UNIVERSIDAD POLITÉCNICA DE VALENCIA

DEPARTAMENTO DE CIENCIA ANIMAL

SUSTITUCIÓN DE ACEITES DE PESCADO EN DIETAS DE ENGORDE DE DORADA (*Sparus aurata*) RICAS EN PROTEÍNAS VEGETALES. EFECTOS SOBRE EL CRECIMIENTO Y LOS PERFILES DE ÁCIDOS GRASOS

Memoria presentada por Laura Benedito Palos para optar al grado de Doctor

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TESIS DIRIGIDA POR:

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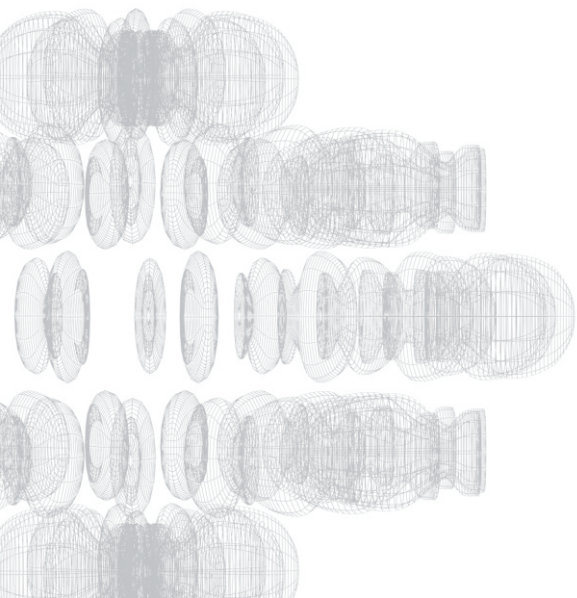
CAPÍTULO 1

INTRODUCCIÓN GENERAL



1.1. Estado actual de la acuicultura

Hasta la primera mitad del siglo XX, el mar se consideraba un reservorio prácticamente inagotable de pescado que se podía explotar de forma ilimitada. Fue a mediados de siglo cuando se empezó a pensar que las poblaciones naturales de peces eran finitas y que el esfuerzo pesquero podía ser demasiado intenso. Esta preocupación pasó a ser una realidad a comienzos de la segunda mitad de siglo. Si bien, aproximadamente en esa misma época, la acuicultura empezó a desarrollarse, manteniendo la esperanza de un aporte suficiente de pescado para la alimentación humana del futuro.

En los tres últimos decenios, la acuicultura ha crecido con rapidez, a un ritmo mayor que otros sectores de producción animal. Así pues, mientras que la pesca de captura se estancó a mediados de la década de 1980, la producción global del sector acuícola ha crecido de un modo sostenido, pasando de 0,6 millones de Tm en 1950 a 65,2 millones de Tm en 2007 (**Figura 1.1**).

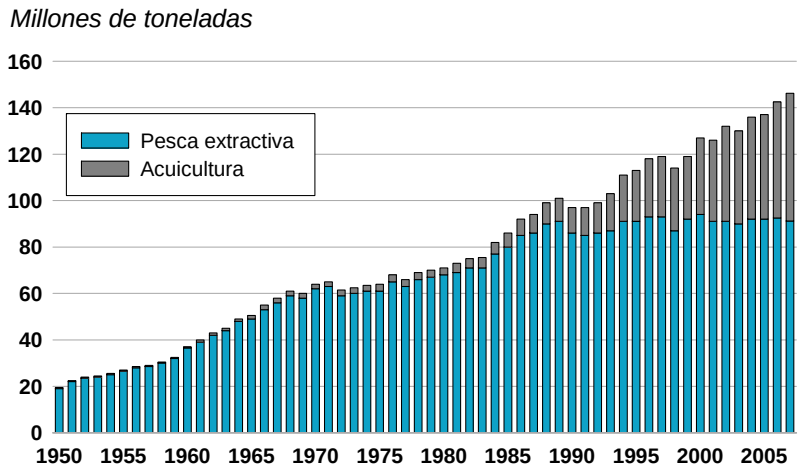


Figura 1.1. Evolución de la producción pesquera en el mundo (FAO, 2008).

Sin embargo, de manera global, la tasa de crecimiento de la acuicultura ha empezado a descender, pasando de un incremento anual del 11,8% en el periodo 1985-1994 al 7,1% en el decenio siguiente. En todo caso, la probabilidad de que las pesquerías aumenten en un futuro es prácticamente nula y la acuicultura sigue siendo una apuesta de futuro a corto-medio plazo. Por otro lado, ante la perspectiva de que la población mundial alcance los 9200 millones de personas en el año 2050 (Cohen, 1995), la cuestión principal es si habrá suficiente alimento para cubrir una demanda creciente. Actualmente, ya hay datos de los efectos negativos del cambio climático sobre las reservas de agua potable (CAWMA, 2007), viéndose afectado el sector de la agricultura, la ganadería, así como la acuicultura continental. En este sentido, la acuicultura marina se vislumbra como una verdadera alternativa ("revolución azul") para cubrir la demanda de alimentos de la humanidad a lo largo del siglo XXI (Duarte y cols., 2009).

El consumo de pescado, como fuente de proteínas animales, es un elemento esencial en la dieta de la población de algunos países densamente poblados. Es más, aunque el consumo medio per cápita sea bajo, los efectos sobre la salud pueden ser de notable relevancia al ser el pescado una fuente importante de aminoácidos esenciales (lisina, triptófano), vitaminas (A, D), minerales (yodo, selenio) y ácidos grasos poli-insaturados omega-3 de cadena larga (ácido eicosapentaenoico, 20:5n-3, EPA; ácido docosahexaenoico, 22:6n-3, DHA).

Según datos de la FAO (2008) se calcula que la contribución de las proteínas de pescado al suministro mundial de proteínas animales en la dieta humana incrementó desde el 13,7% en 1961 hasta un máximo del 16% en 1996, y en la actualidad se ha estabilizado en un 15%. Este incremento del consumo de pescado ha coincidido con el importante desarrollo de la acuicultura durante las últimas dos décadas. No obstante, para que la acuicultura aumente en la medida en que lo hace la demanda, los acuicultores deberán superar diversas limitaciones y obstáculos.

1.1.1. La acuicultura en España

La acuicultura europea representa una fuente importante de pescado de alto valor comercial. Su relevancia económica y social supera a la de la pesca extractiva en algunos países, como también ocurre en algunas Comunidades Autónomas españolas. Por consiguiente, esta actividad desempeña un papel relevante en el desarrollo social y económico, además de preservar la cultura marítima y pesquera de determinadas zonas costeras.

España es el Estado Miembro de la Unión Europea (UE) con una mayor producción en acuicultura (21,6% del total de la UE), seguido por Francia (18,2%) e Italia (13,7%). En relación al montante económico, España se sitúa en el quinto lugar, después de Reino Unido, Italia, Francia y Grecia, con 307 millones de euros lo que supone el 8,6% de la producción acuícola de la UE (Informe de Apromar, 2009).

El cultivo del rodaballo está en auge en la vertiente atlántica española, pero el cultivo de dorada y lubina sigue siendo la base de la piscicultura marina nacional (**Figura1.2**). La dorada es la especie más producida (24.000 Tm en 2008) y, aunque hoy en día sigue llegando cierta cantidad salvaje a los puertos pesqueros (1.100 Tm en 2008), su volumen permanece relativamente constante y con una expectativa prácticamente nula de que aumente en el futuro.

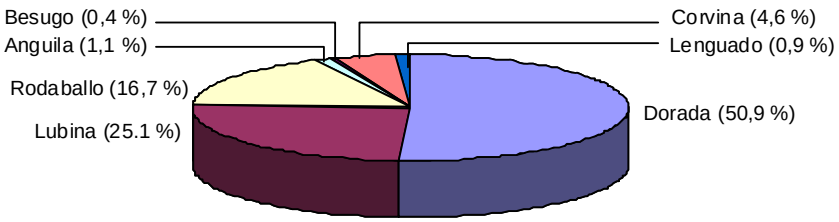


Figura 1.2. Producción de pescados marinos de crianza en España (datos del Informe de Apromar, 2009).

La Comunidad Valenciana encabeza la producción nacional de dorada con un 40% del total, seguida de Murcia (23%), Canarias (20%), Andalucía (10%) y Cataluña (7%) (datos de 2008, Informe de Apromar 2009). Esta producción de dorada descendió de forma global en un 1,5% a lo largo del 2009 y se prevé una caída incluso superior en el 2010. Por consiguiente, la actual crisis económica y financiera está afectando severamente a la acuicultura española. La crisis dificulta el acceso al crédito a un sector con largos ciclos de producción y elevadas necesidades de capital circulante. Además, a pesar de que la cantidad de pescado fresco consumida en España no ha descendido de forma significativa en 2008-2009, el valor de esas compras sí lo ha hecho. A esa coyuntura se suma la apuesta de las cadenas de distribución comercial por pescados baratos aun a costa de la calidad, con el consiguiente cambio en la elección de compra del consumidor. Ante esta situación, el sector acuícola deberá trabajar arduamente para ofrecer un presente y un futuro de sostenibilidad.

1.2. Materias primas

El sector acuícola es una de las actividades industriales que actualmente requiere una mayor demanda de harinas y aceites de pescado (Mundheim y cols., 2004). Las harinas se obtienen mayoritariamente a partir de la cocción, prensado y secado de triturados de pescado con poco valor añadido para el consumo humano directo (anchoveta, capelina y sábalo). Los aceites, por su parte, son subproductos del proceso de prensado. El precio de la harina de pescado alcanzó los 1400 dólares por Tm en 2006, desde entonces se ha mantenido por encima de los 1000 dólares (FAO, 2008). El precio del aceite también ha incrementado de forma extraordinaria, alcanzando la cifra récord de 1700 dólares por Tm en 2008 (FAO, 2008). De este modo, los piscicultores han visto aumentar progresivamente sus costes de producción.

Con el aumento de la producción acuícola, las partidas de harinas y aceites de pescado destinados a la acuicultura han aumentado considerablemente, llegando a suponer el 56% y el 87% de la actual disponibilidad mundial, respectivamente. De hecho, los fabricantes de alimentos para la acuicultura están aumentando la utilización de harina y aceite de pescado a expensas de todos los demás sectores (por ejemplo, consumo humano, industrial y farmacéutico). No obstante, el estancamiento de las pesquerías a nivel mundial pone de manifiesto que la actividad piscícola no puede basarse únicamente en las reservas finitas de pescado (FAO, 2008). Este hecho es especialmente relevante si tenemos en cuenta que, de acuerdo con los actuales cánones, se precisan de 2 a 5 kg de pescado para producir 1 kg de pescado de crianza (Tacon, 1997; Naylor y cols., 2000). Por tanto, la sustitución de harinas y aceites de pescado por fuentes alternativas y sostenibles de materias primas resulta ineludible.

Para la mayor parte de peces marinos los ácidos grasos poli-insaturados omega-3 de cadena larga (EPA y DHA) son ácidos grasos esenciales (Sargent y cols., 1997), ya que no poseen la maquinaria bioquímica para sintetizarlos y elongarlos a partir del ácido linolénico (18:3n-3) (Fountoulaki y cols., 2003). Las diatomeas y los dinoflagelados, ricos en EPA y DHA, son la base de la cadena trófica oceánica, por tanto los peces marinos tienen un superávit de estos ácidos grasos en su dieta natural (Tocher, 2003). Por el contrario, la mayoría de los peces de agua dulce son capaces de realizar la conversión de 18:3n-3 a EPA y DHA (Sargent y cols., 2002) (**Figura 1.3**). Esta diferencia es por tanto fundamental a la hora de determinar los requerimientos en ácidos grasos esenciales. Así pues, la sustitución total de aceites de pescado como ingrediente o como componente de las harinas de pescado no es teóricamente factible en piensos de engorde para peces marinos, mientras que en especies de agua dulce, la sustitución es teóricamente posible en su práctica totalidad.

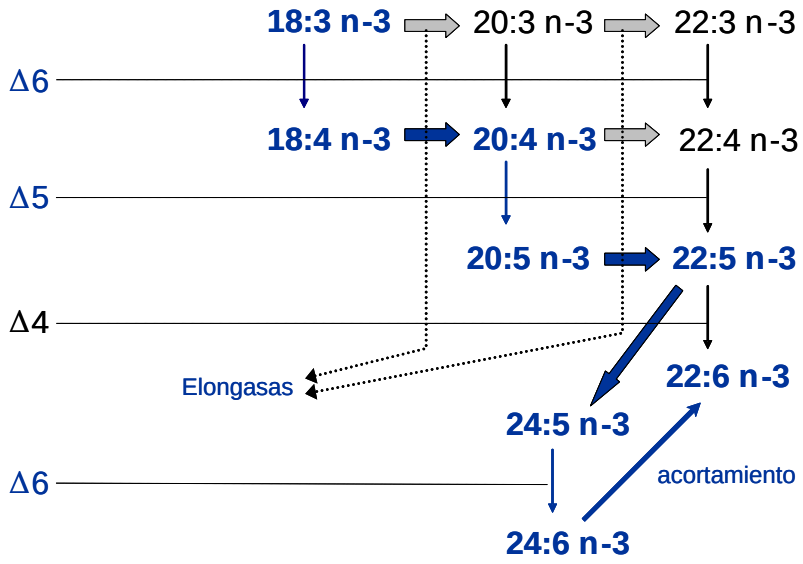


Figura 1.3. Ruta biosintética de ácidos grasos poli-insaturados omega-3 C_{20} y C_{22} a partir de su precursor C_{18}. Desaturasas: Δ4, Δ5, Δ6. El ácido graso 18:3n-3 es esencial para la mayoría de animales incluyendo la mayor parte de peces de agua dulce; la ruta marcada en color azul es la que emplean para la biosíntesis de ácidos grasos de cadena más larga. La actividad de las desaturasas y elongasas en las especies de origen marino es muy limitada y, por tanto, el 20:5n-3 y el 22:6n-3 son ácidos grasos esenciales para estas especies.

Además, la utilización de fuentes alternativas de aceites en piensos de engorde está condicionada por la necesidad de mantener unos niveles elevados de EPA y DHA en el producto final destinado al consumo humano (Sociedad internacional para el estudio de los ácidos grasos y lípidos, www.issfal.org). Los alimentos de origen marino son la fuente más importante de EPA y DHA en la dieta humana (Hunter y Roberts, 2000). Estos ácidos grasos poli-insaturados omega-3 de cadena larga aportan importantes beneficios en la prevención de enfermedades cardiovasculares (Din y cols., 2004). Además, el consumo regular de productos marinos ayuda a mantener el cociente omega-3/omega-6 alto (Simopoulos, 2000), con importantes

propiedades anti-inflamatorias, anti-trombóticas, anti-arritmicas, hipolipidémicas y vasodilatadoras (Simopoulos, 1999). Un adecuado cociente entre estos grupos de ácidos grasos tiene un impacto primordial en la salud, reduciendo la prevalencia tanto de enfermedades cardiovasculares como de otras enfermedades crónicas e inflamatorias (Wijendran y Hayes, 2004; Calder, 2006; El Badry y cols., 2007).

Por otra parte, la sustitución de harinas y aceites de pescado por fuentes alternativas puede resultar beneficiosa desde el punto de vista de la seguridad alimentaria, ya que los productos de origen marino son fuentes potenciales de residuos químicos (dioxinas, policlorobifenilos, furanos) y metales pesados (metilmercurio, cadmio, arsénico) (Jacobs y cols., 2002; Cortazar y cols., 2008; Serrano y cols., 2008). En ocasiones, estos contaminantes son fruto de una contaminación natural, pero en cualquier caso, su eliminación a escala industrial es compleja por lo que se impone la práctica de estrictos criterios de selección y control. Algunos países han hecho públicas recomendaciones para limitar el consumo de ciertos tipos de productos pesqueros, con especial atención a grupos vulnerables como niños y mujeres embarazadas. Sin embargo, estos destinatarios últimos tienen una especial dependencia por una dieta nutricionalmente adecuada a la hora de satisfacer sus requerimientos de vitaminas, yodo y ácidos grasos omega-3, que son especialmente necesarios en las primeras fases de desarrollo del sistema nervioso (Horrocks y Yeo, 1999; Guesnet y Alessandri, 2010). Así pues, la relación entre salud y seguridad alimentaria hace que sea necesario un enfoque más holístico a la hora de hacer recomendaciones destinadas a equilibrar los riesgos y los beneficios del consumo de productos marinos (Simopoulos, 2001; Mariné y Vidal, 2001).

1.3. Fuentes alternativas

1.3.1. Sustitución proteica

El componente proteico en las dietas utilizadas en el cultivo de peces carnívoros es cuantitativamente el más importante y, a su vez, el más costoso. Así pues, la inclusión de harinas de pescado suele oscilar entre un 20% y un 60%, de ahí los esfuerzos por encontrar fuentes alternativas de calidad, sostenibles y de menor coste.

Las posibles alternativas incluyen materias primas derivadas de sub-productos animales, tanto de invertebrados (zooplancton, crisálida de gusano de seda o lombriz) (Kibria y cols., 1999; Plasencia-Jatomea y cols., 2002) como de vertebrados (sangre, hígado, carne o hueso) (Alexis y cols., 1985; Millamena y Golez, 2001), organismos unicelulares (hongos o bacterias) (Oliva-Teles y Gonçalves, 2001; Li y Gatlin III, 2003), algas (Dallaire y cols., 2007), semillas (soja, girasol, colza o algodón) (Kissil y cols., 2000), legumbres (soja, alubias o guisantes) (Pereira y Oliva-Teles, 2002; Thiessen y cols., 2003) y otros productos de origen vegetal (gluten de maíz, trigo o concentrados proteicos) (Regost y cols., 1999; Burel y cols., 2000).

Sin embargo, gran parte de esas alternativas presentan ciertos inconvenientes. Según Tacon (1994), la disponibilidad de proteínas procedentes de organismos unicelulares es limitada, costosa y de calidad variable. Por ejemplo, la utilización de algas como fuente de materias primas está condicionada por su bajo contenido en proteínas digestibles. Además, el uso de productos derivados de animales terrestres también resulta complicado, debido entre otros a la presencia de factores anti-nutricionales y posibles contaminaciones microbianas. Por estos motivos, los mayores esfuerzos a lo largo de las últimas décadas se han centrado en los ingredientes vegetales de origen terrestre (Kaushik y cols., 1995). Un primer criterio es el contenido total de proteínas y el balance en aminoácidos esenciales. Otros criterios son el contenido en carbohidratos y factores anti-

nutricionales (inhibidores de proteasas, taninos, ácido fítico, antitripsina) (Watanabe, 2002).

En última instancia, el éxito de la sustitución de las harinas de pescado por fuentes proteicas de origen vegetal depende de la especie y de la fase del ciclo biológico. Ahora bien, según Kaushik (1990), la composición aminoacídica es el factor más importante a tener en cuenta en la formulación de los piensos, además del contenido total en proteínas y su digestibilidad. De hecho, el pobre perfil aminoacídico de algunas proteínas de origen vegetal aconseja la utilización de diferentes combinaciones para simular el perfil aminoacídico de las harinas de pescado (**Figura 1.4**), siendo necesaria en muchos casos la suplementación de la dieta con preparados comerciales de aminoácidos esenciales (por ejemplo, lisina y metionina). Basándose en ello, los resultados de diversos estudios muestran que la sustitución de las harinas de pescado por ingredientes de origen vegetal es posible en diversas especies de peces sin disminuir así su potencial de crecimiento (Carter y Hauler, 2000; Kaushik y cols., 2004; Gómez-Requeni et al., 2005; Hansen y cols., 2007; Hernández y cols., 2007).

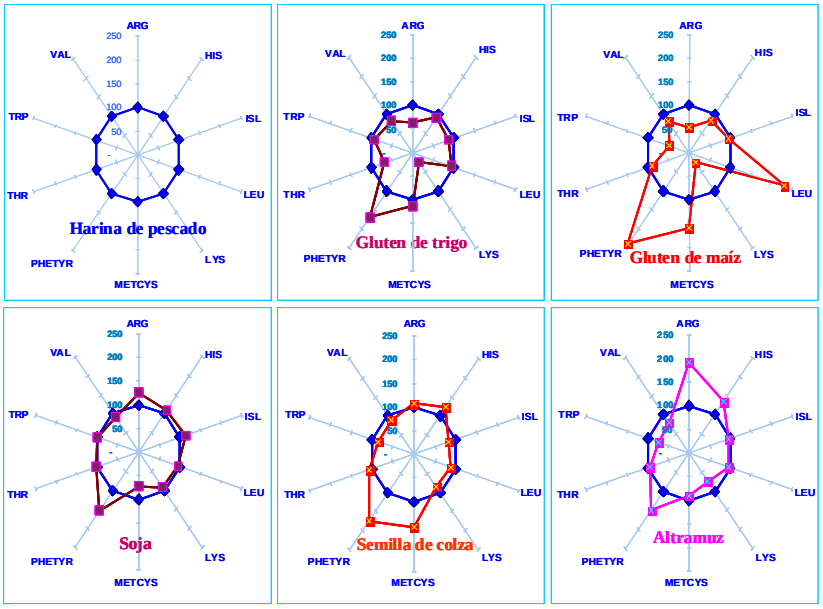


Figura 1.4. Perfil aminoacídico de la harina de pescado y proteínas vegetales.

1.3.2. Sustitución lipídica

El papel de los lípidos en acuicultura ha cobrado especial relevancia en los últimos años con la utilización de dietas de alto contenido energético, que mejoran la producción acortando el periodo de engorde. Tradicionalmente se ha utilizado el aceite de pescado como única fuente lipídica para la fabricación de los piensos de engorde de peces. Sin embargo, el aumento de los precios y la limitación de la disponibilidad de este tipo de aceites también obliga a la industria a buscar fuentes alternativas.

Aceites vegetales

La producción de aceites vegetales ha aumentado considerablemente en las últimas tres décadas, contrariamente al estancamiento que ha sufrido la producción-disponibilidad del aceite de pescado (**Figura 1.5**). Los aceites de palma, soja, colza y girasol son los más abundantes a nivel global y, con la excepción del aceite de girasol, sus precios se han mantenido históricamente por debajo del los del aceite de pescado. No obstante, en 2009 el precio del aceite de soja sobrepasó al del aceite de pescado, debido en parte al auge de los biocombustibles, aunque actualmente ambos se han igualado (www.globefish.org). En todo caso, procesos cíclicos como el fenómeno de El Niño en el Sureste del Pacífico pueden hacer descender el número de capturas y provocar una nueva subida en el precio de los productos derivados del pescado.

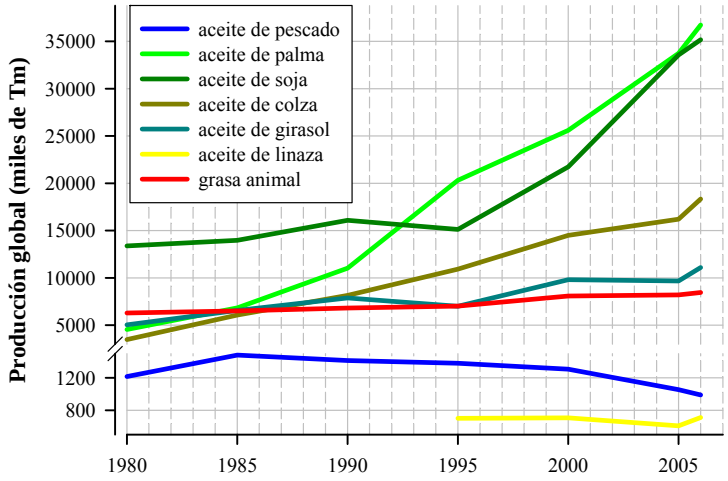


Figura 1.5. Producción global de distintos tipos de aceite de 1980 a 2006 (Modificado de Turchini y cols., 2009).

Al igual que el aceite de pescado, los peces metabolizan fácilmente los aceites vegetales y los utilizan como fuente de energía (Bell y cols., 2001; Regost y cols., 2003; Stubhaug y cols., 2007). Sin embargo, sus características químicas, especialmente su composición en ácidos grasos, limitan su uso como única fuente lipídica. La mayoría de ellos son pobres en ácidos grasos omega-3 y ricos en omega-6 y omega-9, principalmente ácido linoleico (18:2n-6) y oleico (18:1n-9), con la excepción del aceite de linaza que contiene gran cantidad de ácido linolénico (18:3n-3) (**Tabla 1.1**). Su uso como sustituto del aceite de pescado ha sido evaluado, tanto individualmente (Bell y cols., 2002, 2003a, 2004; Regost y cols., 2003; Izquierdo et al., 2005) como con mezclas de varios aceites vegetales (Torstensen y cols., 2005; Mourente y Bell, 2006; Francis y cols., 2007). El perfil de estas mezclas de aceites vegetales se intenta asemejar al del aceite de pescado, en relación a la cantidad relativa de ácidos grasos saturados y monoenos y al cociente de ácidos grasos omega-3/omega-6. Así, la práctica totalidad del aceite de pescado puede sustituirse por aceites vegetales en piensos basados en proteína de pescado, tanto en salmónidos (Bell y cols., 2003b; Brandsden y cols., 2003; Torstensen et al., 2004) como en especies marinas (Regost y cols., 2003; Piedecausa y

cols., 2007). En estos casos, los requerimientos en ácidos grasos esenciales del animal quedan cubiertos por la cantidad de EPA y DHA ligada a las harinas de pescado, puesto que éstas suelen contener entre un 8-10% de aceite de pescado rico en ácidos grasos poli-insaturados omega-3 de cadena larga (Bimbo, 2000).

Tabla 1.1. Composición de ácidos grasos (% total de ácidos grasos) de aceite de pescado, aceites vegetales y grasa animal (modificado de Sauvant y cols., 2004).

Ácido graso	Anchoveta	Palma	Soja	Colza	Girasol	Linaza	Animal
16:0	17,8	43,0	10,5	4,2	6,3	6,4	25,3
18:0	3,9	4,4	3,8	1,8	4,3	3,4	19,2
18:1n-9	12,0	37,1	21,7	58,0	20,3	18,7	37,5
18:2n-6	1,1	9,9	53,1	20,5	64,9	14,7	2,8
18:3n-3	0,8	0,3	7,4	9,8	0,3	54,2	0,6
20:4n-6	0,3	-	-	-	-	-	0,2
20:5n-3	18,3	-	-	-	-	-	-
22:6n-3	8,5	-	-	-	-	-	-
n-3/n-6	22,5	-	0,1	0,6	-	4,2	0,2

Según lo expuesto anteriormente, en la formulación de piensos basados en aceites vegetales se buscará un adecuado balance entre ácidos grasos saturados (ej. 16:0, abundante en el aceite de palma) y monoenos (ej. 18:1n-9, abundante en el aceite de colza) que los peces utilizarán preferentemente como fuente de energía (Henderson y Sargent, 1985; Tocher, 2003). Al mismo tiempo, se restringirá el aporte de 18:2n-6, favoreciendo el balance omega-3/omega-6 (ej. con aceite de linaza, rico en 18:3n-3).

Como alternativa al aceite de linaza, se ha considerado recientemente el uso de aceite de *Camelina sativa*, especie de planta herbácea perteneciente a la familia de las brasicáceas. Esta planta está siendo objeto de varios estudios debido a unos niveles excepcionalmente altos de ácidos grasos omega-3 (hasta un 45%), poco comunes en fuentes vegetales. El perfil característico de este aceite contiene un 35-45% de 18:3n-3, 15-20% de 18:2n-6 y otro tanto de 20:1n-9. Su precio y facilidad de cultivo, puesto que puede crecer en condiciones áridas y que apenas requiere fertilizantes, podrían hacer de esta planta una alternativa altamente competitiva. Algunos autores ya han utilizado este aceite como parte de la mezcla de aceites vegetales en la formulación de piensos para el engorde de salmones (Bell y cols., 2010), habiéndose observado que su empleo con respecto al del aceite de linaza no produce diferencias en términos de crecimiento.

El aceite de ciertas plantas borragináceas como el obtenido a partir de *Echium* sp. también ha despertado un notable interés, debido a su alto contenido en ácido estearidónico (18:4n-3). La generación de este ácido graso es el primer paso en la ruta biosintética de ácidos grasos omega-3 de cadena larga a partir del ácido linolénico (ver sección 1.2, **Figura 1.3**). Este primer paso, donde la enzima Δ6 desaturasa actúa convirtiendo el ácido linolénico en estearidónico, podría ser la principal limitación en la escasa biosíntesis de ácidos grasos poli-insaturados omega-3 de cadena larga por parte de algunos peces marinos (Sargent y cols., 2002; Tocher, 2003). Por ello, este aceite podría suponer una alternativa eficaz en la sustitución del aceite de pescado (Bell y cols., 2006; Díaz-López y cols., 2009; 2010; Bharadwaj y cols., 2010). Sin embargo, los resultados obtenidos hasta la fecha no muestran un aumento eficaz en la biosíntesis de EPA y DHA a partir del ácido estearidónico (Ghioni y cols., 1999; Miller y cols., 2006). En este sentido, los beneficios derivados del empleo de este aceite serían similares a los obtenidos con el aceite de linaza, con alto contenido en 18:3n-3 y mayor disponibilidad comercial. No obstante, el perfil de los distintos ácidos grasos del aceite de *Echium* sp. está más equilibrado y ello podría influir en una reducción saludable de grasa corporal y en una mejor asimilación de EPA y DHA en los tejidos (Miller y cols., 2006).

Grasas animales

Además de los aceites vegetales, una posible alternativa al aceite de pescado son las grasas de origen animal (mantecas de cerdo, restos de aves de corral o grasas de ternera y cordero). El reciente aumento del precio de los aceites vegetales ha hecho que crezca el interés por el uso de grasas de origen animal en acuicultura. El precio de las grasas animales es un 50% menor que el del aceite de pescado. No obstante, su producción (**Figura 1.4**) está muy por debajo que la de los aceites vegetales con una mayor disponibilidad en el mercado (palma, soja y colza). Además, las características químicas de la grasa animal pueden variar ampliamente dependiendo de la historia nutricional, la especie y la edad del animal. Aún así, estas grasas suelen tener altos niveles de ácidos grasos saturados y de monoenos y, aunque pueden contener EPA y DHA, su contenido es muy limitado y pueden considerarse carentes en estos dos ácidos grasos (**Tabla 1.1**). A pesar de ello, el aceite de pescado puede sustituirse en un 50% por grasas de origen animal sin afectar al crecimiento de los peces (Hardy y cols., 1987; Liu y cols., 2004), aunque la digestibilidad de las grasas animales y el balance en ácidos grasos omega-3/omega-6 obtenido es inferior al que suele obtenerse con aceites vegetales (Martins y cols., 2009).

Subproductos de procesado

Los subproductos de las plantas de procesado de pescado de pesca extractiva (como vísceras, cabezas, sangre, pieles, huesos) pueden utilizarse como fuentes de aceite en el engorde de peces. Su bajo coste ofrece un alto potencial y, además, su contenido en EPA y DHA hace de su uso una alternativa interesante. Sin embargo, no existen datos oficiales acerca de la producción, disponibilidad y precio del aceite de pescado derivado de esta práctica. Además, existen algunas reticencias acerca de su uso debido al posible riesgo de transmisión de enfermedades bacterianas y parasitarias. No obstante, se han obtenido buenos resultados, en cuanto a crecimiento y composición corporal, en diversos estudios donde estas nuevas fuentes de materias primas han sido utilizadas para la alimentación de peces en cultivo (Kotzamanis y cols., 2001; Turchini y cols., 2003).

Los subproductos de procesado de pescado de acuicultura también podrían utilizarse para producir aceites. Sin embargo, en este caso, hay que tener en consideración que la alimentación empleada durante el engorde de estos animales puede tener una influencia determinante sobre el perfil de ácidos grasos del aceite generado.

Otros subproductos como el aceite de krill (*Euphausia pacifica*) y calamar también han sido considerados por su alta calidad nutritiva, ya que son fuentes ricas en ácidos grasos esenciales, fosfolípidos y carotenos (Kang y cols., 2005; Maki y cols., 2009). No obstante, los costes de explotación son el principal factor limitante a la hora de considerar su empleo a nivel industrial.

Otras fuentes de origen marino

El alto contenido en ácidos grasos poli-insaturados omega-3 de cadena larga de ciertos organismos marinos como algas unicelulares, organismos pelágicos e invertebrados bentónicos ha hecho que este tipo de organismos sea considerado como fuente alternativa de aceites. Microorganismos como *Schizochytrium* spp., *Crypthecodinium cohnii* y *Phaeodactylum tricornutum* se han utilizado como componente alimenticio de la dorada (Atalah y cols., 2007; Ganuza y cols., 2008). Otros microorganismos y copépodos también parecen ofrecer buenos resultados en piensos para el cultivo del salmón (Olsen y cols., 2004; Miller y cols., 2007). Sin embargo, el coste de producción de este tipo de aceites es extremadamente alto, mermando su potencial comercial y dificultando su uso a gran escala.

Organismos genéticamente modificados

Los últimos avances en ingeniería genética aportan la posibilidad de utilizar organismos genéticamente modificados. La modificación genética de algunas semillas ha permitido obtener aceites vegetales enriquecidos en ácidos grasos poli-insaturados omega-3 de cadena larga que podrían ser útiles en el sector acuícola (Opsahl-Ferstad y cols., 2003; Robert, 2006). No obstante, este tipo de organismos son motivo de controversia y sus detractores opinan que pueden ocasionar riesgos para el

medio ambiente y, posiblemente, para la salud humana. Debido a la polémica generada, el uso de los organismos genéticamente modificados no llega a convencer a la opinión pública, por tanto, a pesar del enorme potencial que ofrece este sector, su aplicación en la UE parece inviable, al menos a corto o medio plazo.

A la vista de todo lo expuesto anteriormente, a pesar de que el crecimiento de los animales es posiblemente el factor más importante a la hora de evaluar posibles fuentes alternativas de materias primas, éste no es el único aspecto que debe tenerse en consideración. El reto en el uso de fuentes alternativas de proteínas y aceites de pescado está en mantener, si no mejorar, los beneficios que el pescado ofrece al consumidor. Simultáneamente, se deberán considerar aspectos de sostenibilidad, rentabilidad, salud y bienestar animal. Para ello, la eficiencia de conversión del alimento, el estado de inmuno-competencia del animal y la calidad y seguridad alimentaria del producto final también deben estudiarse en profundidad. De forma global, todos estos aspectos han sido considerados en la discusión general (capítulo 7) de la presente Tesis Doctoral.

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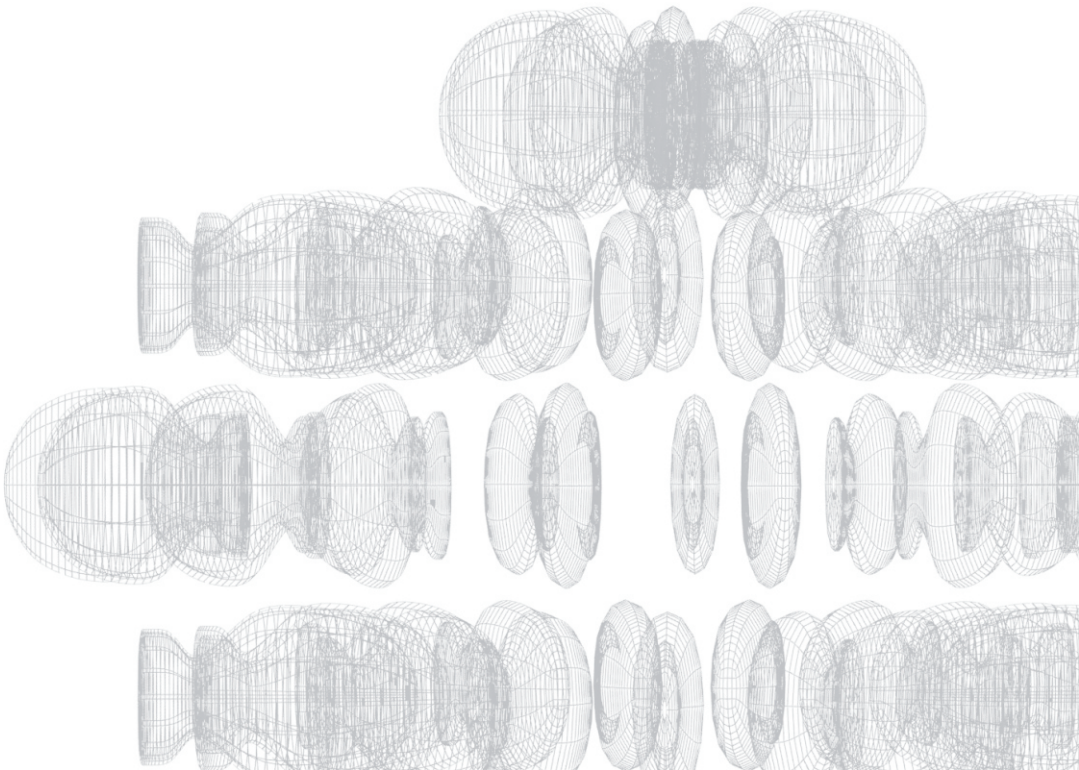
CAPÍTULO 2

OBJETIVOS Y PLAN DE TRABAJO



Ante la necesidad de fuentes alternativas de materias primas para piensos de engorde en acuicultura, la UE financió en su V Programa Marco una serie de proyectos centrados en el desarrollo y explotación de fuentes alternativas a las harinas y los aceites de pescado:

- RAFOA: Researching alternatives to fish oil for aquaculture.
- PEPPA: Perspectives of plan protein usage in aquaculture.
- GUTINTEGRITY: Gastrointestinal functions and feed intake regulation in salmonids: impact of dietary vegetable lipids.
- PUFAFEED: Feed for aquatic animals that contains cultivated marine microorganisms as alternatives for fish oil.
- FPPARS: Cloning and functional analysis of fish peroxisome proliferator-activated receptors: the transcriptional control of lipid metabolism in farmed fish.

Los participantes en estos proyectos constituyeron una red temática denominada FORM (Fish Oil and Meal Replacement) que sirvió de foro de discusión y dio pie al proyecto AQUAMAX (Sustainable aquafeeds to maximise the health and benefits of farmed fish for consumers) en el VI Programa Marco de la UE.

El Grupo de Nutrición y Endocrinología del Crecimiento del Instituto de Acuicultura de Torre de la Sal (IATS) participó activamente en el desarrollo del proyecto PEPPA (2001-2004) y los estudios que se llevaron a cabo indican que las harinas de pescado se pueden sustituir por ingredientes vegetales (hasta un 75%) en una especie típicamente carnívora como la dorada, sin detrimento del crecimiento (Gómez-Requeni y cols., 2004), del estado de inmunocompetencia (Sitjà-Bobadilla y cols., 2005) y de la calidad del producto final destinado al consumo humano (De Francesco y cols., 2007).

La investigación desarrollada en la presente Tesis Doctoral está enmarcada dentro del proyecto AQUAMAX (2006-2010). El objetivo básico de este proyecto ha sido establecer el nivel máximo de sustitución conjunta de proteínas y aceites de pescado en piensos de acuicultura elaborados con materias primas alternativas, sostenibles y con una baja carga de contaminantes, sin afectar al crecimiento, la conversión del alimento, la salud y bienestar animal. Todo ello sin perjuicio de obtener un pescado de alto valor nutritivo, garantizando la seguridad y calidad alimentaria, y con amplia aceptación por parte del consumidor. Este proyecto ha considerado la trucha, la carpa, el salmón atlántico y la dorada como especies modelo. El grupo del IATS ha coordinado activamente los estudios relacionados con dorada, y parte de los trabajos desarrollados en este ámbito constituyen la base de la presente Tesis Doctoral.

En este contexto, **los objetivos de la presente Tesis Doctoral** son:

1. Definir en piensos de engorde de dorada el nivel crítico de sustitución del aceite de pescado por aceites vegetales en dietas basadas en proteínas de origen vegetal.
2. Establecer la viabilidad de dicha sustitución tanto a corto plazo como a lo largo de un ciclo completo de engorde basándose en parámetros de crecimiento, metabólicos y hormonales.
3. Analizar los diferentes patrones tisulares de ácidos grasos en lípidos polares y lípidos de reserva.
4. Establecer las cinéticas de ácidos grasos en músculo de peces alimentados con dietas de sustitución y finalizadoras.

Para abordar estos objetivos se estableció un **plan de trabajo** basado en dos grandes bloques de experimentos de engorde que generaron muestras de sangre y tejidos para diferentes análisis de parámetros biométricos, histológicos, bioquímicos y hormonales (ver **Figura 2.1**).

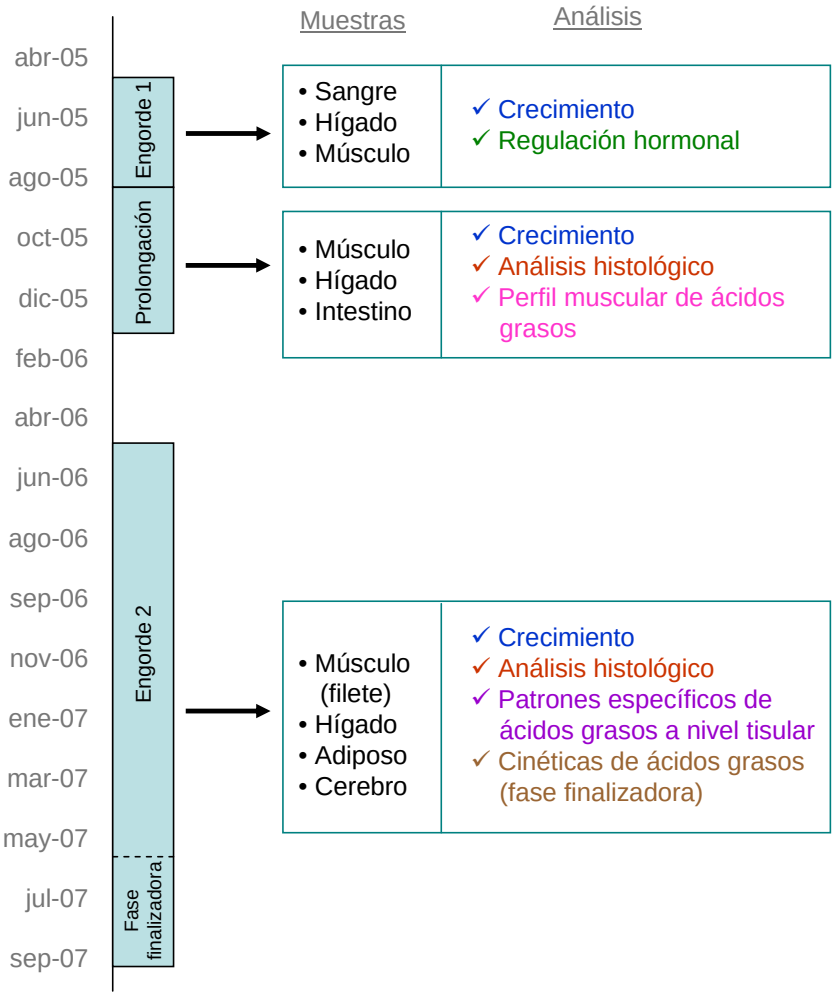


Figura 2.1. Organigrama resumen del plan de trabajo.

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

El **capítulo 3** describe los efectos a corto plazo de dietas basadas en ingredientes vegetales sobre el potencial de crecimiento y regulación hormonal de juveniles de dorada (engorde 1). Para ello se emplearon cuatro dietas basadas en proteínas vegetales (75% de sustitución de la harina de pescado) con una sustitución parcial (0%, 33%, 66%) o total del aceite de pescado de origen escandinavo por una mezcla de aceite de colza, linaza y palma. Como biomarcadores del potencial de crecimiento y del estado nutricional se analizaron diferentes componentes del eje somatotrópico (hormona del crecimiento, receptores de hormona del crecimiento y somatomedinas).

El **capítulo 4** describe un ciclo completo de engorde de 8 meses (engorde 1, prolongación) con los mismos grupos experimentales del capitulo anterior y analiza la relación existente entre la composición de ácidos grasos de la dieta y el músculo esquelético, con especial referencia al perfil de ácidos grasos de la fracción polar. El estudio se completó con el análisis de los posibles efectos histopatológicos de la dieta sobre el hígado y el tracto intestinal.

Con las dietas seleccionadas a partir de parámetros de crecimiento y biomarcadores bioquímicos y hormonales, se diseñó una nueva experiencia de engorde (engorde 2) de 14 meses de duración que fue la base de los estudios descritos en los capítulos 5 y 6.

El **capítulo 5** refiere el análisis de la robustez de los distintos patrones tisulares de ácidos grasos, mostrando el perfil de los triglicéridos y fosfolípidos del músculo esquelético, cerebro, hígado y tejido adiposo mesentérico al finalizar un ciclo completo de engorde con los peces alimentados con las dos dietas extremas.

El **capítulo 6** muestra el análisis de la cinética de los ácidos grasos del filete de doradas de tamaño comercial durante una fase finalizadora con una dieta basada en aceite de pescado. El estudio incluye la validación experimental de un modelo de dilución simple de perfiles de ácidos grasos, considerando además las consecuencias de este modelo sobre el ahorro de aceites de pescado en piensos de engorde de dorada.

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

- Capítulo 3: **Benedito-Palos L**, Saera-Vila A, Calduch-Giner JA, Kaushik S, Pérez-Sánchez J (2007). Combined replacement of fish meal and fish oil in practical diets for fast growing juveniles of gilthead sea bream (*Spaurs aurata* L.): Networking of systemic and local components of GH/IGF axis. Aquaculture 267, 199-212.
- Capítulo 4: **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 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.
- Capítulo 5: **Benedito-Palos L**, Navarro JC, Kaushik S, Pérez-Sánchez J (2010). Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata* L.) fed practical diets with a combined high replacement of fish meal and fish oil. Journal of Animal Science 88, 1759-1770.
- Capítulo 6: **Benedito-Palos L**, Navarro JC, Bermejo-Nogales A, Saera-Vila A, Kaushik S, Pérez-Sánchez J (2008). 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.

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:

- Bermejo-Nogales A, **Benedito-Palos L**, Saera-Vila A, Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J (2008). Confinement exposure induces glucose regulated protein 75 (GRP75/mortalin/mtHsp70/PBP74/HSPA9B) in the hepatic tissue of gilthead sea bream (*Sparus aurata* L.). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 149, 428-438.
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- Nácher-Mestre J, Serrano R, Portolés T, Hernández F, **Benedito-Palos L**, Pérez-Sánchez J (2009). A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time of flight analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities. Rapid Communications in Mass Spectrometry 23, 2075-2086.
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- **Benedito-Palos L**, Bermejo-Nogales A, Karampatos, AI, Ballester-Lozano GF, Navarro JC, Diez A, Bautista JM, Bell, JG, Tocher DR, Obach A, Kaushik S, Pérez-Sánchez J (2011). Modelling the predictable effects of dietary lipid sources on the fillet fatty acid composition of one-year-old gilthead sea bream (*Sparus aurata* L.). Food Chemistry 124, 538-544.
- Estensoro I*, **Benedito-Palos L***, Palenzuela O, Kaushik S, Sitjà-Bobadilla A, Pérez-Sánchez J (*ambos autores han contribuido de igual modo) (2010). The nutritional background of the host alters the disease outcome of a fish-myxosporean system. Veterinary Parasitology, en prensa, doi: 10.1016/j.vetpar.2010.09.015.

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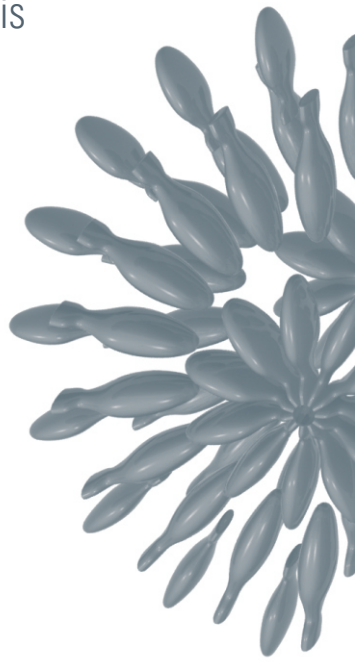
CAPÍTULO 3

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



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Aquaculture (2007) 267: 199-212

ABSTRACT

Growth performance and growth regulatory pathways were examined in juvenile gilthead sea bream fed diets containing largely plant-based ingredients. Four isonitrogenous and isolipidic extruded diets with a low level (20%) of fishmeal inclusion were formulated with graded levels of a vegetable oil mixture (17:58:25 of rapeseed: linseed: palm oils) replacing fish oil at 33, 66 and 100% (33VO, 66VO and VO diets). All diets were supplemented with lysine (0.55%) and contained soy lecithin (1%). Daily growth coefficients and feed efficiency over the course of an 11-week trial were almost identical in fish fed the FO, 33VO and 66VO diets. The VO diet reduced feed intake and growth without significant effects in proximate whole body composition, nitrogen or energy retentions. The highest concentration of plasma levels of insulin-like growth factor-I (IGF-I) was found in fish fed the 33VO diet. The lowest concentration was attained in fish fed the VO diet, whereas intermediate values were found in fish fed FO and 66VO diets. An opposite trend was found for circulating levels of growth hormone (GH), probably as a result of a reduced negative feedback inhibition from circulating IGF-I. Hepatic expression of IGF-I and GH receptor type I (GHR-I) was regulated in concert and mRNA levels paralleled plasma levels of IGF-I. Hepatic IGF-II and GHR-II were expressed in a more constitutive manner and no changes at the mRNA level were detected. In the skeletal muscle, IGF-I and GHR-I mRNAs did not vary significantly among groups. By contrast, IGF-II mRNA was up-regulated in fish fed the control diet, whereas the highest amount of GHR-II mRNA was attained in fish fed the 66VO diet. All together, these results suggest different growth compensatory mechanisms mediated by IGF-II and GHR-II at the local tissue level. These new insights prompted us to propose that practical diets low in marine ingredients can be used over the productive cycle of gilthead sea bream when essential fatty acids are supplied above the requirement levels.

Keywords: Sparidae; Fish oil; Vegetable oil; Plant proteins; Growth hormone; Growth hormone receptors; Insulin-like growth factors; Endocrine disrupters; Contaminants

3.1. Introduction

Currently, aquaculture is the major consumer of fish meal, a protein-dense feedstuff that approximates the ideal amino acid profile of most cultured livestock. However, fish meal is a limited resource whose availability has remained stable from the late 1980s at approximately 6 million metric tonnes per annum, which limits the continuous growth of aquaculture production (FAO, 2004). Furthermore, inherent variability in fish meal composition due to species, season, geographic origin and processing leads to variation in quality (Opstvedt et al., 2003; Bragadóttir et al., 2004), and most of the future changes in developing novel aquafeeds should be focused on alternative protein sources.

The n-3 long-chain highly unsaturated fatty acids (n-3 HUFA) are naturally abundant in the marine environment, and fish oil is the major source of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 20:6n-3) for aquafeeds. Besides the scarcity of fish oil, which is of great concern for marine fish, these animals have a limited capacity to biosynthesize n-3 HUFA from the shorter chain linolenic acid (18:3n-3), and both EPA and DHA become critical dietary constituents to ensure successful survival, growth, and development of these fish (Sargent et al., 1999, 2002). At this standpoint, it must be noted that fish meal also contains certain amounts of oil rich in n-3 HUFA, and the fish oil added to energized diets can be totally replaced by vegetable oils when fish meal is included at a high level in diets for Atlantic salmon (Bell et al., 2003; Bransden et al., 2003; Torstensen et al., 2004), rainbow trout (Richard et al., 2006a), and the freshwater African catfish (Ng et al., 2004). Similar results have been achieved in a typically marine fish such as turbot (Regost et al., 2003). A high fish oil replacement is also feasible in the Murray cod using casein-based diets (Francis et al., 2006). Likewise, up to 60% of fish oil added to diets has been replaced successfully in juvenile European sea bass (Montero et al., 2005; Mourente et al., 2005) and gilthead sea bream (Izquierdo et al., 2005), but the diets used in these studies also contained 35 to 40% fish meal.

Marine derived feedstuffs are also possible vectors of contaminants, such as PCBs, dioxins and other harmful chemicals affecting the safety of farm-raised fish (Jacobs et al., 2002). It is clear that reduction in fish oil levels can lead to a decrease in the contaminant levels of feed and consequently on fish filets (Berntssen et al., 2005; Bethune et al., 2006). Thus, the general consensus is that alternative protein and oil sources are needed to supplement or replace fish meal and fish oil in aquafeeds, contributing to long-term sustainability of the aquaculture industry (Hardy, 2004). In the present study, our objective was hence to maximize the combined replacement of fish meal and fish oil in practical diets for fast growing juveniles of gilthead sea bream. In earlier studies, we had shown that a good proportion of fish meal can be replaced by a mixture of plant protein sources in gilthead sea bream diets (Gómez-Requeni et al., 2003, 2004; Sitjà-Bobadilla et al., 2005). Based on these results, we attempted here to replace fish oil by a blend of vegetable oils, which have been already shown to be very effective in other fish species (Torstensen et al., 2005; Mourente and Bell, 2006; Richard et al., 2006a,b). To address this issue, growth and nutrient retention were analyzed in a conventional manner. Circulating levels of growth hormone (GH) and insulin-like growth factor-I (IGF-I) were used as markers of growth and nutrient status (see Pérez-Sánchez and Le Bail, 1999; Dyer et al., 2004). Also, transcripts of IGFs and GH receptors (GHR) were measured in liver and skeletal muscle by means of realtime PCR assays.

3.2. Materials and methods

3.2.1. Diets

As shown in **Table 1**, three diets (33VO, 66VO and VO) with relatively low fish meal inclusion (20%) levels were formulated with practical plant protein ingredients for the graded replacement (33, 66 and 100%) of the added fish oil by a blend of vegetable oils (rapeseed oil: linseed oil: palm oil). A fish oil-based diet (FO diet) equal in lipid content (220 g/kg) was used as

the reference diet. Diets were supplemented with lysine (0.55%) and contained soy lecithin (1%). EPA plus DHA content varied on a dry matter basis between 2.3% (FO diet) and 0.3% (VO diet), and the DHA/EPA ratio (1.1–1.2) remained constant. All diets were manufactured using a twin-screw extruder (Clextral, BC 45) in the INRA experimental research station of Donzacq (Landes, France), dried under hot air, sealed and kept in air-tight bags until use.

Diet samples were hydrolysed (6N HCl, 110 °C) and amino acid analysis was performed using high-performance liquid chromatography. Tryptophan was determined by the colorimetric method of Basha and Roberts (1977) after alkaline hydrolysis of each sample (see **Table 2**). Fatty acid methyl esters (FAME) were prepared from aliquots of total lipid by acid-catalysed transmethylation for 16 h at 50 °C (Christie, 1982) after the addition of nonadecaenoic fatty acid (19:0) as an internal standard. FAMEs were extracted and separated in a Fisons Instruments GC 8000 Series (Thermo Electron Co., Rodano, Italy) gas chromatograph, equipped with a fused silica 30 m×0.25 mm open tubular column (Tracer TR-WAX, film thickness: 0.25 μm-Teknockroma, Spain) and a cold column injection system, using helium as carrier and 50 to 220 °C thermal gradient. Peaks were recorded with Chrom-Card for Windows software (Fisons CE Instruments, Milan, Italy) and identified by comparison with known standards (see **Table 3**).

3.2.2. Growth trial and fish sampling

Gilthead sea bream (*Sparus aurata* L.) fingerlings of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimated to laboratory conditions for 20 days before the start of the growth study. Fish of 16 g initial mean body weight were distributed into 12 fibreglass tanks (500-l capacity) in groups of 60 fish each. Water (37.5‰ salinity) flow was 20 l/min, and oxygen content of outlet water remained higher than 85% saturation. Day length increased over the course of the trial (May–August) following natural changes at our latitude (40°5′ N; 0°10′ E). Water temperature also varied naturally increasing from 17 to 25 °C.

Table 1. Ingredients and chemical composition of experimental dets.

Ingredient (g/kg)	FO	33VO	66VO	VO
Fish meal (CP 70%) [a]	150	150	150	150
CPSP 90 [b]	50	50	50	50
Corn gluten meal	400	400	400	400
Soybean meal	143	143	143	143
Extruded wheat	40	40	40	40
Fish oil [c]	151	101	51	0
Rapeseed oil	0	8.5	17	25.8
Linseed oil	0	29	58	88
Palm oil	0	12.5	25	38
Soya lecithin	10	10	10	10
Binder (sodium alginate)	10	10	10	10
Mineral premix [d]	10	10	10	10
Vitamin premix [e]	10	10	10	10
$CaHPO_4.2H_2O$ (18%P)	20	20	20	20
L-Lysine	5.5	5.5	5.5	5.5
Proximate composition				
Dry matter (DM, %)	93.4	94.2	94.8	95.4
Crude protein (% DM)	48.9	48.7	49.0	48.6
Crude fat (% DM)	22.2	22.3	22.1	22.3
Ash (% DM)	6.5	6.6	6.6	6.4
EPA + DHA (% DM)	2.3	1.6	0.9	0.3
Gross energy (kJ/g DM)	24.7	24.7	24.6	24.5

[a]Fish meal (Scandinavian LT). [b]Fish soluble protein concentrate (Sopropêche, France). [c]Fish oil (Sopropêche, France). [d]Supplied the following (mg / kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3 (Unité des preparation des aliments expérimentaux (UPAE), Jouy, INRA, France). [e]Supplied the following (mg / kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B_{12} 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500 (UPAE, Jouy, INRA, France).

The growth study was undertaken over 11 weeks (74 days) and each diet was randomly allocated to triplicate groups of fish. Feed was offered by hand to apparent visual satiety in two meals per day (0900 and 1400 h), and feed consumption was recorded daily. Every 3 weeks, fish were counted and group-weighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS 222; 100 μg/ml). Blood and tissue sampling was done at the end of the growth trial from randomly selected fish killed by a blow to the head. Five h after the morning meal (12 animals per diet; 4 animals per tank), blood samples were taken from caudal vessels with heparinised syringes. Following overnight fasting (20 h after the second daily meal), 12 additional fish per dietary treatment were taken for sampling of blood, liver and white skeletal muscle. Plasma was drawn after centrifugation at 3000 ×*g* for 20 min at 4 °C, and stored at −30 °C until further hormone analyses. Liver and white muscle were rapidly excised, frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

3.2.3. Chemical composition analyses

Proximate analysis of diets was made by the following procedures: dry matter by drying at 105 °C for 24 h, ash by combustion at 550 °C for 12 h, protein (N×6.25) by the Kjeldahl method, fat after dichloromethane extraction by the Soxhlet method and gross energy in an adiabatic bomb calorimeter (IKA). Specimens for whole body analyses (a pooled sample of 10 fish at the beginning and pools of 5 fish per tank at the end of trial) were ground, and small aliquots were dried to estimate moisture content. The remaining samples were freeze-dried and chemical analyses were performed as indicated for experimental diets.

Table 2. Amino acid, mineral and trace element composition of the diets along with data on amino acid needs (Kaushik 1998).

Amino acid (%)	FO/VO diets	Needs
Arg	2.18	1.86
His	0.93	0.86
Ile	1.92	1.11
Leu	5.58	1.73
Lys	2.42	2.23
Met	1.05	
Cys	0.61	
Cys + Met	1.66	1.24
Phe	2.47	
Tyr	2.00	
Phe + Tyr	4.47	2.23
Thr	1.69	0.99
Trp	0.39	0.25
Val	2.15	1.49
Ser	2.23	
Ala	3.36	
Asp	3.69	
Glu	8.37	
Gly	2.01	
Pro	3.02	
Minerals (%, µg/g)		
Phosphorous (%)	1.08	
Magnesium (%)	0.18	
Potassium (%)	0.74	
Iron (µg/g)	216	
Copper (µg/g)	15	
Manganese (µg/g)	19	
Zinc (µg/g)	50	
Selenium (µg/g)	0.9	

Table 3. Fatty acid composition of experimental diets (% total FAME).

Fatty acid	FO	33VO	66VO	VO
14:0	5.02	3.70	1.89	0.59
15:0	0.35	0.22	0.13	0.12
16:0	16.7	16.9	16.9	16.7
16:1n-7	4.63	2.97	1.96	0.76
16:1n-9	0.22	0.15	tr	tr
16:3	0.49	0.35	0.26	0.14
16:3n-3	0.19	0.13	0.08	tr
16:4	0.40	0.29	0.17	tr
17:0	0.41	0.29	0.23	0.10
18:0	2.55	2.92	3.43	3.73
18:1n-9	12.5	17.5	21.9	25.9
18:1n-7	1.92	1.69	1.49	1.21
18:2n-6	12.1	15.7	19.2	21.3
18:3n-3	1.58	8.94	16.3	23.2
18:4n-3	2.16	1.47	0.82	0.20
20:0	0.30	0.30	0.31	0.29
20:1n-9	7.24	5.12	3.05	1.06
20:1n-7	0.21	0.16	0.09	tr
20:2n-6	0.17	0.12	0.11	tr
20:3n-3	0.08	0.07	tr	tr
20:4n-6	0.31	0.22	0.13	tr
20:4n-3	0.43	0.28	0.15	tr
20:5n-3 (EPA)	6.86	4.68	2.75	0.94
22:0	tr	0.16	0.16	0.17
22:1n-11	10.19	6.74	3.68	0.74
22:1n-9	0.56	0.43	0.29	0.16
22:2n-6	0.24	0.17	tr	tr
22:5n-3	0.64	0.40	0.18	tr
22:6n-3 (DHA)	8.34	5.68	3.38	1.06
Total	96.9	97.7	98.9	98.4
Saturates	25.3	24.5	22.9	21.7
Monoenes	37.6	34.8	32.4	29.8
n-6 HUFA[a]	0.31	0.22	0.12	0.7
n-3 HUFA[a]	16.3	11.9	6.5	2

Values are means of two determinations; tr = trace value < 0.05

[a]Fatty acids with 20 or more carbon atoms and more than 3 double bonds.

3.2.4. GH and IGF-I radioimmunoassay

Plasma GH levels were assayed by a homologous gilthead sea bream radioimmunoassay (RIA), using recombinant GH as tracer and standard (Martínez-Barberá et al., 1995). Sensitivity and midrange of the assay were 0.1 ng/ml and 2.1 to 2.3 ng/ml, respectively.

After acid-ethanol precipitation, circulating levels of IGF-I were measured with a generic fish IGF-I RIA (Vega-Rubín de Celis et al., 2004). The assay was based on the use of recombinant red sea bream IGF-I (GroPep, Adelaide, Australia) as tracer and standard, and antibarramundi (Asian sea bass) IGF-I serum (GroPep, Adelaide, Australia) (1:8000) as first antibody. A goat anti-rabbit IgG (1:20) (Biogenesis, Poole, UK) was used as precipitating antibody. The sensitivity and midrange of the assay were 0.05 and 0.7 to 0.8 ng/ml, respectively.

3.2.5. RNA extraction and RT procedure

Total RNA extraction was performed with the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, CA, USA). Briefly, liver and white skeletal muscle were homogenized at a ratio of 25 mg/ml with a guanidine-detergent lysis reagent. The reaction mixture was treated with protease K, and RNA purification was achieved by passing the tissue lysate (0.5 ml) through a purification tray containing an application-specific membrane. Wash solutions containing DNase were applied, and total RNA was eluted into a 96-well PCR plate. The RNA yield was 40–50 μg with absorbance measures ($A_{260/280}$) of 1.9 to 2.1.

Reverse transcription (RT) with random decamers was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA were reverse transcribed in a final volume of 100 μl. RT reactions were incubated 10 min at 25 °C and 2 h at 37 °C. Control reactions were run without reverse transcriptase and were used as negative real-time PCR controls.

3.2.6. Real-time PCR

Real-time PCR was performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as previously described (Calduch-Giner et al., 2003). Diluted RT reactions were used for PCR reactions in 25 µl volume. Each PCR-well contained SYBR Green Master Mix (Bio-Rad) with specific primers for target and reference genes at a final concentration of 0.9 µM (see **Table 4**).

The efficiency of PCR reactions for target and reference genes varied between 87 and 97%. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and fluorescence data acquired during the extension phase were normalized to β-actin, using the delta–delta method (Livak and Schmittgen, 2001).

Table 4. Primers for real-time PCR. Forward primer, f; reverse primer, r.

Gene	Accession		Primer sequence	Position
β-actin	X89920	f	5'-TCCTGCGGAATCCATGAGA	811-829
		r	5'-GACGTCGCACTTCATGATGCT	861-841
GHR-I	AF438176	f	5'-ACCTGTCAGCCACCACATGA	1275-1294
		r	5'-TCGTGCAGATCTGGGTCGTA	1373-1354
GHR-II	AY573601	f	5'-GAGTGAACCCGGCCTGACAG	1690-1709
		r	5'-GCGGTGGTATCTGATTCATGGT	1764-1743
IGF-I	AY996779	f	5'-TGTCTAGCGCTCTTTCCTTTCA	112-133
		r	5'-AGAGGGTGTGGCTACAGGAGATAC	195-172
IGF-II	AY996778	f	5'-TGGGATCGTAGAGGAGTGTTGT	406-427
		r	5'-CTGTAGAGAGGTGGCCGACA	514-495

3.2.7. Statistics

Tank average values of growth, feed intake and nutrient retention were used as experimental units in one way analysis of variance followed by Student–Newman–Keuls test at a significance level of $P<0.05$. Plasma levels of GH and IGF-I were analysed by one and two-way analysis of variance, followed by Student–Newman–Keuls test. Correlation analyses between hepatic transcripts and plasma hormone levels were made by Pearson Product Moment correlations ($P<0.05$).

3.3. Results

Diets 33VO and 66VO were well accepted by fish, and animals grew rapidly from 16 to 91–92 g over the course of the 11-week growth study (**Table 5**). No differences in feed intake (69 to 67.5 g/fish), daily growth indices (2.66 to 2.68%), and feed (1.09 to 1.11) or protein (2.21 to 2.25) efficiencies were found among control fish (FO) and fish fed 33VO and 66VO diets. Total replacement of fish oil by the vegetable oil blend (diet VO) reduced feed intake (61 g/fish) and daily growth indices (2.43%) without any significant effect on whole body composition. Nitrogen (35 to 37%) and energy (50 to 52%) retentions were not altered by dietary treatments, remaining high in all experimental groups. Lipid deposition in mesenteric and liver depots was not affected significantly by dietary treatments, although there was a trend for liver fat to increase with fish oil replacement.

Table 5. Data on growth performance, whole body composition, and nutrient gain and retention of gilthead sea bream fed the four experimental diets for 11 weeks.

	FO	33VO	66VO	VO	*P* [1]
IBW (g)[2]	16.1±0.09	16.3±0.01	16.3±0.03	16.1±0.09	*0.31*
FBW (g)[3]	91.7±0.45[b]	91.3±0.90[b]	91.1±1.20[b]	80.9±0.28[a]	*<0.001*
Viscera (g)	8.28±0.45	8.50±0.33	8.37±0.31	8.35±0.46	*0.77*
Mesenteric fat (g)	1.72±0.24	1.66±0.11	1.79±0.15	1.52±0.14	*0.43*
Liver (g)	1.78±0.12	1.82±0.09	1.92±0.08	1.72±0.13	*0.68*
VSI (%) [4]	9.36±0.30	9.12±0.23	9.10±0.24	9.89±0.55	*0.28*
MFI (%) [5]	1.78±0.23	1.73±0.39	1.95±0.17	1.78±0.15	*0.45*
HSI (%) [6]	1.85±0.07	1.93±0.11	2.09±0.09	2.02±0.16	*0.38*
Liver fat (%)	15.9±0.83	17.7±0.94	18.7±1.05	19.3±0.51	*0.06*
DM intake (g/fish)	68.8±0.60[b]	68.9±0.66[b]	67.6±0.25[b]	61.3±0.77[a]	*<0.001*
Weight gain (%)	467.6±6.2[b]	460.3±5.2[b]	460.1±7.1[b]	401.9±3.1[a]	*<0.001*
DGI (%) [7]	2.68±0.03[b]	2.66±0.03[b]	2.66±0.05[b]	2.43±0.02[a]	*<0.001*
FE [8]	1.10±0.01	1.09±0.01	1.11±0.02	1.06±0.01	*0.07*
PER [9]	2.21±0.01	2.23±0.01	2.25±0.04	2.14±0.02	*0.06*
Whole body composition (% wet matter)					
Moisture	64.3±0.29	64.1±0.48	64.1±0.28	63.9±0.32	*0.70*
Crude protein	15.9±0.46	16.0±0.52	16.7±0.43	16.9±0.12	*0.10*
Crude fat	14.1±0.67	14.3±0.34	14.4±0.42	14.4±0.10	*0.95*
Ash	2.88±0.11	3.44±0.27	3.38±0.14	3.61±0.16	*0.09*
Retention (% intake)					
Nitrogen	35.4±1.27	35.1±1.12	36.9±1.92	37.3±0.63	*0.56*
Energy	50.1±2.01	50.3±1.06	52.5±0.55	51.9±0.76	*0.58*

Initial body composition: water, 70.9%; protein, 15.1%; lipid, 9.3%; ash, 3.4%. [1]*P* values result from analysis of variance. Different superscript letters in each row indicate significant differences among dietary treatments (Student Newman-Keuls test, P<0.05). [2]Initial body weight. [3]Final body weight. [4]Vicerosomatix index = (100 × viscera wt) / fish wt. [5]Mesenteric fat index = (100 × mesenteric fat wt) / fish wt. [6]Hepatosomatic index = (100 × liver wt) / fish wt. [7]Daily growth index = [100 × (final fish $wt^{1/3}$ – initial fish $wt^{1/3}$)] / days. [8]Feed efficiency = wet weight gain / dry feed intake. [9]Protein efficiency ratio = wet weight gain / protein intake

At the end of the growth study, plasma levels of IGF-I were decreased over the course of the post-pandrial period (P<0.05) (**Fig. 1**). The highest IGF-I concentration was found in fish fed the 33VO diet and the lowest in fish fed the VO diet irrespective of sampling time (5 to 20 h post-feeding). Intermediate values were found in control fish and fish fed the 66VO diet.

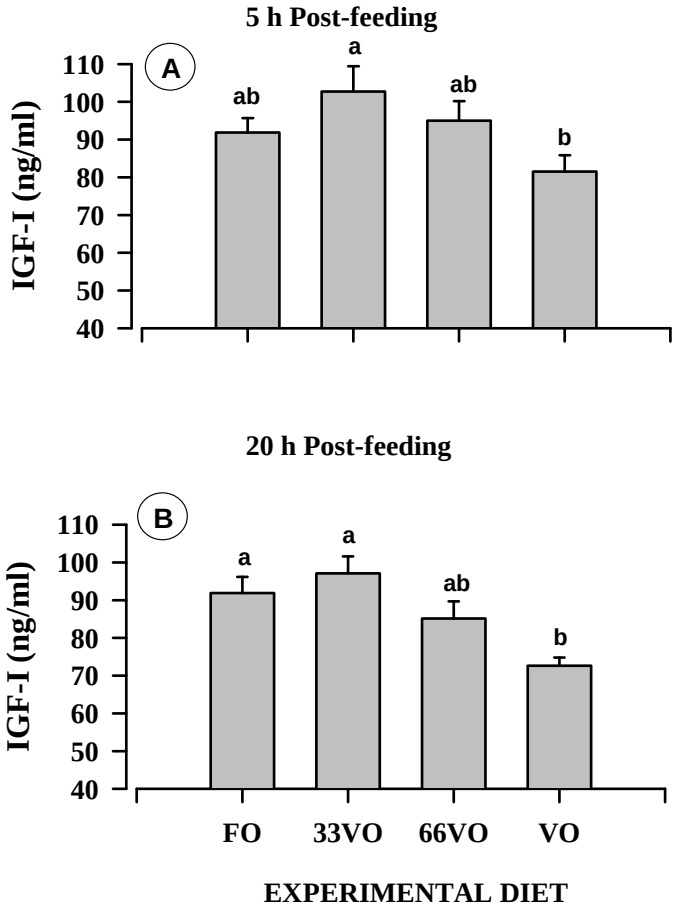

Fig. 1. Plasma levels of insulin-like growth factor-I (IGF-I) in fish fed experimental diets 5 h after the meal (A) and following overnight fasting (B). Each value is the mean ± SEM of 10 to 12 animals. Values with different letters are significantly different (P<0.05).

There was no significant effect of dietary treatment on plasma GH levels (**Fig. 2**). However, the trend was opposite to that of plasma IGF-I levels. First, the overall plasma GH concentration increased over the course of post-pandrial period (P<0.05). Secondly, the lowest GH concentration was found in fish fed the 33VO diet whereas increased values were observed in fish fed the VO diet.

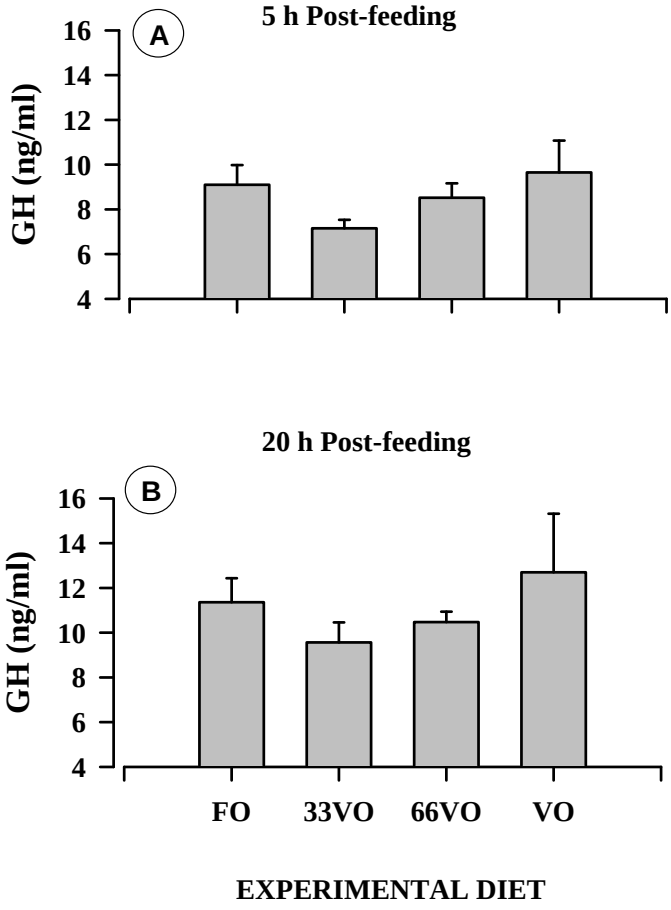

Fig. 2. Plasma growth hormone (GH) levels in fish fed experimental diets 5 h after the meal (A) and following overnight fasting (B). Each value is the mean ± SEM of 10 to 12 animals.

Hepatic IGF-I mRNA and plasma levels of IGF-I (20 h post-feeding) were positively correlated (P<0.05). The highest amount of IGF-I mRNA was found in fish fed the 33VO diet with a progressive and significant decrease with additional fish oil replacement, whereas control fish remained at intermediate values (**Fig. 3A**). IGF-II was expressed at a reduced level and no significant changes were found with dietary treatments, although the trend for IGF-II mRNA was similar to that reported for IGF-I mRNA (**Fig. 3B**).

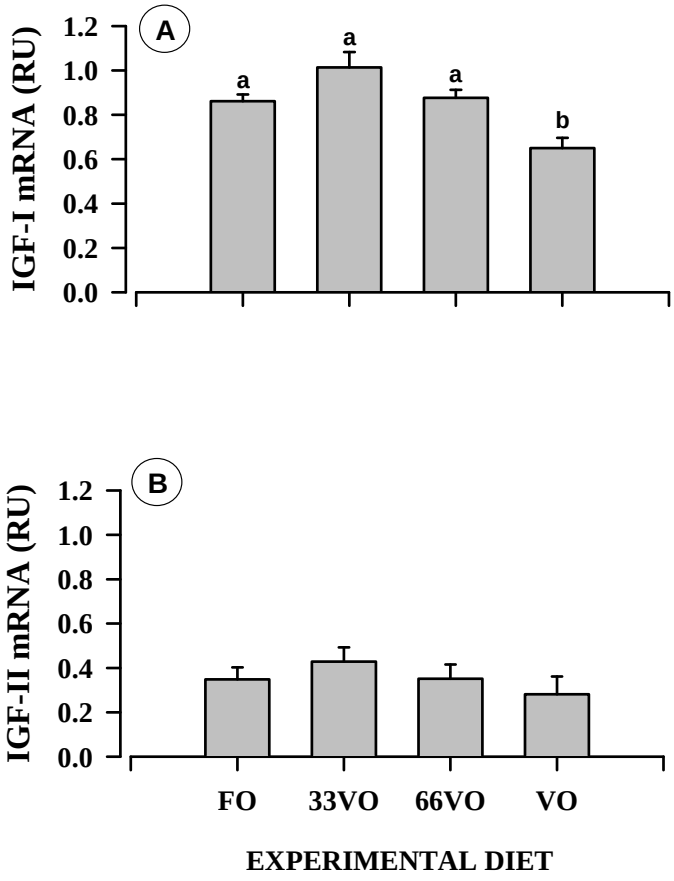

Fig. 3. Normalized mRNA levels of IGF-I (A) and IGF-II (B) in the liver of fish fed experimental diets (20 h post-feeding). Each value is the mean ± SEM of 6 to 8 animals. Values with different letters are significantly different (P<0.05).

Hepatic levels of GHR-I mRNA correlated positively with hepatic transcripts of IGF-I and plasma levels of IGF-I (**Fig. 4A**). Thus, GHR-I mRNA decreased progressively and significantly with the graded replacement of fish oil in fish fed 33VO, 66VO and VO diets. Intermediate values were found in fish fed diet FO. The overall expression of GHR-II was of the same order of magnitude, but no significant changes in GHR transcripts were detected with dietary treatments (**Fig. 4B**).

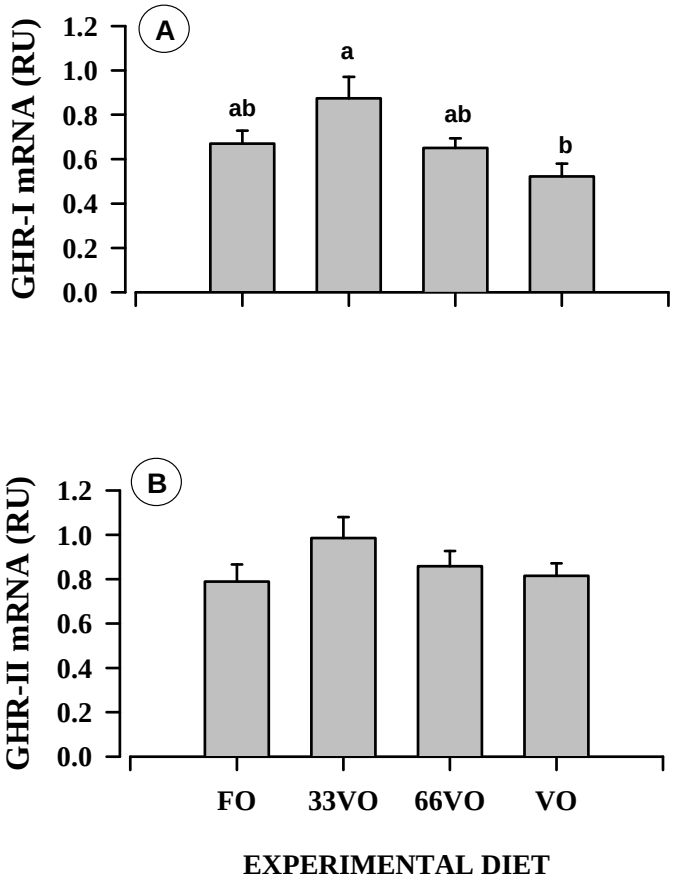

Fig. 4. Normalized mRNA levels of GHR-I (A) and GHR-II (B) in the liver of fish fed experimental diets (20 h post-feeding). Each value is the mean ± SEM of 6 to 8 animals. Values with different letters are significantly different ($P<0.05$).

Muscle expression of IGF-I was lower in comparison to that of IGF-II, and no significant effect of dietary treatments on IGF-I mRNA levels were detected (**Fig. 5A**). By contrast, IGF-II mRNA was down-regulated in fish fed vegetable oils irrespective of the degree of replacement (**Fig. 5B**).

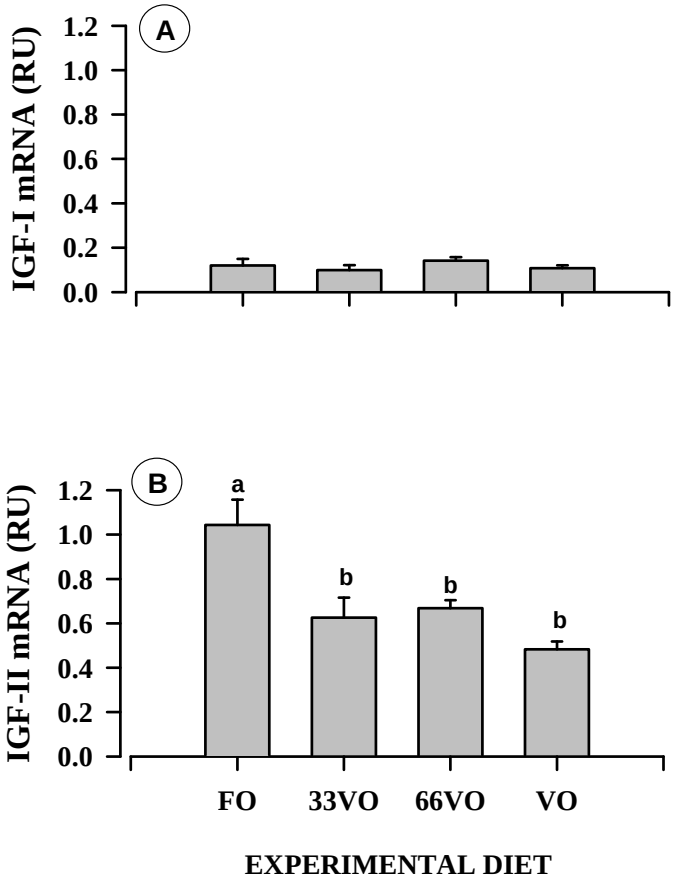

Fig. 5. Normalized mRNA levels of IGF-I (A) and IGF-II (B) in the skeletal muscle of fish fed experimental diets (20 h post-feeding). Each value is the mean ± SEM of 6 to 8 animals. Values with different letters are significantly different (P<0.05).

The overall muscle expression of GHR-I and II was of the same order of magnitude. There was no consistent change on GHR-I mRNAs with dietary treatment (**Fig. 6A**). By contrast, transcripts of GHR-II were progressively up-regulated in fish fed 33VO and 66VO diets, decreasing thereafter with the 100% of replacement of fish oil (VO diet) (**Fig. 6B**).

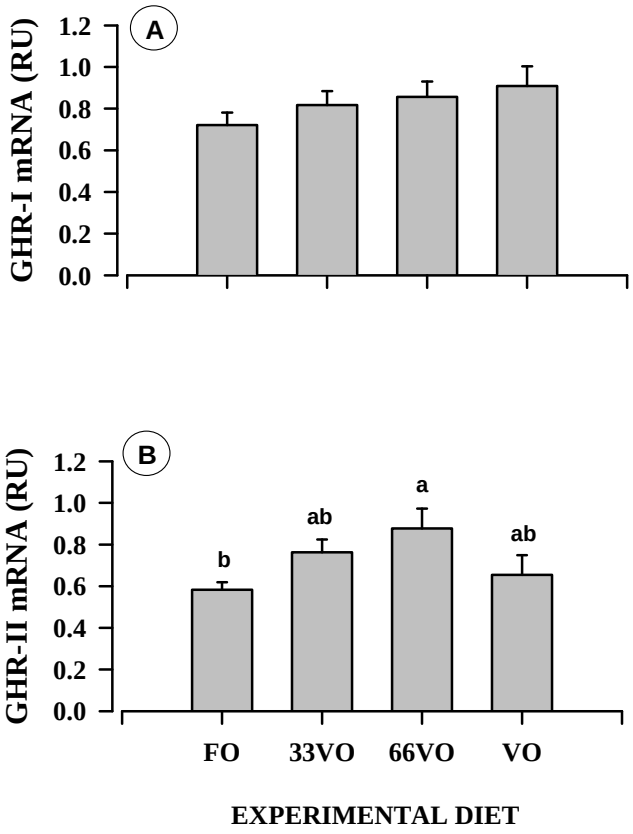

Fig. 6. Normalized mRNA levels of GHR-I (A) and GHR-II (B) in the skeletal muscle of fish fed experimental diets (20 h post-feeding). Each value is the mean ± SEM of 6 to 8 animals. Values with different letters are significantly different (P<0.05).

3.4. Discussion

The overall growth indices attained in the current work by juvenile gilthead sea bream are higher than those reported for fish of the same age under similar light and temperature conditions (Gómez-Requeni et al., 2003, 2004). This excellent growth performance in all experimental groups could be attributed to improved diet formulation, fish management and culture conditions. However, fish fed the VO diet showed a reduced feed intake and increased liver fat deposition, which is characteristic of a wide range of dietary and hormonal imbalances (see McClain et al., 2004; Avramoglu et al., 2006). Indeed, in juvenile gilthead sea bream fed diets with amino acid imbalances, peripheral lipolysis and tissue expression of lipoprotein lipase are regulated in concert to increase the flux of dietary fatty acids through the liver (Albalat et al., 2005; Saera-Vila et al., 2005a). This can be of special relevance during fasting and over-wintering, and extensive work is now underway for this risk assessment.

Quantitative requirements of essential fatty acids (EFA) appear to vary depending on fish species and growth stage (Sargent et al., 2002). Thus, the biological demand for n-3 HUFA was at least 1.3% for flatfish larvae (Le Milinaire et al., 1983), whereas requirements for juvenile and grower fish were reduced to 0.8% (Gatesoupe et al., 1977; Lee et al., 2003; Kim and Lee, 2004) and 0.6% (Léger et al., 1979), respectively. Similar requirements have been reported for juveniles of European sea bass (Skalli and Robin, 2004) and gilthead sea bream (Kalogeropoulos et al., 1992) fed defatted fish meal and casein-based diets, respectively. Likewise, no detrimental growth effects were found in the present study in fish fed the 66VO diet (0.9% EPA+DHA, see **Table 3**), which indicates that fish oil replacement by alternative vegetable oils is feasible at a high level when EFA requirements are covered. Partial fish oil replacement has been conducted successfully in a wide variety of fish species, but this is the first report that maximizes the simultaneous replacement of fish meal and fish oil in practical aquafeeds for fast growing juvenile marine fish.

Fish growth rates vary with season, age and nutritional status and most of these regulatory events are mediated by the GH/IGF axis (Company et al., 2001; Pérez-Sánchez et al., 2002). The wide tissue distribution of GHRs supports the pleiotropic action of GH, although the liver is the most important target tissue of GH and the primary source of systemic IGF-I (endocrine form). In this scenario, changes on the plasma binding capacity of the 33–47 kDa IGF-binding protein represents in rainbow trout an effective mechanism to limit biologically active IGFs (free IGF fraction), keeping growth and GH secretion under control (Gómez-Requeni et al., 2005). Likewise, circulating levels of IGF-I are positively correlated with growth rates and dietary protein levels in Atlantic salmon and Asian sea bass (Dyer et al., 2004). Plasma IGF-I levels are also a good indicator of growth in channel catfish (Silverstein et al., 2000; Li et al., 2006). Similarly, in gilthead sea bream, circulating GH and IGF-I are good markers of nutritional disorders arising from changes in ration size (Pérez-Sánchez et al., 1995, 2002), dietary energy/ratio (Martí-Palanca et al., 1996; Company et al., 1999) and dietary protein source (Gómez-Requeni et al., 2003, 2004). In the current work, the decreased growth of fish fed the VO diet were accordingly paralleled by decreased plasma levels of IGF-I. Since IGF-I mRNA and GHR-I mRNA were also reduced, the reduction in growth could be attributed to a transcriptional defect in the signal transduction of GHR in spite of increased plasma levels of GH. This metabolic feature leads to liver GH resistance as is now widely accepted in several fish species (Pérez-Sánchez et al., 1995; Beckman et al., 2004; Pierce et al., 2005; Wilkinson et al., 2006).

Growth in fish fed the VO diet (0.3% EPA+DHA; see **Table 3**) was only 90% of the maximum observed, and there was no mortality in this group over the course of the study. Similar results were reported for juvenile European sea bass fed defatted fish meal diets (Skalli and Robin, 2004), which suggests that marine fish are relatively tolerant to dietary fish oil restriction despite of the recognized essentiality of n-3 HUFA. As stated very early by Watanabe (1982), the triacyglycerol and polar lipid fractions of lipids, both containing adequate amounts of EPA and DHA, have the same EFA value. Takeuchi and Watanabe (1979) have shown that a level of EFA exceeding four times the

requirement of rainbow trout leads to poor growth and feed utilisation. Detrimental growth effects have also been reported in juvenile flounder when dietary n-3 HUFA becomes excessive (Kim and Lee, 2004), and 25% replacement of fish oil by palm oil fatty acid distillate improved weight gain of African catfish (Ng et al., 2004). In the present study, growth performance of fish fed FO, 33VO and 66VO diets was almost identical, but the balance between endocrine and locally produced IGFs differed depending on dietary treatment. Thus, in fish fed the FO diet, the reduced gene expression and protein production of hepatic IGF-I was apparently compensated by the increased expression of IGF-II at the local tissue level.

In mammals, IGF-II mRNA is detected in many fetal tissues but decreases quickly during post-natal development (Daughaday and Rotwein, 1989). Accordingly, IGF-II null mice are small at birth but continue to growth post-natally at a rate similar to wild-type. By contrast, IGF-I null mice born were small and most died in the early neonatal stages. All this strongly supports the key role of IGF-I during prenatal and post-natal growth. However, hepatic IGF-I is not crucial for postnatal growth in mammals, and liver-specific IGF-I knockout mice show normal growth due to the compensatory action of autocrine/paracrine IGF-I (see Le Roith et al., 2001a,b). As postulated above in the present study, compensatory increases of systemic IGF-I also occur in fish but, in this case, most of these effects are dependent on local IGF-II. Indeed, substantial amounts of IGF-II are expressed later in life in a wide range of fish species, including common carp (Vong et al., 2003), rainbow trout (Chauvigné et al., 2003), Nile tilapia (Caelers et al., 2004), channel catfish (Peterson et al., 2004), and gilthead sea bream as already evidenced in previous studies (Duguay et al., 1996; Radaelli et al., 2003) and confirmed here. Furthermore, as found for IGF-I, the main site for fish IGF-II expression is the liver, but in contrast to IGF-I, other organs such as skeletal muscle also express quite high levels of IGF-II mRNA. Thus, fast growing families of channel catfish express hepatic and muscle IGF-II at a high rate (Peterson et al., 2004), and the growth spurt of juvenile rainbow trout during refeeding could be mediated by muscle IGF-II (Chauvigné et al., 2003). Accordingly, it is reasonable to assume that IGF-II acts in fish as

an important growth-promoting factor through all the life cycle, although most of these regulatory capabilities might have been lost during the evolution of higher vertebrates.

To our knowledge, the precise mechanism(s) regulating the relative contribution of systemic and local IGFs on fish growth remains unexplored. However, we suspect that some results of the current study could be mediated by factors other than dietary fatty acids. One of these factors might be the reduction in unwanted feedborne lipid soluble contaminants with the reduction in fish oil level. This assumption is based on our complementary data (unpublished results) showing that dioxin-like PCBs in the FO diet were markedly reduced with the graded fish oil replacement, as shown in previous studies (Berntssen et al., 2005; Bethune et al., 2006). Experimental evidence also indicates that the wasting syndrome caused in mice by 3-methylcholanthrene is mediated by aromatic hydrocarbon receptors (AHRs) that interact with xenobiotic responsive elements (XREs) in the GHR promoter, disrupting the liver GH signalling pathway (IGF production) (Nukaya et al., 2004). Likewise, several XREs have been identified in the 5′-flanking region of gilthead sea bream GHR-I (unpublished results), although further studies are needed to determine whether these cisregulatory elements are functional in fish. Besides, it has been proven that expression levels of GH and prolactin (PRL) are regulated in rainbow trout by persistent xenoestrogens and antiestrogenic pollutants (Elango et al., 2006), and both estradiol and 4-nonylphenol suppress growth and plasma levels of IGF-I in juvenile Atlantic salmon (Arsenault et al., 2004). It is not surprising, therefore, that gonadal steroids modulate hepatic production of IGF-I and IGFBPs in tilapia (Riley et al., 2004) and coho salmon (Larsen et al., 2004). All this provides regulatory mechanisms for dimorphic growth patterns in fish, but at the same time makes the GH/IGF axis more vulnerable to potential anthropogenic feed-borne contaminants.

Transgenic models in mice also indicate that major effects of GH on growth are dependent on IGF-I expression, which requires intact insulin and IGF-I receptor signalling in skeletal muscle (Kim et al., 2005). However, GH regulates other mitogenic factors, and there is now experimental evidence

supporting the up-regulated expression of GHRs during muscle repair and maintenance (Casse et al., 2003). In fish, it is believed that genetic duplication and divergence of two GHR subtypes (GHR types I and II) would take place on an early ancestor of fish lineage (Saera-Vila et al., 2005b; Jiao et al., 2006). GHR-I (also named somatolactin receptor by Fukada et al., 2005) was first described in non-salmonid fish (Calduch-Giner et al., 2001), conserving most of the structural features of mammalian GHRs. By contrast, GHR-II is unique to teleosts and encompasses most of the published GHR sequences of salmonid fish. These two GHR subtypes are conserved in a wide range of fish species, although apparent silencing and/or genomic loss of GHR-II was reported in the flatfish lineage with the occurrence of truncated variants of GHR-I (Pérez-Sánchez et al., 2002; Saera-Vila et al., 2005b). In this scenario, the current study confirms and extends the notion that major GH effects on growth and hepatic IGF expression are mediated by GHR-I in gilthead sea bream. By contrast, GHR-II emerges as a more constitutive gene that does not necessarily require intact IGF-pathways to exert a protective and/or growth promoting action. This is consistent with the up-regulated expression of GHR-II in skeletal muscle of fish fed the 66VO diet. In this way, we previously reported that GHR-II is up-regulated in the skeletal muscle of fasted juvenile gilthead sea bream (Saera-Vila et al., 2005). Similarly, Fukada et al. (2004) indicated that transcript levels of GHR-II are not related in masu salmon to decreased expression of hepatic IGF-I during fasting.

In summary, our data strongly support that combined replacement at a high level of fish meal and oil is possible in diets of gilthead sea bream, contributing to the development of sustainable aquafeeds. Data also bring new insights on the compensatory regulation of systemic and local components of the GH/IGF axis (see **Fig. 7** for a comprehensive survey) in growing fish. Additional studies are underway to further explore the potential of practical diets low in marine ingredients over the full cycle of gilthead sea bream farming, addressing also issues related to potential feed-borne endocrine disruption of GH/IGF axis.

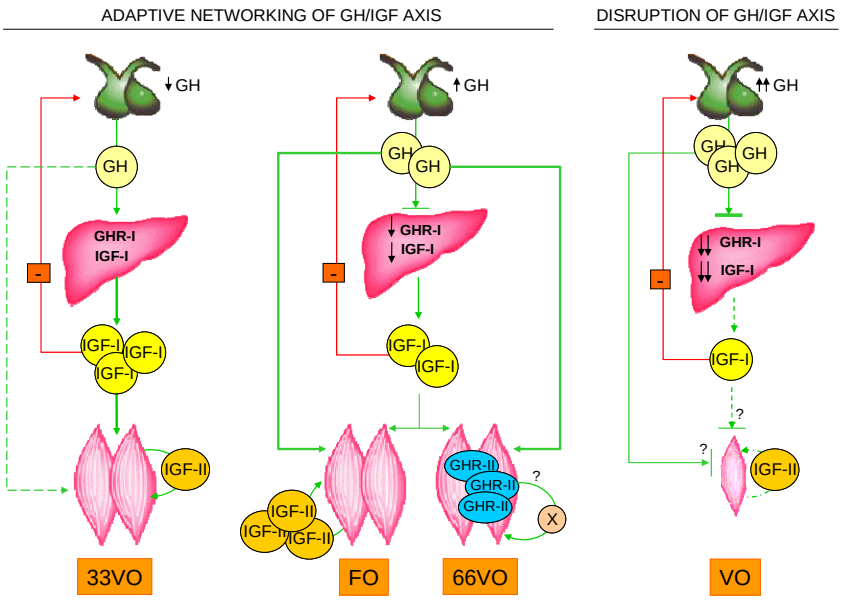

Fig. 7. Proposed model for the balanced regulation of systemic and local components of GH/IGF axis. Growth dysfunction occurs when the reduced production of systemic IGF-I is not compensated at the local tissue level (fish fed VO diet). Compensatory IGF-II production occurs at the local tissue level in fish fed FO diet. Alternatively, other compensatory mechanisms of GH/IGF axis could be mediated at the local tissue level by GHR-II via unknown factors (66VO diet).

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CAPÍTULO 4

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

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ABSTRACT

The feasibility of fish oil (FO) replacement by vegetable oils (VO) was investigated in gilthead sea bream (*Sparus aurata* L.) in a growth trial conducted for the duration of 8 months. Four isolipidic and isoproteic diets rich in plant proteins were supplemented with L-lysine (0·55%) and soya lecithin (1%). Added oil was either FO (control) or a blend of VO, replacing 33% (33VO diet), 66% (66VO diet) and 100% (VO diet) of FO. No detrimental effects on growth performance were found with the partial FO replacement, but feed intake and growth rates were reduced by about 10% in fish fed the VO diet. The replacement strategy did not damage the intestinal epithelium, and massive accumulation of lipid droplets was not found within enterocytes. All fish showed fatty livers, but signs of lipoid liver disease were only found in fish fed the VO diet. Muscle fatty acid profiles of total lipids reflected the diet composition with a selective incorporation of unsaturated fatty acids in polar lipids. The robustness of the phospholipid fatty acid profile when essential fatty acid requirements were theoretically covered by the diet was evidenced by multivariate principal components analysis in fish fed control, 33VO and 66VO diets.

Keywords: Essential fatty acids; Phospholipids; Soya lecithin; Lipoid liver disease

Abbreviations: EFA, essential fatty acid; FO, fish oil; HUFA, highly unsaturated fatty acid; MPCA, multivariate principal components analysis; PL, phospholipid; TL, total lipids; VO diet, diet in which vegetable oil replaces 100% of fish oil; 33VO diet, diet in which vegetable oil replaces 33% of fish oil; 66VO diet, diet in which vegetable oil replaces 66% of fish oil.

4.1. Introduction

Marine fish farming is mostly based on diets containing high levels of n-3 highly unsaturated fatty acids (HUFA), particularly EPA (20:5n-3) and DHA (22:6n-3). However, the continuous expansion of aquaculture and the decreasing global availability of marine oil and fishmeal force the industry to explore alternative and sustainable lipid sources[1,2]. In salmonids, the use of vegetable oils to replace the majority of dietary fish oil (FO) is now feasible in practical aquafeed without loss of growth performance[3–5]. Nevertheless, essential fatty acid (EFA) requirements differ between species. Thus, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) can satisfy the EFA requirements of freshwater fish, whereas marine fish require longer-chain n-3 and n-6 PUFA for optimal growth and health[6]. Supporting this, fatty acid desaturation and elongation of linoleic acid and α-linolenic acid are well established in freshwater and anadromous fish species[7], but marine fish including European sea bass[8] and gilthead sea bream (*Sparus aurata* L.)[9,10] do not show rates for bioconversion of C18 PUFA into C20 and C22 HUFA that would allow n-3 HUFA requirements to be met.

Signs of EFA deficiencies in fish include skin lesions and several neurological alterations linked to reduced growth and survival rates during larval and juvenile on-growing phases[11]. Lipoid liver disease and intense accumulation of intestinal lipid droplets are also documented as metabolic disorders arising from defective supplies of phospholipids (PL)[12–14] and n-3 HUFA[15]. Additionally, fatty acids modulate immune responses and eicosanoids produced from arachidonic acid (20:4n-6) are recognised as inflammatory agents, whereas DHA, and especially EPA-derived eicosanoids, exert anti-inflammatory effects in a wide variety of experimental models[16,17]. However, factors other than dietary ones may influence lipid metabolism, and relative rates of fat deposition and mobilisation vary greatly as a result of environmental factors including parr-smolt transformation in salmonids[18,19]. Likewise, gonadal maturation and spawning have a significant impact in the muscle fatty acid profile of gilthead sea bream females[20]. Deposition rates and

fatty acid profiles also vary seasonally in wild gilthead sea bream[21], but the feeding regimen is a major influence and most of these changes can be overridden by full rations given under intensive aquaculture. Indeed, monitoring studies in various Greek fish farms failed to show a seasonal impact in the muscle fat deposition and profiling of gilthead sea bream[22].

Gilthead sea bream is a major cultured finfish in the Mediterranean area, and extensive research to sustain further growth has proved that vegetable oils can replace up to 60% of the added FO, in fishmeal-based diets, without adverse effects on growth, feed efficiency and survival rates[8,23,24]. Additional studies have addressed the extensive replacement of fishmeal by plant proteins[25,26], and recently growth-compensatory mechanisms of the somatotropic axis have been evidenced in short-term trials when juvenile fish were fed during the summer growth spurt with plant protein-based diets and graded levels of vegetable oils[27]. Indeed, with the total replacement of dietary FO some growth reduction occurred, and it was accompanied by decreased production of hepatic insulin-like growth factor-I not compensated by the local expression (skeletal muscle) of insulin-like growth factor and/or growth hormone receptors. In humans and other animal models, there is also increasing evidence linking endocrine and metabolic dysfunctions resulting in obesity and insulin resistance with steatosic livers and altered fatty acid profiles of PL and stored TAG[28]. In this sense, three major goals were addressed in the present paper in a gilthead sea bream trial conducted over a growth trial of 8 months' duration: (a) the relationship between dietary and muscle fatty acid profiles; (b) the robustness of the PL fatty acid profile when EFA requirements are theoretically covered in the diet; (c) histological alterations of liver and intestine as sensitive target tissues of lipid-metabolism dysregulation.

4.2. Materials and methods

4.2.1. Diets

Four isoproteic, isolipidic and isoenergetic plant protein-based diets were made with a low inclusion level (20%) of fishmeal and fish soluble protein concentrates (**Tables 1 and 2**). All diets were supplemented with L-lysine (0·55%) and contained soya lecithin (1%). Added oil was either Scandinavian FO (control diet) or a blend of vegetable oils, replacing 33% (33VO diet), 66% (66VO diet) and 100% (VO diet) of the FO. The blend of vegetable oils (rapeseed oil–linseed oil–palm oil, 2·5:8·8:3·6, by wt) provided a similar balance of saturates, monoenes and PUFA to that found in FO, but without HUFA[29,30]. All diets were manufactured using a twin-screw extruder (Clextral, BC 45) at the INRA experimental research station of Donzacq (Landes, France), dried under hot air, sealed and kept in air-tight bags until use.

4.2.2. Growth trial and tissue sampling

Juvenile gilthead sea bream of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimatised to laboratory conditions at the Institute of Aquaculture Torre de la Sal (IATS) for 20 d before the start of the growth study. Fish of 16 g initial mean body weight were distributed into twelve fibreglass tanks (500 litres) in groups of sixty fish per tank. Water flow was 20 litres/min, and O_2 content of outlet water remained higher than 85% saturation. The growth study was undertaken over 8 months (23 May to 18 January), and daylength and water temperature (11–27ºC) varied over the course of the trial following natural changes at IATS latitude (40º5'N; 0º10'E).

Each diet was randomly allocated to triplicate groups of fish, and feed was offered by hand to apparent visual satiety twice per day (09.00 and 14.00 hours) from May to September, and once per day (12.00 hours) from October to January. No mortality was registered, and feed intake was recorded daily. At regular intervals, fish were counted and groupweighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS 222;

100 µg/ml). At critical step windows over the growth trial (midsummer, 5 August; early autumn, 27 September; early winter, 18 January), randomly selected fish (four fish per tank; twelve fish per treatment) were killed by a blow on the head before tissue sampling. Portions of dorsal muscle (white muscle) were extracted and rapidly excised, frozen in liquid N_2, and stored at -80ºC until fatty acid analyses of lipid extracts. Liver and intestine samples for fat content determinations and histological samples were taken only in September (20 h after the last feeding) when fish still show an active feeding behaviour. All procedures were carried out according to national and institutional regulations (Consejo Superior de Investigaciones Científicas, Institute of Aquaculture Torre de la Sal Review Board) and the current European Union legislation on handling experimental animals.

4.2.3. Histology and tissue lipid content determinations

Tissue fragments of liver and hindgut were fixed in 10% buffered formalin, embedded in Technovit-7100 resin (Kulzer, Heraeus, Germany), and stained with toluidine blue or haematoxylin-eosin after thin sectioning (1–3mm). Liver and muscle lipids were extracted according to Folch et al.[31], and determined gravimetrically after the evaporation of the organic solvent under a stream of N_2 and overnight desiccation.

Table 1. Ingredients and chemical composition of experimental diets.

	CTRL	33VO	66VO	VO
Ingredient (%)				
Fish meal (CP 70%)[1]	15·00	15·00	15·00	15·00
CPSP 90 [2]	5·00	5·00	5·00	5·00
Corn gluten meal (CP 63%)	40·00	40·00	40·00	40·00
Soybean meal (CP 46%)	14·30	14·30	14·30	14·30
Extruded wheat (CP 15%)	4·00	4·00	4·00	4·00
Fish oil [3]	15·15	10·15	5·15	0·00
Rapeseed oil	0·00	0·85	1·70	2·58
Linseed oil	0·00	2·90	5·80	8·79
Palm oil	0·00	1·25	2·50	3·79
Soya lecithin	1·00	1·00	1·00	1·00
Binder (sodium alginate)	1·00	1·00	1·00	1·00
Mineral premix [4]	1·00	1·00	1·00	1·00
Vitamin premix [5]	1·00	1·00	1·00	1·00
$CaHPO_4.2H_2O$ (18%P)	2·00	2·00	2·00	2·00
L-Lysine	0·55	0·55	0·55	0·55
Proximate composition				
Dry matter (DM, %)	93·43	94·10	94·79	95·38
Protein (% DM)	48·98	48·74	49·03	48·65
Fat (% DM)	22·19	22·26	22·11	22·31
Ash (% DM)	6·54	6·57	6·62	6·41
EPA + DHA (% DM)	2·31	1·61	0·90	0·30
Gross energy (kJ/g DM)	24·72	24·71	24·65	24·49

[1]Fish meal (Scandinavian LT)
[2]Fish soluble protein concentrate (Sopropêche, France)
[3]Fish oil (Sopropêche, France)
[4]Supplied the following (mg / kg diet, except as noted): calcium carbonate (40% Ca) 2·15 g, magnesium hydroxide (60% Mg) 1·24 g, potassium chloride 0·9 g, ferric citrate 0·2 g, potassium iodine 4 mg, sodium chloride 0·4 g, calcium hydrogen phosphate 50 g, copper sulphate 0·3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0·3.
[5]Supplied the following (mg / kg diet): retinyl acetate 2·58, DL-cholecalciferol 0·037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2·5, thiamin 7·5, riboflavin 15, pyridoxine 7·5, nicotinic acid 87·5, folic acid 2·5, calcium pantothenate 2·5, vitamin B_{12} 0·025, ascorbic acid 250, inositol 500, biotin 1·25 and choline chloride 500.

Table 2. Fatty acid composition of experimental diets (% FAME). (Mean values of two determinations)

FA %	CTRL	33VO	66VO	VO
14:0	5·02	3·70	1·89	0·59
15:0	0·35	0·22	0·13	0·12
16:0	16·70	16·90	16·9	16·7
16:1n-7	4·63	2·97	1·96	0·76
16:1n-9	0·22	0·15	tr	tr
16:2	0·49	0·35	0·26	0·14
16:3n-3	0·19	0·13	0·08	tr
16:4	0·40	0·29	0·17	tr
17:0	0·41	0·29	0·23	0·10
18:0	2·55	2·92	3·43	3·73
18:1n-9	12·50	17·50	21·90	25·90
18:1n-7	1·92	1·69	1·49	1·21
18:2n-6	12·10	15·70	19·20	21·30
18:3n-3	1·58	8·94	16·30	23·20
18:4n-3	2·16	1·47	0·82	0·20
20:0	0·30	0·30	0·31	0·29
20:1n-9	7·24	5·12	3·05	1·06
20:1n-7	0·21	0·16	0·09	tr
20:2n-6	0·17	0·12	0·11	tr
20:3n-3	0·08	0·07	tr	tr
20:4n-6	0·31	0·22	0·13	tr
20:4n-3	0·43	0·28	0·15	tr
20:5n-3	6·86	4·68	2·75	0·94
22:0	tr	0·16	0·16	0·17
22:1n-11	10·19	6·74	3·68	0·74
22:1n-9	0·56	0·43	0·29	0·16
22:5n-3	0·64	0·40	0·18	tr
22:6n-3	8·34	5·68	3·38	1·06
Total	96·55	97·58	98·04	98·37
Saturates	25·33	24·33	22·89	21·53
Monoenes	37·47	34·76	32·46	29·83
n-3 HUFA[1]	16·35	11·11	6·46	2·00
n-6 HUFA[2]	0·48	0·34	0·24	tr

[1] Calculated excluding 18 C atoms of the n -3 series.
[2] Calculated excluding 18 C atoms of the n -6 series.

4.2.4. Fatty acid analyses

Muscle total lipids (TL) for fatty acid analyses were extracted by the method of Folch et al.[31], using chloroform–methanol (2:1, v/v) containing 0·01% butylated hydroxytoluene as antioxidant. PL from muscle lipid extracts were isolated by TLC (Silica gel G 60, 20 x 20 cm glass plates; Merck, Darmstadt, Germany) using hexane–diethyl ether–acetic acid (85:15:1·5, by vol.) as a solvent system. PL bands at the bottom of plates were scraped and extracted with chloroform–methanol (2:1, v/v) containing 0·01% butylated hydroxytoluene.

After the addition of nonadecaenoic acid (Sigma, Poole, Dorset, UK) as internal standard, muscle PL and TL extracts were subjected to acid-catalysed transmethylation for 16 h at 50ºC using 1ml toluene and 2ml 1% (v/v) sulfuric acid in methanol[32]. Fatty acid methyl esters were extracted with hexane–diethyl ether (1:1, v/v), and those derived from TL were purified by TLC using hexane–diethyl ether–acetic acid (85:15:1·5, by vol.) as a solvent system. Fatty acid methyl esters were then analysed with a gas chromatograph (Fisons Instruments GC 8000 Series; Rodano, Italy) equipped with a fused silica 30m x 0·25mm open tubular column (Tracer, TR-WAX; film thickness: 0·25mm; Teknokroma, Spain) and a cold on-column injection system. The carrier gas used was He, and temperature programming was from 50 to 180ºC at 40ºC/min and then to 220ºC at 3ºC/min. Peaks were recorded in a personal computer using the Azur software package (version 4.0.2.0; Datalys, Saint-Martin d'Hères, France). Individual fatty acid methyl esters were identified by reference to well-characterised FO standards, and the relative amount of each fatty acid was expressed as a percentage of the total amount of fatty acids in the analysed sample.

4.2.5. Statistical analyses

Growth parameters (tank average values) and the relative amount of fatty acids were checked for normal distribution and homogeneity of variances, and when necessary arcsin transformation was performed. Data were analysed by oneway ANOVA followed by Student–Newman–Keuls test at a significance level of 5%. Also, the percentages of each fatty acid were chemometrically analysed by including them as variables in a multivariate principal components analysis (MPCA) model. With such a parsimonious approach, the dataset of variables (fatty acids) is reduced into a smaller set of factors or components. Parsimony is achieved by explaining the maximum amount of common variance in a correlation matrix using the smallest number of explanatory concepts. Factors are statistical entities that can be visualised as classification axes along which measurement variables can be plotted, giving an idea of their correlation with the corresponding factor (loading). Score plots are a graphical representation of individual (dietary groups) scores in the new subset of measurement variables (factors). They illustrate the relationship among individual cases (dietary groups), and the variables, and help in the analysis of data by showing graphical associations, or through new statistical analyses. In the present study, factor scores were subsequently analysed by one-way ANOVA and Student–Newman–Keuls multiple-comparison tests. All analyses were made using the SPSS package version 13.0 (SPSS Inc., Chicago, IL, USA).

4.3. Results

4.3.1. Growth performance

Fish grew from 16 to 240–270 g over a growth trial of 8 months' duration under natural light and temperature conditions (**Fig. 1**). The final body weight of fish fed the control diet did not differ from that of fish fed 33VO and 66VO diets, with overall specific growth rates ranging between 1·12 and 1·16 (see **Table 3**). By contrast, the total replacement of FO dictated a slight but significant reduction (10 %) of final body weight in fish fed the

VO diet. A concurrent and significant decrease of voluntary feed intake (g DM intake) was found in fish fed the VO diet. Feed efficiency (0·97–1·01) remained high and unchanged irrespective of dietary treatment.

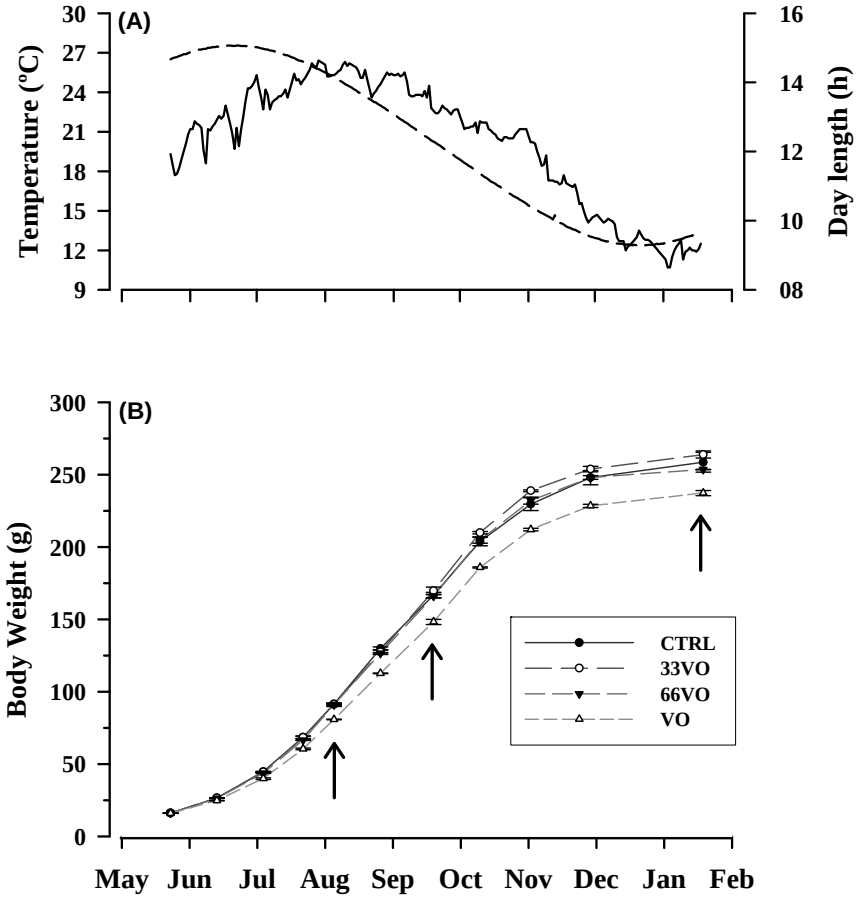

Fig. 1. (A) Seasonal changes of temperature (—) and day length (- - -). (B) Body weight over the course of the trial of fish fed the experimental diets; ↑, tissue sampling times. Values are the means of triplicate tanks, with standard errors represented by vertical bars.

Table 3. Growth performance of fish fed the four experimental diets during 8 months.
(Mean values and standard deviations of triplicate tanks)

	CTRL		33VO		66VO		VO		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P [1]
Initial body weight (g)	16·10	0·09	16·30	0·01	16·30	0·03	16·10	0·09	0·31
Final body weight (g)	257·80^{ab}	11·84	269·57^{b}	2·41	253·72^{a}	0·16	237·39^{c}	3·07	<0·05
DM intake (g/fish)	238·35^{a}	6·68	256·87^{b}	4·42	241·59^{a}	2·69	226·11^{c}	0·62	<0·001
SGR (%) [2]	1·14^{ab}	0·01	1·16^{a}	0·00	1·13^{b}	0·00	1·11^{c}	0·00	<0·05
FE[3]	1·01	0·02	0·98	0·00	0·98	0·01	0·97	0·01	0·07

Mean values within a row with unlike superscript letters were significantly different (P<0·05; Student–Newman–Keuls).

[1] P values result from one-way ANOVA.

[2] SGR = [100 x (ln final fish wt - ln initial fish wt)]/d.

[3] FE = wet wt gain / dry feed intake.

4.3.2. Tissue fat deposition and histological alterations

After the summer replenishment of energy stores, lipid content of dorsal white muscle (6–8 %) was not affected by the dietary treatment. Hepatic fat content in fish fed control and 33VO diets was high and of the same order of magnitude (15% on wet-matter basis; 0·23–0·25 g/100 g body weight). A progressive and significant increase (up to 25%; 0·44 g/100 g body weight) was found with the graded replacement of FO in fish fed 66VO and VO diets (**Fig. 2 (C)**). However, signs of nitial and localised lipoid liver disease were only found with the total replacement of FO with vegetable oils (**Fig. 2 (A) and (B)**). None of the FO-replaced diets produced apparent signs of histological damage in the intestine. Only one fish fed the VO diet had a moderate accumulation of lipid droplets in the intestinal epithelium that was not considered pathological.

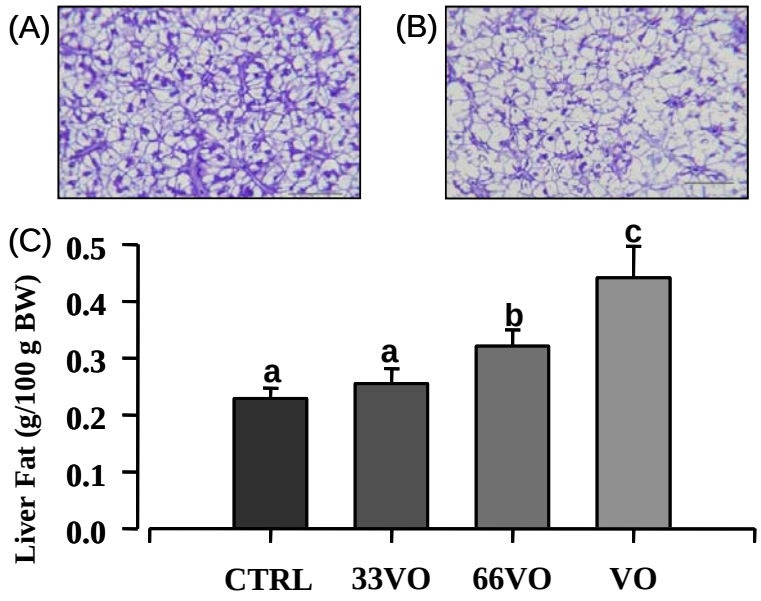

Fig. 2. Representative histological sections of livers of fish sampled in September after 18 weeks of feeding the experimental diets: (A) CTRL diet; (B) VO diet (scale bars ¼ 50mm). Liver fat content (C) of fish fed the four experimental diets (18 weeks). Mean values with unlike letters were significantly different (P<0·05; Student–Newman–Keuls).

4.3.3. Muscle fatty acid profile

The effects of dietary treatment upon muscle fatty acid profiles of TL are shown on a time-course basis (**Table 4**). Overall, fish fed the control diet contained 28% saturates (mainly 16:0 and 14:0), almost 32% monoenes (over half of which were 18:1n-9), 12% n-6 fatty acids (predominantly 18:2n-6) and 18–20% n-3 HUFA (predominantly EPA and DHA). Increased amounts of 18:1n-9, 18:2n-6 and 18:3n-3, in combination with reduced proportions of n-3 HUFA and saturated fatty acids, were found with the progressive replacement of FO by vegetable oils. The two first components of MPCA accounted for the 78% of variation of this dataset, although 67·9% of variation was explained by component 1 itself (**Fig. 3 (A)**). Thus, no grouping was recognised on the basis of sampling time (second factor score), whereas four groups were significantly separated (Student–Newman–Keuls; $P<0{\cdot}05$) and identified as the VO, 66VO, 33VO and control diets in the first factor score (**Fig. 3 (B)**).

The fatty acid profile of muscle PL of fish sampled at the end of the trial (January) is shown in **Table 5.** All experimental groups retained high amounts of SFA, predominantly 16:0 (13%) and 18:0 (8%), but the relative amount of 18:2n-6 increased up to 23% in fish fed the VO diet. A concurrent reduction in n-3 HUFA was also found, decreasing the EPA plus DHA content from 36 to 28% (fish fed control, 33VO and 66VO diets) to 16% (fish fed the VO diet). Thus, when data of PL and TL fractions were analysed by MPCA, the two principal components accounted for 67% of variation (**Fig. 4 (A)**). Component 1 explained 39·6% of variation and separated fatty acids that predominate in TL (on the left) from those characteristic of more unsaturated PL (on the right). Component 2 accounted for 27·8% of variation, and separated fatty acids representative of FO (above the zero line) from those characteristic of vegetable oils (below the zero line). The factor score plot separated TL and PL in the abscise axis, whereas grouping in the ordinate axis was based on the different effects of dietary intervention upon each lipid class. Accordingly, three major clusters were significantly separated (Student–Newman–Keuls; $P<0{\cdot}05$) and identified in

the first factor score plot as: (a) TL group, (b) PL of fish fed the VO diet and (c) a homogeneous group corresponding to PL of fish fed the control, 33VO and 66VO diets (**Fig. 4 (B)**).

Table 4. Effects of the feeding regimen on the muscle fatty acid profile of total lipids (% of total fatty acid methyl esters) in fish sampled in August, September and January.

(Mean values and standard deviations of ten fish)

	CTRL						33VO					
	Aug		Sep		Jan		Aug		Sep		Jan	
Fatty acids	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	3·70^{a}	0·22	3·67^{a}	0·63	4·52^{b}	0·36	2·48	0·41	2·54	0·41	2·60	0·42
16:0	20·40^{a}	0·74	20·10^{a}	0·81	18·30^{b}	0·98	20·60^{a}	0·79	19·00^{b}	0·79	19·00^{b}	0·44
16:1n-7	4·75	0·28	4·58	0·74	5·38	0·52	3·60	0·51	3·65	0·51	3·64	0·50
16:2	0·25	0·02	0·25	0·02	0·28	0·02	0·13	0·05	0·18	0·05	0·15	0·02
16:3	0·19	0·06	0·22	0·04	0·23	0·02	0·15	0·01	0·16	0·01	0·14	0·04
16:4	0·18	0·02	0·15	0·04	0·15	0·01	0·11	0·03	0·11	0·03	0·10	0·02
17:0	0·22	0·02	0·26	0·05	0·23	0·01	0·20	0·04	0·18	0·04	0·19	0·02
18:0	3·82^{a}	0·38	3·96^{a}	0·66	3·00^{b}	0·26	4·57	0·74	4·32	0·74	4·10	0·55
18:1n-9	17·40	0·56	16·00	0·86	16·80	0·98	20·40	1·40	20·60	1·41	18·50	2·85
18:1n-7	1·87	0·08	1·84	0·10	1·93	0·07	1·59	0·20	1·75	0·19	1·55	0·05
18:2n-6	10·70^{a}	0·12	10·60^{a}	0·65	11·80^{b}	0·19	12·80^{a}	0·93	13·40^{ab}	0·93	14·90^{b}	1·56
18:3n-3	1·06	0·12	0·98	0·09	1·07	0·05	5·65	0·83	6·42	0·83	5·80	0·64
18:4n-3	1·28	0·08	1·22	0·22	1·38	0·10	0·89	0·20	1·00	0·20	0·83	0·13
20:0	0·18	0·02	0·18	0·02	0·18	0·01	0·17	0·06	0·20	0·06	0·17	0·02
20:1n-9	4·90	0·40	4·79	0·84	5·53	0·22	3·25	0·50	3·15	0·46	3·25	0·01
20:2n-6	0·22	0·00	0·24	0·02	0·25	0·03	0·23	0·06	0·28	0·02	0·26	0·01
20:3n-6	0·17	0·02	0·12	0·06	0·13	0·01	0·16	0·02	0·16	0·06	0·18	0·03
20:3n-3	0·07	0·00	0·09	0·03	0·08	0·00	0·12^{a}	0·05	0·17^{b}	0·05	0·16^{b}	0·01
20:4n-6	0·49^{a}	0·02	0·54^{a}	0·16	0·38^{b}	0·08	0·49	0·10	0·41	0·02	0·42	0·09
20:4n-3	0·58	0·06	0·59	0·07	0·66	0·04	0·49	0·00	0·52	0·15	0·52	0·06
20:5n-3	6·06	0·42	6·40	0·85	5·02	0·37	4·87	0·93	4·58	0·03	4·34	0·72
22:1n-9	0·62	0·08	0·31	0·08	0·42	0·03	0·22	0·10	0·29	0·93	0·26	0·03
22:1n-11	4·83	0·66	4·73	1·05	5·35	0·51	2·62	0·60	2·91	0·15	2·78	0·46
22:5n-3	1·31	0·10	1·37	0·10	1·51	0·11	1·10	0·07	1·06	0·08	1·25	0·15
22:6n-3	10·80	1·00	12·40	2·79	10·60	2·05	9·74	2·32	8·85	0·63	11·00	2·67
24:1n-9	0·56^{a}	0·08	0·40^{b}	0·04	0·41^{b}	0·04	0·56^{a}	0·03	0·40^{b}	0·07	0·35^{b}	0·08
Saturates	28·32^{a}	1·05	28·17^{a}	0·81	26·23^{b}	0·90	28·02	1·86	26·24	1·22	26·06	0·57
Monoenes	34·93	1·46	32·65	3·61	35·82	1·92	32·24	3·36	32·75	2·93	30·33	4·07
n-3 HUFA[1]	18·82	1·48	20·85	3·59	17·87	2·38	16·32	3·14	15·18	3·26	17·27	3·44
n-6 HUFA[2]	0·88	1·13	0·09	0·11	0·76	0·06	0·88	0·11	0·85	0·22	0·86	0·11

(continued on next page)

Table 4 (continued).

66VO						VO						
Aug		Sep		Jan		Aug		Sep		Jan		
Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Fatty acids
1·79	0·16	1·62	0·15	1·77	0·31	0·90	0·12	0·79	0·12	1·12	0·45	14:0
17·80[a]	0·89	19·00[b]	0·90	17·20[a]	0·62	15·80	0·58	16·20	0·34	16·10	0·53	16:0
2·93	0·18	2·56	0·21	2·85	0·38	1·77	0·34	1·52	0·21	2·15	0·52	16:1n-7
0·08	0·03	0·13	0·04	0·11	0·03	tr		tr		0·11	0·00	16:2
0·12	0·06	0·08	0·02	0·10	0·04	0·08	0·04	0·09	0·01	0·09	0·01	16:3
0·07	0·01	0·06	0·00	0·08	0·06	tr		0·07	0·02	0·13	0·05	16:4
0·12	0·06	0·18	0·01	0·21	0·06	0·14	0·56	0·14	0·56	0·13	0·01	17:0
4·15	0·56	4·88	0·48	3·92	0·64	4·53	0·36	4·92	1·67	4·40	0·66	18:0
25·00	1·61	23·80	0·91	24·50	2·25	28·20	0·78	27·50	0·08	27·30	3·06	18:1n-9
1·38	0·02	1·30	0·04	1·36	0·04	1·10	0·06	1·09	0·85	1·22	0·13	18:1n-7
16·30[a]	0·46	16·60[a]	0·33	17·40[b]	0·15	19·40	0·52	20·40	1·54	20·50	1·66	18:2n-6
12·20	1·15	11·00	1·19	12·10	1·50	17·80	0·76	16·80	0·11	15·80	1·75	18:3n-3
0·81	0·12	0·64	0·15	0·77	0·12	0·63	0·08	0·51	0·03	0·55	0·13	18:4n-3
0·16	0·01	0·16	0·01	0·17	0·01	0·15	0·01	0·17	0·10	0·16	0·01	20:0
1·92	0·53	1·86	0·26	1·91	0·29	0·91	0·04	0·92	0·08	0·93	0·52	20:1n-9
0·27	0·01	0·27	0·03	0·27	0·03	0·28	0·02	0·33	0·03	0·33	0·03	20:2n-6
0·19	0·05	0·19	0·04	0·18	0·07	0·18	0·04	0·23	0·10	0·22	0·10	20:3n-6
0·27[ab]	0·02	0·23[a]	0·04	0·29[b]	0·04	0·40	0·06	0·45	0·07	0·48	0·09	20:3n-3
0·26	0·10	0·30	0·05	0·24	0·12	0·18	0·04	0·17	0·09	0·17	0·09	20:4n-6
0·47	0·03	0·39	0·05	0·45	0·05	0·34	0·04	0·34	0·39	0·35	0·05	20:4n-3
2·83	0·55	3·06	0·29	2·56	0·76	1·41	0·24	1·34	0·05	1·55	0·74	20:5n-3
0·20	0·15	0·11	0·02	0·28	0·12	0·10	0·15	0·09	0·03	0·14	0·04	22:1n-9
1·65	0·31	1·46	0·28	1·62	0·40	0·27	0·12	0·30	0·05	0·33	0·09	22:1n-11
0·80	0·17	0·63	0·10	0·69	0·41	0·36	0·1	0·32	0·17	0·45	0·09	22:5n-3
5·75	1·77	6·54	0·82	6·02	2·58	3·11	0·5	3·15	1·15	3·52	1·82	22:6n-3
0·40[a]	0·04	0·38[a]	0·04	0·32[b]	0·03	0·29	0·02	0·29	0·03	0·35	0·06	24:1n-9
24·02[a]	1·32	25·84[b]	1·24	23·27[a]	0·97	21·52	0·78	22·22	0·77	21·91	0·93	Saturates
33·48	2·43	31·47	1·09	32·84	3·41	32·64	0·96	31·71	1·97	32·42	3·35	Monoenes
10·12	2·49	10·85	1·23	10·01	3·73	5·62	0·81	5·60	1·61	6·35	2·75	n-3 HUFA[1]
0·72	0·15	0·76	0·13	0·69	0·19	0·64	0·07	0·73	0·18	0·72	0·20	n-6 HUFA[2]

tr, trace value<0·05; HUFA, highly unsaturated fatty acids.
Mean values within dietary groups with unlike superscript letters were significantly different (P<0·05; Student–Newman–Keuls).
[1] Calculated excluding 18 C atoms of the n -3 series.
[2] Calculated excluding 18 C atoms of the n -6 series.

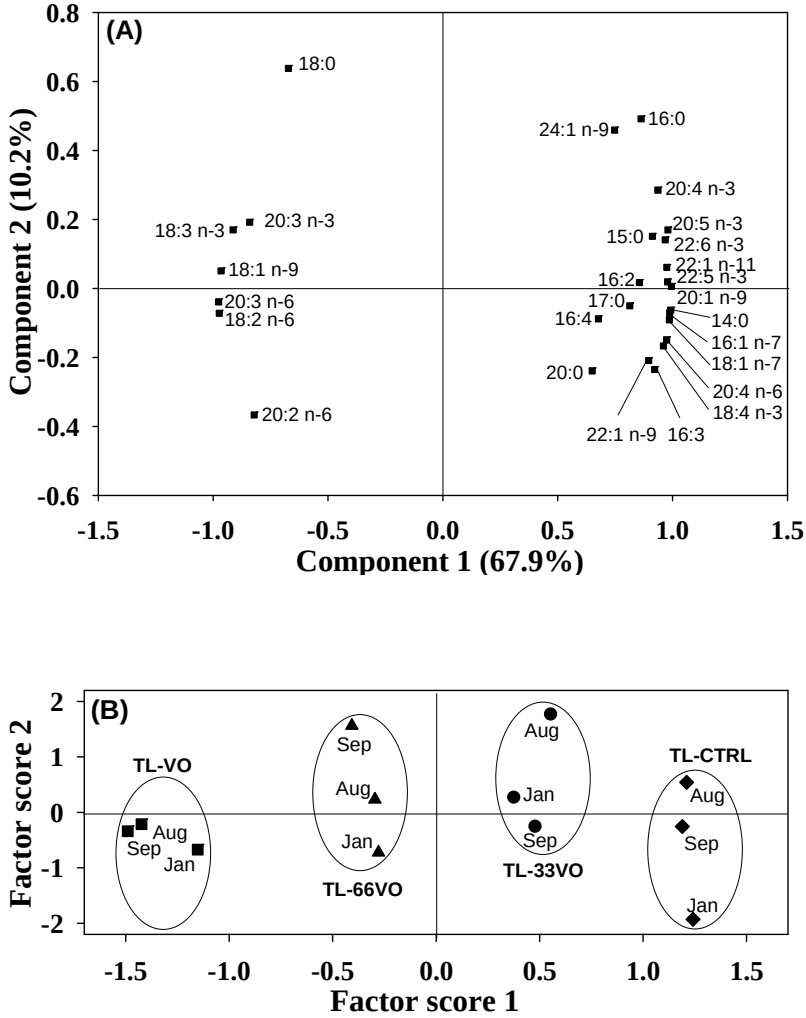

Fig. 3. Component plot (A) and factor score plot (B) of the multivariate principal components analysis for the muscle fatty acid profile of total lipids (TL) in fish sampled in August (Aug), September (Sep) and January (Jan). Mean values are shown in the factor score plot to simplify the graph representation. Ovals stand for different clusters in the factor score 1 (P<0·05; Student–Newman–Keuls).

Table 5. Effects of the feeding regimen on the muscle fatty acid profile of phospholipids (% of total fatty acid methyl esters) in fish sampled at the end of the trial (January)
(Mean values and standard deviations of ten fish)

	CTRL		33VO		66VO		VO	
Fatty acids	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0·62	0·20	0·57	0·51	0·54	0·24	0·23	0·07
16:0	18·4^{a}	1·22	17·50^{ab}	1·19	16·50^{b}	0·96	13·2^{c}	0·38
16:1n-7	1·06	0·36	0·80	0·12	0·70	0·17	0·76	0·23
16:2	0·30^{a}	0·15	0·25^{ab}	0·12	0·23^{b}	0·00	0·22^{b}	0·00
16:3	0·34	0·00	0·20	0·14	0·36	0·06	0·16	0·13
16:3 n-3	1·76	1·43	0·62	0·48	1·12	1·01	0·81	0·33
16:4	0·30	0·07	0·29	0·10	0·39	0·09	0·42	0·07
17:0	0·38	0·13	0·30	0·17	0·34	0·04	0·26	0·14
18:0	10·10	1·24	8·42	0·75	10·20	1·12	8·44	0·69
18:1n-9	7·59^{a}	0·14	9·33^{b}	0·02	10·20^{b}	0·06	13·40^{c}	0·08
18:1n-7	1·84	0·41	1·66	0·62	1·54	0·92	0·82	0·61
18:2n-6	7·26^{a}	0·74	10·90^{b}	1·42	14·20^{c}	0·92	23·30^{d}	1·79
18:3n-3	0·45^{a}	0·26	2·29^{b}	0·18	4·88^{c}	0·41	10·20^{d}	1·11
18:4n-3	0·31	0·25	0·29	0·29	0·30	0·11	0·29	0·11
20:0	0·27	0·00	0·16	0·03	0·26	0·00	0·30	0·13
20:1n-9	2·42^{a}	0·25	1·67^{b}	0·20	1·09^{c}	0·18	0·57^{d}	0·27
20:2 n-6	0·40	0·14	0·44	0·30	0·65	0·26	0·80	0·60
20:3n-6	0·54	0·44	0·38	0·20	0·41	0·08	0·68	0·06
20:3n-3	0·53^{a}	0·48	0·24^{a}	0·21	0·34^{a}	0·12	0·87^{b}	0·19
20:4n-6	0·94	0·05	1·15	0·07	0·87	0·28	0·65	0·08
20:4n-3	0·43	0·43	0·51	0·20	0·57	0·14	0·53	0·18
20:5n-3	7·08^{a}	0·64	7·52^{a}	0·44	6·32^{b}	0·42	3·72^{c}	0·19
22:1n-11	0·68	0·38	0·40	0·31	0·37	0·28	0·36	0·29
22:5n-3	1·93^{a}	0·07	2·05^{a}	0·15	1·64^{b}	0·22	1·23^{c}	0·20
22:6n-3	29·00^{a}	3·62	27·80^{a}	3·26	21·40^{b}	2·06	12·60^{c}	0·52
24:1n-9	0·76	0·24	0·52	0·25	0·59	0·07	0·41	0·18
Saturates	29·77^{a}	1·75	26·95^{b}	0·55	27·84^{ab}	1·94	22·43^{c}	0·95
Monoenes	14·35^{a}	0·83	14·38^{a}	0·73	14·49^{a}	0·84	16·32^{b}	0·33
n-3 HUFA[1]	38·97^{a}	3·39	38·12^{a}	3·17	30·27^{b}	2·62	18·95^{c}	0·78
n-6 HUFA[2]	1·88	0·79	1·97	0·68	1·93	0·47	2·13	0·96
n-3/n-6 ratio[3]	4·34^{a}	0·33	3·16^{b}	0·09	2·19^{c}	0·06	1·15^{d}	0·02

HUFA, highly unsaturated fatty acids.
Mean values within a row with unlike superscript letters were significantly different (P<0·05; Student–Newman–Keuls).
[1] Calculated excluding 18 C atoms of the n-3 series.
[2] Calculated excluding 18 C atoms of the n-6 series.
[3] Calculated taking into account all n-3 and n-6 fatty acid series.

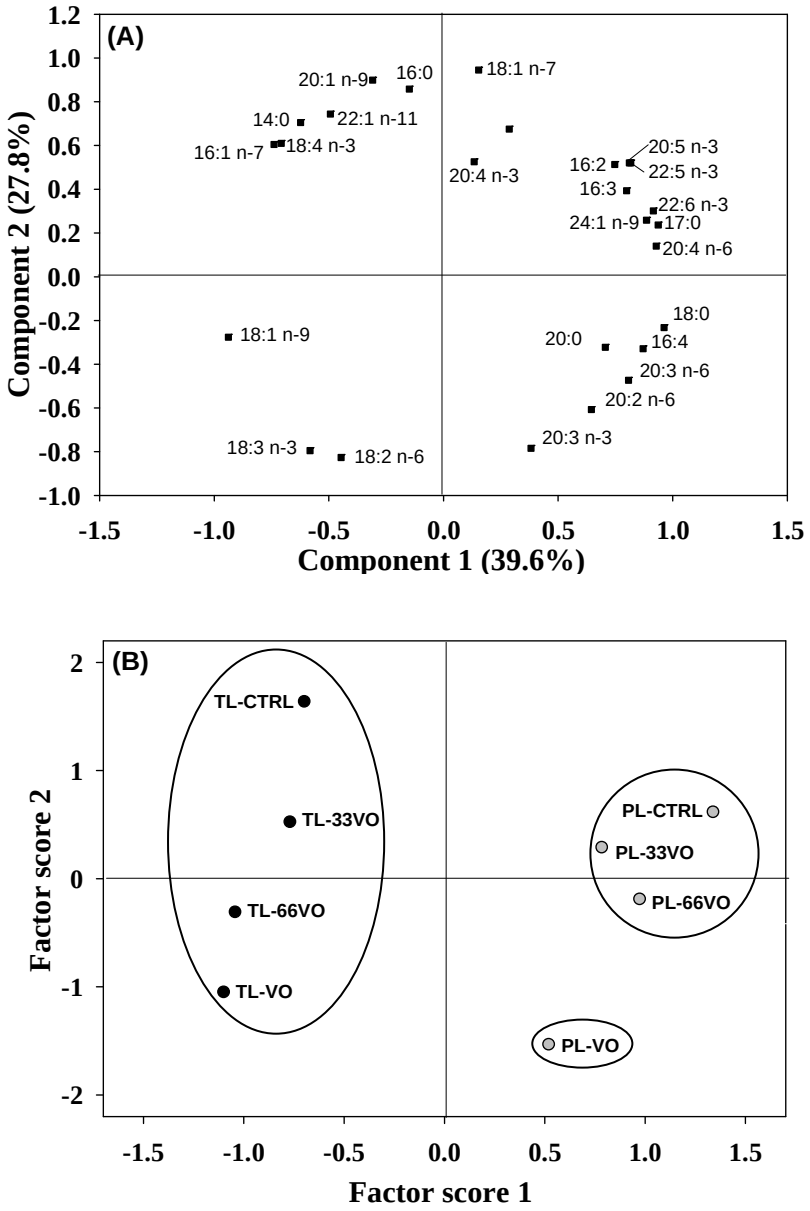

Fig. 4. Component plot (A) and factor score plot (B) of the multivariate principal components analysis for the muscle fatty acid profile of total lipids (TL;●) and phospholipids (PL;○) (January-sampled fish). Mean values are shown in the factor score plot to simplify the graph representation. Ovals stand for different clusters in the factor score 1 (P<0·05; Student–Newman–Keuls).

4.4. Discussion

The demand for feed in intensive aquaculture has increased over recent years and extensive research has been done on alternative raw materials of vegetable origin. However, the main constraint for the use of vegetable oils in marine fish feeds is the lack of n-3 long-chain PUFA, particularly EPA and DHA. Moreover, quantitative requirements depend on species and growth rates, and the biological demand for n-3 HUFA was at least 1·6% of DM for flatfish larvae[33] decreasing to 0·8–0·6% in juvenile[34,35] and grower fish[36]. Similar requirements were reported for juvenile European sea bass[37] and gilthead sea bream[38]. In the present study the theoretical requirements of EFA were met by 33VO (1·6% EPA + DHA) and 66VO (0·9% EPA + DHA) diets, but not by the VO diet (0·3% EPA + DHA). Thereby, in this and in a previous short-term trial[27], no detrimental effects on growth performance were found with the replacement of up to 66% of the added FO, whereas a slight but significant reduction in feed intake and weight gain was found with the total FO replacement, indicating that a dietary supply of 0·3% of EPA + DHA was not sufficient for normal growth and development of gilthead sea bream. However, fishmeal itself contains appreciable amounts of FO, and trials conducted in our experimental facilities show that the total replacement of the added FO is feasible without adverse effects on growth in gilthead sea bream diets with a 30–35% fishmeal inclusion (L Benedito-Palos, JC Navarro, A Sitjà-Bobadilla, JG Bell, S Kaushik and J Pérez-Sánchez, unpublished results). Regost et al.[39] also reported the feasibility of the total replacement of FO by vegetable oils in turbot fed fishmeal-based diets. Similar results were reported in sharpsnout sea bream by Piedecausa et al.[40]. However, in the present study, we report for the first time, over the production cycle of a marine fish, the use of well-balanced plant protein diets with a low inclusion of marine raw materials (20%) just to cover EFA needs.

It is noteworthy that growth rates in the trial conducted in the present study were excellent and even improved upon the values reported for fish of the same size class under similar experimental conditions[25,26,41,42]. This fact can be attributed to

the genetic improvement of fish strains but also to better fish management, culture conditions and dietary formulation. Since fishmeal is also a source of PL, the plant protein mixture in the present study was adequately supplemented with amino acids and PL supplied in the form of soya lecithin. This added component is rich in phosphatidylcholine, a polar lipid molecule that is a natural component of lipoproteins and cellular membranes, adding fluidity and rigidity to cells as well as being required for lipoprotein synthesis, lipid mobilisation and digestibility. Our experimental design does not delineate unequivocally the beneficial effects of soya lecithin, but it must be noted that signs of intestine damage and transport dysfunction (massive accumulation of lipid droplets) were not found in any experimental group. By contrast, intense accumulation of lipid droplets was reported earlier in the hindgut of juvenile gilthead sea bream fed plant protein and FO-based diets without PL supplementation[43]. Similar histological alterations have been reported by other authors using transmission electron microscopy[15] and, interestingly, earlier studies in young larvae demonstrated that dietary lecithin increases the appearance of lipoproteins and enhances the lipid transport through the gut[12,44,45]. Likewise, intense accumulation of lipid droplets was seen in the gastrointestinal tract of salmonids fed with plant oils, but this condition was reversed by PL supplementation[13,14].

Defects in fatty acid storage and oxidation are a central initiating factor for metabolic and endocrine alterations, resulting in enhanced fatty acid flux from adipose tissue towards liver and muscle[46,47]. Ration size by itself is also a major disrupting factor, and long-term feeding close to satiation increases hepatic fat deposition in gilthead sea bream juveniles, leading to lipoid liver disease and enterocyte desquamation in fish fed commercial diets[48]. Dietary inclusion of vegetable oils[49,50] and plant proteins[43] also induces lipoid liver disease, and the role of TNFα and lipoprotein lipase as lipolytic cytokines and rate-limiting enzymes in tissue fatty acid uptake has been reported in gilthead sea bream[51,52]. Precise effects of nutrients on the dysregulation of lipid metabolic pathways still remain largely unknown, but several studies indicate that soyabean phosphatidylcholine may alleviate signs of liver diseases, promoting a healthy lipid metabolism[12,53,54]. This notion is

supported in the present study by the observation that hepatic fat deposition varied between 15 and 25% of wet weight, though signs of initial and focal lipoid liver disease were only found with the total FO replacement. By contrast, clear signs of liver disease have been reported with a liver fat deposition below 15% in fish fed 16% lipid diets[43] (22% lipid diets were used in the present study). This finding suggests that the fat threshold level for liver damage was significantly increased in the present study. However, the extent to which this condition is due to PL supplementation with soya lecithin rather than to other poorly defined dietary factors merits more specific research.

The gilthead sea bream, as other poikilotherms, utilises favourable conditions in summer for rapid growth and replenishment of energy stores, but analyses of fatty acid profiles in this and other fish species including Atlantic salmon[55,56], rainbow trout[57], turbot[39] and European sea bass[58,59] suggest a selective incorporation of n-3 PUFA in polar lipids and perhaps increased oxidation rates of other more easily utilisable fatty acids. Moreover, the seasonal cycling increases in fat storage alter the ratio of polar and neutral lipids, driving the well-reported changes in the muscle fatty acid profile seen in wild gilthead sea bream[21]. In addition, there is experimental evidence linking fatty acid profiles of wild brown trout with the trophic level of the species, the location of the catch, and the size and physiological status of the animal[60]. However, feeding regimens under intensive aquaculture production apparently override the impact of the season on the fatty acid profile of farmed gilthead sea bream[22]. This notion is supported by data from the present study, and the MPCA analysis revealed that the 68% of the total variation in the muscle fatty acid profile of TL is explained by the dietary component. Likewise, alterations in the muscle fatty acid profile of cultured Chinook salmon are viewed as a direct consequence of changes in body weight, fat deposition and ration size[61]. This information is of relevance and highlights important nutritional and quality traits, in particular for meeting human requirements for n-3 PUFA and HUFA, which needs to be considered for a proper timing and use of FO finishing diets for the recovery of a marine fatty acid profile in fish fed vegetable oils through most of the production cycle[29,30,39].

The degree of unsaturation of fatty acids mediates the fluidity and structural integrity of cell membranes, which may exacerbate signs of EFA deficiency during fish over-wintering [1,62,63]. This is the reason why the analysis of PL fatty acid profiles was focused in the present study on the cold season. At this time, the factor score plot showed two major clusters corresponding to PL and TL subgroups. In addition, the PL branch of fish fed the control, 33VO and 66VO diets appeared as a high homogeneous group, which evidenced the robustness of the PL fatty acid profile when EFA requirements were theoretically covered. However, fish fed the VO diet were deficient in EFA, and PL-VO appeared as an outlier group in the MPCA analysis. More detailed analyses revealed the relative enrichment of these fish in 20:2n-6, 20:3n-6 and 20:3n-3. Since vegetable oils are devoid of these fatty acids and they are part of the biosynthetic routes of n-6 and n-3 HUFA, this finding highlights adaptive attempts to alleviate EFA deficiencies. The accumulation of 20:3n-6 indicates increased Δ-6 desaturation and elongationof dietary 18:2n-6 that is driven by increased dietary and tissue levels of this fatty acid, derived from vegetable oils, as well as reduced tissue levels of n-3 HUFA[8]. The increased levels of 20:2n-6 and 20:3n-3, which are 'dead-end' elongation products of 18:2n-6 and 18:3n-3, respectively, reflect increased levels of dietary C18 PUFA although increased levels of 20:3n-9, a marker of EFA deficiency, were not observed. In gilthead sea bream, the expression of Δ-6 desaturase is highly induced in fish fed a HUFA-free diet[10]. There is also now evidence for a regulatory role of conjugated linoleic acid upon the hepatic and intestine expression of fatty acyl elongase and Δ-6 fatty acyl desaturase[64]. However, a low activity of Δ-5 fatty acyl desaturase activity has been reported either in vitro[65] or in vivo[9], which may act as a major constraining factor for bioconversion of C18 PUFA into C20 and C22 HUFA at appreciable rates.

In summary, data on growth performance, tissue histology and fatty acid analysis prompted us to use practical diets with a low inclusion of marine raw materials through most of the production cycle of gilthead sea bream, linking the robustness of the PL fatty acid profile with endocrine, metabolic and somatotropic factors. Precise effects at different

developmental stages need to be further evaluated, and, interestingly, muscle fatty acid profiles and MPCA emerge not only as powerful tools to understand foraging ecology and food webs, but also to evaluate alternative and sustainable aquafeeds in a global change scenario.

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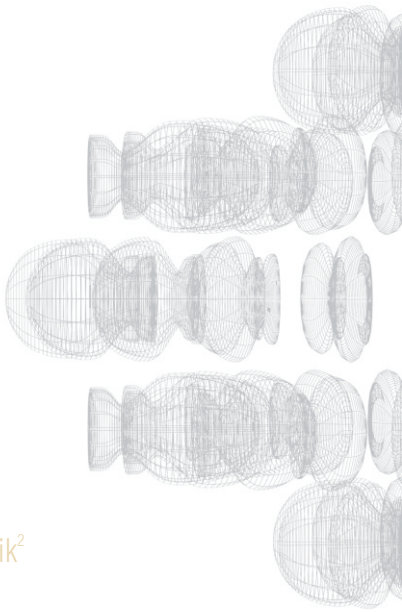
CAPÍTULO 5

Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata* L.) fed practical diets with a combined high replacement of fish meal and oil



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ABSTRACT

The present study aimed to determine the tissue-specific robustness of fatty acid (FA) signatures of gilthead sea bream (*Sparus aurata* L.) by analyzing the changes in lipid class and FA composition of skeletal muscle, brain, liver, and mesenteric adipose tissue. Triplicate groups of fish were fed to visual satiety over a 14-mo production cycle with 2 practical plant protein-based diets formulated with either fish oil or a blend of vegetable oils (66% of fish oil replacement) to contain 53% CP and 21% crude fat on a DM basis. Growth rates ($P = 0.22$) and tissue lipid class composition were not altered by the dietary treatment ($P = 0.34$ and 0.52 for neutral lipids and phospholipids, respectively). The FA signatures of neutral lipids reflected the composition of the diet, although the output of principal components analysis revealed a divergent FA profile for liver compared with skeletal muscle, brain, and mesenteric adipose tissue. Because the theoretical EFA needs were met by the 2 diets, the FA composition of phospholipids remained almost unaltered in all tissues. Interestingly, however, the brain showed the greatest robustness and regulatory capacity to preserve the phenotype of fish fed fish oil-based diets. The FA signatures of total lipids are a combinatory result of neutral and polar lipids, and the most relevant fat storage tissues (mesenteric adipose tissue and skeletal muscle) were more easily influenced by dietary FA composition. The present study provides new insights into fish tissue FA composition and reinforces the use of FA signatures as useful criteria in determining whether EFA requirements for a wide range of physiological processes, including those of neural tissues, can be met with practical fish feeds.

Keywords: fatty acid biomarker, fish composition, fish oil, neutral lipid, phospholipid, vegetable oil.

5.1. Introduction

The use of fatty acids (**FA**) as biomarkers in river and marine ecosystems has received considerable attention in the last few decades (Sargent et al., 1988; Iverson et al., 1997; Kaushik et al., 2006; Stowasser et al., 2009). Most of these FA compounds are presented in all the available foods, but prey species usually differ in the relative proportions of FA, and the overall profile across several FA is termed the FA signature. Thus, although FA dynamics in the predator-prey process are complex and the composition of predator tissue will not exactly match that of the prey, certain FA patterns can be conservatively transferred through aquatic food webs in a predictable way (Iverson et al., 2004; Berge and Barnathan, 2005; Thiemann, 2008). This is more evident under controlled feeding conditions. The available literature in farmed fish collectively supports a close association between diet and fillet FA composition, as reviewed by Turchini et al. (2009).

The gilthead sea bream, *Sparus aurata* L., is a highly cultured fish in the Mediterranean region. Most seasonal changes in muscle FA composition are overwritten under nonrestricted feeding conditions (Grigorakis et al., 2002; Benedito-Palos et al., 2008), following a simple FA dilution model for fillet FA (Benedito-Palos et al., 2009). However, n-3 long-chain PUFA (**LC-PUFA**) are selectively incorporated into phospholipids (**PL**), and their muscle FA signatures show a high robustness when the theoretical FA requirements are met in the diet (Benedito-Palos et al., 2008). Experimental evidence describing compensatory mechanisms (Benedito-Palos et al., 2007), the kinetics of feed-borne contaminants (Nacher-Mestre et al., 2009), and complex adjustments of the antioxidant and hepatic-detoxifying system (Saera-Vila et al., 2009) with increased dietary content of vegetable oils (**VO**) already exists in the recent literature on gilthead sea bream. However, the tissue robustness of FA signatures still remains unresolved in fish fed limited amounts of EFA. The present study aimed to determine how conservative the tissue-specific FA signatures are by analyzing the changes in lipid class and FA composition of skeletal muscle, brain, liver, and mesenteric adipose tissue of gilthead sea bream

fed plant protein-based diets with either fish oil (**FO**) or VO as the most important dietary oil source.

5.2. Materials and methods

All procedures were consistent with the national and institutional regulations (Consejo Superior de Investigaciones Cientificas, Institute of Aquaculture Torre la Sal Review Board, Castellon, Spain) and the current European Union legislation on handling experimental animals.

5.2.1. Diets

Two practical plant protein-based diets (fish meal inclusion, 150 g/kg) with either VO or marine FO were formulated to contain 53% CP and 21% crude fat on a DM basis. Fish oil from the southern hemisphere was the lipid source in the FO diet, whereas a blend of VO (17:58:25 rapeseed oil:linseed oil:palm oil) replaced 66% of FO in the **66VO** diet. The relative proportion of n-3 LC-PUFA, predominantly 20:5n-3 (eicosapentaenoic acid, **EPA**) and 22:6n-3 (docosahexaenoic acid, **DHA**), varied from 19.4% in the FO diet to 6.6% in the 66VO diet (**Table 1**). Diets were manufactured using a twin-screw extruder (BC-45, Clextral, Firminy, France) at the INRA experimental research station in Donzacq (Landes, France), dried under hot air, sealed, and kept in airtight bags until use. Details on ingredients and proximate composition have been presented by Benedito-Palos et al. (2009).

5.2.2. Growth trial and tissue sampling

Juvenile gilthead sea bream of Atlantic origin (Ferme Marine de Douhet, Ile d'Oleron, France) were acclimated to laboratory conditions at the Institute of Aquaculture Torre de la Sal for 20 d before the beginning of the study. Fish of 18 g initial mean BW were distributed into 6 fiberglass tanks (3,000 L) in groups of 150 fish/tank. Water flow was 20 L/min and the oxygen content of the outlet water remained greater than 5 mg/L. The growth study was undertaken over 14 mo (July 2006 to September 2007), and day length and water temperature (10 to 27°C) varied over the course of the trial following natural changes at our latitude (40°5′ N; 0°10′ E).

Table 1. Fatty acid composition of experimental diets (% of total fatty acid methyl esters)[1].

Fatty acids %	FO[2]	66VO[3]
14:0	7.18	2.7
15:0	0.12	tr
16:0	22.26	18.48
16:1n-7	7.06	2.62
16:2	0.47	0.15
16:3	1.66	0.46
16:3n-3	0.11	0.03
16:4	1.8	0.47
17:0	0.96	0.32
18:0	4.27	3.55
18:1n-9	12.49	24.59
18:1n-7	2.97	tr
18:2n-6	10.35	17.48
18:3 n-6	0.34	0.09
18:3n-3	0.81	17.33
18:4n-3	1.8	0.62
20:0	0.07	0.06
20:1n-9	0.92	1.03
20:2n-6	0.6	0.19
20:3 n-6	0.07	tr
20:4n-6	0.69	0.18
20:4n-3	0.3	0.15
20:5n-3	13.57	4.38
22:1n-11	0.97	0.76
22:5n-3	0.81	0.23
22:6n-3	4.78	1.88
Total	97.83	97.81
Saturates	34.86	25.11
Monoenes	24.41	29
n-3 LC-PUFA[4]	19.46	6.64
n-6 LC-PUFA[5]	1.36	0.37

[1]Values are the means of 2 determinations (trace values <0.05). [2]FO diet = diet formulated with fish oil. [3]66VO diet = diet in which vegetable oil replaced 66% of fish oil. [4]LC-PUFA = long-chain PUFA; calculated excluding 16-C and 18-C atom n-3 series. [5]Calculated excluding 18-C atom n-6 series.

Triplicate groups of fish were randomly allocated to each diet. Feed was offered by hand to apparent visual satiety twice a day (0900 and 1400 h; 6 to 7 d/wk) from July 2006 to September 2006, and once a day (1200 h, 4 to 6 d/wk) from October 2006 to September 2007. At regular intervals, fish were counted and group-weighed under moderate anesthesia (3-aminobenzoic acid ethyl ester, 100 µg/mL; MS 222, Sigma-Aldrich, Madrid, Spain). Feed intake and mortalities (total mortality, <2%) were recorded daily. At the end of the study, randomly selected fish (9 fish/treatment) were anesthetized and killed by cervical section before tissue sampling. Brain, liver, mesenteric fat, and fillet portions (Norwegian Quality Cut) without skin and bone were rapidly excised, frozen in liquid nitrogen, and stored at −80°C until lipid analysis.

5.2.3. Lipid analysis

Lipids from target tissues were extracted by the method of Folch et al. (1957), using chloroform:methanol (2:1) containing 0.01% butylated hydroxytoluene as antioxidant. Lipid content was determined gravimetrically (0.1 mg, Mettler Toledo, Barcelona, Spain), and total lipids (**TL**) were fractionated by TLC (Silica gel G 60, 20 × 20 cm glass plates; Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic acid (85:15:1.5, vol/vol) as a solvent system. Phospholipid bands were scraped and extracted with chloroform:methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene. Neutral lipids (**NL**) were scraped and extracted with hexane:diethyl eter (1:1, vol/vol). After the addition of nonadecanoic FA (Sigma, Poole, Dorset, UK) as an internal standard, fractions of TL, NL, and PL were subjected to acid-catalyzed transmethylation for 16 h at 50°C using toluene (1 mL) and 1% (vol/vol) sulphuric acid in methanol (2 mL; Christie, 1982). The FA methyl esters (**FAME**) were extracted with hexane:diethyl ether (1:1, vol/vol), and those derived from TL were purified by TLC using hexane:diethyl ether:acetic acid (85:15:1.5, vol/vol) as a solvent system. The FAME were then analyzed with a gas chromatograph (GC 8000 Series, Fisons Instruments, Rodano, Italy) equipped with a fused-silica 30 m × 0.25 mm open tubular column (film thickness: 0.25 µm; Tracer, TR-WAX, Teknokroma, Barcelona, Spain) and a cold on-column injection

system. Helium was used as a carrier gas and temperature programming was from 50 to 180°C at 40°C/min and then to 220°C at 3°C/min. Peaks were recorded in a personal computer using a software package (Azur, Datalys, St Martin d'Heres, France). Individual FAME were identified by reference to well-characterized FO standards, and the relative amount of each FA was expressed as a percentage of the total amount of FA in the analyzed sample.

The analysis of lipid classes was performed by high performance TLC using a one-dimensional double development as described by Olsen and Henderson (1989). Briefly, TL were separated on high-performance TLC plates [10 × 10 cm, silica gel (200 μm), Merck] using methyl acetate:isopropanol:chloroform:methanol:0.25% aqueous potassium chloride (25:25:25:10:9, vol/vol) and hexane:diethyl ether:acetic acid (85:15:1.5, vol/vol) as developing solvent mixtures for PL and NL, respectively. The separated lipid classes were charred for 10 min at 160°C after spraying the plate with 3% (wt/vol) cupric acetate in 8% (vol/vol) phosphoric acid (Fewster et al., 1969) and were quantified by calibrated scanning densitometry (Bio-Rad, San Francisco, CA). The proportions of each lipid class were expressed as a percentage of the total lipid classes in the analyzed sample.

5.2.4. Statistical analysis

Growth and biochemical variables were checked for normal distribution and homogeneity of variances, and, when necessary, arcsine transformation was performed. Data on PL and NL classes were compared by 2-way ANOVA (with diet and tissue as factors) at a significance level of 5%. Student's *t*-test was used to analyze the specific effects of dietary treatment on the FA composition of each particular tissue. Overall data on tissue FA composition were analyzed chemometrically by multivariate principal components analysis (**PCA**), with the score plots being used as a pattern recognition tool. Similarities between the FA profiles of tissue PL from the dietary treatments were studied using the *D* coefficient of distance (McIntire et al., 1969),

$$D(h-j) = (\sum_{i=1}^{n} (Pih - Pij)^2)^{1/2}$$

where $D(h - j)$ is the distance between treatments h and j, and Pih and Pij are the percentages of FA i in treatments h and j, for each i FA. All analyses were completed using the SPSS package (SPSS Inc., Chicago, IL).

5.3. Results

5.3.1. Growth performance

Growth performance data have been presented by Benedito-Palos et al. (2009). Growth and feed efficiency were not affected (P = 0.22 and 0.33, respectively) by dietary treatments over the 14-mo production cycle (data not shown). Overall, fish grew from 18 g to 520 to 530 g, with a final feed efficiency (wet mass gain/dry feed intake) of 0.80 to 0.81. Hepato-somatic index (%, liver mass/fish mass) and mesenteric fat index (%, mesenteric fat mass/fish mass) remained unaltered, with average values of 1.2 to 1.4% and 1.5 to 1.6%, respectively (data not shown).

5.3.2. Tissue lipid content

The tissue rates of lipid deposition were not altered by the dietary treatment (skeletal muscle, P = 0.06; adipose tissue, P = 0.52; liver, P = 0.15; and brain P = 0.14; **Table 2**). Two-way ANOVA revealed that differences were due to tissue factors ($P <$ 0.001), with the lipid class composition not being altered by dietary treatment (Σ NL, P = 0.34; Σ PL, P = 0.52). The NL were over-represented in mesenteric fat and muscle, whereas increased amounts of PL were found in brain and liver tissues.

Table 2. Tissue lipid content (%, wet weight basis) and lipid class composition (%) in fish fed the experimental diets[1]

	Skeletal muscle			Adipose tissue			Liver			Brain		
	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM
Lipid content	11.37	11.40	0.52	83.73	85.69	2.87	15.40	17.99	1.63	44.42	39.84	2.86
Lipid class[4]												
CE	2.19	1.55	0.67	0.06	0.45	0.01	2.06	3.25	0.68	1.74	3.80	0.54
TAG	87.18	85.77	2.75	97.91	96.85	0.59	78.72	76.33	1.48	55.67	33.36	2.72
FFA	0.97	1.22	0.03	< 0.1	0.57	-	2.08	2.93	0.40	3.41	16.69	1.43
COL	1.97	2.34	0.23	1.11	1.92	0.07	4.14	4.42	0.34	14.13	16.94	1.17
DG	1.25	0.75	0.17	0.37	0.59	0.16	0.76	1.22	0.08	2.32	5.04	0.21
MG	2.05	0.71	0.42	0.15	1.07	0.01	1.12	0.75	0.36	0.29	1.66	0.29
SL	< 0.1	< 0.1	-	< 0.1	< 0.1	-	< 0.1	< 0.1	-	0.57	0.78	0.18
PE	0.99	1.44	0.27	< 0.15	< 0.2	-	3.69	3.65	0.32	10.44	11.83	0.82
PA/CL	0.78	0.41	0.16	< 0.1	< 0.1	-	0.97	0.89	0.12	0.59	0.77	0.15
PI	0.24	0.20	0.04	< 0.1	< 0.1	-	0.21	0.91	0.16	0.8	0.80	0.22
PS	< 0.16	< 0.1	-	< 0.1	< 0.1	-	0.24	< 0.2	0.01	2.51	1.84	0.51
PC	3.05	2.51	0.31	< 0.19	< 0.3	-	5.87	5.83	0.36	6.75	5.45	0.28
SM	< 0.1	< 0.1	-	< 0.1	< 0.1	-	< 0.1	< 0.1	-	< 0.1	0.42	0.04
Σ NL[5]	95.61	92.4	1.93	99.61	99.5	0.25	88.88	88.9	1.01	78.14	77.50	1.43
Σ PL[5]	5.02	4.7	0.62	0.34	0.5	0.10	10.98	11.4	0.88	21.09	21.90	2.00

[1]Means and pooled SEM are presented (n = 8). [2]FO diet = diet formulated with fish oil. [3]66VO diet = diet in which vegetable oil replaced 66% of fish oil. [4]CE = cholesterol esters; TAG = triacylglycerols; COL = cholesterol; DG = diacylglycerols; MG = monoacylglycerols; SL = sphingolipids; PE = phosphatidylethanolamine; PA/CL = phosphatidic acid/cardiolipin; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin; NL = neutral lipids; PL = phospholipids. [5]Dietary treatment did not affect the tissue-specific content of NL (Σ NL, $P = 0.34$) and PL (Σ PL, $P = 0.52$). Differences were found among all tissues ($P < 0.001$).

5.3.3. FA composition

The FA profile of NL is shown in **Table 3**. Fish fed the FO diet showed increased proportions of n-3 LC-PUFA (P < 0.001 for all tissues) in combination with reduced amounts of 18:1n-9, 18:2n-6, and 18:3n-3 (P < 0.001 for all tissues) as compared with fish fed the 66VO diet. Within tissues, the greatest (P = 0.001) concentration of DHA was found in the brain. The liver shared the greatest (P < 0.001; for 4 FA: 16:0, 18:0, 22:0, and 18:1n-9) concentrations of SFA (16:0, 18:0, and 22:0) and 18:1n-9, whereas the muscle and mesenteric fat had an increased (P = 0.006) concentration of 18:2n-6. The PCA (**Figure 1A**) revealed that the first 2 components accounted for 80% of the total variation. Sixty-two percent of the variation was explained by component 1 itself, which separated the most characteristic marine FO (right quadrant) and VO (left quadrant). Thus, factor score plot 1 recognized 2 major groups on the basis of dietary treatment: tissues of fish fed the FO diet on the right vs. tissues of fish fed the 66VO diet on the left. The second factor score plot separated liver from the other tissues analyzed, which was more evident in the case of fish fed the FO diet (**Figure 1B**).

The FA profile of PL is shown in **Table 4.** The influence of dietary regimen on FA profiles was less in PL than in NL, as evidenced by the paired Student's *t*-test. This was more apparent in brain tissue, where only 5 FA (18:2n-6, 18:3n-3, 20:0, 20:4n-6, and 20:5n-3) showed differences (P < 0.05) between treatments. When all data on PL FA profiles were analyzed by PCA, the 2 principal components accounted for 60% of the total variation, with 40% being explained by component 1 (**Figure 2A**), leading to separate tissues with no potential grouping associated with dietary treatment in the factor score plot (**Figure 2B**). However, some tissues were more conservative than others, and the coefficient of distance D for a given tissue varied from 13.28 in muscle to 1.67 in brain. Intermediate values were found (P < 0.001) in mesenteric fat (9.27) and liver (7.33).

The FA composition of TL reflected the combined composition of NL and polar lipids (**Table 5**). Again, the greatest (P < 0.001; for 2 FA: DHA and 24:1n-9) concentrations of DHA

and 24:1n-9 were found in the brain, and the concentration of 18:0 was greater ($P < 0.001$) in the liver and brain than in other tissues. Liver showed the greatest ($P < 0.001$, $P = 0.5$, and $P < 0.001$; for 16:0, 20:0, and 18:1n-9, respectively) concentrations of SFA (16:0, 20:0) and 18:1n-9, whereas 18:2n-6 and 18:3n-3 were greater ($P < 0.001$ and $P = 0.009$ for 18:2n-6 and 18:3n-3, respectively) in mesenteric adipose tissue. The PCA, including data on FA composition of the 2 fish feeds, revealed that the first 2 components accounted for 56% of the total variation, with 40% of the variation being explained by component 1 (**Figure 3A**). Component 1 separated the most characteristic variables of marine FO vs. VO, located on the right and left quadrants, respectively. On this basis, 2 groups were clearly recognized in the first factor score plot: FO feed and tissues of fish fed the FO diet on the right vs. 66VO feed and tissues of fish fed the 66VO diet on the left. The second factor score plot separated liver and brain from muscle, mesenteric fat, and feeds **(Figure 3B**).

Table 3. Fatty acid profile of skeletal muscle, mesenteric adipose tissue, liver, and brain neutral lipids (% of fatty acid methyl esters) in fish fed the experimental diets[1]

	Skeletal muscle			Adipose tissue			Liver			Brain		
Fatty acid, %	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM
14:0	4.71	2.49*	0.06	5.13	2.58*	0.06	3.34	2.02*	0.22	4.10	2.17*	0.17
15:0	0.13	0.06*	0.01	0.13	0.08*	0.01	0.08	0.05*	0.01	0.19	0.10*	0.04
16:0	16.38	14.08*	0.22	15.85	14.59*	0.20	19.20	19.19	0.74	15.63	15.78	0.24
16:1n-7	8.65	4.79*	0.08	8.47	4.28*	0.10	6.52	3.51*	0.24	6.98	3.65*	0.30
16:2	1.05	0.42*	0.01	1.13	0.42*	0.02	0.80	0.30*	0.04	0.94	0.37*	0.04
16:3	1.53	0.62*	0.02	1.67	0.61*	0.02	0.99	0.36*	0.07	1.37	0.52*	0.06
16:4	1.17	0.40*	0.02	1.35	0.41*	0.03	0.62	0.15*	0.09	1.05	0.33*	0.05
17:0	0.26	0.16*	0.01	0.26	0.16*	0.01	0.31	0.21*	0.01	0.33	0.24	0.04
18:0	3.27	2.47*	0.23	3.17	3.08	0.14	6.38	6.41	0.60	4.68	5.48	0.82
18:1 n-9	17.84	23.28*	0.37	17.11	25.12*	0.37	21.56	30.06*	1.29	15.65	24.25*	0.55
18:1 n-7	3.45	2.14*	0.05	3.45	2.15*	0.04	3.85	2.41*	0.11	3.37	2.39*	0.08
18:2 n-6	9.33	16.32*	0.14	9.82	15.89*	0.13	7.42	11.10*	0.60	8.58	13.45*	0.40
18:3 n-3	0.76	16.77*	0.20	0.86	15.49*	0.16	0.66	9.02*	0.51	0.79	12.07*	0.38
18:3 n-6	0.38	0.14*	0.00	0.42	0.14*	0.01	0.42	0.13*	0.01	0.35	0.11*	0.02
18:4 n-3	1.47	0.91*	0.03	1.63	0.78*	0.03	0.93	0.63*	0.08	1.39	0.67*	0.08
20:0	0.20	0.09*	0.02	0.20	0.17*	0.01	0.19	0.18	0.01	0.22	0.23	0.02
20:1 n-7	0.26	0.07*	0.01	0.26	0.10*	0.01	0.23	0.12*	0.01	0.24	0.10*	0.02
20:1 n-9	1.56	0.84*	0.04	1.56	1.17*	0.03	1.46	1.37	0.07	1.44	1.27*	0.06
20:2 n-6	0.33	0.41*	0.02	0.35	0.39*	0.02	0.46	0.65*	0.04	0.56	0.64	0.10
20:3 n-6	0.22	0.20	0.01	0.23	0.18*	0.01	0.31	0.31	0.03	0.23	0.18*	0.02
20:3 n-3	0.05	0.48*	0.02	0.05	0.44*	0.01	0.06	0.65*	0.03	0.05	0.41*	0.02
20:4 n-6	0.63	0.24*	0.01	0.65	0.20*	0.02	0.57	0.20*	0.02	0.93	0.40*	0.19
20:4 n-3	0.76	0.60*	0.02	0.80	0.52*	0.01	0.84	0.71*	0.05	0.71	0.27*	0.02
20:5 n-3	9.72	3.89*	0.10	9.78	3.17*	0.11	6.83	2.21*	0.50	9.28	3.45*	0.38
22:0	0.05	0.02*	0.00	0.04	0.04*	0.00	0.08	0.09	0.01	0.02	0.05	0.01
22:1 n-9	0.35	0.14*	0.02	0.32	0.25*	0.01	0.39	0.39*	0.02	0.25	0.25*	0.04
22:1 n-11	0.97	0.27*	0.02	0.91	0.46*	0.03	0.65	0.38*	0.06	0.77	0.54*	0.06
22:5 n-3	3.35	1.72*	0.09	3.45	1.44*	0.06	4.14	1.60*	0.20	3.25	1.64*	0.14
22:6 n-3	6.16	2.91*	0.07	5.97	2.33*	0.07	5.36	2.10*	0.40	8.20	4.86*	0.41

[1]Means and pooled SEM are presented (n = 8). Within a tissue, means between dietary groups with an asterisk are different ($P < 0.05$). [2]FO diet = diet formulated with fish oil. [3]66VO diet = diet in which vegetable oil replaced 66% of fish oil.

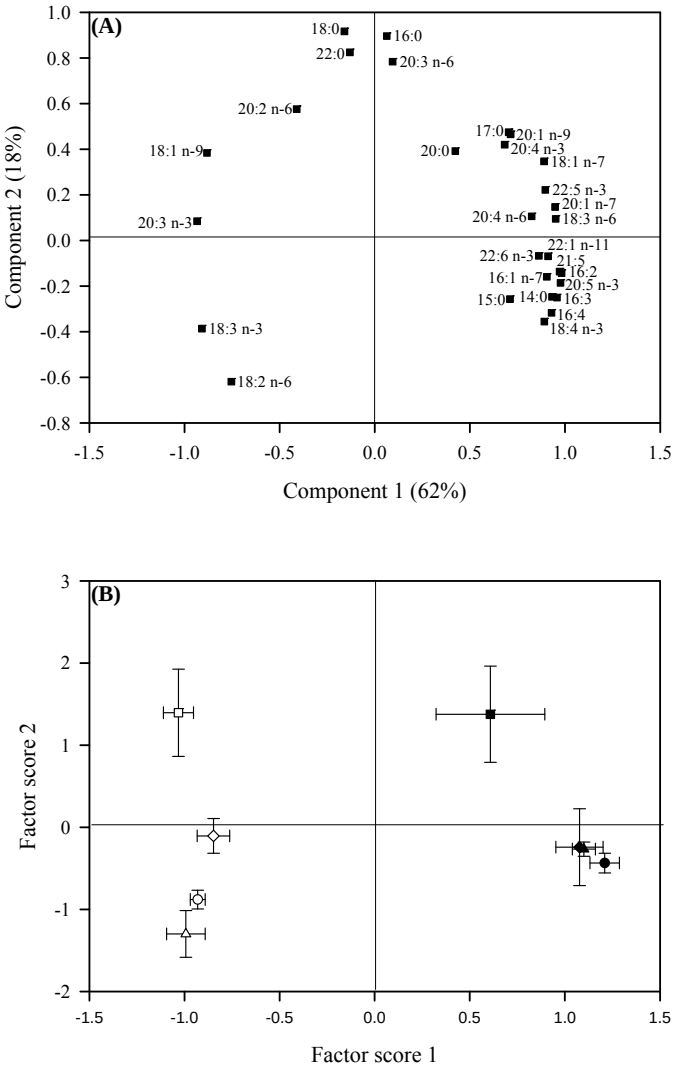

Figure 1. Component plot (A) and factor score plot (B) of the principal components analysis for the fatty acid profile of neutral lipids in skeletal muscle (triangles), mesenteric adipose tissue (circles), liver (squares), and brain (diamonds) of fish fed diets formulated with either fish oil (FO) or a vegetable oil blend replacing 66% of fish oil (66VO). Solid symbols refer to the FO diet; open symbols refer to the 66VO diet. Data values are represented in the factor score plot as mean ± SEM (n = 8).

Table 4. Fatty acid profile of skeletal muscle, mesenteric adipose tissue, liver, and brain phospholipids (% of fatty acid methyl esters) in fish fed the experimental diets[1].

	Skeletal muscle			Adipose tissue			Liver			Brain		
Fatty acids, %	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM
14:0	2.01	1.49	0.21	5.75	3.62*	0.58	2.04	1.38*	0.19	0.83	0.84	0.18
15:0	-	-	-	-	-	-	0.22	0.21	0.04	0.10	0.07	0.03
16:0	20.79	17.87*	0.36	15.90	14.32*	0.55	19.94	18.83	0.56	18.20	18.13	1.62
16:1n-7	2.69	1.71*	0.38	5.12	4.18*	0.30	2.30	1.55*	0.17	2.28	2.08	0.14
16:2	1.08	1.00	0.12	5.50	4.21*	0.50	1.23	1.14	0.14	0.29	0.43	0.10
16:3	0.36	0.31	0.06	1.42	0.74	0.31	0.55	0.50	0.06	0.74	0.65	0.41
16:4	0.36	0.40	0.07	-	-	-	0.20	0.22	0.03	3.98	3.47	0.54
17:0	-	-	-	-	-	-	0.33	0.22	0.03	0.31	0.24	0.04
18:0	8.09	7.73	0.17	9.97	8.02*	0.54	10.97	10.68	0.45	16.20	16.57	0.78
18:1 n-9	11.73	15.81*	0.50	10.48	14.94*	1.15	6.17	8.46*	0.13	15.56	15.42	1.51
18:1 n-7	2.89	1.34*	0.26	-	-	-	3.26	2.34*	0.12	1.80	1.51	0.13
18:2 n-6	5.42	11.55*	0.24	6.14	7.96	1.06	3.96	8.11*	0.25	1.06	1.60*	0.30
18:3 n-3	0.41	6.30*	0.22	1.64	7.00*	0.64	0.20	3.09*	0.07	0.36	1.24*	0.28
18:4 n-3	0.40	0.28	0.10	0.65	0.61	0.17	0.13	0.15*	0.01	0.28	0.30	0.07
20:0	0.17	0.18	0.04	0.57	0.66	0.11	0.72	0.70	0.10	0.36	0.40*	0.06
20:1 n-9	0.86	0.73	0.20	1.63	3.17	0.68	0.41	0.44	0.02	0.44	0.37	0.05
20:2 n-6	0.20	0.50	0.12	1.37	1.20	0.12	0.34	0.62*	0.03	0.09	0.09	0.01
20:3 n-6	0.40	0.56	0.12	0.65	0.78	0.07	0.79	1.33*	0.09	0.13	0.18	0.02
20:3 n-3	-	-	-	-	-	-	0.45	0.62*	0.14	0.09	0.11	0.02
20:4 n-6	2.41	1.73*	0.07	2.31	1.11*	0.21	3.75	2.40*	0.13	1.45	1.27*	0.07
20:4 n-3	0.47	0.49	0.02		0.70	0.04	0.62	1.23*	0.08	0.14	0.15	0.03
20:5 n-3	13.24	9.23*	0.32	5.85	3.26*	0.35	12.91	9.71*	0.52	3.12	2.39*	0.16
22:1 n-9	-	-	-	-	-	-	0.08	0.91	0.36	0.14	-	0.02
22:1 n-11	0.26	0.26	0.11	1.75	1.75	0.10	0.32	0.28	0.04	0.15	0.18	0.03
22:5 n-3	3.53	3.09*	0.15	2.07	1.72*	0.08	2.80	2.63	0.21	1.04	0.95	0.13
22:6 n-3	17.42	13.61*	0.53	7.42	4.40*	0.44	17.13	14.63*	0.56	21.57	22.29	1.55
24:1 n-9	-	-	-	-	-	-	1.55	1.50*	0.05	2.19	2.34	0.44

[1]Means and pooled SEM are presented (n = 8). Within a tissue, means between dietary groups with an asterisk are different ($P < 0.05$). [2]FO diet = diet formulated with fish oil. [3]66VO diet = diet in which vegetable oil replaced 66% of fish oil.

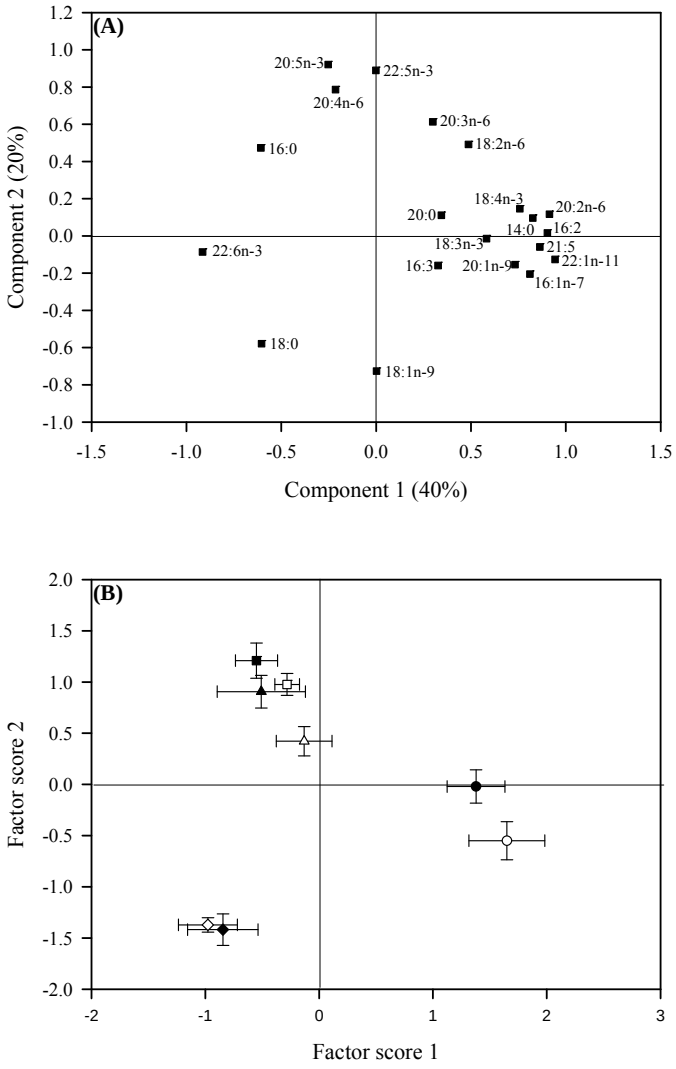

Figure 2. Component plot (A) and factor score plot (B) of the principal components analysis for the fatty acid profile of phospholipids in skeletal muscle (triangles), mesenteric adipose tissue (circles), liver (squares), and brain (diamonds) of fish fed diets formulated with either fish oil (FO) or a vegetable oil blend replacing 66% of fish oil (66VO). Solid symbols refer to the FO diet; open symbols refer to the 66VO diet. Data values are represented in the factor score plot as mean ± SEM (n = 8).

Table 5. Fatty acid profile of skeletal muscle, mesenteric adipose tissue, liver, and brain total lipids (% fatty acid methyl esters) in fish fed the experimental diets[1].

	Skeletal muscle			Adipose tissue			Liver			Brain		
Fatty acids, %	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM	FO[2]	66VO[3]	SEM
14:0	5.67	2.54*	0.10	6.23	2.94*	0.10	3.43	2.08*	0.24	3.14	1.58*	0.24
16:0	19.60	18.33*	0.49	19.21	17.50*	0.23	22.16	21.56	0.76	16.59	17.77	0.70
16:1n-7	8.98	4.42*	0.20	9.11	4.31*	0.11	6.13	3.56*	0.27	5.90	3.04*	0.23
16:2	0.62	0.16*	0.09	1.13	0.42*	0.01	0.52	0.38	0.10	0.70	0.30*	0.04
16:3	1.30	0.47*	0.02	0.20	0.10*	0.01	0.85	0.13*	0.05	0.11	0.11	0.01
16:4	0.93	0.25*	0.02	0.72	0.28*	0.10	0.37	0.10*	0.06	2.23	1.11	0.66
17:0	1.11	0.33*	0.06	1.44	0.63*	0.08	0.74	0.35*	0.06	0.97	0.39*	0.05
18:0	3.42	3.52	0.24	3.67	3.66	0.14	7.18	7.41	0.65	6.96	8.82*	0.69
18:1 n-9	18.43	26.95*	0.32	18.95	27.47*	0.46	20.54	28.85*	0.90	18.40	21.97*	1.05
18:1 n-7	4.00	3.22*	0.20	2.96	2.13	0.35	4.23	2.26*	0.54	2.94	2.09*	0.15
18:2 n-6	8.77	13.57*	0.27	9.52	15.06*	0.14	6.77	10.48*	0.60	6.39	9.32*	0.55
18:3 n-3	0.80	11.53*	0.18	0.77	13.30*	0.11	0.79	7.69*	0.53	0.53	8.51*	0.28
18:4 n-3	1.23	0.55*	0.03	1.29	0.62*	0.03	0.82	0.50*	0.08	1.07	0.53*	0.06
20:0	0.21	0.20	0.03	0.20	0.19	0.01	0.26	0.19*	0.03	0.23	0.21	0.03
20:1 n-9	1.20	0.99*	0.05	1.38	1.08*	0.06	1.09	0.96	0.13	0.90	0.74*	0.05
20:2 n-6	0.33	0.25	0.05	0.37	0.27*	0.01	0.38	0.52	0.08	0.30	0.22	0.04
20:3 n-6	0.31	0.19*	0.02	0.20	0.28*	0.01	0.34	0.49*	0.05	0.19	0.23	0.03
20:3 n-3	0.07	0.31*	0.02	0.06	0.36*	0.01	0.06	0.53*	0.04	0.07	0.29*	0.02
20:4 n-6	0.64	0.22*	0.02	0.51	0.18*	0.03	0.53	0.33	0.09	1.04	0.77*	0.06
20:4 n-3	0.58	0.34*	0.02	0.63	0.39*	0.01	0.70	0.65	0.08	0.51	0.33*	0.03
20:5 n-3	7.59	2.35*	0.15	7.60	2.32*	0.09	6.33	2.30*	0.35	7.72	3.12*	0.31
22:1 n-9	0.40	-	0.01	0.32	0.33	0.02	0.35	0.34	0.03	0.36	0.23*	0.04
22:1 n-11	0.86	0.74*	0.07	1.00	0.56*	0.02	0.61	0.42	0.08	0.51	0.31*	0.04
22:5 n-3	2.36	0.97*	0.08	2.63	1.07*	0.06	3.17	1.34*	0.20	2.42	1.30*	0.10
22:6 n-3	4.41	1.98*	0.12	4.17	1.56*	0.08	5.29	2.38*	0.32	11.42	12.32	1.10
24:1 n-9	0.31	0.13	0.06	0.31	0.29	0.03	0.30	0.31	0.06	1.51	0.88	0.42

[1]Means and pooled SEM are presented (n = 8). Within a tissue, means between dietary groups with an asterisk are different ($P < 0.05$). [2]FO diet = diet formulated with fish oil. [3]66VO diet = diet in which vegetable oil replaced 66% of fish oil.

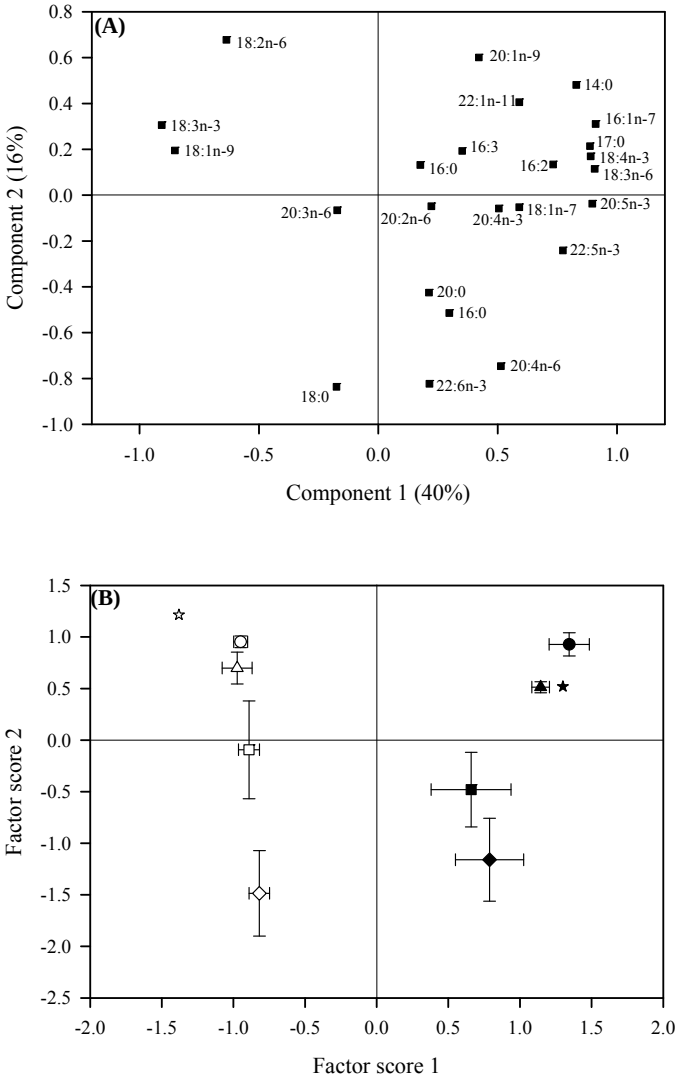

Figure 3. Component plot (A) and factor score plot (B) of the principal components analysis for the fatty acid profile of total lipids in feeds (stars) and tissue samples from skeletal muscle (triangles), mesenteric adipose tissue (circles), liver (squares), and brain (diamonds) of fish fed diets formulated with either fish oil (FO) or a vegetable oil blend replacing 66% of fish oil (66VO). Solid symbols refer to the FO diet; open symbols refer to the 66VO diet. Tissue data values are represented in the factor score plot as mean ± SEM (n = 8).

5.4. Discussion

Fish meal usually contains 8 to 10% residual fat, which often includes 20 to 35% n-3 LC-PUFA (Bimbo, 2000; Bransden et al., 2003). Hence, if as little as 350 g/kg of fish meal is included in the diet, it will provide the general EFA requirements (EPA + DHA: 0.7 to 0.9% of DM) of most commonly farmed finfish, including those of marine fish with extremely limited capacities for the elongation and desaturation of C18 PUFA into C20 PUFA (Tocher and Ghioni, 1999; Sargent et al., 2002; Tocher, 2003). However, in the absence of a noticeable capacity for EFA biosynthesis, the risk of a net deficiency is possible with a substantial replacement of the fish meal fraction along with a reduction in FO. For instance, the growth performance of the turbot *Psetta maxima* L. (Regost et al., 2003), the red sea bream *Diplodus puntazzo* (Cetti; Piedecausa et al., 2007), and the gilthead sea bream (Bouraoui et al., 2010) is not compromised by the complete replacement of FO with VO in fish meal-based diets. Nevertheless, the complete replacement of FO is not feasible with the inclusion of 150 g/kg of fish meal, and the threshold concentration without negative effects on growth is experimentally fixed at 66% for juvenile gilthead sea bream, which supplies approximately 0.9% of EPA + DHA (Benedito-Palos et al., 2007, 2009). The robustness of muscle PL FA profiles is also a valuable criterion to assess the feasibility of this partial FO replacement in gilthead sea bream (Benedito-Palos et al., 2008). In the present study, the conservative feature of PL was highlighted in all the tissues analyzed (muscle, brain, liver, and mesenteric fat) regardless of tissue-specific differences in FA composition. Moreover, given that DHA is strictly controlled in the brains of fish and higher vertebrates (Tocher and Harvie, 1988; Mourente et al., 1991; Furuita et al., 1998), it is reasonable to assume that a wide range of neurally associated functions, including social behavior and visual functions, are preserved in gilthead sea bream with the maximized replacement of FO and fish meal.

Increased replacement concentrations of FO (Caballero et al., 2004; Wassef et al., 2007) and fish meal (Sitja-Bobadilla et al., 2005) often induce lipoid liver diseases in gilthead sea bream. However, well-balanced plant protein mixtures,

supplemented with soy lecithin, prevent the appearance of severe signs of hepatic diseases, even in the case of full FO replacement (Benedito-Palos et al., 2008). This assumption was highlighted by a lack of changes in fat storage capacity and tissue lipid class composition. In the European sea bass *Dicentrarchus labrax* L. (Pagliarani et al., 1986), the Atlantic cod *Gadus morhua* L. (Bell et al., 2006), and the Atlantic salmon *Salmo salar* L. (Nanton et al., 2007), shifts in lipid class composition have not been ascribed to changes in dietary oil sources. However, fat storage location differs within and between fish species (Tocher and Harvie, 1988; Stoknes et al., 2004; Bell et al., 2006), and data currently available indicate that in 2-yr-old gilthead sea bream (300 to 500 g of body mass), approximately 50% of body fat depots are in the muscle tissue. Storage lipids are thereby abundant in the skeletal muscle of gilthead sea bream, and NL are overrepresented in muscle samples devoid of skin and bone, as shown here with 11% muscle lipid content on a wet weight basis. This increased lipid content would be indicative of increased intramuscular adipocyte infiltration because it has been documented by electronic microscopy in Atlantic salmon with a one-half muscle fat deposition ratio (Nanton et al., 2007).

From our results, it is also conclusive that the central nervous system is rich in polar lipids. However, it must be noted that the lipid content in the brain tissue is greater than initially envisaged (39 to 44%, wet weight basis). Again, this fact is related to an increased content of lipids because the NL fraction represented more than 75% of TL regardless of diet. Increased amounts of triacylglycerols (**TAG**) in retina and neural tissues have also been observed in the rainbow trout *Oncorhynchus mykiss* (Walbaum; Tocher and Harvie, 1988). Interestingly, interspecies differences in TAG abundance in these and other tissues also match the lean- and fatty-fish classification well (Stoknes et al., 2004).

The FA compositions of wild and farmed fishes have been analyzed in various tissues (primarily skeletal muscle and liver) of gilthead sea bream (Grigorakis et al., 2002; Ibarz et al., 2005; Izquierdo et al., 2005; Mnari et al., 2007; Fountoulaki et al., 2009) and closely related sparid fishes such as the black sea bream *Acanthopagrus schlegeli* (Bleeker; Peng et al., 2008), the

black sea bream *Spondyliosoma cantharus* L. (Rodriguez et al., 2004), the white sea bream *Diplodus sargus* L. (Cejas et al., 2004), and the red sea bream (Huang et al., 2007). However, this is the first study to determine the FA signatures of skeletal muscle, brain, liver, and mesenteric adipose tissue in the same fish fed practical diets with an increased replacement of FO. It must be noted that the FA composition of NL reflected the composition of the diet regardless of the tissue. Similar results have been reported in European sea bass (Skalli et al., 2006), Atlantic salmon (Jobling and Bendiksen, 2003), turbot (Regost et al., 2003), the pike perch *Sander lucioperca* L. (Schulz et al., 2005), and other commonly farmed fish, as reviewed by Sargent et al. (2002). A secondary source of variation can be specifically ascribed to the nature of tissue, as clearly evidenced by the outcome of the PCA. Indeed, liver appeared as an independent group in the upper quadrant of the NL factor score plot. This differential FA signature can be primarily attributed to 20:3n-6 and SFA (16:0, 18:0, and 22:0), which are indicative of the well-recognized lipogenic activity of liver in fish (Dias et al., 1998). This metabolic feature is not a discriminatory factor for brain, skeletal muscle, and mesenteric adipose tissue, and factor score 2 grouped these non-lipogenic tissues together, especially in fish fed the FO diet. However, the presence of C18 FA (18:2n-6 and 18:3n-3) is limited in a broad sense in the marine environment, and their increased feed intake in fish fed the 66VO diet accounted for a tissue dietary-specific phenotype that separated brain from adipose tissue and skeletal muscle.

The FA composition of the PL fraction is quite regulated and contributes to maintaining the structure and functional integrity of cell membranes (Henderson and Tocher, 1987; Sargent et al., 2002). Therefore, it is not surprising that, in the present study, FA composition of PL was modestly affected by dietary regimen, although the coefficient of distance *D* indicated that tissues with increased content of n-3 LC-PUFA were also more conservative than others. Hence, brain tissue shared the greatest concentration of DHA and this tissue also displayed the greatest capacity to regulate it, preserving functionality with FO replacement. The exact mode of action of DHA-containing PL is not known, but it is possible that they exert their beneficial effects by regulating the blood-brain barrier, membrane fluidity,

the activity of certain enzymes, ionic channels, and the nerve growth factor, as has been reported in mammals (Hussain and Roots, 1994; Vreugdenhil et al., 1996; Ikemoto et al., 2000). This wide range of functions would explain the greater robustness of brain PL in comparison with other tissues. However, this notion is not supported by all studies in fish, and Pagliarani et al. (1986) reported in European sea bass that structural lipids of the brain and liver appear to be similarly sensitive to dietary input. This might be an artifactual result because the latter authors assumed that the FA profile of TL was equivalent in the brain to that of PL. Indeed, in a more recent study, Skalli et al. (2006) clearly showed that PL in neural tissues of European sea bass had the greatest regulation capacity to preserve DHA content in juveniles fed n-3 LC-PUFA-deficient diets. In salmonids, Atlantic cod, and flat fishes, DHA is also known to be present in elevated amounts in the brain (Kreps et al., 1969; Tocher and Harvie, 1988; Mourente et al., 1991), but it remains to be established whether the influence of fish feeds based on VO is less in neural tissues than in the other ones.

Last, it must be highlighted that in gilthead sea bream, the FA composition of TL specifically reflects dietary inputs because of the relatively increased content of the TAG fraction in all the analyzed tissues. This was underlined by the outcome of the PCA, although a minor source of variation was supported by the second component according to the nature of the tissue. Accordingly, tissues with elevated TAG content (muscle and mesenteric adipose tissue) also showed a major influence from the diet, as reported elsewhere in humans and other animal models (Beynen et al., 1980; Raclot, 2003), including fish (dos Santos et al., 1993; Jobling et al., 2002). All these findings offer new insights into tissue-specific FA signatures and reinforce the use of FA signatures as easy and useful criteria for qualitatively and quantitatively evaluating whether EFA requirements are being met in practical fish feeds with a decreased inclusion of marine raw materials. In addition, such approaches can answer some important questions on preserving a wide range of functions in neuronal tissues that otherwise can be difficult to monitor and evaluate.

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CAPÍTULO 6

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

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ABSTRACT

The aim of the study was to determine whether changes in the tissue fatty acid (FA) profile follows a simple test dilution model after changing the dietary oil sources in gilthead sea bream. A 14-month trial was conducted with juvenile fish of 18 g initial body weight fed either a fish oil-based diet (FO diet) or vegetable oils replacing 33% (33VO) and 66% (66VO) of fish oil. The trial included 3 months feeding a fish oil finishing diet to follow the restoration of the FA profile with the FO diet. Fish oil replacement with/without a finishing phase of fish oil re-feeding did not affect growth and all groups reached 520–531 g body weight. Changes in body composition with weight gain did not modify the FA profile of fish continuously fed FO, 33VO or 66VO diets. Increased amounts of oleic acid (18:1n−9), linoleic acid (18:2n−6) and linolenic acid (18:3n−3), in combination with reduced proportions of n−3 long chain polyunsaturated FAs, were found with the partial replacement of fish oil. Hence, multivariate component analysis highlighted a gradient of fish oil load determined by the total intake of fish oil over the entire production cycle. The simple dilution model was a good descriptor of these tissue FA changes, and excellent correlations between observed and predicted values were found at the end of finishing period in fish grow out with either 33VO or 66VO diets.

Keywords: Fish; Growth; Flesh; Fatty acids; Plant proteins.

6.1. Introduction

Fish oil supplies are finite (FAO, 2006) and the continuous increase in global aquaculture production has necessitated research on alternative lipid sources for fish feeds (Watanabe, 2002). Since fish oils are also highly susceptible to contamination with persistent organic pollutants, the use of vegetable oils can contribute toward a reduction in contaminant loadings in the tissue of farmed fish (Sargent et al., 1995; Bell et al., 2005). However, vegetable oils are devoid of n−3 long chain polyunsaturated fatty acids (LC-PUFA), and can adversely affect the tissue fatty acid (FA) composition if added at high inclusion levels (Sargent and Tacon, 1999; Torstensen et al., 2005). Thus, it may be desirable to use finishing diets formulated with uncontaminated or decontaminated fish oils to restore the wild tissue FA profile of farmed fish. For instance, southern hemisphere fish oils are cleaner than northern hemisphere fish oils and can deliver similar levels of n−3 LC-PUFA at lower dietary inclusion levels (Pratoomyot et al., 2008).

Lipid tailoring is, however, a fish-specific process and marine fish show extremely low capabilities for the bioconversion of C_{18} polyunsaturated FAs into C_{20} and C_{22} PUFA (Sargent et al., 2002). Despite this, the essential FA requirements of fast growing juvenile gilthead sea bream are met in practical diets by a 25% inclusion of marine ingredients (fish meal plus fish oil) (Benedito-Palos et al., 2007). Besides, eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3) are selectively incorporated into the polar lipid fraction resulting in a high membrane phospholipid robustness (Benedito-Palos et al., 2008). The same study pointed out the fact that muscle fat depots highly reflect the composition of the diet regardless of season. Earlier studies in gilthead sea bream have also monitored the effect of fish oil re-feeding on the tissue FA profile (Izquierdo et al., 2005). In turbot and brown trout, a simple dilution model was proposed and validated by Robin et al. (2003) to follow the time-course of FA changes after shifting levels in dietary lipid sources. The same model was re-evaluated with different success in Atlantic salmon (Jobling, 2004a,b), red sea bream (Jobling, 2004a), Murray cod (Turchini et al., 2006)

and Atlantic cod (Jobling et al., 2008). The rationale for the present study was to investigate whether dietary FAs are incorporated in the tissue of gilthead sea bream following similar patterns. Specifically, we monitored FA dynamics after fish oil refeeding in fish previously fed plant protein-based diets at two levels of fish oil replacement. Economy of fish oil usage was analysed for the entire 14-month production cycle, including both grow-out and finishing periods.

6.2. Matherials and methods

6.2.1. Diets

Three plant protein diets with pellet size increasing (2–5 mm) according to fish weight were coated with vegetable oils and fish oil from the southern hemisphere to contain 53% crude protein and 21% crude lipid on a dry weight basis (**Table 1**). Fish oil was the only lipid source in the reference/finishing diet (FO). The other two diets contained a blend of vegetable oils (17:58:25 of rapeseed oil: linseed oil: palm oil), replacing 33% (33VO) and 66% (66VO) of fish oil. The fatty acid composition of diets is reported in **Table 2**; reduction in fish oil levels decreased the proportion of n−3 LC-PUFA (predominantly EPA and DHA) from 19.4% in the FO diet to 6.6% in the 66VO diet. All diets were manufactured using a twin-screw extruder (Clextral, BC 45) at the INRA experimental research station of Donzacq (Landes, France), dried under hot air, sealed and kept in air-tight bags until use.

6.2.2. Growth-out trial

Juvenile gilthead sea bream (*Sparus aurata* L.) of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimated to laboratory conditions at the Institute of Aquaculture Torre de la Sal (IATS) for 20 days before the start of the study. Fish of 18 g initial mean body weight were distributed into 9 fibreglass tanks (3000 l) in groups of 150 fish per tank. Water flow was 20 l/min, and oxygen content of outlet water remained higher than 5 mg/l. The growth study was

undertaken over 11 months (July 11th 2006–June 6th 2007), and daylength and water temperature (10–26 °C) varied over the course of the trial following natural changes (40° 5′N; 0° 10′E).

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

Ingredient (%)	FO	33VO	66VO
Fish meal (CP 70%) [1]	15	15	15
CPSP 90 [2]	5	5	5
Corn gluten	40	40	40
Soybean meal	14.3	14.3	14.3
Extruded wheat	4	4	4
Fish oil [3]	15.15	10.15	5.15
Rapeseed oil	0	0.85	1.7
Linseed oil	0	2.9	5.8
Palm oil	0	1.25	2.5
Soya lecithin	1	1	1
Binder	1	1	1
Mineral premix [4]	1	1	1
Vitamin premix [5]	1	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2	2
L-Lys	0.55	0.55	0.55
Proximate composition			
Dry matter (DM, %)	93.13	92.9	92.77
Crude protein (% DM)	53.2	52.81	52.62
Crude fat (% DM)	21.09	21	20.99
Ash (% DM)	6.52	6.69	6.57

[1]Fish meal (Scandinavian LT). [2]Fish soluble protein concentrate (Sopropêche, France). [3]Fish oil (Sopropêche, France). [4]Supplied the following (mg/kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3. [5]Supplied the following (mg/kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B12 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500.

Each diet was randomly allocated to triplicate groups of fish. Feed was offered by hand to apparent visual satiety twice a day (0900 and 1400 h, 6–7 days per week) from July 2006 to September 2006, and once a day (1200 h, 4–6 days per week)

from October 2006 to June 2007. At regular intervals, fish were counted and group-weighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS 222, 100 µg/ml) (Sigma-Aldrich, Madrid, Spain). Feed distributed and mortalities (<5% during the course of the whole 14-month period) were registered daily.

Table 2. FA composition of the experimental diets (% of total FAME).

FA %	FO	33VO	66VO
14 :0	7.18	5	2.7
16:0	22.26	20.30	18.48
16:1n-7	7.06	4.85	2.62
16:2	0.47	0.31	0.15
16:3	1.66	1.09	0.46
16:3n-3	0.11	0.07	0.03
16:4	1.8	1.1	0.47
17:0	0.96	0.64	0.32
18:0	4.27	3.92	3.55
18:1n-9	12.49	20.39	24.59
18:1n-7	2.97	0.23	tr
18:2n-6	10.35	14.03	17.48
18:3 n-6	0.34	0.21	0.09
18:3n-3	0.81	9.16	17.33
18:4n-3	1.8	1.17	0.62
20:0	0.07	0.07	0.06
20:1n-9	0.92	0.97	1.03
20:2n-6	0.6	0.16	0.19
20:3 n-6	0.07	0.11	tr
20:4n-6	0.69	0.43	0.18
20:4n-3	0.3	0.21	0.15
20:5n-3	13.57	8.84	4.38
22:1n-11	0.97	0.82	0.76
22:5n-3	0.81	0.56	0.23
22:6n-3	4.78	3.3	1.88
Total	97.83	98.25	97.81
Saturates	34.86	30.02	25.11
Monoenes	24.41	27.26	29
n-3 LC-PUFA[1]	19.46	12.91	6.64
n-6 LC-PUFA[1]	1.36	0.7	0.37

tr = trace values. [1]Calculated excluding 16 C and 18 C.

6.2.3. Fish oil finishing trial and sampling protocol

To follow the restoration of marine FA profile in fish fed vegetable oils, two of the three replicates of fish fed 33VO and 66VO diets were fed the FO diet once a day (6 days per week) from June 2007 to September 2007 (12 weeks). These duplicate groups became 33VO/FO and 66VO/FO, respectively. The remaining fish continued to be fed with the FO (3 tanks), 33VO (1 tank) and 66VO (1 tank) diets.

At regular intervals after the start of the finishing diet period (zero time, 27, 55 and 88 days), 8 randomly selected fish per dietary treatment were killed by a blow on the head. The right-hand side whole fillet (denuded from skin and bone) was excised, vacuum packed and stored at −80 °C until analyses. All procedures were carried out according to institutional regulations (Consejo Superior de Investigaciones Científicas, IATS Review Board) and the current European Union legislation on handling experimental animals.

6.2.4. Proximate analyses

The proximate composition of diets and fillets was analysed by standard procedures (AOAC, 1990). Moisture content was determined by drying in an oven at 105 °C for 24 h. Subsequently, diets and fillets were freeze-dried and blended for protein, lipid and ash determinations. Crude protein content (N×6.25) was determined in 75–100 mg samples using the automated Kjeldahl method (Kjeldahl Auto 4002430 Analyser, Selecta, Barcelona, Spain). Samples (0.5 g) for lipid analyses were desiccated (105 °C for 3 h) in porous recipients before Soxhlet extraction with 50 ml diethyl ether at 120 °C (Soxhlet 4001046 Auto extraction apparatus; Selecta, Barcelona, Spain). Ash content was determined after heating at 600 °C in a muffle furnace for 2 h.

6.2.5. FA analyses

Total lipids for FA analyses were extracted by the method of Folch et al. (1957), using chloroform:methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. After the addition of nonadecanoic FA (19:0) as

internal standard, total lipids (TL) were subjected to acid catalysed transmethylation for 16h at 50 °C using 1 ml toluene and 2 ml of 1% (v/v) sulphuric acid in methanol (Christie, 1982). FA methyl esters (FAME) were extracted with hexane:diethyl ether (1:1), and purified by thin layer chromatography (Silica gel G 60, 20×20 cm glass plates, Merck, Darmstadt, Germany) using hexane:diethyl-ether:acetic acid (85:15:1.5) as a solvent system. FAME were then analyzed with a Fisons Instruments GC 8000 Series (Rodano, Italy) gas chromatograph, equipped with a fused silica 30 m×0.25 mm open tubular column (Tracer, TR-WAX; film thickness: 0.25 μm, Teknokroma, Spain) and a cold on-column injection system. Helium was used as a carrier gas, and temperature programming was from 50 to 180 °C at 40 °C/min and then to 220 °C at 3 °C/min. Peaks were recorded in a personal computer using the Azur software package (version 4.0.2.0. Datalys, France). Individual FAME were identified by reference to well characterized fish oil standards, and the relative amount of each FA was expressed as a percentage of the total amount of FA in the analysed sample.

BHT and internal standard (19:0) were obtained from Sigma-Aldrich (Madrid, Spain). All solvents in lipid extraction and FA analyses were HPLC grade and were obtained from Merck (Darmstadt, Germany).

6.2.6. Dilution model

Changes in the tissue FA profile as a result of fish oil re-feeding were described according to Robin et al. (2003) by the following equation:

$$P_T = P_{RT} + [(P_0 - P_{RT}) / (Q_T / Q_0)]$$

where P_T is the percentage at time T of a given FA, P_0 is the FA percentage at the start of the finishing period, and P_{RT} is the FA percentage at time T in fish continuously fed the reference/finishing diet. Q_0 and Q_T represent the initial and final (at time T) tissue lipid content, respectively.

In the present study, P_T is the predicted FA percentage at a given time T in finishing groups (33VO/FO, 66VO/FO), P_0 is the FA percentage of a given FA at the start of the finishing period in 33VO and 66VO groups, P_{RT} represents at time T that

of the reference group always fed the finishing FO diet. Q_0 and Q_T are the initial and final tissue lipid content in the respective group. The adequacy of the dilution model was evaluated by direct comparisons of model predictions with the observed values.

6.2.7. Statistical analysis

Growth parameters (fish average values per tank) and the relative amount of FA were checked for normal distribution and homogeneity of variances, and when necessary arcsin transformation was performed. Data were analysed by one-way ANOVA followed by Student-Newman-Keuls (SNK) test at a significance level of 5%. The percentages of each FA were chemometrically analysed by multivariate principal components analysis (MPCA). All analyses were made using the SPSS package version 14.0 (SPSS Inc, Chicago, USA).

6.3. Results

6.3.1. Growth performance

Growth, feed intake and feed conversion ratios were not affected by the dietary treatment over the course of the feeding trial. Hence, at each sampling point, all data on body weight and feed intake were pooled and represented in the fitting plot as the mean of the 9 experimental tanks (**Fig. 1B**). Overall, fish grew during the 11-month grow-out period from 18 g to 284–294 g with a feed efficiency (wet wt. gain/dry feed intake) of 0.82–0.86 over this period. The subsequent trial (3-month period) was conducted over the course of summer, and the cumulative feed intake (g/fish) was of the same order of magnitude as that of the initial period (324 vs 307 g). At the end of the finishing diet period, mean body weight of fish among tanks varied between 520 and 531 g with a feed efficiency for the finishing period of 0.73–0.79.

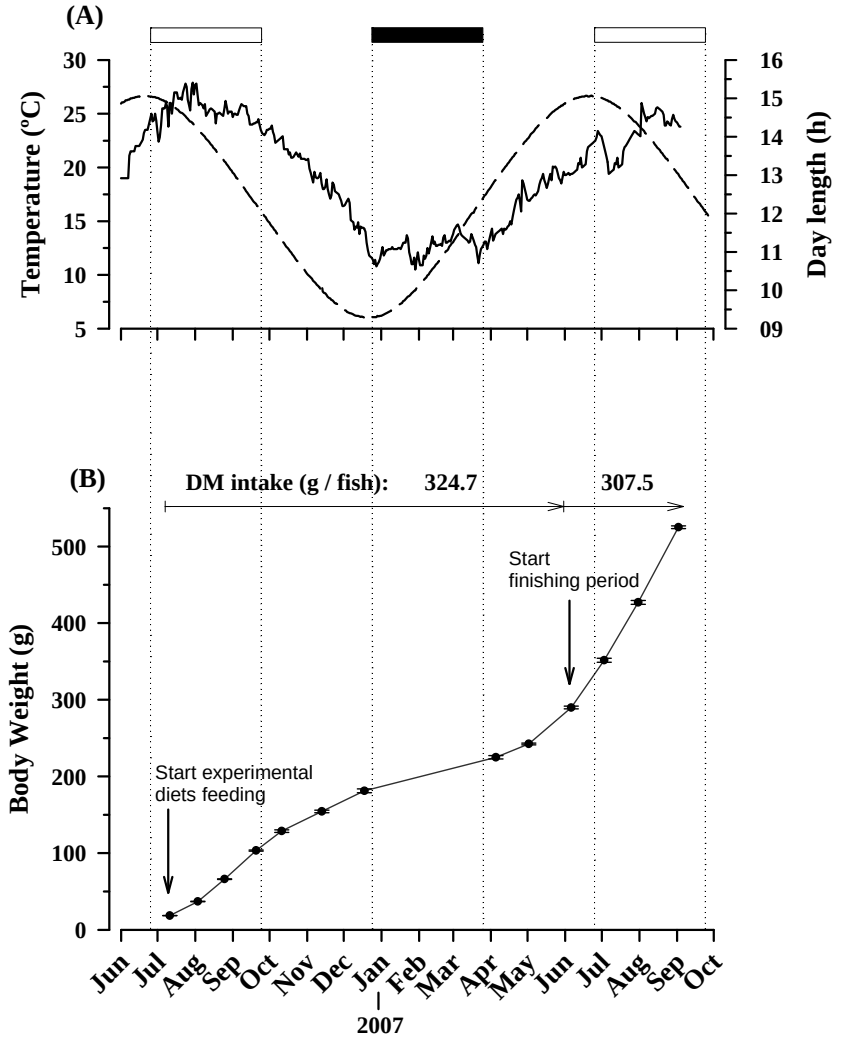

Fig 1. (A) Seasonal changes on temperature (solid line) and day length (dashed line). White and black boxes at the top of figure refer to summer and winter period, respectively. (B) Body weight representation as mean±SEM of all experimental groups in the experimental design. Arrows indicate the start of the study and finishing periods. Cumulative feed intake is indicated at the top of figure for each period.

In absolute terms, fish oil usage (g feed intake × ingredient percentage of fish oil in the diet) in fish always fed FO, 33VO and 66VO was concordant with the percentage of replacement (**Fig. 2**). At the end of the finishing diet period, fish oil usage in fish fed 33VO became equal to that found in the 66VO/FO group. In the 33VO/FO group, fish oil usage was reduced by 15% in comparison to fish always fed the FO diet.

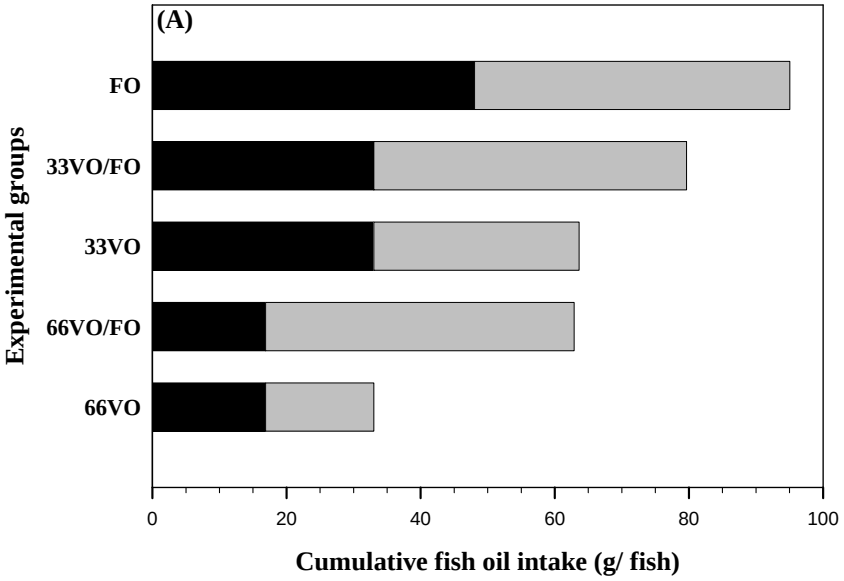

Fig. 2. Effects of diet composition and feeding protocol on fish oil intake (g feed intake×ingredient percentage of fish oil in the diet) per fish through grow-out (330 days, black bars) and finishing (88 days, grey bars) periods.

6.3.2. Lipid content and tissue FA profile

Fillet yield and lipid content of skinned fillets was not altered by dietary intervention. However, lipid deposition increased by a 20–30% over the course of the finishing period regardless of dietary treatment (**Tables 3–5**).

As shown in **Table 3**, no consistent changes in the FA profile were found over the course of the finishing period in fish continuously fed the same diet (FO, 33VO and 66VO groups),

and only a few FAs (16:1 n−7, 17:0, 18:2 n−6) showed significant differences (less than 5–30% of variation) in one or two of the three experimental groups. Regarding the effect of dietary treatment, fish fed the FO diet contained 29% saturates (mainly 16:0 and 14:0), almost 32% monoenes (over half of which were 18:1n−9), 1% n−6 LC-PUFA, and 17% n−3 LC-PUFA (predominantly EPA and DHA). Increased amounts of 18:1n−9, 18:2n−6 and 18:3n−3, in combination with reduced proportions of n−3 LC-PUFA and saturated FAs were found with the progressive replacement of fish oil by vegetable oils.

The time course of changes through the finishing period on the tissue FA profile of fish previously fed vegetable oils are shown in **Tables 4 and 5**. Both in 33VO/FO and 66VO/FO groups, the finishing diet caused a progressive increase in the FAs present in higher amounts in fish oil (i.e. 14:0, 16:1n−7, 20:1n−9, 22:1n−11, EPA and DHA), while those characteristic of vegetable oils (i.e. 18:1n−9, 18:2n−6 and 18:3n−3) decreased in proportion to the degree of fish oil replacement in the diet.

The MPCA analysis of fillet FA profiles before, during and at the end of the finishing period revealed that the two first components accounted for 62% of the total variation, with 52.5% of the variation being explained by component 1 itself (**Fig. 3A**). Some of the most characteristic variables of marine versus vegetable oils had the highest loadings on function 1 and were located at the extremes. The results of the score plot are represented only for the first component since it accounted for the majority of the variation (**Fig. 3B**). The plot revealed that the three invariable groups (FO, 33VO and 66VO) were well separated from each other, with 66VO and FO at the extremes. The finishing 33VO/FO and 66VO/FO groups were also clearly separated from each other on a time-FO intake-manner (**Fig. 3B**). Thus, a gradient of fish oil load caused either by the amount of this ingredient in the diet, or by the total intake per unit of body weight, could be easily distinguishable. At the end of the finishing period, the resulting FA profile of the 66VO/FO became equal to that of fish always fed the 33VO diet, and intermediate values between 33VO and FO groups were found for the 33VO/FO group.

Table 3. Fillet weight, wet lipid content and FA profile (% of total FAME) in fish always fed FO, 33VO and 66VO diets.

	FO				33VO				66VO			
	June		September		June		September		June		September	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fillet (g)	61.50[a]	12.5	114.7[b]	14.4	59.52[a]	5.73	113.1[b]	20.5	58.23[a]	13.2	124.2[b]	14.9
Lipid (%)	9.19[a]	2.01	11.36[b]	1.21	10.97[a]	3.20	12.84[b]	3.24	9.33[a]	2.02	11.40[b]	0.88
Σ FAs (mg/g)	53.99	6.61	61.67	10.8	62.46	18.8	66.78	16.5	59.76	14.2	60.54	7.86
FA (%)												
14:0	5.34	0.47	5.72	0.51	4.24	0.52	4.21	0.43	2.78	0.32	2.92	0.42
15:0	0.20	0.09	0.17	0.05	0.27	0.14	0.19	0.08	0.27	0.22	0.21	0.18
16:0	17.78	1.37	19.34	0.51	17.62	0.70	18.65	0.99	16.58	0.36	18.00	0.68
16:1n-7	7.96	0.64	8.36	0.25	5.96	0.47	5.94	0.18	3.85[a]	0.14	4.08[b]	0.17
16:2	1.03	0.20	0.90	0.29	0.60	0.10	0.69	0.15	0.27	0.09	0.35	0.08
16:3	1.21	0.21	1.35	0.06	0.82	0.08	0.91	0.17	0.43	0.23	0.55	0.14
16:4	1.09	0.30	0.95	0.08	0.52	0.06	0.53	0.07	0.24	0.02	0.26	0.02
17:0	0.66[a]	0.31	0.45[b]	0.37	0.24	0.02	0.25	0.01	0.25	0.09	0.25	0.14
18:0	3.70	0.38	3.71	0.30	3.76	0.17	3.90	0.19	3.85	0.12	3.84	0.12
18:1 n-9	18.13	2.24	18.11	0.77	23.21	1.05	23.27	0.59	27.06	0.71	26.97	0.59
18:1 n-7	3.23	0.25	3.30	0.14	2.56	0.12	2.52	0.10	2.00	0.12	1.98	0.19
18:2 n-6	9.39[a]	0.83	8.95[b]	0.31	12.32[a]	0.75	11.96[b]	0.55	15.02	0.31	14.34	0.31
18:3 n-6	0.38	0.06	0.39	0.02	0.25	0.02	0.25	0.01	0.14	0.01	0.15	0.03
18:3 n-3	1.16	1.20	0.71	0.04	7.39	0.93	7.11	0.26	12.94	0.33	10.82	4.33
18:4 n-3	1.16	0.14	1.23	0.07	0.82	0.02	0.82	0.05	0.60	0.06	2.03	4.07
20:0	0.20	0.02	0.20	0.01	0.19	0.01	0.22	0.06	0.18	0.02	0.19	0.01
20:1 n-7	0.21	0.02	0.22	0.01	0.15	0.01	0.16	0.01	0.11	0.01	0.11	0.01
20:1 n-9	1.14	0.08	1.21	0.02	1.02	0.05	1.08	0.03	0.96	0.03	1.02	0.03
20:1 n-11	0.24	0.02	0.24	0.02	0.20	0.02	0.20	0.02	0.17	0.02	0.17	0.01
20:2 n-6	0.25	0.08	0.26	0.04	0.28	0.06	0.29	0.05	0.32	0.07	0.32	0.07
20:3 n-6	0.26	0.07	0.28	0.03	0.23	0.04	0.25	0.07	0.24	0.11	0.21	0.06
20:3 n-3	0.16	0.20	0.23	0.25	0.23	0.09	0.26	0.09	0.34	0.04	0.36	0.05
20:4 n-6	0.57	0.18	0.53	0.19	0.40	0.12	0.36	0.09	0.23	0.03	0.24	0.03
20:4 n-3	0.61	0.06	0.62	0.03	0.51	0.03	0.51	0.03	0.42	0.02	0.40	0.03
20:5 n-3	8.49	0.89	8.84	0.36	5.57	0.39	5.25	0.38	2.88	0.18	2.98	0.31
22:0	0.17	0.06	0.16	0.06	0.16	0.06	0.15	0.05	0.14	0.03	0.15	0.03
22:1 n-9	0.32	0.02	0.32	0.03	0.31	0.02	0.30	0.02	0.29	0.01	0.28	0.02
22:1 n-11	0.89	0.06	0.95	0.03	0.72	0.05	0.73	0.05	0.58	0.01	0.59	0.02
22:5 n-3	2.66	0.33	2.63	0.15	1.92	0.12	1.94	0.19	1.19	0.04	1.20	0.17
22:6 n-3	5.48	0.90	5.16	0.20	3.92	0.21	3.58	0.27	2.57	0.21	2.29	0.18
24:1 n-9	0.35	0.06	0.28	0.11	0.31	0.03	0.26	0.11	0.31	0.04	0.30	0.03
Saturates	28.04	1.92	29.75	0.74	26.47	1.64	27.26	1.29	24.04	0.67	25.56	0.90
Monoenes	32.53	2.58	33.22	0.84	34.45	1.81	34.51	0.64	35.18	0.84	35.32	0.73
n-3 LC-PUFA[1]	17.40	2.06	17.47	0.60	12.15	0.84	11.54	0.76	7.40	0.22	7.23	0.62
n-6 LC-PUFA[2]	1.09	0.21	1.07	0.18	0.92	0.22	0.90	0.09	0.79	0.06	0.78	0.06

[1]Calculated excluding 18 C n-3 series.
[2]Calculated excluding 18 C n-6 series.
Fish were sampled at the beginning (June 2007) and at the end of the finishing period (September 2007). Mean values and standard deviations of individual fish are presented (n=8).
Mean values within dietary groups with unlike superscript letters are significantly different ($P<0.05$).

Table 4. Effects of finishing diet on fillet weight, wet lipid content and FA profile (% of total FAME) in fish fed 33VO diet and then FO diet (33VO/FO group).

	Jun (+0)		Jul (+27)		Aug (+55)		Sep (+88)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fillet (g)	59.52^{a}	5.73	74.58^{b}	14.8	98.66^{c}	8.59	126.1^{d}	12.5
Lipid (%)	10.97	3.20	11.44	1.82	13.19	1.73	12.36	1.62
Σ FAs (mg/g)	62.46	18.8	68.26	23.5	69.64	12.6	63.84	19.9
FA (%)								
14 :0	4.24^{a}	0.52	4.39^{ab}	0.36	4.55^{ab}	0.35	4.98^{b}	0.32
15:0	0.27	0.14	0.17	0.08	0.17	0.07	0.15	0.08
16:0	17.62^{a}	0.70	18.29^{b}	0.40	18.77^{b}	0.52	19.30^{b}	0.36
16:1n-7	5.96^{a}	0.47	6.48^{ab}	0.20	6.97^{b}	0.32	7.15^{b}	0.12
16:2	0.60^{a}	0.10	0.78^{b}	0.14	0.74^{b}	0.25	0.72^{b}	0.22
16:3	0.82^{a}	0.08	0.94^{ab}	0.07	1.01^{b}	0.06	1.08^{b}	0.10
16:4	0.52^{a}	0.06	0.55^{ab}	0.24	0.62^{ab}	0.21	0.71^{b}	0.12
17:0	0.24	0.02	0.26	0.03	0.40	0.28	0.30	0.05
18:0	3.76	0.17	3.89	0.08	3.70	0.18	3.93	0.10
18:1 n-9	23.21^{a}	1.05	22.61^{ab}	0.98	21.84^{ab}	0.88	21.58^{b}	0.78
18:1 n-7	2.56^{a}	0.12	2.75^{b}	0.12	2.89^{bc}	0.14	2.94^{c}	0.12
18:2 n-6	12.32^{a}	0.75	11.61^{b}	0.60	10.77^{c}	0.29	10.16^{d}	0.17
18:3 n-6	0.25^{a}	0.02	0.29^{b}	0.01	0.31^{c}	0.01	0.33^{d}	0.01
18:3 n-3	7.39^{a}	0.93	5.78^{ab}	0.45	4.74^{bc}	0.12	3.61^{c}	0.13
18:4 n-3	0.82	0.02	0.76	0.30	0.97	0.07	0.90	0.34
20:0	0.19	0.01	0.19	0.01	0.18	0.01	0.20	0.02
20:1 n-7	0.15^{a}	0.01	0.17^{ab}	0.01	0.18^{b}	0.01	0.19^{b}	0.01
20:1 n-9	1.02^{a}	0.05	1.11^{b}	0.09	1.14^{b}	0.05	1.11^{b}	0.04
20:1 n-11	0.20	0.02	0.21	0.01	0.22	0.03	0.22	0.02
20:2 n-6	0.28	0.06	0.32	0.03	0.29	0.04	0.25	0.07
20:3 n-6	0.23	0.04	0.24	0.03	0.24	0.03	0.24	0.03
20:3 n-3	0.23^{a}	0.09	0.22^{a}	0.13	0.15^{b}	0.01	0.17^{b}	0.13
20:4 n-6	0.40	0.12	0.43	0.11	0.47	0.03	0.46	0.11
20:4 n-3	0.51	0.03	0.55	0.03	0.58	0.03	0.55	0.02
20:5 n-3	5.57^{a}	0.39	6.06^{a}	0.52	6.83^{b}	0.31	6.91^{b}	0.22
22:0	0.16	0.06	0.14	0.03	0.13	0.05	0.17	0.07
22:1 n-9	0.31	0.02	0.30	0.01	0.31	0.03	0.30	0.01
22:1 n-11	0.72^{a}	0.05	0.77^{ab}	0.03	0.80^{b}	0.05	0.80^{b}	0.02
22:5 n-3	1.92	0.12	2.10	0.09	2.20	0.20	2.16	0.11
22:6 n-3	3.92	0.21	4.08	0.41	4.08	0.28	4.19	0.21
24:1 n-9	0.31	0.03	0.33	0.03	0.32	0.04	0.30	0.02
Saturates	26.47^{a}	1.64	27.34^{a}	0.19	27.89^{ab}	0.88	29.03^{b}	0.46
Monoenes	34.45	1.81	34.72	1.11	34.71	0.79	34.67	0.86
n-3 LC-PUFA[1]	12.15^{a}	0.84	13.00^{ab}	0.83	13.85^{ab}	0.65	13.97^{b}	0.46
n-6 LC-PUFA[2]	0.92	0.22	0.99	0.10	1.01	0.05	0.95	0.11

[1]Calculated excluding 18 C n-3 series. [2]Calculated excluding 18 C n-6 series. Fish were sequentially sampled through the finishing period (June, +0; July, +27; August, +55; September, +88). Mean values and standard deviations of individual fish are presented (n=8). Raw values with unlike superscript letters are significantly different over sampling time (P<0.05).

Table 5. Effects of finishing diet on fillet weight, wet lipid content and FA profile (% of total FAME) in fish fed 66VO diet and then FO diet (66VO/FO group).

	Jun (+0)		Jul (+27)		Aug (+55)		Sep (+88)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fillet (g)	58.23^{a}	13.2	71.61^{b}	11.1	97.65^{c}	9.33	116.2^{d}	12.9
Lipid (%)	9.33	2.02	10.30	3.27	11.81	2.51	11.15	1.11
Σ FAs (mg/g)	59.76	14.2	56.27	20.6	67.46	17.6	55.14	7.23
FA (%)								
14 :0	2.78^{a}	0.32	3.27^{a}	0.34	3.68^{ab}	0.27	4.38^{b}	0.39
15:0	0.27	0.22	0.10	0.06	0.18	0.09	0.16	0.09
16:0	16.58^{a}	0.36	17.63^{b}	0.62	18.14^{b}	0.54	18.81^{c}	0.61
16:1n-7	3.85^{a}	0.14	4.81^{ab}	0.31	5.60^{bc}	0.19	6.20^{c}	0.13
16:2	0.27^{a}	0.09	0.39^{ab}	0.11	0.55^{b}	0.20	0.59^{b}	0.19
16:3	0.43^{a}	0.23	0.63^{a}	0.09	0.70^{ab}	0.21	0.91^{b}	0.07
16:4	0.24^{a}	0.02	0.31^{ab}	0.15	0.50^{bc}	0.05	0.60^{c}	0.08
17:0	0.25	0.09	0.28	0.17	0.28	0.12	0.34	0.20
18:0	3.85	0.12	3.86	0.18	3.81	0.13	3.85	0.21
18:1 n-9	27.06^{a}	0.71	25.89^{b}	1.43	24.62^{c}	0.58	22.80^{d}	0.90
18:1 n-7	2.00^{a}	0.12	2.31^{b}	0.11	2.46^{c}	0.16	2.66^{d}	0.09
18:2 n-6	15.02^{a}	0.31	13.81^{b}	0.48	12.67^{c}	0.31	11.69^{d}	0.34
18:3 n-6	0.14^{a}	0.01	0.20^{ab}	0.02	0.23^{bc}	0.01	0.27^{c}	0.02
18:3 n-3	12.94^{a}	0.33	10.35^{b}	0.47	8.36^{c}	0.45	6.31^{d}	0.50
18:4 n-3	0.60^{a}	0.06	0.61^{ab}	0.23	0.83^{b}	0.03	0.92^{c}	0.07
20:0	0.18	0.02	0.19	0.02	0.18	0.00	0.20	0.02
20:1 n-7	0.11^{a}	0.01	0.13^{bc}	0.01	0.14^{c}	0.01	0.17^{d}	0.01
20:1 n-9	0.96^{a}	0.03	1.00^{ab}	0.06	1.05^{bc}	0.03	1.08^{c}	0.06
20:1 n-11	0.17	0.02	0.20	0.02	0.18	0.02	0.20	0.02
20:2 n-6	0.32	0.07	0.30	0.13	0.24	0.09	0.30	0.04
20:3 n-6	0.24	0.11	0.25	0.06	0.22	0.04	0.24	0.05
20:3 n-3	0.34^{a}	0.04	0.30^{ab}	0.03	0.26^{ab}	0.05	0.24^{b}	0.09
20:4 n-6	0.23^{a}	0.03	0.32^{ab}	0.10	0.33^{ab}	0.06	0.39^{b}	0.08
20:4 n-3	0.42^{a}	0.02	0.47^{b}	0.06	0.49^{bc}	0.02	0.52^{c}	0.02
20:5 n-3	2.88^{a}	0.18	3.90^{b}	0.61	5.01^{c}	0.22	5.86^{d}	0.31
22:0	0.14	0.03	0.17	0.06	0.13	0.04	0.18	0.04
22:1 n-9	0.29	0.01	0.30	0.02	0.29	0.01	0.30	0.02
22:1 n-11	0.58^{a}	0.01	0.65^{b}	0.03	0.69^{c}	0.03	0.76^{d}	0.03
22:5 n-3	1.19^{a}	0.04	1.40^{b}	0.09	1.61^{c}	0.06	1.87^{d}	0.11
22:6 n-3	2.57^{a}	0.21	3.14^{b}	0.38	3.28^{b}	0.26	3.65^{c}	0.42
24:1 n-9	0.31	0.04	0.33	0.03	0.32	0.04	0.32	0.03
Saturates	24.04^{a}	0.67	25.50^{b}	1.10	26.39^{b}	0.74	27.92^{b}	0.92
Monoenes	35.18	0.84	35.50	1.66	35.29	0.46	34.47	0.86
n-3 LC-PUFA[1]	7.40^{a}	0.22	9.21^{b}	0.65	10.65^{c}	0.47	12.13^{d}	0.75
n-6 LC-PUFA[2]	0.79	0.06	0.86	0.18	0.80	0.09	0.93	0.09

[1]Calculated excluding 18 C n-3 series. [2]Calculated excluding 18 C n-6 series. Fish were sequentially sampled through the finishing period (June, +0; July, +27; August, +55; September, +88). Mean values and standard deviations of individual fish are presented (n=8). Raw values with unlike superscript letters are significantly different over sampling time (P<0.05).

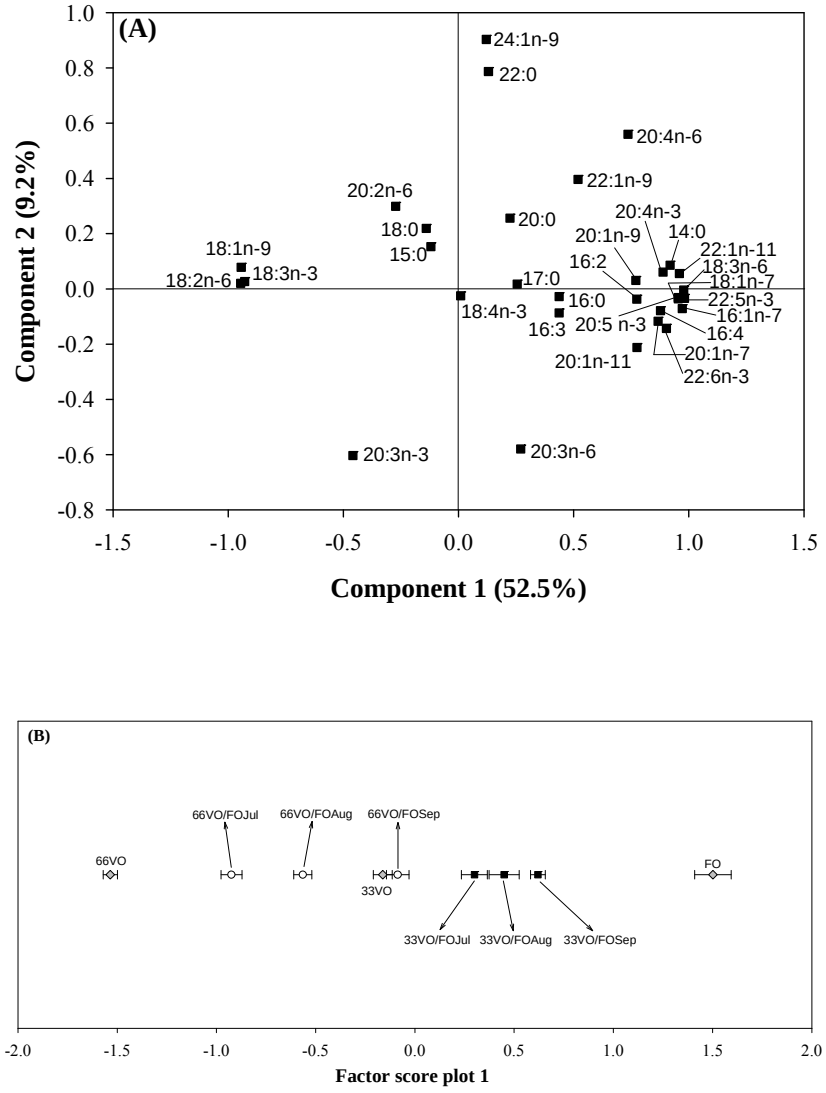

Fig. 3. Component plot (A) and factor score plot (B) of the MPCA for the fillet FA profile through the 3-month finishing period (June–September). Temporal series (July, August and September) derived from 33VO/FO and 66VO/FO fish are represented in factor score 1 as mean±SEM (n=8). No temporal changes were found for FO, 33VO and 66VO groups and data from June and September (initial and final steps of finishing period) are represented as one point for each group.

Regardless of nutritional background (33VO and 66VO diets), the concordance between the observed FA values (x-axis) and those predicted by the dilution model (y-axis) was extremely high at the end of the finishing period (**Fig. 4**). This gave a regression line with a slope very close to 1 (0.96–0.95) when 32 FAs were considered in the models derived from both 33VO/FO and 66VO/FO fish. Similar slopes (1.04–1.05) were obtained when calculations were repeated for 14 selected FAs having a high weight in the MPCA.

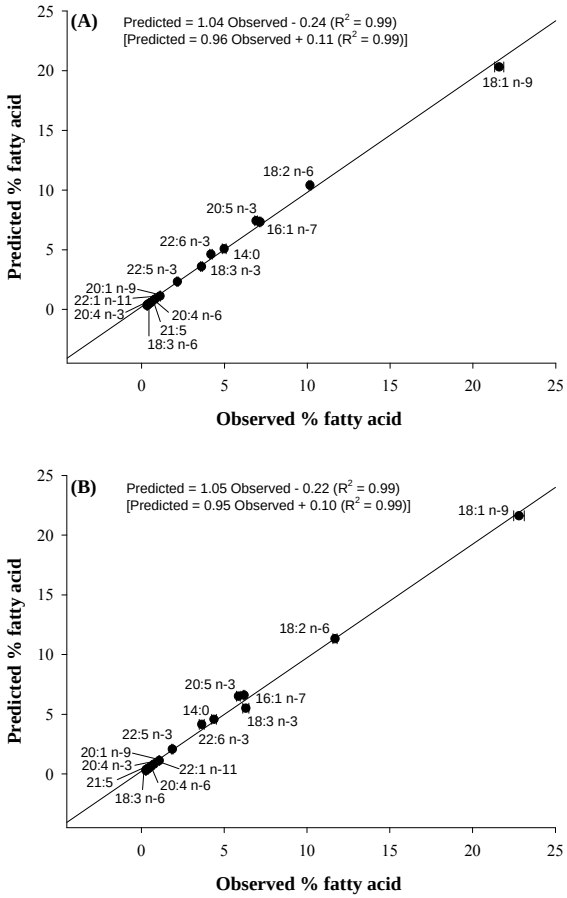

Fig. 4. Plot prediction (dilution model) in 33VO/FO (A) and 66VO/FO (B) groups of the tissue FA profile at the end of the finishing period. Observed values are the mean±SEM of 8 fish per treatment. The solid line is the plotted regression. The equations were calculated considering both the selected and all (square brackets) the identified FAs.

6.4. Discussion

Data reported here, along with those of Benedito-Palos et al. (2008) over an 8-month feeding trial, convincingly demonstrate that dietary fish oils of northern and southern origin can be replaced by up to 66% without negative effects on the growth performance of gilthead sea bream. These data are novel in a stenohaline marine teleost maximizing the simultaneous replacement of fish meal and fish oil by alternative plant ingredients without histopathological signs of liver and intestine damage (Benedito-Palos et al., 2008). Other metabolic effects are complex, interconnected and, to date, not fully understood. However, it must be noted that growth-compensatory mechanisms are orchestrated at the local tissue level (skeletal muscle) by the somatotropic axis when rapidly growing gilthead sea bream juveniles are fed high levels of vegetable oils (Benedito-Palos et al., 2007). Besides, n−3 LC-PUFA are selectively incorporated into polar lipids, and the stability of muscle phospholipid FA composition is a useful and complementary criterion to assess the suitability of the replacement strategy in fish feeds with low levels of marine derived ingredients (Benedito-Palos et al., 2008).

In fish and higher vertebrate species, neutral lipids are less conservative than phospholipids (Tocher, 2003; Skalli and Robin, 2004; Schulz et al., 2005). This is because they are the fat storage form and its FA profile highly reflects that of the diet. In the present study, the muscle lipid content was greater than 10% on a wet matter basis, and the FA profile of total lipids and thereby that of triacylglycerols (TAG) remained mostly unchanged through the finishing period in fish always fed either FO, 33VO or 66VO diets. The result of these temporal series agrees with data on a previous seasonal study (Benedito-Palos et al., 2008), and reinforces the idea that accelerated growth of farmed fish might override most of the changes in the flesh FA profile (Grigorakis, 2007). However, it should be born in mind that the tissue-specific FA profile varies in salmonids with the size and age of fish (Bell et al., 2002, 2003a,b). Fish oil replacement by alternative lipid sources has also a pronounced effect on the tissue FA profile of fish, and we found in fish always fed 33VO and 66VO diets a 22–36% increase of 18:1n−9

and 18:2n−6 with a concurrent 20–65% reduction in EPA and DHA. Similar results have been reported in gilthead sea bream (Izquierdo et al., 2005) and a wide variety of fish species, including Atlantic salmon (Bell et al., 2002, 2003b; Bransden et al., 2003; Bell, 2004; Torstensen et al., 2004; Nanton et al., 2007), rainbow trout (Drew et al., 2007), turbot (Regost et al., 2003), European sea bass (Montero et al., 2005; Mourente and Bell, 2006), Murray cod (Francis et al., 2007a,b), red sea bream (Huang et al., 2007; Piedecausa et al., 2007) and black sea bream (Peng et al., 2008). Since this feature can compromise the beneficial effects of seafood (Din et al., 2004; Psota et al., 2006) as the main source of EPA and DHA in the human diet, there is increased interest in finfish aquaculture for modelling the time-course of FA changes during fish oil re-feeding.

Gilthead sea bream shows, in our latitude, a pronounced growth seasonality (Mingarro et al., 2002), and for the most effective restoration of EPA and DHA, the finishing window should take place in the broadly active feeding period of May–October. Thus, after the growth stop of winter, one month was spent before the start of the finishing diet period that then was continued through the summer (June–September). Several variables, including among others the growth and lipid deposition rates, need to be considered when analysing the effectiveness of fish oil wash-out. Therefore, one must be cautious before drawing a definitive conclusion, but the literature is prolific on studies in which a complete restoration of the FA profile was not fully achieved after fish oil re-feeding: 32 days in red sea bream (Glencross et al., 2003), 8 weeks in turbot (Regost et al., 2003); 8 weeks in brown trout (Robin et al., 2003), 12–25 weeks in Atlantic salmon (Bell et al., 2003a,b; Bell, 2004; Torstensen et al., 2004); 3 months in gilthead sea bream (Izquierdo et al., 2005); 4 months in Murray cod (Turchini et al., 2006); and 5 months in European sea bass (Montero et al., 2005). Most evidence points toward a dilution model, which was proven in turbot, brown trout and Murray cod. Using original data and those derived from red sea bream (Glencross et al., 2003) and Atlantic salmon (Bell et al., 2003a) studies, Jobling (2004a,b) concluded that a dilution process also plays a key role in governing the muscle FA profile of these fish species. Our work in gilthead sea bream points clearly toward the same direction,

and gradual changes in the FA profiles of the 33VO/FO and 66VO/FO groups were found during the finishing period, making them increasingly similar to the FO group. This is particularly highlighted by the results of the MPCA that shows the gradient of fish oil load along the ordinate axis.

Therefore, changes in the FA profile arise because the existing stores become diluted as fish grow and deposit increasing amounts of dietary derived FAs. In other words, nutritional background in fish with no apparent signs of FA deficiencies has a marginal role on FA turnover and tissue FA profiles, although age- and nutritional condition affect the expression pattern of cytokines and key limiting enzymes on tissue FA uptake and mobilization (Saera-Vila et al., 2005, 2007). Thus, using the simple dilution model, a reliable FA prediction was found herein at the end of the fish oil finishing period regardless of the level of fish oil replacement. The model is in fact a good general descriptor of FAs, and regression curves (predicted vs observed values) give slopes nearby to the line of equality when either selected or almost all FAs were considered in the model. In Atlantic salmon, Jobling (2004b) tested three FAs (18:1 isomers, 18:2 n−6 and 18:3 n−3) and confirmed closely the predictions made with the dilution model. Jobling (2004a) again evaluated the dilution model with data from red sea bream studies (Glencross et al., 2003), and a high degree of concordance was found between the predicted and observed values. However, lipid retention is a tissue and fish-specific process, and probably the concordance with the dilution model will be higher in species with high lipid tissues, which may explain why the predictions are better in salmonids and sparid fish than in Murray cod (Turchini et al., 2006) or Atlantic cod (Jobling et al., 2008). Thus, our gilthead sea bream study highlights that fish grown-out with the 33VO diet need more than 12 weeks to revert back the FA composition toward the normal variability of fish fed fish oil-based diets. Besides, low and intermediate levels of fish oil replacement can produce equally acceptable fillets when the latter is accompanied by a fish oil finishing phase. This is because temporal changes on fish oil intake gave a minor effect on the muscle FA profile if the absolute amount becomes equal at the end of the trial. In our experimental model, this was the case for the 33VO and

66VO/FO groups, and the tissue FA profile at the end of the 3-month finishing period was very close in both groups.

Savings on fish oil resources are therefore limited to a simple dilution, and new approaches are required to improve any mobilisation or turnover of pre-existing FAs. Intake of conjugate linoleic acid complex (CLA) has a lipid-lowering effect in gilthead sea bream juveniles, promotes the diversion of dietary-derived TAG from muscle and adipose tissue to liver, and increase hepatic peroxisomal β oxidation (Diez et al., 2007). At the same time, however, LC-PUFA biosynthesis is reduced and changes in the muscle FA profile indicate that the inclusion of CLA in aquaculture diets would be of little benefit in gilthead sea bream. Nevertheless, the use of more specific agonists/antagonists of peroxisome proliferator-activated receptors (PPARs) cannot be excluded to improve the retention of n−3 LC-PUFA. Attention also needs to be focused on the transfer from fish oil of PCBs, dioxins and other harmful lipophilic organic chemicals that are now ubiquitous contaminants in the marine ecosystems (Sargent et al.,1995; Jacobs et al., 2002; Bell et al., 2005; Domingo, 2007). The effects of feeding strategies on toxic-kinetics will be reported separately to have a more complete framework of nutritional fish tailoring, and to gain public acceptance for the fish fed with alternative and sustainable diets.

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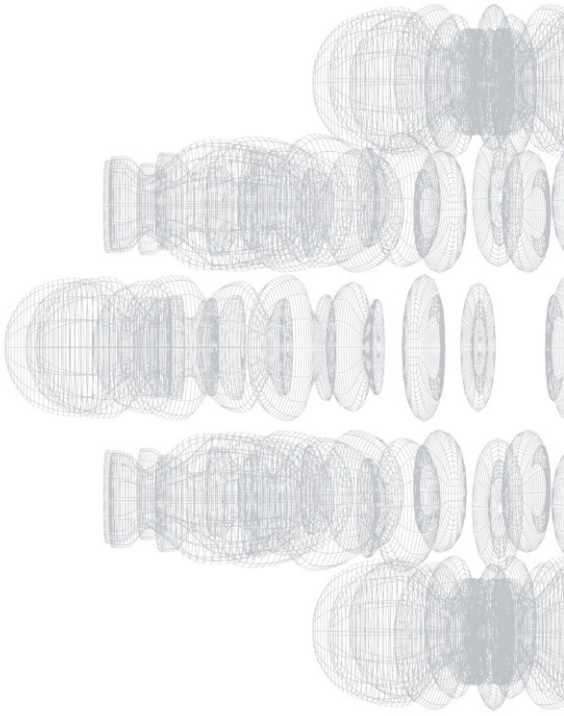
CAPÍTULO 7

DISCUSIÓN GENERAL



Los resultados de la presente Tesis Doctoral han puesto de manifiesto que las harinas y los aceites de pescado de los piensos de engorde de dorada pueden sustituirse en gran medida por ingredientes de origen vegetal. Por tanto, se pueden utilizar piensos de alto contenido en ingredientes vegetales sin afectar al crecimiento del animal. Sin embargo, para establecer el nivel máximo de sustitución conjunta de harinas y aceites de pescado, hay otros factores que deben tenerse en consideración, puesto que en último término el producto final va destinado a un consumidor cada vez más exigente y mejor informado que demanda un producto con unas determinadas propiedades organolépticas y claros efectos beneficiosos sobre la salud. De este modo, resulta necesario un adecuado control de todos los eslabones de la cadena alimentaria (**Figura 7.1**). En este sentido, los resultados descritos en los capítulos 3-6, junto con otros derivados del proyecto AQUAMAX, se han abordado de forma conjunta en el último capítulo de la presente Tesis Doctoral.

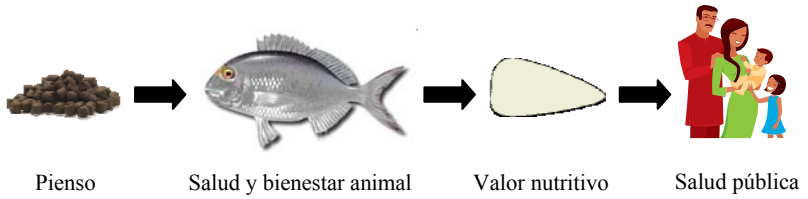


Figura 7.1. Cadena alimentaria del pescado de crianza.

7.1. Crecimiento y nivel de sustitución de aceites de pescado

Los resultados obtenidos en la primera experiencia de engorde (**capítulos 3 y 4**) pusieron de relieve que es posible un alto nivel de sustitución del aceite de pescado con dietas basadas en proteínas vegetales (15-20% de inclusión de harinas de pescado, ~75% de sustitución de las harinas de pescado por proteínas vegetales) sin detrimento aparente del potencial de crecimiento. Según Kalogeroupolos y cols. (1992), los requerimientos de EPA+DHA en juveniles de dorada son del 0,9% en peso seco. Por tanto, no es de extrañar que la dieta

66VO, con un contenido similar de EPA+DHA, no provocase efectos negativos sobre el crecimiento de los animales. Sin embargo, la sustitución total del aceite de pescado en la dieta VO provocó un descenso del contenido de EPA+DHA hasta un 0,3%, con la consiguiente reducción de la ingesta y de las tasas de crecimiento.

En todo caso, los requerimientos de EPA y DHA en juveniles de dorada están posiblemente sobrestimados. De hecho, se ha podido comprobar que una dieta con 0,7% en peso seco de EPA+DHA no provoca efectos negativos sobre el crecimiento (Bouraoui y cols., 2010). Dicha experiencia se realizó en el IATS con una dieta basada en una sustitución del 50% de las harinas de pescado y el 100% de los aceites de pescado por ingredientes vegetales (dieta PP50:VO).

El engorde con la dieta PP50:VO se llevó a cabo conjuntamente con la primera experiencia de cultivo, descrita en los **capítulos 3 y 4**. Los resultados no mostraron diferencias significativas entre el crecimiento de los animales alimentados con la dieta PP50:VO y los alimentados con la dieta FO, utilizada como control (**Figura 7.2**). Ello es debido a que las harinas de pescado también contienen cierta cantidad de aceites, que al final del proceso de fabricación se estima en un 8-10% por término medio (Bimbo, 2000). De este modo, y con independencia de la capacidad biosintética de la especie, tanto en salmónidos (Bell y cols., 2003a; Brandsden y cols., 2003; Torstensen y cols., 2004) como en especies marinas (Regost y cols., 2003; Piedecausa y cols., 2007), es posible sustituir totalmente el aceite de pescado por aceites vegetales, siempre y cuando la cantidad de harinas de pescado sea suficiente para cubrir los requerimientos en ácidos grasos esenciales y fosfolípidos del animal.

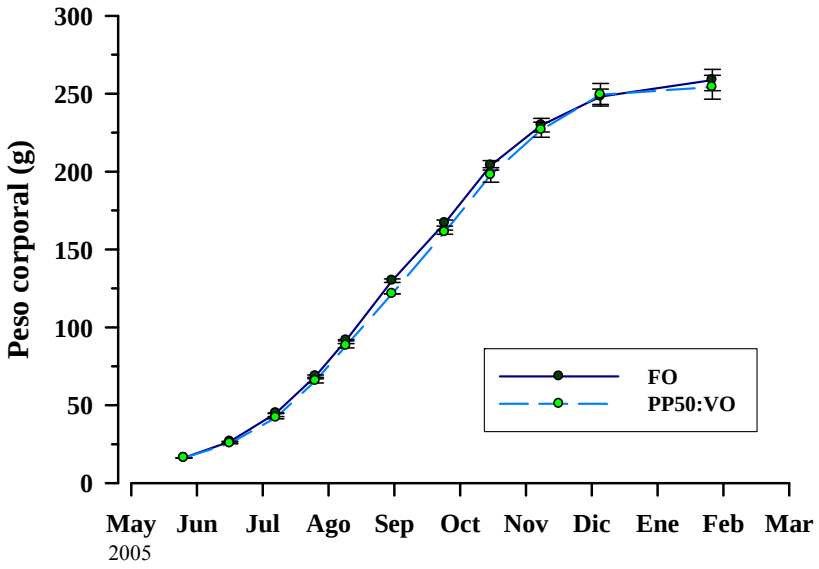


Figura 7.2. Crecimiento de juveniles de dorada en las instalaciones de cultivo del IATS. FO, animales alimentados con una dieta basada en aceite de pescado y 15% de inclusión de harinas de pescado. PP50:VO, animales alimentados con una dieta basada en aceites vegetales (100% de sustitución) y 35% de inclusión de harinas de pescado. En ningún punto de muestreo se encontraron diferencias significativas entre ambos grupos experimentales (p<0,05).

Con independencia de la cantidad total de EPA y DHA, hay múltiples evidencias en la bibliografía sobre los efectos del cociente EPA/DHA sobre el crecimiento de peces en cultivo. La influencia de este cociente es especialmente evidente durante las primeras fases del desarrollo larvario (Mourente y cols., 1993; Ibeas y cols., 1997; Rodríguez y cols., 1998), aunque no están totalmente descartados ciertos efectos del cociente EPA/DHA sobre el crecimiento de juveniles-adultos de dorada u otras especies de peces. Desde un punto de vista práctico, este hecho podría modificar el nivel óptimo de sustitución de acuerdo con el origen del aceite de pescado. Es bien sabido que el aceite de pescado procedente del hemisferio norte contiene mayor cantidad de DHA que de EPA, mientras que en el aceite del sur esta relación es inversa (Sargent y cols., 1997; Dalsgaard y cols.,

2003; Pratoomyot y cols., 2008). Estos cambios también afectan al cociente EPA/DHA, tal y como se observa al comparar la composición de las dietas del primer y segundo engorde, formuladas con aceite de pescado de distinta procedencia. Sin embargo, en términos de crecimiento, este hecho no limitó la viabilidad de la estrategia de sustitución de la dieta 66VO. Así pues, parece concluyente que, con independencia del origen del aceite de pescado, se puede sustituir un alto porcentaje del mismo (66%) sin afectar negativamente el crecimiento de la dorada.

Además de criterios meramente biométricos, la viabilidad de la estrategia de sustitución también se validó mediante el uso de diferentes marcadores hormonales, histopatológicos y bioquímicos.

Marcadores hormonales

El sistema somatotrópico juega un papel fundamental en la regulación del crecimiento, habiéndose demostrado en la dorada que este sistema hormonal responde rápidamente a cambios en la ración (Pérez-Sánchez y cols., 1995; Company y cols., 1999), edad del animal (Saera-Vila y cols., 2007) y variaciones estacionales de fotoperiodo y temperatura (Pérez-Sánchez y cols., 1994; Mingarro y cols., 2002; Pérez-Sánchez y cols., 2002a; Saera-Vila y cols., 2007).

Como patrón general, en estados catabólicos se desencadena una resistencia hepática a la acción anabólica de la hormona del crecimiento (GH), comportando en primera instancia una disminución del número de receptores hepáticos de GH (GHR). Ello supone una disminución de la expresión hepática de somatomedina (IGF-I), que es la fuente más importante de IGF-I circulante. El descenso de IGF-I circulante se traduce, a su vez, en un pronunciado aumento de los niveles circulantes de GH, como consecuencia de un menor efecto en la retroalimentación negativa de la IGF-I sobre la síntesis y liberación hipofisiaria de GH al torrente circulatorio (Pérez-Sánchez y cols., 1992) (**Figura 7.2.**).

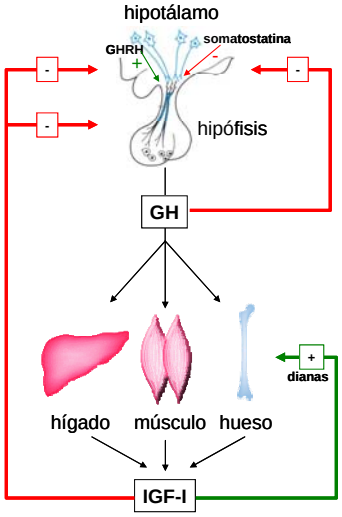


Figura 7.2. Secreción y modos de accción de la GH. La somatostatina y la hormona liberadora de GH (GHRH) se sintetizan en el hipotálamo y controlan la secreción hipofisiaria de GH. La GH puede unirse a sus receptores de membrana en tejidos diana. Ello se traduce en un aumento de la IGF-I circulante, provocando respuestas promotoras de crecimiento sobre los propios tejidos e inhibiendo la liberación de GH y GHRH.

En el **capítulo 3** se describen los efectos del régimen dietario sobre los diferentes componentes del eje somatotrópico. Para ello, se utilizaron juveniles de doradas del primer engorde alimentadas con las cuatro dietas experimentales (FO, 33VO, 66VO y VO) tras 3 meses de experimentación, coincidiendo con la época estival de máximo crecimiento. Los resultados pusieron de relieve una compleja regulación del sistema somatotrópico, que posiblemente responde a diferentes requerimientos nutricionales y cargas de biocontaminantes en la dieta.

Las deficiencias en ácidos grasos esenciales de la dieta VO, como resultado de la sustitución total del aceite de pescado por aceite vegetal, estuvieron acompañadas de una reducción del crecimiento, de los niveles circulantes de IGF-I y de la expresión hepática de las IGF y los GHR a nivel hepático y muscular. Los niveles circulantes de GH no variaron significativamente aunque sí se observó una tendencia al aumento en consonancia con el descenso de la IGF-I circulante.

En ausencia de alteraciones significativas sobre el crecimiento (FO, 33VO, 66VO), los niveles más altos de IGF-I circulante se alcanzaron en los peces alimentados con la dieta 33VO. Ello sugiere la existencia de ciertos mecanismos de acción compensatoria del crecimiento, en los peces alimentados con las dietas 66VO y FO entre los diferentes componentes del eje somatotrópico a nivel local (para mejor comprensión, ver **Figura 7 del capítulo 3**):

- Como respuesta a una menor ingesta de ácidos grasos esenciales, los peces alimentados con la dieta 66VO mostraron una reducción en la expresión hepática de GHR-I e IGF-I, que aparentemente se vio compensada por un aumento de la expresión del GHR-II a nivel muscular.

- En los peces alimentados con la dieta FO, la reducción de la expresión hepática de GHR-I e IGF-I fue aparentemente compensada por una mayor expresión a nivel muscular de la IGF-II. En este caso, la dieta presenta unos niveles en ácidos grasos esenciales que cubren con creces los requerimientos del animal. Sin embargo, la actividad hepática puede verse comprometida por una mayor actividad de detoxificación, como resultado de la mayor carga de contaminantes de las dietas basadas en aceites de origen marino (ver apartado 7.5). Como prueba de ello, los estudios de expresión génica con biomarcadores del metabolismo xenobiótico muestran una disminución progresiva de la transcripción hepática del citocromo P450-1A1 y del receptor de dioxinas AhR-1 con la sustitución del aceite de pescado en la dietas 33VO, 66VO y VO. Al mismo tiempo, aumentó el cociente glutatión reducido/oxidado, lo que evidencia un menor riesgo de daño oxidativo en los animales alimentados con mayor cantidad de aceites vegetales (ver Saera-Vila y cols., 2009).

Marcadores histopatológicos

La arquitectura del hígado y del intestino puede verse notablemente alterada, tanto por el aumento elevado de la ración alimenticia (Sitja-Bobadilla y col., 2003), como por la carencia de ciertos nutrientes en la dieta. En este sentido, se ha documentado la aparición de desórdenes metabólicos en peces alimentados con dietas deficientes en fosfolípidos (Olsen et al. 1999; 2003) y ácidos grasos poli-insaturados omega-3 de cadena larga (Caballero et al. 2003; Mourente y cols., 2005). El resultado es la presencia masiva de gotas lipídicas en el intestino y un excesivo acúmulo graso en el hígado que puede acabar convirtiéndose en esteatosis hepática.

En el caso de los salmónidos, se ha observado que la acumulación de gotas lipídicas en el intestino de peces alimentados con dietas basadas en proteínas vegetales desaparece con la suplementación de la dieta con fosfolípidos en forma de lecitina de soja (Olsen y cols., 1999; 2003). En el caso de la dorada, estudios previos también sugieren la aparición de deficiencias en fosfolípidos cuando se alimenta a juveniles de rápido crecimiento con dietas donde las harinas de pescado se sustituyen totalmente por proteínas vegetales (Sitjà-Bobadilla y cols., 2005). Basado en ello, el componente proteico de las dietas utilizadas en la presente Tesis Doctoral fue adecuadamente suplementado con un 1% de lecitina de soja que actúa como fuente natural de fosfatidilcolina. En estas circunstancias, a pesar del alto contenido energético de las dietas experimentales (22% de lípidos), no se detectaron daños histopatológicos en el tejido intestinal, relacionado con altas tasas de conversión del alimento de todos los grupos experimentales analizados en el primer engorde.

Respecto al hígado, el porcentaje de lípidos varió de un 15% a un 25% a medida que aumentó la sustitución de aceite de pescado, aunque sólo se encontraron signos evidentes de esteatosis hepática en unos pocos peces alimentados con la dieta VO, teóricamente deficiente en ácidos grasos esenciales (ver **Figura 2 del capítulo 4**). Por el contrario, en trabajos anteriores, en ausencia de una suplementación de fosfolípidos, los síntomas

de esteatosis hepática ya fueron evidentes con un acúmulo graso del orden del 15% (Sitjà-Bobadilla et al. 2005).

En el segundo engorde (**capítulos 5 y 6**), con las dietas de sustitución parcial seleccionadas a partir de los resultados obtenidos tras el primer engorde, tampoco se detectaron daños histopatológicos en ninguno de los tejidos analizados (datos no mostrados).

Parámetros bioquímicos (ácidos grasos)

En el **capítulo 4** se describe el efecto de la sustitución del aceite de pescado sobre el perfil de ácidos grasos muscular tras 8 meses de cultivo (primer engorde completo) con las dietas FO, 33VO, 66VO y VO, alcanzando un peso final del orden de 300 g. La composición de ácidos grasos de los lípidos totales se mantuvo prácticamente invariable durante verano-otoño-invierno y se mostró como un reflejo del perfil de la dieta en cada uno de los grupos experimentales (ver **figura 3 del capítulo 4**). Por el contrario, el perfil de ácidos grasos de los fosfolípidos es mucho más robusto y menos influenciable por la dieta, debido a la retención selectiva de ciertos ácidos grasos por parte de la fracción polar, con el fin de mantener la fluidez de las membranas celulares (Henderson y Tocher, 1987; Sargent y cols., 2002). De este modo, la sustitución parcial no provocó un cambio significativo en el perfil de ácidos grasos fosfolipídico, ya que los requerimientos teóricos en ácidos grasos esenciales estaban teóricamente cubiertos por las dietas 33VO y 66VO. En contraposición, el análisis multivariante mostró el perfil de los fosfolípidos de los animales alimentados con la dieta VO como un grupo diferencial, debido a la deficiencia en ácidos grasos esenciales (ver **figura 4 del capítulo 4**).

Llegados a este punto, basándose en criterios biométricos, hormonales, histológicos y bioquímicos, se puede afirmar que la sustitución parcial del aceite de pescado (dietas 33VO y 66VO) en dietas basadas en proteínas vegetales, no produce alteraciones fisiológicas aparentes en juveniles de dorada con altas tasas de crecimiento y de conversión del alimento.

Aún así, existen otras funciones fisiológicas que podrían verse alteradas con la sustitución del aceite de pescado. Por ello, al finalizar el segundo ciclo completo de engorde (**capítulo 5**), se examinó la robustez de los distintos patrones tisulares de ácidos grasos en peces alimentados con las dos dietas extremas utilizadas durante este engorde (FO, 66VO). Para ello, se analizaron los cambios en las clases lipídicas y el perfil de ácidos grasos de músculo esquelético, cerebro, hígado y tejido adiposo mesentérico. Los resultados indicaron que, mientras que los lípidos neutros reflejan el perfil de ácidos grasos de la dieta con independencia del tejido analizado (ver **Figura 1 del capítulo 5**), el perfil de los fosfolípidos es característico de cada tejido y se mantiene prácticamente invariable frente a la dieta (ver **Figura 2 del capítulo 5**). Esta robustez de los fosfolípidos confirmaría que los requerimientos en ácidos grasos esenciales están cubiertos por la estrategia de sustitución de la dieta 66VO. De hecho, el cerebro fue el tejido que menos varió su perfil, mostrando una gran capacidad para preservar un fenotipo típicamente marino. Así pues, a pesar de ser el tejido con mayores requerimientos en DHA, el tejido neural se mostró especialmente conservativo. Estos resultados sugieren que la dieta 66VO no sólo cubre los requerimientos en ácidos grasos esenciales en términos de crecimiento, sino que también podría preservar un amplio rango de funciones neurológicas con efectos sobre el comportamiento que de otro modo resultan difíciles de evaluar.

7.2. Criterios de sostenibilidad. Análisis "FIFO"

El balance FIFO (del inglés "*Fish-in : Fish-out ratio*") es un parámetro que se ha introducido recientemente para calcular la cantidad de pescado salvaje necesaria para producir 1 kg de pescado de acuicultura (Jackson, 2009; Kaushik y Troell, 2010). Basándose en la recuperación y en las proporciones de harinas y aceites de pescado utilizados para la producción de piensos, el balance FIFO se calcula de la siguiente manera:

$$FIFO = \left[\frac{harina\ en\ pienso}{RH} + \frac{aceite\ en\ pienso}{RA} \right] \times ICA$$

RH, rendimiento de la extracción de harina de pescado (24%).
RA, rendimiento de la extracción de aceite de pescado (5%).
ICA, conversión del alimento (pienso ingerido/incremento biomasa).

En los piensos comerciales de dorada, el nivel de inclusión de harinas y aceites de pescado es del orden de 400 g/kg y 150 g/kg, respectivamente (Kaushik y Troell, 2010). Con esta formulación y un índice ICA del 1,2, el valor del FIFO es 5,6 o lo que es lo mismo, para producir 1 kg de dorada de crianza se requieren 5,6 kg de pescado salvaje. Tras los resultados de la presente Tesis Doctoral, el FIFO obtenido a lo largo de un ciclo completo de engorde de 14 meses con la dieta 66VO, que contiene 150 g/kg de harina de pescado y 51,5 g/kg de aceite, es de 1,9. De este modo, con menos de 2 kg de pescado salvaje se puede obtener 1 kg de pescado de crianza de un alto valor añadido en el mercado (**Tabla 7.1**).

En relación con lo expuesto en el apartado anterior, otra alternativa con resultados similares de FIFO es la que se obtiene con la dieta PP50:VO, en la que se sustituye totalmente el aceite de pescado por aceite vegetal a expensas de una mayor inclusión de harina de pescado en la dieta.

Tabla 7.1. Cantidad de harina y aceite de pescado (g/kg) en diferentes piensos y resultado del cálculo del balance FIFO.

Dieta	Harina de pescado	Aceite de pescado	**FIFO**
Comercial	400	150	**5,6**
FO	150	150	**4,3**
33VO	150	101	**3,1**
66VO	150	51,5	**1,9**
PP50:VO	350	0	**1,7**

En todo caso, la estrategia de sustitución de las harinas y los aceites de pescado, individualmente o de modo combinado, dependerá de la disponibilidad y los valores de mercado de las diferentes fuentes de materias primas. En este sentido, el aumento actual del precio de los aceites vegetales ha hecho que los productores de piensos primen la sustitución de las harinas de pescado con ingredientes vegetales frente a la de los aceites. Así, la multinacional Skretting (Stavanger, Noruega) cambió recientemente la línea de piensos de engorde de dorada "Excel", con 27% de harina de pescado y un 9% de aceite de pescado, por la línea "Optibream" formulada íntegramente con aceite de pescado y un 21% de harina de pescado.

7.3. Valor nutritivo del producto final. Cinéticas de ácidos grasos

En el apartado anterior, el valor del FIFO se ha fijado únicamente basándose en criterios de crecimiento. No obstante, si se quiere garantizar la calidad y el valor nutritivo del pescado como la fuente más importante de EPA y DHA para el consumo humano, se deben considerar los efectos del régimen alimenticio sobre el contenido graso y la composición en ácidos grasos de los filetes de peces de talla comercial. Por ello, ante el uso cada vez más generalizado de aceites vegetales en piensos de engorde de peces marinos, se recomienda el uso de dietas finalizadoras con aceites de pescado durante la última fase de producción para revertir, al menos en parte, el perfil del filete a un patrón típicamente marino (Bell y cols., 2003b; Regost y cols., 2003; Mourente y cols., 2005). En este sentido, como demanda clara del sector, el estudio de las cinéticas de ácidos grasos en aquellas porciones destinadas al consumo humano resulta necesario.

Comparar adecuadamente pescado salvaje y pescado de crianza es una tarea enormemente compleja, dada la notable variabilidad que existe a nivel local y estacional en el contenido graso de los peces salvajes (ver página web de composición nutricional de los productos acuícolas: www.nutraqua.com). En general, la denominación de pescado azul y pescado blanco alude

esencialmente a la proporción de grasa inserta en la carne del animal. El pescado azul contiene más de un 5% de grasa, mientras que el pescado blanco contiene cantidades próximas o inferiores al 2%. Existe una clase intermedia, los pescados semigrasos, como la lubina y la dorada, que contienen cantidades de grasa entre un 2% y un 5%. Sin embargo, el cultivo intensivo o semi-intensivo de estas especies comporta un incremento notable del acúmulo graso, por lo que el filete de estos peces en el mercado suele contener entre un 6-10% de grasa (Grigorakis y cols., 2002; Orban y cols., 2003).

En la medida en que el perfil de ácidos grasos se mantenga invariable (peces salvajes *vs* peces cultivo), el mayor contenido graso del pescado de crianza disminuye la ingesta recomendada de pescado en la dieta humana según los requerimientos de EPA y DHA (Cahu y cols, 2004). Sin embargo, el perfil de ácidos grasos del filete de especies grasas o semigrasas es altamente dependiente de la composición de la dieta, por lo que un incremento del contenido graso del animal no comporta necesariamente un mayor contenido en términos absolutos de EPA y DHA. Estudios epidemiológicos y de intervención nutricional indican que el consumo de estos ácidos grasos produce efectos beneficiosos para la salud, especialmente al reducir el riesgo de padecer enfermedades cardiovasculares (revisado por Din y cols., 2004; He, 2009). En este sentido, la Sociedad Internacional para el Estudio de Ácidos Grasos y Lípidos (ISSFAL) sugiere una ingesta diaria de 500 mg de EPA+DHA en adultos y un mínimo de 200 mg de DHA en el caso de mujeres embarazadas y lactantes. Además, en caso de padecer enfermedades coronarias, la Sociedad Americana del Corazón (AHA) recomienda 1 g diario de EPA+DHA y de 2 a 4 g diarios para pacientes con hipertrigliceridemia.

Ahora bien, si se tienen en cuenta las recomendaciones de ingesta de EPA+DHA, no existe pescado suficiente para asumirlas a nivel global. Con una población de 6.000 millones de personas y unas recomendaciones diarias de ingesta de EPA+DHA de 500 mg, el requerimiento global es de 3 billones de mg de EPA+DHA al día, lo que supone un requerimiento de 1.095.000 Tm de EPA+DHA al año. Si la disponibilidad global

de pescado es de 100 millones de Tm al año, del cual sólo el 50% es comestible con un contenido graso del 5% y un 15% de EPA+DHA, se obtendrían tan solo 375.000 Tm/año de EPA+DHA, por lo que el déficit global es del orden de 750.000 Tm/año (**Tabla 7.2**).

Tabla 7.2. Requerimiento y disponibilidad global de EPA+DHA.

Demanda de EPA+DHA	**1.095.000 Tm/año**
Pescado disponible	100.000.000 Tm/año
Comestible (50%)	50.000.000 Tm/año
Contenido graso (5%)	2.500.000 Tm/año
EPA+DHA (15%) disponible	**375.000 Tm/año**
Déficit global	**>700.000 Tm/año**

Ante este déficit de EPA y DHA para el consumo humano, el margen de maniobra es bastante limitado. Algunas de las alternativas planteadas (por ejemplo, re-utilización de subproductos y uso de microalgas y plantas transgénicas como fuente adicional de EPA y DHA) ya han sido tratadas en la introducción general de la presente Tesis Doctoral. No obstante, estas medidas son sólo paliativas, ya que la tecnología actual no permite la producción a gran escala de fuentes adicionales de EPA y DHA para su uso en la cadena de producción animal. Es más, la actual legislación europea de organismos genéticamente modificados limita enormemente cualquier cambio en la actual estructura productiva.

En tal caso, todas aquellas medidas que comporten un ahorro de los ácidos grasos poli-insaturados omega-3 de cadena larga como fuente de energía para el organismo comportarán, en última instancia, una mayor disponibilidad de EPA y DHA para el consumo humano. Por sí mismo este argumento justifica el uso de aceites vegetales en la formulación de piensos de engorde de peces marinos. No obstante, aunque se lleguen a cubrir los requerimientos en ácidos grasos esenciales del pez, se corre el riesgo de producir un producto final con un bajo contenido en

EPA y DHA (revisado por Turchini y cols., 2009). Es por ello, que con el uso de dietas basadas en fuentes alternativas de aceites de pescado, el estudio de las cinéticas de ácidos grasos cobra especial relevancia.

Idealmente, los cambios que se producen en el perfil de ácidos grasos con el uso de aceites vegetales deberían poderse revertir rápidamente con dietas finalizadoras ricas en EPA y DHA. El **capítulo 6** de la presente Tesis Doctoral describe la experiencia realizada a tal efecto, con el fin revertir el perfil de los peces alimentados con las dietas 33VO y 66VO a un perfil más próximo al de peces alimentados permanentemente con aceite de pescado. Tras un periodo de engorde de 11 meses con las dietas 33VO y 66VO en el que los peces alcanzaron un peso de aproximadamente 300 g, éstos fueron alimentados con la dieta finalizadora (FO) durante 3 meses hasta alcanzar un peso final de aproximadamente 530 g. El cambio en el perfil de ácidos grasos del filete fue progresivo y el análisis de componentes principales lo mostró como un gradiente de la cantidad ingerida de aceite de pescado a lo largo de todo el ciclo de producción (ver **Figura 3 del capítulo 6**).

Estos cambios en el perfil de ácidos grasos reflejan un modelo de dilución simple, similar al inicialmente descrito por Robin y cols. (2003) en rodaballo y trucha marina. En el caso de la dorada, el modelo fue extensible a la práctica totalidad del conjunto de ácidos grasos identificados en las muestras de filetes de tamaño comercial con un alto nivel de confianza (ver F**igura 4 del capítulo 6**). Estos resultados indican que el resultado final en el perfil de ácidos grasos del filete de doradas en cultivo depende exclusivamente de la cantidad total ingerida de cada ácido graso durante todo el engorde, con escasa incidencia de la secuencia temporal. Consecuentemente, tras la fase finalizadora, los peces alimentados con la dieta 66VO presentaron un perfil prácticamente indistinguible del mostrado por animales alimentados continuamente (sin dieta finalizadora) con la dieta 33VO, ya que en ambos grupos experimentales el consumo de aceite de pescado al final de la experiencia fue del mismo orden (ver **Figura 2 del capítulo 6**).

Así pues, para conseguir un nivel alto de EPA y DHA en el filete de pescado, los peces deberán alimentarse inevitablemente con una cantidad significativa de aceites marinos, bien utilizando una dieta de alto contenido en aceite marino, o bien con el uso de dietas finalizadoras de alto contenido en EPA y DHA. El resultado final de ambas estrategias (33VO, 66VO/FO) es un contenido absoluto de EPA+DHA en el filete similar, o incluso superior, al de las doradas salvajes que presentan un contenido graso notablemente inferior al de las doradas en cultivo.

La generación de este conocimiento constituye por sí mismo una herramienta de gran utilidad frente a posibles controles de calidad del valor nutritivo del pescado (perfil de ácidos grasos) destinado al consumo humano. Así, ante cambios no programados en la composición de la dieta, existe cierta capacidad de respuesta con el uso de una adecuada dieta finalizadora. En todo caso, la propia naturaleza del modelo de dilución simple hace que la reversión total a un perfil típicamente marino sea prácticamente imposible en un periodo limitado de tiempo.

Los programas de selección genética pueden ayudar a mejorar la utilización de los lípidos y el crecimiento en general (Glencross y cols., 2007). En este sentido, algunos autores sugieren un control genético para favorecer la deposición de ácidos grasos poli-insaturados de cadena larga durante el proceso de finalización aunque, en estos casos, la rápida incorporación inicial de estos ácidos grasos en los animales seleccionados acaba igualándose con la del grupo control (Bell y cols., 2010). En otros estudios se ha intentado promover la biosíntesis de ácidos grasos omega-3 durante el cultivo larvario de peces marinos con dietas deficientes en EPA y DHA, sin embargo, esa capacidad no ha podido mantenerse hasta el estado juvenil (Vagner y cols., 2009). Por consiguiente, los resultados obtenidos hasta la fecha son menos esperanzadores de lo que cabría esperar en un principio, por lo que es necesario un mayor esfuerzo investigador para comprender los mecanismos últimos de regulación del metabolismo lipídico en peces.

7.4. Análisis instrumental "nariz electrónica"

Las propiedades organolépticas o sensoriales son percibidas directamente por el consumidor al comprar y comer el producto. Los consumidores juegan un papel fundamental en la aceptación de los alimentos y, aunque muchos otros aspectos influyen en su decisión (precio, publicidad, cultura), si las propiedades sensoriales se alteran, el producto no tendrá cabida en el mercado. Por tanto, cuando se producen cambios en el régimen alimenticio de los animales, la calidad sensorial e instrumental del producto final no debe verse significativamente comprometida.

La alimentación del pescado puede influir en sus características organolépticas, como el color, la textura, el aroma, o el sabor. La apariencia externa del pescado refleja en cierto modo su historia alimenticia. La pigmentación de la piel de la dorada está relacionada con la ingesta de carotenoides (Gomes y cols., 2002; Gouveia y cols., 2010). Sin embargo, al contrario de lo que sucede con los salmónidos (Storebakken y No, 1992; Wathne y cols., 1998), los carotenos no parecen acumularse en el músculo de la dorada (Gouveia y cols., 2010) y su color está más relacionado con el nivel de grasa, otorgándole un color más blanco cuanto mayor es su contenido graso (Grigorakis y cols., 2002). La textura y la jugosidad del filete también son propiedades correlacionadas con el contenido graso (Izquierdo y cols., 2003; Grigorakis, 2007). Además, la contribución lipídica influye en el aroma y el sabor, ya que los lípidos por sí mismos tienen un sabor característico y los ácidos grasos poli-insaturados actúan como precursores de algunos compuestos aromáticos volátiles (Grigorakis, 2007).

Por tanto, si se comparan doradas salvajes con doradas de cultivo, el diferente contenido graso de ambos grupos puede alterar notablemente las propiedades organolépticas del producto final (Grigorakis y cols., 2003). Sin embargo, cabe reseñar que los resultados de los paneles sensoriales suelen relacionar el mayor contenido graso de los animales en cultivo con una percepción de mayor frescura (Pérez-Sánchez y cols., 2002b).

Entre las doradas de cultivo, cuando los requerimientos en aminoácidos esenciales están cubiertos por la dieta, la incorporación de proteínas vegetales en el pienso no provoca cambios en las propiedades sensoriales (de Francesco y cols., 2007). La sustitución parcial del aceite de pescado por aceites vegetales en piensos basados en harinas de pescado tampoco parece provocar grandes cambios en las propiedades organolépticas del filete (Menoyo y cols., 2004; Fountoulaki y cols., 2009), aunque estudios previos revelan una ligera influencia del aceite de soja en el aroma y el sabor del filete, junto con una leve reducción de la dureza y aumento de la jugosidad (Izquierdo y cols., 2003; 2005).

Con el fin de evaluar la calidad sensorial del filete de pescado, dentro del marco del proyecto AQUAMAX, la empresa Alpha M.O.S. (Toulouse, Francia) analizó los efectos de las dietas 33VO y 66VO (con y sin fase finalizadora) sobre la percepción olfatoria del producto. Los análisis se realizaron basándose en el método objetivo de "la nariz electrónica" como alternativa a los paneles sensoriales. Con los equipos Fox 4000 y Heracles (Flash E-Nose), se analizaron 15 muestras de filete por grupo experimental. Estos sistemas detectaron ligeras variaciones aromáticas que podrían asociarse con la cantidad de aceites vegetales de la dieta. Sin embargo, la discriminación entre grupos no fue suficiente como para identificar aromas específicos ligados a un determinado régimen alimenticio, especialmente después de la fase finalizadora (**Figura 7.4**).

Por consiguiente, a pesar de su notable sensibilidad, estas medidas instrumentales no fueron capaces de discernir diferencias en el aroma entre los filetes de las doradas alimentadas con las diferentes dietas y procedimientos experimentales empleados en el segundo engorde, contemplado en la presente Tesis Doctoral. Estos resultados han sido confirmados mediante pruebas de cata (análisis descriptivo cuantitativo, QDA) con panelistas semi-entrenados realizadas por el grupo de Fisiología de la Facultad de Biología de la Universidad de Barcelona (García de la Serrana, 2009).

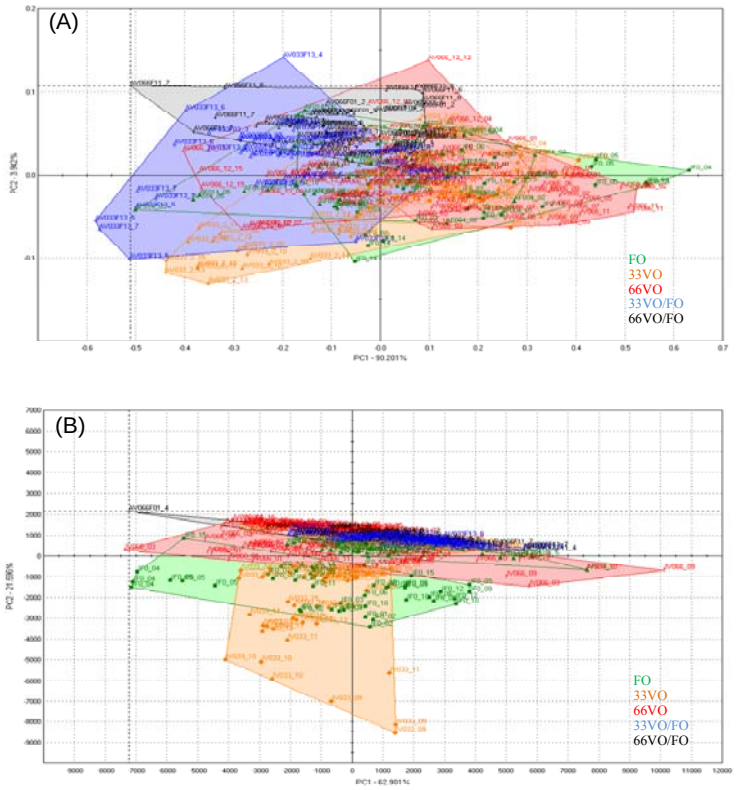


Figura 7.4. Análisis de componentes principales de los compuestos volátiles de doradas pertenecientes a los grupos FO, 33VO, 66VO, 33VO/FO y 66VO/FO analizados según el sistema Fox (A) y Heracles (B). Con ambos sistemas de medida, los efectos del régimen de alimentación no son significativos y la variación intra-grupos es comparable a la variación inter-grupos.

7.5. Seguridad alimentaria

Frente a los beneficios que presenta su consumo, uno de los principales problemas que afecta al pescado es el nivel de contaminantes químicos (tanto orgánicos como inorgánicos) que pueden incorporar las distintas especies comerciales (Hites y cols., 2004; Corsolini y cols., 2005; Dórea, 2008). Este hecho podría llegar a limitar su consumo en determinados grupos de riesgo (niños, mujeres embarazadas y lactantes).

En los piensos comerciales de uso en acuicultura, el aceite de pescado es la fuente más importante de contaminantes orgánicos persistentes (policlorobifenilos, policlorobifenilos de tipo dioxina, pesticidas organoclorados e hidrocarburos policíclicos aromáticos), mientras que las harinas de origen marino suelen contener metales pesados (mercurio, cadmio, arsénico, etc.) (Smith y Gangolli, 2002; Dórea, 2009). Por consiguiente, para garantizar la seguridad alimentaria de los consumidores, la UE ha establecido unos límites máximos para la mayoría de estos compuestos, tanto en el pienso como en el pescado destinado al consumo (Reglamento (CE) número 199/2006 de la Comisión de 3 de febrero de 2006).

Por todo ello, el control de las materias primas utilizadas en la formulación de los piensos es esencial para garantizar la disminución, si no la ausencia, de contaminantes que podrían acumularse en el pescado. En este sentido, la sustitución de las harinas y los aceites de pescado por ingredientes vegetales en los piensos puede reducir el nivel de compuestos orgánicos persistentes y metales pesados en el pescado. De este modo, diversos estudios revelan una reducción sustancial de los niveles de residuos de organoclorados tanto en salmón atlántico (Bell y cols., 2005; Berntssen y cols., 2005) como en trucha arcoíris (Drew y cols., 2007). Como ya se ha expuesto reiteradamente en apartados anteriores, el inconveniente en estos casos es la reducción en el nivel de EPA y DHA. Por tanto, debe existir un balance entre los riesgos y los beneficios derivados del consumo de pescado (Mariné y Vidal, 2001).

Como parte de los objetivos del proyecto AQUAMAX, los piensos y filetes de las doradas utilizadas en los trabajos que constituyen la presente Tesis Doctoral fueron analizados por el Instituto Universitario de Plaguicidas y Aguas (IUPA) de la Universitat Jaume I de Castellón.

De los resultados obtenidos se deduce que la sustitución del aceite de pescado por aceites vegetales redujo significativamente el nivel de compuestos orgánicos persistentes en los piensos utilizados en la primera experiencia de engorde (Saera-Vila y cols., 2009) (**Tabla 7.3**). La misma tendencia se observó cuando se analizaron los piensos del segundo engorde (Nácher-Mestre y cols., 2009; 2010) (**Figura 7.5**). Aún así, en todas las dietas experimentales, la carga de la mayor parte de biocontaminantes fue baja, y similar o inferior a los valores previamente descritos en piensos de salmónidos (Easton y cols., 2002; Hites y cols., 2004) y especies marinas como la lubina (Serrano y cols., 2003).

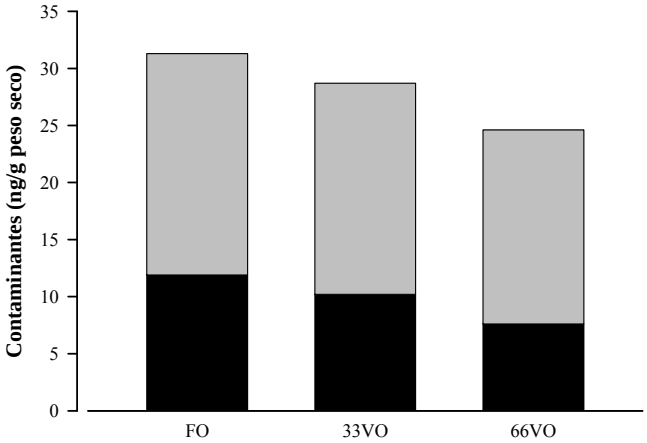


Figura 7.5. Contaminantes en las dietas experimentales del segundo engorde. La suma de policlorobifenilos, policlorobifenilos de tipo dioxina y pesticidas organoclorados aparece en color negro (modificado de Nacher-Mestre y cols., 2009); el contenido de hidrocarburos policíclicos aromáticos aparece en color gris (modificado de Nácher-Mestre y cols., 2010).

Tabla 7.3. Concentración de policlorobifenilos (PCB), policlorobifenilos de tipo dioxina (*), pesticidas organoclorados (OCP), polibromodifenil éteres (PBDE) e hidrocarburos policíclicos aromáticos (PAH) en las dietas experimentales del primer engorde (modificado de Saera-Vila y cols., 2009).

Compuesto (ng/g peso seco)	FO	33VO	66VO	VO
PCB 28+31	0,2	0,1	0,1	< 0,1
PCB 52	0,5	0,3	0,2	0,1
PCB 101	1	0,7	0,4	0,2
PCB 77*	< 0,2	< 0,2	< 0,2	< 0,2
PCB 118*	1	0,8	0,5	0,2
PCB 153	2,5	2	1,5	0,6
PCB 105*	0,4	0,3	0,2	< 0,1
PCB 138	1,4	1,3	0,8	0,4
PCB 126*	< 0,2	< 0,2	< 0,2	< 0,2
PCB 128	0,3	0,2	0,2	< 0,1
PCB 156 *	0,3	0,2	< 0,1	0,1
PCB 180	0,7	0,8	0,5	0,3
PCB 169*	0,2	0,1	< 0,1	< 0,1
PCB 170	0,3	0,3	0,2	< 0,2
HCB	0,6	0,4	0,4	0,2
p,p´-DDT	5,8	4,1	2,6	0,9
p,p´-DDE	2,2	1,7	1,1	0,3
p,p´-DDD	1,2	1,1	0,7	0,3
PBDE 28	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 71	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 47	0,13	0, 12	< 0,1	< 0,1
PBDE 66	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 100	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 99	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 85	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 154	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 153	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 138	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 183	< 0,1	< 0,1	< 0,1	< 0,1
PBDE 209	< 0,1	< 0,1	< 0,1	< 0,1
Naphthaleno	< 0,5	< 0,5	< 0,5	< 0,5
Fluoreno	< 0,3	< 0,3	< 0,3	< 0,3
Fenantreno+Antraceno	0,62	1,50	0,38	< 0,2
Fluoranteno	< 0,2	< 0,2	< 0,2	< 0,2
Pireno	1,33	0,83	< 0,2	< 0,2
Benzo [a] pireno	< 0,2	< 0,2	< 0,2	< 0,2
Benzo [b] fluoranteno	2,68	2,01	2,20	2,06
Indeno [1,2,3-cd] pireno	< 0,2	< 0,2	< 0,2	< 0,2
∑PCB + PCB*	8,66	7,03	4,71	2,22
∑OCP	9,8	7,3	4,8	1,6
∑PAH	9,5	6,5	4,7	4,1

El análisis de contaminantes en las muestras de filete utilizadas para el análisis de las cinéticas de ácidos grasos (descrito en **capítulo 6**) también mostró niveles traza para la mayor parte de contaminantes detectados en los piensos. Como se esperaba, los niveles más altos de organoclorados (pesticidas organoclorados y policlorobifenilos) se encontraron en los filetes de los peces alimentados con la dieta FO (Nacher-Mestre y cols., 2009) (ver **Figura 7.6**).

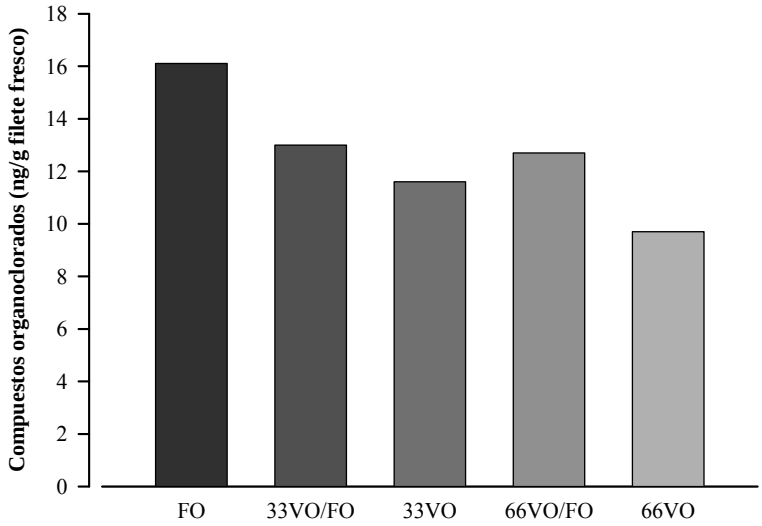


Figura 7.6. Compuestos organoclorados en filete de doradas pertenecientes a los diferentes grupos experimentales tras el periodo de finalización (Modificado de Nacher-Mestre y cols., 2009).

La misma tendencia se observó cuando se analizaron los niveles de hidrocarburos policíclicos aromáticos (PAH), aunque en este caso el uso de la dieta finalizadora hizo aumentar los niveles de PAH por encima de los niveles detectados en los peces alimentados continuamente con la dieta FO (Nacher-Mestre y cols., 2010) (ver **Figura 7.7**).

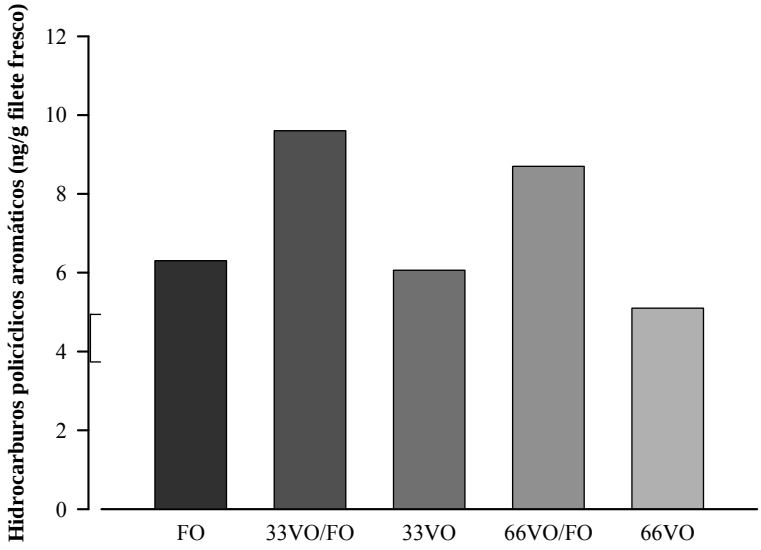


Figura 7.7. Hidrocarburos policíclicos aromáticos en el filete de doradas pertenecientes a los diferentes grupos experimentales tras el periodo de finalización (Modificado de Nacher-Mestre y cols., 2010).

En todo caso, para ambos grupos de contaminantes, el uso de la dieta finalizadora hizo aumentar su concentración en el filete (66VO/FO *vs* 33VO) para una misma ingesta final de aceite de pescado (ver **Figura 2** del **capítulo 6**). Esto sugiere que la capacidad de detoxificación del animal puede ser mayor cuando éste ha sido expuesto previamente a niveles relativamente elevados de contaminantes. Como consecuencia práctica, desde el punto de vista de la seguridad alimentaria, el uso de una única fuente de materias primas durante todo el

engorde parece ser la mejor estrategia. Para solventar este problema, algunos investigadores han propuesto el uso de dietas finalizadoras basadas en aceites de pescado descontaminados (Pratoomyot y cols., 2008). El uso actual de este aceite es todavía limitado, pero esta situación podría cambiar en el futuro si se demuestra que el proceso de descontaminación no comporta una pérdida selectiva de EPA y DHA. En este sentido, se han obtenido resultados satisfactorios en estudios realizados con salmón del atlántico, en los que el proceso de descontaminación no afectó significativamente a la composición del aceite (Pratoomyot y cols., 2008), ni tampoco al crecimiento de los animales (Bell y cols., 2010; Berntssen y cols., 2010; Sprague y cols., 2010).

7.6. Salud y bienestar animal

Los cambios en el régimen de alimentación pueden provocar diferentes alteraciones con efectos sobre la salud y el bienestar del animal, como la aparición de cataratas, deformidades óseas, descamaciones en la piel o alteraciones histopatológicas (Lall and Tibbetts, 2009). Estos síntomas pueden verse agravados por una alta densidad de cultivo, estados de hipoxia y estrés térmico, con efectos directos o indirectos sobre el estado de inmunocompetencia que pueden aumentar la susceptibilidad frente a diferentes patógenos oportunistas (Hosfeld y cols., 2009; Kunttu y cols., 2009).

La complejidad de los diferentes mecanismos que regulan la homeostasis del animal ha sido evaluada recientemente en el IATS con un modelo de estrés por confinamiento mediante estudios de genómica funcional (Calduch-Giner y cols., 2010). En este estudio, el uso de chips de ADN (*microarrays*) específicos de dorada con más de 6000 genes anotados ha permitido definir los efectos del estrés sobre el transcriptoma hepático de dorada. De acuerdo con ello, se ha definido un modelo de respuesta ante el estrés por confinamiento en el que se diferencian de forma secuencial tres grandes etapas:

- 6 h post-confinamiento: rápida movilización de las reservas lipídicas para aumentar la disponibilidad energética del animal.
- 24 h post-confinamiento: respuesta másica en su mayor parte mediada por el retículo endoplasmático rugoso para reparar el daño tisular sufrido.
- 72 h post-confinamiento: re-ajuste de la homeostasis del animal para disminuir el riesgo de daño oxidativo, disminuyendo la producción de radicales libres y aumentado su secuestro.

Actualmente, los efectos del historial nutricional (FO *vs* 66VO) sobre la capacidad de respuesta al estrés por confinamiento están siendo evaluados. Asimismo, ya se han obtenido resultados de los efectos de la dieta sobre el trancriptoma del tracto intestinal, que es una de las primeras barreras del organismo frente al ataque de organismos patógenos (Sitjà-Bobadilla y cols., 2007). Cabe reseñar que la dieta 66VO no provoca cambio alguno sobre el transcriptoma del intestino. No obstante, existen evidencias experimentales de que la progresión de la enfermedad ante un reto infeccioso con el parasito intestinal *Enteromyxum leei* se ve afectada por el estado nutricional del animal.

El parasito *Enteromyxum leei* invade el intestino de la dorada y la infección crónica que conlleva provoca una pérdida progresiva de peso, anorexia, caquexia y finalmente la muerte del animal. Su transmisión puede producirse por contacto directo con animales infectados o por contaminación de los efluentes (revisado por Sitjà-Bobadilla y cols., 2007). Este parásito se utilizó como modelo de reto infeccioso para evaluar la viabilidad de las dietas empleadas en la presente Tesis Doctoral. Los resultados pusieron de relieve que la enfermedad progresa más rápidamente en las doradas alimentadas con la dieta 66VO que en las alimentadas con la dieta control (FO) basada en aceite de pescado (Estensoro y cols., 2010). A nivel trancripcional, este hecho va acompañado de importantes cambios en los patrones de expresión génica (datos no publicados), poniendo de relieve la necesidad de desarrollar a corto-medio plazo diferentes pruebas fisiológicas para evaluar la capacidad de respuesta del animal

ante diferentes perturbaciones de la homeostasis del organismo en distintos modelos experimentales.

A pesar de todo, es importante destacar que mediante estudios más convencionales, se ha podido concluir la posibilidad de un alto grado de sustitución conjunta de harinas y aceites de pescado en los piensos de engorde de dorada sin comprometer, a grandes rasgos, la salud y el bienestar animal. Como se ha mencionado en el **capítulo 4**, únicamente se detectaron hallazgos histopatológicos en el hígado de los peces alimentados con la dieta de sustitución total del aceite de pescado por aceites vegetales (dieta VO). Aún así, la esteatosis hepática se presentó de forma incipiente y esporádica, y el daño histopatológico fue mínimo. Por otro lado, la observación con microscopía óptica de cortes de intestino no reveló cambios aparentes en la estructura del epitelio en ninguno de los grupos experimentales.

La ausencia de signos patológicos en el tejido hepático e intestinal puede ser atribuible, al menos en parte, a la incorporación de la lecitina de soja como suplemento fosfolipídico en las dietas experimentales. De forma general, se asume que los peces en estado juvenil y adulto no presentan requerimientos específicos de fosfolípidos (revisado por Tocher y cols., 2008). No obstante, esta asunción podría estar enmascarada por el hecho de que las dietas basadas en harinas de pescado contienen gran cantidad de fosfolípidos y por tanto, de existir requerimientos, éstos estarían cubiertos sobradamente por la dieta. Sin embargo, la sustitución de las harinas de pescado por proteínas vegetales puede reducir el contenido de fosfolípidos en la dieta y provocar situaciones carenciales, por lo que a corto-medio plazo deberían reevaluarse los requerimientos de fosfolípidos de los peces en cultivo.

Todo ello pone de relieve la compleja relación entre la nutrición, el estado de inmunocompetencia y la susceptibilidad frente a procesos infecciosos, que posiblemente varía a lo largo del ciclo biológico y la naturaleza del agente infeccioso.

7.7. Análisis global

Tal y como se detalla en el apartado **7.2** de la discusión general, se puede alcanzar un valor FIFO de 1,7–1,9 con dietas de engorde basadas en ingredientes vegetales sin afectar al crecimiento de doradas en cultivo hasta que alcanzan la talla comercial. El valor FIFO con piensos comerciales actualmente en uso es del orden de 5–6 (ver **Tabla 7.1**), por lo que la estrategia propuesta con la dieta 66VO, o equivalentes, supone un notable ahorro de materias primas de origen marino. Sin embargo, esta estrategia de sustitución está basada enteramente en los efectos sobre el crecimiento de los animales sin tener en cuenta los efectos que la dieta conlleva sobre la calidad y seguridad alimentaria del producto final.

Por otro lado, en el apartado **7.5** se indica que la concentración de contaminantes del pienso disminuye progresivamente con la sustitución de las materias de origen marino por ingredientes vegetales de origen terrestre. Otra cuestión es que el uso de materias primas alternativas comporte la aparición de contaminantes emergentes. Aún así, podemos asumir que la sustitución de harinas y aceites de pescado en los piensos de engorde en acuicultura mejora la seguridad alimentaria del producto final. Por consiguiente, no parece factible que éste sea un criterio que limite la estrategia de sustitución contemplada en la presente Tesis Doctoral.

Las medidas de calidad sensorial e instrumental también pueden verse afectadas por la composición de la dieta. No obstante, las dietas seleccionadas según criterios de crecimiento tienen una escasa incidencia sobre estos parámetros (apartado **7.4**). Posiblemente, ello es indicativo de la notable capacidad de la dorada para mantener unas determinadas propiedades organolépticas una vez cubiertos los requerimientos nutricionales. Por tanto, *a priori*, éste tampoco es un factor limitante de la estrategia de sustitución conjunta de harinas y aceites de pescado.

Cada vez son más las iniciativas para mejorar la información relativa al valor nutritivo de los productos acuícolas. Un buen ejemplo de ello es la página web de AQUIMER donde se puede obtener información sobre la composición nutricional de más de 50 productos acuícolas (www.nutraqua.com). No obstante, no existe una normativa específica que garantice al consumidor una determinada composición y valor nutritivo del producto destinado al mercado. En parte, ello es debido a que el pescado y los productos acuícolas en general, gozan de una buena imagen para el consumidor, debido principalmente a los beneficios que aporta su alto contenido en ácidos grasos poli-insaturados omega-3 de cadena larga. Cualitativamente, las dietas basadas en aceites de pescado no deben producir grandes cambios en el valor nutritivo del pescado. No obstante, con el uso cada vez más generalizado de aceites vegetales en los piensos de engorde, esta situación puede cambiar significativamente a corto-medio plazo con los efectos que ello comporta en el perfil de ácidos grasos del filete, especialmente en el caso de especies grasas o semigrasas.

En el caso de la dorada, los efectos de la dieta sobre los perfiles tisulares de ácidos grasos se han descrito ampliamente en varios capítulos de la presente Tesis Doctoral, habiéndose concluido según el "modelo de dilución simple" que el perfil de ácidos grasos del filete depende de la ingesta total de cada ácido graso con independencia de su secuencia temporal a lo largo del ciclo de engorde. La sencillez de este modelo facilita enormemente la predicción de la composición final de ácidos grasos del filete. Sin embargo, este hecho también restringe el umbral de sustitución por debajo del cual no se puede sustituir el aceite de pescado por aceites vegetales sin afectar drásticamente a su valor nutritivo para el consumo humano (contenido en EPA+DHA).

Por tanto, en la medida de lo posible y teniendo en cuenta la actual disponibilidad de materias primas, los productos derivados de la acuicultura deberán promover un desarrollo sostenible del sector, a la vez que preservar las propiedades nutritivas propias de la especie en el medio salvaje. Con la incorporación de aceites vegetales en la dieta es inevitable la

presencia en el filete de cantidades relativamente importantes de ácidos grasos poli-insaturados de 18 átomos de carbono, propios de estos aceites. Al mismo tiempo, la sustitución del aceite de pescado produce una disminución relativa (% de ácidos grasos totales) del contenido de los ácidos grasos omega-3 poli-insaturados de cadena larga, especialmente EPA y DHA. Ahora bien, desde un punto de vista dietético, la cantidad de EPA y DHA por gramo de filete de pescado de crianza debe ser similar o comparable a la del salvaje.

En el caso de la dorada, a partir de los resultados de contenido graso y perfiles de ácidos grasos obtenidos, se establece como dieta de referencia, para todo el proceso de engorde, aquella con un contenido mínimo de 150 y 100 g/kg de harinas y aceites de pescado. Estos valores se corresponden con la dieta 33VO u otras equivalentes modificando la proporción de harinas y aceites de pescado.

Dentro del marco del proyecto AQUAMAX se han contemplado aproximaciones similares en otras especies como el salmón atlántico, la trucha arcoíris y la carpa (www.aquamaxip.eu). En todos los casos, se ha conseguido una importante reducción de los valores FIFO con respecto a los del pienso comercial (**Tabla 7.4**). La reducción para la trucha y la carpa ha sido mayor, puesto que se trata de especies de agua dulce que no se alimentan de organismos marinos en su hábitat natural y por consiguiente no tienen requerimientos específicos de EPA y DHA.

Las especies anádromas como el salmón viven gran parte de su vida en el océano alimentándose de moluscos, crustáceos y otros peces. Por tanto, al igual que para la dorada, el uso de materias primas de origen marino en los piensos de engorde resulta necesario para preservar un determinado perfil de ácidos grasos, con independencia de la capacidad biosintética de EPA y DHA a partir del ácido linolénico.

Tabla 7.4. Comparativa del balance FIFO entre dietas basadas en formulaciones comerciales y la estrategia más conservativa de la sustitución conjunta en el proyecto Aquamax. Ingredientes (g/kg).

	Harina de pescado	Aceite de pescado	**FIFO**
Piensos comerciales			
Salmón	350	250	**7,7**
Trucha	300	200	**6,3**
Carpa	200	50	**2,2**
Dorada	400	150	**5,6**
Piensos Aquamax			
Salmón	160	120	**3,6**
Trucha	50	50	**1,4**
Carpa	0	0	**0**
Dorada	150	100	**3,1**

Basado en todo ello, la posibilidad de poder predecir el perfil de ácidos grasos del pescado en función de la composición de la dieta está cobrando especial relevancia. Al igual que sucede con los animales monogástricos terrestres, los peces absorben los ácidos grasos de los alimentos sin modificarlos y por tanto su perfil tisular estará altamente influido por ellos (Chesworth y cols., 1998). En este sentido, ha podido observarse una correlación entre el perfil de ácidos grasos del pienso y el muscular, tanto en salmón (Bell y cols., 2001, 2002, 2003b) como en bacalao del atlántico (Karalazos y cols., 2007). Sin embargo, los resultados obtenidos con estas especies son todavía insuficientes para poder establecer un patrón de predicción realmente fiable. En el caso de la dorada, se ha iniciado la construcción de una base de datos a partir de diferentes experiencias de engorde con perfiles de ácidos grasos de peces de diferente origen alimentados con dietas de distinta composición. Como primer resultado, se ha publicado recientemente un modelo matemático de predicción de la composición en ácidos grasos del filete de doradas de primer año a partir del perfil de la dieta (Benedito-Palos y cols., 2011).

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CONCLUSIONES

PRIMERA: En piensos de engorde de dorada, la sustitución conjunta de harinas y aceites de pescado por ingredientes vegetales no compromete el crecimiento del animal una vez cubiertos los requerimientos teóricos en aminoácidos y ácidos grasos esenciales.

SEGUNDA: La sustitución parcial del aceite de pescado por aceites vegetales modifica la contribución relativa de las IGF y de los GHR de origen hepático y muscular.

TERCERA: Las deficiencias en ácidos grasos esenciales van acompañadas de una inhibición del sistema somatotrópico tanto a nivel sistémico como a nivel muscular.

CUARTA: Las dietas experimentales de sustitución parcial del aceite de pescado no alteran de forma aparente la arquitectura del hígado y del intestino.

QUINTA: El perfil de ácidos grasos del filete de doradas en cultivo de un determinado grupo de edad refleja la composición de la dieta con una escasa influencia estacional.

SEXTA: El perfil de ácidos grasos de los fosfolípidos es característico de cada tejido y se mantiene relativamente constante una vez cubiertos los requerimientos teóricos en ácidos grasos esenciales con las dietas de sustitución parcial.

SÉPTIMA: Los tejidos con mayores requerimientos en ácidos grasos esenciales son a su vez los más conservativos ante cambios en la composición de la dieta.

OCTAVA: Durante la fase finalizadora, los cambios originados en los perfiles de ácidos grasos siguen un modelo de dilución simple, de modo que el perfil final del filete depende de la cantidad total ingerida de cada ácido graso con independencia de la secuencia temporal.

NOVENA: La estrategia más conservativa de sustitución consigue un ahorro de materias primas de origen marino de más del 40% en relación a los actuales piensos comerciales.

RESÚMENES

El sector acuícola es una de las actividades industriales con más demanda de harinas y aceites de pescado. Sin embargo, debido al estancamiento de las pesquerías a nivel mundial, la actividad piscícola no puede estar basada únicamente en las reservas finitas de pescado. Por tanto, la sustitución de harinas y aceites de pescado por fuentes alternativas de materias primas resulta ineludible.

Para abordar esta problemática, se analizó el efecto de dietas basadas en una sustitución conjunta de proteínas y aceites de pescado por ingredientes vegetales sobre el crecimiento y el perfil de ácidos grasos de doradas (*Sparus aurata*). El diseño de las dietas se realizó sustituyendo el aceite de pescado por una mezcla de aceites vegetales (17:58:25 de colza:linaza:palma) en un 0% (FO), 33% (33VO), 66% (66VO), 100% (VO). Todas las dietas se formularon con alta proporción de proteínas vegetales y se suplementaron con 1% de lecitina de soja y 0,55% de L-lisina.

No se encontraron diferencias en el crecimiento de los animales alimentados con las dietas FO, 33VO y 66VO. Sin embargo, los peces alimentados con la dieta VO mostraron un leve pero significativo descenso de la biomasa y una menor ingesta del alimento. La dieta VO produjo una reducción en los niveles circulantes de somatomedina (IGF-I) y en la expresión de IGF a nivel hepático y muscular. Los niveles más altos de IGF-I se encontraron en los peces alimentados con la dieta 33VO, mientras que los alimentados con las dietas FO y 66VO mostraron valores intermedios. La tendencia de los niveles circulantes de hormona del crecimiento (GH) fue opuesta a la de la IGF-I, posiblemente como resultado de la retroalimentación negativa. En los peces alimentados con las dietas FO y 66VO, se observó una regulación compensatoria entre los componentes del eje somatotrópico, mediada a nivel local por la IGF-II y el receptor de la GH tipo II. En cuanto a los parámetros histopatológicos, la dieta no alteró la integridad del tejido intestinal en ninguno de los grupos experimentales y sólo se observaron signos de esteatosis hepática leve en los peces alimentados con la dieta VO. El perfil de ácidos grasos de los lípidos totales del músculo varió, reflejando la composición de la dieta, y se mantuvo estable con independencia de la estación. La sustitución del aceite de pescado produjo un aumento de 18:2n-6, 18:3n-3 y 18:1n-9 junto con una reducción de 20:5n-3 y 22:6n-3. Sin embargo, el perfil de ácidos grasos de los fosfolípidos fue más constante entre los grupos experimentales y sólo se encontraron cambios significativos en el perfil de los peces alimentados con la dieta VO, posiblemente debido a unos niveles insuficientes de ácidos grasos

esenciales en la misma. La robustez de los distintos patrones tisulares de ácidos grasos se examinó en músculo, hígado, tejido adiposo mesentérico y cerebro de peces alimentados con las dietas FO y 66VO. El perfil de ácidos grasos de los fosfolípidos fue característico del tejido analizado y se mantuvo prácticamente invariable frente al efecto de la dieta. El perfil de ácidos grasos del cerebro fue el más conservativo y no se vio alterado por la dieta, pudiendo indicar indirectamente que las funciones neurológicas no se vieron afectadas. Con el fin de revertir el perfil de ácidos grasos del filete de los peces alimentados con las dietas 33VO y 66VO a uno más próximo al de peces alimentados permanentemente con aceites marinos, durante la última fase de producción se utilizó la dieta FO como finalizadora. Los cambios originados en el perfil de ácidos grasos del filete se produjeron como resultado de un proceso de dilución, dependiendo expresamente de la cantidad absoluta de los distintos ácidos grasos ingeridos durante todo su ciclo de producción. Como conclusión general, es posible la sustitución del 66% del aceite de pescado por aceites vegetales en piensos basados en proteínas vegetales sin afectar al crecimiento de la dorada. Aún así, hay otros factores que deben tenerse en consideración como la calidad sensorial, seguridad alimentaria, salud y bienestar animal.

El sector aqüícola és una de les activitats industrials amb més demanda de farines i olis de peix. No obstant això, a causa de l'estancament de les pesqueries a nivell mundial, l'activitat piscícola no pot estar basada únicament en les reserves finites de peix. Per tant, la substitució de farines i olis de peix per fonts alternatives de matèries primeres resulta ineludible.

Per abordar esta problemàtica, es va analitzar l'efecte de dietes basades en una substitució conjunta de proteïnes i olis de peix per ingredients vegetals sobre el creixement i el perfil d'àcids grassos de daurades (*Sparus aurata*). El disseny de les dietes es va realitzar substituint l'oli de peix per una mescla d'olis vegetals (17:58:25 de colza:llinós:palma) en un 0% (FO), 33% (33VO), 66% (66VO), 100% (VO). Totes les dietes es van formular amb alta proporció de proteïnes vegetals i es van suplementar amb 1% de lecitina de soja i 0,55% d'L-lisina.

No es van trobar diferències en el creixement dels animals alimentats amb les dietes FO, 33VO i 66VO. No obstant això, els peixos alimentats amb la dieta VO van mostrar un lleu però significatiu descens de la biomassa i una menor ingesta de l'aliment. La dieta VO va produir una reducció en els nivells circulants de somatomedina (IGF-I) i en l'expressió d'IGF a nivell hepàtic i muscular. Els nivells més alts d'IGF-I es van trobar en els peixos alimentats amb la dieta 33VO, mentre que els alimentats amb les dietes FO i 66VO van mostrar valors intermedis. La tendència dels nivells circulants d'hormona del creixement (GH) va ser oposada a la de l'IGF-I, possiblement com a resultat de la retroalimentació negativa. En els peixos alimentats amb les dietes FO i 66VO, es va observar una regulació compensatòria entre els components de l'eix GH/IGF, mediada a nivell local per l'IGF-II i el receptor de la GH tipus II. Quant als paràmetres histopatològics, la dieta no va alterar la integritat del teixit intestinal en cap dels grups experimentals i només es van observar signes d'esteatosi hepàtica lleu en els peixos alimentats amb la dieta VO. El perfil d'àcids grassos dels lípids totals del múscul va variar, reflectint la composició de la dieta, i es va mantindre estable amb independència de l'estació. La substitució de l'oli de peix va produir un augment de 18:2n-6, 18:3n-3 y 18:1n-9 junt amb una reducció de 20:5n-3 i 22 :6n-3. No obstant això, el perfil d'àcids grassos dels fosfolípids va ser més constant entre els grups experimentals i només es van trobar canvis significatius en el perfil dels peixos alimentats amb la dieta VO, possiblement a causa d'uns nivells insuficients d'àcids grassos essencials en aquesta. La robustesa

dels distints patrons tissulars d'àcids grassos es va examinar en múscul, fetge, teixit adipós mesentèric i cervell de peixos alimentats amb les dietes FO i 66VO. El perfil d'àcids grassos dels fosfolípids va ser característic del teixit analitzat i es va mantindre pràcticament invariable enfront de l'efecte de la dieta. El perfil d'àcids grassos del cervell va ser el més conservatiu i no es va veure alterat per la dieta, podent indicar indirectament que les funcions neurològiques no es van veure afectades. A fi de revertir el perfil d'àcids grassos del filet dels peixos alimentats amb les dietes 33VO i 66VO a un perfil més pròxim al de peixos alimentats permanentment amb olis marins, durant l'última fase de producció es va utilitzar la dieta FO com finalitzadora. Els canvis originats en el perfil d'àcids grassos del filet es van produir com a resultat d'un procés de dilució, depenent expressament de la quantitat absoluta dels distints àcids grassos ingerits durant tot el seu cicle de producció. Com a conclusió general, és possible la substitució del 66% de l'oli de peix per olis vegetals en pinsos basats en proteïnes vegetals sense afectar el creixement de la daurada. Encara així, hi ha altres factors que han de tindre's en consideració com la qualitat sensorial, seguretat alimentària, salut i benestar animal.

Aquaculture is one of the major consumers of fish meal and fish oil. However, the continuous expansion of aquaculture and the decreasing global availability of marine oil and fish meal force the industry to explore alternative and sustainable alimentary sources for fish feeds.

To address this issue, the feasibility of combined replacement of fish meal and oil by vegetable ingredients was investigated in gilthead sea bream (*Sparus aurata*) and dietary effect on growth performance and tissue fatty acid profiles were examined. Four diets with a low level of fish meal inclusion were supplemented with lysine (0.55%) and contained soy lecithin (1%). Added oil was either fish oil (FO diet) or a blend of a vegetable oil mixture (17:58:25 of rapeseed:linseed:palm oils) replacing fish oil at 33, 66 and 100% (33VO, 66VO and VO diets).

No detrimental effects on growth performance were found with the partial fish oil replacement (FO compared to 33VO and 66VO diets), but feed intake and growth rates were slightly although significantly reduced in fish fed the VO diet. The VO diet reduced plasma levels and muscle and hepatic expression of insulin-like growth factor-I (IGF-I). The highest concentration of plasma levels of IGF-I was attained in fish fed 33VO diet, whereas intermediate values were found in fish fed FO and 66VO diets. An opposite trend was found for circulating levels of growth hormone (GH), probably as a result of a reduced negative feedback inhibition from circulating IGF-I. In fish fed FO and 66VO diets different growth compensatory mechanisms mediated by IGF-II and GH receptor type II were observed at the local tissue level. Regarding to histological alterations, the replacement strategy did not damage the intestinal epithelium and signs of lipoid liver disease were only found in fish fed the VO diet. Muscle fatty acid profiles of total lipids reflected the diet composition regardless of season. Increased amounts of 18:2n-6, 18:3n-3 and 18:1n-9 in combination with reduced proportions of 20:5n-3 and 22:6n-3, were found with the progressive replacement of fish oil by vegetable oils. However, fatty acid composition of phospholipids was more constant among FO, 33VO and 66VO groups, and only significant changes were found in VO group, probably because this is an essential fatty acids deficient diet. Tissue-specific robustness of fatty acid signatures was analysed in muscle, liver, mesenteric adipose tissue and brain of fish fed FO and 66VO diets. The fatty acid composition of phospholipids showed tissue-specific differences although it remained almost unaltered by the diet effect. Brain showed the greatest

robustness to preserve its specific profile being reasonable to assume that a wide range of neural associated functions were preserved. Attempting to restore the fillet fatty acid profile of fish fed 33VO and 66VO to the original optimal fatty acid make-up of fish continuously fed marine oils, FO diet was used as a finishing diet. Changes in the tissue fatty acid profile followed a dilution process and depending specifically on the absolute amount of different fatty acids consumed during complete production cycle. In summary, 66% of fish oil replacement by vegetable oils is possible in diets containing largely plant-based ingredients without adverse effects on gilthead sea bream growth. Even then, other factors must be borne in mind as sensorial quality, food safety, fish health and welfare.