzar, M., McPhail, L., Millet, P., Rao, A., et al. (2012). Fueling the flame: bioenergy couples metabolism and inflammation. J. Leukoc. Biol. 92, 499–507. doi: 10.1189/jlb.0212078

Liu, T. F., Vachharajani, V., Millet, P., Bharadwaj, M. S., Molina, A. J., and McCall, C. E. (2015). Sequential actions of SIRT1-RELB-SIRT3 coordinate nuclear-mitochondrial communication during immunometabolic adaptation to acute inflammation and sepsis. J. Biol. Chem. 290, 396–408. doi: 10.1074/jbc.M114.566349

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT. Methods 25, 402–408. doi: 10.1006/meth.2001.1262

Lundby, A., Lage, K., Weinert, B. T., Bekker-Jensen, D. B., Secher, A., Skovgaard, T., et al. (2012). Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. Cell Rep. 2, 419–431. doi: 10.1016/j.celrep.2012.07.006

Mailloux, R. J., and Harper, M. E. (2011). Uncoupling proteins and the control of mitochondrial reactive oxygen species production. Free Radic. Biol. Med. 51, 1106–1115. doi: 10.1016/j.freeradbiomed.2011.06.022

Martínez-Álvarez, R. M., Morales, A. E., and Sanz, A. (2005). Antioxidant defenses in fish: biotic and abiotic factors. Rev. Fish Biol. Fish. 15, 75–88. doi: 10.1007/s11160-005-7846-4

Martínez-Barberá, J. P., Pendón, C., Martí-Palanca, H., Calduch-Giner, J. A., Rodríguez, R. B., Valdivia, M.M., et al. (1995). The use of recombinant gilthead sea bream (*Sparus aurata*) growth hormone for radioiodination

and standard preparation in radioimmunoassay. Comp. Biochem. Physiol. A Physiol. 110, 335–340. doi: 10.1016/0300-9629(94)00178-V

Martin, S. A. M., and Król, E. (2017). Nutrigenomics and immune function in fish: new insights from omics technologies. Dev. Comp. Immunol. 75, 86–98. doi: 10.1016/j.dci.2017.02.024

Martos-Sitcha, J. A., Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2017). Gene expression profiling of whole blood cells supports a more efficient mitochondrial respiration in hypoxia-challenged gilthead sea bream (*Sparus aurata*). Front. Zool. 14:34. doi: 10.1186/s12983-017-0220-2

Masri, S. (2015). Sirtuin-dependent clock control: new advances in metabolism, aging and cancer. Curr. Opin. Clin. Nutr. Metab. Care 18, 521–527. doi: 10.1097/MCO.00000000000 00219

McAndrew, B., and Napier, J. (2011). Application of genetics and genomics to aquaculture development: current and future directions. J. Agric. Sci. 149, 143–151. doi: 10.1017/S0021859610001152

Mingarro, M., de Celis, S. V. R., Astola, A., Pendón, C., Valdivia, M. M., and Pérez-Sánchez, J. (2002). Endocrine mediators of seasonal growth in gilthead sea bream (*Sparus aurata*): the growth hormone and somatolactin paradigm. Gen. Comp. Endocrinol. 128, 102–111. doi: 10.1016/S0016-6480(02)00042-4

Moon, Y. A., Hammer, R. E., and Horton, J. D. (2009). Deletion of ELOVL5 leads to fatty liver through activation of SREBP-1c in mice. J. Lipid Res. 50, 412–423. doi: 10.1194/jlr.M800383-JLR200

Nabben, M., and Hoeks, J. (2008). Mitochondrial uncoupling protein 3 and its role in cardiac-and skeletal muscle metabolism. Physiol. Behav. 94, 259–269. doi: 10.1016/j.physbeh.2007.11.039

Nasrin, N., Wu, X., Fortier, E., Feng, Y., Bare, O. C., Chen, S., et al. (2010). SIRT4 regulates fatty acid oxidation and mitochondrial gene expression in liver and muscle cells. J. Biol. Chem. 285, 31995–32002. doi: 10.1074/jbc.M110.124164

Nei, M. (1973). Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. U.S.A. 70, 3321–3323. doi: 10.1073/pnas.70.12.3321

Olesen, I., Bentsen, H. B., Phillips, M., and Ponzoni, R. W. (2015). Can the global adoption of genetically improved farmed fish increase beyond 10%, and how? J. Mar. Sci. Eng. 3, 240–266. doi: 10.3390/jmse3020240

Otero-Rodiño, C., Librán-Pérez, M., Velasco, C., Álvarez-Otero, R., López-Patiño, M. A., Míguez, J. M., et al. (2016). Glucosensing in liver and Brockmann bodies of rainbow trout through glucokinase-independent

mechanisms. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 199, 29–42. doi: 10.1016/j.cbpb.2015.09.008

Pacitti, D., Wang, T., Martin, S. A. M., Sweetman, J., and Secombes, C. J. (2014). Insights into the fish thioredoxin system: expression profile of thioredoxin and thioredoxin reductase in rainbow trout (*Oncorhynchus mykiss*) during infection and in vitro stimulation. Dev. Comp. Immunol. 42, 261–277. doi: 10.1016/j.dci.2013.09.013

Peres, H., Santos, S., and Oliva-Teles, A. (2013). Selected plasma biochemistry parameters in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630–636. doi: 10.1111/j.1439-0426.2012.02049.x

Pérez-Sánchez, J. (2000). The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): a short review. Fish Physiol. Biochem. 22, 135–144. doi: 10.1023/A:1007816015345

Pérez-Sánchez, J., Benedito-Palos, L., Estensoro, I., Petropoulos, Y., Calduch-Giner, J. A., Browdy, C. L., et al. (2015). Effects of dietary NEXT ENHANCER 150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). Fish Shellfish Immunol. 44, 117–128. doi: 10.1016/j.fsi.2015.01.039

Pérez-Sánchez, J., Marti-Palanca, H., and Kaushik, S. J. (1995). Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (*Sparus aurata*). J. Nutr. 125, 546–552.

Piazzon, M. C., Calduch-Giner, J. A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto, M., et al. (2017). Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. Microbiome 5:164. doi: 10.1186/s40168-017-0390-3

Piazzon, M. C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J. A., Gomez-Casado, E., et al. (2016). Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a Perciform fish. Front. Immunol. 7:637. doi: 10.3389/fimmu.2016.00637

Qin, K., Han, C., Zhang, H., Li, T., Li, N., and Cao, X. (2017). NAD+ dependent deacetylase Sirtuin 5 rescues the innate inflammatory response of endotoxin tolerant macrophages by promoting acetylation of p65. J. Autoimmun. 81, 120–129. doi: 10.1016/j.jaut.2017.04.006

Rajan, K. E., Thangaleela, S., and Balasundaram, C. (2015). Spatial learning associated with stimulus response in goldfish *Carassius auratus*:

relationship to activation of CREB signalling. Fish. Physiol. Biochem. 41, 685–694. doi: 10.1007/s10695-015-0038-9

Raymond, M., and Rousset, F. (1995). Genepop (Version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86, 248–249. doi: 10.1093/oxfordjournals.jhered.a111573

Rimoldi, S., Benedito-Palos, L., Terova, G., and Pérez-Sánchez, J. (2016). Wide-targeted gene expression infers tissue-specific molecular signatures of lipid metabolism in fed and fasted fish. Rev. Fish Biol. Fish. 26, 93–108. doi: 10.1007/s11160-015-9408-8

Rise, M. L., Hall, J. R., Nash, G. W., Xue, X., Booman, M., Katan, T., et al. (2015). Transcriptome profiling reveals that feeding wild zooplankton to larval Atlantic cod (*Gadus morhua*) influences suites of genes involved in oxidation-reduction, mitosis, and selenium homeostasis. BMC Genomics 16:1016. doi: 10.1186/s12864-015-2120-1

Robledo, D., Rubiolo, J. A., Cabaleiro, S., Martínez, P., and Bouza, C. (2017). Differential gene expression and SNP association between fastand slow-growing turbot (*Scophthalmus maximus*). Sci. Rep. 7:12105. doi: 10.1038/s41598-017-12459-4

Rotllant, J., Balm, P. H.M., Pérez-Sánchez, J.,Wendelaar-Bonga, S. E., and Tort, L. (2001). Pituitary and interrenal function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement stress. Gen. Comp. Endocrinol. 121, 333–342. doi: 10.1006/gcen.2001.7604

Rousset, F. (2008). Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Resour. 8, 103–106. doi: 10.1111/j.1471-8286.2007.01931.x

Rui, L. (2014). Energy metabolism in the liver. Compr. Physiol. 4, 177–197. doi: 10.1002/cphy.c130024

Saera-Vila, A., Calduch-Giner, and, J., Pérez-Sánchez. (2007). Co-expression of IGFs and GH receptors (GHRs) in gilthead sea bream (*Sparus aurata* L.): sequence analysis of the GHR-flanking region. J. Endocrinol. 194, 361–372. doi: 10.1677/JOE-06-0229

Sala-Rabanal, M., Sánchez, J., Ibarz, A., Fernández-Borràs, J., Blasco, J., and Gallardo, M. A. (2003). Effects of low temperatures and fasting on hematology and plasma composition of gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 29, 105–115. doi: 10.1023/B:FISH.0000035904.1 6686.b6

Santos, L., Escande, C., and Denicola, A. (2016). Potential modulation of sirtuins by oxidative stress. Oxid. Med. Cell. Longev. 2016:9831825. doi: 10.1155/2016/9831825

Saravanan, S., Schrama, J.W., Figueiredo-Silva, A. C., Kaushik, S. J., Verreth, J. A., and Geurden, I. (2012). Constraints on energy intake in fish: the link between diet composition, energy metabolism, and energy intake in rainbow trout. PLoS ONE 7:e34743. doi: 10.1371/journal.pone.0034743

Schadinger, S. E., Bucher, N. L., Schreiber, B. M., and Farmer, S. R. (2005). PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. Am. J. Physiol. Endocrinol. Metab. 288, E1195–E1205. doi: 10.1152/ajpendo.00513.2004

Schmeisser, K., Mansfeld, J., Kuhlow, D., Weimer, S., Priebe, S., Heiland, I., et al. (2013). Role of sirtuins in lifespan regulation is linked to methylation of nicotinamide. Nat. Chem. Biol. 9, 693–700. doi: 10.1038/nchembio.1352

Schrauwen-Hinderling, V. B., Schrauwen, P., Hesselink, M. K. C., Van Engelshoven, J. M. A., Nicolay, K., Saris, W. H. M., et al. (2003). The increase in intramyocellular lipid content is a very early response to training. J. Clin. Endocr. Metab. 88, 1610–1616. doi: 10.1210/jc.2002-021464

Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., et al. (2006). Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. J. Biol. Chem. 281, 40236–40241. doi: 10.1074/jbc.M608048200

Schwer, B., and Verdin, E. (2008). Conserved metabolic regulatory functions of sirtuins. Cell Metab. 7, 104–112. doi: 10.1016/j.cmet.2007.11.006

Sharples, A. P., Hughesb, D. C., Deane, C. S., Saini, A., Selman, C., and Stewart, C. E. (2015). Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging cell 14, 511–523. doi: 10.1111/acel.12342

Shimizu, M., Swanson, P., Fukada, H., Hara, A., and Dickhoff, W. W. (2000). Comparison of extraction methods and assay validation for salmon insulinlike growth factor-I using commercially available components. Gen. Comp. Endocrinol. 119, 26–36. doi: 10.1006/gcen.2000.7498

Shimizu, N., Maruyama, T., Yoshikawa, N., Matsumiya, R., Ma, Y., Ito, N., et al. (2015). A muscle-liver-fat signalling axis is essential for central control of adaptive adipose remodeling. Nat. Commun. 6:6693. doi: 10.1038/ncomms7693

Silbernagel, G., Kovarova, M., Cegan, A., Machann, J., Schick, F., Lehmann, R., et al. (2012). High hepatic SCD1 activity is associated with low liver fat content in healthy subjects under a lipogenic diet. J. Clin. Endocrinol. Metab. 97, E2288–E2292. doi: 10.1210/jc.2012-2152

Simó-Mirabet, P., Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2017a). Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 187, 153–163. doi: 10.1007/s00360-016-1014-0

Simó-Mirabet, P., Piazzon, M. C., Calduch-Giner, J. A., Ortiz, Á., Puyalto, M., Sitjà-Bobadilla, A., et al. (2017b). Sodium salt medium-chain fatty acids and Bacillus-based probiotic strategies to improve growth and intestinal health of gilthead sea bream (*Sparus aurata*). PeerJ 5:e4001. doi: 10.7717/peerj.4001

Srivastava, S., Diaz, F., Iommarini, L., Aure, K., Lombes, A., and Moraes, C. T. (2009). PGC-1a/b induced expression partially compensates for respiratory chain defects in cells from patients with mitochondrial disorders. Hum. Mol. Genet. 18, 1805–1812. doi: 10.1093/hmg/ddp093

Stanton, D. A., Alway, S. E., and Mohamed, J. S. (2017). The role of Sirtuin 2 in the regulation of myogenesis. FASEB J. 31(1 Suppl.), 877.13–877.13. doi: 10.1096/fasebj.31.1_supplement.877.13

Tannahill, G. M. (2013). Succinate is an inflammatory signal that induces IL-1 through HIF-1 a. Nature 496, 238–242. doi: 10.1038/nature11986

Tarantino, G., Finelli, C., Scopacasa, F., Pasanisi, F., Contaldo, F., Capone, D., et al. (2014). Circulating levels of sirtuin 4, a potential marker of oxidative metabolism, related to coronary artery disease in obese patients suffering from NAFLD, with normal or slightly increased liver enzymes. Oxid. Med. Cell. Longev. 2014:920676. doi: 10.1155/2014/920676

Teigen, L. E., Orczewska, J. I., McLaughlin, J., and O'Brien, K. M. (2015). Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in threespine stickleback. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 188, 139–147. doi: 10.1016/j.cbpa.2015.06.028

Tripathy, S., Lytle, K. A., Stevens, R. D., Bain, J. R., Newgard, C. B., Greenberg, A. S., et al. (2014). Fatty acid elongase-5 (Elovl5) regulates hepatic triglyceride catabolism in obese C57BL/6J mice. J. Lipid Res. 55, 1448–1464. doi: 10.1194/jlr.M050062

Vakhrusheva, O., Smolka, C., Gajawada, P., Kostin, S., Boettger, T., Kubin, T., et al. (2008). Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. Circ. Res. 102, 703–710. doi: 10.1161/CIRCRESAHA.107.164558

de Celis,L S. V., Gómez-Requeni, P., and Pérez-Sánchez, J. (2004). Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and

insulin-like growth factor-I in European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 139, 266–277. doi: 10.1016/j.ygcen.2004.09.017

Vélez, E. J., Azizi, S., Millán-Cubillo, A., Fernández-Borràs, J., Blasco, J., Chan, S. J., et al. (2016). Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*). Am. J. Physiol. Regul. Integr.Comp. Physiol. 310, R313–R322. doi: 10.1152/ajpregu.00230.2015

Verdin, E., Hirschey, M. D., Finley, L. W., and Haigis, M. C. (2010). Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. Trends Biochem. Sci. 35, 669–675. doi: 10.1016/j.tibs.2010.07.003

Wahli, W., and Michalik, L. (2012). PPARs at the crossroads of lipid signaling and inflammation. Trends Endocrinol. Metab. 23, 351–363. doi: 10.1016/j.tem.2012.05.001

Wang, F., Wang, K., Xu, W., Zhao, S., and Ye, D., Wang, et al. (2017). SIRT5 desuccinylates and activates pyruvate kinase M2 to block macrophage IL-1b production and to prevent DSS-induced colitis in mice. Cell Rep. 19, 2331–2344. doi: 10.1016/j.celrep.2017.05.065

Wang, X., Buechler, N. L., Martin, A., Wells, J., Yoza, B., McCall, C. E., et al. (2016). Sirtuin-2 regulates sepsis inflammation in ob/ob mice. PLoS ONE 11:e0160431. doi: 10.1371/journal.pone.0160431

Wellen, K. E., and Thompson, C. B. (2010). Cellular metabolic stress: considering how cells respond to nutrient excess. Mol. Cell 40, 323–332. doi: 10.1016/j.molcel.2010.10.004

Wenz, T. (2013). Regulation of mitochondrial biogenesis and PGC-1a under cellular stress. Mitochondrion 13, 134–142. doi: 10.1016/j.mito.2013. 01.006

Wu, G., Song, C., Lu, H., Jia, L., Yang, G., Shi, X., et al. (2014). Sirt2 induces C2C12 myoblasts proliferation by activation of the ERK1/2 pathway. Acta Biochim. Biophys. Sin. 46, 342–345. doi: 10.1093/abbs/ gmt151

Yúfera, M., Perera, E., Mata-Sotres, J. A., Calduch-Giner, J., Martínez-Rodríguez, G., and Pérez-Sánchez, J. (2017). The circadian transcriptome of marine fish (*Sparus aurata*) larvae reveals highly synchronized biological processes at the whole organism level. Sci. Rep. 7:12943. doi: 10.1038/s41598-017-13514-w

Zhang, Y. A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., et al. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat. Immunol. 11, 827–835. doi: 10.1038/ni.1913

Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., et al. (2010). Regulation of cellular metabolism by protein lysine acetylation. Science 327, 1000–1004. doi: 10.1126/science.1179689

CAPÍTULO 5- Sodium salt medium-chain fatty acids and *Bacillus*-based probiotic strategies to improve growth and intestinal health of gilthead sea bream (*Sparus aurata*)

Paula Simó-Mirabet[1], M. Carla Piazzon[2], Josep Alvar Calduch-Giner[1], Álvaro Ortiz[3], Mónica Puyalto[4], Ariadna Sitjà-Bobadilla[2], Jaume Pérez-Sánchez[1]

[1]Nutrigenomics and Fish Growth Endocrinology Group, Institute of Aquaculture Torre de la Sal, CSIC, Castellón, Spain.

[2]Fish Pathology Group, Institute of Aquaculture Torre de la Sal, CSIC, Castellón, Spain.

[3]Evonik Nutrition and Care GmbH, Hanau-Wolfgang, Germany.

[4]NOREL S.A., Madrid, Spain.

PeerJ (2017) 5:e4001

Abstract

Background. The increased demand for fish protein has led to the intensification of aquaculture practices which are hampered by nutritional and health factors affecting growth performance. To solve these problems, antibiotics have been used for many years in the prevention, control and treatment against disease as well as growth promoters to improve animal performance. Nowadays, the use of antibiotics in the European Union and other countries has been completely or partially banned as a result of the existence of antibiotic cross-resistance. Therefore, a number of alternatives, including enzymes, prebiotics, probiotics, phytonutrients and organic acids used alone or in combination have been proposed for the improvement of immunological state, growth performance and production in livestock animals. The aim of the present study was to evaluate two commercially available feed additives, one based on medium-chain fatty acids (MCFAs) from coconut oil and another with a *Bacillus*-based probiotic, in gilthead sea bream (GSB, *Sparus aurata*), a marine farmed fish of high value in the Mediterranean aquaculture.

Methods. The potential benefits of adding two commercial feed additives on fish growth performance and intestinal health were assessed in a 100-days feeding trial. The experimental diets (D2 and D3) were prepared by supplementing a basal diet (D1) with MCFAs in the form of a sodium salt of coconut fatty acid distillate (DICOSAN®; Norel, Madrid, Spain), rich on C-12, added at 0.3% (D2) or with the probiotic *Bacillus amyloliquefaciens* CECT 5940, added at 0.1% (D3). The study integrated data on growth performance, blood biochemistry, histology

and intestinal gene expression patterns of selected markers of intestinal function and architecture.

Results. MCFAs in the form of a coconut oil increased feed intake, growth rates and the surface of nutrient absorption, promoting the anabolic action of the somatotropic axis. The probiotic (D3) induced anti-inflammatory and anti-oxidant effects with changes in circulating cortisol, immunoglobulin M, leukocyte respiratory burst, and mucosal expression levels of cytokines, lymphocyte markers and immunoglobulin T.

Discussion. MCFA supplementation showed positive effects on GSB growth and intestinal architecture acting mainly in the anterior intestine, where absorption takes place. The probiotic *B. amyloliquefaciens* CECT 5940 exhibited key effects in the regulation of the immune status inducing anti-inflammatory and anti-oxidant effects which can be potentially advantageous upon infection or exposure to other stressors. The potential effects of these feed additives in GSB are very promising to improve health and disease resistance in aquaculture.

Keywords: Medium-chain fatty acid; Teleost; Probiotic; Intestinal health; *Bacillus amyloliquefaciens*; DICOSAN.

5.1. Introduction

Aquaculture is an expanding industry with 73.8 million tonnes produced globally in 2014 (FAO, 2016). The increased demand for fish protein has led to the intensification of aquaculture practices increasing the risk of infectious diseases (Segner et al., 2012). In this regard, the growth of the aquaculture industry is hampered by nutritional and health factors which affect growth performance. To sort out these problems, antibiotics have been used for many years in the prevention, control and treatment against disease as well as growth promoters to improve animal performance (Done, Venkatesan & Halden, 2015). However, due to the occurrence of antibiotic cross-resistance, the European Union and other countries have completely or partially banned their use for growth and disease prevention purposes (January 2006; Regulation 1831/2003/EC). Hence, a number of alternatives, including enzymes, prebiotics, probiotics, phytonutrients and organic acids used alone or in combination have been proposed for the improvement of host immunity or animal growth and production (Seal et al., 2013).

The use of organic acids as feed additives has been in progress for over four decades, and several studies have proved their antimicrobial and growth-promoting action in swine and poultry (Suiryanrayna & Ramana, 2015; Khan & Iqbal, 2016). Currently, there is also commercial and research interests in the use of organic acids in aquafeeds to improve growth performance, nutrient utilization and disease resistance in commercially important farmed fish. Many studies have reported that some short-chain fatty acids (SCFAs) and their salts or mixtures can significantly enhance growth performance and health

status of fish (Ng & Koh, 2016) or be beneficial in reverting detrimental effects produced by low inclusion levels of fish meal and fish oil (Benedito-Palos et al., 2016; Estensoro et al., 2016). Another type of organic acids often used as nutritional supplements are medium-chain fatty acids (MCFAs), which are highly abundant in oil distillates from coconut oil, palm kernels and milk. MCFAs have been suggested to have a role in immunological regulation (Wang et al., 2006), antibacterial activity (Bergsson et al., 2001; Skřivanová et al., 2009) and can also improve gut development, enhancing performance in piglets (Hanczakowska et al., 2016). The physiological consequences of supplementing fish aquafeeds with medium-chain triglycerides are poorly studied. However, a recent study in GSB demonstrated the up-regulation of different immune-related genes in skin of fish feed with a palm oil-supplemented diet, which could be considered a good mechanism to enhance humoral immunity in fish skin (Cerezuela et al., 2016). In the same way, stimulation of several immune parameters has been detected in European sea bass (*Dicentrarchus labrax*) fed with a diet enriched with extracts obtained from the date palm fruits (Guardiola et al., 2016).

Another potential strategy to replace antibiotics is the use of probiotics. Although the first application of probiotics in aquaculture feeds was more than three decades ago, their use has recently regained considerable attention due to their ability to act as growth promoters and their positive effects in disease control, nutrient digestion, reproduction, stress tolerance as well as in the improvement of water quality (Zorriehzahra et al., 2016). Among probiotics, the genus *Bacillus* has been used in humans and animals due to their ability to produce antimicrobial substances and their sporulation capacity, conferring them a double

advantage in terms of survival in different habitats (Abriouel et al., 2011). Specifically, the spores of *Bacillus amyloliquefaciens* have been used as probiotics in poultry feeds, because they reduce the effect of pathogenic bacteria such as *Clostridium perfringens*, *Escherichia coli* and *Yersinia* and thus decrease poultry mortality (Diaz, 2007; Commission Regulation (EC) No. 1292/2008). Among the few studies addressing the use of *B. amyloliquefaciens* in aquafeeds, improved growth performance (Ridha & Azad, 2012) and enhanced immune status and disease resistance (Selim & Reda, 2015) have been described in Nile tilapia (*Oreochromis niloticus*). Similar results have also been reported for European eel (*Anguilla anguilla*) (Lu et al., 2011), catfish (*Ictalurus punctatus*) (Ran et al., 2012) and Indian major carp (*Catla catla*) (Das et al., 2013).

The aim of the present study was to evaluate the potential benefits of two commercially available feed additives, one based on MCFAs from coconut oil and another a *Bacillus*-based probiotic, in GSB, a marine farmed fish of high value for the Mediterranean aquaculture. To pursue this issue, we assessed their effects on growth performance and intestinal health in a 14-weeks feeding trial. To our knowledge, this is the first report addressing in the same study the effects of MCFAs with great growth-promoting action, and a *Bacillus*-based probiotic with promising effects on immune maintenance and disease resistance in a farmed fish.

5.2. Material and methods

5.2.1. Experimental diets

The basal control diet (D1) and two different experimental diets (D2, D3) were formulated and delivered by SPAROS LDA (Portugal) (**Table**

5.1). D1 diet (45% protein and 18% fat) contained fish meal, blood proteins and poultry meal as the main source of dietary proteins. Plant ingredients as a blend of soy protein, wheat gluten, corn gluten, soybean meal, rapeseed meal, wheat meal and pea starch were included at 42%. Fish oil (SAVINOR, Portugal) was added at 13.9%. The experimental diets (D2 and D3) were prepared by supplementing D1 diet with two different commercial preparations: (i) medium-chain fatty acids in the form of a sodium salt of coconut fatty acid distillate (DICOSAN®, Norel) rich on C-12 was added as a powder to D2 diet at 0.3%; (ii) the probiotic *Bacillus amyloliquefaciens* CECT 5940 was added "top coated" to D3 diet, being mixed with oil and sprayed at the appropriate rate to match the final dose at 0.1%. All diets were supplemented with antioxidants, a mineral-vitamin mix and DL-methionine.

Table 5.1 Experimental diet composition. Ingredients of basal/control diet (D1). Experimental diets were formulated on the same composition of D1 with 0.3% DICOSAN for diet D2 or 0.1% probiotic (*Bacillus amyloliquefaciens*) for diet D3.

Ingredient (%)	D1
Fishmeal LT Diamante	7.5
Fishmeal 60	20
CPSP 90	2.5
Porcine blood meal	5
Poultry meal 65	7
Soy protein concentrate	5
Wheat Gluten	6
Corn gluten	5
Soybean meal 48	9
Rapeseed meal	5
Wheat meal	7
Pea starch	5
Fish oil - SAVINOR	13.9
Vit & Min Premix	1
Binder (Kieselghur)	0.5
Antioxidant powder (Paramega)	0.2
Sodium propionate	0.1
DL-Methionine	0.3

5.2.2. Fish, feeding trial and sampling collection

Juvenile GSB of Atlantic origin (Tina Menor, Santander, Spain) of 4-5 g initial body weight (May 2015) were acclimatized for more than two months to the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC, Spain). During this initial period, fish were fed with standard diets (Inicio Forte 824 1.9 mm; BioMar, Dueñas, Spain). Then, fish of 29.6 ± 0.32 g initial mean body weight (±SEM) were distributed in 500 l tanks in triplicate groups of 50 fish each. Each experimental diet was offered to visual satiety (one time per day) six days per week from June to August and five days per week from September to October in a 100-days feeding trial. Feed intake was recorded weekly and fish were counted and group-weighed every 4-6 weeks. Oxygen content of outlet water remained higher than 75% saturation, and day-length and water temperature followed the natural changes at IATS latitude (40º5'N; 0º10'E), varying from 24 ºC in later June to 22 ºC in early October with a maximum peak of 28 ºC at the end of July.

At the end of the trial, overnight fasted fish (6 fish per tank, 18 per experimental condition) were randomly sampled and anaesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 0.1 g/l; Sigma, St. Louis, MO, USA) for blood and tissue collection. Blood was quickly drawn from caudal vessels with heparinized syringes. One aliquot was directly used for measurements of leukocyte respiratory burst and hematocrit/hemoglobin determinations. The remaining blood was centrifuged at 3,000 x g for 20 min at 4 ºC, and plasma samples were frozen and stored at -80 ºC until biochemical and immunological assays were done. Prior to tissue collection, anaesthetized fish were killed by

cervical section, and liver, viscera and mesenteric fat were weighed. Intestine was taken for weight and length measurements. Representative portions of anterior and posterior intestinal segments were rapidly taken, frozen in liquid nitrogen and stored at -80 ºC until RNA isolation. Additional samples of liver and intestinal segments were taken for histological processing.

All procedures were approved by the Ethics and Animal Welfare Committee of the Institute of Aquaculture Torre de la Sal according to national (Royal Decree RD53/2013) and EU legislation (2010/63/EU) on the handling of experimental animals.

5.2.3. Hematological, biochemical and immunological blood analyses

Hemoglobin concentration was determined with a HemoCue B-Hemoglobin Analyser® (Aktiebolaget Leo Diagnostics, Helsigborg, Sweden), which uses a modified azide methemoglobin reaction for Hb quantification. The hematocrit (Hc) was measured after centrifugation of blood in heparinized capillary tubes at 13,000 x g for 10 min. Plasma glucose was determined by the glucose oxidase method (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Plasma triglycerides (TGs) were determined using lipase/glycerol kinase/glycerol-3-phosphate oxidase reagent (ThermoFisher Scientific, Waltham, MA, USA). Total cholesterol was measured using cholesterol esterase/cholesterol dehydrogenase reagent (ThermoFisher Scientific, Waltham, MA, USA). Total plasma proteins were measured using BioRad protein reagent (Hercules, CA, USA) with bovine serum albumin as standard. Total antioxidant capacity was measured as Trolox activity

using a microplate assay kit (709,001, Cayman Chemical, Ann Arbor, MI, USA).

Induction of the respiratory burst (RB) activity of blood leukocytes was measured directly from heparinized blood as previously reported (Saera-Vila et al., 2009). Briefly, 4 µl of heparinized blood were diluted with 96 µl Hank's balanced salt solution pH 7.4 with calcium and magnesium (HBSS++; Gibco, ThermoFisher Scientific, Waltham, MA, USA) in duplicates in white 96 well plates (NUNC). One hundred µl of a luminol (Fluka, Sigma, St. Louis, MO, USA) solution 2 mM in borate buffer (0.2 M pH 9.2) with 2 µg/ml phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA) were added to each well and the luminescence was measured immediately every 3 min for 1 h at 25 ºC in a microplate luminescence reader (Ultra Evolution; Tecan, Männedorf, Zürich, Switzerland). The integral luminescence in relative light units (RLU) was calculated using HBSS++ as a blank.

Plasma lysozyme was measured by a turbidimetric assay as previously described (Sitjà-Bobadilla et al., 2005). Briefly, 5 µl of serum were diluted with 5 µl of 50 mM sodium phosphate buffer pH 6.2 (PB) and incubated with 200 µl of a 0.3 mg/ml *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) suspension in PB. The reduction of the absorbance at 450 nm was determined in a microplate reader (Ultra Evolution; Tecan, Männedorf, Zürich, Switzerland) after 0.5 and 4.5 min and a unit of lysozyme activity was calculated as the amount of enzyme that caused a decrease in absorbance of 0.001 per min.

The alternative complement pathway (ACP) activity was determined using a modification of the method described in Sitjà-Bobadilla et al., (2005) using sheep red blood cells (SRBC; Durviz,

Valencia, Spain) as a target. Briefly, 25 µl of a suspension of 2.85 x 10^8 SRBC/ml in 10 mM EGTA, 10 mM Mg^{2+} HBSS (without calcium and magnesium; Gibco, ThermoFisher Scientific, Waltham, MA, USA) were incubated with 100 µl of different dilutions of serum (1:5, 1:10, 1:20, 1:40, 1:80 and 1:100) in duplicates for 1 h at 20 ºC with constant shaking. After spinning down the remaining SRBC, 75 µl of supernatants were transferred to a new 96 well plate and the absorbance at 415 nm was measured. Finally, the dilution of serum that caused 50% hemolysis (CH50) was calculated.

Serum immunoglobulin M (IgM) was analyzed using an ELISA assay. ELISA plates were coated with 50 µl of a 1:6,000 dilution of serum in carbonate/bicarbonate buffer 0.1 M pH 9.6, incubated overnight at 4 ºC and, after washing, blocked with 200 µl Tris 20 mM 0.5 M NaCl pH 7.4 (TBS) 5% non-fat dry milk (BioRad, Hercules, CA, USA) for 1 h at 37 ºC. Then, plates were washed, incubated with 50 µl of rabbit polyclonal anti-GSB IgM (Palenzuela, Sitjà-Bobadilla & Álvarez Pellitero, 1996) diluted 1:20,000 in TBS, 0.05% Tween 20 and 3% non-fat dry milk (3% TTBS) for 1 h at 37 ºC, washed again and incubated for another hour with 50 µl of a 1:1,000 dilution of goat anti-rabbit-horseradish peroxidase (HRP) (Sigma, St. Louis, MO, USA) in 3% TTBS. After careful washing, the reaction was developed using the TMB substrate kit (BioRad, Hercules, CA, USA) following the manufacturer's instructions, the reaction was stopped after 20 min with 1 N H_2SO_4 and the absorbance was measured at 450 nm. Each serum was tested in triplicates and a blank with no serum was included as a background control.

Plasma growth hormone (GH) levels were determined by a homologous GSB radioimmunoassay (RIA) (Martínez-Barberá et al., 1995). The sensitivity and midrange (ED50) of the assay were 0.15 and 2.5-3 ng/ml, respectively. Plasma insulin-like growth factors (IGFs) were extracted by acid-ethanol cryoprecipitation and the concentration of IGF-I was measured using a generic fish IGF-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The sensitivity and midrange of the assay were 0.05 and 0.7-0.8 ng/ml respectively. Cortisol levels were analyzed using an EIA kit (kit RE52061, IBL International GmbH, Hamburg, Germany) following the manufacturer's instructions. The limit of detection was 50 pg/ml with an assay midrange of 700 pg/ml.

5.2.4. Histological analyses

For histological examination, pieces of liver, anterior (immediately after the pyloric caeca) and posterior (immediately before the rectum) intestinal segments were fixed in 10% buffered formalin, embedded in paraffin, 4 µm-sectioned and stained with hematoxylin-eosin (H&E), Giemsa and periodic acid-Schiff (PAS) following standard procedures. For each dietary group, a total of 9 fish were examined (three fish per tank replicate).

5.2.5. Immunohistochemical analyses

In order to further characterize the observations performed in the histological analyses, 4 µm thick paraffin sections of anterior and posterior intestine samples were collected on Super-Frost-plus microscope slides (Menzel-Gläser, Braunschweig, Germany) and dried

overnight. To stain histamine positive cells we used the protocol described in Estensoro et al. (2014) with some modifications. Briefly, samples were deparaffinized and hydrated and the endogenous peroxidase activity was blocked by incubation in methanol:hydrogen peroxide 0.3% v/v in a 9:1 proportion for 40 min. All incubations were performed in a humid chamber, at room temperature and the washing steps consisted of 5 min immersion in TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.4) and 5 min immersion in TBS (without Tween 20) unless stated otherwise. Slides were washed and blocked twice for 30 min, first with TBS 5% bovine serum albumin (BSA) and secondly with 1.5% normal goat serum (Vector Laboratories, Burlingame, CA, USA). After washing, they were incubated with a rabbit anti-histamine antibody (Sigma, St. Louis, MO, USA) 1:50 in TBS overnight at 4 ºC, washed again and incubated with a biotinylated goat anti-rabbit antibody 1:200 in TBS (Vector Laboratories, Burlingame, CA, USA) for 1 h. The slides were subsequently washed, incubated for 1 h with the avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA.), washed and developed by incubating with 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB; Sigma, St. Louis, MO, USA) for 2 min. The reaction was stopped with deionized water and the slides were counterstained for 5 min with Gill's hematoxylin before being dehydrated and mounted for light microscopy examination.

5.2.6. RNA extraction, reverse transcription and gene expression analyses

RNA from anterior and posterior intestine samples was extracted using a MagMAX-96 total RNA isolation kit (Life Technologies, Madrid, Spain) as described before (Bermejo-Nogales, Calduch-Giner & Pérez-Sánchez, 2015). The RNA yield was 50-100 mg and RIN (RNA integrity number) values were 8-10 with the Agilent 2100 Bioanalyzer. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers, using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Negative control reactions were performed excluding the reverse transcriptase.

A 96-well PCR array layout was used for the simultaneous profiling under uniform cycling conditions of 35 selected markers of intestinal epithelial barrier, enterocyte mass and nutrient absorption, mucin production and goblet cell differentiation and immunological status (**Table 5.2**). The primers were designed to obtain amplicons of 50-150 bp in length (**Table 5.3**). Each PCR reaction of 25 µl contained the equivalent of 660 pg of total input RNA, 12.5 µl of 2 x SYBR Green Master Mix (BioRad, Hercules, CA, USA) and 0.9 µM of specific primers. The pipetting and liquid manipulations required to construct the arrays were performed by use of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany), and the real-time quantitative PCR was carried out on an Eppendorf Mastercycler Ep Realplex Real-Time PCR Detection System (Eppendorf, Hamburg, Germany). The PCR amplification program consisted of an initial denaturation step at

95ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95 ºC and annealing/extension for 60 s at 60 ºC. The efficiency of PCRs (>90%) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 ºC/10 s over a temperature range of 55-95 ºC) and linearity of serial dilutions of RT reactions. Fluorescence data acquired were analyzed using the delta-delta Ct method (Livak & Schmittgen, 2001). Ct values of β actin were consistent among groups (with a minimum of 21.88 ± 0.14 and a maximum of 22.21 ± 0.19) in spite of the dietary treatment or the intestine section, and it was used as housekeeping gene. To compare mRNA expression levels, all values were referenced to the expression level of *sirt1* (with a Ct value corresponding to the median of Ct values from all genes analyzed) in the anterior intestine of D1 fish, for which a value 1 was arbitrarily assigned.

5.2.7. Statistical analyses

Data on growth performance and blood biochemistry of the different experimental diets were analyzed by one-way analysis of variance followed by Holm-Sidak test. Two-way analysis of variance, followed by the Student-Newman-Keuls (SNK) test, was carried out to analyze the effect of dietary treatment on the intestine gene expression (with intestine segment and diet as variable factors). The significance level was set at $P < 0.05$. All analyses were made using the SigmaPlot version 13.0 (Systat Software, San Jose, CA, USA). Hierarchical Clustering to assess the gene expression pattern across intestine between experimental diets was carried out by means of Genesis software (Sturn, Quackenbush & Trajanoski, 2002).

Table 5.2 Genes included in the PCR-array.

Category	Gene name	Symbol	Accession No.
Energy sensing	*Sirtuin 1*	*sirt1*	KF018666
	Sirtuin 2	*sirt2*	KF018667
	Sirtuin 3	*sirt3*	KF018668
	Sirtuin 4	*sirt4*	KF018669
	Sirtuin 5	*sirt5*	KF018670
	Sirtuin 6	*sirt6*	KF018671
	Sirtuin 7	*sirt7*	KF018672
Intestinal epithelial barrier	*Occludin*	*ocln*	KF861990
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
Enterocyte mass and nutrient absorption	*Intestinal-type alkaline phosphatase*	*alpi*	KF857309
	Liver-type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
	Ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production and goblet cell differentiation	*Mucin 2*	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
	Intestinal mucin	*i-muc*	JQ277712
	Transcription factor hes-1-b	*hes1-b*	KF857344
	Krueppel-like factor 4	*klf4*	KF857346

(Continued)

Table 5.2 Continued

Immunological/inflammatory status	*Tumor necrosis factor-alpha*	*tnfα*	AJ413189
	Interleukin-1 beta	*il1β*	AJ419178
	Interleukin-6	*il6*	EU244588
	Interleukin-8	*il8*	JX976619
	Interleukin-10	*il10*	JX976621
	Cd4	*cd4*	AM489485
	Cd8 alpha	*cd8α*	EU921630
	Cd8 beta	*cd8β*	KX231275
	Galectin-1	*lgals1*	KF862003
	Galectin-8	*lgals8*	KF862004
	Secreted Immunoglobulin M	*sIgM*	JQ811851
	Secreted Immunoglobulin T	*sIgT*	KX599200
	Membrane Immunoglobulin M	*mIgM*	KX599199
	Membrane Immunoglobulin T	*mIgT*	KX599201

Table 5.3 Forward and reverse primers used for real-time qPCR.

Gene name	Symbol		Primer sequence
Sirtuin1	*sirt1*	F	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC
		R	CCT CAG AAT GGT CCT CGG ATC GGT CTC
Sirtuin2	*sirt2*	F	GAA CAA TCC GAC GAC AGC AGT GAA G
		R	AGG TTA CGC AGG AAG TCC ATC TCT
Sirtuin3	*sirt3*	F	CTG CCA AGT CCT CAT CCC
		R	CTT CAC CAG ACG AGC CAC
Sirtuin4	*sirt4*	F	GGC TGG CGG AGT CGG ATG
		R	TCC TGA ATA CAC CTG TGA CGA AGA C
Sirtuin5	*sirt5*	F	CAG ACA TCC TAA CCC GAG CAG AG
		R	CCA CGA GGC AGA GGT CAC A

(Continued)

Table 5.3 Continued

Sirtuin6	*sirt6*	F	ACT CCA CCA CCA CCG ATG TCA A
		R	CTC CTC CTC CTT CAC CTT TCG CTT TG
Sirtuin7	*sirt7*	F	CTG GAG CAA CCT CTA AAC TGG AA
		R	CAC CTT CAG ACT GGA GCC TAA
Occludin	*ocln*	F	GTG TCA GAA CCT CTA CCA GAC CAG CTA CTC
		R	GAA AGC CTC CCA CTC CTC CCA TCT
Claudin-12	*cldn12*	F	CTC TCA GGG CTA CAC ATC TAC CTA TGC
		R	ACA TTC GTG AGC GGC TGG AG
Claudin-15	*cldn15*	F	CCG ATT GTG GAA GTA GTG GCT CTG GT
		R	CAG CAT CAC CCA ACC GAC GAA CC
Cadherin-1	*cdh1*	F	TGC TCC ATA CAG CGT CAC CTT ACA
		R	CTC GTT CAT CCT AGC CGT CCA GTT
Cadherin-17	*cdh17*	F	GAT GCC CGC AAC CCA GAG
		R	CCG TTG ATT CAC TGC CGT AGA C
Intestinal-type alkaline phosphatase	*alpi*	F	CCG CTA TGA GTT GGA CCG TGA T
		R	GCT TTC TCC ACC ATC TCA GTA AGG G
Liver type fatty acid-binding protein	*fabp1*	F	GTC CTC GTC AAC ACC TTC ACC AT
		R	CGC CTT CAT CTT CTC GCC AGT
Intestinal fatty acid-binding protein	*fabp2*	F	CGA GCA CAT TCC GCA CCA AAG
		R	CCC ACG CAC CCG AGA CTT C
Ileal fatty acid-binding protein	*fabp6*	F	ACC CAG GAC GGC AAT ACC
		R	CGA CGG TGA AGT TGT TGG T
Mucin 2	*muc2*	F	ACG CTT CAG CAA TCG CAC CAT
		R	CCA CAA CCA CAC TCC TCC ACA T
Mucin 13	*muc13*	F	TTC AAA CCC GTG TGG TCC AG
		R	GCA CAA GCA GAC ATA GTT CGG ATA T

(Continued)

Table 5.3 Continued

Intestinal mucin	*i-muc*	F	GTG TGA CCT CTT CCG TTA
		R	GCA ATG ACA GCA ATG ACA
Transcription factor HES-1-B	*hes1-b*	F	GCC TGC CGA TAT GAT GGA A
		R	GGA GTT GTG TTC ATG CTT GC
Krueppel-like factor 4	*klf4*	F	ACA TCA CCG CAC GCA CAC
		R	AAC CAC AGC CCT CCC AGT C
Tumor necrosis factor-alpha	*tnfα*	F	CAG GCG TCG TTC AGA GTC TC
		R	CTG TGG CTG AGA GCT GTG AG
Interleukin-1 beta	*il1β*	F	GCG ACC TAC CTG CCA CCT ACA CC
		R	TCG TCC ACC GCC TCC AGA TGC
Interleukin-6	*il6*	F	TCT TGA AGG TGG TGC TGG AAG TG
		R	AAG GAC AAT CTG CTG GAA GTG AGG
Interleukin-8	*il8*	F	CAG CAG AGT CTT CAT CGT CAC TAT TG
		R	AGG CTC GCT TCA CTG ATG G
Interleukin-10	*il10*	F	AAC ATC CTG GGC TTC TAT CTG
		R	GTG TCC TCC GTC TCA TCT G
CD4	*cd4*	F	TCC TCC TCC TCG TCC TCG TT
		R	GGTGTCTCATCTTCCGCTGTCT
CD8 alpha	*cd8α*	F	GCA GCA ACG GTA ACA CGA ACG
		R	CCAGTATGAGCGGAGTACAGAACA
CD8 beta	*cd8β*	F	CCG AAA TGT GGA AGA CTG GAA CTC
		R	CTTTGGAGGTAAGGTTGGAGGGAT
Galectin-1	*lgals1*	F	GTG TGA GGA GGT CCG TGA TG
		R	ACT GTA GAG CCG TCC GAT AGG
Galectin-8	*lgals8*	F	GGC GGT GAA CGG CGG TCA
		R	GCT CCA GCT CCA GTC TGT GTT GAT AC

(Continued)

Table 5.3 Continued

Secreted Immunoglobulin M	*sIgM*	F	ACC TCA GCG TCC TTC AGT GTT TAT GAT GCC
		R	CAG CGT CGT CGT CAA CAA GCC AAG C
Secreted Immunoglobulin T	*sIgT*	F	GCT GTC AAG GTG GCC CCA AAA G
		R	CAA CAT TCA TGC GAG TTA CCC TTG GC
Membrane Immunoglobulin M	*mIgM*	F	GCTATGGAGGCGGAGGAAGATAACA
		R	GCAGAGTGATGAGGAAGAGAAGGATGAA
Membrane Immunoglobulin T	*mIgT*	F	AGA CGA TGC CAG TGA AGA GGA TGA GT
		R	CGA AGG AGG AGG CTG TGG ACC A
β-Actin	*actb*	F	TCCTGCGGAATCCATGAGA
		R	GACGTCGCACTTCATGATGCT

5.3. Results

5.3.1. Growth performance

To assess the effects of experimental diets on growth performance, data on feed intake, body weight and organo-somatic indexes were calculated (**Table 5.4**). Fish fed D1 and D3 diets showed equally good growth performance across the feeding trial, growing from an initial body weight of 29.66 ± 0.49 g to 146.56 ± 1.11 g with overall feed efficiencies of 0.82-0.84 that were not significantly altered by dietary treatment. Conversely, fish fed D2 diet showed increased feed intake and grew significantly faster when compared to the fish fed D1 with a final body weight (167.7 ± 2.26 g) and weight gain (468 ± 6.10%) that were significantly improved by a 12% and 15%, respectively. This yielded specific growth rates that increased significantly from 1.61 ± 0.01 (D1) and 1.58 ± 0.04 (D3) to 1.74 ± 0.01 in fish fed D2 diet.

Table 5.4 Effects of experimental diets on growth performance and organo-somatic indexes. Effects of dietary treatment on growth performance of gilthead sea bream juveniles fed to visual satiety with different experimental diets for 14 weeks. Data on body weight, feed intake and growth indices are the mean ± SEM of triplicate tanks. Data on liver and viscera weight are the mean ± SEM of 18 fish. Data on intestine indexes are the mean ± SEM of nine fish. Different superscript letters in each row indicate significant differences among dietary treatments (Holm-Sidak test $P < 0.05$).

	D1-CTRL	D2-DICOSAN	D3-Probiotic	P[1]
Initial body weight (g)	29.2 ± 0.41	29.5 ± 0.14	30.1 ± 0.88	0.149
Final body weight (g)	147.9 ± 1.95[a]	167.7 ± 2.26[b]	145.8 ± 1.82[a]	**0.003**
Final fork length (cm)	17.7 ± 0.17	17.9 ± 0.22	17.4 ± 0.22	0.148
Feed intake (g DM/fish)	148.7 ± 3.48[a]	163.3 ± 3.29[b]	139.9 ± 3.26[a]	**0.003**
CF[2]	2.84 ± 0.07	2.99 ± 0.01	2.87 ± 0.07	0.326
Weight gain (%)[3]	400.0 ± 3.24[a]	468.8 ± 6.10[b]	384.6 ± 18.2[a]	**0.010**
SGR (%)[4]	1.61 ± 0.01[a]	1.74 ± 0.01[b]	1.58 ± 0.04[a]	**0.014**
FE (%)[5]	0.82 ± 0.01	0.85 ± 0.01	0.84 ± 0.01	0.207
Liver weight (g)	2.74 ± 0.11[a]	3.51 ± 0.13[b]	2.69 ± 0.15[a]	**<0.001**
Viscera weight (g)	10.5 ± 0.38[a]	11.9 ± 0.47[b]	10.1 ± 0.35[a]	**0.008**
Mesenteric fat (g)	1.85 ± 0.21	2.30 ± 0.25	2.21 ± 0.32	0.438
Intestine weight (g)	2.65 ± 0.34	2.60 ± 0.19	2.32 ± 0.23	0.631
Intestine length (cm)	11.0 ± 0.81	10.0 ± 0.46	11.6 ± 0.86	0.264
HSI (%)[6]	1.70 ± 0.06[a]	2.03 ± 0.06[b]	1.77 ± 0.07[a]	**0.004**
VSI (%)[7]	6.62 ± 0.15	6.92 ± 0.25	6.78 ± 0.25	0.648
MSI (%)[8]	1.18 ± 0.14	1.36 ± 0.15	1.22± 0.12	0.652
IWI (%)[9]	1.59 ± 0.15	1.57 ± 0.09	1.53 ± 0.15	0.951
ILI[10]	10.9 ± 1.78[a]	19.9 ± 2.12[b]	12.3 ± 2.78[ab]	**0.029**

[1]Values resulting from one-way analysis of variance.
[2]Condition factor = (100 x body weight) / fork length^3.
[3]Weight gain (%) = (100 x body weigh increase) / initial body weight.
[4]Specific growth rate = 100 x (ln final body weight - ln initial body weight) / days.
[5]Feed efficiency = wet weight gain / dry feed intake.
[6]Hepatosomatic index = (100 x liver weight) / fish weight.
[7]Viscerosomatix index = (100 x viscera weight) / fish weight.
[8]Mesenteric fat index = (100 x mesenteric fat weight) / fish weight.
[9]Intestine weight index = (100 x intestine weight) / fish weight.
[10]Intestine length index = (100 x fish weight) / intestine length^3.

Organo-somatic indexes calculated as the ratio of tissue to body weight were determined for liver, viscera, mesenteric fat and intestine (**Table 5.4**). The resulting viscerosomatic (VSI) and mesenteric (MSI) indexes were not altered by dietary treatment, whereas hepatosomatic index (HSI) of fish fed D2 (2.03 ± 0.06) was significantly higher than those of D1 (1.70 ± 0.06) and D3 (1.77 ± 0.07) fed fish. Dietary intervention did not alter the intestine weight index calculated as the ratio of organ weight to fish weight (IWI). However, when it was determined as the quotient of fish weight to intestine length (ILI), this relative intestine length index was significantly increased from 10.9 ± 1.78 in D1 fish to 19.9 ± 2.12 in fish fed D2. Intermediate ILI values (12.3 ± 2.78), that did not differ significantly from D1 values, were achieved in fish fed D3 diet.

5.3.2. Blood biochemistry and immunological parameters

Different biochemical and immunological analyses were performed on fish blood to assess the effects of experimental diets (**Table 5.5**). Dietary intervention did not alter the measured hematological parameters yielding values in the range of 39-43% for Hc and 9.6-10.3 g/dl for Hb concentration. Likewise, plasma levels of triglycerides, cholesterol and proteins were not altered by diet, whereas plasma glucose concentration was significantly increased in fish fed D2 in comparison to D1. Plasma total antioxidant capacity remained unaltered by dietary treatment. A significant diet effect was not found on circulating levels of growth factors (GH, IGF-I), although the highest IGF-I/GH ratio was coincident with the enhanced feed intake and growth found in fish fed D2.

Table 5.5 Effects of experimental diets on blood biochemistry and immunological parameters. Gilthead sea bream juveniles were fed to visual satiety with different experimental diets for 14 weeks. Data are the mean ± SEM of nine fish. Different superscript letters in each row indicate significant differences among dietary treatments (Holm-Sidak test P <0.05).

	D1-CTRL	D2-DICOSAN	D3-Probiotic	P[1]
Haemoglobin (g/dl)	10.3 ± 0.24	10.3 ± 0.35	9.6 ± 0.26	0.198
Haematocrit (%)	43.5 ± 1.35	39.6 ± 1.47	38.5 ± 1.82	0.072
Glucose (mg/dl)	36.9 ± 1.38[a]	43.7 ± 2.21[b]	42.5 ± 1.94[ab]	**0.049**
Triglycerides (mM)	6.95 ± 1.30	4.73 ± 1.00	3.48 ± 0.70	0.084
Total cholesterol (mg/dl)	188.5 ± 10.2	191.6 ± 6.48	169.9 ± 6.22	0.130
Total proteins (g/l)	40.5 ± 1.12	40.3 ± 1.22	38.9± 1.39	0.629
Antioxidant capacity (Trolox mM)	0.90 ± 0.02	0.88 ± 0.03	0.86 ± 0.02	0.446
GH (ng/ml)	11.4 ± 1.30	10.9 ± 0.99	13.8 ± 1.36	0.157
IGF-I (ng/ml)	83.7 ± 3.4	94.4 ± 6.9	82.7 ± 7.8	0.101
IGF-I/GH	7.73 ± 0.62	8.97 ± 0.56	6.73 ± 0.89	0.250
Respiratory burst (IRLU)	1462.2 ± 239.8[a]	1032.5 ± 197.5[ab]	686.6 ± 182.2[b]	**0.044**
Lysozyme (Units/ml)	93.7 ± 49.7	29.2 ± 11.1	13.4 ± 9.5	0.153
Alternative complement pathway (ACH50)	25.8 ± 5.37	24.5 ± 8.84	29.1 ± 5.18	0.886
IgM (OD 450 nm)	1.27 ± 0.04[a]	1.17 ±0.06[ab]	1.04 ± 0.09[b]	**0.024**
Cortisol (ng/ml)	8.27 ± 0.96[a]	6.92 ± 0.67[ab]	4.74 ± 1.03[b]	**0.038**

[1]Values resulting from one-way analysis of variance.

Regarding blood immunological parameters, D3 fed fish showed an anti-inflammatory profile characterized by significantly decreased respiratory burst on PMA stimulated leukocytes and reduced levels of circulating IgM and cortisol when compared to D1 diet. D2 diet yielded intermediate values in all cases. Serum lysozyme values were not significantly different among groups. The alternative complement pathway (ACH50) was completely unaffected by the different diets.

5.3.3. Histological observations

The effect of the different diets was evaluated by histology. Liver and intestinal samples of all groups were observed to determine differences in general appearance, tissue integrity and cell types present. The liver did not show any pathological effect or significant difference among groups (**Fig. 5.1A-1C**). Fat accumulation was similar in the three groups and no steatosis was observed in hepatocytes. Glycogen deposition was similar and moderate in all groups.

In the intestine, goblet cells showed similar histochemical characteristics in all groups and their abundance was slightly higher in the anterior intestine of D3 fed fish (**Fig. 5.1G-1L**). Interesting differences were observed in the intestinal architecture of the different dietary groups (**Fig. 5.1D-1F**). Anterior intestines of D3 fed fish showed clearly higher intestinal folds or villi, whereas D2 diets induced denser and more complex folds. Another remarkable difference was the presence of eosinophilic granulocytes in the submucosa of the different groups. The irregular shape and size of these cells make exact quantification difficult, thus we only refer to abundance based on overall observations. Eosinophilic granulocytes appeared in almost double amounts in the submucosa of the anterior intestine of fish fed with D2 and were clearly less abundant in the posterior intestine of the D3 group, when compared to the other two groups (**Fig. 5.1G-1L**).

5.3.4. Immunohistochemistry

The presence of a differential number of eosinophilic granulocytes in the intestinal submucosa of fish from the different dietary challenges

required a further characterization of the cell types. Fish granulocytes differ from those of mammals in their staining properties, which also vary among fish species (Reite & Evensen, 2006). GSB eosinophilic granulocytes can be either acidophils or mast cells, and from these two, only mast cells contain histamine (Mulero et al., 2007). Thus, an anti-histamine antibody was used to further characterize these cells. This allowed detecting histamine-positive cells with similar shape, size and location to those of the observed eosinophilic granulocytes in **Fig. 5.1G-1L** (**Fig. 5.2**), and therefore we conclude that at least part of the observed eosinophilic granulocytes were histamine-positive mast cells. As stated before, reliable quantification of these cells could not be performed, thus we only refer to significant changes that can be observed by microscopic observation. The anterior intestine of D1 and D2 fed fish had large numbers of mast cells, whereas at least half the amount was found in D3 fed fish (**Fig. 5.2A-2C**). In the posterior intestine, mast cells were more abundant in D2 fed fish and clearly lower in D3 fed fish when compared to the control animals (**Fig. 5.2D-2F**). Although the presence of another type of eosinophilic granulocytes (acidophils) cannot be discarded, it is clear that, at least, the mast cell population in the intestinal submucosa is affected by the different dietary treatments.

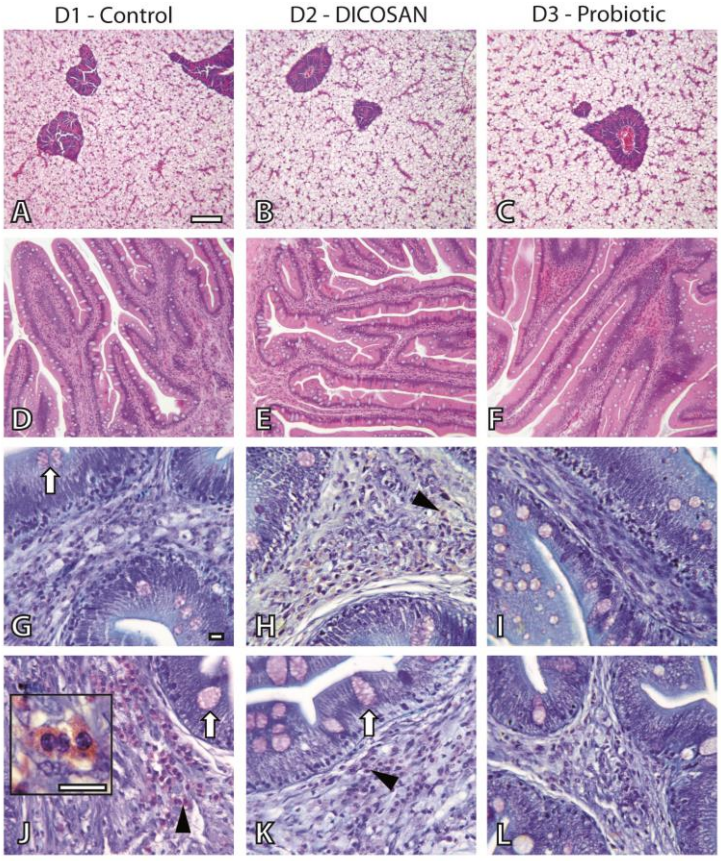


Figure 5.1 Histological effects of experimental diets. Photomicrographs of liver (A-C), anterior (D-I) and posterior (J-L) intestinal segments of gilthead sea bream fed with D1 (control) (A, D, G, J), D2 (supplemented with DICOSAN) (B, E, H, K) or D3 (supplemented with the probiotic *Bacillus amyloliquefaciens*) (C, F, I, L). Staining, scale bars: A-F D H&E, 100 µm; G-L = Giemsa, 10 µm. White arrows point to goblet cells. Black arrowheads point to submucosa with abundant eosinophilic granulocytes (pink cells). Inset in J (scale bar = 10 µm) shows the characteristic irregular and granular shaped eosinophilic granulocytes.

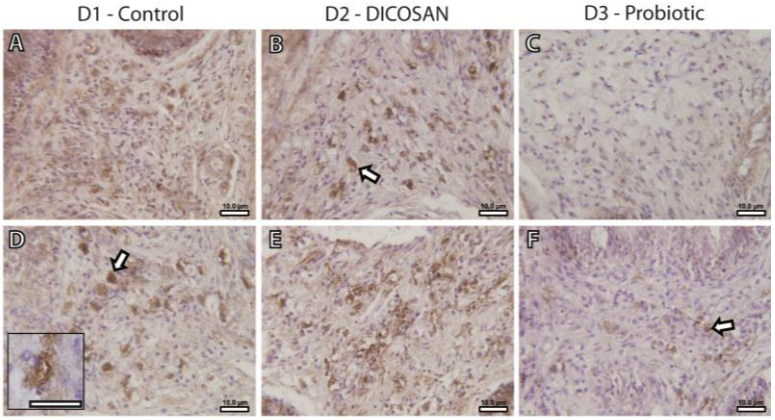


Figure 5.2 Effects of experimental diets on mast cell abundance in the intestinal submucosa. Representative photomicrographs of anterior (A-C) and posterior (D-F) intestinal segments of gilthead sea bream fed with D1 (control) (A, D), D2 (supplemented with DICOSAN) (B, E) or D3 (supplemented with the probiotic *Bacillus amyloliquefaciens*) (C, F) and stained with an anti-histamine antibody. Histamine positive cells are located in the submucosa and appear brown. All pictures were taken using the same magnification (40x) and the scale bars are 10 µm. White arrows point to some representative mast cells in the submucosa. Inset in D (scale bar = 10 µm) shows a typical histamine positive cell with irregular shape and cytoplasmic granules.

5.3.5. Gene expression profiles

The gene expression profile of selected markers was studied in samples of anterior and posterior intestines of fish fed the different experimental diets in order to characterize the general intestinal status of these fish. For most studied genes, a clear expression pattern across intestinal segments was found, regardless of the diet. The structural genes *cdh1*, *cdh17*, had higher expression in anterior intestine, whereas *cldn15*, *ocln* and *cldn12*, were mostly expressed in posterior intestine (as indicated by the low *P* values in the intestinal segment column of **Table 5.6**). Markers of

enterocyte mass and nutrient absorption were also differentially expressed across the intestine, with higher expression of *alpi* in the anterior intestine and *fabp6* in the posterior intestine. Some markers of mucus production (*muc13*, *i-muc*) also showed a pronounced spatial pattern. Markers of immunological status (*il1 β*, *il8*, *il10*, *cd4*, *cd8* α, *cd8 β*, *lgals1*, *mIgM* and *mIgT*) were significantly higher expressed in posterior intestine.

Regarding the diet effect, fish fed D2 and D3 diets showed a reduced expression of energy sensing molecules (*sirt1*, *sirt2*, *sirt3*), *cdh1*, *cd4* and *lgals8* in anterior intestine, compared to fish fed D1 diet. Fish fed D2 diet showed the highest expression of *alpi*, significantly in the anterior intestine when compared to D3 fed fish. The expression of *fabp2* was higher in D2 than in D1 fish in both anterior and posterior intestinal segments, whereas intermediate values were found in D3 fish. Overall, the expression level of mucin genes was not significantly altered by diet, although the diet x intestine section interaction was statistically significant for *muc13*, and its expression was significantly down-regulated in D3 fish in comparison to D1 fish. Regarding the named *i-muc* gene, a diet effect was not statistically significant for this exclusive fish gene that was mostly expressed in the posterior intestine of GSB. D2 induced a significant down-regulation of *il8* in anterior intestine, and both D2 and D3 diets down-regulated the expression of *cd4* and *lgals8* in the anterior intestine segment. D3 diet did not modify significantly the expression of membrane and secreted IgM isoforms, although this diet significantly increased the expression of the membrane IgT (*mIgT*) in posterior intestine.

Table 5.6 Relative gene expression of anterior and posterior intestinal sections of gilthead sea bream fed experimental diets. Data are the mean SEM of 9 fish. All values are referred to sirt1 with an arbitrary value of 1 in the anterior intestine of fish fed D1. P-values are the result of two-way analysis of variance followed by SNK test.

	Anterior intestine			Posterior intestine			ANOVA II, P-value		
	D1-CTRL	D2-DICOSAN	D3-Probiotic	D1-CTRL	D2-DICOSAN	D3-Probiotic	Int. Segment	Diet	Interaction
sirt1	1.03 ± 0.09	0.84 ± 0.04*	0.77 ± 0.04*	0.85 ± 0.09	0.84 ± 0.09	0.89 ± 0.15	0.847	0.441	0.283
sirt2	1.22 ± 0.11	1.05 ± 0.03*	0.93 ± 0.06*	1.09 ± 0.10	1.06 ± 0.11	1.12 ± 0.13	0.741	0.378	0.253
sirt3	0.52 ± 0.07	0.35 ± 0.02*	0.34 ± 0.03*	0.46 ± 0.08	0.42 ± 0.04	0.52 ± 0.08	0.187	0.203	0.129
sirt4	0.15 ± 0.02	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.02	0.549	0.642	0.569
sirt5	1.08± 0.10	1.04 ± 0.05	0.96 ± 0.10	1.11 ± 0.12	1.04 ± 0.058	1.24 ± 0.09	0.171	0.768	0.264
sirt6	0.24 ± 0.03	0.21 ± 0.01	0.20 ± 0.01	0.21 ± 0.03	0.19 ± 0.02	0.25 ± 0.03	0.939	0.465	0.162
sirt7	0.26 ± 0.02	0.24 ± 0.01†	0.18 ± 0.01**	0.25 ± 0.02	0.23 ± 0.02	0.26 ± 0.05	0.360	0.453	0.219
ocln	6.36 ± 0.66	6.02 ± 0.23	6.73 ± 0.61	8.52 ± 0.54	10.2 ± 0.83	9.71 ± 1.35	**<0.001**	0.583	0.431
cldn12	0.94 ± 0.10	1.05 ± 0.05	0.95 ± 0.04	1.02 ± 0.12	1.29 ± 0.13	1.16 ± 0.13	**0.035**	0.154	0.716
cldn15	27.3 ± 3.69	23.5 ± 0.76	26.5 ± 1.95	40.1 ± 3.73	48.7 ± 6.90	48.7 ± 9.13	**<0.001**	0.772	0.493
cdh1	22.8 ± 2.76	14.4 ± 0.68**	14.8 ± 0.77**	12.22 ± 1.17	15.4 ± 1.63	12.5 ± 0.92	**0.001**	**0.032**	**0.001**
cdh17	72.0 ± 7.72	62.7 ± 1.77	71.9 ± 4.14	45.9 ± 2.07	53.03 ± 4.42	49.9 ± 4.06	**<0.001**	0.759	0.150
alpi	112.7 ± 9.78	124.4 ± 7.99†	97.7 ± 8.36	28.8 ± 3.79	40.8 ± 6.60	35.5 ± 3.35	**<0.001**	**0.041**	0.313
fabp1	159.5 ± 16.5	155.4 ± 8.64	147.8 ± 6.87	119.8 ± 18.3	136.6 ± 30.2	142.4 ± 23.8	0.113	0.882	0.872
fabp2	364.0 ± 94.9	734.4 ± 104.9*	556.2 ± 73.8	606.9 ± 83.5	1208.5 ± 210.6*	799.2 ± 189.8	**0.006**	**0.003**	0.599
fabp6	< 0.05	< 0.05	< 0.05	3423.5 ± 507.1	2563.6 ± 440.6	2874.4 ± 435.9	**<0.001**	0.424	0.424
muc2	41.5 ± 3.3	44.4 ± 3.03	39.06 ± 2.60	37.9 ± 3.24	32.6 ± 2.84	39.5 ± 4.02	0.063	0.923	0.142
muc13	149.2 ± 17.1	118.7 ± 5.49	107.9 ± 6.22*	101.6 ± 9.47	121.7 ± 10.2	98.7 ± 7.93	**0.027**	0.067	**0.037**
i-muc	0.14 ± 0.02	0.15 ± 0.04	0.13 ± 0.02	7.29 ± 3.10	26.6 ± 16.9	29.1 ± 18.2	**0.015**	0.513	0.513
hes1-b	3.82 ± 0.67	3.3 ± 0.36	3.59 ± 0.39	4.04 ± 0.45	4.61 ± 0.70	5.05 ± 1.17	0.078	0.808	0.636
klf4	2.88 ± 0.25	2.68 ± 0.17	3.15 ± 0.28	2.21 ± 0.18	2.90 ± 0.19*	2.87 ± 0.22*	0.186	0.136	0.143
tnfα	0.13 ± 0.02	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.16 ± 0.02	0.094	0.356	0.108
il1β	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	**0.002**	0.849	0.964
il6	0.03 ± 0.01	0.02 ± 0.00†	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.615	**0.024**	0.596
il8	0.25 ± 0.04	0.13 ± 0.01**	0.17 ± 0.03	0.32 ± 0.09	0.21 ± 0.03	0.22 ± 0.04	**0.050**	**0.038**	0.939
il10	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.16 ± 0.01	0.16 ± 0.02	0.17 ± 0.03	**0.001**	0.916	0.817
cd4	0.53 ± 0.12	0.35 ± 0.02*	0.32 ± 0.02*	0.55 ± 0.04	0.52 ± 0.06	0.45 ± 0.05	**0.032**	**0.038**	0.446
cd8α	0.80 ± 0.09	0.86 ± 0.09	0.71 ± 0.05	0.80 ± 0.13	1.10 ± 0.15	1.15 ± 0.19	**0.036**	0.382	0.259
cd8β	0.12 ± 0.02	0.13 ± 0.02	0.10 ± 0.01	0.14 ± 0.02	0.18 ± 0.03	0.18 ± 0.04	**0.008**	0.522	0.458
lgals1	5.70 ± 0.70	5.92 ± 0.37	5.31 ± 0.50	10.2 ± 0.97	10.4 ± 0.83	9.86 ± 1.23	**<0.001**	0.741	1.000
lgals8	3.77 ± 0.40	2.76 ± 0.15*	2.72 ± 0.24*	3.00 ± 0.33	4.23 ± 0.70	3.92 ± 0.78	0.131	0.935	0.074
sIgM	10.1 ± 3.59	4.14 ± 0.86	5.77 ± 0.66	11.9 ± 3.87	8.09 ± 2.90	8.36 ± 1.67	0.180	0.134	0.906
sIgT	0.10 ± 0.03	0.08 ± 0.01†	0.12 ± 0.02	0.05 ± 0.02	0.08 ± 0.02	0.12 ± 0.04	0.403	0.146	0.518
mIgM	0.20 ± 0.07	0.15 ± 0.02	0.13 ± 0.01	0.40 ± 0.09	0.23 ± 0.04	0.38 ± 0.10	**0.002**	0.231	0.41
mIgT	0.45 ± 0.03	0.44 ± 0.05	0.58 ± 0.11	0.59 ± 0.06	0.83 ± 0.09	1.56 ± 0.46*	**<0.001**	**0.004**	**0.038**

Globally across the intestine, the dietary-mediated effects affected the expression of 15 out of the 35 genes studied (43%). The heatmap in **Fig. 5.3** was constructed with the expression values of those differentially regulated genes in order to obtain a visual overview of the changes. Fish fed D2 diet revealed an overall down-regulation of genes related to intestinal integrity (*cdh1*) and energy sensing (*sirt1-3*) mainly affecting the anterior intestine. Fish fed D3 diet showed more prominent effects on posterior intestine with up-regulation of genes related to immune response (*IgT*, *il6*).

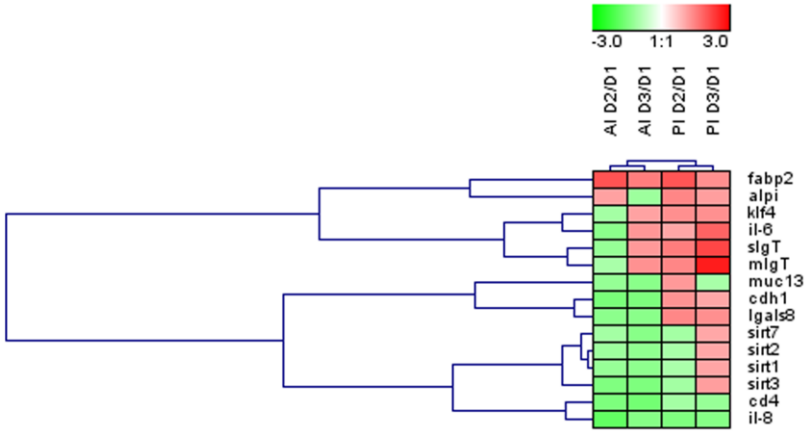


Figure 5.3 Effects of experimental diets on genes related to intestinal architecture, absorption and immune response. Hierarchical heatmap of fold-changes (experimental group (D2, D3) vs. control (D1)) for differentially expressed genes in at least one of the intestinal sections. AI, anterior intestine; PI, posterior intestine.

5.4. Discussion

Evidence in mammals indicates that the metabolic utilization of ingested nutrients determines satiety and feeding behavior (Scharrer & Langhans, 1986). Since MCFAs can enter directly in the portal vein and their transport across the mitochondrial membrane does not require carnitine palmitoyltransferase-1, their final tissue uptake and oxidation is accelerated, acting as satiety factors. Hence, in comparison to long-chain triglycerides, preloading of oral emulsions of medium-chain triglycerides decreased feed intake in rats (Ooyama et al., 2009). High dietary inclusion levels of medium-chain triglycerides (15-30%) also decreased feed intake in polka-dot grouper (*Cromileptes altivelis*) (Williams et al., 2006), and a strong inverse relationship between dietary medium-chain triglycerides (0.4-15%) and feed intake was found in Atlantic salmon (*Salmo salar*) (Nordrum et al., 2003). By contrast, feed intake was not affected by medium-chain triglyceride supplementation (5-15%) in sunshine bass (female white bass *Morone chrysops* x male striped bass *Morone saxatilis*) (Trushenski, 2009) and rainbow trout (*Oncorhynchus mykiss*) (Figueiredo-Silva et al., 2012), which indicates that other factors, besides metabolic fuel availability, regulate feed intake in fish. Indeed, our results showed that dietary supplementation with MCFAs in the form of a sodium salt of coconut fatty acid distillate (D2 diet) was able to enhance the overall feed intake and growth rates of GSB with no signs of histopathological damage in liver and intestine tissues. This growth-promoting action was supported by histological and gene expression data at the intestine level (see below) and by a slight, although non-significant, increase of the circulating IGF/GH quotient, which can be viewed as an

increased liver GH-responsiveness to the anabolic action of GH. This agrees with the observation that dietary medium-chain triglycerides act in young and growing pigs via stimulation of somatotropic endocrine pathways, minimizing weaning-associated disorders such as slow growth and diarrhea (Miller et al., 2016). Other potential benefits could be mediated by changes in the intestinal microbiota (Lai et al., 2014), although the therapeutic potential of MCFAs and their potential benefits on feed intake and key performance indicators seems to be highly dependent on the intake dose and age of piglets (Lai et al., 2014; Zhang et al., 2016). In this regard, it is noteworthy that the gene expression of several sirtuins was down-regulated in the anterior intestine of fish fed D2 or D3 diets. Since sirtuins are NAD^+-dependent deacetylases that act as energy sensors, it can be argued that both D2 and D3 diets regulate energy metabolism at the intestinal local level by means of epigenetic-related mechanisms. Recently, the tissue-specific regulation of the seven mammalian sirtuin counterparts of GSB has been described (Simó-Mirabet et al., 2017). The present study provides further evidence for a functional regulation of sirtuins at intestinal level in farmed fish, which can be viewed as a consequence of a reduced energy demand in fish with an increased feed intake (D2 diet) or as a part of an adaptive hypo-metabolic conditioning (D3 diet) in fish with a slight reduction of feed intake.

Absorption of lipids occurs mostly in the first segments of fish intestines, where bile salts are secreted to hydrolyze triglycerides to free fatty-acids and glycerol (Sundell & Rønnestad, 2011) resembling mammalian small intestines. In accordance, expression of *fabp6*, involved in reabsorption of bile acids and exclusively expressed in mammalian

ileums (Besnard et al., 2002), can only be found in the posterior intestinal segment of GSB and other fish species (Alves-Costa et al., 2008; Pérez-Sánchez et al., 2015; Calduch-Giner, Sitjà-Bobadilla & Pérez-Sánchez 2016). FABP2, involved in regulation of the intracellular concentration of free fatty acids, preferentially binds long-chain fatty acids (LCFAs) (Lowe et al., 1987), but can also bind MCFAs (Huang et al., 2002). Several studies in mammals associated FABP2 expression or genetic variants to insulin resistance and type 2 diabetes in individuals fed different sources of MCFAs or LCFAs (Rubin et al., 2012). Our results show a clear up-regulation of *fabp2* and an increase in plasma glucose levels upon MCFA intake (D2 diet). In agreement with the current results, rainbow trout fed high levels of coconut oil also showed increased plasma glucose levels (Figueiredo-Silva et al., 2012). Thus, there is a clear relationship among lipid source, *fabp2* expression and glucose tolerance both in mammals and teleost fish, and the underlying mechanisms deserve further study.

In our study, additional changes associated to coconut oil supplementation are the increase in intestinal complexity and gene expression of *alpi* in anterior intestine, which are related to intestinal architecture and nutrient absorption. In fish, a decrease in Alpi activity has been linked to malnutrition (Bakke-McKellep et al., 2000; Ducasse-Cabanot et al., 2007). Conversely, unchanged Alpi activity or gene expression levels in intestine were related with good growth performance values (Estensoro et al., 2016). In the current study, MCFAs (D2 diet) increased *alpi* gene expression which, together with the increased complexity of intestinal folds in the main absorptive intestinal segment, can be directly related to the increased intake and growth performance of

this dietary group. Moreover, the intestine of D2 fed fish was shorter than that of the other two experimental groups. This trend was also found when comparing carnivorous with omnivores and herbivorous fish, since the total intestinal surface required for the absorption of nutrients is directly linked to digestive efficiency, increasing gut length allometrically with the body length (Karachle & Stergiou, 2010).

A recent study performed in mice also showed that MCFA addition to fish oil formulations is able to induce an anti-inflammatory profile, decreasing the host response to inflammatory challenge (Carlson et al., 2015). In view of these results, MCFA supplementation has been proposed as a candidate to formulate optimized diets to be used as therapeutic intervention for diseases derived from chronic inflammation. Coconut oil as a source of MCFA in GSB (D2 diet) induced no significant changes in serum lysozyme, complement activity, IgM levels or respiratory burst of stimulated blood leukocytes. However, significant down-regulation of *cd4, il8* and *lgals8* was observed in the intestine, hinting to some anti-inflammatory effects on GSB intestinal immunity. By contrast, an increase in eosinophilic granulocytes, particularly in mast cells, was observed in these intestines. Nonetheless, the lack of any prototypical pro-inflammatory signal, such as *tnf* α or *il1 β* up-regulation, suggests that these cells have been recruited and reside inactive in the intestinal submucosa as a kind of surveillance machinery ready to be activated in case of threat. Although in the present study no challenge was performed to evaluate the readiness of the immune system of GSB upon pathogen exposure, there is evidence that coconut oil supplementation can increase survival of catfish upon *Aeromonas hydrophila* challenge (Vargas et al., 2013).

Probiotics modulate the gastrointestinal microbial communities having diverse effects, such as inhibition or suppression of pathogen growth, improvement of stress tolerance, stimulation of growth or modulation of the host immune status (Balcázar et al., 2006; Wang, Li & Lin, 2008). Accordingly, oral administration of *B. amyloliquefaciens* in mice showed a protective effect against *Clostridium difficile* and has been proposed to be used in humans (Geeraerts et al., 2015). It also showed alleviating effects on immune stress induced by lipopolysaccharide (LPS) challenge in broilers, by ameliorating the growth performance and inducing an anti-inflammatory shift down-regulating pro-inflammatory gene expression and increasing *IL10* expression (Li et al., 2015). Its use in aquaculture has also been proposed, and *B. amyloliquefaciens* showed potential to control vibriosis in turbot (*Scophthalmus maxima*) (Chen et al., 2016a) and other fish pathogens (Chen et al., 2016b). The present study does not deal with any pathological challenge, but aims to define the effect of this probiotic administration on the basal health of GSB in order to assess their readiness to face a threat. In addition, the *Bacillus*-based probiotic-supplemented diet (D3) did not induce negative effects on the growth performance of GSB. Likewise, beneficial effects upon infection, but no interference with growth, have been documented in similar trials with different *Bacillus* species in Nile tilapia (Aly, Mohamed & John, 2008) and rainbow trout (Raida et al., 2003). Many studies have demonstrated the beneficial effects of *B. amyloliquefaciens* on broiler performance parameters (Sánchez et al., 2006; Mallo et al., 2010), but in some cases, probiotics supplementation might not affect growth parameters, and a protective effect on growth can only be observed upon challenge (Li et al., 2015).

Our results with fast growing juveniles of GSB also showed clear interesting outcomes of the *Bacillus*-based probiotic (D3 diet) on intestinal architecture and mucus production. Anterior intestine showed higher intestinal folds in agreement with the results obtained in Nile tilapia fed the same probiotic bacteria (Silva et al., 2015). In addition, D3 diet correlated with low numbers of goblet cells and down-regulation of the expression of the transmembrane *muc13*, a major enterocyte component of GSB (Pérez-Sánchez et al., 2013). It must be argued that *muc13* does not contribute significantly to the extended glycocalyx of GSB when the mucin secretion from goblet cells remains high. Otherwise, studies on Muc13 -/- mice suggest that this mucin has an anti-inflammatory function and anti-apoptotic effects in epithelial cells (Sheng et al., 2011; Sheng et al., 2013). In this regard, in our experimental model, the down-regulation of *muc13* could be viewed as a consequence rather than a cause of an overall anti-inflammatory status, which comprised a decrease in *lgals8* and *cd4* transcripts in anterior intestine, a lower respiratory burst activity of blood leukocytes, lower amounts of circulating IgM and cortisol, and lower numbers of eosinophilic granulocytes, in particular mast cells, in the intestinal submucosa.

The anti-inflammatory effects of *B. amyloliquefaciens* as a probiotic have been already reported in birds and mammals. This probiotic ameliorated the damage caused by inflammation in dextran sulphate sodium (DSS)-induced colitis in mice (Hairul Islam et al., 2011) and LPS-induced stress in broilers (Li et al., 2015) by down-regulating expression of pro-inflammatory cytokines such as *TNF* α, *IL1 β* and *IL2* and up-regulating the anti-inflammatory cytokine *IL10* upon exacerbated inflammation. In addition, a decrease in neutrophil myeloperoxidase and

an increase in antioxidant molecules (superoxide dismutase and catalase) was observed (Hairul Islam et al., 2011). In the present study, superoxide dismutase and catalase were not measured, but the observed decrease in oxidative radicals measured with the respiratory burst assay indirectly reflects this antioxidant effect. A similar decrease in respiratory burst was observed in European sea bass fed a different probiotic, which also induced an anti-oxidant status (Guardiola et al., 2016). Opposite to the decrease in circulating levels of IgM, a significant up-regulation of *mIgT* expression was observed in posterior intestine. IgT is the key mucosal immunoglobulin in teleosts (Zhang et al., 2010) and the importance of its fine regulation upon infection in GSB intestines has been recently described (Piazzon et al., 2016). The presence of higher number of *mIgT* transcripts can be related to higher numbers of IgT^+ B cells. The availability of this larger pool of IgT^+ B cells can be critical upon infection or any other threat, as these cells are already in the local environment ready to be activated and exert their function as effector cells. In fact, when the transcription of *IgT* in GSB intestines is impaired, as it happens when fish are fed diets with high plant ingredient substitution, the disease outcome upon parasitic infection is worse (Piazzon et al., 2016). The results of the current study with *B. amyloliquefaciens* CECT 5940 supplementation as a probiotic (D3 diet) point to a fine-tuning of the mucosal immunity by increasing the number of resident mucosal related IgT^+ B cells to improve the surveillance mechanisms in the posterior intestine, where intestinal immune responses mainly take place.

5.5. Conclusions

Altogether, we observed differential beneficial effects induced by these dietary supplements that are related to their different nature. MCFAs induced positive effects on GSB growth and intestinal architecture mainly affecting the anterior intestinal segment where absorption mostly takes place. Conversely, *B. amyloliquefaciens* CECT 5940 supplementation had key effects in the regulation of the immune status inducing anti-inflammatory and antioxidant effects that can potentially be advantageous upon infection or stressful situations. Separately, the two additives showed interesting effects posing a promising way to improve health and disease resistance in aquaculture.

Funding

This work has been carried out with financial support from NOREL S.A. Additional funding has been received by Spanish MINECO project no. AGL2013-48560-R and Generalitat Valenciana (PROMETEO-FASE II/2014/085). M. Carla Piazzon was supported by MINECO through grant FPDI-2013-15741 and by CSIC PIE project no. 201740E013. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

The authors thank J Monfort and L Rodríguez for histological processing, MA González for technical assistance with gene expression analyses, and I Vicente for technical assistance with fish husbandry and samplings.

References

Abriouel H, Franz CMAP, Omar NB, Galvez A. 2011. Diversity and applications of *Bacillus bacteriocins*. FEMS Microbiology Reviews 35:201-232 DOI 10.1111/j.1574-6976.2010.00244.x.

Alves-Costa FA, Denovan-Wright EM, Thisse C, Thisse B, Wright JM. 2008. Spatiotemporal distribution of fatty acid-binding protein 6 (fabp6) gene transcripts in the developing and adult zebrafish (*Danio rerio*). FEBS Journal 275:3325-3334 DOI 10.1111/j.1742-4658.2008.06480.x.

Aly SM, Mohamed MF, John G. 2008. Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (*Oreochromis niloticus*). Aquaculture Research 39:647-656 DOI 10.1111/j.1365-2109.2008.01932.x.

Bakke-McKellep AM, McL Press C, Baeverfjord G, Krogdahl Å, Landsverk T. 2000. Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. Journal of Fish Diseases 23:115-127 DOI 10.1046/j.1365-2761.2000.00218.x.

Balcázar JL, Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Múzquiz JL. 2006. The role of probiotics in aquaculture. Veterinary Microbiology 114:173-186 DOI 10.1016/j.vetmic.2006.01.009.

Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz Á, Calduch-Giner J, Pérez-Sánchez J. 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). Aquaculture 454:8-18 DOI 10.1016/j.aquaculture.2015.12.008.

Bergsson G, Arnfinnsson J, Steingrímsson Ó, Thormar H. 2001. Killing of Grampositive cocci by fatty acids and monoglycerides. APMIS 109:670-678 DOI 10.1034/j.1600-0463.2001.d01-131.x.

Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. 2015. Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. PLOS ONE 10:e0122889 DOI 10.1371/journal.pone.0122889.

Besnard P, Niot I, Poirier H, Clément L, Bernard A. 2002. New insights into the fatty acid-binding protein (FABP) family in the small intestine. Molecular and Cellular Biochemistry 239:139-147 DOI 10.1023/A:1020505512364.

Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J. 2016. Gene expression profiling reveals functional specialization along the intestinal tract of a carnivorous teleostean fish (*Dicentrarchus labrax*). Frontiers in Physiology 7:Article 359 DOI 10.3389/fphys.2016.00359.

Carlson SJ, Nandivada P, Chang MI, Mitchell PD, O'Loughlin A, Cowan E, Gura KM, Nose V, Bistrian B, Puder M. 2015. The addition of medium-chain triglycerides to a purified fish oil-based diet alters inflammatory profiles in mice. Metabolism: Clinical and Experimental 64:274-282 DOI 10.1016/j.metabol.2014.10.005.

Cerezuela R, Guardiola FA, Cuesta A, Esteban MA. 2016. Enrichment of gilthead seabream (*Sparus aurata* L.) diet with palm fruit extracts and probiotics: effects on skin mucosal immunity. Fish and Shellfish Immunology 49:100-109 DOI 10.1016/j.fsi.2015.12.028.

Chen Y, Li J, Xiao P, Li GY, Yue S, Huang J, Zhu WY, Mo ZL. 2016a. Isolation and characterization of *Bacillus* spp. M001 for potential application in turbot (*Scophthalmus maximus* L) against *Vibrio anguillarum*. Aquaculture Nutrition 22:374-381 DOI 10.1111/anu.12259.

Chen Y, Li J, Xiao P, Zhu W, Mo ZL. 2016b. The ability of marine *Bacillus* spp. isolated from fish gastrointestinal tract and culture pond sediment to inhibit growth of aquatic pathogenic bacteria. Iranian Journal of Fisheries Sciences 15:701-714.

Das A, Nakhro K, Chowdhury S, Kamilya D. 2013. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). Fish and Shellfish Immunology 35:1547-1553 DOI 10.1016/j.fsi.2013.08.022.

Diaz D. 2007. Effect of *Bacillus amyloliquefaciens* CECT-5940 spores on broiler performance and digestibility. Available at http:// en.engormix.com/MA-poultry-industry/ articles/ effect-bacillus-amyloliquefaciens-cect5940-t795/ p0.htm.

Done HY, Venkatesan AK, Halden RU. 2015. Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? AAPS Journal 17:513-524 DOI 10.1208/s12248-015-9722-z.

Ducasse-Cabanot S, Zambonino-Infante J, Richard N, Medale F, Corraze G, Mambrini M, Robin J, Cahu C, Kaushik S, Panserat S. 2007. Reduced lipid intake leads to changes in digestive enzymes in the intestine but has minor effect on key enzymes of hepatic intermediary metabolism in

rainbow trout (*Oncorhynchus mykiss*). Animal 1:1272-1282 DOI 10.1017/s1751731107000596.

Estensoro I, Ballester-Lozano G, Benedito-Palos L, Grammes F, Martos-Sitcha JA, Mydland L-T, Calduch-Giner JA, Fuentes J, Karalazos V, Á Ortiz., Øverland M, Sitjà-Bobadilla A, Pérez-Sánchez J. 2016. Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. PLOS ONE 11:e0166564 DOI 10.1371/journal.pone.0166564.

Estensoro I, Mulero I, Redondo MJ, Alvarez-Pellitero P, Mulero V, Sitjà-Bobadilla A. 2014. Modulation of leukocytic populations of gilthead sea bream (*Sparus aurata*) by the intestinal parasite *Enteromyxum leei* (Myxozoa: Myxosporea). Parasitology 141:425-440 DOI 10.1017/S0031182013001789.

FAO. 2016. The state of world fisheries and aquaculture. Contributing to food security and nutrition for all. Rome. Available at http:// www.fao.org/ 3/ a-i5555e.pdf .

Figueiredo-Silva C, Kaushik S, Terrier F, Schrama JW, Médale F, Geurden I. 2012. Link between lipid metabolism and voluntary food intake in rainbow trout fed coconut oil rich in medium-chain TAG. British Journal of Nutrition 107:1714-1725 DOI 10.1017/S0007114511004739.

Geeraerts S, Ducatelle R, Haesebrouck F, Van Immerseel F. 2015. *Bacillus amyloliquefaciens* as prophylactic treatment for *Clostridium difficile*-associated disease in a mouse model. Journal of Gastroenterology and Hepatology 30:1275-1280 DOI 10.1111/jgh.12957.

Guardiola FA, Porcino C, Cerezuela R, Cuesta A, Faggio C, Esteban MA. 2016. Impact of date palm fruits extracts and probiotic enriched diet on antioxidant status, innate immune response and immune-related gene expression of European seabass (*Dicentrarchus labrax*). Fish and Shellfish Immunology 52:298-308 DOI 10.1016/j.fsi.2016.03.152.

Hanczakowska E, Świątkiewicz M, Natonek-Wiśniewska M, Okoń, K. 2016. Medium chain fatty acids (MCFA) and/or probiotic *Enterococcus faecium* as a feed supplement for piglets. Livestock Science 192:1-7 DOI 10.1016/j.livsci.2016.08.002.

Hairul Islam VI, Prakash Babu N, Pandikumar P, Ignacimuthu S. 2011. Isolation and characterization of putative probiotic bacterial strain, *Bacillus amyloliquefaciens*, from north east Himalayan soil based on in vitro and in vivo functional properties. Probiotics and Antimicrobial Proteins 3:175-185 DOI 10.1007/s12602-011-9081-8.

Huang H, Starodub O, McIntosh A, Kier AB, Schroeder F. 2002. Liver fatty acid-binding protein targets fatty acids to the nucleus. Real time confocal and multiphoton fluorescence imaging in living cells. The Journal of Biological Chemistry 277:29139-29151 DOI 10.1074/jbc.M202923200.

Karachle PK, Stergiou KI. 2010. Gut length for several marine fish: relationships with body length and trophic implications. Marine Biodiversity Records 3:e106 DOI 10.1017/S1755267210000904.

Khan SH, Iqbal J. 2016. Recent advances in the role of organic acids in poultry nutrition. Journal of Applied Animal Research 44:359-369 DOI 10.1080/09712119.2015.1079527.

Lai WK, Yen HC, Lin CS, Chiang SH. 2014. The effects of dietary medium-chain triacylglycerols on growth performance and intestinal microflora in young pigs. Journal of Animal and Feed Sciences 23:331-336 DOI 10.22358/jafs/65669/2014.

Li Y, Zhang H, Chen YP, Yang MX, Zhang LL, Lu ZX, Zhou YM, Wang T. 2015. *Bacillus amyloliquefaciens* supplementation alleviates immunological stress and intestinal damage in lipopolysaccharide-challenged broilers. Animal Feed Science and Technology 208:119-131 DOI 10.1016/j.anifeedsci.2015.07.001.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408 DOI 10.1006/meth.2001.1262.

Lowe JB, Sacchettini JC, Laposata M, McQuillan JJ, Gordon JI. 1987. Expression of rat intestinal fatty acid-binding protein in *Escherichia coli*. Purification and comparison of ligand binding characteristics with that of *Escherichia coli*-derived rat liver fatty acid-binding protein. Journal of Biological Chemistry 262:5931-5937.

Lu L, Cao H, He S, Wei R, Diong M. 2011. *Bacillus amyloliquefaciens* G1: a potential antagonistic bacterium against eel-pathogenic *Aeromonas hydrophila*. Evidence-Based Complementary and Alternative Medicine 2011:824104 DOI 10.1155/2011/824104.

Mallo JJ, Gracia MI, Honrubia P, Sedano G. 2010. Use of a *Bacillus amyloliquefaciens* probiotic in broiler farms. Poultry Science 89: E-Suppl.815.

Martínez-Barberá JP, Pendón C, Martí-Palanca H, Calduch-Giner JA, Rodríguez RB, Valdivia MM, Pérez-Sánchez J. 1995. The use of recombinant gilthead sea bream (*Sparus aurata*) growth hormone for radioiodination and standard preparation in radioimmunoassay.

Comparative Biochemistry and Physiology Part A: Physiology 110:335-340 DOI 10.1016/0300-9629(94)00178-V.

Miller DW, Prosser Z, Chee EYW, Hansen CF, Dunshea FR, Mullan BP, Pluske JR. 2016. Dietary stimulation of the endogenous somatotropic axis in weaner and grower-finisher pigs using medium chain triglycerides and cysteamine hydrochloride. Journal of Animal Science and Biotechnology 7:Article 61 DOI 10.1186/s40104-016-0121-9.

Mulero I, Sepulcre MP, Meseguer J, García-Ayala A, Mulero V. 2007. Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. Proceedings of the National Academy of Sciences of the United States of America 104:19434-19439 DOI 10.1073/pnas.0704535104.

Ng WK, Koh CB. 2016. The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. Reviews in Aquaculture 0:1-27 DOI 10.1111/raq.12141.

Nordrum S, Olli JJ, Røsjø C, Holm H, Krogdahl Å. 2003. Effects of graded levels of medium chain triglycerides and cysteine on growth, digestive processes and nutrient utilization in sea water reared Atlantic salmon (*Salmo salar*, L.) under ad libitum feeding regime. Aquaculture Nutrition 9:263-274 DOI 10.1046/j.1365-2095.2003.00252.x.

Ooyama K, Kojima K, Aoyama T, Takeuchi H. 2009. Decrease of food intake in rats after ingestion of medium-chain triacylglycerol. Journal of Nutritional Science and Vitaminology 55:423-427 DOI 10.3177/jnsv.55.423.

Palenzuela O, Sitjà-Bobadilla A, Álvarez Pellitero P. 1996. Isolation and partial characterization of serum immunoglobulins from sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). Fish & Shellfish Immunology 6:81-94 DOI 10.1006/fsim.1996.0010.

Pérez-Sánchez J, Benedito-Palos L, Estensoro I, Petropoulos Y, Calduch-Giner JA, Browdy CL, Sitjà-Bobadilla A. 2015. Effects of dietary NEXT ENHANCE® 150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). Fish & Shellfish Immunology 44:117-128 DOI 10.1016/j.fsi.2015.01.039.

Pérez-Sánchez J, Estensoro I, Redondo MJ, Calduch-Giner JA, Kaushik S, Sitjà-Bobadilla A. 2013. Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. PLOS ONE 8:e65457 DOI 10.1371/journal.pone.0065457.

Piazzon MC, Galindo-Villegas J, Pereiro P, Estensoro I, Calduch-Giner JA, Gomez-Casado E, Novoa B, Mulero V, Sitjà-Bobadilla A, Pérez-Sánchez J. 2016. Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a Perciform fish. Frontiers in Immunology 7:Article 637 DOI 10.3389/fimmu.2016.00637.

Raida MK, Larsen JL, Nielsen ME, Buchmann K. 2003. Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). Journal of Fish Diseases 26:495-498 DOI 10.1046/j.1365-2761.2003.00480.x.

Ran C, Carrias A, Williams MA, Capps N, Dan BCT, Newton JC, Kloepper JW, Ooi EL, Browdy CL, Terhune JS, Liles MR. 2012. Identification of *Bacillus* strains for biological control of catfish pathogens. PLOS ONE 7:e45793 DOI 10.1371/journal.pone.0045793.

Reite OB, Evensen O. 2006. Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish & Shellfish Immunology 20:192-208 DOI 10.1016/j.fsi.2005.01.012.

Ridha MT, Azad IS. 2012. Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative probiotic bacteria. Aquaculture Research 43:843-852 DOI 10.1111/j.1365-2109.2011.02899.x.

Rubin D, Helwig U, Pfeuffer M, Auinger A, Ruether A, Matusch D, Darabaneanu S, Freitag-Wolf S, Nothnagel M, Schreiber S, Schrezenmeir J. 2012. The effect of FABP2 promoter haplotype on response to a diet with medium-chain triacylglycerols. Genes and Nutrition 7:437-445 DOI 10.1007/s12263-012-0280-z.

Saera-Vila A, Benedito-Palos L, Sitjà-Bobadilla A, Nácher-Mestre J, Serrano R, Kaushik S, Pérez-Sánchez J. 2009. Assessment of the health and antioxidant trade-off in gilthead sea bream (*Sparus aurata* L.) fed alternative diets with low levels of contaminants. Aquaculture 296:87-95 DOI 10.1016/j.aquaculture.2009.07.028.

Sánchez J, Quiles A, Espinel AE, Díaz D, Gracia MI. 2006. Effect of supplementing a probiotic feed additive on performance and digestibility of broilers. Poultry Science 85:Article 11.

Scharrer E, Langhans W. 1986. Control of food intake by fatty acid oxidation. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 250:1003-1006.

Seal BS, Lillehoj HS, Donovan DM, Gay CG. 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production.

Animal Health Research Reviews/Conference of Research Workers in Animal Diseases 14:78-87 DOI 10.1017/S1466252313000030.

Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, Ruane N, Jutfelt F, Toften H, Vaughan L. 2012. Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish Physiology and Biochemistry 38:85-105 DOI 10.1007/s10695-011-9517-9.

Selim KM, Reda RM. 2015. Improvement of immunity and disease resistance in the Nile tilapia, *Oreochromis niloticus*, by dietary supplementation with *Bacillus amyloliquefaciens*. Fish and Shellfish Immunology 44:496-503 DOI 10.1016/j.fsi.2015.03.004.

Sheng YH, Lourie R, Lindén SK, Jeffery PL, Roche D, Tran TV, Png CW, Waterhouse N, Sutton P, Florin THJ, McGuckin MA. 2011. The MUC13 cell-surface mucin protects against intestinal inflammation by inhibiting epithelial cell apoptosis. Gut 60:1661-1670 DOI 10.1136/gut.2011.239194.

Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, Sutton P, McGuckin MA. 2013. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. Mucosal Immunology 6:557-568 DOI 10.1038/mi.2012.98.

Silva TFA, Petrillo TR, Yunis-Aguinaga J, Marcusso PF, Claudiano GS, Moraes FR, Moraes JRE. 2015. Effects of the probiotic *Bacillus amyloliquefaciens* on growth performance, hematology and intestinal morphometry in cage-reared Nile tilapia. Latin American Journal of Aquatic Research 43:963-971 DOI 10.3856/vol43-issue5-fulltext-16.

Simó-Mirabet P, Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. 2017. Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*). Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 187:153-163 DOI 10.1007/s00360-016-1014-0.

Sitjà-Bobadilla A, Peña Llopis S, Gómez-Requeni P, Médale F, Kaushik S, Pérez-Sánchez J. 2005. Effect of fish meal replacement by plant protein sources on non-specific defense mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249:387-400 DOI 10.1016/j.aquaculture.2005.03.031.

Skřivanová E, Molatová Z, Skřivanová V, Marounek M. 2009. Inhibitory activity of rabbit milk and medium-chain fatty acids against enteropathogenic *Escherichia coli* O128. Veterinary Microbiology 135:358-362 DOI 10.1016/j.vetmic.2008.09.083.

Sturn A, Quackenbush J, Trajanoski Z. 2002. Genesis: cluster analysis of microarray data. Bioinformatics 18:207-208 DOI 10.1093/bioinformatics/18.1.207.

Suiryanrayna MVAN, Ramana JV. 2015. A review of the effects of dietary organic acids fed to swine. Journal of Animal Science and Biotechnology 6:1-11 DOI 10.1186/s40104-015-0042-z.

Sundell KS, Rønnestad I. 2011. Integrated function and control of the gut|Intestinal absorption. Encyclopedia of Fish Physiology 2:1311-1321 DOI 10.1016/B978-0-12-374553-8.00072-1.

Trushenski JT. 2009. Saturated lipid sources in feeds for sunshine bass: Alterations in production performance and tissue fatty acid composition. North American Journal of Aquaculture 71:363-373 DOI 10.1577/A09-001.1.

Vargas RJ, Dotta G, Mouriño JL, Silva BC, Fracalossi DM. 2013. Dietary lipid sources affect freshwater catfish jundiá, *Rhamdia quelen*, survival, when challenged with *Aeromonas hydrophila*. Acta Scientiarum. Animal Sciences 35:349-355 DOI 10.4025/actascianimsci.v35i4.19617.

Vega-Rubín de Celis S, Rojas P, Gómez-Requeni P, Albalat A, Gutiérrez J, Médale F, Kaushik SJ, Navarro I, Pérez-Sánchez J. 2004. Nutritional assessment of somatolactin function in gilthead sea bream (*Sparus aurata*): concurrent changes in somatotropic axis and pancreatic hormones. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 138:533-542 DOI 10.1016/j.cbpb.2004.06.007.

Wang J, Wu X, Simonavicius N, Tian H, Ling L. 2006. Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. Journal of Biological Chemistry 281:34457-34464 DOI 10.1074/jbc.M608019200.

Wang YB, Li JR, Lin J. 2008. Probiotics in aquaculture: challenges and outlook. Aquaculture 281:1-4 DOI 10.1016/j.aquaculture.2008.06.002.

Williams I, Williams KC, Smith DM, Jones M. 2006. Polka-dot grouper, *Cromileptes altivelis*, can utilize dietary fat efficiently. Aquaculture Nutrition 12:379-387 DOI 10.1111/j.1365-2095.2006.00437.x.

Zhang H, Li Y, Hou X, Zhang L, Wang T. 2016. Medium-chain TAG improve energy metabolism and mitochondrial biogenesis in the liver of intra-uterine growth-retarded and normal-birth-weight weanling piglets. The British Journal of Nutrition 115:1521-1530 DOI 10.1017/S0007114516000404.

Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, LaPatra SE, Bartholomew J, Sunyer JO. 2010. IgT, a primitive immunoglobulin class specialized in

mucosal immunity. Nature Immunology 11:827-835 DOI 10.1038/ni.1913.

Zorriehzahra MJ, Delshad ST, Adel M, Tiwari R, Karthik K, Dhama K, Lazado CC. 2016. Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. Veterinary Quarterly 36:228-241 DOI 10.1080/01652176.2016.1172132.

CAPÍTULO 6- Local DNA methylation helps to regulate muscle sirtuin 1 gene expression across season and advancing age in gilthead sea bream (*Sparus aurata*)

Paula Simó-Mirabet, Erick Perera, Josep Alvar Calduch-Giner, Jaume Pérez-Sánchez

Nutrigenomics and Fish Growth Endocrinology Group, Institute of Aquaculture Torre de la Sal, CSIC, Castellón, Spain.

En revisión en Frontiers in Zoology (2020)

Abstract

Background. Sirtuins (SIRTs) are master regulators of metabolism and their expression patterns in gilthead sea bream (GSB) disclose different tissue-metabolic capabilities and changes in energy status. Since little is known on their transcriptional regulation, the aim of this work was to study for the first time in fish the effect of age (one- and three-year-old) and season (winter and summer) on *sirt* gene expression, correlating expression patterns with local changes of DNA methylation in liver and white skeletal muscle (WSM).

Methods. The genomic organization of the seven members of the *sirt* gene family was analyzed by blat searches in the IATS-CSIC genomic database (www.nutrigroup-iats.org/seabreamdb/). The presence of CpG islands (CGIs) was mapped by means of the MethPrimer software. DNA methylation analyses were made by bisulfite pyrosequencing. A PCR-array was designed for the simultaneous gene expression profiling of *sirts* and related markers (*cs*, *cpt1a*, *pgc1α*, *ucp1, ucp3)* in liver and WSM of one- and three-year-old fish during winter and summer.

Results. The occurrence of CGIs in close association with SP1 binding sites was evidenced in *sirt1* and *sirt3* promoters. This latter CGI remained hypomethylated regardless of tissue, age and season. Conversely, the methylation signature of *sirt1* varied with age and season in the WSM. Methylation at CpG positions containing SP1 binding sites was negatively correlated with *sirt1* expression, which was lower in younger fish. DNA methylation of *sirt1* promoter at six CpG positions negatively correlated with the summer decrease of *sirt1* expression. These changes in *sirt1* regulation match well with variations in feed intake and energy

metabolism, as judged by the concurrent changes in the analyzed markers. This was supported by discriminant analyses, which identified *sirt1* as a highly responsive element to energy changes imposed by age and season in WSM.

Conclusions. The genomic organization of *SIRTs* is highly conserved through vertebrate evolution, and the presence of CGIs agrees with a more highly regulated expression of *sirt1* and *sirt3* across GSB tissues, especially in the case of *sirt1*. Correlation studies highlighted the involvement of epigenetic mechanisms in the regulation of *sirt1* expression, which reinforces its value as a fish biomarker.

Keywords: Fish; Sirtuins; DNA methylation; Epigenetic marks; Skeletal muscle; Age; Season.

6.1. Introduction

Aquaculture is a fast growing food production sector [1], but the current growing trend will rely on a deeper understanding of genetic and downstream physiological mechanisms affecting productive traits. Biomarkers that identify and follow up desired traits are, thereby, especially appropriated for the selection of environmental conditions and genotypes that promote or exhibit better physiological performances [2-4]. This is of particular relevance for productive traits related with intermediary metabolism that are not easy to measure (e.g. feed efficiency, energy status, redox homeostasis). Thus, gene expression patterns of growth-promoting factors, antioxidant markers or lipid- and energy-metabolism related markers become highly informative to disclose different metabolic features in challenged fish and higher vertebrates [5-7], but in parallel, there is a growing interest for epigenetics markers as they are relatively stable and provide information about gene function and environment interactions [8, 9].

Epigenetic mechanisms include changes in DNA methylation, histone modifications and non-coding RNAs regulation that collectively affect chromatin architecture and accessibility of the transcriptional machinery to genetic loci [10, 11]. Concretely, DNA methylation at promoter regions reduces gene expression by impairing the binding of transcriptional activators, whereas histone acetylation generally provides a permissive environment for transcription [12, 13], also as part of the DNA demethylation machinery [14]. As a consequence, differentiated cells develop a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription and the precise tuning of different

biological processes, particularly under conditions where the environment can be manipulated or natural variation exists through life cycle or livestock production [15]. In fish, good examples of this are sex determination in European sea bass [16] and tongue sole [17], early maturation [18] and muscle development in Atlantic salmon [19], larvae metamorphosis in the sea lamprey [20], growth traits and osmotic regulation in the tongue sole [21, 22], migration propensity in rainbow trout [23], as well as the adaptive plasticity to freshwater and marine conditions in stickleback [24].

Sirtuins (SIRTs), a conserved family of enzymes that couple protein deacylation with the energy status of the cell via the cellular NAD^+/NADH ratio, are part of this complex puzzle linking nutrition and energy status with epigenetic regulation [25-27]. Thus, it is known that SIRT1, 2, 6 and 7 exert different epigenetic actions via deacetylation of histones, transcription factors (TFs) or other enzymes with epigenetic roles, whereas the involvement of mitochondrial SIRTs (SIRT3-5) in epigenetic mechanisms remains still under debate [27]. Particularly in gilthead sea bream (*Sparus aurata*) (GSB), the patterns of *sirt* gene expression are powerful metabolic biomarkers at the tissue-specific level, as they disclose different energy status resulting from nutrient availability or growth potentiality [4, 28, 29]. In this fish, there is also abundant literature showing the effects of temperature over different aspects of intermediary metabolism, including changes in the expression of TFs, membrane translocases, molecular chaperones, and rate limiting enzymes of fatty acid β-oxidation and the tricarboxylic acid cycle [30, 31]. It is also known that the methylation level of genome is higher in polar fish than in temperate and tropical fish [32]. However, how temperature and other

biotic and abiotic factors affect the local DNA methylation of *sirts* or other key regulatory genes of energy metabolism remains poorly studied in fish. In contrast, in humans and rodents, there is abundant literature on the aging-mediated effects of DNA methylation and SIRT regulation and function [33]. Since methylated cytosines are found primarily at CpG dinucleotides, and CpG-rich regions (the called CG islands, CGIs) span a number of promoters of annotated genes in higher vertebrates [34], the double aim of the present study was: i) to map CGIs across the genomic sequences of GSB *sirts*, and ii) to correlate changes in gene expression and CGI methylation signatures, using as experimental model one- and three-year-old fish sampled in winter and summer. Liver and white skeletal muscle (WSM) were chosen as target tissues, providing this survey new insights in the regulation and action of *sirts* in the protandric hermaphroditic GSB, which is now highly cultured through the Mediterranean region.

6.2. Material and methods

6.2.1. Experimental fish, husbandry conditions and sampling

One- (+1) and three- (+3) year-old GSB of Atlantic origin ('strain 1' in [4]) were reared at the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC) in 3,000 L tanks under natural photoperiod and temperature conditions at the IATS-CSIC latitude (40º5'N; 0º10'E). Water temperature ranged from 10ºC in winter to 27ºC in summer. Water oxygen concentration was always higher than 75% saturation, and unionized ammonia remained below toxic levels (< 0.02

mg/L) irrespective of season. Fish were fed with a standard commercial diet (EFICO YM 568; BioMar, Dueñas, Spain) once a day until visual satiety (3, 5 or 6 times per week depending on season and fish size). In winter and summer sampling points, 10 fish per each class of age (class +1, 50-115 g body weight; class +3, 1 kg body weight) were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 100 μg/mL), and liver and WSM were rapidly excised, frozen in liquid nitrogen and stored at -80ºC until RNA and DNA extraction.

6.2.2. *In silico* analyses

The genomic organization of GSB *sirts* was analyzed by blat searches in the IATS-CSIC genomic database of GSB (http://nutrigroup-iats.org/seabreamdb/). The retrieved sequences were manually curated by aligning genome sequences (Clustal X) with GSB *sirts* transcripts [28], using the online tool FGENESH from softberry for predicting gene structure [35]. For comparative purposes, the seven human and zebrafish SIRT counterparts were obtained from ENSEMBL database (http//www.ensembl.org). Graphical representations were carried out with the online tool Exon-Intron Graphic Maker (http://wormweb.org/exonintron). Polyadenylation sites were identified by means of the Softberry POLYAH (http://www.softberry.com). Predictions of putative transcription start sites (TSSs) were done by means of Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/) [36]. Core promoter regions were predicted by using two complementary approaches: Easy Promoter Prediction Program (EP3), which uses GC content and structural features of DNA to identify promoter regions [37] and MatInspector (www.genomatrix.de), which searches transcription

factor binding sites (TFBSs) and TFBSs containing-promoter modules. In addition to TFBSs retrieved from MatInspector, searches in a ~1 kb region upstream TSS, and in the first exon, for TFBSs known to be present in *SIRTs* promoters of higher vertebrates were done by ConTra v3 (http://bioit2.irc.ugent.be/contra/v3/#/step/1) [38] using the TRANSFAC database, with sensitivity and accuracy set at core match = 0.95 and matrix match = 0.85. Predictions of CGIs through the entire gene, including a 2 kb region upstream TSS, were done by means of MethPrimer software (http://www.urogene.org/methprimer/). Search parameters used were: length ≥ 200, C+G content ≥ 50%, ratio of observed/expected CpGs ≥ 0.60 and window size = 100.

6.2.3. DNA isolation and bisulfite conversion

DNA was extracted using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. Quantity and quality of DNA were assessed by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and DNA integrity was analyzed in a 1% agarose gel. Extracted DNA was bisulfite converted using the EZ DNA Methylation Gold bisulfite conversion kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions.

6.2.4. PCR of bisulfite-converted DNA

Primers were designed using the PyroMark Assay Design 2.0.01.15 (Qiagen, Hilden, Germany) to hybridize CpG-free sites at the highest melting temperature (**Table 6.1**). Reverse primers were labelled with biotin at the 5'-end and bisulfite-converted DNA was amplified by PCR,

using the Invitrogen™ Platinum™ Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with forward- and reverse-specific primers at 1 μM each in a total volume of 25 μL. The reaction was performed in a Touchgene Gradient Thermal Cycler (Techne, Cambridge, UK) as follows: 95ºC for 5 min, followed by 35 cycles of 95ºC for 45 s, 60ºC for 45 s, and 72ºC for 1.5 min with a final extension at 72ºC for 5 min. PCR products were checked by 1% agarose gels to ensure specificity before pyrosequencing.

6.2.5. Pyrosequencing and DNA methylation analyses

Pyrosequencing analysis was performed as described before [39]. Briefly, primers for pyrosequencing (**Table 6.1**) were designed using the PyroMark assay design 2.0.01.15. The Vacuum Prep Tool (Biotage, Uppsala, Sweden) was used to prepare single-stranded PCR products according to manufacturer's instructions. Pyrosequencing reactions were performed in a PyroMark Q24 System version 2.0.6 (Qiagen, Hilden, Germany). Data were analyzed using PyroMark Q24 software and the quantification of methylation was attained from the average of individual CpGs included in the analyzed sequence.

Table 6.1 Forward and reverse PCR primers, pyrosequencing primers and sequence to analyze.

Gene name	Primers	Sequence	CpGs
sirt1	Forward outer	AGGGATGTATTTATAAAGTTTATATGTTGT	
	Reverse outer	TTAATAAAACAAACAATTCCCACTC	
	Forward inner 1p1 (nested)	TGTTTTATGTAAATGAGTTAGTTGT	
	Reverse inner 1p1 (nested)	CTCCCAACTCTCAATAACCCC *BIOTIN	
	Pyrosequencing left	GAGAGGAAGGATTTGTTTA	
	Sequence to analyze left	G**YG**GTTTTG**Y G**GGTTGAAGA TGG**YG**GA**YG**G AGAGAGTAGT TT**YG**GAA**YG**G TTTTTTTAGG	6
	Pyrosequencing right	AAAAAGGTRGAAAATTAG	
	Sequence to analyze right	TT**YG**TTGATT AATTA**YG**GAT TTAAAGT**YG**T TAAAGYGGAT TAGTTATTAG GTTTTT	3
	Forward inner 1p2 (nested)	GGGTTATTGAGAGTTGGGAGG	
	Reverse inner 1p2 (nested)	TAATAAAACAAACAATTCCCACTCC *BIOTIN	
	Pyrosequencing left	TTGAGAGTTGGGAGG	
	Sequence to analyze left	**YG**G**YG**GTGGA TTGTG**YG**TAG TTAG**YG**GAGA AGGAAG**YG**AA GT**YG**GTGATG G**YG**GTAGAGT AGGTTTTAG	7
	Pyrosequencing right	GTATAAATTAGTAGTGAAATTAGA	
	Sequence to analyze right	**YG**A**YG**G**YG**TT GTGTT**YG**GGA TAAT**YG**AGGA GGGTGTTGGT ATGTAAAGAG	5
sirt3	Forward outer	ATTGTTGAAATGTATTTTTTGTTGGT	
	Reverse outer	CCTCACCTACCTAACTCTCCAATTA	
	Forward inner (nested)	AGAAAGAAGTTAAGTGAAGTATAAATATTT	
	Reverse inner (nested)	CATAAACTCCAACAACAATAAAAAC *BIOTIN	
	Pyrosequencing	GTGATAGTTTTGTTTTTTAAAGT	
	Sequence to analyze	TTT**YG**GTTTT AGGT**YG**TTG**Y G**TGTAGATAA AGTT**YG**TGTT TTTTAGGAGA AGGAGA	4

*Biotinylated and HPLC purified.

6.2.6. Gene expression analyses

RNA was extracted using the MagMAX-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50-100 µg, with absorbance ratios (A260/A280) of 1.9-2.1. RNA integrity number (RIN) values of 8-10 (Agilent 2100 Bioanalyzer; Agilent, Santa Clara, CA, USA) were indicative of clean and intact RNA. Reverse transcription of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Negative control reactions were run without reverse transcriptase. A 96-well PCR-array of 11 markers of metabolic condition was designed for the simultaneous gene expression profiling of liver and WSM. Two housekeeping genes (β-Actin and rRNA 18S) and controls of PCR performance were included in each array. Briefly, 660 pg of total cDNA was used in 25 µL PCR reactions. PCR wells contained 2x SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 µM (**Table 6.2**). All pipetting operations for the PCR-arrays were performed by an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. Real-time quantitative PCR was carried out in an Eppendorf Mastercycler Ep Realplex (Eppendorf, Hamburg, Germany). The PCR amplification program consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. The efficiency of the PCR reactions was consistently higher than 90% and similar among all the genes. The specificity of the reactions was verified by melting curve analysis (ramping rates of 0.5°C/10 s over a temperature range of 55-

95°C). Negative controls without a template were performed for each primer set. Gene expression was calculated using the delta-delta Ct method [40]. For multi-gene analysis, all values for a given tissue were referenced to the summer expression level of *sirt1* in +3 fish, for which a value of 1 was arbitrarily assigned. Fold-changes in gene expression were calculated as the expression ratio between +3/+1 fish. A value > 1 indicates higher expression levels in +3 fish, and values < 1 indicate lower expression levels in +3 fish.

6.2.7. Statistical analysis

Normality and equal variance of data were tested by Shapiro-Wilk and Levene tests, respectively. Changes in DNA methylation at individual CpG sites were analyzed by Student's t-test. The effect of age and season on gene expression of *sirts* and related markers in liver and WSM was analyzed by Student's t-test and two-way analysis of variance. The relationship between site-specific DNA methylation and gene expression was assessed by Pearson correlation analysis. The significance level was set to $P < 0.05$ for all tests performed. These analyses were made using SigmaPlot version 13.0 (Systat Software, San Jose, CA, USA). Supervised multivariate analysis partial least-squares discriminant analysis (PLS-DA) was applied using EZ-INFO® v3.0 (Umetrics, Umeå, Sweden) to depict the contribution of analyzed genes to the group discrimination. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To discard the possibility of over-fitting of the supervised model, a validation test consisting in 500 random permutations was performed using SIMCA-P+ v11.0 (Umetrics, Umeå, Sweden). The

relative relevance of genes in the discriminant functions was assessed by the Variable Importance in the Projection (VIP) values. A VIP score > 1 was considered to be an adequate threshold to determine discriminant variables in the PLS-DA model [41-43].

Table 6.2 Forward and reverse primers for liver and white skeletal muscle pathway-focused qPCR array.

Gene name	Symbol		Primer sequence
Sirtuin1	*sirt1*	F	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC
		R	CCT CAG AAT GGT CCT CGG ATC GGT CTC
Sirtuin2	*sirt2*	F	GAA CAA TCC GAC GAC AGC AGT GAA G
		R	AGG TTA CGC AGG AAG TCC ATC TCT
Sirtuin3	*sirt3*	F	CTG CCA AGT CCT CAT CCC
		R	CTT CAC CAG ACG AGC CAC
Sirtuin4	*sirt4*	F	GGC TGG CGG AGT CGG ATG
		R	TCC TGA ATA CAC CTG TGA CGA AGA C
Sirtuin5	*sirt5*	F	CAG ACA TCC TAA CCC GAG CAG AG
		R	CCA CGA GGC AGA GGT CAC A
Sirtuin6	*sirt6*	F	ACT CCA CCA CCA CCG ATG TCA A
		R	CTC CTC CTC CTT CAC CTT TCG CTT TG
Sirtuin7	*sirt7*	F	CTG GAG CAA CCT CTA AAC TGG AA
		R	CAC CTT CAG ACT GGA GCC TAA
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	F	CGT GGG ACA GGT GTA ACC AGG ACT C
		R	ACC AAC CAA GGC AGC ACA CTC TAA TTC T

(Continued)

Table 6.2 Continued

Citrate synthase	*cs*	F	TCC AGG AGG TGA CGA GCC
		R	GTG ACC AGC AGC CAG AAG AG
Uncoupling protein1	*ucp1*	F	GCA CAC TAC CCA ACA TCA CAA G
		R	CGC CGA ACG CAG AAA CAA AG
Uncoupling protein3	*ucp3*	F	AGG TGC GAC TGG CTG ACG
		R	TTC GGC ATA CAA CCT CTC CAA AG
Carnitine palmitoyltransferase 1A	*cpt1a*	F	GTG CCT TCG TTC GTT CCA TGA TC
		R	TGA TGC TTA TCT GCT GCC TGT TTG
rRNA 18S	*rRNA 18S*	F	GCA TTT ATC AGA CCC AAA ACC
		R	AGT TGA TAG GGC AGA CAT TCG
β-Actin	*actb*	F	TCC TGC GGA ATC CAT GAG A
		R	GAC GTC GCA CTT CAT GAT GCT

6.3. Results

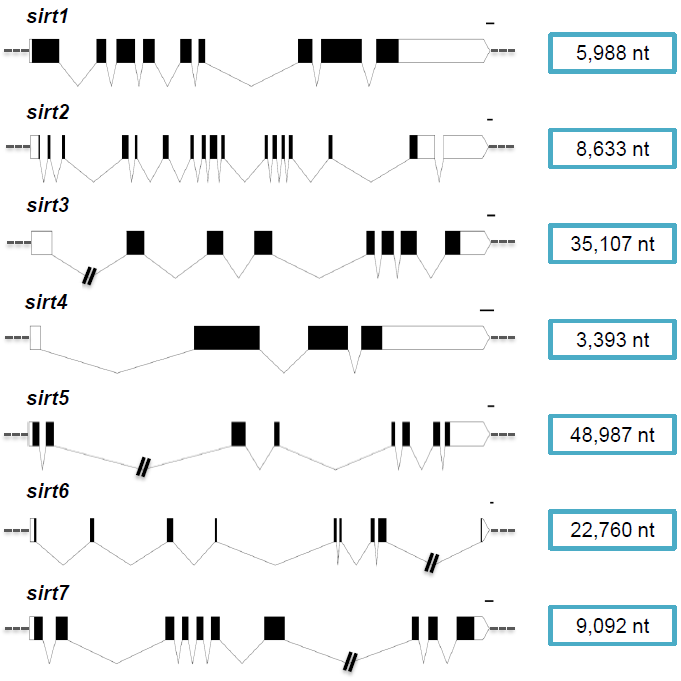
6.3.1. Sirtuin gene structure and regulatory elements

The seven *sirt* gene sequences of GSB were uploaded to GenBank with accession numbers MN123792-MN123798. These genes have a variable number of exons that ranges from 3 in *sirt4* to 16 in *sirt2* (**Fig. 6.1A**). When comparisons were made within and among the seven *SIRT* orthologous genes of human, zebrafish and GSB, the entire gene length varies from 1.4 kb to 48.3 kb, with the exception of *sirt6* of zebrafish, which contains several long introns that increase the gene length from

the start to the stop codon up to 136.5 kb. Despite this, the number and length of exons seems to be highly conserved for each *SIRT* gene through vertebrate evolution (**Fig. 6.1B**).

The occurrence of CGIs close to TSSs of GSB *sirts* was evidenced in the case of *sirt1* and *sirt3*. The CGI of GSB *sirt1* is 473 base pairs (bp) in length, comprising 100 bp of the 5' untranslated region (5'UTR), 349 bp downstream the ATG start codon and 24 bp from the first intron (**Fig. 6.2A**). The GSB *sirt3* have a shorter CGI (229 pb), which comprises 118 bp from the 5'flanking region and 111 bp downstream the TSS, corresponding to the first non-coding exon (**Fig. 6.3A**). Further analysis highlighted a *sirt1* gene structure with an open reading frame (ORF) of 2093 bp and a 3'UTR of 1583 bp until the predicted canonical polyadenylation signal. Likewise, *sirt3* contains an ORF of 1355 bp and a 3'UTR of 411 bp until the predicted canonical polyadenylation signal. Searches for regulatory elements in the promoter region of *sirt1* (~1 kb upstream TSS) and in the first exon, predict a wide-range of multiple *cis*- regulatory elements (i.e., HIF1, P53, C/EBP-α, GATA2, MYOD, FOXO1, AML1, PPARγ, GATA1, HNF1, NFκB, ETS, SP1, OCTAMER, PIT1, XBP1, MYC and CHREBP) (**Fig. 6.2B**). Some of them were also retrieved in the promoter region of *sirt3* (i.e., GATA1, GATA2, OCTAMER, HNF1, AML1, NFκB, MYC, SP1), while others seem to be exclusive of *sirt3* (i.e., TBP, AP1, PBX1, CREB, NRF2, HTF, CHREBP, ZF5, SOX6, ERR-α, and MYF) (**Fig. 6.3B**).

A



B

CODIFYING EXONS	*sirt1*	*sirt2*	*sirt3*	*sirt4*	*sirt5*	*sirt6*	*sirt7*
GSB	9 (4.8)	16 (7.3)	7 (4.5)	3 (1.4)	8 (48.3)	9 (22.4)	10 (8.9)
HUMAN	9 (31.9)	16 (20.3)	7(19.6)	3 (9.5)	8 (27.8)	8 (7.9)	10 (5.7)
ZEBRAFISH	9 (17.4)	16 (19.8)	8 (23.5)	3 (4.9)	9 (13.6)	8 (136.5)	10 (10.4)

Figure 6.1 Gene organization of gilthead sea bream *sirts*. A Schematic representation of the exon-intron structure of the seven *sirt* genes of gilthead sea bream. White and black boxes represent the non-codifying and codifying exons, respectively. Introns are shown as connecting lines. Numbers show total length of the sequences in our database. Scale bars are 100 bp. **B** Number of coding exons and gene size (in kb) from ATG to stop codon in brackets.

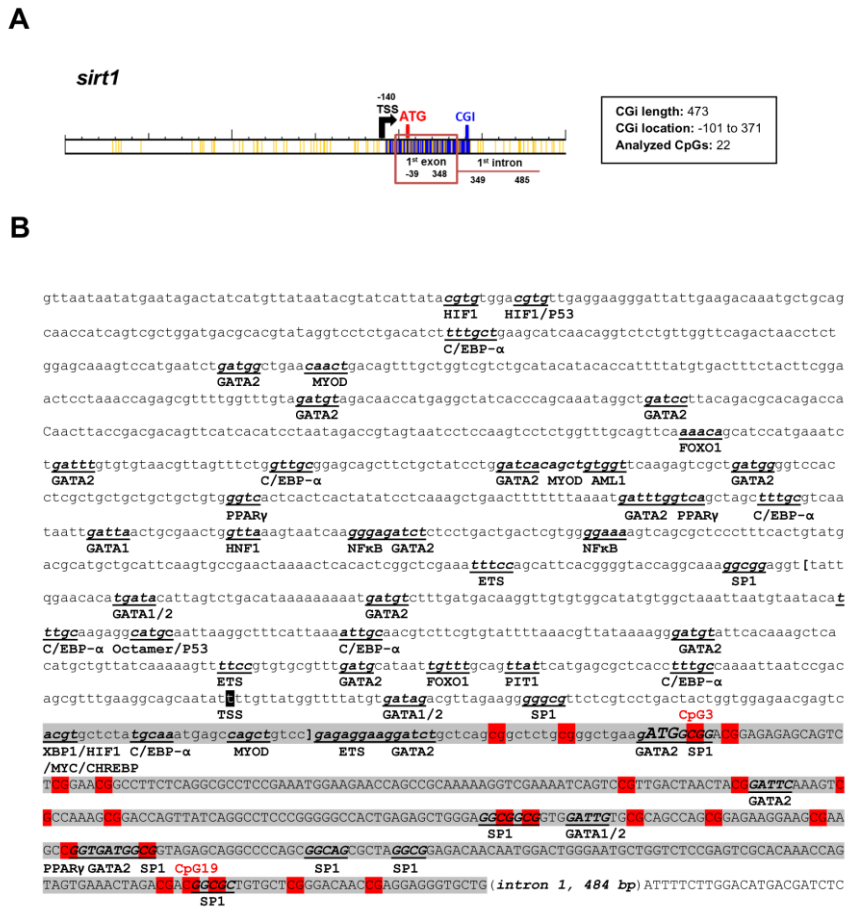


Figure 6.2 CpG islands and regulatory elements in *sirt1* of gilthead sea bream. A Schematic representation of CpG island (CGI) in *sirt1* of gilthead sea bream. Yellow lines represent CpG sites. Numbers indicate the position of the putative transcription start site (TSS, black arrow) and the start and the end of the first exon and intron respect to the start codon (ATG, red). **B** Regulatory elements in *sirt1* of gilthead sea bream. First intron sequence was replaced by indication to their length. Lower case letters indicate a 2 kb sequence upstream the translational start site ATG, and includes the 5' UTR and the 5' flanking region. Predicted core promoter region is flanked by brackets. Putative TSS is shaded in black. Predicted regulatory elements are indicated in bold, underlined, and lower case italics. CGI is shaded in grey. The analyzed CpG positions are shaded in red. This CGI spans the first exon and expands into the first intron (not shown).

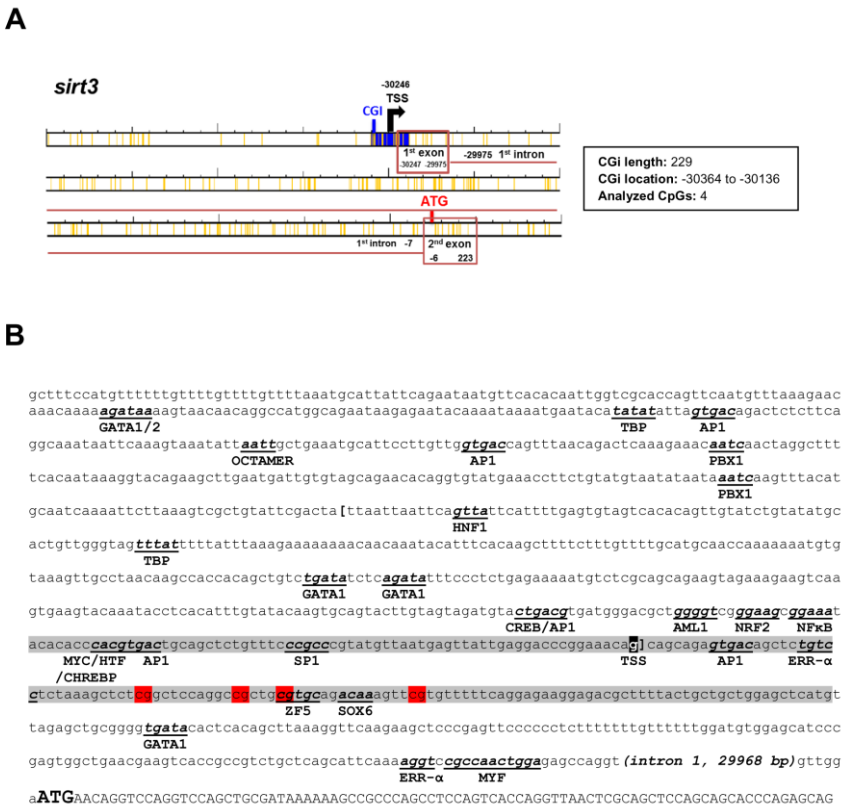


Figure 6.3 CpG islands and regulatory elements in *sirt3* of gilthead sea bream. A Schematic representation of CpG island (CGI) in *sirt3* of gilthead sea bream. Yellow lines represent CpG sites. Numbers indicate the position of the putative transcription start site (TSS, black arrow) and the start and the end of the first exon and intron respect to the start codon (ATG, red). **B** Regulatory elements in *sirt3* of gilthead sea bream. First intron sequence was replaced by indication to their length. Lower case letters indicate a 2.37 kb sequence upstream the translational start site ATG, and includes the first exon and the first intron, as well as the 5' UTR and the 5' flanking region. Putative TSS is shaded in black. Predicted regulatory elements are indicated in bold, underlined, lower case italics. CGI is shaded in grey. The analyzed CpG positions are shaded in red.

6.3.2. Sirtuin 1 and 3 promoter methylation

Up to 4 CpG sites at the CGI of *sirt3* promoter were chosen for methylation analyses of liver and WSM in fish of two classes of age at two critical windows along the production cycle (winter and summer). These CpGs remained in general hypomethylated regardless of age, tissue and season (**Fig. 6.4**).

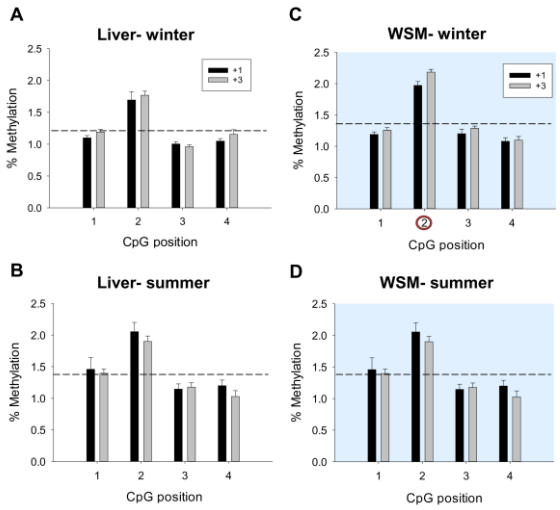


Figure 6.4 Age and seasonal changes in DNA methylation of gilthead sea bream *sirt3*. Site-specific-DNA methylation (%) of *sirt3* in liver (**A**, **B**) and white skeletal muscle (WSM) (**C**, **D**) of fish with different ages (+3, three-year-old; +1, one-year-old) in winter and summer. Data are the mean ± SEM of 8-10 fish. CpG position with a circle means statistical differences between ages by t-test ($P < 0.05$). Dashed lines indicate the mean methylation of all individuals and positions.

However, analyzing individual positions, the CpG2 site consistently showed the highest methylation level though it did not match with key TFBSs (**Fig. 6.3B**). The CGI of *sirt1* promoter was also hypomethylated without a clear pattern of methylation at individual CpG sites for the 22 analyzed positions in liver and WSM (**Fig. 6.5**). Moreover, this CGI

remained hypomethylated in all the CpG sites when comparisons are made at the hepatic level between the two groups of age in both winter and summer (**Fig. 6.5A**, **B**). The same was found in the WSM for fish sampled in winter (**Fig. 6.5C**). However, in the summer sampling point, 15 out of 22 CpG sites (69%) shared an increased methylation in young fish (**Fig. 6.5D**). This observation was more evident for the first three CpG sites, reaching CpG2 and CpG3 sites the highest level of methylation (~ 4%).

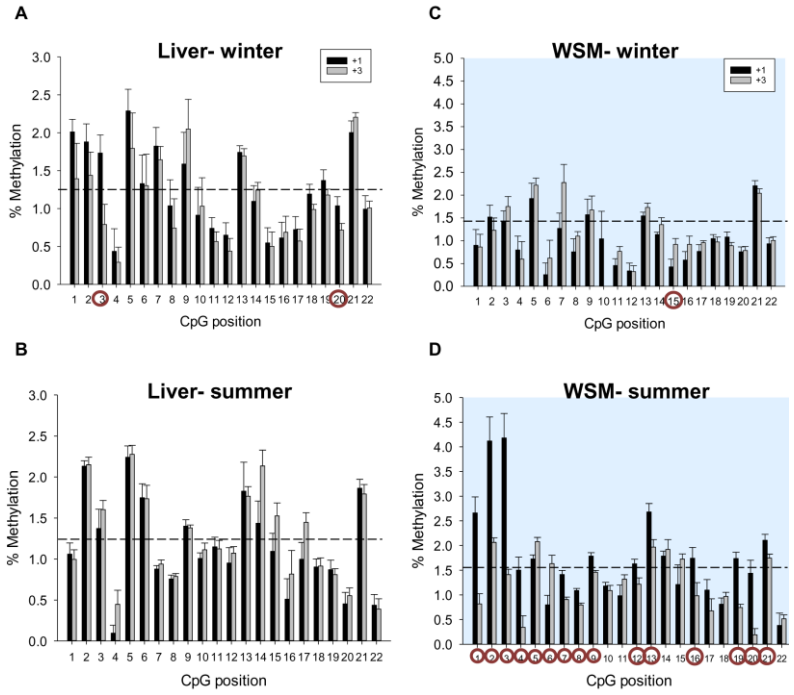


Figure 6.5 Age and seasonal changes in DNA methylation of gilthead sea bream *sirt1*. Site-specific-DNA methylation (%) of *sirt1* in liver (**A**, **B**) and white skeletal muscle (WSM) (**C**, **D**) of fish with different ages (+3, three-year-old; +1, one-year-old) in winter and summer. Data are the mean ± SEM of 8-10 fish. CpG position with a circle means statistical differences between ages by t-test ($P < 0.05$). Dashed lines indicate the mean methylation of all individuals and positions.

The methylation level of CpG19 and CpG20 sites was also reduced by age, being SP1 binding sites close or within all these responsive positions. This observation was reinforced by regression and Pearson correlation analyses, which showed across individuals a statistically significant negative correlation between WSM *sirt1* gene expression and the methylation level of *sirt1* CGI promoter at CpG3 (R = -0.66; P = 0.01) (**Fig. 6.6**). A negative correlation was also found at CpG19 (R = -0.74; P = 0.004) site despite of its lower methylation level. When comparisons were made on a seasonal basis, the DNA methylation was increased in summer (**Fig. 6.7A**). This was especially evident in class +1, and a consistent negative correlation was found between *sirt1* gene expression and the averaged methylation level of CpG2, 3 sites (R = -0.658; P = 0.008) (**Fig. 6.7B**). The same trend with a lower level of methylation was reported for CpG12-14, 16 sites (R = -0.679; P = 0.002), being these positions close or within SP1 binding sites.

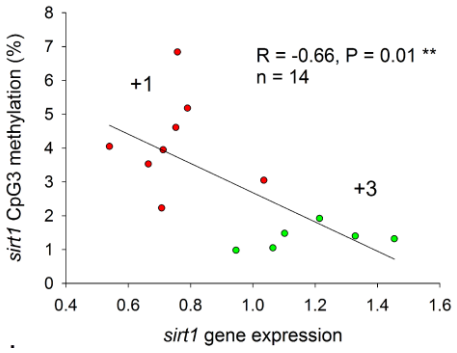


Figure 6.6 Summer correlation of local DNA methylation and *sirt1* gene expression in gilthead sea bream muscle. Correlation between DNA methylation at CpG3 in *sirt1* gene promoter region and *sirt1* gene expression in white skeletal muscle (WSM) of gilthead sea bream during summer. Green and red points represent data from +3, three-year-old and +1, one-year-old, respectively.

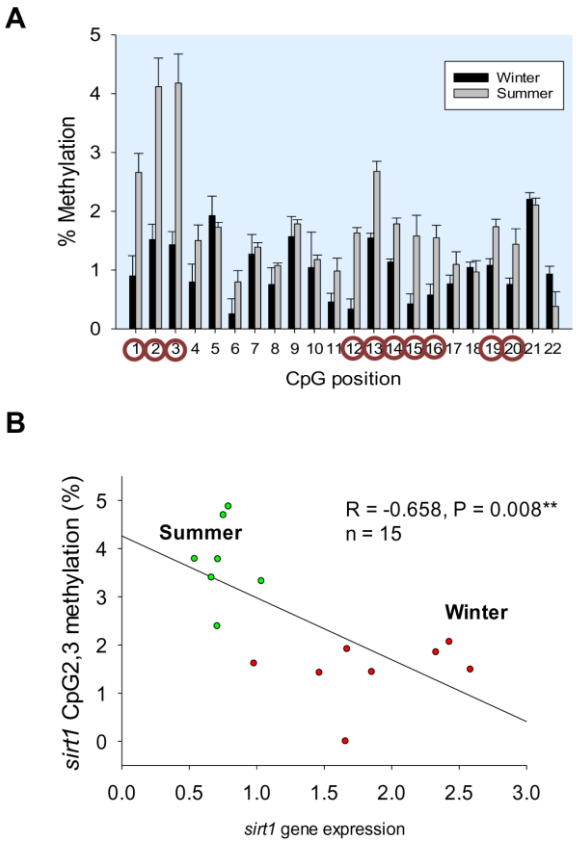


Figure 6.7 Seasonal local DNA methylation and correlation with *sirt1* gene expression in muscle of one-year-old fish. A Site-specific-DNA methylation (%) of *sirt1* in white skeletal muscle (WSM) of one-year-old fish in winter (black bars) and summer (grey bars). Data are the mean ± SEM of 8-10 fish. CpG position with a circle means statistical differences between seasons by t-test ($p < 0.05$). **B** Correlation between mean DNA methylation of SP1-related CpG sites (CpG2, 3) in *sirt1* gene promoter region and *sirt1* gene expression in white skeletal muscle (WSM) of one-year-old fish. Red and green points represent data from winter and summer, respectively.

6.3.3. Gene expression profiling

Gene expression profiling of all members of the *sirt* gene family in combination with related markers of energy metabolism is summarized in **Table 6.3**. A statistically significant effect of season (two-way ANOVA) was found in both liver and WSM for almost all the analyzed genes, with the exception of *sirt7* and *pgc1α* in liver and *sirt2* in WSM. However, the age effects became more evident for *sirt* genes in WSM rather than in liver. Conversely, markers of fatty acid β-oxidation (*cpt1a*) and mitochondrial uncoupling respiration (*ucp1*) were more responsive to age-mediated changes in liver than in WSM. Also, as a general feature, most genes included in the array were down-regulated by age at the hepatic level (**Fig. 6.8A**, **6.8B**). This was especially evident for *sirt1*, *sirt2, sirt5, cpt1a* and *ucp1*, which were consistently down-regulated in class +3 in both winter and summer. In contrast, in WSM, most of the genes included in the array were up-regulated by age in winter (**Fig. 6.8C**). This also applied in summer to *sirt1, sirt2, sirt5, sirt6* and *sirt7*, but the opposite was found for *cpt1a* and *ucp3* (**Fig. 6.8D**). Of note, most of these age-mediated changes in gene expression were accentuated in winter as indicates the statistically significant interaction of age and season in the two-way ANOVA (**Table 6.3**).

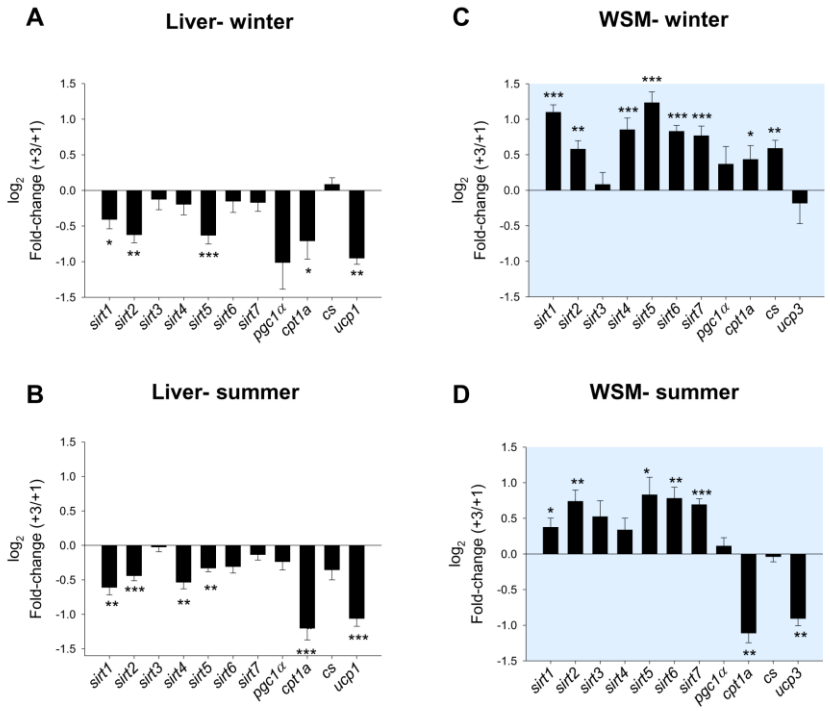


Figure 6.8 Age and seasonal gene expression changes of *sirts* and related markers in gilthead sea bream. For each season (winter and summer), fold-changes (+3, three-year-old/ +1, one-year-old) of expression in liver (**A**, **B**) and white skeletal muscle (WSM) (**C**, **D**) is shown. Asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01, ***P < 0.001) between ages. Values > 1 indicate up-regulated genes in +3 fish; values < 1 indicate down-regulated genes in +3 fish.

Table 6.3 Relative gene expression of liver and white skeletal muscle (WSM) of gilthead sea bream. Data are the mean ± SEM of 10 fish of different ages (+3, three-year-old; +1, one-year-old) sampled in winter (W) and summer (S). P-values are the result of two-way analysis of variance.

	Winter		Summer		Two-way ANOVA		
	W+3	W+1	S+3	S+1	Season	Age	Interaction
LIVER							
sirt1	2.53 ± 0.24	3.22 ± 0.21	1.02 ± 0.08	1.52 ± 0.11	**<0.001**	**0.002**	0.597
sirt2	4.15 ± 0.33	6.20 ± 0.46	5.21 ± 0.27	6.99 ± 0.27	**0.012**	**<0.001**	0.697
sirt3	1.54 ± 0.16	1.60 ± 0.12	0.81 ± 0.04	0.82 ± 0.04	**<0.001**	0.762	0.799
sirt4	0.59 ± 0.06	0.64 ± 0.07	0.34 ± 0.02	0.48 ± 0.03	**<0.001**	0.081	0.406
sirt5	6.86 ± 0.54	10.3 ± 0.52	5.47 ± 0.21	6.83 ± 0.26	**<0.001**	**<0.001**	**0.014**
sirt6	0.69 ± 0.08	0.72 ± 0.05	0.42 ± 0.03	0.51 ± 0.05	**0.001**	0.531	0.935
sirt7	1.07 ± 0.08	1.17 ± 0.07	1.11 ± 0.06	1.20 ± 0.04	0.177	0.385	0.795
pgc1α	3.29 ± 0.90	5.03 ± 0.63	4.14 ± 0.33	4.75 ± 0.32	0.633	0.058	0.350
cpt1a	20.1 ± 3.21	28.7 ± 2.37	3.94 ± 0.48	8.57 ± 0.98	**<0.001**	**0.004**	0.357
cs	30.1 ± 1.95	27.9 ± 1.18	16.3 ± 1.84	19.9 ± 1.32	**<0.001**	0.666	0.077
ucp1	261.4 ± 16.1	496.2 ± 44.8	603.2 ± 45.9	1228.3 ± 105.9	**<0.001**	**<0.001**	**0.003**
WSM							
sirt1	3.90 ± 0.30	1.78 ± 0.17	1.04 ± 0.09	0.77 ± 0.05	**<0.001**	**<0.001**	**<0.001**
sirt2	3.23 ± 0.27	2.10 ± 0.14	3.44 ± 0.39	1.96 ± 0.08	0.884	**<0.001**	0.488
sirt3	0.65 ± 0.06	0.58 ± 0.06	0.35 ± 0.06	0.22 ± 0.01	**<0.001**	0.081	0.548
sirt4	0.60 ± 0.06	0.31 ± 0.03	0.39 ± 0.04	0.29 ± 0.03	**0.017**	**<0.001**	**0.044**
sirt5	12.9 ± 1.37	5.19 ± 0.43	5.48 ± 1.02	2.72 ± 0.31	**<0.001**	**<0.001**	**0.011**
sirt6	0.55 ± 0.03	0.31 ± 0.03	0.32 ± 0.03	0.18 ± 0.02	**<0.001**	**<0.001**	0.092
sirt7	1.77 ± 0.16	1.00 ± 0.07	0.71 ± 0.04	0.43 ± 0.03	**<0.001**	**<0.001**	**0.014**
pgc1α	6.32 ± 0.83	4.40 ± 0.80	2.82 ± 0.27	2.55 ± 0.25	**0.002**	0.050	0.500
cpt1a	71.6 ± 8.58	49.2 ± 5.78	4.69 ± 0.48	9.78 ± 1.17	**<0.001**	0.112	**0.020**
cs	235.2 ± 19.4	152.0 ± 12.8	63.9 ± 3.11	64.9 ± 4.01	**<0.001**	**0.002**	**0.006**
ucp3	173.0 ± 33.1	165.1 ± 10.8	25.1 ± 1.74	46.1 ± 2.38	**<0.001**	0.794	0.498

This temporal and tissue-specific regulation of gene expression is reinforced by multivariate analysis. In liver tissue, *ucp1, cpt1a* and *cs* loadings predicted most of the observed variance, with a poor contribution of *sirts,* which was reduced to *sirt5* after considering five components in the PLS-DA (**Fig. 6.9**). In contrast, in WSM, the first three components showed cumulative values for R2Y (explained variance) and Q2 (predicted variance) of 69% and 59%, respectively (**Fig. 6.10A**). With this dataset, the separation along the first component explained 28% of total variance separating groups by season (winter *vs*

summer), whereas component 2 explained 27% of variance separating groups by age in both winter and summer (**Fig. 6.10B**). In this scenario, genes with a contribution to VIP > 1 in component 1 are a total of 6 with a main contribution of *sirt1, sirt5, sirt6* and *sirt7*. When the second component was also considered, a total of 4 genes (*sirt2, sirt4, ucp3* and *cpt1a)* showed VIP values > 1 (**Fig. 6.10C**).

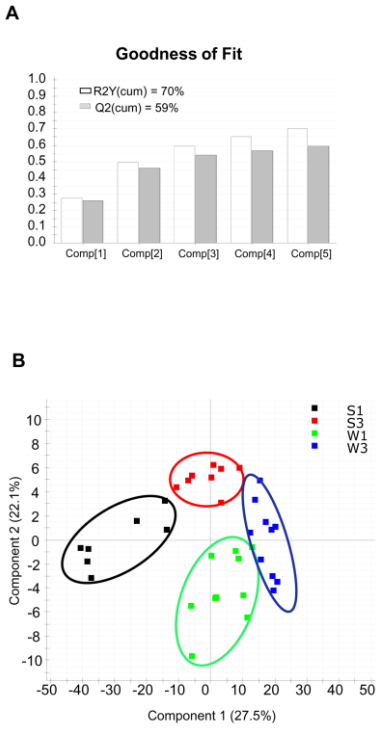
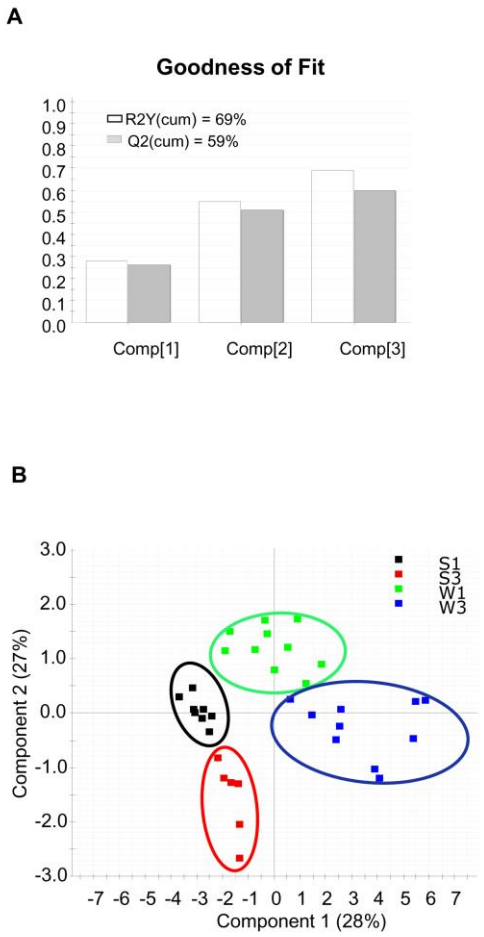


Figure 6.9 Discriminant analysis (PLS-DA) of molecular signatures in liver of gilthead sea bream. Data consist of relative expression of the 11 genes included in the array from fish of different ages (3, three-year-old; 1, one-year-old) in two seasons (summer, S, and winter, W). **A** Cumulative coefficients of goodness of fit (R2Y, white bars) and prediction (Q2, grey bars) by each component. **B** PLS-DA score plot of acquired data from fish of +3 and +1 years in summer and winter along the two main components, explaining 49.6% of total variance. **C** Ordered list of markers by variable importance (VIP) in the projection of PLS-DA model for group differentiation.



C

Genes, VIP > 1

After Comp 1	
sirt1	1.21
sirt7	1.17
cs	1.15
sirt5	1.10
sirt6	1.10
cpt1a	1.06
After Comp 2	
sirt2	1.43
ucp3	1.22
sirt4	1.04
cpt1a	1.03
After Comp 3	
sirt2	1.53
sirt3	1.21
ucp3	1.15

Figure 6.10 Discriminant analysis (PLS-DA) of molecular signatures in white skeletal muscle of gilthead sea bream. Data consist of relative expression of the 11 genes included in the array from fish of different ages (3, three-year-old; 1, one-year-old) in two seasons (summer, S, and winter, W). **A** Cumulative coefficients of goodness of fit (R2Y, white bars) and prediction (Q2, grey bars) by each component. **B** PLS-DA score plot of acquired data from fish of +3 and +1 years in summer and winter along the two main components, explaining 55% of total variance. **C** Ordered list of markers by variable importance (VIP) in the projection of PLS-DA model for group differentiation.

6.4. Discussion

6.4.1. Genomic organization of vertebrate *SIRTs* is highly conserved

SIRTs are widely conserved among living organisms with a number that ranges from one in bacteria to seven in vertebrates [44], evidencing this gene expansion the important role of SIRTs as key components of energy metabolism in all the studied living organisms. Certainly, the seven GSB counterparts of mammalian SIRTs have a conserved Rossman fold domain, commonly found in proteins that bind NAD^+ or $NADP^+$ [45]. Furthermore, phylogenetic analysis of GSB Sirts rendered monophyletic clusters [28] according to the four SIRT classes described by [46]. This conserved feature across vertebrate evolution is also extensive to genomic organization as it was illustrated herein by the comparisons made among GSB, zebrafish and human *SIRTs*, with an exon length that is within the averaged size (170 nt) for the annotated genes in higher vertebrate species [47, 48]. In contrast, the size of introns for the members of the *SIRT* gene family is highly variable within and among species probably due to the less functional constraints of non-codifying DNA regions [49]. Of note, the recognized top TFBSs for human *SIRT1* (C/EBP-α, MYOD and MYC) and *SIRT3* (AML1, CREB, HTF, NRF2, PBX1, TBP) [50] were also retrieved in GSB close to the predicted TSS of *sirt1* and *sirt3*. Furthermore, in different models of human and rodent metabolic homeostasis, it has been proven that SIRT1 deacetylase activity modulates the function of most transcriptional regulators (e.g. NF-κB, p53, FOXO1, PPARγ, CHREBP, HIF1 and C/EBP-α) forming negative-feedback loops [51] which

probably also occurs in fish. This notion is reinforced by the large number of enzymes of intermediary metabolism (more than 4,000 acetylated proteins in rat tissues) that are susceptible to SIRT deacetylation [52, 53], making possible the complex crosstalk between SIRTs and a wide-range of biological processes [54].

6.4.2. CGIs in *SIRT* promoters appeared early through vertebrate evolution

The number and location of CGIs is very similar in humans and mouse [55], acting most "orphan" CGIs (far from annotated promoters) as alternative promoters for novel transcripts (e.g. miRNAs) that might have regulatory significance [56]. In contrast, the number and density of CGIs is highly variable among fish genomes [57, 58], though interspecies experiments in human, mouse and zebrafish demonstrate that classical CGI sequences can be interpreted as an evolutionary conserved mechanism that protects DNA for methylation to shape the usual epigenome during development [59]. This notion fits well with the early appearance of CGIs during vertebrate evolution, being associated this feature with an increased concentration from cold-blooded vertebrates to warm-blooded vertebrates of CGIs close to TSSs [60]. To the best of our knowledge this general trend has not been assessed in *SIRT* genes, but interestingly the occurrence of CGIs has been reported in *SIRT* promoters of humans [61-65], mouse [66] and ruminants [67-69]. In contrast, in GSB, the occurrence of CGIs, in close association with SP1 binding sites, was only found in the case of *sirt1* and *sirt3*. With the same search criteria, CGIs were also retrieved in the *sirt1* promoter of fugu (Gene ID: 101061405), zebrafish (Gene ID: 797132), tilapia (Gene ID:

100700447), Atlantic salmon (Gene ID: 106576833) and Australian ghostshark (Gene ID: 103181092), which has a basal position in vertebrate evolution respect to bony fish. However, the CGI of GSB *sirt3* promoter did not appear to be conserved in all fish species, which can evidence a different regulation of energy metabolism through the fish lineage.

6.4.3. CGIs allow differential *sirt* gene regulation in GSB

CGIs allow promoter function by destabilizing nucleosomes and attracting proteins that create a transcriptionally permissive chromatin state [55]. Indeed, CGIs co-localize with a lot of promoters in both the human and mouse genomes, which is compatible with the idea that CGI promoters support a transcriptionally permissive state [55, 70, 71]. According to this, CGIs should be appropriate for modulating genes that are required to be expressed ubiquitously irrespective of cell type. In contrast, promoters without CGIs should be more suitable for mounting responses to external/internal cues, because their transcriptional on/off status could be more strictly controlled depending on the situation [60]. However, this feature is more complex than initially expected because *sirts* with/without GGI promoters apparently co-exist in GSB to assure a ubiquitous and perhaps highly regulated activity in different tissues and cellular locations. Certainly, all *sirts* are expressed at detectable levels in 14 analyzed GSB tissues [28], and multivariate analysis highlighted a higher expression level not only of *sirt1* but also of *sirt2* and *sirt5* (without a CGI promoter). However, *sirt1* appeared as one of the most ubiquitous and highly expressed isotype when considering as a whole the entire set of analyzed tissues [28]. Conversely, *sirt3,* as well as *sirt4, 6* and *7,* was

categorized as a *sirt* isotype with a relatively low expression level, though it is able to achieve a high expression in immune relevant fish tissues (e.g. head kidney, posterior intestine) [28, 29]. Of note, the human *SIRT3* shares a bidirectional promoter with *PSMD13* (Proteasome 26S Subunit, Non-ATPase 13), being this head-to-head organization conserved in birds, rat, mouse, dog and chimpanzee as a transcriptional mechanism that would facilitate the co-regulated expression of *SIRT3* and *PSMD13* [72]. How fish genomes and *sirt* genes in particular benefit of bidirectional promoters remains intriguing in fish [73]. In any case, both in this and other previous study [4], a high expression level of *sirt3* is not substantiated in metabolically active tissues of GSB (e.g. liver and WSM). It must be argued that this tissue-specific dualism in *sirt3* expression might be favored by CGI promoters, which might also contribute to enable Sirt abundance at different cellular locations (Sirt1, nucleus; Sirt3, mitochondria) as part of the functional Sirt diversification in fish.

6.4.4. Local DNA methylation contributes to regulate *sirt1* gene expression

The connection between metabolism and epigenetics through the action of SIRTs has been widely demonstrated in higher vertebrates [26]. In fact, the deacylase activity of SIRTs over histones, TFs and epigenetic enzymes, and their requirement of NAD^+ as a co-substrate, makes SIRTs to transduce energy metabolic signals into the epigenetic regulation of gene expression, chromatin biology and genome stability [25]. However, the epigenetic regulation of *SIRTs* is less understood and contradictory results have been reported in different experimental models, with no correlation between *SIRT* expression and local DNA

methylation of CGIs in both humans [61-65] and mouse [66]. In contrast, a clear negative correlation between gene expression and CGI hypermethylation has been reported for human *SIRT1* [62], and demethylation of bovine *SIRT4-6* promoters enhanced their transcriptional activity favoring the binding of specific TFs to their promoters [67-69]. Likewise, in the present study, the analyzed CpG sites of *sirt3* were hypomethylated irrespective of tissue, age and season, which may indicate that the observed changes in *sirt3* expression (mainly related to season) were not regulated in this fish species by changes in DNA methylation at the analyzed CpG sites. At the hepatic level, the 22 examined CpG sites of CGI *sirt1* were also highly refractory to methylation. The same for WSM in winter, but importantly, slight but consistent changes in DNA methylation were found in summer when fish of class +1 and class +3 were compared. It is known that intermediate DNA methylation levels are fine-tuned, and the association of DNA methylation with an observed phenotype can occur through small differences in the methylation level, often only 1-5% [74]. Indeed, less than 1% changes in methylation affect the binding of NRF1 and E2F1 to SIRT6 promoter in bovine adipocytes [69].

In mammals, there is a growing body of literature supporting an age-specific drift of methylation patterns, which includes global hypomethylation and region-specific hypermethylation [75, 76]. The nature of local hypermethylation changes is complex, but it has been shown that some of them co-occur near tumor suppressor genes with functional effects that might be linked to cell cycle regulation. Hence, the difference between DNA methylation age and chronological age is a promising tool in predicting disease risk and longevity potential in early

life [77], being associated the accelerated increases in DNA methylation at particular local sites with the early onset of most age-related diseases in humans [78]. Although there is so far no proof that changes in specific DNA methylation patterns of *SIRTs* can extent lifespan, it is noteworthy that SIRT1 (the orthologous counterpart of Sir2 in higher vertebrates) is a master regulator of aging, inflammation and metabolism [79, 80], mediating most of the healthy and anti-aging effects related with caloric restriction and the resveratrol induced SIRT activation in humans and rodents [81]. However, whereas alterations of the epigenome in adult somatic tissues might reflect aging-associated deleterious events, developmental and seasonal changes of the epigenome are necessary and fine-tuned by environmental cues. In our experimental model, this observation fits well with the summer age-related changes in *sirt1* expression at the WSM, resulting in a close negative association between *sirt1* expression and DNA methylation at CpG3 and CpG19 positions, which is considered adaptive to adjust the changing metabolic needs and feed intake that normally occurs with advancing age and growth deceleration (see below).

Interestingly, our results also indicate that the summer decrease of *sirt1* expression was concurrent with the increased DNA methylation of *sirt1* promoter, which was especially evident at CpG positions containing SP1 binding sites (CpG2, 3 and CpG12-14, 16). Since this observation was particularly evident in young fish, one possible explanation is that the epigenetic program resulting in a tissue-specific methylome is reset with advancing age as part of the overall hypomethylation program of aged animals. The ultimate mechanisms remain unexplored, but it seems that it occurs via SP1 interactions with

other TFs as reported for PPARβ for the enhanced expression of human *SIRT1* [82].

6.4.5. *sirt* gene expression enables changes in lipogenic and growth energy demanding processes

The combined gene expression profiling of biomarkers of energy demand (*pgc1α*, *cpt1a*, *cs*, *ucp1/3*) and energy status (*sirt1-7*) helps to better discriminate phenotypes with different growth potentiality in GSB [4]. Such approaches are also highly informative of the metabolic status of animals across season and normal development. Certainly, in the present study, the hepatic expression of almost all the analyzed markers of energy metabolism was down-regulated by age, especially in the cold season. Indeed, differences in feed intake between young and less active growing fish are exacerbated in our latitude from November to March, with the practical feeding stop in adult fish [83]. As a consequence, catabolic states resulting of short-term fasting [28] or natural starvation during winter are sensed by a wide range of energy sensors, including *sirts* that reflect the energy status rather energy demand. In fact, the most energy demanding process of liver is the lipogenic activity [84], and reduced lipid biosynthetic capabilities during fasting or temperature drops are linked with a pronounced down-regulated expression of the enzyme subunits of mitochondrial respiration chain (oxidative phosphorylation pathway, OXPHOS) [30, 85]. This contrasts with the up-regulated expression of markers of OXPHOS in the WSM of fasted or feed-restricted GSB [85, 86], which reflects an increased tissue-energy demand to preserve the protein muscular mass when fish facing a reduced supply of nutrients or an enhanced energy demand for growth

of the still fast growing individuals during the summer season [83]. This metabolic feature was illustrated herein by an increased expression in young fish of markers of fatty acid β-oxidation (*cpt1a*) and muscle respiration uncoupling (*ucp3*), which evolved to protect mitochondria against oxidative stress in a highly oxidative cellular milieu [87, 88]. Certainly, SIRT1 acts as a major repressor of UCP3 in muscle tissues of rodents [89], also inhibiting the progression of different antioxidant responses mediated by NF-κB and NRF2 [51]. Conversely, the down-regulation of *SIRT1* enhances the myogenic gene program to adjust it to energetic demands driven by changing growth, nutrient availability or increased muscle activity [90]. Human studies also indicate that SIRT2 enhance myoblast proliferation [91] and differentiation [92], being related the enhanced muscle expression of *sirt2* in juvenile fish of GSB with a higher growth potentiality [4]. Since these findings are apparently contradictory with the observed up-regulated expression of *sirt2* with advancing age, we consider that this age-mediated response highlights additional *sirt* functions related in other animal models to genome maintenance and the avoidance of cell senescence in general [89, 93, 94].

As a corollary of this complex and sometimes controversial puzzle, the multivariate analyses of gene expression patterns indicated that changes in *sirt* gene expression at the level of WSM are particularly responsive to physiological changes mediated by age and season. Certainly, six *sirts* out of seven collectively have a discriminant role (in particular *sirt1*) to disclose the seasonal-related changes in feed intake and growth performance, as well as the switch of metabolism from glucose utilization to fatty acid oxidation [95]. However, further studies are needed to cope changes in environmental cues and epigenetic

modifications as a promising tool to fully exploit the growth potentiality of currently farmed fish. In any case, collectively our results point out an important role of *sirt1* in the adjustment of energy metabolism in GSB.

6.5. Conclusions

This study demonstrates that the gene structure of *sirts* is highly conserved through vertebrate evolution in the fish lineage. *In silico* analyses also highlighted the presence of common regulatory elements in *sirt1* and *sirt3* promoter regions in fish and higher vertebrates, being associated the presence of CGIs in the promoter region of these two *sirts* with a fine adjustment of gene expression across metabolically active and/or immunologically relevant tissues. Correlation analysis of *sirt* gene expression and local DNA methylation was done for the first time in a marine fish, revealing the increase of local DNA methylation a less permissive condition for *sirt1* gene expression at the level of WSM. In particular, we showed that the methylation level of CpG positions containing SP1 binding sites contributes to explain either seasonal and age related changes in *sirt1* expression. The functional significance of changes in *sirt1* methylation deserves further investigation, but importantly it appears that not all the changes in *sirt1* gene expression are mediated by epigenetic DNA methylation mechanisms. This is not surprising given that most changes in energy metabolism require a fast response for fish to cope with a poorly predictable environment. Collectively, all these findings confirm and extend the idea that *sirts* are good markers of metabolic condition in farmed fish, contributing to define the ideal gene expression pattern at a tissue-specific level (see **Fig. 6.11**).

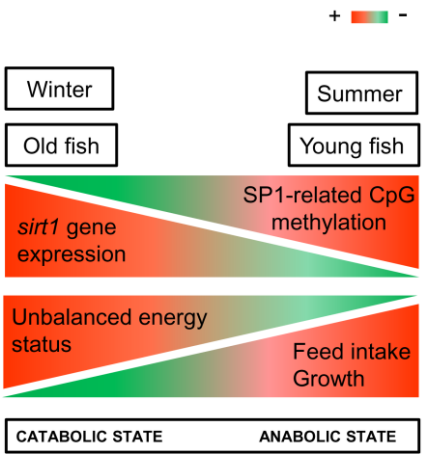


Figure 6.11 Integrative scheme of DNA methylation and gene expression of *sirt1*. Different energy status induces changes in methylation and gene expression of *sirt1* in muscle of young and old gilthead sea bream in winter and summer.

Funding

This work was funded by a Spanish MICINN project (BreamAquaINTECH, RTI2018-094128-B-I00) and the PerformFISH (Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain) EU Project (H2020-SFS-2016-2017; 727610). This publication reflects the views only of the authors and the European Commission cannot be held responsible for any use which may be made of the information contained therein. PS-M was supported by a predoctoral research fellowship (BES-2014-069250) from Spanish MINECO. EP was founded by a Postdoctoral Research Fellow (Juan de la Cierva-Incorporación, Reference IJCI-2016-27637) from MINECO.

Acknowledgments

The authors thank M. A. González for technical assistance with gene expression analyses. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

References

1. FAO. The state of world fisheries and aquaculture 2018- Meeting the sustainable development goals. Rome 2018.
2. Chen Y, Gondro C, Quinn K, Herd RM, Parnell PF, Vanselow B. Global gene expression profiling reveals genes expressed differentially in cattle with high and low residual feed intake. Anim Genet. 2011;42:475–490.
3. Choi MJ, Kim GD, Kim JM, Lim HK. Differentially-expressed genes associated with faster growth of the Pacific Abalone, *Haliotis discus hannai*. Int J Mol Sci. 2015;16:27520–27534.
4. Simó-Mirabet P, Perera E, Calduch-Giner JA, Afonso JM, Pérez-Sánchez J. Co-expression analysis of sirtuins and related metabolic biomarkers in juveniles of gilthead sea bream (*Sparus aurata*) with differences in growth performance. Front Physiol. 2018;9:608.
5. Pérez-Sánchez J, Borrel M, Bermejo-Nogales A, Benedito-Palos L, Saera-Vila A, Calduch-Giner JA, et al. Dietary oils mediate cortisol kinetics and the hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream (*Sparus aurata*) exposed to crowding stress. Implications on energy homeostasis and stress susceptibility. Comp Biochem Physiol Part D Genomics Proteomics. 2013;8(2):123–130.
6. Benedito-Palos L, Ballester-Lozano GF, Pérez-Sánchez J. Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*) Gene. 2014;547:34–42.
7. Umehara T, Murase T, Abe Y, Yamashita H, Shibaike Y, Kagawa S, et al. Identification of potential markers of fatal hypothermia by a body temperature-dependent gene expression assay. Int J Legal Med. 2019;133(2):335–345.
8. Ryu R, Choi MS. Differences in metabolic biomarkers in the blood and gene expression profiles of peripheral blood mononuclear cells among

normal weight, mildly obese and moderately obese subjects. Br J Nutr. 2016;116(6):1022–1032.
9. García-Giménez JL, Seco-Cervera M, Tollefsbol TO, Romá-Mateo C, Peiró-Chova L, Lapunzina P, et al. Epigenetic biomarkers: Current strategies and future challenges for their use in the clinical laboratory. Crit Rev Clin Lab Sci. 2017;54(7–8):529–550.
10. Jablonka E, Lamb MJ. The changing concept of epigenetics. Ann N Y Acad Sci. 2002;981:8296.
11. Ibeagha-Awemu EM, Zhao X. Epigenetic marks: regulators of livestock phenotypes and conceivable sources of missing variation in livestock improvement programs. Front Genet. 2015;6:302.
12. Kouzarides T. Chromatin modifications and their function. Cell. 2007;128:693–705.
13. Moore LD, Le T, Fan G. DNA methylation and its basic function. Neuropsychopharmacology. 2013;38:23–38.
14. D' Alessio AC, Weaver IC, Szyf M. Acetylation-induced transcription is required for active DNA demethylation in methylation-silenced genes. 2007;27:7462–7474.
15. Gavery MR, Roberts SB. Epigenetic considerations in aquaculture. PeerJ. 2017;5:e4147.
16. Navarro-Martín L, Viñas J, Ribas L, Díaz N, Gutiérrez A, Di Croce L, et al. DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genet. 2011;7:e1002447.
17. Shao C, Li Q, Chen S, Zhang P, Lian J, Hu Q, et al. Epigenetic modification and inheritance in sexual reversal of fish. Genome Res. 2014;24:604–615.
18. Morán P, Pérez-Figueroa A. Methylation changes associated with early maturation stages in the Atlantic salmon. BMC Genet. 2011;12:86.
19. Burgerhout E, Mommens M, Johnsen H, Aunsmo A, Santi N, Andersen Ø. Genetic background and embryonic temperature affect DNA methylation and expression of myogenin and muscle development in Atlantic salmon (*Salmo salar*). PLoS One. 2017;12: e0179918.
20. Covelo-Soto L, Saura M, Morán P. Does DNA methylation regulate metamorphosis? The case of the sea lamprey (*Petromyzon marinus*) as an example. Comp Biochem Physiol B Biochem Mol Biol. 2015;185:42–46.
21. Si Y, He F, Wen H, Li J, Zhao J, Ren Y, et al. Genetic polymorphisms and DNA methylation in exon 1 CpG–rich regions of PACAP gene and

its effect on mRNA expression and growth traits in half smooth tongue sole (*Cynoglossus semilaevis*). Fish Physiol Biochem. 2016;42(2):407–421.

22. Li S, He F, Wen H, Li J, Si Y, Liu M, et al. Low salinity affects cellularity, DNA methylation, and mRNA expression of igf1 in the liver of half smooth tongue sole (*Cynoglossus semilaevis*). Fish Physiol Biochem. 2017;43(6):1587–1602.
23. Baerwald MR, Meek MH, Stephens MR, Nagarajan RP, Goodbla AM, Tomalty KMH, et al. Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. Mol Ecol. 2016;25:1785–1800.
24. Artemov AV, Mugue NS, Rastorguev SM, Zhenilo S, Mazur AM, Tsygankova SV, et al. Genome-wide DNA methylation profiling reveals epigenetic adaptation of stickleback to marine and freshwater conditions. Mol Biol Evol. 2017;34:2203–2213.
25. Bosch-Presegué L, Vaquero, A. Sirtuin-dependent epigenetic regulation in the maintenance of genome integrity. FEBS J. 2015;282:1745–1767.
26. Jing H, Lin H. Sirtuins in epigenetic regulation. Chem Rev. 2015;115:2350–2375.
27. Kosciuk T, Wang M, Hong JY, Lin H. Updates on the epigenetic roles of sirtuins. Curr Opin Chem Biol. 2019;51:18–29.
28. Simó-Mirabet P, Bermejo-Nogales A, Calduch-Giner J, Pérez-Sánchez J. Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*). J Comp Physiol B Biochem Syst Environ Physiol. 2017;187:153–163.
29. Simó-Mirabet P, Piazzon MC, Calduch-Giner JA, Ortiz Á, Puyalto M, Sitjà-Bobadilla A, et al. Sodium salt medium–chain fatty acids and *Bacillus*–based probiotic strategies to improve growth and intestinal health of gilthead sea bream (*Sparus aurata*). Peer J. 2017;5:e4001.
30. Bermejo-Nogales A, Nederlof M, Benedito-Palos L, Ballester-Lozano GF, Folkedal O, Olsen RE, et al. Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping. Gen Comp Endocrinol. 2014;205:305–315.
31. Melis R, Sanna R, Braca A, Bonaglini E, Cappuccinelli R, Slawski H, et al. Molecular details on gilthead sea bream (*Sparus aurata*) sensitivity to low water temperatures from 1H NMR metabolomics. Comp Biochem Physiol A Mol Integr Physiol. 2017;204:129–136.

32. Varriale A, Bernardi G. DNA methylation and body temperature in fishes. Gene. 2006;385:111–121.
33. Wątroba M, Dudek I, Skoda M, Stangret A, Rzodkiewicz P, Szukiewicz D. Sirtuins, epigenetics and longevity. Ageing Res Rev. 2017;40:11–19.
34. Illingworth RS, Gruenewald-Schneider U, Webb S, Kerr ARW, James KD, Turner DJ, et al. Orphan CpG islands identify numerous conserved promoters in the mammalian genome. PLoS Genet. 2010;6(9):e1001134.
35. Solovyev V, Kosarev P, Seledsov I, Vorobyev D. Automatic annotation of eukaryotic genes, pseudogenes and promoters. Genome Biol. 2006;7 Suppl 10:1–12.
36. Knudsen S. Promoter 2.0: for the recognition of PolII promoter sequences. Bioinformatics. 1999;15:356–361.
37. Abeel T, Saeys Y, Bonnet E, Rouzé P, Van de Peer Y. Generic eukaryotic core promoter prediction using structural features of DNA. Genome Res. 2008;18:310–323.
38. Kreft L, Soete A, Hulpiau P, Botzki A, Saeys Y, De Bleser P. ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. Nucleic Acids Res. 2017;45:W490-W494.
39. Sandoval J, Mendez-Gonzalez J, Nadal E, Chen G, Carmona FJ, Sayols S, et al. A prognostic DNA methylation signature for stage I non-small-cell lung cancer. J Clin Oncol. 2013;31:4140–4147.
40. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402–408.
41. Wold S, Sjöström M, Eriksson L. PLS-regression: a basic tool of chemometrics. Chemom Intell Lab Syst. 2001;58:109–130.
42. Li H, Ma M–L, Luo S, Zhang R–M, Han P, Hu W. Metabolic responses to ethanol in *Saccharomyces cerevisiae* using a gas chromatography tandem mass spectrometry-based metabolomics approach. Int J Biochem Cell Biol. 2012;44:1087–1096.
43. Kieffer DA, Piccolo BD, Vaziri ND, Liu S, Lau WL, Khazaeli M, et al. Resistant starch alters gut microbiome and metabolomic profiles concurrent with amelioration of chronic kidney disease in rats. Am J Physiol Renal Physiol. 2016;310:F857–871.
44. Greiss S, Gartner A. Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. Mol Cells. 2009;28:407–415.
45. Sauve AA, Wolberger C, Schramm VL, Boeke JD. The biochemistry of sirtuins. Annu Rev Biochem. 2006;75:435–465.

46. Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2–like proteins. Biochem Biophys Res Commun. 2000;273:793–798.
47. Zhang MQ. Statistical features of human exons and their flanking regions. Hum Mol Genet. 1998;7:919–932.
48. Sakharkar MK, Perumal BS, Sakharkar KR, Kangueane P. An analysis on gene architecture in human and mouse genomes. In Silico Biol. 2005;5:347–365.
49. Li W, Graur D. Fundamentals of Molecular Evolution. Sunderland, MA: Sinauer Associates; 1991.
50. Stelzer G, Rosen R, Plaschkes I, Zimmerman S, Twik M, Fishilevich S, et al. The GeneCards Suite: from gene data mining to disease genome sequence analysis. Curr Protoc Bioinformatics. 2016;54 Suppl 30:1–33.
51. Buler M, Andersson U, Hakkola J. Who watches the watchmen? Regulation of the expression and activity of sirtuins. FASEB J. 2016;30(12):3942–3960.
52. Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, et al. Regulation of cellular metabolism by protein lysine acetylation. Science. 2010;327(5968):1000–1004.
53. Lundby A, Lage K, Weinert BT, Bekker-Jensen DB, Secher A, Skovgaard T, et al. Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. Cell rep. 2012;2(2):419–431.
54. Correia M, Perestrelo T, Rodrigues AS, Ribeiro MF, Pereira SL, Sousa MI, et al. Sirtuins in metabolism, stemness and differentiation. Biochim Biophys Acta. 2017;1861:3444–3455.
55. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev. 2011;25:1010–1022.
56. Koerner MV, Chhatbar K, Webb S, Cholewa-Waclaw J, Selfridge J, De Sousa D, et al. An orphan CpG island drives expression of a let–7 miRNA precursor with an important role in mouse development. Epigenomes. 2019;3:7.
57. Han L, Su B, Li WH, Zhao Z. CpG island density and its correlations with genomic features in mammalian genomes. Genome Biol. 2008;9(5):R79.
58. Han L, Zhao Z. Comparative analysis of CpG islands in four fish genomes. Comp Funct Genomics. 2008:565631.
59. Long HK, King HW, Patient RK, Odom DT, Klose RJ. Protection of CpG islands from DNA methylation is DNA-encoded and evolutionarily conserved. Nucleic Acids Res. 2016;44(14):6693–6706.

60. Sharif J, Endo TA, Toyoda T, Koseki H. Divergence of CpG island promoters: A consequence or cause of evolution? Dev Growth Differ. 2010;52(6):545–554.
61. Furuya TK, da Silva PN, Payão SL, Rasmussen LT, de Labio RW, Bertolucci PH, et al. SORL1 and SIRT1 mRNA expression and promoter methylation levels in aging and Alzheimer's Disease. Neurochem Int. 2012;61:973e5.
62. Hou Y, Chen H, He Q, Jiang W, Luo T, Duan, J, et al. Changes in methylation patterns of multiple genes from peripheral blood leucocytes of Alzheimer's disease patients. Acta Neuropsychiatr. 2013;25(2):66–76.
63. Sahin K, Yilmaz S, Gozukirmizi N. Changes in human sirtuin 6 gene promoter methylation during aging. Biomed Rep. 2014;2:574e8.
64. Wang L, Zhou H, Wang Y, Cui G, Di LJ. CtBP maintains cancer cell growth and metabolic homeostasis via regulating SIRT4. Cell Death Dis. 2015;6:e1620.
65. Kurylowicz A, Owczarz M, Polosak J, Jonas MI, Lisik W, Jonas M, et al. SIRT1 and SIRT7 expression in adipose tissues of obese and normal-weight individuals is regulated by microRNAs but not by methylation status. Int J Obes (Lond). 2016;40(11):1635–1642.
66. Zullo A, Simone E, Grimaldi M, Gagliardi M, Zullo L, Matarazzo MR, et al. Effect of nutrient deprivation on the expression and the epigenetic signature of sirtuin genes. Nutr Metab Cardiovasc Dis. 2018;28(4):418–424.
67. Hong J, Wang X, Mei C, Wang H, Zan L. DNA methylation and transcription factors competitively regulate SIRT4 promoter activity in bovine adipocytes: roles of NRF1 and CMYB. DNA Cell Biol. 2019;38(1):63–75.
68. Hong J, Wang X, Mei C, Zan L. Competitive regulation by transcription factors and DNA methylation in the bovine SIRT5 promoter: Roles of E2F4 and KLF6. Gene. 2019;684:39–46.
69. Hong JY, Mei CG, Li SJ, Wang HB, Zhao CP, Zan LS. Coordinate regulation by transcription factors and DNA methylation in the core promoter region of SIRT6 in bovine adipocytes. Arch Biochem Biophys. 2018;659:1–12.
70. Saxonov S, Berg P, Brutlag DL. 2006. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A. 3;103(5):1412–1417.
71. Smith ZD, Meissner A. DNA methylation: roles in mammalian development. Nature Reviews Genetics. 2013;14(3):204.

72. Bellizzi D, Dato S, Cavalcante P, Covello G, Di Cianni F, Passarino G, et al. Characterization of a bidirectional promoter shared between two human genes related to aging: SIRT3 and PSMD13. Genomics. 2007;89:143–150.
73. Yang MQ, Taylor J, Elnitski L. Comparative analyses of bidirectional promoters in vertebrates. BMC Bioinformatics. 2008;9 Suppl 6:S9.
74. Plagemann A, Harder T, Brunn M, Harder A, Roepke K, Wittrock-Staar M, et al. Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome. J Physiol. 2009; 587:4963-4976.
75. Jung M, Pfeifer GP. Aging and DNA methylation. BMC Biol. 2015;13:7.
76. Xiao F, Kong QP, Perry B, He YH. Progress on the role of DNA methylation in aging and longevity. Brief Funct Genomics. 2016;15:454–459.
77. Vaiserman A, Koliada A, Lushchak O. Developmental programming of aging trajectory. Ageing Res Rev. 2018;47:105–122.
78. Xiao F-H, Wang H-T, Kong Q-P. Dynamic DNA methylation during aging: A "prophet" of age-related outcomes Front Genet. 2019;10:107.
79. Bellet MM, Sassone-Corsi P. Mammalian circadian clock and metabolism - the epigenetic link. J Cell Sci. 2010;123:3837–3848.
80. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. Nat Rev Mol Cell Biol. 2012;13:225–238.
81. Stacchiotti A, Favero G, Rezzani R. Resveratrol and SIRT1 activators for the treatment of aging and age-related diseases. In: Badria FA, editor. Resveratrol. Rijeka: IntechOpen; 2019. p. 117-135.
82. Okazaki M, Iwasaki Y, Nishiyama M, Taguchi T, Tsugita M, Nakayama S, et al. PPARbeta/delta regulates the human SIRT1 gene transcription via Sp1. Endocr J. 2010;57(5):403–413.
83. Pérez-Sánchez J, Simó-Mirabet P, Naya-Català F, Martos-Sitcha JA, Perera E, Bermejo-Nogales A, et al. Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. Front Endocrinol. 2018;9:687.
84. Rui L. Energy metabolism in the liver. Compr Physiol. 2014;4:177–197.
85. Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. PLoS One. 2015;10:e0122889.

86. Calduch-Giner JA, Echasseriau Y, Crespo D, Baron D, Planas JV, Prunet P, et al. Transcriptional assessment by microarray analysis and large-scale meta-analysis of the metabolic capacity of cardiac and skeletal muscle tissues to cope with reduced nutrient availability in gilthead sea bream (*Sparus aurata* L.). Mar Biotechnol. 2014;16:423–435.
87. Schrauwen-Hinderling VB, Schrauwen P, Hesselink MKC, Van Engelshoven JMA, Nicolay K, Saris WHM, et al. The increase in intramyocellular lipid content is a very early response to training. J Clin Endocr Metab. 2003;88:1610–1616.
88. Bermejo-Nogales A, Benedito-Palos L, Calduch-Giner JA, Pérez-Sánchez J. Feed restriction up-regulates uncoupling protein 3 (UCP3) gene expression in heart and red muscle tissues of gilthead sea bream (*Sparus aurata* L.): new insights in substrate oxidation and energy expenditure. Comp Biochem Phys A. 2011;159:296–302.
89. Shoba B, Lwin ZM, Ling LS, Bay BH, Yip GW, Kumar SD. Function of sirtuins in biological tissues. Anat Rec. 2009;292(4):536–543.
90. Pardo PS, Boriek AM. The physiological roles of Sirt1 in skeletal muscle. Aging. 2011;3(4):430–437.
91. Wu G, Song C, Lu H, Jia L, Yang G, Shi X, et al. Sirt2 induces C2C12 myoblasts proliferation by activation of the ERK1/2 pathway. Acta Biochim Biophys Sin. 2014;46:342–345.
92. Stanton DA, Alway SE, Mohamed JS. The role of Sirtuin 2 in the regulation of myogenesis. FASEB J. 2017; 31 Suppl 1:877.13.
93. Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA. Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol Cell Biol. 2003;23(9):3173–3185.
94. Anwar T, Khosla S, Ramakrishna G. Increased expression of SIRT2 is a novel marker of cellular senescence and is dependent on wild type p53 status. Cell Cycle. 2016;15:1883–1897.
95. Lomb DJ, Laurent G, Haigis MC. Sirtuins regulate key aspects of lipid metabolism. Biochim Biophys Acta. 2010;1804(8):1652–1657.

CAPÍTULO 7- Discusión

A lo largo de las dos últimas décadas se han conseguido notables avances en el cultivo de peces a escala industrial, por lo que la producción de muchas especies ha pasado en pocos años de unos pocos cientos a miles de toneladas. Sin embargo, el disponer de unas infraestructuras de referencia para evaluar diferentes condiciones de cultivo de forma fiable y fácilmente reproducible sigue siendo un reto a la hora de establecer el máximo potencial de crecimiento o el grado de domesticación de las especies susceptibles de cultivo. De hecho, el mantener a los peces en unas condiciones distintas a las de su medio natural comporta unos estados de riesgo que requieren el establecimiento de programas de prevención y tratamiento de enfermedades, así como procedimientos apropiados de gestión, nutrición, manipulación y sacrificio que garanticen el bienestar animal, al igual que la calidad y seguridad alimentaria del producto final destinado al consumo. Es por todo ello, que para para poder cumplir los objetivos de la agenda 2030 es necesario el establecimiento de estándares de calidad, basados en buenas prácticas de alimentación y cultivo, así como en un uso adecuado del conocimiento generado a partir de las investigaciones en curso. Por consiguiente, a lo largo de este último capítulo de la Tesis Doctoral se han intentado mostrar algunos de los últimos avances en el cultivo de la dorada y el impacto a corto-medio plazo que puede tener el conocimiento generado sobre la estructura, función y regulación de las *sirts* como marcadores del estado metabólico y bienestar de los peces en cultivo.

7.1. Últimos avances en el cultivo de la dorada

7.1.1. Las dietas basadas en ingredientes vegetales no limitan el potencial de crecimiento en condiciones experimentales de cultivo

La industria del salmón noruego ha disminuido hasta un 30% los niveles de inclusión de ingredientes marinos en los piensos de engorde (Ytrestøyl et al., 2015; Shepherd et al., 2017) y resultados similares o incluso mejores se han obtenido a escala experimental en dorada, como especie modelo de la acuicultura mediterránea. En gran parte, ello ha sido posible gracias al esfuerzo investigador de los últimos 15-20 años en el marco de diferentes proyectos europeos (PEPPA, AQUAMAX, ARRAINA) en los que se han abordado, primero por separado y luego conjuntamente, la sustitución de harinas y aceites de pescado por ingredientes vegetales. Todo ello ha permitido disminuir progresivamente la dependencia de las pesquerías al ir reduciendo, sin efectos negativos sobre el crecimiento, el nivel mínimo de inclusión de harinas y aceites de pescado desde un 35% (Gómez-Requeni et al., 2004) hasta un 7,5-10% (Benedito-Palos et al., 2016) (**Fig. 7.1**). Para ello, se han llevado a cabo toda una serie de engordes de corta-larga duración en condiciones naturales de fotoperiodo y temperatura en las instalaciones de cultivo del Instituto de Acuicultura Torre de la Sal (IATS).

En cualquier caso, las formulaciones más extremas requieren un periodo inicial de adaptación o de programación nutricional que, si no se contempla, comporta una ligera merma del crecimiento (6-7%) que se arrastra a lo largo de todo el ciclo de producción, aunque con

posterioridad el patrón de expresión génica de marcadores seleccionados de crecimiento a nivel hepático y muscular no muestra diferencias relevantes entre el grupo control (D1), con un 25% de harina de pescado y 15% de aceite de pescado, y los grupos experimentales (D2-D4) con tan solo un 5% de harinas y una sustitución progresiva del aceite de pescado (6,5%-2,5%) por una mezcla de aceites vegetales (**Fig. 7.2A**). Es más, después de completar un ciclo de producción de 3 años, nuestros resultados muestran que todos los grupos experimentales (D1-D4) crecen de forma eficiente y con altas tasas de crecimiento para los estándares de la especie. De acuerdo con ello, y con independencia de la dieta, la eficiencia de conversión global del alimento es de 1, 0,8 y 0,7 para doradas de 300 g, 1 kg y 1,5 kg (Simó-Mirabet et al., 2018, **Fig. 7.2B**).

Desde un punto de vista práctico, estos valores pueden ser considerados como referencia para la especie, aunque la extrapolación a la industria no es fácil dada la heterogeneidad de las poblaciones actualmente en cultivo, además del mayor nivel de estrés asociado a las prácticas de cultivo a escala industrial (Bermejo-Nogales et al., 2014). Ahora bien, cabe destacar que los buenos resultados con las nuevas formulaciones del proyecto ARRAINA se han visto reiteradamente corroborados en otros engordes. Es más, cuando se combina el uso de ingredientes vegetales con el de hidrolizados proteicos, los resultados de los parámetros de crecimiento pueden ser incluso mejores que los obtenidos con formulaciones estándar o comerciales basadas en harinas y aceites de pescado (Martos-Sitcha et al., 2018), lo que indica que el nivel máximo de sustitución de los ingredientes típicamente marinos en dietas de engorde de peces marinos carnívoros es mayor del inicialmente

esperado. De forma indirecta, ello facilita el uso de otro tipo de ingredientes basados en proteínas microbianas, de insectos u otros subproductos de proteínas animales, siguiendo al mismo tiempo los principios de economía circular y deshechos cero. En esta línea ya se han conseguido importantes avances dentro del proyecto europeo GAIN, aunque el conocimiento generado no está todavía suficientemente maduro para ser transferido a la industria.

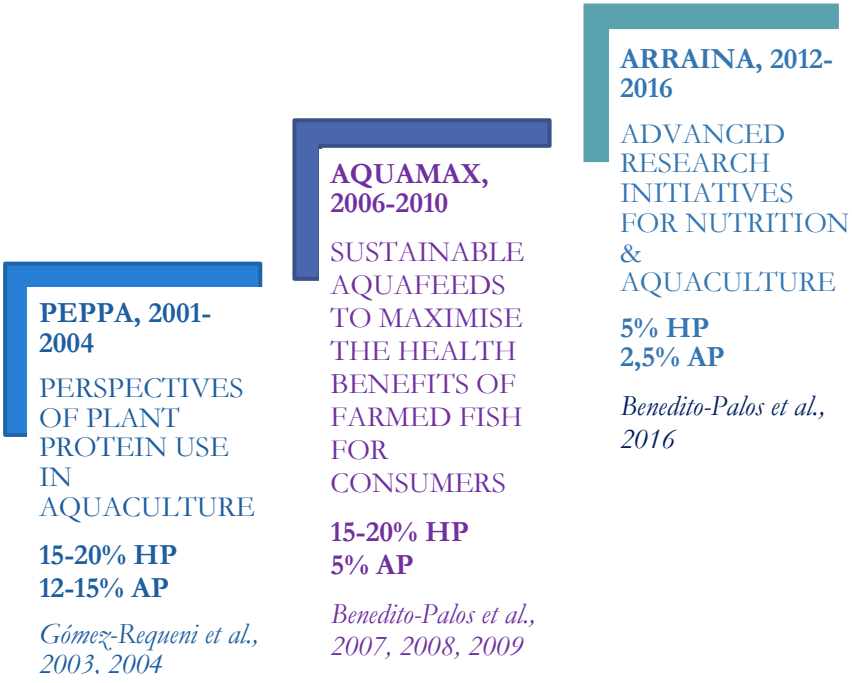
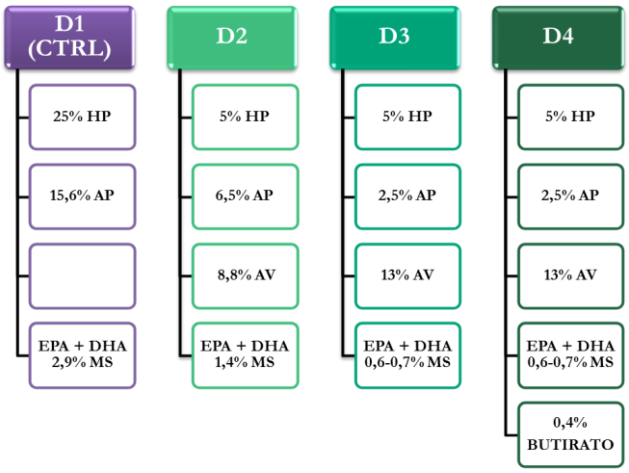


Figura 7.1 Avances en los niveles sustitución de ingredientes marinos por vegetales de las dietas de dorada. En la figura se indican los proyectos donde se han desarrollado las dietas y los artículos resultantes. *Abreviaturas: AP, aceite de pescado; HP, harina de pescado.*

A



B

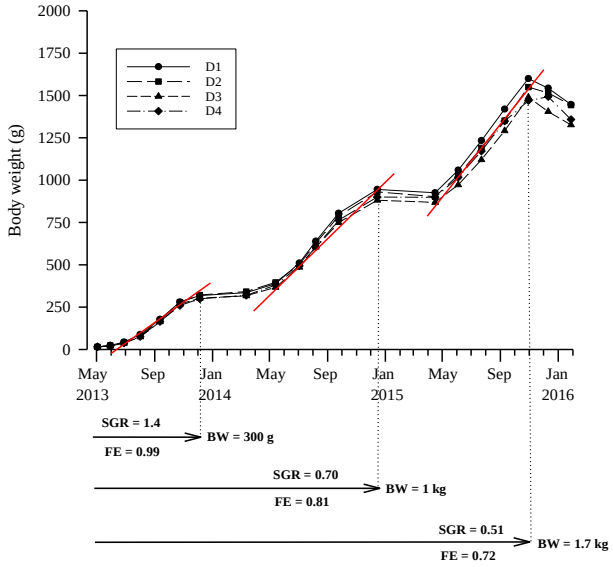


Figura 7.2 A Composición en harinas y aceites de pescado de las dietas experimentales D1-D4. **B** Rendimiento del crecimiento de la dorada alimentada con estas dietas desde estadios tempranos hasta la madurez sexual en un ciclo de producción de 3 años. Modificada de Simó-Mirabet et al., 2018. *Abreviaturas: AP, aceite de pescado; AV, aceite vegetal; BW, peso corporal; FE, eficiencia alimentaria; HP, harina de pescado; MS, materia seca; SGR, tasa específica de crecimiento.*

7.1.2. Las dietas vegetales alteran la proporción de sexos como consecuencia de un falso efecto feminizante

Los peces muestran una gran variedad de estrategias reproductivas en cuanto al determinismo sexual y a la expresión de su sexualidad, presentando todos los tipos de reproducción conocidos en vertebrados: unisexualidad, gonocorismo y hermafroditismo sincrónico o secuencial (Devlin y Nagahama, 2002; Kobayashi et al., 2013). Esta diversidad en los tipos de reproducción de los peces implica orígenes evolutivos muy diversos además de un alto grado de plasticidad evolutiva, destacando el hermafroditismo secuencial como claro ejemplo de plasticidad fenotípica en respuesta a un medio ambiente en continuo cambio (West-Eberhard, 2003; Aubin-Horth y Renn, 2009; Moczek, 2015).

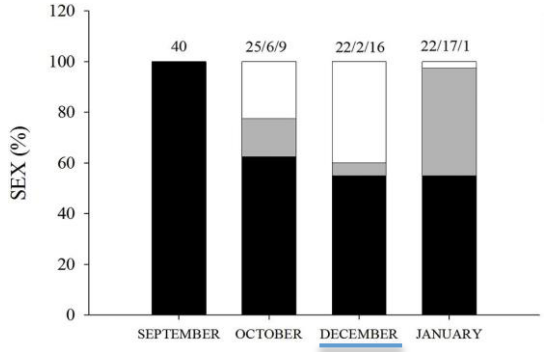
La teoría dominante que explica la ventaja adaptativa del hermafroditismo secuencial es el modelo de "la ventaja de la talla" (Todd et al., 2016), en base a lo cual el cambio de sexo es ventajoso cuando un individuo se reproduce con mayor eficiencia como miembro de un sexo cuando es joven o pequeño, y como miembro del otro sexo cuando es grande o de mayor edad, maximizando el éxito reproductivo a lo largo del ciclo biológico (Warner, 1988). Por tanto, para cada especie, la talla o la edad de cambio de sexo está genéticamente determinada. Así pues, estudios recientes en dorada han identificado dos QTLs, uno para peso corporal y otro que afecta a la determinación sexual (Loukovitis et al., 2011). Ambos están localizados en el mismo grupo de ligamiento, lo que añade el componente genético a la determinación del sexo en esta especie. De hecho, en las instalaciones del IATS, el peso de los animales

que revirtieron a hembras a los 3 años fue un 10% mayor que el de los machos (Simó-Mirabet et al., 2018).

Además de la influencia de los factores genéticos, el cambio de sexo está determinado por las interacciones sociales (Lamm et al., 2015). Concretamente en dorada, las hembras inhiben la reversión sexual de los machos y el hecho de quitar hembras de los tanques provoca la feminización de los machos con el fin de asegurar la reproducción (Zohar et al., 1978; Happe y Zohar, 1988). Como consecuencia, aunque la dorada suele cambiar de sexo un año después de su primera maduración como macho (D'Ancona, 1941; Lasserre, 1976), la reversión de macho a hembra puede ocurrir más tarde, tanto en condiciones naturales (Chaoui et al., 2006; Hadj-Taieb et al., 2013) como en cautividad (Simó-Mirabet et al., 2018).

Este proceso de reversión sexual también está regulado nutricionalmente, y doradas cultivadas en el IATS durante más de 3 años con una formulación estándar (D1), mostraron una proporción equilibrada de sexos (50% machos, 50% hembras) en el momento de la puesta, mientras que en los grupos de peces alimentados con dietas vegetales (D2, D3) se vio favorecida la reversión de machos a hembras por un falso efecto feminizante (**Fig. 7.3**). Sin embargo, este proceso fue revertido por la adición de butirato (D4), gracias a los efectos de este aditivo sobre el patrón de esteroides sexuales (ver más adelante).

A



B

Diet	Male	Female	% Female
D1	46	46	50
D2	34	62	64.6
D3	38	64	62.7
D4	40	48	54.5
D2+D3	72	126	63.6*

Figura 7.3 A Evolución de la proporción de sexos en peces de 3 años durante el periodo reproductivo (setiembre 2015-enero 2016). Los datos corresponden a 40 peces por mes y el número de hembras (negro), machos (blanco) e intersexos (gris) está indicado en cada punto de muestreo. **B** Efectos de la dieta sobre la proporción de sexos en diciembre de 2015. Las diferencias significativas en la proporción de sexos (grupo experimental; D2, D3, D4 respecto al control; D1) se determinaron mediante el test de Chi cuadrado; *, P <0,06. Modificada de Simó-Mirabet et al., 2018.

En la **Figura 7.4**, se muestra con más detalle la evolución de la proporción de sexos y el estado del desarrollo gonadal a lo largo de la fase reproductiva. En septiembre (**Fig. 7.4A**), se observó la presencia de hembras con oocitos previtelogénicos y de algunos individuos con testículos remanentes, unidos al tejido ovárico, con espermatogonias no diferenciadas. En octubre (**Fig. 7.4B**), un 63% eran hembras (con oocitos vitelogénicos), un 15% intersexos (con oocitos previtelogénicos cercanos al tejido masculino con espermatocitos u espermatozoides y muy pocas espermatogonias) y un 22% machos (con avanzada

espermatogénesis). En diciembre (**Fig. 7.4C**), los machos aumentaron hasta el 40% mientras que los intersexos disminuyeron hasta el 5% y las hembras hasta el 55%. En ese momento, el tejido ovárico de las hembras mostró oocitos en estadios tempranos y tardíos de la vitelogénesis y también postvitelogénicos mientras que los machos presentaron espermatozoides y espermatocitos. En enero (**Fig. 7.4D**), tras el periodo de puesta, el % de hembras fenotípicas se mantuvo igual mientras que los intersexos aumentaron hasta un 42% y solo quedó un individuo macho, lo que muestra que tras la época de puesta y con la falta de señales femeninas (feromonas), los machos pasan rápidamente a intersexos y, dependiendo del tipo de señales que reciban en la próxima etapa reproductiva, serán machos o ya de forma irreversible se convertirán en hembras funcionales.

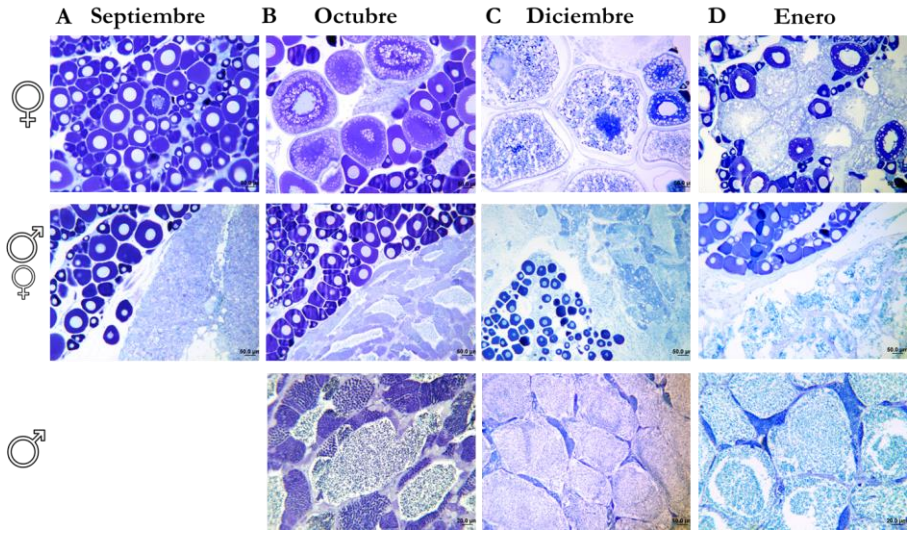


Figura 7.4 Desarrollo gonadal a lo largo de la fase reproductiva de la dorada. Modificada de Simó-Mirabet et al., 2018.

Es un hecho bien conocido que la inclusión de ingredientes de origen vegetal en las dietas provoca un aumento de sustancias estrogénicas no esteroideas, principalmente fitoestrógenos (Pelissero y Sumpter, 1992; Miyahara et al., 2003) que pueden influir sobre la reproducción de los peces. En nuestro caso, y como era de esperar, los niveles de esteroides sexuales aumentaron a lo largo del proceso reproductivo con picos, en diciembre, de estradiol y 11-kestosterona en hembras y machos, respectivamente (Simó-Mirabet et al., 2018). Igualmente, es de reseñar que la presencia de vitelogenina mediante Western blot solo dio positivo en hembras.

El análisis de los efectos de la dieta sobre el perfil de esteroides sexuales es más complejo de abordar, aunque como patrón general, el análisis multivariante mostró un perfil masculinizado de esteroides sexuales en los animales alimentados con dietas vegetales, que fue revertido al menos en parte con la adición de butirato en las dietas vegetales. Por consiguiente, los efectos de las dietas vegetales sobre el potencial de crecimiento y el proceso reproductivo son diferentes, ya que -en las condiciones optimizadas de crecimiento en la infraestructura de cultivo del IATS- las formulaciones basadas en dietas vegetales no comprometen el potencial de crecimiento y la suplementación con butirato no tiene un efecto estimulador del crecimiento. Por el contrario, las dietas vegetales afectan a la proporción de sexos y posiblemente también a la capacidad reproductora y a la calidad de la progenie, aspectos que, al menos en parte, son revertidos por el butirato, un aditivo que también ha mostrado tener un efecto castrante en machos durante el primer periodo reproductivo. Además, como veremos más adelante, este aditivo contribuye a mitigar otros efectos negativos de las

dietas vegetables, preservando el fenotipo salvaje propio de peces alimentados con dietas basadas en harinas y aceites de pescado.

7.1.3. La viabilidad del cultivo con dietas vegetales no se ve comprometida por la actual heterogeneidad de las poblaciones en cultivo

El último paso en la validación de nuevas formulaciones de piensos incluye el estudio de la interacción nutrición-genoma, para lo cual se ha analizado -en el marco del programa de selección genética PROGENSA- la respuesta de peces seleccionados por su diferente potencial de crecimiento a una formulación estándar y a una dieta experimental de composición intermedia a la de las dietas D2-D3 del proyecto ARRAINA (**Fig. 7.2A**). Los resultados de crecimiento en un modelo "common garden" han puesto de relieve la ausencia de una interacción significativa nutrición-genoma en las 11 familias analizadas, lo que se evidencia por la alta correlación existente entre las tasas de crecimiento de los peces alimentados con las dos dietas a lo largo del ciclo de producción (**Fig. 7.5**). Esto no implica que no existan diferencias con formulaciones más extremas, que no se han analizado en nuestro modelo experimental por los efectos que ello conlleva sobre el crecimiento y la composición en AGPs del filete (Benedito-Palos et al., 2009; Vasconi et al., 2017). De hecho, diferentes trabajos en trucha han mostrado una interacción genotipo-ambiente en peces alimentados con dietas con una sustitución alta o total de harinas y aceites de pescado por ingredientes vegetales (Pierce et al., 2008; Le Boucher et al., 2011), lo que posiblemente pone de manifiesto una diferente capacidad de elongación y desaturación a partir del AG 18:3n-3. Esta vía estaría limitada en peces

marinos por las deficiencias a nivel enzimático (*elovl2*, Δ5 desaturasa) en la maquinaria de síntesis de AGPs de cadena larga. En cualquier caso, la programación nutricional del metabolismo lipídico a nivel de reproductores o en los primeros estadios del desarrollo mejora la respuesta de la progenie a dietas de alto contenido en aceites vegetales, tal y como se ha puesto de manifiesto recientemente en dorada con modificaciones epigenéticas de la expresión de la Δ9 desaturasa (Perera et al., 2020), lo que permite una regulación más precisa de una enzima lipogénica que a su vez aumenta el nivel de instauración al promover la síntesis de AGMs.

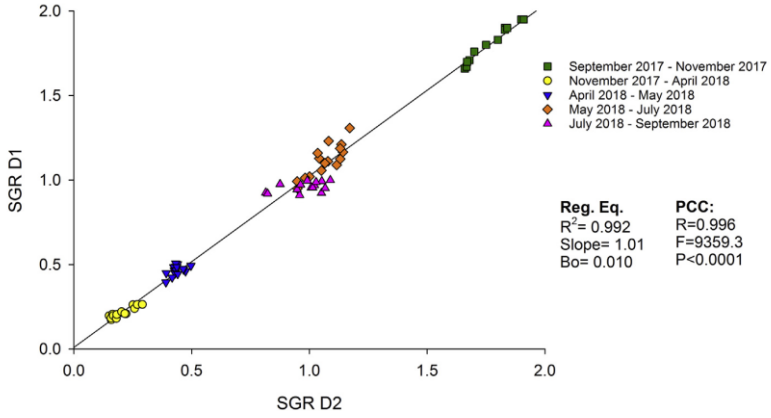


Figura 7.5 Tasas específicas de crecimiento (SGR) de las familias de crecimiento rápido y lento alimentadas con las dietas experimentales (D1-control, D2-vegetal) en distintos periodos. Los valores de la regresión de la ecuación (Reg. eq.) y el coeficiente de correlación de Pearson (PCC) corresponden al global de todos los periodos. Modificado de Perera et al., 2019.

A diferencia de los indicadores de crecimiento, otros parámetros biométricos relacionados con el almacenamiento de energía como el tamaño del hígado y la cantidad de tejido adiposo sí que mostraron una interacción nutrición-genotipo. Por un lado, las dietas vegetales provocaron un aumento del índice hepatosomático (HSI), lo cual es común en la dorada (Cruz-García et al., 2011; Benedito-Palos et al., 2016) y otros peces carnívoros alimentados con dietas vegetales (Riche y Williams, 2011; Cabral et al., 2013). En contraposición, disminuyó la deposición grasa (y el índice mesentérico, MSI), siendo esta reordenación de los depósitos grasos un síntoma de una ingesta limitada en AGs omega-3 de cadena larga (Ballester-Lozano et al., 2015), así como de un diferente potencial de crecimiento, tal y como se indica en la **Tabla 4.5** cuando se analiza la expresión de *sirts* en doradas de dos líneas de peces en cultivo genéticamente diferenciadas.

Por otro lado, la función intestinal está especialmente regulada a nivel genético, ya que las familias de rápido crecimiento del programa PROGENSA aumentaron la longitud del intestino y, por tanto, su capacidad digestiva y de absorción cuando se alimentaron con la dieta vegetal; mientras que las familias de crecimiento lento siempre mostraron un intestino alargado con independencia de la dieta (Perera et al., 2019). Actualmente, está en fase de evaluación cómo se ve afectada la composición de la microbiota intestinal en estos mismos animales, como parte del metanálisis encaminado a establecer los efectos de la edad, la estación, el sexo y la dieta sobre la microbiota y la funcionalidad intestinal en el marco de proyectos nacionales (Bream-aquaINTECH, 2019-2022) y europeos (AQUAIMPACT, 2019-2022) (Piazzon et al., 2017; 2019; Naya-Català et al., resultados no publicados).

7.1.4. Los ácidos grasos de cadena corta y media tienen efectos diferentes sobre el crecimiento y la salud intestinal

El incremento de la demanda de proteína de pescado y la consecuente intensificación de la acuicultura ha comportado el aumento del uso de aditivos en la dieta para minimizar los posibles efectos negativos de las nuevas formulaciones de piensos. Buenos ejemplos de ello son los AGs de cadena corta y media que son compuestos naturales con menos de 6 o de 6-12 átomos de carbono que juegan un papel importante en los procesos energéticos mitocondriales y en la señalización intracelular (Schönfeld y Wojtczak, 2016). Ambos tipos de compuestos proporcionan rápidamente energía gracias a su transporte directo a través de la vena portal hasta el hígado como AGs libres o unidos a albúmina. Además, su absorción celular y el transporte intracelular no requiere (o lo hace en menor cantidad) proteínas de unión a AGs (FABP) y el transporte a través de la membrana mitocondrial es independiente de la CPT-1, considerada una enzima limitante en la oxidación mitocondrial de AGs de cadena larga (Friedman et al., 1990).

El **butirato** (AG de 4 átomos de carbono) es posiblemente uno de los aditivos de acción pleiotrópica mejor estudiados, siendo capaz de mitigar los efectos pseudofeminizantes de las dietas vegetales en dorada (Simó-Mirabet et al., 2018), de la misma manera que la disminución de los niveles circulantes de colesterol y hemoglobina, así como el aumento de la expresión de citoquinas inflamatorias y de marcadores musculares de la morfogénesis celular y la degradación de proteínas (Benedito-Palos et al., 2016). La adición de butirato también ayuda a conservar la función

intestinal, preservando la integridad del epitelio intestinal (Estensoro et al., 2016), la biodiversidad de la flora intestinal, a la vez que limita la proliferación de determinadas especies de Photobacterias, que pueden actuar como patógenos secundarios (Piazzon et al., 2017). Además, ayuda a revertir los cambios observados en la composición del proteoma del mucus que suelen ir asociados, en peces alimentados con dietas vegetales, a una menor abundancia de ciertas mucinas y proteínas relacionadas con la digestión, con los efectos que ello comporta sobre la susceptibilidad a patógenos y la propia función digestiva, que requiere acciones compensatorias como el ya descrito aumento de la longitud del intestino. Esto también es extensible a nivel transcripcional, estando la suplementación con butirato de las dietas vegetales acompañada de una reversión al fenotipo salvaje del patrón de expresión intestinal de varios marcadores inflamatorios, de permeabilidad epitelial y de producción de mucus (Estensoro et al., 2016). Todos estos cambios acaban teniendo un alto valor predictivo sobre la susceptibilidad a enfermedades, mejorando el uso del butirato en dietas vegetales la progresión de la enteritis parasitaria en peces infectados experimentalmente con el myxozoo *E. leii* (Piazzon et al., 2017).

A diferencia del butirato, el **heptanoato** (AG de 7 átomos de carbono) no revierte la hipocolesterolemia causada por las dietas vegetales, pero con independencia de la composición de la dieta aumenta los niveles circulantes de cortisol, lo que a su vez facilita más si cabe la rápida producción de energía con una alta disponibilidad de sustratos rápidamente metabolizables (Haase et al., 2016). Sin embargo, por sí mismo ello aumenta el riesgo de estrés oxidativo y deben existir mecanismos compensatorios que lo minimicen sin prejuicio de la

disponibilidad de energía en estados de alta demanda. De hecho, la adición de heptanoato mejora transitoriamente la conversión del alimento en peces alimentados con dietas estándar y la respuesta al ejercicio en cámaras metabólicas. Sin embargo, no se observa una mejora adicional con dietas vegetales suplementadas con hidrolizados de proteínas de peces, que por sí mismos aumentan la disponibilidad de sustratos fácilmente metabolizables (Martos-Sitcha et al., 2018).

Como se muestra en el **Capítulo 5**, la suplementación con **AG de 12 átomos de carbono** (extraídos a partir del aceite de coco) ejerció una acción estimuladora del crecimiento de la dorada por sus efectos sobre la ingesta, lo que estaría mediado, al menos en parte, por la activación de la acción anabólica del eje somatotrópico. Sin embargo, la acción a nivel macroscópico, microscópico y transcripcional sobre la arquitectura y la función intestinal fue más limitada, a pesar del papel de los AGCMs como agentes antiinflamatorios (Carlson et al., 2015). Este hecho es más evidente cuando se comparó, en el mismo experimento, con la acción de un **probiótico comercial** basado en *Bacillus amyloliquefaciens*, que presentó una amplia variedad de efectos antioxidantes y antiinflamatorios que incluyeron la disminución de la producción de mucus, del número de células goblet, del estallido respiratorio, de los niveles circulantes de IgM y de cortisol, además de cambios en la expresión de citoquinas y marcadores de linfocitos a nivel mucoso. Al mismo tiempo, la adición de este probiótico en la dieta aumentó la expresión intestinal de la *igt* (inmunoglobulina mucosa de peces), cuya regulación en dorada se ha estudiado en respuesta a infecciones víricas, bacterianas y parasitarias (Piazzon et al., 2016). El significado fisiológico de esta importante observación no está totalmente

dilucidado, aunque posiblemente está relacionado con un mayor número de células IgT^{+} B residentes, que estarían listas para ser activadas y ejercer sus funciones bajo una infección o cualquier otra situación estresante.

Para resumir, en la **Fig. 7.6** se muestra la acción diferencial de los distintos aditivos tratados en este apartado.

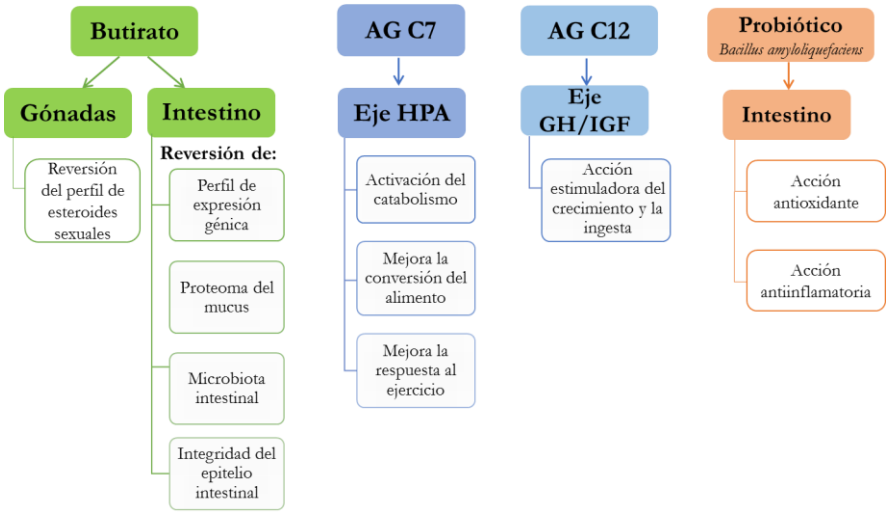


Figura 7.6 Dianas y acción diferencial de los distintos aditivos basados en ácidos grasos de cadena corta (butirato), cadena media (C7, C12) y un probiótico (*Bacillus amyloliquefaciens*).

7.2. Las sirtuínas, del gen a la función

7.2.1. Las sirtuínas están altamente conservadas a lo largo de la evolución

El análisis de las secuencias aminoacídicas de las SIRTs confirma que estas proteínas tienen una estructura y unas funciones muy conservadas evolutivamente (**Capítulo 3**). La parte más conservada es la región catalítica que consta de un dominio central formado por unos 250 aminoácidos que contiene un dominio grande Rossman fold y uno pequeño de unión al Zn^{+2} (**Fig. 3.1** y **Fig. 3.2**). Esta zona central está rodeada por regiones N- y C-terminales que son variables en longitud y secuencia, por lo que son las dianas de las modificaciones postraduccionales que confieren cierta especificidad a las funciones y a la localización celular de los distintos miembros de la familia (Flick y Luscher, 2012). Así pues, a pesar de estas regiones divergentes, las 7 *sirts* de la dorada presentan una alta identidad con sus respectivos ortólogos en humanos, con un porcentaje que oscila entre el 60,7% (Sirt3a) y el 73% (Sirt2). Ello confirma el alto grado de conservación de este grupo de enzimas a lo largo de la evolución de los vertebrados y de forma indirecta evidencia la importancia de las funciones de las SIRTs como reguladoras del metabolismo energético.

El análisis filogenético de las SIRTs de varias especies de vertebrados (incluidas las de dorada), las clasifica de acuerdo con la presente jerarquía de los vertebrados y las clases descritas por Frye (2000): clase I, SIRT1-3; clase II, SIRT4; clase III, SIRT5 y clase IV, SIRT6-7 (**Fig. 3.3**). Todas las ramas del árbol filogenético forman agrupaciones monofiléticas y las secuencias de las *sirts* de peces se

agrupan entre ellas con la excepción de la *sirt3*. En este caso, la *sirt3* de la dorada parece estar más relacionada con la de la rana que con la del pez cebra, lo que podría ser indicativo de la existencia de copias múltiples más que de una verdadera divergencia evolutiva (ver más adelante).

El análisis de las secuencias de las *sirts* de dorada se ha completado a nivel de organización génica mediante búsquedas blat en la base de datos del genoma de dorada del IATS-CSIC (**Fig. 6.1**), disponible a través del siguiente enlace http://nutrigroup-iats.org/seabreamdb/ (Pérez-Sánchez et al., 2019). De forma general, las distintas *sirts* de dorada presentan una gran variabilidad en el número de exones, que va desde 3 en la *sirt4* hasta 16 en la *sirt2*. Sin embargo, cuando las comparaciones se hacen con los ortólogos de las SIRTs de pez cebra y humanos, el número y la longitud de los exones está altamente conservado a lo largo de la evolución. Por el contrario, la longitud de los intrones es altamente variable, probablemente como consecuencia de unas restricciones funcionales menos estrictas en el caso de las regiones no codificantes (Li y Graur, 1991).

7.2.2. La familia de las sirtuínas como ejemplo de la alta tasa de duplicación del genoma de la dorada

Además de los 7 ortólogos de las SIRTs de mamíferos, descritos inicialmente en la **Tabla 3.2**, búsquedas más exhaustivas en el segundo borrador del genoma de la dorada -publicado en diciembre de 2019 (Pérez-Sánchez et al., 2019)- han puesto de relieve la existencia de secuencias completas correspondientes a 2 copias o parálogos de *sirt3* y *sirt5* (**Fig. 7.7**). La *sirt3b,* con una longitud de 4,5 kb y 7 exones es la duplicación con la que se han llevado a cabo los diferentes estudios de

expresión génica a lo largo de la presente Tesis Doctoral. La *sirt3a,* que tiene una longitud de 4,3 kb es la nueva variante y consta de 9 exones que codifican para una proteína de 393 aminoácidos. En el caso de la *sirt5,* la variante *sirt5a* formada por 8 exones y con una longitud de 48,3 kb es la que se detectó inicialmente, mientras que la *sirt5b* es la nueva variante con una longitud de 11,7 kb y formada por 8 exones que codifican para una proteína de 315 aminoácidos.

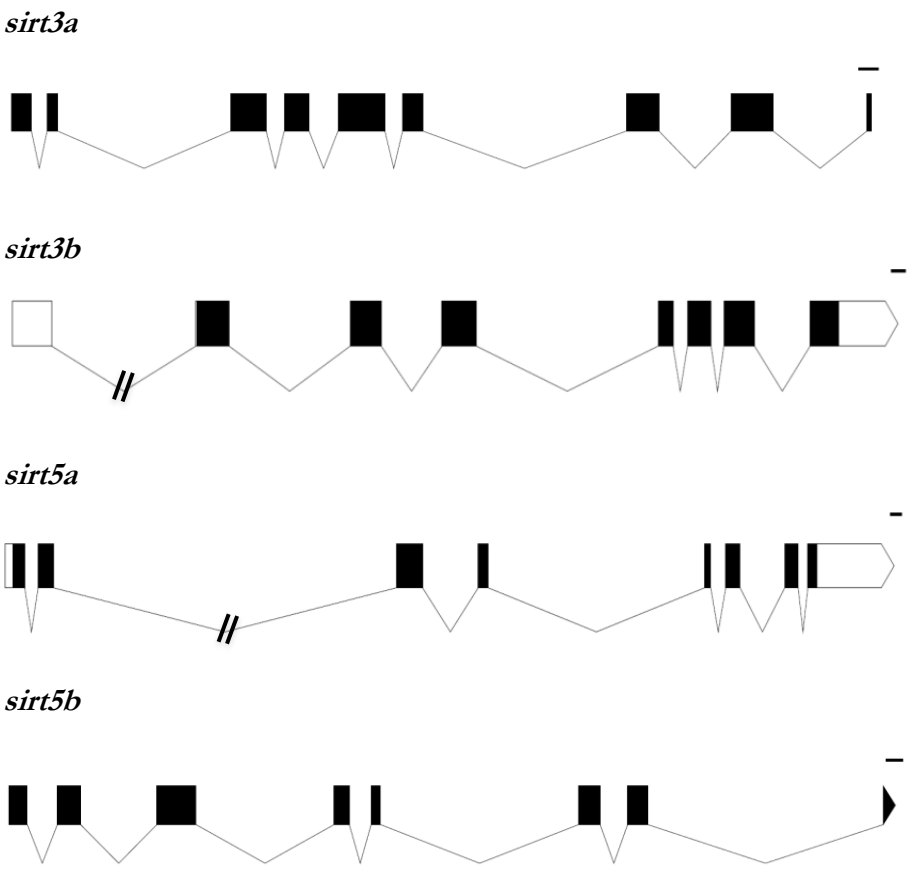


Figura 7.7 Estructura génica de las copias de la *sirt3* y *sirt5* de la dorada. La escala corresponde a 100 pares de bases.

Con estas nuevas secuencias de Sirts de dorada, junto con otras SIRTs de vertebrados presentes en las bases de datos públicas, se han construido dos nuevos árboles filogenéticos (**Fig. 7.8** y **Fig. 7.9**) como complemento al ya mostrado en el **Capítulo 3** (**Fig. 3.3**). En estos nuevos árboles se ha incluido la lamprea (*Petromyzon marinus*) que representa la unión entre invertebrados y vertebrados mandibulados. También se ha añadido un representante de los gnatostomados condrictios (*Callorhinchus milii*). Dentro de los sarcopterigios se han incluido: el celacanto (*Latimeria chalumnae*), un organismo fósil vivo que hace de puente entre las especies acuáticas y terrestres, y varios representantes del grupo de los tetrápodos (reptiles, anfibios, aves y mamíferos). Finalmente, se han incluido distintas especies de teleósteos y otras especies de actinopterigios que divergieron con anterioridad a los teleósteos (el polipteriforme *Erpetoichthys calabaricus* y el lepisosteiforme *Lepisosteus oculatus*).

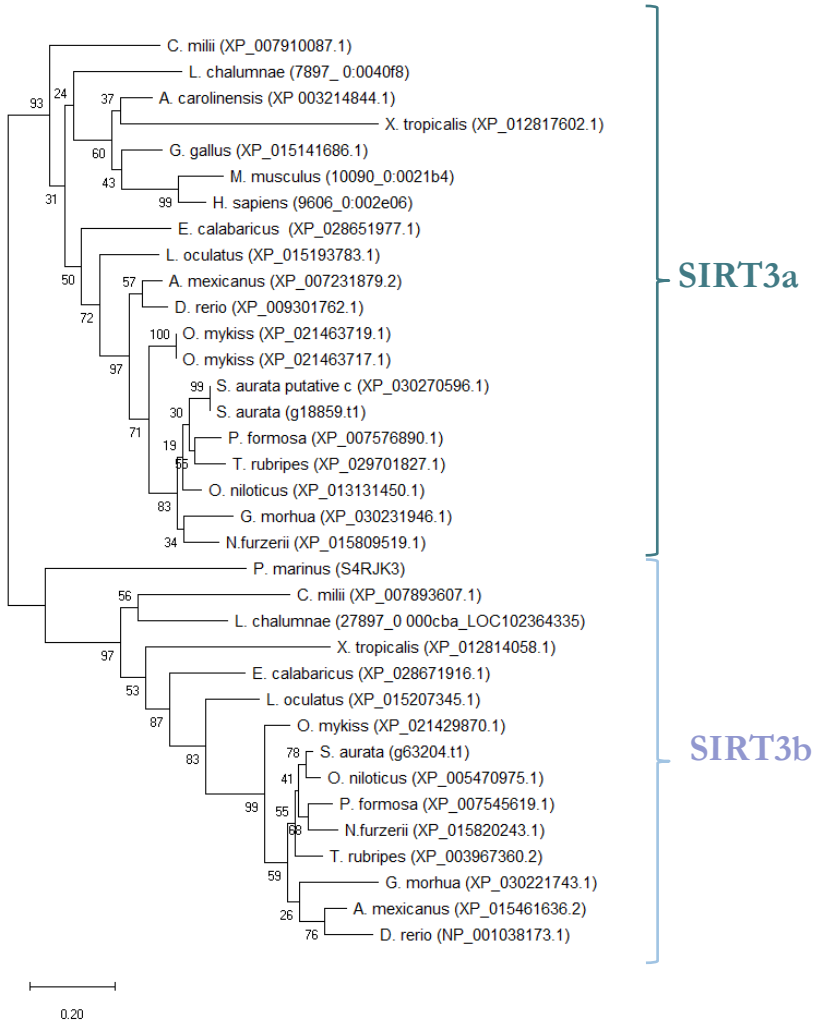


Figura 7.8 Árbol filogenético de las distintas copias de la SIRT3 (SIRT3a y SIRT3b) de vertebrados. Los números de acceso de GenBank, OrthoDB y del navegador del genoma de dorada del IATS-CSIC se incluyen entre paréntesis. El análisis se ha realizado mediante el método de máxima verosimilitud con el programa MEGA-X. La barra de escala representa el número de sustituciones por aminoácido.

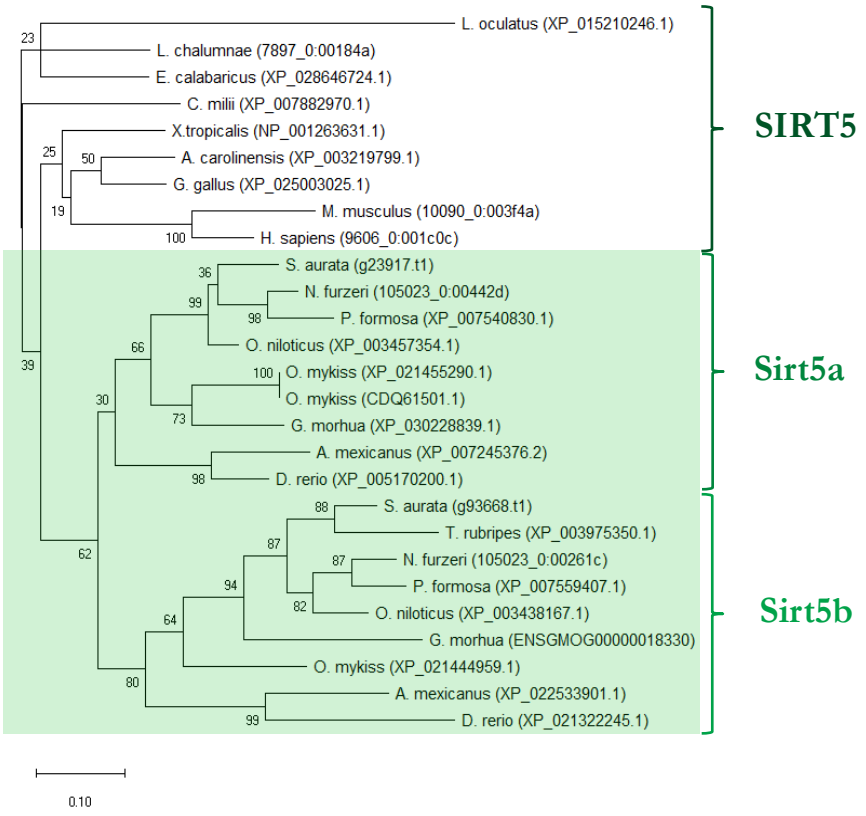


Figura 7.9 Árbol filogenético de las distintas copias de la SIRT5 (SIRT5, Sirt5a y Sirt5b) de vertebrados. Los números de acceso de GenBank, OrthoDB y del navegador del genoma de dorada del IATS-CSIC se incluyen entre paréntesis. El fondo verde indica las especies de teleósteos. El análisis se ha realizado mediante el método de máxima verosimilitud con el programa MEGA-X. La barra de escala representa el número de sustituciones por aminoácido.

A lo largo de la evolución de los vertebrados han ocurrido dos eventos de duplicación genómica global (2R). Una tercera duplicación, específica de teleósteos (3R), ocurrió en un ancestro común de los teleósteos hace unos 350 millones de años (Meyer y Van de Peer, 2005). Esa duplicación que se produjo durante la evolución temprana de los teleósteos aumentó extraordinariamente el número de genes y favoreció la expansión de uno de los grupos de vertebrados con mayor número de especies. Por tanto, la presencia de dos copias de *sirt3* en casi todos grupos analizados en el árbol filogenético de la **Fig. 7.8** podría indicar que la segunda copia se originó tras la duplicación del genoma del ancestro común de todos los vertebrados (2R) y que las especies que no la presentan (reptiles, aves y mamíferos) la han perdido posteriormente. Sin embargo, la presencia de dos parálogos relacionados con un solo gen ortólogo en otras especies de vertebrados es común en teleósteos, debido a la duplicación 3R específica de este grupo de vertebrados (Christoffels et al., 2004; Vandepoele et al., 2004). Esto es lo que ocurre con las copias de la *sirt5* de dorada, que se habrían generado con la duplicación 3R, conservándose en la mayor parte de peces modernos (**Fig. 7.9**).

Además de las ya mencionadas duplicaciones genómicas, hay una cuarta duplicación propia de los salmónidos y de otras especies de peces tetraploides. Esto explicaría, al menos en parte, que el tamaño de los genomas secuenciados de peces varíe entre 342 Mb en *Tetraodon nigroviridis* y 2,90 Gb en *Salmo salar* (Yuan et al., 2018), mostrando el último borrador del genoma de dorada un tamaño intermedio (1,24 Gb) (Pérez-Sánchez et al., 2019). Este tamaño es superior al de otros genomas de peces cultivados como el rodaballo o la lubina, donde el

tamaño estimado es de 0,54-0,68 Gb (Tine et al., 2014; Figueras et al., 2016), que fue similar al estimado en el primer borrador del genoma de dorada publicado en 2018 (Pauletto et al., 2018). Sin embargo, el mayor tamaño de nuestro genoma podría explicarse por el empleo de técnicas de no enmascaramiento durante el proceso de ensamblaje, tras lo cual se ha puesto de manifiesto que más del 55% de los 55.000 genes de dorada son genes duplicados con una tasa media de duplicación de 2,01 (0,3 excluyendo expansiones). Es más, el análisis del filoma ha evidenciado que muchas de estas duplicaciones son recientes, lo que habría contribuido a la notable plasticidad fenotípica de una especie hermafrodita protándrica, euriterma y eurihalina con una gran capacidad de adaptación al cultivo intensivo y a las nuevas formulaciones de piensos. Por tanto, no podemos descartar que se hayan producido más duplicaciones de los genes de la familia de las *sirts* en una especie con una alta tasa de duplicación génica, lo que está especialmente asociado a la respuesta inmune y a una rápida percepción de otros posibles cambios del medio en el que se encuentra el animal (Pérez-Sánchez et al., 2019).

7.2.3. Las islas CpG no están igualmente conservadas en las regiones promotoras de las sirtuínas

Las CGIs están conservadas en número y localización en vertebrados superiores (Deaton y Bird, 2011), pero el número y densidad de las CGIs son variables entre los genomas de peces (Han et al., 2008; Han y Zhao, 2008). Ello se podría deber a dificultades metodológicas, aunque está claro que la aparición de las CGIs se da de forma temprana durante la evolución y que su número en las regiones promotoras incrementa

progresivamente desde los peces (vertebrados de sangre fría) hasta los vertebrados de sangre caliente (Sharif et al., 2010).

Existen pocos estudios que analicen con detalle la regulación de los promotores de las *SIRTs*, aunque se ha descrito la presencia de CGIs en estas regiones en humanos (Furuya et al., 2012; Hou et al., 2013; Sahin et al., 2014; Wang et al., 2015; Kurylowicz et al., 2016), ratón (Zullo et al., 2018) y rumiantes (Hong et al., 2018; 2019a; 2019b). En la presente Tesis Doctoral se ha analizado *in silico* la presencia de CGIs en la región promotora de las distintas *sirts* de dorada y solo se han detectado en la *sirt1* y la *sirt3* (**Fig. 6.2** y **Fig. 6.3**). La CGI de la *sirt1* parece estar conservada desde condrictios como el tiburón elefante hasta diferentes especies de peces teleósteos (pez globo, pez cebra, tilapia y salmón atlántico). Sin embargo, la presencia de CGIs no está conservada en la *sirt3* de peces, lo que podría ser indicativo de una diferente regulación del metabolismo energético de los peces.

En cualquier caso, es interesante reseñar que estudios sobre la actividad de más de 4.500 promotores de humanos han permitido establecer que los promotores CGIs tienden a estar enriquecidos en lugares de unión a factores de transcripción para SP1, NRF1, E2F, BoxA, CRE, E-box y ETS (Rozenberg et al., 2008; Landolin et al., 2010). Estos lugares de unión a factores de transcripción también se han encontrado en las regiones promotoras de la *sirt1* y la *sirt3* de la dorada (**Fig. 6.2** y **Fig. 6.3**), lo que demuestra la conservación de la organización génica de las *sirts* y de su regulación a nivel transcripcional. No obstante, por el momento, solo en humanos y roedores se ha demostrado que la SIRT1 es capaz de modular mediante

retroalimentación negativa los factores de transcripción que la regulan (Buler et al., 2016).

7.2.4. La expresión de las sirtuínas de dorada es ubicua y con un patrón específico propio de cada de tejido

Las 7 *sirts* analizadas a nivel transcripcional en la presente Tesis Doctoral se detectaron en al menos 14 tejidos (**Tabla 3.3**). Resultados similares se han obtenido en humanos (Michishita et al., 2005), cerdos (Jin et al., 2009) y más recientemente en el pez teleósteo killi (*Nothobranchius furzeri*) (Kabiljo et al., 2019). Sin embargo, en pez cebra, las *sirt2*, *4* y *7* no se han detectado en todos los tejidos analizados (Pereira et al., 2011). De hecho, nuestros resultados mostraron una expresión ubicua a la vez que específica de cada tejido para cada uno de los miembros de la familia. Es más, el análisis multivariante de los patrones de expresión de las *sirts* mostró unos niveles altos de expresión de las *sirt1*, *2* y *5*, en contraposición a una expresión baja o de valor intermedio de las *sirt3*, *4*, *6* y *7* (**Fig. 3.4**).

Un análisis más detallado muestra que la *sirt1* está igualmente expresada en todos los tejidos analizados de dorada, pez cebra y killi. Por el contrario, la *sirt2* está claramente sobreexpresada en el hígado de la dorada o a nivel muscular en el pez killi. En cambio, la *sirt5* se clasifica entre las *sirts* con niveles bajos de expresión en pez cebra, mientras que en killi y en dorada muestra niveles altos de expresión en varios tejidos. Concretamente en dorada, los tejidos con una mayor expresión de *sirt5* fueron el hígado y los tejidos aeróbicos musculares (corazón y músculo esquelético rojo). De acuerdo con ello, en humanos y ratón, la *SIRT5* presenta unos niveles altos de expresión en corazón y cerebro

(Nakagawa et al., 2009; Matsushita et al., 2011), que son tejidos con una alta demanda energética. Ello podría ser debido al hecho de que la SIRT5 es única por su robusta actividad lisina demalonilasa, desuccinilasa y deglutarilasa, estando estas modificaciones de lisina presentes en proteínas relacionadas con varias rutas metabólicas incluyendo la glucólisis, gluconeogénesis, la β-oxidación y la fosforilación oxidativa (Du et al., 2011; Peng et al., 2011; Tan et al., 2014). Ahora bien, los dos parálogos de la *sirt5* del pez killi presentan una expresión similar en todos los tejidos analizados, con excepción del riñón, estando altamente expresados en hígado, corazón, intestino y músculo, mientras que los niveles más bajos se dan en cerebro y testículos (Kabiljo et al., 2019). Actualmente, se están llevando a cabo estudios similares en dorada, para confirmar un posible patrón de expresión diferencial de los parálogos *sirt5a* y *sirt5b* de peces.

El análisis transcripcional de la *sirt3*, la deacetilasa mitocondrial por excelencia en mamíferos, mostró niveles bajos de expresión en todos los tejidos de la dorada excepto en riñón anterior e intestino posterior, que son tejidos inmunorelevantes en peces (Rombout et al., 2011; Secombes y Wang, 2012; Calduch-Giner et al., 2016). En pez cebra, las *sirts* mitocondriales también muestran niveles de expresión bajos (Pereira et al., 2011), aunque de todas las variantes mitocondriales, la *sirt3* es la que presenta niveles más altos en músculo y riñón. De manera similar, en el pez killi la expresión de la *sirt3* es alta en músculo y baja en hígado y corazón. Curiosamente, estos tejidos están más expuestos a determinadas condiciones de estrés (p. ej. hipoxia o estrés oxidativo) que afectan a la función mitocondrial, lo que refuerza el papel de la SIRT3 en el mantenimiento de la homeostasis celular después de episodios de estrés

(Hirschey et al., 2010). En cualquier caso, es importante recordar que la *sirt3* (*sirt3b*) que hemos estudiado a lo largo de esta Tesis Doctoral es aparentemente la copia que se ha perdido en mamíferos y por tanto el patrón de expresión y las funciones pueden ser distintas de las descritas para la SIRT3 típica de mamíferos, con una expresión alta en tejidos con una alta demanda metabólica como el corazón, el hígado, el riñón, el tejido adiposo marrón y el músculo esquelético (Jin et al., 2009).

Considerado como un todo, estos resultados muestran la complejidad de los patrones de expresión de las SIRTs en los distintos tejidos y, lo más importante, cómo éstos pueden diferir entre grupos taxonómicos con diferentes presiones evolutivas a lo largo de su ciclo vital. En todo caso, los resultados disponibles muestran perfiles de expresión específicos de tejido para cada miembro de la familia, lo que unido a diferentes actividades enzimáticas y localizaciones subcelulares, es la base de una subfuncionalización y complementariedad que todavía está lejos de haberse elucidado completamente, tal y como se muestra en los apartados siguientes.

7.2.5. La expresión de las sirtuínas está notablemente alterada por la disponibilidad de nutrientes

Muchos animales viven en ambientes donde la disponibilidad de alimento no es constante y para hacer frente a los periodos de escasez reducen su demanda de energía con el fin de mantener sus funciones fisiológicas vitales (Secor y Carey, 2016). En este tipo de respuestas, la modificación o alteración del metabolismo mitocondrial es un componente esencial (Monternier et al., 2014; Bermejo-Nogales et al., 2015; Chausse et al., 2015), estando la fosforilación oxidativa

altamente regulada a nivel nutricional en dorada (Bermejo-Nogales et al., 2015). Por tanto, la falta de nutrientes causada por una situación de restricción calórica o ayuno natural induce respuestas adaptativas que comportan una reducción de los requerimientos energéticos provocando una disminución de los depósitos grasos del animal, como se muestra en el modelo de ayuno objeto de estudio en la presente Tesis Doctoral (**Tabla 7.1**). En todo caso, las respuestas adaptivas que se desencadenan son propias o específicas de cada tejido y la diferente expresión de las *sirts* a nivel tisular puede facilitar el fenotipado metabólico de cada uno de los distintos tejidos, de acuerdo con sus diferentes capacidades metabólicas. Así pues, el ayuno disminuyó la expresión de las *sirts* en tejidos con capacidad de acúmulo graso y la aumentó en tejidos con una alta demanda energética como músculo rojo, cerebro, riñón anterior y corazón. Especial mención merece la activación generalizada de la expresión de la *sirt1*, lo que está en consonancia con una importante participación en una amplia variedad de procesos fisiológicos. Asimismo, cabe destacar la respuesta de la *sirt2,* cuya expresión se vio fuertemente disminuida en tejidos de acúmulo graso (hígado, piel y tejido adiposo) y de absorción de nutrientes (intestino anterior). Sin embargo, la expresión tanto de *sirt1* como de *sirt2* aumentó con el ayuno en cerebro y riñón anterior, posiblemente con la finalidad de proteger a estos tejidos del riesgo de estrés oxidativo, tal y como se ha evidenciado en otros modelos experimentales en los que esta acción está mediada por los factores de transcripción FOXO (Wang et al., 2007).

Tabla 7.1 Parámetros biométricos y de crecimiento de los peces alimentados (grupo CTRL) y ayunados durante 10 días. Cada valor corresponde a la media ± error estándar de 8 peces. Modificada de Bermejo-Nogales et al., 2015.

	CTRL	Ayunado	*P*[a]
Peso final (g)	109,5 ± 3,42	79,9 ± 1,82	*<0,001*
Vísceras (g)	9,35 ± 0,49	4,34 ± 0,23	*<0,001*
Hígado (g)	2,31 ± 0,13	0,52 ± 0,03	*<0,001*
VSI (%)[b]	8,52 ± 0,23	5,41 ± 0,19	*<0,001*
HSI (%)[c]	2,10 ± 0,06	0,64 ± 0,02	*<0,001*
Ingesta MS (g/pez)	17,25	-	

El peso medio inicial de los peces fue de 86 ± 0,08 g.
[a]Valores P resultantes de un test t-Student.
[b]Índice viscerosomátco = (100 × peso vísceras)/peso pez.
[c]Índice hepatosomático = (100 × peso hígado)/peso pez.

El hígado es un tejido metabólicamente activo (Guderley et al., 2003; Wang et al., 2006) que responde al ayuno de manera rápida y drástica. Así, por ejemplo, un ayuno de dos semanas en juveniles de trucha común (*Salmo trutta*) va acompañado de una reducción del peso del hígado y de su actividad mitocondrial (Salin et al., 2018). En dorada, un ayuno similar desencadena una importante disminución de la expresión de enzimas lipogénicas (incluyendo elongasas y desaturasas de AGs) (Benedito-Palos et al., 2014) y de 72 de los 88 genes analizados pertenecientes a los 5 complejos enzimáticos de la cadena respiratoria mitocondrial (Bermejo-Nogales et al., 2015), lo que no es de extrañar dado que la lipogénesis hepática está considerada el proceso más demandante de energía a nivel hepático (Rui, 2014). Por tanto, tal y como se muestra en la **Fig. 3.5**, la reducción de la demanda energética del hígado con el ayuno estaría sustentada, al menos en parte, por una menor expresión de 5 de las 7 *sirts* analizadas. Entre ellas no se incluye la *sirt1* a pesar de que varios estudios en ratones han demostrado que el

ayuno activa positivamente la transcripción de la *SIRT1* y de genes gluconeogénicos a través de la formación del complejo CREB/CRTC2 (Noriega et al., 2011). Además, con la prolongación del ayuno, la SIRT1 también deacetila la PGC-1α y la FOXO-1, permitiendo que continúe la gluconeogénesis en ausencia de CREB a la vez que se estimula la β-oxidación (Brunet et al., 2004; Rodgers et al., 2005). Esta diferente regulación a nivel de especie podría deberse a que los modelos de ayuno de mamíferos no son totalmente equiparables en tiempo ni intensidad a los de peces (p. ej. en roedores un ayuno prolongado consiste en 24h). En cualquier caso, la respuesta conjunta de varias *sirts* es un hecho probado tanto en dorada como en modelos de humanos y roedores (Lai et al., 2013; Ghiraldini et al., 2013), lo que es indicativo de un importante grado de complementariedad y posiblemente también de solapamiento.

El músculo esquelético suele representar más del 60% del peso corporal, por lo que es un gran consumidor de energía en condiciones óptimas de crecimiento (Johnston et al., 2011). De acuerdo con ello, estudios previos en dorada han demostrado que la disponibilidad de alimento influye en los cambios relacionados con el recambio de proteínas musculares y la actividad mitocondrial (Calduch-Giner et al., 2014; Bermejo-Nogales et al., 2015). Es más, contrariamente a lo que ocurre a nivel hepático, el ayuno estimula la fosforilación oxidativa del músculo esquelético y cardiaco tanto en dorada como en cerdos (da Costa et al., 2004) y ratones (Suzuki et al., 2002). Por tanto, cada tejido genera respuestas distintas frente al ayuno que también se ven reflejadas en una diferente regulación de las *sirts*, pero en todo caso la respuesta a nivel muscular es más heterogénea que a nivel hepático. De hecho, la expresión de *sirt1* aumentó en todos los tejidos musculares estudiados,

pero la *sirt5* solo lo hizo de forma significativa en el músculo esquelético blanco y la *sirt6* en el corazón.

En el músculo de los mamíferos, el cambio del uso de los carbohidratos por el de los lípidos como fuente principal de energía durante el ayuno está mediado por la acción de la SIRT1 (Chang y Guarente, 2014) que deacetila el PGC-1α para activar la maquinaria lipolítica. Ello va acompañado de la activación del AMPK que aumenta la expresión de PGC-1α, lo que comporta un aumento de la biogénesis mitocondrial y de la oxidación de AGs en el músculo (Gerhart-Hines et al., 2007; Jager et al., 2007; Hardie et al., 2012). Sin embargo, la participación de la *sirt5* en la regulación del metabolismo del músculo no está muy estudiada, aunque como se ha comentado previamente en el **Capítulo 1** sus distintas actividades enzimáticas tienen como diana proteínas de varias rutas metabólicas (Du et al., 2011; Peng et al., 2011; Tan et al., 2014). En cambio, la *sirt6* tiene una función cardioprotectora que parece estar compartida por otras *sirts*. De hecho, la *sirt1* previene el estrés oxidativo (Calabrese et al., 2008; Hsu et al., 2010) y la hipertrofia cardiaca (Planavila et al., 2011), además de promover la oxidación aeróbica de la glucosa manteniendo, de este modo, la homeostasis cardiaca (Khan et al., 2018).

El tejido adiposo participa en la homeostasis energética del organismo, regulando la movilización/deposición de energía y la secreción de adipocinas que tienen una función endocrina y actúan sobre el sistema nervioso central y otros tejidos periféricos (Salmeron, 2018). Los mecanismos últimos todavía son objeto de debate, pero en el caso de la dorada nuestros resultados de expresión (**Tabla 3.4**) indican que el aumento de la *sirt1* podría mediar, al menos en parte, la activación de la

lipolisis del tejido adiposo durante el ayuno, tal y como se ha descrito previamente por otros autores en ratones (Picard et al., 2004). Por el contrario, tanto en dorada como en ratones (Jing et al., 2007), el alto nivel de expresión de la *sirt2* en el tejido adiposo de estos animales sugiere un importante papel en la regulación de la deposición grasa, habiéndose demostrado su participación en los procesos conducentes a la diferenciación de los adipocitos (Jing et al., 2007). Al igual que la *sirt2*, la *sirt7* de dorada también disminuyó en nuestro modelo de ayuno, lo que está en consonancia con el aumento de la expresión de la *SIRT7* en el tejido adiposo de pacientes obesos (Kurylowicz et al., 2016). Estudios recientes en ratón han demostrado que la SIRT7 reprime la actividad de la SIRT1 y, por consiguiente, el bloqueo de la SIRT7 permite la acción de la SIRT1, que bloquea el PPARγ y la diferenciación de los adipocitos (Fang et al., 2017). Por lo cual, tanto en dorada como en vertebrados superiores, la *sirt1*, *2* y *7* parecen jugar un importante papel en la regulación del metabolismo lipídico del tejido adiposo.

A nivel intestinal, 4 de las 7 *sirts* (*sirt1*, *5-7*) analizadas aumentaron su expresión tanto en la región anterior, más relacionada con la absorción de nutrientes, como en la posterior, especialmente relacionada con la función inmune. En todo caso, la respuesta global es una activación de la expresión de las *sirts* que favorecería un perfil antiinflamatorio típico de una situación de restricción calórica (González et al., 2012; Youm et al., 2015). De hecho, la SIRT1 ha demostrado tener efectos antinflamatorios en respuesta a la inflamación intestinal aguda y reprime la tumorigénesis intestinal y el cáncer de colon asociado a la colitis (Larrosa et al., 2009; Bereswill et al., 2010; Hofseth et al., 2010). En otros tejidos de mamíferos, también se ha descrito una acción

antiinflamatoria de las SIRTs, como lo demuestra el hecho que la SIRT6 regula la actividad del NF-κB, controlando así las reacciones inflamatorias del corazón (Yu et al., 2013) y del tejido óseo (Wu et al., 2015). También se ha demostrado que la SIRT5 regula la respuesta inflamatoria de macrófagos (Wang et al., 2017) y que la SIRT7 tiene un papel antiinflamatorio, aunque los mecanismos de acción están menos estudiados (Mendes et al., 2017).

A modo de resumen, en la **Fig. 7.10** se muestra la diferente regulación de las *sirts* en respuesta a la falta de nutrientes, con el consiguiente reajuste metabólico propio de un estado hipometabólico. Tal reajuste también se da en un estado de hipoxia moderada, caracterizada por una inhibición de la ingesta que conlleva una reducción de la producción de ROS y un menor riesgo de estrés oxidativo (Martos-Sitcha et al., 2019). A nivel transcripcional, ello comporta cambios en los sistemas de detección de energía del músculo esquelético, pero sobre todo en el corazón, donde la expresión de varias *sirts* (*sirt1*, *5-7*) se ve disminuida en los peces sometidos a hipoxia, en especial si se encuentran en condiciones de alta densidad de cultivo. Respecto a una situación de ayuno, las *sirts* que responden son las mismas, aunque la dirección del cambio es contraria, demostrando una vez más que son buenos marcadores metabólicos al responder de manera específica a diferentes tipos de estrés metabólico.

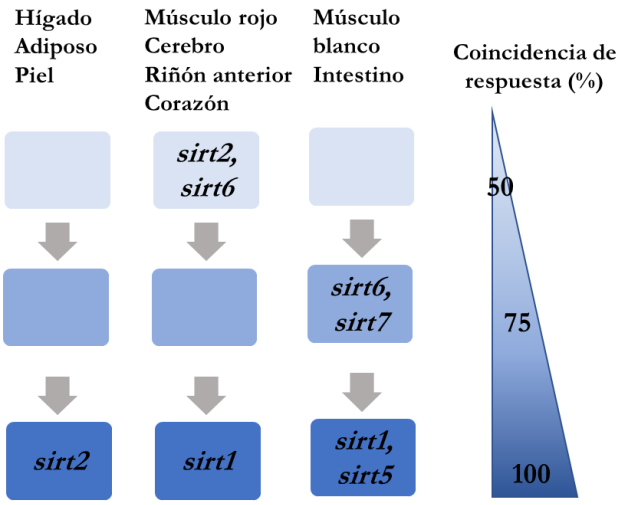


Figura 7.10 Diagrama resumen en un modelo de ayuno de la respuesta de los isotipos de *sirts* en el que se indica el % de tejidos en los que se detecta un mismo patrón de respuesta para cada grupo de tejidos. Los tejidos están clasificados según sus capacidades metabólicas: almacenamiento de energía (hígado, tejido adiposo, piel), alta demanda energética (músculo rojo, cerebro, riñón anterior, corazón) y absorción de nutrientes y acreción proteica (músculo blanco e intestino). Modificada de la Fig. 3.5.

7.2.6. Las sirtuínas contribuyen al reajuste metabólico asociado a la aceleración del crecimiento

Como resultado del aumento de la ingesta también aumenta la actividad mitocondrial y la producción de ROS, lo que acaba afectando a la ingesta voluntaria de alimento y al crecimiento (Saravanan et al., 2012; Rise et al., 2015). Por tanto, los peces con altas ingestas y tasas de crecimiento deben afrontar un mayor riesgo de estrés oxidativo y la regulación diferencial de las *sirts* puede contribuir al reajuste y preservación de la homeostasis metabólica y del crecimiento, siendo el eje GH/IGF uno de sus actores principales (ver la revisión de Pérez-Sánchez et al., 2018). Los mecanismos últimos por los que cambios en la expresión y actividad de

las SIRTs modulan la acción de las hormonas metabólicas y del sistema GH/IGF están poco estudiados tanto en vertebrados inferiores como superiores. Sin embargo, estudios en ratones demuestran que la supresión *in vivo* de la *Sirt1* hepática reestablece la caída de los niveles de IGF-I provocada por el ayuno, a la vez que aumenta la producción de IGF-I en respuesta a la acción anabólica de la GH (Yamamoto et al., 2013). Así pues, los "knockdown" de *Sirt1* en ratón aumentan el nivel de acetilación y de fosforilación de la STAT5, lo que indica que la SIRT1 regula negativamente la producción de IGF-I dependiente de GH a través de la regulación postranscripcional de la STAT5 (Yamamoto y Takahahi, 2018). La SIRT1 también actúa a nivel cerebral como nexo de unión entre las señales propias del eje GH/IGF y las desencadenadas por un determinado nivel de ingesta (Satoh et al., 2010), mostrando los "knockout" de *Sirt1* de cerebro signos de enanismo y niveles bajos de GH e IGF-I circulantes (Cohen et al., 2009). Por el contrario, la activación de la SIRT1 con resveratrol inhibe la síntesis hipofisaria de GH en ratas, al reducir la disponibilidad de PIT-1 para el promotor de la *Gh* mediante la supresión transcripcional de CREB (Monteserin-García et al., 2013). A pesar de esta aparente contradicción, el efecto neto de la activación de SIRT1 continúa siendo la supresión del tono GH/IGF, con la finalidad de disminuir la demanda de energía para crecimiento en un escenario con baja disponibilidad de sustratos metabólicos.

Como se ha discutido en el apartado anterior, el ayuno no altera en el caso de dorada la expresión hepática de *sirt1*. Sin embargo, doradas de crecimiento acelerado sí que muestran una menor expresión hepática de *sirt1* (**Fig. 4.1**), a la vez que una mayor ingesta y niveles plasmáticos más altos de Igf-i (**Tabla 4.5**). Es más, hay estudios en ratones que

indican que con el aumento de la disponibilidad de nutrientes, el factor de transcripción ChREBP regula negativamente la expresión de la *SIRT1* (Chalkiadaki y Guarente, 2011; Noriega et al., 2011). Análisis *in silico* también nos han permitido mostrar que los lugares de unión al factor de transcripción ChREBP están conservados en la región promotora de la *sirt1* de dorada (**Fig. 6.2**), lo que podría indicar unos mecanismos de regulación parecidos.

La expresión de las *sirts* también está altamente regulada por el estado energético en el músculo esquelético blanco de la dorada (**Fig. 4.1**). Por un lado, la expresión de *sirt2* está aumentada en peces de rápido crecimiento, mientras que la *sirt5* parece especialmente sensible a los cambios de estado energético propios de un ayuno forzado (**Tabla 3.4**) o natural (**Fig. 6.8**), cuando se compararon en este último caso peces de diferentes edades durante la temporada invernal (ver más adelante). En cualquier caso, ello comporta la activación de la maquinaria lipolítica y una mayor eficiencia energética, evidenciado por cambios en la expresión de las proteínas de desacoplamiento de la respiración mitocondrial (*ucps*) y de varios marcadores del metabolismo lipídico y de la fosforilación oxidativa, al igual que en trabajos previos con juveniles de dorada alimentados con una ración de mantenimiento (Calduch-Giner et al., 2014). Por otra parte, es importante resaltar que hay evidencias experimentales de que la SIRT2 está implicada en la proliferación y diferenciación de células musculares (Dryden et al., 2003; Wu et al., 2014; Stanton et al., 2017) y que polimorfismos de un solo nucleótido se han asociado a caracteres relacionados con el de peso en ganado vacuno (Gui et al., 2015).

El nivel de adiposidad es también otro de los factores relacionados con cambios en la expresión de las SIRTs. En concreto, la disminución de la actividad de la SIRT6 está asociada con la obesidad en ratones (Kuang et al., 2017), de la misma manera que la disminución de la SIRT5 ha demostrado estar relacionada con el aumento de la adiposidad, la inflamación y la resistencia a la insulina en humanos (Jukarainen et al., 2016). De acuerdo con ello, la mayor expresión de la *sirt5* y *sirt6* en el tejido adiposo de peces de rápido crecimiento (**Fig. 4.1**) podría mediar, al menos en parte, la menor deposición grasa de estos animales en el tejido adiposo, con la consiguiente movilización de AGs hacia el músculo esquelético y el hígado, como características propias de un fenotipo delgado, que se considera especialmente valioso en animales de cultivo, tanto en términos de salud como de eficiencia energética (Kause et al., 2016). Ahora bien, la movilización grasa propia del ayuno estaría mediada por otras *sirts*, lo que es indicativo de una regulación específica propia de cada situación, en la que también participaría el sistema endocrino representado por el eje GH/IGF con la doble finalidad de: i) evitar o minimizar la pérdida de masa muscular durante las etapas de balance energético negativo y ii) ajustar los procesos de crecimiento que demandan energía de los organismos con la disponibilidad de sustratos metabólicos (ver a modo de resumen la **Fig. 7.11**).

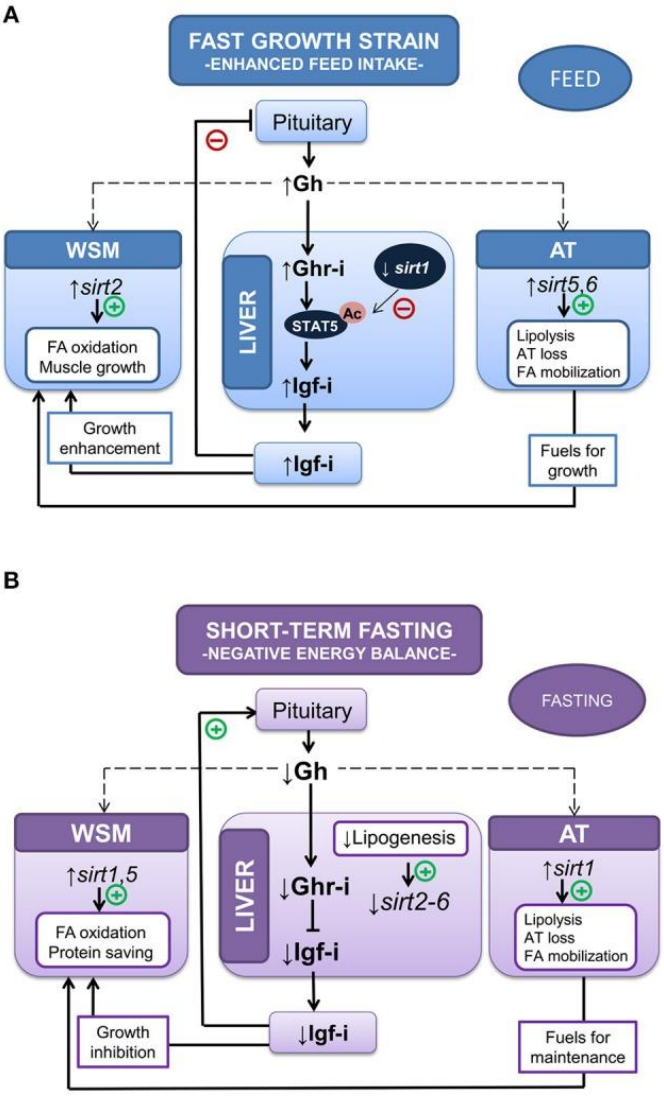


Figura 7.11 Relación entre las *sirts* y el sistema Gh/Igf en el hígado, el músculo esquelético (WSM) y el tejido adiposo (AT) de dorada en dos modelos experimentales correspondientes los **Capítulos 3** y **4** de la presente Tesis Doctoral. **A** Cepa de rápido crecimiento con un aumento de la ingesta. **B** Ayuno de 10 días que ocasiona un balance energético negativo. Modificado de Pérez-Sánchez et al., 2018.

7.2.7. Las sirtuínas responden a nivel intestinal sin mostrar un patrón espacial concreto

Tanto en lubina como en dorada se ha demostrado que existe una especialización funcional a lo largo del intestino (Pérez-Sánchez et al., 2015; Calduch-Giner et al., 2016; Estensoro et al., 2016) y por este motivo, varios grupos de genes muestran patrones de expresión espaciales, destacando en la región anterior aquellos marcadores relacionados con el transporte y la absorción de nutrientes mientras que en la región posterior están sobreexpresados marcadores inmunitarios (**Tabla 5.6**). Sin embargo, no parece ser éste el caso de las *sirts* de dorada al no mostrar un patrón de expresión diferencial a lo largo del intestino. A pesar de ello, la respuesta ante un determinado estímulo sí que puede ser diferente a lo largo del tracto intestinal en función de la intensidad o naturaleza de dicho estímulo. Así por ejemplo, la respuesta ante el ayuno es bastante uniforme a lo largo del tracto intestinal (**Fig. 3.5**), mientras que la respuesta diferencial en nuestro modelo de peces de diferente crecimiento queda reducida a los cambios mostrados por las *sirt2*, *3*, *5* y *7* en la sección posterior del intestino (**Fig. 4.2**), posiblemente en relación con las acciones antiinflamatorias descritas para la mayor parte de SIRTs (Zhou et al., 2018), lo que cobra especial relevancia en una región del intestino con una mayor concentración de células del sistema inmune. Sin embargo, con la suplementación dietaria de AGCMs o probióticos (**Fig. 7.6**), los mayores cambios en la expresión de *sirts* a nivel intestinal se observaron en la región anterior del intestino, lo que es indicativo de la dificultad de establecer un patrón general de regulación de las *sirts* que,

como no puede ser de otra manera en marcadores del estado energético, está influenciado por un gran número de factores bióticos y abióticos.

7.2.8. Las sirtuínas varían su expresión con los cambios metabólicos asociados a la edad y a la temperatura

El potencial de crecimiento propio de una determinada clase de edad varía con la disponibilidad de alimento. Las fluctuaciones de la temperatura asociadas a los cambios de estación también afectan al crecimiento y a varios procesos metabólicos implicados en la producción de energía (Donaldson et al., 2008; Ibarz et al., 2010; Kyprianou et al., 2010; Bermejo-Nogales et al., 2014), quedando ello reflejado a nivel hormonal en cambios en los patrones circulantes de las hormonas de la familia GH/PRL. Estas hormonas presentan un marcado patrón estacional, que en el caso de la Gh es indicativo del disparo del crecimiento durante el periodo estival, mientras que las variaciones de los niveles circulantes de prolactina (Prl) y somatolactina (Sl) proporcionarían las señales que promueven la reposición de los depósitos grasos durante periodos de alta ingesta, actuando también como factores desencadenantes del proceso reproductor, una vez alcanzado un determinado tamaño y nivel de adiposidad (**Fig. 7.12**). Estos cambios a nivel metabólico y hormonal también se ponen de relieve a nivel de microbiota, tal y como se ha demostrado en estudios recientes llevados a cabo con animales criados en nuestras instalaciones experimentales, que evidencian que la microbiota intestinal cambia con la edad y la reversión de macho a hembra en un hermafrodita protándrico como la dorada (Piazzon et al., 2019). Es más, los cambios observados

en la composición de la microbiota son indicativos de una mayor propensión a estados proinflamatorios con el avance de la edad.

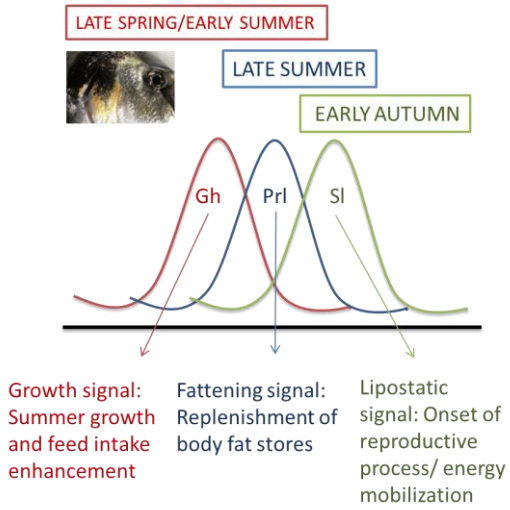


Figura 7.12 Patrones estacionales de los niveles plasmáticos de Gh, Prl, y Sl en juveniles de dorada. Modificada de Pérez-Sánchez et al., 2018.

En este escenario, los resultados de expresión de las *sirts* con animales de 1 y 3 años (**Capítulo 6**) muestran un carácter marcadamente estacional a nivel hepático y muscular, que es indicativo del diferente estado energético de los peces en cultivo con los cambios de estación y edad. Así pues, de forma generalizada, la expresión de las *sirts* aumentó durante el periodo invernal, siendo mayores las diferencias entre juveniles y adultos en esta época del año, ya que si bien en nuestra latitud (40°5N; 0°10E) los primeros conservan cierto nivel de ingesta durante el invierno, la ingesta es nula o casi nula en peces de más de dos años. Ahora bien, esta diferente regulación con la edad es claramente dependiente del tejido, ya que la edad disminuye la expresión de las *sirts* a nivel hepático, mientras que ejerce el efecto contrario a nivel muscular (**Fig. 6.8**). Esta diferente regulación también está sustentada por la

diferente regulación de otros marcadores del metabolismo energético, habiéndose concluido que el aumento de la expresión de las *sirts* analizadas es más informativo del estado energético que de la demanda de energía para crecimiento (músculo) o actividad lipogénica (hígado).

Tomando estos resultados como un todo, la *sirt1* resulta de especial relevancia para interpretar los cambios metabólicos asociados con la estación y la edad, habiéndose considerado que su mayor expresión con la edad y en el periodo invernal podrían estar relacionados con la participación de esta *sirt* en la inhibición de la miogénesis (Pardo et al., 2011). A su vez, el aumento de la expresión de la *sirt2* con la edad podría estar relacionado con la preservación de la integridad genómica y de la progresión del ciclo celular y el mantenimiento de la senescencia celular (Serrano et al., 2013; Anwar et al., 2016), más que con la proliferación y la diferenciación de los mioblastos (Wu et al., 2014; Stanton et al., 2017) que podría explicar también su alta expresión en peces con una genética que promueve un crecimiento acelerado.

7.2.9. La familia de las sirtuínas combina la presencia de promotores con y sin islas CpG

La presencia de CGIs en las regiones promotoras (promotores CGIs) está relacionada con genes expresados de manera ubicua, mientras que los genes sin estos elementos reguladores (promotores no-CGI) están más relacionados con genes que responden a señales ambientales y que requieren una activación/inhibición rápida bajo un estricto control transcripcional (Sharif et al., 2010). En el caso de las *sirts* de dorada, existen ambos tipos de promotores, probablemente para asegurar una expresión ubicua a la par que altamente regulada y que sea capaz de

responder a distintas situaciones o estímulos ambientales desde diferentes tejidos o localizaciones celulares. Como se ha descrito anteriormente, el patrón de expresión de las distintas *sirts* de dorada es específico de tejido y las que se expresan con un nivel más alto en el conjunto de los tejidos son la *sirt1*, *2* y *5* (**Fig. 3.4**). A pesar de esto, solo la *sirt1*, que presenta unos valores altos y más estables en los distintos tejidos, presentó un promotor con CGI. Por el contrario, el otro miembro de la familia con un promotor CGI es la *sirt3* que, junto con la *sirt4*, *6* y *7*, ha sido clasificada dentro de los isotipos con un menor nivel de expresión en los distintos tejidos de dorada, excepto en tejidos inmunológicamente relevantes. Si esto puede tener alguna relación con la existencia de copias múltiples para algunas de las variantes de *sirts* de peces, está todavía por determinar.

7.2.10. Mecanismos de regulación epigenética participan en la regulación de la *sirt1* a nivel muscular

La metilación del ADN genómico en la región promotora está relacionada con la inhibición de la expresión génica, mientras que la metilación en el cuerpo del gen no la bloquea e incluso puede estimular la elongación de la transcripción o influir en el tipo de "splicing" (Jones, 2012). En cualquier caso, el patrón de metilación del ADN es muy diverso y se ha demostrado que depende del tejido (Jones et al., 2015) y de la edad (Jung y Pfeifer, 2015), así como de factores externos como la temperatura en el caso de los peces (Varriale y Bernardi, 2006; Navarro-Martín et al., 2011; Shao et al., 2014).

En el caso que nos ocupa, las CGIs de la región promotora de la *sirt3* de dorada mostraron unos niveles de metilación bajos en todas las

posiciones analizadas con independencia de la edad, estación y tejido (hígado y músculo). Por tanto, esto es indicativo de que los cambios observados en la expresión de la *sirt3* no están aparentemente regulados por cambios en el nivel de metilación de la región promotora. Un resultado similar se obtuvo con la *sirt1* a nivel hepático. Sin embargo, a nivel muscular, algunas de las 22 posiciones estudiadas mostraron cambios significativos del nivel de metilación al comparar, durante el periodo estival, juveniles y adultos (**Fig. 6.5**). En concreto, 15 de las 22 posiciones estudiadas, mostraron una disminución del nivel de metilación con la edad, lo que puede interpretarse como un cambio adaptativo para ajustar las necesidades metabólicas y de ingesta propias de cada edad, habiéndose observado en posiciones cercanas al factor de transcripción SP1 una correlación negativa entre porcentaje de metilación y nivel de expresión de la *sirt1*. De la misma manera, comparando entre estaciones, también se observó una disminución de la expresión de la *sirt1* (sobre todo en juveniles) en verano que se correlacionó con el aumento de la metilación en CpGs cercanas a lugares de unión al factor de transcripción SP1, que es uno de los activadores transcripcionales mejor caracterizados (Pugh y Tjian, 1990; Suske, 1999; Wierstra, 2008; Chu, 2012). Este factor de transcripción se une a secuencias ricas en GC que son necesarias para la expresión y regulación de una gran variedad de genes, sobre todo "housekeeping" y otros genes sin promotores TATA.

La conexión entre el metabolismo y la epigenética a través de la acción de las SIRTs se ha demostrado ampliamente en vertebrados superiores (Jing y Lin, 2015). De hecho, la actividad deacilasa de las SIRTs sobre las histonas, los factores de transcripción y las enzimas

epigenéticas, y su requerimiento de NAD^+ como cosustrato, hace que las SIRTs conviertan las señales metabólicas en modificaciones epigenéticas para regular la expresión génica, la cromatina y la estabilidad del genoma (Bosch-Presegué et al., 2015). Sin embargo, la regulación epigenética de las SIRTs está menos estudiada y se han encontrado resultados contradictorios en diferentes modelos experimentales, mientras que algunos describen una correlación negativa entre la expresión génica y la metilación de las CGIs de las SIRTs (Hou et al., 2013; Hong et al., 2018; 2019a; 2019b), otros no encuentran ninguna correlación (Furuya et al., 2012; Sahin et al., 2014; Wang et al., 2015; Kurylowicz et al., 2016; Zullo et al., 2018). En el caso de la dorada, ello parece restringido de momento a la *sirt1* y a un tejido y condición estacional, que podría interpretarse como un mecanismo de salvaguardia para mantener regulada la expresión de una *sirt* con una expresión de carácter ubicuo y relativamente alta. Los mecanismos implicados no se han estudiado, aunque, al igual que en humanos, el PPAR β/δ podría participar en la regulación transcripcional de la SIRT1 vía SP1 (Okazaki et al., 2010).

7.2.11. Consideraciones finales

Los perfiles de expresión génica contribuyen a la comprensión de los mecanismos biológicos y facilitan la identificación de genes candidatos para un carácter de interés. Concretamente, los patrones de expresión específicos de las *sirts* en los distintos tejidos y su regulación diferencial en función de la intensidad y el tipo de estímulo o situación de demanda energética (**Fig. 7.13**) hacen de estas enzimas unos buenos marcadores para diferenciar distintos estados metabólicos en una amplia variedad de organismos. Por tanto, la información generada sobre estos sensores del

estado energético es valiosa para profundizar en investigaciones e incluirlos en paneles de marcadores para predecir el potencial genético de crecimiento, el nivel de adiposidad o el estado inflamatorio, con la finalidad última de establecer un fenotipo metabólico ideal.

El uso de biomarcadores es particularmente relevante para rasgos difíciles de medir, como los relacionados con el metabolismo intermediario. En este sentido, las *sirts* han demostrado que su regulación en peces está relacionada con respuestas específicas de tejido en diferentes situaciones energéticas, como la disponibilidad de alimento, el potencial de crecimiento, la temperatura y la edad. Muchos de los caracteres de interés en los programas de selección genética son cuantitativos y suelen estar controlados por varios genes que presentan efectos pequeños pero aditivos. Por tanto, la identificación de marcadores relacionados con uno de estos loci puede aumentar la tasa de mejora genética mediante la selección asistida por marcadores (MAS). Curiosamente, se han detectado en ganado bovino polimorfismos de las SIRT1-3 asociadas con características de la carcasa de alto valor productivo (Gui et al., 2015). Ello es indicativo de las ventajas del uso de las *SIRTs* como marcadores genéticos en la mejora de programas de selección genética. En este sentido, a lo largo de esta Tesis Doctoral se han puesto de manifiesto notables diferencias entre dos familias de diferente potencial de crecimiento, aunque no necesariamente el patrón encontrado se ha de reproducir exactamente al seleccionar individuos procedentes de otros cruces u orígenes de animales con diferente potencial de crecimiento.

En cualquier caso, ha quedado claro que la regulación funcional de las *sirts* no sigue un patrón general, sino que es variable en función de

factores bióticos y abióticos, lo que supone una dificultad adicional, aunque ello acaba siendo un valor añadido por su importante papel en diferentes estados o situaciones fisiológicas. Además, el hecho de que coexistan *sirts* de dorada con y sin CGIs, con una acción reguladora sobre el nivel de expresión, es una prueba indirecta de la variedad y complejidad de mecanismos reguladores de la función de estas enzimas como piezas claves del metabolismo energético e intermediario. Sin embargo, son necesarios más estudios para establecer cómo regular las SIRTs de forma efectiva mediante activadores de éstas, programas de selección genética o programación ambiental y nutricional. En definitiva, es necesario establecer la mejor estrategia para promover el desarrollo sostenible de la acuicultura, en cuyo caso las SIRTs acaban siendo una pieza más de un complicado puzzle para promover la selección de animales de alta eficacia de conversión del alimento, aunque sea a expensas de una merma de las tasas de crecimiento.

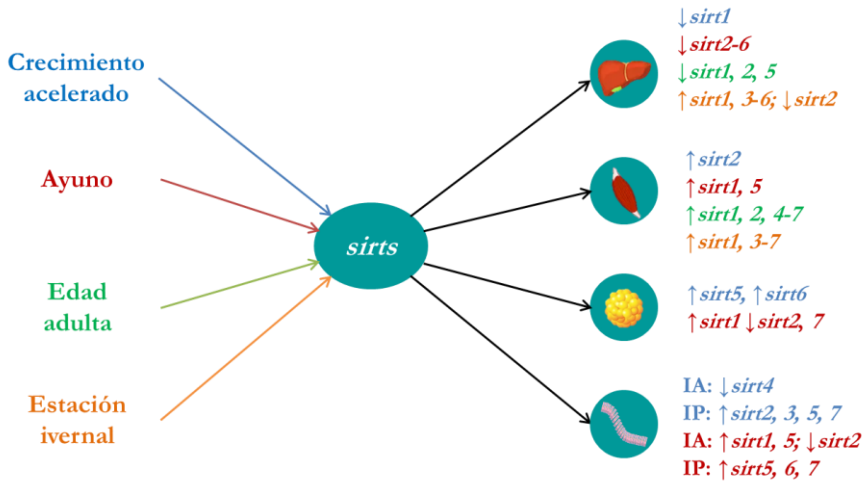


Figura 7.13 Respuesta específica de las *sirts* en los distintos tejidos (hígado, músculo, adiposo y intestino anterior (IA) y posterior (IP)) en los distintos modelos experimentales estudiados en la presente Tesis Doctoral.

Bibliografía

Anwar T, Khosla S, Ramakrishna G. 2016. Increased expression of SIRT2 is a novel marker of cellular senescence and is dependent on wild type p53 status. *Cell Cycle* 15(14):1883-1897.

Aubin-Horth N, Renn SC. 2009. Genomic reaction norms: Using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology* 18(18):3763-3780.

Ballester-Lozano GF, Benedito-Palos L, Estensoro I, Sitjà-Bobadilla A, Kaushik S, Pérez-Sánchez J. 2015. Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets. *British Journal of Nutrition* 114(5):713-726.

Benedito-Palos L, Saera-Vila A, Calduch-Giner JA, Kaushik S, Pérez-Sánchez J. 2007. Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis. *Aquaculture* 267(1-4):199-212.

Benedito-Palos L, Navarro JC, Sitjà-Bobadilla A, Bell JG, Kaushik S, Pérez-Sánchez J. 2008. High levels of vegetable oils in plant-protein rich diets fed to gilthead sea bream (*Sparus aurata* L.): Growth performance, muscle fatty acid profiles and histological alterations of target tissues. *British Journal of Nutrition* 100:992-1003.

Benedito-Palos L, Navarro JC, Bermejo-Nogales A, Saera-Vila A, Kaushik S, Pérez-Sánchez J. 2009. The time course of fish oil wash-out follows a simple dilution model in gilthead sea bream (*Sparus aurata* L.) fed graded levels of vegetable oils. *Aquaculture* 288:98-105.

Benedito-Palos L, Ballester-Lozano G, Pérez-Sánchez J. 2014. Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene* 547:34-42.

Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz Á, Calduch-Giner J, et al. 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). *Aquaculture* 454:8-18.

Bereswill S, Muñoz M, Fischer A, Plicket R, Haag LM, Otto B, et al. 2010. Anti-inflammatory effects of resveratrol, curcumin and simvastatin in acute small intestinal inflammation. *PLOS ONE* 5:e15099.

Bermejo-Nogales A, Nederlof M, Benedito-Palos L, Ballester-Lozano GF,

Folkedal O, Olsen RE, et al. 2014. Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping. *General and Comparative Endocrinology* 205:305-315.

Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. 2015. Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. *PLOS ONE* 10:e0122889.

Bosch-Presegué L, Vaquero A. 2015. Sirtuin-dependent epigenetic regulation in the maintenance of genome integrity. *FEBS Journal* 282:1745-1767.

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. 2004. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 303(5666):2011-2015.

Buler M, Andersson U, Hakkola J. 2016. Who watches the watchmen? Regulation of the expression and activity of sirtuins. *The FASEB Journal* 30(12):3942-3960.

Cabral EM, Fernandes TJR, Campos SD, Castro-Cunha M, Oliveira MBPP, Cunha LM, et al. 2013. Replacement of fish meal by plant protein sources up to 75% induces good growth performance without affecting flesh quality in ongrowing Senegalese sole. *Aquaculture* 380:130-138.

Calabrese V, Cornelius C, Mancuso C, Pennisi G, Calafato S, Bellia F, et al. 2008. Cellular stress response: A novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. *Neurochemical research* 33(12):2444-2471.

Calduch-Giner JA, Echasseriau Y, Crespo D, Baron D, Planas JV, Prunet P, et al. 2014. Transcriptional assessment by microarray analysis and large-scale meta-analysis of the metabolic capacity of cardiac and skeletal muscle tissues to cope with reduced nutrient availability in gilthead sea bream (*Sparus aurata* L.). *Marine Biotechnology* 16:423-435.

Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J. 2016. Gene expression profiling reveals functional specialization along the intestinal tract of a carnivorous teleostean fish (*Dicentrarchus labrax*). *Frontiers in Physiology* 7:359.

Carlson SJ, Nandivada P, Chang MI, Mitchell PD, O'Loughlin A, Cowan E, et al. 2015. The addition of medium-chain triglycerides to a purified fish oil-based diet alters inflammatory profiles in mice. *Metabolism: Clinical and Experimental* 64:274-282.

Chalkiadaki A, Guarente L. 2011. Metabolic signals regulate SIRT1 expression. *EMBO Reports* 12(10):985-986.

Chang HC, Guarente L. 2014. SIRT1 and other sirtuins in metabolism. *Trends in Endocrinology & Metabolism 25*(3):138-145.

Chaoui L, Kara MH, Faure E, Quignard JP. 2006. Growth and reproduction of the gilthead seabream Sparus aurata in Mellah Lagoon (north-eastern Algeria). Scien. Mar. 70: 545–552

Chaoui L, Kara MH, Faure E, Quignard JP. 2006. Growth and reproduction of the gilthead seabream *Sparus aurata* in Mellah Lagoon (north-eastern Algeria). *Scientia Marina* 70:545-552.

Chausse B, Vieira-Lara MA, Sanchez AB, Medeiros MH, Kowaltowski AJ. 2015. Intermittent fasting results in tissue-specific changes in bioenergetics and redox state. *PLOS ONE* 10(3):e0120413.

Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S, Venkatesh B. 2004. Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Molecular Biology and Evolution* 21(6):1146-1151.

Chu S. 2012. Transcriptional regulation by post-transcriptional modification—role of phosphorylation in Sp1 transcriptional activity. *Gene* 508(1):1-8.

Cohen DE, Supinski AM, Bonkowski MS, Donmez G, Guarente LP. 2009. Neuronal SIRT1 regulates endocrine and behavioral responses to calorie restriction. *Genes & Development* 23(24):2812-2817.

Cruz-García L, Sánchez-Gurmaches J, Bouraoui L, Saera-Vila A, Pérez-Sánchez J, Gutiérrez J, et al. 2011. Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata* L.). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 158(4):391-399.

D'Ancona U. 1941. Ulteriori osservazioni sull'ermafroditismo e il differenziamento sessuale dell'orata (*Sparus aurata* L.) (Completamento delle ricerche della Dott. A. Pasquali). *Pubblicazioni della Stazione Zoologica di Napoli* 18:313-336.

Da Costa N, McGillivray C, Bai Q, Wood JD, Evans G, Chang KC. 2004. Restriction of dietary energy and protein induces molecular changes in young porcine skeletal muscles. *Journal of Nutrition* 134:2191-2199.

Deaton AM, Bird A. 2011. CpG islands and the regulation of transcription. *Genes & development* 25(10):1010-1022.

Devlin RH, Nagahama Y. 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* 208:191-364.

Donaldson MR, Cooke SJ, Patterson DA, Macdonald JS. 2008. Cold shock and

fish. *Journal of Fish Biology* 73(7):1491-1530.

Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA. 2003. Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Molecular and Cellular Biology* 23:3173-3185.

Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. 2011. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. *Science* 334:806-809.

Estensoro I, Ballester-Lozano GF, Benedito-Palos L, Grammes F, Martos-Sitcha JA, Mydland L-T, et al. 2016. Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. *PLoS ONE* 11:e0166564.

Fang J, Ianni A, Smolka C, Vakhrusheva O, Nolte H, Kruger M, et al. 2017. SIRT7 promotes adipogenesis in the mouse by inhibiting autocatalytic activation of SIRT1. *Proceedings of the National Academy of Sciences USA* 114:E8352-E8361.

Figueras A, Robledo D, Corvelo A, Hermida M, Pereiro P, Rubiolo JA, et al. 2016. Whole genome sequencing of turbot (*Scophthalmus maximus*; Pleuronectiformes): A fish adapted to demersal life. *DNA research* 23(3):181-192.

Flick F, Lüscher B. 2012. Regulation of sirtuin function by posttranslational modifications *Frontiers in Pharmacology* 3:29.

Friedman MI, Ramirez I, Bowden C R, Tordoff MG. 1990. Fuel partitioning and food intake: Role for mitochondrial fatty acid transport. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 258(1):R216-R221.

Frye RA. 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochemical and Biophysical Research Communications* 273:793798.

Furuya TK, da Silva PN, Payão SL, Rasmussen LT, de Labio RW, Bertolucci PH, et al. 2012. SORL1 and SIRT1 mRNA expression and promoter methylation levels in aging and Alzheimer's Disease. *Neurochemistry International* 61:973e5.

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, et al. 2007. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. *The EMBO journal* 26(7):1913-1923.

Ghiraldini FG, Crispim ACV, Mello MLS. 2013. Effects of hyperglycemia and aging on nuclear sirtuins and DNA damage of mouse hepatocytes. *Molecular Biology of the Cell* 24(15):2467-2476.

Gómez-Requeni P, Mingarro M, Calduch-Giner JA, Médale F, Martin SAM, Houlihan DF, et al. 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture* 232:493-510.

González OA, Tobia C, Ebersole JL, Novak MJ. 2012. Caloric restriction and chronic inflammatory diseases. *Oral Diseases* 18(1):16-31.

Guderley H, Lapointe D, Bédard M, Dutil JD. 2003. Metabolic priorities during starvation: Enzyme sparing in liver and white muscle of Atlantic cod, *Gadus morhua* L. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 135(2):347-356.

Gui L, Hao R, Zhang Y, Zhao X, Zan L. 2015. Haplotype distribution in the class I sirtuin genes and their associations with ultrasound carcass traits in Qinchuan cattle (*Bos taurus*). *Molecular and Cellular Probes* 29:102-107.

Haase CG, Long AK, Gillooly JF. 2016. Energetics of stress: Linking plasma cortisol levels to metabolic rate in mammals. *Biology Letters* 12:20150867.

Hadj-Taieb A, Ghorbel M, Hadj-Hamida NB, Jarboui O. 2013. Proporción de sexos, reproducción y crecimiento de la dorada, *Sparus aurata* (Pisces: Sparidae), en el golfo de Gabes, Túnez. *Ciencias Marinas* 39(1):101-112.

Han L, Su B, Li WH, Zhao Z. 2008. CpG island density and its correlations with genomic features in mammalian genomes. *Genome Biology* 9:R79.

Han L, Zhao Z. 2008. Comparative analysis of CpG islands in four fish genomes. *Comparative and Functional Genomics* 2008:565631.

Happe A, Zohar Y. 1988. Self-fertilization in the protandrous hermaphrodite *Sparus aurata*: development of the technology. In: Reproduction in fish: Basic and applied aspects in endocrinology and genetics: Proceedings of the French-Israeli symposium Tel Aviv (Israel). INRA, Paris, France, pp 177-180.

Hardie DG, Ross FA, Hawley SA. 2012. AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews Molecular Cell Biology* 13(4):251.

Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, et al. 2010. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature* 464(7285):121.

Hofseth LJ, Singh UP, Singh NP, Nagarkatti M, Nagarkatti PS. 2010. Taming the beast within: resveratrol suppresses colitis and prevents colon cancer. *Aging* 2:183-184.

Hong JY, Mei CG, Li SJ, Wang HB, Zhao CP, Zan LS. 2018. Coordinate regulation by transcription factors and DNA methylation in the core

promoter region of SIRT6 in bovine adipocytes. *Archives of Biochemistry and Biophysics* 659:1-12.

Hong J, Wang X, Mei C, Wang H, Zan L. 2019a. DNA methylation and transcription factors competitively regulate SIRT4 promoter activity in bovine adipocytes: Roles of NRF1 and CMYB. *DNA Cell Biology* 38(1):63-75.

Hong J, Wang X, Mei C, Zan L. 2019b. Competitive regulation by transcription factors and DNA methylation in the bovine SIRT5 promoter: Roles of E2F4 and KLF6. *Gene* 684:39-46.

Hou Y, Chen H, He Q, Jiang W, Luo T, Duan, J, et al. 2013. Changes in methylation patterns of multiple genes from peripheral blood leucocytes of Alzheimer's disease patients. *Acta Neuropsychiatrica* 25:66-76.

Hsu CP, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, et al. 2010. Silent information regulator 1 protects the heart from ischemia/reperfusion. *Circulation* 122(21):2170-2182.

Ibarz A, Padrós F, Gallardo MÁ, Fernández-Borràs J, Blasco J, Tort L. 2010. Low-temperature challenges to gilthead sea bream culture: Review of cold-induced alterations and 'Winter Syndrome'. *Reviews in Fish Biology and Fisheries* 20(4):539-556.

Jäger S, Handschin C, Pierre JS, Spiegelman BM. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proceedings of the National Academy of Sciences* 104(29):12017-12022.

Jin D, Tan HJ, Lei T, Gan L, Chen XD, Long QQ, et al. 2009. Molecular cloning and characterization of porcine sirtuin genes. *Comparative Biochemistry and Physiology Part B Biochemistry & Molecular Biology* 153(4):348-358.

Jing E, Gesta S, Kahn CR. 2007. SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. *Cell Metabolism* 6:105-114.

Jing H, Lin H. 2015. Sirtuins in epigenetic regulation. *Chemical Reviews* 115(6):2350-2375.

Johnston IA, Bower NI, Macqueen DJ. 2011. Growth and the regulation of myotomal muscle mass in teleost fish. *Journal of Experimental Biology* 214(10):1617-1628.

Jones MJ, Goodman SJ, Kobor MS. 2015. DNA methylation and healthy human aging. *Aging cell* 14(6):924-932.

Jones PA. 2012. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics* 13(7):484-492.

Jukarainen S, Heinonen S, Ramo JT, Rinnankoski-Tuikka R, Rappou E,

Tummers M, et al. 2016. Obesity is associated with low NAD(+)/SIRT pathway expression in adipose tissue of BMI-discordant monozygotic twins. *The Journal of Clinical Endocrinology and Metabolism* 101:275-283.

Jung M, Pfeifer GP. 2015. Aging and DNA methylation. *BMC Biology* 13:7.

Kabiljo J, Murko C, Pusch O, Zupkovitz G. 2019. Spatio-temporal expression profile of sirtuins during aging of the annual fish *Nothobranchius furzeri*. *Gene Expression Patterns* 33:11-19.

Kause A, Kiessling A, Martin SAM, Houlihan D, Ruohonen K. 2016. Genetic improvement of feed conversion ratio via indirect selection against lipid deposition in farmed rainbow trout (*Oncorhynchus mykiss Walbaum*). *British Journal of Nutrition* 116:1656-1665.

Khan D, Sarikhani M, Dasgupta S, Maniyadath B, Pandit AS, Mishra S, et al. 2018. SIRT6 deacetylase transcriptionally regulates glucose metabolism in heart. *Journal of Cellular Physiology* 233(7):5478-5489.

Kobayashi Y, Nagahama Y, Nakamura M. 2013. Diversity and plasticity of sex determination and differentiation in fishes. *Sexual Development* 7.1-3:115-125.

Kuang J, Zhang Y, Liu Q, Shen J, Pu S, Cheng S, et al. 2017. Fat-specific Sirt6 ablation sensitizes mice to high-fat diet-induced obesity and insulin resistance by inhibiting lipolysis. *Diabetes* 66:1159-1171.

Kurylowicz A, Owczarz M, Polosak J, Jonas MI, Lisik W, Jonas M, et al. 2016. SIRT1 and SIRT7 expression in adipose tissues of obese and normal-weight individuals is regulated by microRNAs but not by methylation status. *International Journal of Obesity* (Lond) 40:1635-1642.

Kyprianou TD, Pörtner HO, Anestis A, Kostoglou B, Feidantsis K, Michaelidis B. 2010. Metabolic and molecular stress responses of gilthead seam bream *Sparus aurata* during exposure to low ambient temperature: An analysis of mechanisms underlying the winter syndrome. *Journal of Comparative Physiology B* 180(7):1005-1018.

Lai CC, Lin PM, Lin SF, Hsu CH, Lin HC, Hu ML, et al. 2013. Altered expression of SIRT gene family in head and neck squamous cell carcinoma. *Tumor Biology* 34(3):1847-1854.

Lamm MS, Liu H, Gemmell NJ, Godwin JR. 2015. The need for speed: Neuroendocrine regulation of socially-controlled sex change. *Integrative and Comparative Biology* 55(2):307-322.

Landolin JM, Johnson DS, Trinklein ND, Aldred SF, Medina C, Shulha H, et al. 2010. Sequence features that drive human promoter function and tissue specificity. *Genome Research* 20:890-898.

Larrosa M, Yanez-Gascon MJ, Selma MV, González-Sarrias A, Toti S, Ceron

JJ, et al. 2009. Effect of low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. *Journal of Agricultural and Food Chemistry* 57:2211-2220.

Lasserre G. 1976. Dynamique des populations ichthyologiques lagunaires. Application à *Sparus aurata*. Ph. D. thesis, Univ. Montpellier II.

Le Boucher R, Quillet E, Vandeputte M, Lecalvez JM, Goardon L, Chatain B, et al. 2011. Plant-based diet in rainbow trout (*Oncorhynchus mykiss* Walbaum): Are there genotype-diet interactions for main production traits when fish are fed marine vs. plant-based diets from the first meal? *Aquaculture* 321:41-48.

Li W, Graur D. 1991. Fundamentals of molecular evolution. Sinauer Associates, Sunderland, MA.

Loukovitis D, Sarropoulou E, Tsigenopoulos CS, Batargias C, Magoulas A, Apostolidis AP, et al. 2011. Quantitative trait loci involved in sex determination and body growth in the gilthead sea bream (*Sparus aurata* L.) through targeted genome scan. *PLOS ONE* 6(1):e16599.

Martos-Sitcha JA, Simó-Mirabet P, Piazzon MC, de las Heras V, Calduch-Giner JA, Puyalto M, et al. 2018. Dietary sodium heptanoate helps to improve feed efficiency, growth hormone status and swimming performance in gilthead sea bream (*Sparus aurata*). *Aquaculture Nutrition* 24(6):1638-1651.

Martos-Sitcha JA, Simó-Mirabet P, de Las Heras V, Calduch-Giner JÀ, Pérez-Sánchez J. 2019. Tissue-specific orchestration of gilthead sea bream resilience to hypoxia and high stocking density. *Frontiers in Physiology* 10:840.

Matsushita N, Yonashiro R, Ogata Y, Sugiura A, Nagashima S, Fukuda T, et al. 2011. Distinct regulation of mitochondrial localization and stability of two human Sirt5 isoforms. *Genes Cells* 16:190-202.

Mendes KL, de Farias Lelis D, Santos SHS. 2017. Nuclear sirtuins and inflammatory signaling pathways. *Cytokine & Growth Factor Reviews* 38:98-105.

Meyer A, Van de Peer Y. 2005. From 2R to 3R: Evidence for a fish-specific genome duplication (FSGD). *Bioessays* 27(9):37-945.

Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. 2005. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Molecular Biology of the Cell* 16(10):4623-4635.

Miyahara M, Ishibashi H, Inudo M, Nishijima H, Iguchi T, Guillette LJJ, et al. 2003. Estrogenic activity of a diet to estrogen receptors -α and -β in an

experimental animal. *Journal of Health Sciences* 49:1-11.

Moczek AP. 2015. Developmental plasticity and evolution-quo vadis?. *Heredity* 115(4):302.

Monternier PA, Marmillot V, Rouanet JL, Roussel D. 2014. Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *Journal of Experimental Biology* 217(15):2691-2697.

Monteserin-Garcia J, Al-Massadi O, Seoane LM, Alvarez CV, Shan B, Stalla J, et al. 2013. Sirt1 inhibits the transcription factor CREB to regulate pituitary growth hormone synthesis. *FASEB Journal* 27:1561-1571.

Nakagawa T, Lomb DJ, Haigis MC, Guarente L. 2009. SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. *Cell* 137(3):560-570.

Navarro-Martín L, Viñas J, Ribas L, Díaz N, Gutiérrez A, Di Croce L, Piferrer F. 2011. DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLOS Genetics* 7(12):e1002447.

Noriega LG, Feige JN, Canto C, Yamamoto H, Yu J, Herman MA, et al. 2011. CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. *EMBO Reports* 12(10):1069-1076.

Okazaki M, Iwasaki Y, Nishiyama M, Taguchi T, Tsugita M, Nakayama S, et al. 2010. PPARbeta/delta regulates the human SIRT1 gene transcription via Sp1. *Endocrine Journal* 57(5):403-413.

Pardo PS, Boriek AM. 2011. The physiological roles of Sirt1 in skeletal muscle. *Aging* 3(4):430-437.

Pauletto M, Manousaki T, Ferraresso S, Babbucci M, Tsakogiannis A, Louro B, et al. 2018. Genomic analysis of *Sparus aurata* reveals the evolutionary dynamics of sex-biased genes in a sequential hermaphrodite fish. *Communications Biology* 1(1):119.

Pelissero C, Sumpter JP. 1992. Steroids and "steroid-like" substances in fish diets. *Aquaculture* 107:283-301.

Peng C, Lu Z, Xie Z, Cheng Z, Chen Y, Tan M, et al. 2011. The first identification of lysine malonylation substrates and its regulatory enzyme. *Molecular & Cellular Proteomics* 10(12):M111-012658.

Pereira TCB, Rico EP, Rosemberg DB, Schirmer H, Dias RD, Souto AA, et al. 2011. Zebrafish as a model organism to evaluate drugs potentially able to modulate sirtuin expression. *Zebrafish* 8(1):9-16.

Perera E, Simó-Mirabet P, Shin HS, Rosell-Moll E, Naya-Catalá F, de las Heras V, et al. 2019. Selection for growth is associated in gilthead sea bream (*Sparus aurata*) with diet flexibility, changes in growth patterns and higher

intestine plasticity. *Aquaculture* 507:349-360.

Perera E, Turkmen S, Simó-Mirabet P, Zamorano MJ, Xu H, Naya-Català F, et al. 2020. Stearoyl-CoA desaturase (*scd1a*) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). *Epigenetics*. doi: 10.1080/15592294.2019.1699982.

Pérez-Sánchez J, Benedito-Palos L, Estensoro I, Petropoulos Y, Calduch-Giner JA, Browdy CL, et al. 2015. Effects of dietary NEXT ENHANCE®150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). *Fish & Shellfish Immunology* 44:117-128.

Pérez-Sánchez J, Simó-Mirabet P, Naya-Català F, Martos-Sitcha JA, Perera E, Bermejo-Nogales A, et al. 2018. Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Frontiers in Endocrinology* 9:687.

Pérez-Sánchez J, Naya-Català F, Soriano B, Piazzon MC, Hafez A, Gabaldón T, et al. 2019. Genome sequencing and transcriptome analysis reveal recent species-specific gene duplications in the plastic gilthead sea bream (*Sparus aurata*). *Frontiers in Marine Science* 6:760.

Piazzon MC, Galindo-Villegas J, Pereiro P, Estensoro I, Calduch-Giner JA, Gomez-Casado E, et al. 2016. Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a Perciform fish. *Frontiers in Immunology* 7:637.

Piazzon MC, Calduch-Giner JA, Fouz B, Estensoro I, Simó-Mirabet P, Puyalto M, et al. 2017. Under Control: How a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiom*e 5:164.

Piazzon MC, Naya-Català F, Calduch-Giner JA, Simó-Mirabet P, Picard-Sánchez A, Sitjà-Bobadilla A, et al. 2019. Sex, age and bacteria: How the intestinal microbiota is modulated in a protandrous hermaphrodite farmed fish. *Frontiers in Microbiology* 10:2512.

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, et al. 2004. SIRT1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* 429:771-776.

Pierce LR, Palti Y, Silverstein JT, Barrows FT, Hallerman EM, Parsons JE. 2008. Family growth response to fishmeal and plant-based diets shows genotype x diet interaction in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 278(1-4):37-42.

Planavila A, Iglesias R, Giralt M, Villarroya F. 2011. Sirt1 acts in association with PPARα to protect the heart from hypertrophy, metabolic

dysregulation, and inflammation. *Cardiovascular Research* 90(2):276-284.

Pugh BF, Tjian R. 1990. Mechanism of transcriptional activation by Sp1: Evidence for coactivators. *Cell* 61(7):1187-1197.

Riche M, Williams TN. 2011. Fish meal replacement with solvent-extracted soybean meal or soy protein isolate in a practical diet formulation for Florida pompano (*Trachinotus carolinus*, L.) reared in low salinity. *Aquaculture Nutrition* 17(4):368-379.

Rise ML, Hall JR, Nash GW, Xue X, Booman M, Katan T, et al. 2015. Transcriptome profiling reveals that feeding wild zooplankton to larval Atlantic cod (*Gadus morhua*) influences suites of genes involved in oxidation-reduction, mitosis, and selenium homeostasis. *BMC Genomics* 16:1016.

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. 2005. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature* 434(7029):113.

Rombout JH, Abelli L, Picchietti S, Scapigliati G, Kiron V. 2011. Teleost intestinal immunology. *Fish and Shellfish Immunology* 31:616-626.

Rozenberg JM, Shlyakhtenko A, Glass K, Rishi V, Myakishev MV, FitzGerald PC, et al. 2008. All and only CpG containing sequences are enriched in promoters abundantly bound by RNA polymerase II in multiple tissues. *BMC Genomics* 9(1):67.

Rui L. 2014. Energy metabolism in the liver. *Comprehensive Physiology* 4:177-197.

Sahin K, Yilmaz S, Gozukirmizi N. 2014. Changes in human sirtuin 6 gene promoter methylation during aging. *Biomedical Reports* 2:574e8.

Salin K, Villasevil EM, Anderson GJ, Auer SK, Selman C, Hartley RC, 2018. Decreased mitochondrial metabolic requirements in fasting animals carry an oxidative cost. *Functional Ecology* 32(9):2149-2157.

Salmerón C. 2018. Adipogenesis in fish. *Journal of Experimental Biology* 221(Suppl 1):jeb161588.

Saravanan S, Schrama JW, Figueiredo-Silva AC, Kaushik SJ, Verreth JA, Geurden I. 2012. Constraints on energy intake in fish: The link between diet composition, energy metabolism, and energy intake in rainbow trout. *PLOS ONE* 7(4):e34743.

Satoh A, Brace CS, Ben-Josef G, West T, Wozniak DF, Holtzman DM, et al. 2010. SIRT1 promotes the central adaptive response to diet restriction through activation of the dorsomedial and lateral nuclei of the hypothalamus. *The Journal of Neuroscience* 30:10220-10232.

Schönfeld P, Wojtczak L. 2016. Short-and medium-chain fatty acids in energy metabolism: The cellular perspective. *Journal of Lipid Research* 57:943-954.

Secombes CJ, Wang T. 2012. The innate and adaptive immune system of fish. In: Austin B (ed.) Infectious disease in aquaculture: Prevention and control. Woodhead Publishing, Oxford, Cambridge, Philadelphia, New Delhi, pp 3-68.

Secor SM, Carey HV. 2016. Integrative physiology of fasting. *Comprehensive Physiology* 6(2):773-825.

Serrano L, Martínez-Redondo P, Marazuela-Duque A, Vazquez BN, Dooley SJ, Voigt P, et al. 2013. The tumor suppressor SirT2 regulates cell cycle progression and genome stability by modulating the mitotic deposition of H4K20 methylation. *Genes & Development* 27(6):639-653.

Shao C, Li Q, Chen S, Zhang P, Lian J, Hu Q, et al. 2014. Epigenetic modification and inheritance in sexual reversal of fish. *Genome Research* 24(4):604-615.

Sharif J, Endo TA, Toyoda T, Koseki H. 2010. Divergence of CpG island promoters: A consequence or cause of evolution? *Development Growth & Differentiation* 52:545-554.

Shepherd CJ, Monroig O, Tocher DR. 2017. Future availability of raw materials for salmon feeds and supply chain implications: The case of Scottish farmed salmon. *Aquaculture* 467:49-62.

Simó-Mirabet P, Felip A, Estensoro I, Martos-Sitcha JA, De las Heras V, Calduch-Giner JA, et al. 2018. Impact of low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle. *Aquaculture* 490:64-74.

Stanton DA, Alway SE, Mohamed JS. 2017. The role of Sirtuin 2 in the regulation of myogenesis. *FASEB Journal* 31(1):77.13.

Suske G. 1999. The Sp-family of transcription factors. *Gene* 238(2):291-300.

Suzuki J, Shen WJ, Nelson BD, Selwood SP, Murphy GM, Kanehara H, et al. 2002. Cardiac gene expression profile and lipid accumulation in response to starvation. *American Journal of Physiology-Endocrinology and Metabolism* 283:E94-E102.

Tan M, Peng C, Anderson KA, Chhoy P, Xie Z, Dai L, et al. 2014. Lysine glutarylation is a protein post-translational modification regulated by SIRT5. *Cell Metabolism* 19:605-617.

Tine M, Kuhl H, Gagnaire PA, Louro B, Desmarais E, Martins RS, et al. 2014. European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature Communications* 5:5770.

Todd EV, Liu H, Muncaster S, Gemmell NJ. 2016. Bending genders: The biology of natural sex change in fish. *Sexual Development* 10(5-6):223-241.

Vandepoele K, De Vos W, Taylor JS, Meyer A, Van de Peer Y. 2004. Major events in the genome evolution of vertebrates: Paranome age and size differ considerably between ray-finned fishes and land vertebrates. *Proceedings of the National Academy of Sciences* 101(6):1638-1643.

Varriale A, Bernardi G. 2006. DNA methylation and body temperature in fishes. *Gene* 385:111-121.

Vasconi M, Caprino F, Bellagamba F, Moretti VM. 2017. Fatty acid composition of gilthead sea bream (*Sparus aurata*) fillets as affected by current changes in aquafeed formulation. *Turkish Journal of Fisheries and Aquatic Sciences* 17:451-459.

Wang F, Nguyen M, Qin FX, Tong Q. 2007. SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. *Aging Cell* 6:505-514.

Wang F, Wang K, Xu W, Zhao S, Ye, D, Wang Y, et al. 2017. SIRT5 desuccinylates and activates pyruvate kinase M2 to block macrophage IL-1β production and to prevent DSS-induced colitis in mice. *Cell Reports* 19(11):2331-2344.

Wang J, Wu X, Simonavicius N, Tian H, Ling L. 2006. Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. *Journal of Biological Chemistry* 281:34457-34464.

Wang L, Zhou H, Wang Y, Cui G, Di LJ. 2015. CtBP maintains cancer cell growth and metabolic homeostasis via regulating SIRT4. *Cell Death & Disease* 6:e1620.

Warner RR. 1988. Sex change and the size-advantage model. *Trends in Ecology & Evolution* 3(6):133-136.

West-Eberhard MJ. 2003. Developmental plasticity and evolution. Oxford University Press, USA.

Wierstra I. 2008. Sp1: Emerging roles-beyond constitutive activation of TATA-less housekeeping genes. *Biochemical and Biophysical Research Communications* 372:1-13.

Wu G, Song C, Lu H, Jia L, Yang G, Shi X, et al. 2014. Sirt2 induces C2C12 myoblasts proliferation by activation of the ERK1/2 pathway. *Acta Biochimica et Biophysica Sinica* 46:342-345.

Wu Y, Chen L, Wang Y, Li W, Lin Y, Yu D, et al. 2015. Overexpression of Sirtuin 6 suppresses cellular senescence and NF-*κ*B mediated inflammatory responses in osteoarthritis development. *Scientific Reports* 5:17602.

Yamamoto M, Iguchi G, Fukuoka H, Suda K, Bando H, Takahashi M, et al. 2013. SIRT1 regulates adaptive response of the growth hormone-insulin-like growth factor-I axis under fasting conditions in liver. *Proceedings of the*

National Academy of Sciences USA 110:14948-14953.

Yamamoto M, Takahahi Y. 2018. The essential role of SIRT1 in hypothalamic-pituitary axis. *Frontiers in Endocrinology* 9:605.

Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, et al. 2015. The ketone metabolite β-hydroxybutyrate blocks NLRP3 inflammasome–mediated inflammatory disease. *Nature Medicine* 21(3):263.

Ytrestøyl T, Aas TS, Åsgård T. 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture* 448:365-374.

Yu SS, Cai Y, Ye JT, Pi RB, Chen SR, Liu PQ, et al. 2013. Sirtuin 6 protects cardiomyocytes from hypertrophy in vitro via inhibition of NF-ϰB-dependent transcriptional activity. *British Journal of Pharmacology* 168(1):117-128.

Yuan Z, Liu S, Zhou T, Tian C, Bao L, Dunham R, et al. 2018. Comparative genome analysis of 52 fish species suggests differential associations of repetitive elements with their living aquatic environments. *BMC Genomics* 19:141.

Zhou S, Tang X, Chen HZ. 2018. Sirtuins and insulin resistance. *Frontiers in Endocrinology* 9:748.

Zohar Y, Abraham M, Gordon H. 1978. The gonad cycle of the captivity-reared hermaphroditic teleost *Sparus aurata* during the first two years of life. *Annales de Biologie Animale Biochimie Biophysique* 18:877-882.

Zullo A, Simone E, Grimaldi M, Gagliardi M, Zullo L, Matarazzo MR, et al. 2018. Effect of nutrient deprivation on the expression and the epigenetic signature of sirtuin genes. *Nutrition, Metabolism & Cardiovascular Diseases* 28(4):418-424.

CONCLUSIONES

PRIMERA: Las dietas basadas en ingredientes vegetales permiten un crecimiento eficiente en la dorada a lo largo de todo el ciclo de producción.

SEGUNDA: El butirato revierte los efectos negativos sobre la reproducción y la salud intestinal causados por las dietas vegetales.

TERCERA: La suplementación con ácidos grasos de cadena media (C7, C12) mejora el crecimiento y la respuesta frente al ejercicio.

CUARTA: Los efectos de los ácidos grasos de cadena media y los probióticos sobre el patrón de expresión de las sirtuínas y marcadores de la salud intestinal son específicos del aditivo.

QUINTA: La secuencia y la estructura exón-intrón de los siete ortólogos de las sirtuínas de mamíferos está altamente conservada en peces.

SEXTA: Los árboles filogenéticos de las sirtuínas, incluyendo las secuencias de dorada, están en consonancia con la actual jerarquía de los vertebrados.

SÉPTIMA: Los peces, y la dorada en particular, conservan múltiples copias de la sirtuína 3 y la sirtuína 5 que podrían haberse originado en diferentes momentos a lo largo de la evolución.

OCTAVA: Solo se ha descrito la presencia de islas CG en las regiones promotoras de las sirtuínas 1 y 3, asegurando la coexistencia de ambos tipos de promotores una expresión ubicua a la par que altamente regulada de las sirtuínas en respuesta a estímulos específicos.

NOVENA: El patrón de expresión de las sirtuínas de dorada es ubicuo y específico de tejido para cada uno de los miembros de la familia

analizados, con niveles altos de expresión de las sirtuínas 1, 2 y 5 y bajos o moderados en el caso de las sirtuínas 3, 4, 6 y 7.

DÉCIMA: Las sirtuínas 1, 5, 6 y 7 están sobreexpresadas en una situación de ayuno mostrando, en general, unos mayores niveles de expresión en tejidos con una alta demanda energética.

UNDÉCIMA: El patrón de expresión de las sirtuínas frente a un aumento de la demanda energética fruto del ayuno o de un diferente potencial de crecimiento es diferente dependiendo del tejido y del tipo de estímulo.

DUODÉCIMA: El patrón de expresión hepático y muscular de las sirtuínas es claramente dependiente de la estación y la edad, estando los cambios de la expresión de la sirtuína 1 regulados a nivel muscular por mecanismos epigenéticos que involucran cambios en la metilación a nivel de promotor.

RESÚMENES

La acuicultura es el sector de producción animal con mayor crecimiento a nivel mundial, pero para cubrir la demanda creciente de alimentos de la agenda 2030 es necesario duplicar la producción en los próximos años, lo que debe basarse en la generación de nuevo conocimiento. Para ello es condición indispensable disponer de unas infraestructuras de referencia donde evaluar diferentes condiciones de cultivo de forma fiable y fácilmente reproducible estableciendo unos estándares de calidad, basados en buenas prácticas sanitarias, de alimentación y cultivo. Los últimos avances en el cultivo de la dorada (*Sparus aurata*) han demostrado que las dietas basadas en ingredientes vegetales no comprometen el potencial de crecimiento a lo largo del ciclo de producción. Es más, los efectos negativos relacionados con procesos inflamatorios y cambios en la proporción de sexos, pueden revertirse, al menos en parte, mediante la suplementación con ácidos grasos de cadena corta (butirato). En cambio, los beneficios de la suplementación con ácidos grasos de 7-12 átomos de carbono están más asociados con el crecimiento y la respuesta al ejercicio. Ello pone de relieve la importancia de conocer en todo momento el estado metabólico de los animales en cultivo, por lo que se requieren marcadores que sean capaces de relacionar la demanda de energía con la activación de determinados procesos o vías metabólicas. Las sirtuínas son unos buenos candidatos al ser estas enzimas una familia de proteínas dependientes de NAD^+ que deacilan histonas, factores de transcripción y un gran número de enzimas del metabolismo intermediario. Las sirtuínas están altamente conservadas tanto a nivel génico como proteico a lo largo de la evolución de los vertebrados. La dorada, además de las 7 sirtuínas de vertebrados, presenta copias

adicionales de las sirtuínas 3 y 5, que también se han identificado en otras especies de peces.

Las sirtuínas de dorada se expresan de forma ubicua con patrones de expresión específicos de tejido que suelen coincidir con niveles altos de expresión de las sirtuínas 1, 2 y 5 y bajos o moderados de las sirtuínas 3, 4, 6 y 7. Las sirtuínas están reguladas a nivel nutricional, respondiendo tanto a la inclusión de distintos aditivos en la dieta como a la falta de nutrientes. En el caso del ayuno, las sirtuínas 1, 5, 6 y 7 están sobreexpresadas y en general muestran niveles altos de expresión en tejidos con una alta demanda energética. Sin embargo, el patrón de expresión de los distintos isotipos de sirtuínas es diferente cuando se compara un estado de demanda energética desencadenado por la falta de nutrientes o la aceleración del crecimiento. En este sentido, animales de crecimiento acelerado y fenotipo delgado mostraron una mayor expresión de las sirtuínas con roles antiinflamatorios en el tejido adiposo (sirtuínas 5 y 6) e intestino (sirtuínas 2, 3, 5 y 7) y una disminución de la expresión de la sirtuína 1 a nivel hepático y un aumento de la sirtuína 2 a nivel muscular. La edad y la estación son otros de los factores que afectan de forma diferencial la expresión de las sirtuínas a nivel hepático y muscular. Por tanto, las sirtuínas responden a los cambios del estado energético de forma diferente en función de la intensidad y naturaleza del estímulo que modifique el estado de demanda o disponibilidad de energía. Esta heterogeneidad de la respuesta también va asociada a la presencia (sirtuínas 1 y 3) o ausencia (sirtuínas 2, 4-7) de promotores con islas CG, habiéndose observado a nivel muscular que el aumento de la expresión de la sirtuína 1 está asociado a una menor metilación del promotor con la edad y en época invernal.

L' aqüicultura és el sector de producció animal amb major creixement a nivell mundial, però per aconseguir cobrir la creixent demanda d' aliments de l' agenda 2030 és necessari duplicar la producció en els propers anys, la qual cosa ha de basar-se en la generació de nou coneixement. Per aquest motiu, és necessari disposar d' unes infraestructures de referència on avaluar diferents condicions de cultiu de forma fiable i fàcilment reproduïble establint estàndards de qualitat basats en bones pràctiques sanitàries, d' alimentació i cultiu. Els últims avenços en el cultiu de l' orada (*Sparus aurata*) han demostrat que les dietes basades en ingredients vegetals no comprometen el potencial de creixement al llarg del cicle de producció. A més, els efectes negatius relacionats amb processos inflamatoris i canvis en la proporció de sexes, poden revertir-se, en part, mitjançant la suplementació amb àcids grassos de cadena curta (butirat). En canvi, els beneficis de la suplementació amb àcids grassos de 7-12 àtoms de carboni estan més associats amb el creixement i la resposta a l' exercici. Tot això posa en relleu la importància de conèixer en tot moment l' estat metabòlic dels animals en cultiu, per la qual cosa es requereixen marcadors que siguin capaços de relacionar la demanda d' energia amb l' activació de determinats processos o vies metabòliques. Les sirtuïnes són uns bons candidats, ja que aquests enzims son una família de proteïnes dependents de NAD^+ que deacilen histones, factors de transcripció i un gran nombre d' enzims del metabolisme intermediari. Les sirtuïnes estan altament conservades tant a nivell gènic com proteic al llarg de l' evolució dels vertebrats. L' orada, a més de les 7 sirtuïnes de vertebrats, presenta còpies addicionals de les sirtuïnes 3 i 5, que també s' han identificat en altres espècies de peixos.

Les sirtuïnes d' orada s' expressen de forma ubiqua amb patrons d' expressió específics de teixit que acostumen a coincidir amb nivells alts d' expressió de les sirtuïnes 1, 2 i 5 i baixos o moderats de les sirtuïnes 3, 4, 6 i 7. Les sirtuïnes estan regulades a nivell nutricional, responent tant a la inclusió de diferents additius a la dieta com a la manca de nutrients. En el cas del dejú, les sirtuïnes 1, 5, 6 i 7 estan sobreexpressades i, en general, mostren nivells alts d' expressió en teixits amb una alta demanda energètica. Tanmateix, el patró d' expressió de les diferents sirtuïnes varia quan es compara un estat de demanda energètica degut a la falta de nutrients o a l' acceleració del creixement. En aquest sentit, animals de creixement accelerat i fenotip prim van mostrar una major expressió de les sirtuïnes amb funcions antiinflamatòries en el teixit adipós (sirtuïnes 5 i 6) i l' intestí (sirtuïnes 2, 3, 5 i 7) a més d' una disminució de l' expressió de la sirtuïna 1 a nivell hepàtic i un augment de la sirtuïna 2 al múscul. L' edat i l' estació son altres factors que afecten de forma diferencial l' expressió de les sirtuïnes a nivell hepàtic i muscular. Per tant, les sirtuïnes responen als canvis de l' estat energètic de forma diferent en funció de la intensitat i naturalesa de l' estímul que modifiqui l' estat de demanda o disponibilitat d' energia. Aquesta heterogeneïtat de la resposta també va associada a la presència (sirtuïnes 1 i 3) o absència (sirtuïnes 2, 4-7) de promotors amb illes CG, havent-se observat a nivell muscular, que l' augment de l' expressió de la sirtuïna 1 està associat a una menor metilació del promotor amb l' edat i a l' hivern.

Aquaculture is the fastest growing food-producing sector worldwide, but the capacity of aquaculture to meet the future demand for seafood to deliver the goals by 2030 will largely depend on doubling production in the coming years, which should be based on the generation of new knowledge. For that purpose, it is crucial to have quality facilities to assess different farming conditions in a reliable and easily reproducible way, establishing quality standards based on good aquaculture practices (i.e. feeding, sanitation, management). The latest achievements in gilthead sea bream (*Sparus aurata*) have demonstrated that plant-based diets do not compromise fish growth potential over the production cycle. Moreover, the negative effects related to inflammatory processes and sex proportion can be reverted, at least in part, by the supplementation with short chain fatty acids (butyrate). In contrast, the benefits of adding 7-12 carbon fatty acids are more associated with growth and swimming performance. This highlights the importance of knowing the metabolic state of farmed fish and the interest of developing new biomarkers that link the energy demand with the activation of specific metabolic pathways. Sirtuins are good candidates because these enzymes belong to a NAD^+-dependent protein family that deacylates histones, transcription factors and a wide range of metabolic enzymes. Sirtuins are highly conserved both at the gene and protein levels throughout the evolution of vertebrates. In addition to the 7 vertebrate sirtuins, gilthead sea bream also has additional copies of sirtuins 3 and 5, which have also been identified in other fish species.

Gilthead sea bream sirtuins revealed a ubiquitous gene expression pattern that was tissue-specific for each sirtuin with relatively high levels of sirtuins 1, 2 and 5 and moderate or low in the case of sirtuins 3, 4, 6

and 7. In addition, sirtuins are nutritionally regulated, responding both to the inclusion of different additives in the diet and nutrient availability. In the case of fasting, sirtuins 1, 5, 6 and 7 are up-regulated showing, in general, high gene expression levels in tissues with a high energy demand. However, the expression pattern of sirtuins is different when comparing an energy demand state caused by fasting or fast growth. In this sense, fast-growing fish with a lean phenotype showed a higher expression of sirtuins with anti-inflammatory roles in adipose tissue (sirtuins 5 and 6) and intestine (sirtuins 2, 3, 5 and 7), as well as a reduced hepatic sirtuin 1 expression and an increased sirtuin 2 expression in white skeletal muscle. Age and season are other factors that affect the expression pattern of sirtuins at hepatic and muscular level. Therefore, the differential regulation of sirtuins according to changes in energy status depends on the intensity and nature of the stimulus that modifies the state of demand or energy availability. This heterogeneity of the response is also associated with the presence (sirtuins 1 and 3) or lack (sirtuins 2, 4-7) of promoters with CG islands, being associated at muscular level the increased expression of sirtuin 1 with a lower promoter methylation with age and in winter season.