Efecto de las dietas de doble sustitución y las condiciones de cultivo sobre la capacidad digestiva y absortiva de dorada y lubina

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EFECTOS DE LAS DIETAS DE DOBLE SUSTITUCIÓN Y LAS CONDICIONES DE CULTIVO SOBRE LA CAPACIDAD DIGESTIVA Y ABSORTIVA DE DORADA Y LUBINA

Tesis Doctoral

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Memoria presentada por

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Tesis realizada bajo la dirección de la Dra. Mª Ángeles Gallardo Romero del Departamento de Fisiología e Inmunología, Facultad de Biología.

Adscrita al Programa de Acuicultura

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A mi marido y a mis padres

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Introducción

1. Introducción

Según la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), la acuicultura se define como: “la cría de organismos acuáticos como los peces, moluscos, crustáceos y las plantas acuáticas. Esta actividad supone la intervención en el proceso de producción, a través por ejemplo, de reposición constante, alimentación, protección contra los depredadores, etc. También supone la propiedad individual o conjunta de la población”.

1.1. Estado actual de la acuicultura

El sector acuícola ha crecido de forma espectacular, desde una producción de 0,6 millones de toneladas (Mt) en 1950 a los 90,4 Mt en 2012 (SOFIA, 2014). Así, por ejemplo, entre 1980 y 2012, el volumen de producción acuícola mundial incrementó a una tasa media anual del 8,6%. A pesar de ello, en los últimos años el crecimiento del sector se ha decelerado, aunque se mantiene por encima del 6% en el periodo 2000-2012. En este sentido, la acuicultura continental ha crecido a mayor ritmo que la marina, representando un 63% de la producción total. Actualmente, más de la mitad de los productos de origen acuático consumidos provienen de las granjas acuícolas; de hecho, la FAO estima que para antes del 2030 ese porcentaje sea superior al 65%, ya que, por un lado, la pesca extractiva se mantiene estancada desde mediados de los 80 debido a la sobreexplotación o explotación plena (80,1% de los caladeros en 2011) y por otro lado, la demanda por parte del consumidor sigue en aumento. Así, en los 60 el consumo de pescado per cápita fue de 9,9 kg y desde entonces ha ido en aumento hasta alcanzar los 19,2 kg en 2012. Este incremento se ha debido, además de al crecimiento demográfico, al aumento de los ingresos y la urbanización, la expansión de la producción pesquera y la mayor eficacia en su distribución (SOFIA, 2014).

Concretamente, en 2012 el 48,8% de la producción mundial de acuicultura fue de pescado; pero se ha incrementado en todos los grupos de especies. La producción de vegetales representó el 26,3% de las toneladas producidas, los moluscos el 16,8%, los crustáceos el 7,1%, el grupo de anfibios y reptiles el 0,5% y otros invertebrados el 0,4% restante (Informe APROMAR-ESACUA-OPP, 2014).

A nivel mundial cabe destacar que Asia proporciona cerca del 88% del volumen de la producción acuícola; siendo China el mayor productor (SOFIA, 2014). A nivel europeo la producción ha caído un 44% desde 1988, sin poder compensar la reducción de la pesca extractiva y haciendo necesaria la importación (58% del consumo en 2012) para cubrir la demanda (23,3 Kg/año en la UE). España es el principal productor de la UE (21% del volumen total); sin embargo considerando el valor de la producción se sitúa en quinto lugar.

La ingestión, incluso de pequeñas cantidades de pescado (150g) puede tener una repercusión nutricional positiva considerable mejorando la salud y la calidad de vida; ya que constituye una fuente concentrada de proteínas (50-60% de la necesidad diaria), de ácidos grasos poliinsaturados de la serie omega-3 (ácido eicosapentanoico, EPA, y ácido docosahexanoico, DHA) y micronutrientes esenciales, además el pescado tiene un bajo contenido de grasas saturadas, carbohidratos y colesterol (SOFIA, 2014; Informe APROMAR-ESACUA-OPP, 2014). En 2010, el pescado representó el 16,7% del aporte de proteínas animales de la población mundial y el 6,5% de todas las proteínas consumidas.

En 2012, más del 86% de la producción pesquera mundial se destinó a consumo humano y el 75% de la cantidad restante se redujo a harinas y aceites de pescado. De hecho, la producción de harina y aceite de pescado para piensos en acuicultura se está reduciendo desde 2005. En el periodo 2008-2012, el pescado destinado a la elaboración de harina y aceite de pescado constituyó aproximadamente el 9-12% de la producción pesquera mundial (pesca extractiva y acuicultura). Estos ingredientes pueden producirse a partir de pescado entero (principalmente anchoveta), de sus deshechos o de otros subproductos como cabezas, colas, espinas y otros despojos. La producción del 35% de las harinas de pescado en 2012 se realizó a partir de subproductos de pescado que antes se desechaban mermando su calidad y composición. Esto se debe principalmente a las fluctuaciones producidas en la captura de anchoveta y otras especies causadas por el fenómeno de El Niño que además, han contribuido a incrementar los costes de ambos ingredientes (SOFIA, 2014). Los precios de la harina de pescado alcanzaron máximos históricos en 2013; presentando un incremento del 206% desde 2005. En cambio, el precio de harinas vegetales con buen valor biológico, como la soja, se mantuvo estable y fue más económico durante este periodo; incentivando a la sustitución. Los precios del aceite de pescado también están incrementando debido a la demanda como ingrediente de los piensos para la producción de peces carnívoros, al descenso de producción en América Latina y al aumento de su demanda como complemento alimenticio humano (SOFIA, 2014).

1.1.1. La dorada y la lubina: especies de interés en el Mediterráneo

Ambas especies se distribuyen por las costas orientales del océano Atlántico y por todo el Mediterráneo (Figura 1). Son peces hermafroditas protándricos, euritermos y eurihalinos; cualidades que permiten su cultivo en aguas salobres y marismas, además de las jaulas en mar abierto (Programa de información de especies acuáticas, FAO).

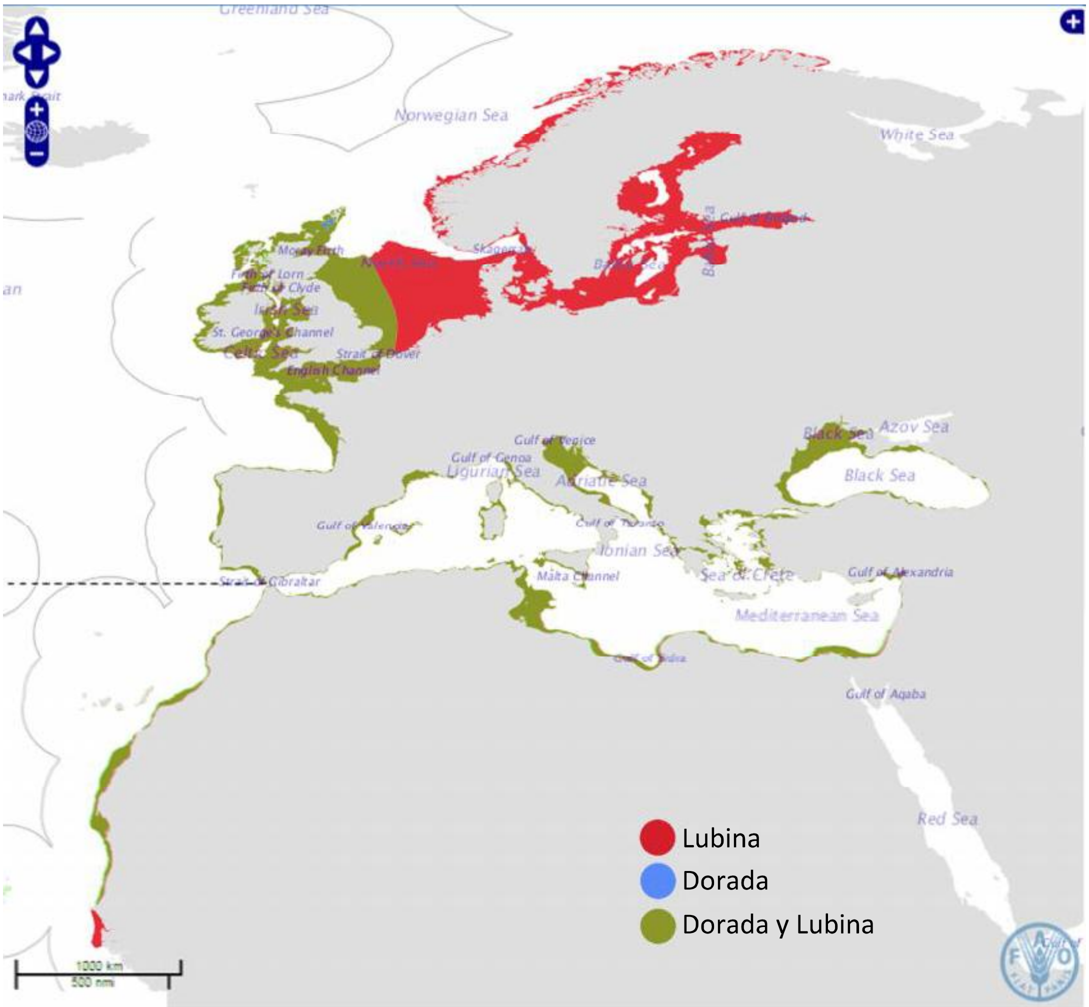


Figura 1. Distribución del cultivo de dorada y lubina a nivel mundial (Fuente: Fisheries and Aquaculture Department, FAO 2014).

La dorada (*Sparus aurata*) (Figura 2) pertenece a la familia Sparidae. Es una especie muy apropiada para acuicultura extensiva en el mediterráneo, debido a su alta tasa de supervivencia y sus hábitos alimentarios, principalmente moluscos, crustáceos y pequeños peces (Cuadernos de Acuicultura, Cultivo de Dorada, 2008). Es un pez bastante sensible a las bajas temperaturas, dejando de alimentarse si la temperatura baja de 12-13 ºC, aunque puede soportar temperaturas inferiores, siendo su mínimo letal del orden de 5-7 ºC (Cuadernos de Acuicultura, Cultivo de Dorada, 2008). Concretamente en nuestras latitudes, en el Mediterráneo Norte, las bajas temperaturas son las causantes de la enfermedad de invierno que hace que el cultivo sea casi inexistente debido al bajo rendimiento de los sistemas de producción (Ibarz et al., 2010). Por el contrario aguanta temperaturas bastante elevadas, creciendo muy rápido a temperaturas de 25-26 ºC y soportando temperaturas de hasta 32-33 ºC. (Cuadernos de Acuicultura, Cultivo de Dorada, 2008).

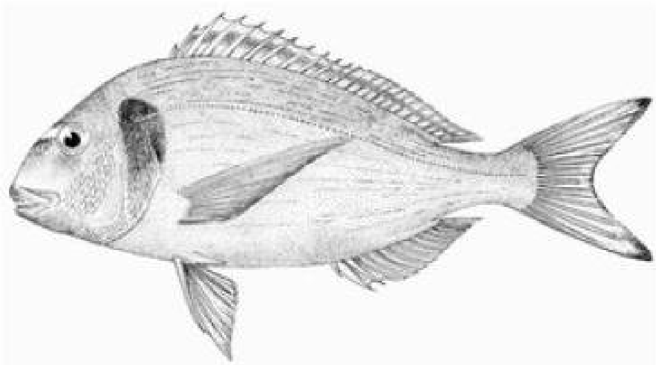


Figura 2. Dorada (*Sparus aurata*)
(Fuente: Programa de información de especies acuáticas, FAO)

La reproducción artificial se logró en Italia en 1981-82 y la producción a gran escala de juveniles de dorada se consiguió por primera vez en 1988-1989 en España, Italia y Grecia. Esta especie se adaptó rápidamente a las condiciones de crianza intensiva, marcando un antes y un después en el negocio de la acuicultura (Programa de información de especies acuáticas, FAO).

La captura de dorada procedente de la pesca extractiva es relativamente constante desde hace algunos años, situándose entre las 7000 y 8000 t., mientras que la dorada de crianza supuso el 95,1% del total en 2012. Los principales productores a nivel europeo en 2013, fueron Grecia (41,7% de la producción total), Turquia (23,2%) y España (9,3%), aunque se cultivó también en otros 16 países. A nivel mundial la producción de dorada en 2013 (179.924 t.) fue un 11,4% superior a la de 2012; sin embargo, la producción de dorada en España ha sido de 16.795 t., un 13,6% menor que en 2012. En nuestro país, la producción está encabezada por la Comunidad Valenciana (42% del total), seguida de Murcia (22%), Canarias (18%), Andalucía (11%) y Cataluña (1,8%) (Informe APROMAR-ESACUA-OPP, 2014).

La lubina (*Dicentrarchus labrax*) pertenece a la familia Moronidae (Figura 3), está ampliamente cultivada en el área mediterránea y fue la primera especie no salmónida cultivada y comercializada en Europa. Se trata de depredadores muy voraces, que se alimentan de peces pequeños, gambas, cangrejos y jibias; y a diferencia de la dorada no son particularmente sensibles a las bajas temperaturas (Programa de información de especies acuáticas, FAO).

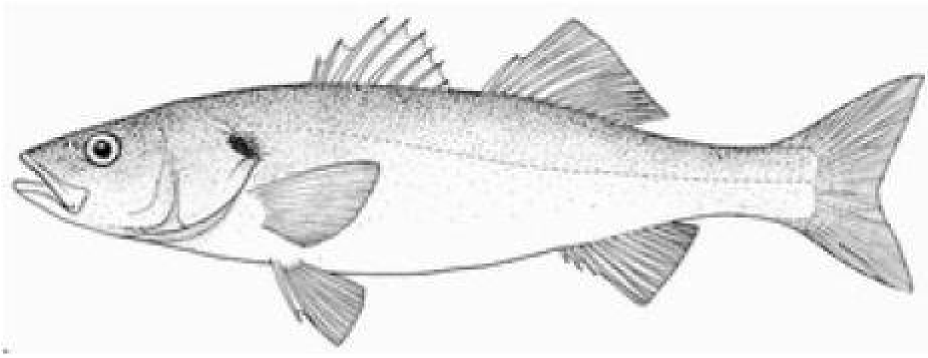


Figura 3. Lubina (*Dicentrarchus labrax*)
(Fuente: Programa de información de especies acuáticas, FAO)

El cultivo de lubina estuvo inicialmente asociado con la producción de sal en lagunas costeras y embalses de marea; hasta que en la década de los 60 se inició la producción masiva de juveniles en Francia e Italia y hacia finales de los 70 se logró controlar la reproducción (Programa de información de especies acuáticas, FAO).

La lubina procedente de la pesca extractiva es superior a la captura de dorada y se mantiene entre las 8000 y 12000 t. anuales. La lubina de crianza supuso el 94.5% del total de la comercializada en 2012. Los principales productores de lubina en 2013, fueron Turquía (37,5% de la producción total), Grecia (34,9%) y España (10,7%), produciéndose además en otros 16 países. En 2013, la producción mundial de lubina (137.723 t.) fue un 4,1% inferior a la de 2012; posiblemente debido a que las estadísticas de producción declaradas por Turquía y Grecia fueran inferiores a las reales (Informe APROMAR-ESACUA-OPP, 2014). La producción española de lubina en 2013 (14.707 t.) fue un 3,1% mayor a la de 2012 y está encabezada por la región de Murcia (34% del total), seguida de Canarias (29%), Andalucía (26%), Comunidad Valenciana (11%) y Cataluña (2,7%) (Informe APROMAR-ESACUA-OPP, 2014).

La industria de dorada y lubina está entrando en su fase madura, de hecho está sufriendo una transformación desde una industria de altos márgenes y bajos volúmenes a una de bajos márgenes y altos volúmenes, también conocida como economía de escala. Para ambas especies, el rápido desarrollo de producción en jaulas marinas ha llevado a una declinación de los precios, debido a la saturación del mercado, que disminuyeron en aproximadamente un 60 por ciento entre 1990 y 2000 y aún siguen disminuyendo. En marzo del presente año, el precio de mercado para tamaños inferiores a los 600g fluctúa alrededor de los 5,10 €/kg y 4,80 €/Kg para dorada y lubina, respectivamente (FIS-Reportes de Mercado). Para continuar creciendo, la industria acuícola, debería adoptar métodos de comercialización más sofisticados y organizados, acceder a nuevos mercados y ampliar los existentes, así como a diversificar el producto y que tenga un valor añadido (peces de mayor calidad o mayor tamaño, permitiendo así el fileteado).

1.2. Anatomía del aparato digestivo de los peces

La anatomía del tubo digestivo en las familias primitivas de peces es bastante homogénea, en cambio es muy variable en las familias más evolucionadas, incluso dentro de los géneros que parecen homogéneos desde el punto de vista de la anatomía externa, presentando una gran plasticidad en función de la dieta (Smith, 1989; Buddington et al., 1997). El esófago, generalmente es ancho y corto, y presenta una musculatura estriada de contracción voluntaria que, a diferencia de los vertebrados superiores, les permite regurgitar el alimento con más facilidad. En teleósteos, el epitelio del esófago es estratificado y en su región posterior, gradualmente, pasa a ser columnado (Yamamoto y Hirano, 1978; Meister et al., 1983). El epitelio estratificado presenta muchas células secretoras de mucus, cuya finalidad además de proteger el esófago, es lubricarlo permitiendo así el paso del alimento (Humbert et al., 1984; Shephard, 1994; Abaurrea-Esquisoain y Ostos-Garrido, 1996). Normalmente, a continuación del esófago se encuentra el estómago, aunque no siempre, ya que las larvas y algunas especies de peces micrófagos, herbívoros o especies que se alimentan de coral carecen de él (Guillaume y Choubert, 2001). En los peces que lo presentan, el epitelio estomacal, de origen endodérmico, está formado por células columnares secretoras de mucus cuya finalidad es proteger el propio epitelio y los tejidos subyacentes de los jugos gástricos (Allen y Felmström, 2005). Generalmente, la región anterior o cardial del estómago no presenta actividad secretora; mientras que el fundus, la región central y la distal presentan glándulas gástricas dispersas entre el epitelio. Éstas glándulas, contienen dos tipos celulares: las células oxinticopepticas, secretoras de ácido clorhídrico (HCl) y pepsinógeno, que están situadas en la base (Buddington y Kuz'mina, 2000b) y las células caliciformes, secretoras de mucus, alineadas con las anteriores (Morrison y Wright, 1999). La mucosa gástrica es la más diferenciada de las presentes en el tracto gastrointestinal (Harder, 1975). Aproximadamente el 60% de los peces poseen a la salida del estómago unos divertículos ciegos, los ciegos pilóricos

(Hossain y Dutta, 1996), que varían en diámetro y longitud, y cuyo número es altamente variable dependiendo de la especie y la temperatura del agua durante el desarrollo (Guillaume y Choubert, 2001). La mucosa que presenta ésta región, es muy similar a la del intestino proximal o anterior y no presenta ningún tipo de secreción u absorción en particular (Kapoor et al., 1975; Buddington y Diamond, 1987); parece ser que su función principal es aumentar la superficie de digestión y absorción, aunque algunas especies presentan en ellos floras intestinales activas (Guillaume y Choubert, 2001). A continuación de los ciegos pilóricos, encontramos el intestino propiamente dicho, un órgano multifuncional que además de digerir y absorber el alimento, participa en la osmorregulación y en la regulación endocrina de la propia digestión (Buddington et al., 1997). Su longitud relativa parece estar generalmente relacionada con las características de su dieta natural y la cantidad de proteína del pienso (Kramer y Bryant, 1995; Horn, 1997; Clements y Raubenheimer, 2005). En este sentido, el intestino de los peces carnívoros es más corto que el de los herbívoros (Kramer y Bryant, 1995). Únicamente los estudios histológicos permiten identificar las tres regiones intestinales existentes; es por ello que la terminología utilizada es variable y confusa: algunos autores hablan de las zonas anterior, media y posterior o distal, mientras que otros únicamente distinguen una zona proximal y una distal (Guillaume y Choubert, 2001). El intestino de los peces, a diferencia del de los mamíferos y las aves, presenta únicamente 3 capas histológicas: la mucosa, la muscular y la serosa, ya que la ausencia de muscularis mucosae no permite diferenciar entre mucosa y submucosa (Guillaume y Choubert, 2001). Los peces presentan pseudovellosidades intestinales ya que a diferencia de las de vertebrados superiores no están bien individualizadas. Como en mamíferos y aves contribuyen a aumentar la superficie de absorción (Figura 4).

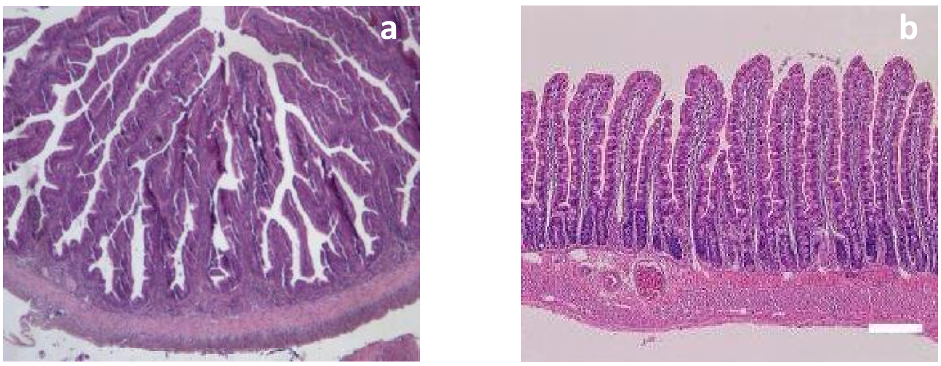


Figura 4. Corte histológico transversal del intestino proximal de dorada (a) y de rata (b). Tinción hematoxilina-eosina 4x. (Fuente: Inoue et al., 2006).

El epitelio intestinal está, principalmente, constituido por células columnares de tipo absortivo, los enterocitos; aunque también encontramos esparcidos por él, células mucosas de tipo caliciforme, linfocitos y células enteroendocrinas (Kapoor et al., 1975; Wilson y Castro, 2011). Las células que conforman este epitelio se originan en la base de las pseudovellosidades y se diferencian durante la migración hacia el ápice de las mismas, dónde acaban descamándose (Figura 5) (Trier y Moxey, 1980; Guillaume y Choubert, 2001).

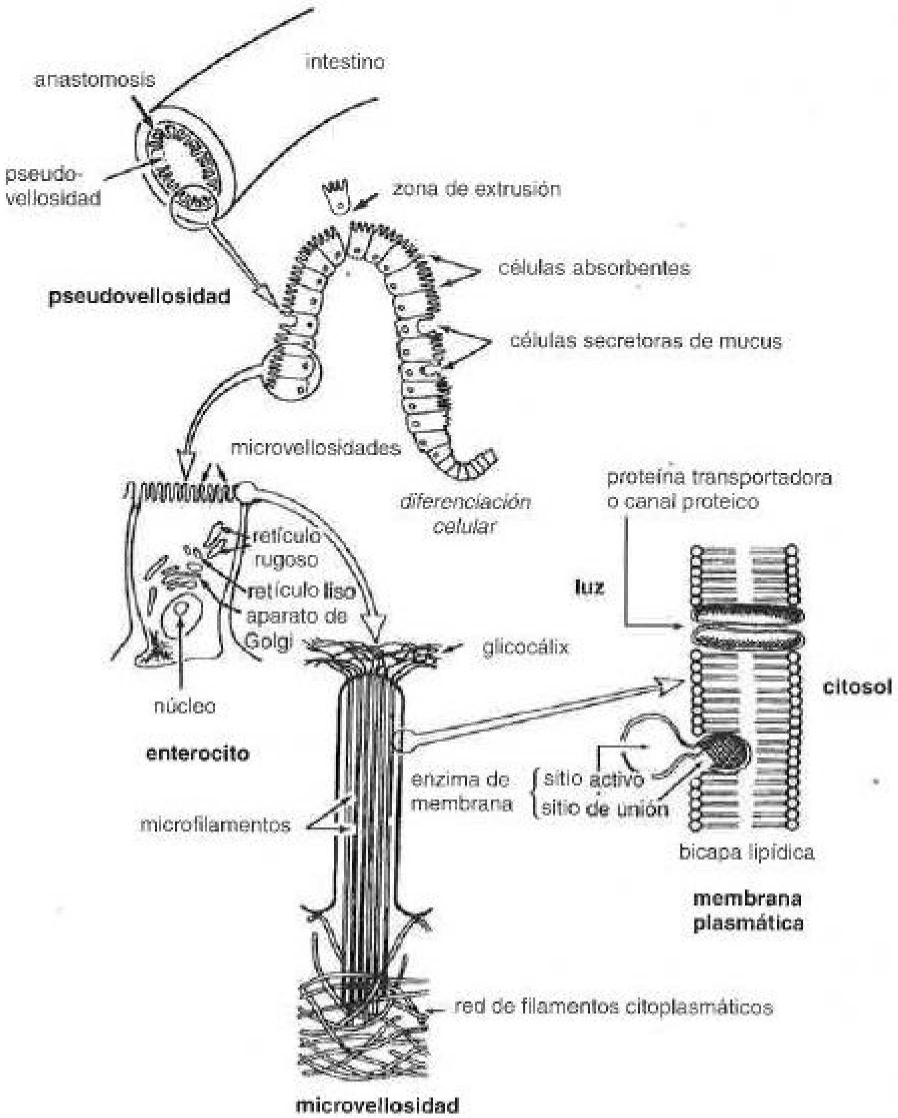


Figura 5. Representación esquemática de la anatomía y la histología del intestino, desde el corte del órgano (escala microscópica) hasta la estructura molecular de la membrana plasmática de las microvellosidades (Fuente: Guillaume y Choubert, 2001)

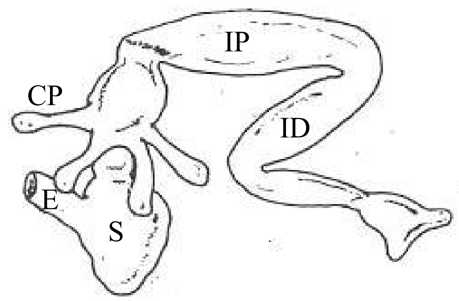
Los enterocitos presentan una clara polarización (Ezeasor y Stokoe, 1981; Buddington et al., 1997); su membrana apical se caracteriza por la presencia de microvilli que constituyen el borde en cepillo, contribuyendo a aumentar la superficie intestinal un 90%. Estos microvilli forman una interfaz digestiva/absortiva, y un microambiente donde se localizan las enzimas que intervienen en la rotura final de los nutrientes y donde tendrá lugar la absorción (Kuz'mina y Gelman, 1997). La membrana basolateral de los enterocitos presenta estructuras lamelares (Ezeasor y Stokoe, 1981), que incrementan su superficie y presentan una gran expresión de Na^+/K^+ ATPasa, enzima esencial para llevar a cabo los procesos de transporte transepitelial, la captación de nutrientes y la regulación iónica (Bakke et al., 2011). Las células caliciformes son células mucosas que se encuentran distribuidas uniformemente a lo largo de todo el tracto intestinal (Buddington et al., 1997). Su núcleo se sitúa en la base, haciendo que se ensanche y luego se estreche formando un poro apical por el que se descarga el moco, de ahí su nombre (Figura 5). En el intestino medio, además de enterocitos y células mucosas, encontramos células encargadas de la absorción de material proteico por endocitosis, que presentan muchas invaginaciones en la base de las microvellosidades y grandes vacuolas en su citoplasma. En el caso de mamíferos y aves estas células se encuentran únicamente en neonatos, perdiéndose esta capacidad posteriormente, mientras que en peces se mantienen durante toda la vida del animal (Guillaume y Choubert, 2001). La región distal es la encargada principalmente de la osmorregulación y absorción de minerales, por ello los enterocitos de éste segmento presentan microvilli más cortos, muchas mitocondrias y pocas características de células absortivas (Buddington et al., 1997).

El hígado y el páncreas son las denominadas glándulas anexas ya que también contribuyen al proceso digestivo. El hígado es un órgano clave tanto a nivel anabólico como catabólico. En peces está bien desarrollado y, aunque no presenta lóbulos, su función es equivalente a la de vertebrados superiores. Se trata de una glándula mixta; su parte endocrina segrega hormonas esteroideas y peptídicas al flujo sanguíneo; su parte exocrina, produce bilis que se secreta a través del canal colédoco a los ciegos pilóricos o intestino proximal facilitando la digestión de los lípidos (Guillaume y Choubert, 2001). Normalmente el hígado; acumula glucógeno como sustrato energético (El-Bakary y El-Gammal, 2010), y/o lípidos como reserva, llegando a adquirir un gran tamaño en algunos peces (Guillaume y Choubert, 2001). Por otro lado, el páncreas, generalmente es discreto o difuso según la especie, formando pequeños islotes que se sitúan entre la grasa que se encuentra alrededor de los ciegos pilóricos e incluso penetrando en el hígado formando un hepatopáncreas (Nejedli y Tlak Gajger, 2013). Sin embargo, en algunas especies es compacto, y puede estar compuesto por lóbulos, normalmente dos. El páncreas consta de dos partes que se diferencian histológicamente: la exocrina y la endocrina (Nejedli y Tlak Gajger, 2013). El páncreas exocrino contiene gránulos de zimógeno que producen y almacenan enzimas digestivas que son liberadas a los ciegos pilóricos, intestino proximal o al conducto biliar (Kurokawa y Suzuki, 1995; Krogdahl y Sundby, 1999; Morrison et al., 2004); mientras que el páncreas endocrino secreta hormonas, como la insulina y el glucagón entre otras (Youson et al., 2006; Nejedli y Tlak Gajger, 2013).

1.2.1. El aparato digestivo de dorada

La dorada es un pez carnívoro, que puede comportarse como omnívoro ocasional (De Sostoa, 1990). Presenta un esófago corto y ancho y un estómago sifonal (Grassé, 1958). El intestino presenta 4 ciegos pilóricos en su región anterior y se dobla sobre sí mismo dos veces (Elbal y Agulleiro, 1986) (Figura 6). Su región proximal es ancha y está constituida principalmente por células absortivas, mientras que en la distal encontramos células con función pinocítica (Elball y Agulleiro, 1986). El intestino va estrechándose desde la región proximal hacia el esfínter, incrementando su diámetro posteriormente para dar lugar al recto; que tiene una longitud relativa de aproximadamente 1:10 parte de la longitud intestinal (Elbal y Agulleiro, 1986). Las células mucosas se encuentran distribuidas de forma uniforme por el epitelio intestinal, sin formar glándulas, a diferencia de lo que ocurre en otros teleósteos (Bishop y Odense, 1966).

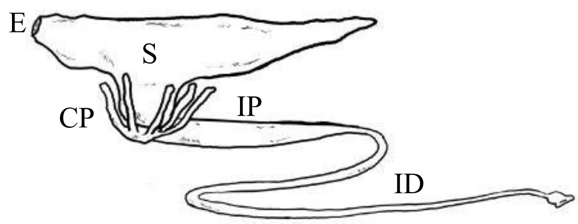
Figura 6. Esquema del tracto digestivo de dorada. Se puede diferenciar el esófago (E), estómago (S), ciegos pilóricos (CP), intestino proximal (IP), intestino distal (ID). (Fuente: Elbal y Agulleiro, 1986).



1.2.2. El aparato digestivo de lubina

La lubina es una especie carnívora, que en su etapa juvenil se alimenta principalmente de langostinos y moluscos y a medida que crece incrementa el consumo de peces (Hampel et al., 2005). Su estómago presenta una rama glandular descendente y una pilórica no-glandular ascendente, correspondiéndose con el tipo cecal descrito por Grassé (1958). El intestino presenta 4 o 5 ciegos pilóricos en su región anterior y, como el de dorada, se dobla sobre sí mismo dos veces (García-Hernández et al., 2001) (Figura 7). La región proximal presenta pseudovellosidades más largas que la distal y está constituida principalmente por células absortivas, mientras que en la distal se encuentran células con función pinocítica. El número de células mucosas, a diferencia de lo que ocurre en dorada, van aumentando hacia la zona caudal del intestino (García-Hernández et al., 2001).

Figura 7. Esquema del tracto digestivo de lubina. Se puede diferenciar el esófago (E), estómago (S), ciegos pilóricos (CP), intestino proximal (IP), intestino distal (ID).



1.3. Digestión

La digestión es el proceso de hidrólisis y solubilización de las macromoléculas ingeridas en metabolitos simples y elementos que pueden ser transportados a través de la membrana intestinal. Para llevarla a cabo, en el tracto digestivo se producen diversas secreciones (ácidas, alcalinas, enzimáticas o endocrinas) cuyas funciones principales son: 1) facilitar el paso y la mezcla del alimento con las enzimas digestivas y otros componentes permitiendo la digestión, 2) solubilizar los nutrientes para asegurar la digestión óptima y la absorción por parte de la mucosa intestinal, 3) proteger ésta mucosa de componentes duros de la dieta, así como de las propias secreciones, y 4) proteger al organismo frente a microbios o químicos que podrían afectar al bienestar del animal (Shephard, 1994).

El estómago es el órgano responsable del almacenamiento y la primera rotura física y enzimática del alimento. Concretamente, el ácido secretado por las células oxinticopepticas, contribuye a iniciar la digestión desnaturalizando la proteína y convirtiendo el pepsinógeno en pepsina (forma activa del enzima) (Yúfera et al., 2004; Wu et al., 2009); iniciando así la ruptura de la proteína ingerida. La llegada del quimo al intestino estimula la secreción de electrolitos, colecistoquinina (CCK), ácidos biliares y enzimas pancreáticas. Los electrolitos son secretados por el páncreas y por el propio intestino. Principalmente se secreta bicarbonato, que neutraliza el pH ácido del quimo procedente del estómago haciéndolo más alcalino, permitiendo así la actuación de las enzimas a un pH más cercano a su óptimo (entre 7 y 9). La CCK, secretada por las células endocrinas intestinales, estimula la liberación de las enzimas pancreáticas y la bilis por parte del páncreas y la vesícula biliar, respectivamente. Los ácidos biliares, secretados por el hígado, emulsionan los lípidos procedentes de la dieta y las vitaminas liposolubles permitiendo una acción más eficiente por parte de las lipasas y la formación de micelas. Al mismo tiempo, el páncreas secreta las enzimas pancreáticas que incluyen: 1) serin-proteasas (tripsina, quimotripsina, elastasa, colagenasa, carboxipeptidasa A y B) que hidrolizan la proteína; 2) α-amylasa que hidroliza el almidón en cadenas lineales; 3) DNAasa y RNAasa y 4) enzimas lipolíticas (lipasa, co-lipasa, fosfolipasa y colesteril ester hidrolasa) (Kurokawa y Suzuki, 1995; Pivnenko et al., 1997; Krogdahl y Sundby, 1999; Kurtovic et al., 2009). El tejido pancreático secreta lipasas dependientes de colipasa (PL) o carboxil ester lipasas dependientes de sales biliares (CEL); las primeras presentes mayormente en especies de agua dulce y las segundas en peces marinos (Kurtovic et al., 2009). Todas estas enzimas permiten que las proteínas, los carbohidratos y los lípidos ingeridos se rompan en metabolitos más simples que pueden ser digeridos por las enzimas de membrana presentes en el borde en cepillo de los enterocitos (leucina aminopeptidasa, maltasa, isomaltasa, esterasas, fosfatasa alcalina, entre otras) liberando así moléculas suficientemente pequeñas para que sean absorbidas. (Figura 8).

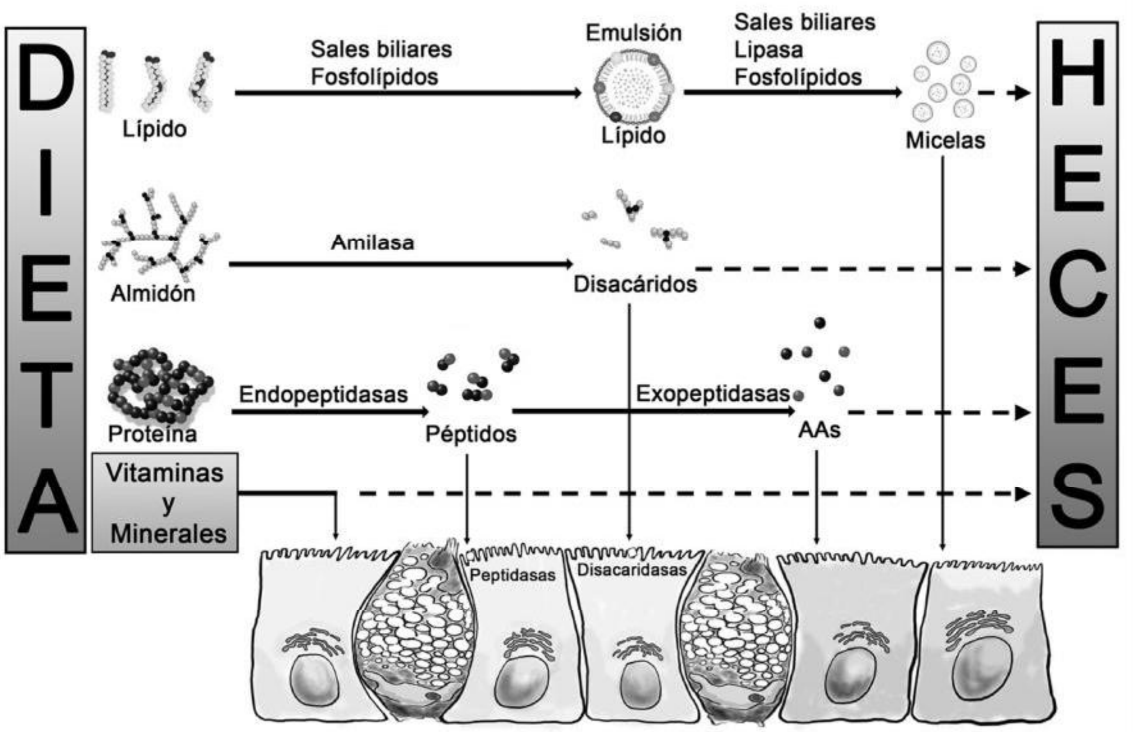


Figura 8. Esquema de los procesos digestivos a lo largo del tracto intestinal de los peces. La localización de las diversas enzimas y otros componentes digestivos, así como sus respectivos procesos en el lumen intestinal se indican en contraposición a la mucosa intestinal.

La α-amilasa y la lipasa se secretan en su forma activa, mientras que las proteasas y la colipasa se secretan como proenzimas que son activadas en el lumen intestinal, por acción de la enteroquinasa. Este enzima activa el tripsinógeno a tripsina (Ogiwara y Takahashi, 2007) y ésta, a su vez, se encarga de activar el resto de pro-proteasas (Krogdahl y Sundby, 1999). En lo que respecta a los carbohidratos, los peces presentan 2 tipos de enzimas para la digestión de almidón y glucógeno, la α-amilasa pancreática y las disacaridasas presentes en el borde en cepillo de los enterocitos. La α-amilasa hidroliza los enlaces α(1-4) produciendo maltosa y oligosacáridos ramificados. Los carbohidratos de bajo peso molecular presentes en dieta, sacarosa y trealosa, son hidrolizados directamente por las enzimas de membrana (Bakke et al., 2011). Por otro lado, las lipasas de peces presentan una alta afinidad por los gliceroles con cadenas largas y altamente insaturadas, a diferencia de lo que ocurre en mamíferos, produciendo principalmente ácidos grasos libres (Gjellesvik, 1991; Gjellesvik et al., 1994). Algunos peces pueden, además, hidrolizar ceras, aunque en menor proporción que triglicéridos y fosfolípidos (Tocher y Sargent, 1984; Olsen et al., 2004). La cantidad y actividad de las enzimas pancreáticas secretadas difiere entre especies y/o en función de su dieta natural; y también en función de la composición nutricional, el procesado y la digestibilidad de la dieta administrada (Adamidou et al., 2009; Borghesi et al., 2009; Tiril et al., 2009). Así, la digestibilidad de la proteína y el lípido puede modificarse en función de la especie y el momento de desarrollo del pez, la tasa de alimentación y la composición principal de la dieta y la temperatura del agua (Røsjø et al., 2000; Refstie et al., 2006; Hansen et al., 2008; Sales et al., 2009; Venou et al., 2009). Como ejemplos: 1) una dieta con alto contenido proteico, pero baja digestibilidad y/o inhibidores de proteasas estimula la secreción de tripsina por el páncreas (Péres et al., 1996; 1998; Krogdahl et al., 1999; 2003) y 2) Bogevik et al., (2009) demostraban la regulación al alza de lipasa, y de las sales biliares en salmón alimentado con una dieta de baja digestibilidad lipídica respecto a una control. Además, en el caso de los lípidos puede verse afectada por la longitud de la cadena, el grado de desaturación y el punto de fusión de los ácidos grasos (Røsjø et al., 2000; Hansen et al., 2008). Por último, la digestibilidad de los carbohidratos se ve modificada por la cantidad de lípido y su calidad, y la fuente de almidón y su cantidad (Fountoulaki et al, 2005; Venou et al., 2003) haciendo que la capacidad de digestión y absorción de los carbohidratos solubles sea muy variable. En herbívoros y omnívoros la secreción de α-amilasa está regulada por la ingesta de almidón; sin embargo la relación no es tan clara en el caso de los carnívoros (Krogdahl et al., 2005). En trucha se detectó una disminución de actividad α-amilasa cuando incrementaba el nivel de almidón en dieta (Spannhof y Plantikow, 1983), que podría deberse a la presencia de factores antinutricionales (Krogdahl et al., 2005). A pesar de los estudios aquí citados, la relación entre el lípido y el carbohidrato presentes en dieta con la secreción enzimática para su digestión no está tan estudiada como en el caso de la proteína (Morais et al., 2007; Zambonino Infante y Cahu, 2007; Bogevik et al., 2009); de hecho, un mejor conocimiento de los efectos de las diferentes condiciones de cultivo y/o dietas sobre la digestión lipídica y glucídica, así como su regulación, permitiría un mejor aprovechamiento de las fuentes de lípidos y carbohidratos procedentes de la dieta.

1.4. Absorción

La absorción de las moléculas resultantes de la digestión tiene lugar en la membrana apical del enterocito. Después los nutrientes salen de la célula a través de la membrana basolateral para entrar al sistema circulatorio. Los procesos de absorción tienen lugar por difusión y transporte facilitado o activo (Mailliard et al., 1995) y siguen una cinética no asintótica que depende de la presencia de los transportadores inmersos en la bicapa lipídica (Palacín et al., 1998). La absorción de nutrientes en los peces, como en otros poiquilotermos es más lenta que en mamíferos, por ello sus transportadores presentan una mayor afinidad por los nutrientes (Bakke et al., 2011). La capacidad de absorción es específica de especie, dependiente de la temperatura y la salinidad (Ferraris y Ahearn, 1984; Collie 1985; Buddington et al., 1987, 1997; Collie y Ferraris, 1995; Lionetto et al., 1996; Houpe et al., 1997; Bakke-McKellep et al., 2000; Nordrum et al., 2000a; Bakke et al., 2010) y de la composición de la dieta natural, la formulación del pienso que se administra y su disponibilidad, y de la ración (Buddington y Hilton, 1987; Nordrum et al., 2000a; Berge et al., 2004; Jutfelt et al., 2007; Terova et al., 2009). A diferencia de lo que ocurre en mamíferos, este proceso absortivo tiene lugar a lo largo de todo el intestino y presenta diferencias entre las distintas regiones intestinales (Ferraris y Ahearn, 1984; Collie, 1985; Bakke-Mckellep et al., 2000). En este sentido, la absorción es más rápida en las regiones proximales del intestino que en las distales y por lo tanto su contribución es mayor (Diaz et al., 1997; Olsen et al., 1999; Nordrum et al., 2000b; Denstadli et al., 2004) y además, se produce antes en el caso de los aminoácidos libres y pequeños péptidos que para las proteínas intactas (Ambardekar et al., 2009). En la región distal la permeabilidad de pequeñas moléculas solubles en agua es más elevada que en la región proximal (Collie, 1985; Schep et al., 1997; Bakke-McKellep et al., 2000; Jutfelt et al., 2007). Además, la especies carnívoras presentan mayor captación de nutrientes en la región de intestino distal que las especies omnívoras o herbívoras (Ferraris y Ahearn, 1984), pudiendo llegar a representar entre un 5-10% de la absorción total (Krogdahl et al., 1999). Esta superior captación de nutrientes parece ser un mecanismo de adaptación a su menor longitud intestinal relativa; y en el caso de peces marinos, se debe además a la menor tasa de transporte de la región proximal que los peces de agua dulce (Ferraris y Ahearn, 1984).

Los aminoácidos y los carbohidratos son absorbidos por transporte activo, facilitado, y por difusión; y su absorción presenta una clara relación con la dieta. En este sentido, las especies herbívoras y omnívoras presentan transportadores de aminoácidos con mayor afinidad por el sustrato que los de carnívoros; y las tasas de transporte de glucosa son superiores en herbívoros (Ferraris y Ahearn, 1984; Buddington et al., 1987). Esta adaptación a la dieta natural facilita el transporte del nutriente más común a través de un mayor número de transportadores o una mayor tasa de transporte; asegurando también la absorción del nutriente menos común (Ferraris y Ahearn, 1984). En peces, se ha descrito la presencia de al menos 4 sistemas de transporte de aminoácidos (Tabla 1) (Storelli et al., 1989); uno acídico, uno básico y uno neutro, análogo al sistema presente en mamíferos X^-_{AG}, y^+ y B^0, respectivamente, y un sistema responsable del transporte de prolina y aminoácidos N-metilados (similar al sistema IMINO y/o PAT), diferente al de mamíferos, e inhibido totalmente por alanina (Vilella et al., 1989a) y parcialmente por fenilalanina (Storelli et al., 1989). También se han encontrado sistemas de transporte Na^+-independientes para alanina, glicina y lisina, análogos a los sistemas para aminoácidos neutros y básicos de mamíferos; mientras que la prolina y el glutamato no son transportados por estos procesos (Storelli et al., 1989). Por otro lado, la L-histidina presenta un transportador altamente específico (Glover y Wood, 2008b). Se ha demostrado también la existencia de transporte de péptidos a través de PepT1 y PepT2 (Thamotharan et al., 1996a; 1996b; Maffia et al., 1997; 2003; Verri et al., 2000; 2003; Romano et al., 2006; Goncalves et al., 2007; Hakim et al., 2009; Sangaletti et al., 2009; Terova et al., 2009). Finalmente, se ha sugerido que la fatty-acid-binding-protein intestinal (I-FABP) puede estar implicada en la unión de péptidos y proteínas para su endocitosis (Concha et al., 2002). La D-glucosa y D-galactosa son transportadas por SGLT1 (Sala-Rabanal et al., 2004; Geurden et al., 2007; Bakke-McKellep et al., 2008); la fructosa también se absorbe pero se desconoce su transportador (Golovanova, 1993) y también se han detectado tránscritos de GLUT2 y GLUT4 en algunas especies de peces (Krasnov et al., 2001; Panserat et al., 2001; Planas et al., 2000; Hall et al., 2006).

No se conoce mucho sobre la translocación apical de productos lipolíticos en peces, pero se cree que es similar a la de mamíferos (Tocher, 2003). Parece ser que la absorción de ácidos grasos tiene lugar por difusión o transporte facilitado; y aunque I-FABP puede estar implicada en la internalización de los ácidos grasos, la presencia de transportadores no ha sido verificada aún (Andre et al., 2000; Concha et al., 2002). En peces, la absorción lipídica se considera inferior a la de mamíferos (Morais et al., 2005a), y tiene lugar principalmente en intestino proximal y ciegos pilóricos (Diaz et al., 1997; Olsen et al., 1999; Røsjø et al., 2000; Denstalli et al., 2004; Hernández-Blázquez et al., 2006). Depende de la clase de lípido, la longitud de la cadena y el grado de saturación (Røsjø et al., 2000; Denstadli et al., 2004; Morais et al., 2005a; 2005b; Oxley et al., 2007). Las gotas de lípido acumuladas en la región supranuclear del enterocito (Sire et al., 1981; Fontagne et al., 1998; Olsen et al., 1999;

Hernández-Blázquez et al., 2006) se metabolizan y empaquetan formando pequeñas lipoproteínas similares a quilomicrones y lipoproteínas de muy baja densidad (VLDL) (Caballero et al., 2003) que abandonan el enterocito por exocitosis (Hernández-Blázquez et al., 2006). La fuente de lípido de la dieta, principalmente los lípidos neutros, favorecen la acumulación de gotas lipídicas en los enterocitos y de lipoproteínas en los espacios intercelulares (Diaz et al., 1997; Fontagne et al., 1998; Olsen et al., 1999; Caballero et al., 2003); reduciendo el metabolismo, y como consecuencia el transporte de lípidos a través de la membrana basolateral, limitando así la absorción de lípidos a través del borde en cepillo. Esto hecho podría estar implicado en la menor digestibilidad de los lípidos cuando su contenido en dieta es muy elevado (Morais et al., 2005b).

Tabla 1. Sistemas de transporte de hexosas, aminoácidos y péptidos descritos de la membrana apical del enterocito de algunos peces (presentado con la nomenclatura de mamíferos) (Bakke et al., 2011).

Sistema	Sustrato	Dependencia iónica	Especie	Referencia
Hexosas				
SGLT1	D-Glucosa	Na^+	Dorada *Sparus aurata*	Sala-Rabanal et al., 2004
	D-Galactosa		Salmón del Atlántico *Salmo salar*	Bakke-McKellep et al., 2008
			Misgurno *Misgurnus anguillicaudatus*	Goncalves et al., 2007
			Rocote cobrizo *Sebates caurinus*	Ahearn et al., 1992
Aminoácidos y péptidos				
B^0	Aminoácidos neutros	Na^+, Cl^-?	Anguila europea *Anguilla anguilla*	Storelli et al., 1989
			Boops salpa	Boge et al., 2002
			Lubina *Dicentrarchus labrax*	Balocco et al., 1993
IMINO/PAT	Iminoácidos	Na^+	Anguila europea	Storelli et al., 1989; Vilella at al., 1989a
X_{AG-}	Aminoácidos ácidos	Na^+	Anguila europea	Storelli et al., 1989
y^+	Aminoácidos básicos		Anguila europea	Storelli et al., 1989
L	Ala, Gly, Lys	?	Anguila europea	Storelli et al., 1989
PepT1	Dipéptidos	H^+	Tilapia *Oreochromis mossambicus*	Thamotharan et al., 1996a
			Zebrafish *Danio rerio*	Verri et al., 2003
			Rocote cobrizo *Sebastes caurinus*	Thamotharan et al., 1996a
			Anguila europea	Verri et al., 2000
			Icefish *Chionodraco hamatus*	Maffia et al., 2003
			Lubina	Hakim et al., 2009; Sangaletti et al., 2009; Terova et al., 2009
PepT2	Dipéptidos	H+	Misgurno	Goncalves et al., 2007

Finalmente, la absorción de vitaminas liposolubles (A, D, E y K), carotenoides y retinol (vitamina A) está mediada por transportadores saturables (Buddington et al., 2002), como se ha demostrado en mamíferos. En cambio, para las vitaminas hidrosolubles (ácido ascórbico, riboflavina, biotina y ácido fólico) la captación está mediada por transportadores no saturables (Vilella et al., 1989b; Rose y Chou, 1990; Buddington et al., 1993; Casirola et al., 1995; Casirola y Ferraris, 1997), aunque parece que existen diferencias interespecíficas en los mecanismos de absorción (Bakke et al., 2011). La absorción de minerales en peces puede tener lugar a través de las branquias modificando la captación a nivel del tracto gastrointestinal (Bury et al., 2003). Está captación tiene lugar a través de canales iónicos o transportadores específicos; mientras que los oligoelementos (como Mn, Co, Se, etc.) normalmente se fijan a un aminoácido que les sirve de ligando (Bakke et al., 2011).

1.5. Dietas de sustitución

En Europa, la mayor parte de las especies cultivadas son carnívoras, por ello, tradicionalmente, las harinas y aceites de pescado han sido los ingredientes más ampliamente utilizados para la elaboración de piensos, debido a sus excelentes propiedades nutricionales; concretamente el buen balance aminoacídico y la presencia de ácidos grasos poliinsaturados (PUFA) y altamente poliinsaturados (HUFA). La utilización de las harinas de pescado en el sector ganadero, el uso de los aceites como complemento nutricional humano y la menor producción de ambos ingredientes ha contribuido al incremento de sus precios e incluso afectado a su calidad (Aksnes et al., 1997; Degara, 1997; Vergara et al., 1999). Todo ello ha hecho necesario testar ingredientes alternativos con la finalidad de abaratar los costes de producción, pero permitiendo un crecimiento óptimo de los animales y asegurando la calidad del producto final (SOFIA, 2014).

Durante los últimos 15 años, la inclusión de ingredientes de origen vegetal en el pienso para peces de acuicultura ha aumentado considerablemente para permitir el crecimiento del sector. De hecho, su elevada disponibilidad, tanto de harinas como de aceites vegetales, y su menor coste comparado con las harinas y aceites de pescado, hace que puedan ser una buena alternativa (Bell and Waagbø, 2008; Turchini et al., 2009) ya que el efecto sobre el crecimiento es nulo o mínimo (Izquierdo et al., 2003; Albrekten et al., 2006; Richard et al., 2006; De Francesco et al., 2007; Wassef et al., 2007; Benedito-Palos et al., 2007). Las harinas de origen vegetal pueden utilizarse siempre y cuando, se cubran los requerimientos de aminoácidos esenciales, sobre todo la lisina y/o metionina, se mejore la palatabilidad de las dietas y el nivel de factores antinutricionales (ANFs) presentes sea bajo (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). Los ANFs (Tabla 2) afectan a la utilización del alimento, a la salud del pez y a la producción (Makkar, 1993). Además, un mismo ingrediente puede contener más de un factor antinutricional y sus efectos son muy variables en función de la especie, la edad, el tamaño, el sexo, el estado de salud, la nutrición y los posibles factores estresantes a los que los animales estén sometidos (Francis et al., 2001; Krogdahl et al., 2010). Sin embargo, también pueden tener efectos beneficiosos, como propiedades antioxidantes, immunoestimuladoras, e incluso en el caso de los aceites vegetales reducir el nivel de contaminantes, como las dioxinas o PCBs, que se presentan en mayores concentraciones en los aceites de pescado (Turchini et al., 2009; Krogdahl et al., 2010).

Tabla 2. Tipos de factores antinutricionales presentes en ingredientes vegetales, efectos y procedimientos para minimizar su presencia. (Fuente: Adaptación de Francis et al., 2001; Gatlin et al., 2007 y Krogdahl et al., 2010)

Factor antinutricional	Vegetales donde se encuentra	Efecto en peces	Procedimientos de inhibición
Inhibidores de proteasas	Leguminosas y cereales	Menor crecimiento. Menor digestibilidad de proteína y lípido, correlacionada con una menor actividad tripsina y quimotripsina. Supresión del feed-back pancreático por afectación de las proteasas y como consecuencia imbalance de aminoácidos.	Calor húmedo Autoclavado
Oligosacáridos y Polisacáridos distintos del almidón	Leguminosas y cereales	Menor digestibilidad de la dieta, por aumento de la viscosidad del quimo. Inhibición de enzimas digestivas, unión a ácidos biliares y minerales. Pueden inducir enteritis en salmónidos.	Cocción al vapor Extrusión a altas temperaturas Fermentación bacteriana o fúngica
Fitatos	Soja, colza, sésamo, altramuz	Menor crecimiento. Reducción de la disponibilidad de minerales, sobre todo de fósforo. Disminución de la digestibilidad proteica por formación de complejos fitato-proteína. Anormalidades en tiroides, riñón e hipertrofia de ciegos pilóricos por vacuolización.	Adición de fitasas al alimento Selección genética Fermentación
Saponinas	Leguminosas	Menor crecimiento. Daña el epitelio branquial, aumentan la permeabilidad intestinal, inhibiendo el transporte, e interfieren en la absorción de esteroles y limitan el reciclaje de sales biliares. Disminuyen la digestibilidad de la proteína por acción de la quimotripsina, probablemente por la formación de complejos saponina-proteína.	Extracción acuosa Selección genética
Lectinas	Leguminosas y guisantes	Disrupción de la integridad intestinal, hipertrofia, hiperplasia, disminución de la absorción de nutrientes, de la actividad enzimática intestinal; aumento de la longitud y la masa intestinal, mayor proliferación de células mucosas y mayor secreción pancreática.	Calor húmedo, autoclavado Selección genética

Continua

Factor antinutricional	Vegetales donde se encuentra	Efecto en peces	Procedimientos de inhibición
Taninos	Gramíneas, leguminosas, crucíferas	Interferencia con procesos digestivos por unión de enzimas, unión a proteínas y a minerales. Reducción de la absorción de vitamina B12. Toxicidad en hígado y riñón.	Descascarillado, autoclavado, tratamiento con base Fermentación bacteriana Selección genética. Tratamiento con agentes oxidantes, acomplejar con saponinas
Glucosinolatos	Crucíferas	Hemorragía hepática y daños en hígado y riñón. Hiperplasia e hipertrofia de la tiroides. Menor crecimiento.	Cocción y presión húmeda Remojo Selección genética
Gosipoles	Género *Gossipium* y orden malvales	Menor crecimiento y anormalidades intestinales y en órganos internos. Menor digestibilidad de proteína por la formación de complejos proteína-gosipol. Problemas a nivel del sistema reproductor.	Selección genética Calor húmedo Pre-presión y extracción
Fitoestrógenos	Soja, algodón, linaza,…	Efectos estrogénicos e inducción de vitelogénesis, que provocan un menor crecimiento.	
Alcaloides	Semillas de legumbres (altramuz)	Disminución de la ingesta causada por su baja palatabilidad afectando al crecimiento.	Selección genética Extracción acuosa, extrusión 120-145ºC
Sustancias antigénicas	Semillas de legumbres y cereales	Lesiones de la mucosa intestinal, enteritis, pseudovellosidades anormales, movimiento anormal del quimo y respuestas inmunes específicas y no específicas que contribuyen a un menor crecimiento.	Tratamiento por calor y sistemas de procesado de pienso comunes
Glucósidos cianógenos	Leguminosas, semillas de aceite, raíces de cosecha (mandioca)	Menor crecimiento. Supresión de la respiración natural y parada cardiaca.	Remojar y secar Acomplejar con taninos

La necesidad de sustituir el aceite de pescado ha llevado a la utilización de aceites de origen vegetal, principalmente soja y colza, ya que se consideran unas buenas alternativas a los aceites de pescado (Lee y Lee, 2006; Turchini et al., 2009), ya que ambos aceites presentan una disponibilidad elevada y su precio es competitivo, además, son ricos en ácidos grasos poliinsaturados, especialmente linoleico (18:2n-6) y oleico (18:1n-9), aunque son pobres en n-3 HUFA y se caracterizan por tener una relación n-6/n-3 elevada (Tabla 3) (Fountoulaki et al., 2009; Turchini et al., 2009). El aceite de soja es una fuente energética de alta calidad y de ácidos grasos por su rico contenido en n-6 PUFA (Brown y Steven, 2011). Por otro lado, el aceite de colza es rico en ácidos grasos monoinsaturados (MUFA), propiedad que lo hace fácilmente digerible, es también una buena fuente energética y sus efectos sobre la calidad del filete son menores que los de otros aceites vegetales (Turchini et al., 2009).

La inclusión de ingredientes de origen vegetal provoca cambios en la capacidad digestiva y absortiva de los animales. En una primera instancia, la capacidad digestiva disminuye; pero posteriormente los animales muestran como mecanismos compensatorios una regulación al alza de la actividad tripsina y un incremento de la longitud relativa intestinal y, además, modulan la capacidad de absorción de los nutrientes para alcanzar un balance absortivo que les permita mantener un crecimiento similar al de los peces alimentados con harinas y aceites de pescado (Santigosa et al., 2008; 2011a; 2011b). Actualmente, más del 50% de la harina de pescado de las dietas comerciales está reemplazada por proteínas de origen vegetal (SOFIA, 2014) y en el caso de dorada (*Sparus aurata*) se ha demostrado que el 75% de la harina de pescado puede ser sustituida (Gomez-Requeni et al., 2004; 2005 Santigosa et al., 2008).

El uso de harinas y aceites de pescado también puede reducirse mediante el ahorro de proteína en la formulación de las dietas, conocido como efecto sparing de proteína. Éste, además de abaratar los costes de producción, puesto que la proteína es el ingrediente más caro, contribuiría a la sostenibilidad del sector acuícola reduciendo las pérdidas de nitrógeno al medio. Lípidos y carbohidratos pueden utilizarse como fuentes energéticas alternativas para reducir el contenido proteico de las dietas para peces (Enes et al., 2011); y además la propiedad aglutinante del almidón es un factor a tener en cuenta en la formulación de piensos (Bakke et al., 2011). Los lípidos juegan un importante papel como fuente energética y su efecto sparing de proteína está bien establecido en peces, incluyendo la dorada y la lubina (García-Alcazar et al., 1994; Pérez et al., 1997; Dias et al., 1998; Company et al., 1999; Vergara et al., 1996; 1999); alcanzándose en el caso de dorada unos crecimientos óptimos con un 48% de proteína y un 22.5% de lípido (Lupatsch et al., 2001). Sin embargo, la utilización de aceites de origen vegetal disminuye la calidad del producto final, que concretamente presenta menor contenido de ácidos grasos n-3 HUFA (EPA, ARA y DHA); mientras que tiende a acumular sobre todo linoleico (18:2 n-6) (Izquierdo et al., 2003; 2005; De Francesco et al., 2007; Fountoulaki et al., 2009). Para intentar minimizar estos efectos sobre la calidad del filete, en las granjas de producción se utilizan dos estrategias alimentarias: 1) inclusión en dieta de niveles moderados de aceites vegetales o 2) inicialmente alimentación con dietas altamente sustituidas y posteriormente la administración de dietas de finalización o "wash out" (dietas con aceite de pescado como única fuente de lípidos) durante los últimos dos-tres meses de cultivo. La inclusión de aceites vegetales, incluso a niveles moderados (como máximo un 60% de sustitución), hacen incrementar los niveles de ácidos grasos C18, como el linoleico, linolénico y oleico; y disminuir el contenido de n-3 HUFA en pez entero, en los distintos órganos y en el filete (Turchini et al, 2009). El uso de dietas de finalización proporciona una solución paliativa, ya que disminuyen los niveles de linoleico y oleico, principalmente por el modelo de dilución (Turchini et al., 2009), pero los niveles de EPA, ARA y DHA aunque incrementan, no llegan a alcanzar la concentración que presentan en peces alimentados con aceite de pescado (Fountoulaki et al., 2009; Turchini et al., 2009).

Tabla 3. Composición de ácidos grasos (% ácidos grasos totales) de aceite de pescado, aceite de soja y aceite de colza/canola utilizados en la formulación de dietas para peces. (Fuente: Turchini et al., 2009).

Aceite	SFA	MUFA	LA	AA	ALA	EPA	DHA	n-6 PUFA	n-3PUFA	n-3/n-6 ratio
Anchoa	28.8	24.9	1.2	0.1	0.8	17.0	8.8	1.3	31.2	24
Menhaden	30.5	24.8	1.3	0.2	0.3	11.0	9.1	1.5	25.1	16.7
Soja	14.2	23.2	51.0	-	6.8	-	-	51.0	6.8	0.1
Colza/Canola	4.6	62.3	20.2	-	12.0	-	-	20.2	12	0.6

SFA: Ácidos grasos saturados, MUFA: Ácidos grasos monoinsaturados, LA: Ácido linoleico, AA: Ácido araquidónico, ALA: Ácido linolenico, EPA: Ácido eicosapentaenoico, DHA: Ácido docohexaenoico, n-6 PUFA: Ácidos grasos poliinsaturados que presentan el primer doble enlace en el sexto átomo de carbono, n-3 PUFA: Ácidos grasos poliinsaturados con el primer doble enlace en el tercer átomo de carbono.

Por otro lado, el uso de los carbohidratos como fuente energética en salmónidos y otras especies carnívoras parece ser limitado (Wilson, 1994; Hemre et al., 2002; Stone et al., 2003); aunque se ha demostrado que presentan toda la maquinaria necesaria para su utilización (Cowey y Walton, 1989; Dabrowski y Guderley, 2002; Enes et al., 2009). En general, las especies carnívoras, incluidos los salmónidos y muchos peces marinos, toleran niveles más bajos de carbohidratos que las especies omnívoras y herbívoras (Wilson, 1994). Enes et al., (2011) revisó los estudios en relación al efecto sparing de proteína por carbohidratos en dorada y lubina. Los estudios realizados muestran un éxito variable en función del nivel de inclusión, la complejidad molecular del glúcido y/o al tratamiento tecnológico al que se someten los piensos, así como la estrategia alimentaria utilizada y la temperatura de cultivo. En lo que se refiere al nivel de inclusión, está descrito que a mayor inclusión de almidón menor digestibilidad del mismo (Enes et al., 2011); en este sentido la estrategia alimentaria utilizada también puede modular la carga de carbohidratos ingerida por el animal. Además, la relación amilasa/amilopectina que presente el almidón utilizado lo hará más o menos susceptible a la degradación enzimática. En este sentido, el trigo ha sido descrito como una fuente de carbohidratos altamente digeribles para la dorada debido a su bajo ratio amilosa/amilopectina (Venou et al., 2003). En el caso del procesamiento del pienso, se ha demostrado que tanto la extrusión como la gelatinización favorecen la digestibilidad del almidón frente al almidón en crudo (Spannhof and Plantikov, 1983; Venou et al., 2003). Los efectos de la temperatura sobre la digestibilidad de los carbohidratos son menos conocidos, en este sentido, en lubina se ha visto que un aumento de la temperatura mejora la digestibilidad (Moreira et al., 2008); sin embargo este efecto no se ha encontrado en dorada (Couto et al., 2008b).

Esta tesis se ha centrado en el estudio de la capacidad de digestión y absorción de la dorada y la lubina, las principales especies marinas cultivadas de la región Mediterránea. A pesar de que su producción está bien establecida, el conocimiento de los efectos que puedan tener las dietas de sustitución sobre estos procesos es escaso. Por ello son necesarios estudios como los englobados en esta tesis, dónde se analiza la actividad de las enzimas digestivas (pepsina, proteasa alcalina, amilasa y lipasa), así como la caracterización de la actividad tripsina/quimotripsina, la actividad de enzimas presentes en la membrana intestinal (peptidasas) y la capacidad de absorción de aminoácidos, esenciales y no esenciales, y D-Glc en las diferentes regiones del tracto intestinal (ciegos pilóricos, intestino proximal e intestino distal) en condiciones tanto post-prandiales como a 24h post-ingesta. Los procesos digestivos y absorptivos son clave para que el aprovechamiento de la dieta sea óptimo y un mejor conocimiento de cómo están regulados ayudaría a la formulación de dietas de elevada calidad para obtener crecimientos óptimos y buenas eficiencias alimentarias; minimizando la pérdida de alimento y contribuyendo al desarrollo sostenible de la industria acuícola.

En el capítulo I se estudia el efecto de la inclusión de distintos niveles de almidón en la dieta. Doradas de un peso inicial de 115g se cultivaron durante 14 semanas, de agosto a noviembre, con dietas que contenían un 65% de ingredientes de origen vegetal. Los animales se alimentaron *ad libitum* 2 veces al día y se estudió el efecto de: 1) dietas isoproteicas e isolipídicas (46%, 17%) con porcentajes crecientes de almidón (5, 14 y 22%); 2) dietas isoproteicas (46%) que presentan niveles crecientes de almidón y decrecientes de lípidos (5, 11, 17, 22% y 23, 20, 19, 17%, respectivamente); y 3) dietas isolipídicas (17%) que presentan niveles crecientes de almidón y decrecientes de proteína (22, 29, 33% y 46, 42, 40%, respectivamente).

El capítulo II pretende establecer el porcentaje óptimo de proteína y lípido de la dieta para juveniles de lubina. Se llevaron a cabo 2 experimentos: 1º) en primavera (Abril – Junio) y 2º) en invierno (Diciembre – Enero); en los cuales juveniles de lubina con un peso inicial de 5g se alimentan 8 semanas con dietas que presentan un 38% de sustitución por ingredientes vegetales y cuya fuente lipídica fue el aceite de pescado. Los animales fueron alimentados *ad libitum*. Primero, se testaron dietas con un contenido proteico ascendente (40, 45, 50, 55 y 59%) y de lípido y almidón decreciente; y segundo, se administraron dietas isoproteicas (52%) con un contenido lipídico que incrementa (12, 14, 16, 18 y 20%) a medida que disminuye el almidón (13, 12, 10, 9 y 8%).

El capítulo III trata de establecer la mejor relación proteína/energía en dietas de elevado contenido lipídico; minimizando así el contenido de proteína mediante la inclusión de lípido y almidón. Juveniles de dorada con un peso medio de 70g se alimentan 2 veces al día a saciedad durante 12 semanas (Mayo – Julio) con dietas con un 50% de sustitución de harina de pescado por ingredientes vegetales (trigo, gluten de trigo y concentrado de proteína de soja) y cuya única fuente lipídica era el aceite de pescado. Se formularon 7 dietas isoenergéticas con diferente contenido proteico (35, 38, 41, 44, 47, 50 y 53%) y en las que los niveles de lípido y almidón son variables (27 al 19% y 21 al 10%, respectivamente).

El capítulo IV estudia el efecto sparing de proteína por carbohidratos con la finalidad de determinar el mejor momento del día para la administración de los mismos. Se cultivaron juveniles de dorada, de unos 21g durante 8 semanas (Noviembre- Enero) con una dieta comercial (C) (48% de proteína y 20% de lípido) y una dieta experimental con carbohidratos altamente digeribles (CH) (37% de proteína, 12.5% lípido y 40% de carbohidratos). En la dieta C más de un 60% de los ingredientes eran de origen vegetal; mientras que en la dieta CH el único componente vegetal era el trigo. Se establecieron tres regímenes dietarios: C, CH-M (Dieta CH por la mañana) y CH-A (Dieta CH por la tarde), administrando una ración del 1.6% del peso corporal de la dieta correspondiente por la mañana y un 1% por la tarde.

Finalmente, en el capítulo V se pretende determinar el efecto de un régimen alimentario dónde los lípidos provenían del aceite de pescado (dieta FO) o en un 75% de aceites vegetales (dieta VO: aceite de soja y colza al 50%) durante el crecimiento de dorada (de 62g a 305g) y tras un cambio, tanto a corto como a largo plazo, de una dieta por la otra en diferentes momentos del crecimiento. Durante un periodo de 19 semanas (Mayo-Octubre); los animales se alimentaron dos veces al día a saciedad con la dieta correspondiente. Los piensos eran isoproteicos e isoenergéticos, con un 75% de sustitución de harina de pescado por harinas de origen vegetal y sólo diferían en su fuente de lípidos.

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Objetivo

2. Objetivo

Los capítulos que constituyen esta tesis se plantearon enmarcados en los proyectos titulados "Caracterización y regulación de marcadores de adipogénesis y deposición lipídica; digestión y absorción de grasas y metabolismo de ácidos grasos poliinsaturados en el engorde de trucha y dorada" (AGL2008-00783/ACU) y "Caracterización de los efectos de nutrientes e ingredientes de la dieta sobre la funcionalidad del adipocito y la fisiología intestinal; regulación de los transportadores de ácidos grasos" (AGL2011-24961/ACU).

El objetivo global de la tesis ha sido determinar la capacidad de modulación de las actividades digestivas y absortivas en dorada (*Sparus aurata*) y lubina (*Dicentrarchus labrax*), peces carnívoros y de gran interés comercial en el área Mediterránea, en diferentes condiciones de cultivo y alimentadas con diversas dietas de sustitución de harinas de pescado o dietas doble sustitución. Se han estudiado los efectos de la inclusión tanto de harinas como de aceites de origen vegetal en diferentes proporciones, el efecto sparing de proteína mediante la inclusión de lípidos y/o carbohidratos en dieta, así como la determinación de la mejor relación proteína/energía para cada una de las especies. Finalmente, para dorada se ha buscado el mejor momento del crecimiento para realizar un cambio en la composición de aceites de la dieta, y también para administrar una dieta con alto contenido de carbohidratos (mañana o tarde), siempre buscando minimizar los efectos negativos que pudiera tener a nivel digestivo y absortivo, y presenvando el crecimiento.

Informe del director

3. Informe del director

La Dra. **Mª Ángeles Gallardo Romero**, como directora de la Tesis Doctoral titulada "Efectos de las dietas de doble sustitución y las condiciones de cultivo sobre las capacidad digestiva y absortiva de dorada y lubina" realizada por **Irene García Meilán**, manifiesto la veracidad del factor de impacto y la implicación de la doctoranda en cada uno de los artículos científicos que se presentan en esta tesis doctoral.

Artículo I (Capítulo I)

Título: Changes in digestive and absorptive processes caused by dietary carbohydrate inclusion in sea bream (*Sparus aurata*). Three different strategies tested

Autores (p.o. de firma): García-Meilán, I., Ordóñez-Grande, B., Fontanillas, R., Gallardo, M.A.

Revista: Enviado a Animal Feed Science and Technology Julio 2015

Participación de la doctoranda: Irene García Meilán ha obtenido las muestras y las ha analizado en su totalidad, colaborando en la interpretación de los resultados. Ha redactado la primera versión del manuscrito y ha colaborado en el proceso de revisión por igual.

Artículo II (Capítulo II)

Título: Effects of dietary protein-to-lipid ratio on digestive and absorptive processes in sea bass fingerlings

Autores (p.o. de firma): García-Meilán, I., Ordóñez-Grande, B., Machahua, C., Buenestado., S., Fontanillas, R., Gallardo, M.A.

Revista: Enviado a Animal Feed Science and Technology Julio 2015

Participación de la doctoranda: Irene García Meilán ha obtenido las muestras y ha colaborado en su análisis y en la interpretación de los resultados, en la redacción de la primera versión del manuscrito y en el proceso de revisión por igual.

Artículo III (Capítulo III)

Título: Different protein to energy ratio diets for gilthead sea bream (*Sparus aurata*): Effects on digestive and absorptive processes

Autores (p.o. de firma): García-Meilán, I., Valentín, J.M., Fontanillas, R., Gallardo, M.A.

Revista: Aquaculture 412-413: 1-7 (2013)

I.F. 1.828, in Fisheries 13/50 Q2 **5 Años I.F.** 2.586

Participación de la doctoranda: Irene García Meilán ha obtenido las muestras y las ha analizado en su totalidad, colaborando en la interpretación de los resultados. Ha redactado la primera versión del manuscrito y ha colaborado en el proceso de revisión por igual.

Artículo IV (Capítulo IV)

Título: Meal timing affects protein-sparing effect by carbohydrates in sea bream: Effects on digestive and absorptive processes

Autores (p.o. de firma): García-Meilán, I., Ordóñez-Grande, B., Gallardo, M.A.

Revista: Aquaculture 434, 121-128 (2014)

I.F. 1.878, in Fisheries 12/52 Q1 **5 Años I.F.** 2.341

Participación de la doctoranda: Irene García Meilán ha obtenido las muestras y ha contribuido a su análisis, colaborando en la interpretación de los resultados. Ha redactado la primera versión del manuscrito y ha colaborado en el proceso de revisión por igual.

Artículo V (Capítulo V)

Título: Modulation of digestive and absorptive processes with age and/or after a lipid dietary change in gilthead sea bream

Autores (p.o. de firma): García-Meilán, I., Ordóñez-Grande, B., Valentín, J.M., Hernández, A., Hernández, M.D., Fontanillas, R., Gallardo, M.A.

Revista: Enviado a Animal Feed Science and Technology Julio 2015

Participación de la doctoranda: Irene García Meilán ha obtenido las muestras y ha contribuido a su análisis, colaborando en la interpretación de los resultados. Ha redactado la primera versión del manuscrito y ha colaborado en el proceso de revisión por igual.

Mª Ángeles Gallardo Romero

Resumen de las Publicaciones

Capítulo I

Cambios en los procesos digestivos y absortivos causados por la inclusión de carbohidratos en dietas para dorada. Tres estrategias diferentes a prueba

Se formularon 8 dietas con diferentes niveles de almidón (5% a 33%) para alimentar a juveniles de dorada durante 14 semanas. Se llevaron a cabo tres experimentos simultáneamente. Al final del crecimiento, las actividades proteasa alcalina, α-amilasa y lipasa se midieron a 7h post-ingesta, y a 24h, la actividad fosfatasa alcalina (AP), leucina aminopeptidasa (LAP) y la capacidad de absorción de nutrientes.

En el primer experimento, se utilizaron dietas isoproteicas (46%) e isolipídicas (17%) con diferente contenido de almidón (5, 14 y 22%). Los animales S22$_{L_{17}P_{46}}$ mostraron una mayor actividad proteasa en ciegos pilóricos e intestino proximal. La zimografía corroboró estos datos; mostrando una disminución de la actividad tripsina-like, mientras que la actividad quimotripsina-like no se detectó en los animales alimentados con un 5 y un 14% de almidón. En cambio, los animales alimentados con la dieta de menor contenido de almidón (S5$_{L_{17}P_{46}}$) presentaron la actividad α-amilasa más elevada. No se encontraron diferencias en las actividades lipasa, AP y LAP; aunque AP tiende a disminuir y LAP a aumentar con la inclusión de almidón en dieta. Además, en los animales S14$_{L_{17}P_{46}}$ se encontró la mayor capacidad de absorción de L-Ala y D-Glc. No se detectaron diferencias en la capacidad de captación de L-Lys ni en el peso final por la incorporación del almidón en dieta.

En el segundo experimento, se utilizaron dietas isoproteicas (46%) donde el lípido se reemplaza por almidón. No se encontraron cambios significativos en la actividad de los enzimas pancreáticos cuando el lípido se sustituye por almidón; aunque la actividad lipasa y proteasa tienden a disminuir. De hecho, la zimografía reveló una reducción proporcional en todas las proteasas alcalinas por el aumento de almidón. El reemplazo de lípido por almidón afecta de forma diferencial a la actividad AP y LAP; mostrando en los peces S22L17$_{P_{46}}$ una tendencia a una menor capacidad de absorción de nutrientes que en los animales S5L23$_{P_{46}}$ y S11L20$_{P_{46}}$ y las doradas S17L19$_{P_{46}}$ mostraron la mayor captación de L-Lys y la menor de D-Glc.

El tercer experimento estudia el efecto sparing de proteína por carbohidratos en dietas isolipídicas (17%). Se detectó una disminución significativa de las actividades proteasa alcalinas y α-amilasa en los animales alimentados con las dietas de menor proteína y mayor contenido de almidón (S29$_{L_{17}}$P42 y S33$_{L_{17}}$P40). La zimografía corroboró los resultados de proteasa alcalina. La actividad lipasa y LAP y la capacidad de absorción de L-Lys y L-Ala no se modificaron por la sustitución de proteína por almidón; mientras que provocó un incremento significativo en AP y una disminución significativa del influjo de D-Glc. Finalmente, las doradas S33$_{L17}$P40 presentaron un SGR y una ingesta voluntaria significativamente menor que los animales S29$_{L17}$P42 y S22$_{L17}$P46.

Capítulo II

Efectos de la relación proteína-lípido de la dieta sobre los procesos digestivos y absortivos en alevines de lubina

Se llevaron a cabo dos experimentos, uno en primavera y otro en otoño-invierno, de 8 semanas de duración con alevines de lubina. Tras el periodo de crecimiento, se estudió la síntesis pancreática de enzimas digestivas en ciegos pilóricos (PC), la adaptación de la actividad anticipatoria al alimento en intestino proximal (PI) y la capacidad de absorción de nutrientes en intestino distal a 24h post-ingesta.

En primavera, se formularon 5 dietas isoenergéticas con diferente nivel de proteína (40, 45, 50, 55 y 59%) cambiando simultáneamente el contenido de lípido y almidón. Los animales alimentados con las dietas de menor contenido proteico presentaron una menor acumulación de proteasas alcalinas en los peces de 40 a 50%, lipasa para los animales de 40 y 45% y α-amilasa en las lubinas del 40 al 55%; mientras que las actividades enzimáticas digestivas en intestino proximal fueron más elevadas en los peces alimentados con un bajo y un elevado contenido proteico, siendo significativas para las actividades proteasas alcalinas en los animales 40 y 59%, y para la α-amilasa en los 40 y 45%. Con la zimografía en PC se detectaron 4 proteasas con actividad tripsina-like (23, 22, 20 y 17KDa) y una proteasa de 18KDa con actividad quimotripsina-like, que se modularon de forma diferencial de acuerdo con la dieta administrada. Los ratios PC/PI para las proteasas alcalinas fueron los más elevados; en cambio los más bajos se determinaron para α-amilasa; mostrando diferentes patrones de respuesta a los cambios en la composición de la dieta: más elevados para las proteasas en los animales 45, 50 y 55% y para la α-amilasa en los 59%. Una regulación a la baja de la capacidad de captación de L-Lys y D-Glc fue detectada a medida que el contenido proteico de la dieta disminuía, mientras que no se detectaron cambios para la capacidad de absorción de L-Met y L-Trp. De acuerdo con los datos arriba mencionados y teniendo en cuenta que los animales alimentados con un 50% de proteína eran los que presentaban un mejor crecimiento, se llevó a cabo un experimento sobre el efecto de la relación proteína/energía.

Las lubinas se alimentaron con 5 dietas isoproteicas que diferían en el contenido lipídico (12, 14, 16, 18 y 20%). La síntesis de proteasas alcalinas y lipasa en PC presentó una regulación al alza para las dietas poco energéticas (12, 14, y 16% y 12 y 14%, respectivamente) y no se detectaron cambios en α-amilasa. La zimografía de PC se correspondía con la cuantificación de proteasas. Sólo la actividad lipasa anticipatoria del alimento se reguló al alza en los animales alimentados con un 12% de lípido. La capacidad de absorción de L-Met y D-Glc se regularon al alza por el incremento de lípido de la dieta y la L-Lys y el L-Trp no se modificaron. No se detectaron cambios en el peso final por el contenido de lípido de la dieta.

Finalmente, se estudiaron los efectos estacionales. Las síntesis de proteasas alcalinas en PC fue significativamente mayor en primavera y los animales mostraron un mayor crecimiento. Sin embargo la adaptación de la actividad anticipatoria del alimento en PI fue mayor en invierno. No se encontraron diferencias en la capacidad de absorción de nutrientes entre estas estaciones.

Capítulo III

Dietas con diferente relación proteína energía para dorada (*Sparus aurata*): Efectos sobre los procesos digestivos y absortivos

Durante 12 semanas juveniles de dorada fueron alimentados con 7 dietas isoenergéticas con diferente contenido proteico (35, 38, 41, 44, 47, 50 y 53%). La proteína de la dieta fue sustituida por lípido (del 27 al 19%) y almidón (del 21 al 10%). Tras el periodo de crecimiento, se estudió la actividad enzimática digestiva a 5h post-ingesta y la capacidad de absorción de nutrientes a 24h post-ingesta. Se detectó un incremento progresivo de la actividad proteasa total (APT) a medida que aumentaba el contenido proteico de la dieta (de 35 a 41%) y una disminución de esta actividad en doradas alimentadas con las dietas de mayor contenido de concentrado de soja (50 y 53% de proteína en dieta). La actividad lipasa no se vio afectada por la composición de la dieta. Se observó una regulación al alza de la actividad α-amilasa y la capacidad de absorción de D-Glc y L-Ala en los peces alimentados con las dietas de menor contenido de carbohidratos, que se correspondían con las que presentaban un 50 y 53% de proteína. Además, se detectó una regulación al alza de la capacidad de absorción de L-Lys en los animales alimentados con las dietas de menor contenido proteico (35, 38, 41 y 44%). Estos cambios en la composición de la dieta modularon la actividad enzimática y la capacidad de absorción de nutrientes, para mejorar la utilización del alimento y asegurar un buen crecimiento. En este sentido, sólo las doradas alimentadas con las dietas de menor contenido proteico (35%) presentaron un SGR significativamente inferior (<1), a pesar de que su ingesta voluntaria incrementó. Las doradas alimentadas con las dieta P_{44} y P_{47} presentaron cambios adaptativos mínimos a la dieta y mostraron un buen crecimiento.

Capítulo IV

El efecto sparing por carbohidratos está afectado por el momento de administración del alimento en dorada (*Sparus aurata*): Efectos sobre los procesos digestivos y absortivos

Una dieta comercial (C) (48% proteína y 20% lípido) y una dieta con carbohidratos altamente digeribles (CH) (37% proteína, 12.5% lípido y 40% de carbohidrato altamente digerible) se administraron a juveniles de dorada durante 8 semanas. En la dieta comercial, más del 60% de los ingredientes procedían de diferentes fuentes de origen vegetal, mientras que el único componente vegetal en la dieta CH era el trigo. Para determinar el mejor momento de administración de los carbohidratos y su posible efecto sparing de proteína, se establecieron 3 regímenes dietarios distintos: C, CH-M (Carbohidratos por la mañana) y CH-A (Carbohidratos por la tarde), y se les administraba la dieta correspondiente por la mañana (1.6% del peso corporal) y por la tarde (1% del peso corporal), según las cantidades de alimento consumido durante el periodo de aclimatación. Al final del periodo de crecimiento, se determinó la tasa de crecimiento específica (SGR), se midió la longitud relativa intestinal y el pH del contenido intestinal y se estudiaron las actividades enzimáticas digestivas (gástrica y pancreática) y la capacidad de absorción de nutrientes a 5h post-ingesta después de cada una de las comidas (mañana y tarde). La actividad proteasa ácida medida fue anticipatoria y era mayor cuando la siguiente comida contenía más proteína. No se encontraron diferencias ni en la longitud intestinal relativa ni en la capacidad de tamponamiento de la dieta. Al administrar una menor ración por la tarde se observaba una menor liberación de proteasa alcalina y α-amilasa y una regulación al alza de la capacidad de absorción de D-Glc y L-Ala. Se detectó un tránsito intestinal más rápido cuando los animales se alimentaban con la dieta CH. Cuando los carbohidratos altamente digeribles se administraban por la mañana y la dieta comercial por la tarde, se observó una mejor asimilación de ambas dietas debido a los mecanismos compensatorios, como el aumento de la capacidad de absorción de L-Lys, D-Glc y L-Ala después de la alimentación de la mañana, y una mayor liberación pancreática de proteasa alcalina y α-amilasa tras la alimentación de la tarde. Por el contrario, cuando los carbohidratos altamente digeribles se administran por la tarde, sólo se observó una regulación al alza significativa en la capacidad de absorción de L-Lys. Por lo tanto, la inclusión de carbohidratos altamente digeribles en la dieta mejora los procesos digestivos y absortivos cuando se administran por la mañana, permitiendo un efecto sparing de proteína que permite alcanzar un crecimiento comparable al de los peces alimentados exclusivamente con la dieta comercial.

Capítulo V

Modulación de los procesos digestivos y absortivos con la edad y/o tras un cambio de lípidos de la dieta en dorada

Juveniles de dorada se alimentaron durante 19 semanas con 2 dietas isonitrogenadas e isoenergéticas en las cuales el 75% de la harina de pescado había sido reemplazada por fuentes proteicas vegetales y que sólo diferían en la fuente lipídica: aceite de pescado (Dieta FO) o aceite vegetal (Dieta VO). Tras el periodo de crecimiento, se estudió el pH del contenido intestinal, la actividad enzimática digestiva, la capacidad de absorción de nutrientes y el peso final tras un ayuno de más de 24h y justo antes de la alimentación en peces alimentados con la dieta FO o VO y después de un cambio de dieta a diferentes pesos (92g, 147g, 233g), tanto a corto como a largo plazo.

El pH del contenido intestinal, las actividades proteasa alcalina, α-amilasa y lipasa y la capacidad de absorción de nutrientes disminuyeron con el crecimiento del animal. Además, los animales VO mostraron una tendencia a aumentar la actividad proteasa y α-amilasa, mientras la actividad lipasa era superior en las doradas FO; estos cambios fueron significativos para los peces de menor tamaño. Los aminoácidos esenciales presentaron una mayor capacidad de captación que los no esenciales y la D-Glc. Además la capacidad de absorción era superior en los animales pequeños VO versus los FO. No se detectaron cambios en la tasa de crecimiento al final del experimento, sin embargo los animales FO mostraron un mayor crecimiento durante la primera mitad del experimento para descender después, mientras que para los animales VO fue más baja pero se mantuvo.

Tras un cambio de dieta a corto plazo, los animales pequeños no fueron capaces de adaptar su actividad enzimática digestiva ni su capacidad de absorción hacia la nueva dieta administrada ni a la de origen, mientras que los peces de mayor tamaño generalmente sí se adaptaron.

A las 9-15 semanas de un cambio de dieta a FO, los animales fueron capaces de adaptar su actividad proteasa y su capacidad de absorción a la nueva situación; pero la actividad α-amilasa disminuyó. Sim embargo, tras un cambio a largo plazo a la dieta VO, los animales mostraron una regulación al alza de la capacidad de absorción de nutrientes, mientras que la actividad proteasa alcalina tendió a disminuir y la α-amilasa a aumentar. Al final del experimento el crecimiento no se vio afectado por el tratamiento dietario.

Publicaciones

CAPÍTULO I

Changes in digestive and absorptive processes caused by dietary carbohydrate inclusion in gilthead sea bream. Three different strategies tested

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Abstract

Eight diets with different levels of starch (5% to 33%) were formulated to feed sea bream juveniles for a 14-week period. Three different trials were conducted simultaneously. At the end of each trial, alkaline protease, α-amylase and lipase activities were measured 7 h post-feeding, and alkaline phosphatase (AP) and leucine amino peptidase (LAP) activities and nutrient absorption capacities 24 h post-feeding.

In the first trial, isoproteic (46%) and isolipidic (17%) diets with different starch levels (5, 14 and 22%) were used. S22$L_{17}P_{46}$ animals showed the highest protease activity in both the pyloric caeca and proximal intestine. Zymography corroborated these data, showing a reduction in trypsin-like activity and no chymotrypsin-like activity in fish fed 5 and 14% starch. Animals fed the diet with the lowest starch content (S5$L_{17}P_{46}$) had the highest α-amylase activity. No differences were found in lipase, AP and LAP activities; however AP tended to decline and LAP to increase with dietary starch inclusion. The highest L-Ala and D-Glc absorption capacities were found in S14$L_{17}P_{46}$. No differences in L-Lys uptake or final growth were detected with dietary starch incorporation.

In the second trial, isoproteic (46%) diets in which lipid were replaced with starch were used. No significant changes were detected in pancreatic enzyme activities when dietary lipid was substituted for starch, although lipase and protease activities tended to decrease. In fact, zymography revealed a proportional reduction in all alkaline protease as dietary starch increased. Lipid replacement with starch affected AP and LAP activities in different ways; S22L17P_{46} fish had the lowest AP values and the highest LAP values. There were no differences in final growth, although S22L17P_{46} fish tended to show lower nutrient absorptive capacities than S5L23P_{46} and S11L20P_{46} animals and S17L19P_{46} fish showed the highest L-Lys and the lowest D-Glc uptake.

The third trial studied the protein sparing effect by carbohydrates in isolipidic (17%) diets. A significant reduction in alkaline protease and α-amylase activities was detected in animals fed diets with lower protein and higher starch contents (S29L_{17}P42 and S33L_{17}P40). Alkaline protease results were corroborated by zymography. Lipase and LAP activities and L-Lys and L-Ala absorption capacities were not modified by the replacement of dietary protein with starch, but there was a significant increase in AP and a significant decrease in D-Glc influx. Finally, significantly lower SRG and voluntary feed intake and higher FCR were found in S33L_{17}P40 fish versus S29L_{17}P42 and S22L_{17}P46 animals.

Keywords: Amylase, brush border membrane enzymes, brush border membrane vesicles, lipase, protease, starch

1. Introduction

More than 50% of the protein in commercial diets currently comes from plant material due to the low availability and high prices of fish meal (SOFIA, 2014). However, the use of plant protein sources could imply lower amino acid availability, mainly of lysine and methionine; or the presence of antinutritional factors (ANFs) and low palatability (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). Some vegetable meals, such as wheat and corn, contain a large amount of carbohydrates (Gatlin et al., 2007), with a potential protein sparing effect. In order to minimize the effects that vegetable dietary inclusion could have a combination of plant-derived feed ingredients is usually used to replace fish meal, and supplements such as amino acids, flavourings and exogenous enzymes, like phytases, may be added to achieve good growth rates and reduce production costs (Gatlin et al., 2007). Moreover, the high price of fish oil and its low availability make the replacement of fish oil by plant oils necessary, but this reduces final product quality (De Francesco et al., 2007; Fountoulaki et al., 2009; Izquierdo et al., 2005; Menoyo et al., 2004).

The use of plant ingredients in the diet may modify digestive and absorptive capacities differently according to dietary composition, with subsequent effects on metabolism and growth. For example, the presence of vegetable ingredients depresses digestive enzyme activities, but this reduction could be compensated by the higher relative intestinal length in fish and by up-regulating trypsin secretion, which modifies the trypsin/chymotrypsin ratio (García-Meilán et al., 2013, 2014; Santigosa et al., 2011a, 2008). Moreover, increased α-amylase activity was detected in a diet with a high starch content (40%) (García–Meilán et al., 2014). Modifications in nutrient absorption capacity may be due to the presence of free amino acids, which leads to asynchronous utilization (Ambardekar et al., 2009; Santigosa et al., 2011b), or to changes in gut histology due to differences in protein composition (Santigosa et al., 2008). Moreover, the administration of low starch diets could lead to the up-regulation of D-Glc and L-Ala absorption capacities (Garcia-Meilán et al., 2013, 2014; Santigosa et al., 2011b).

The digestion and metabolization of carbohydrates by fish seems to be limited, particularly in carnivorous species (Wilson, 1994), even though the whole enzymatic machinery required for their use is present (Cowey and Walton, 1989; Dabrowski and Guderley, 2002; Enes et al., 2009). The digestibility of carbohydrates is affected by their molecular complexity, different dietary inclusion levels and feeding rate, rearing temperature and technological treatments applied (Enes et al., 2011). In this sense, Venou et al. (2003) demonstrated that wheat was a good carbohydrate source even without extrusion processes, but the same was not true for corn due to its higher amylose/amylopectin ratio and structure. All these factors make studies on the sparing effect of carbohydrates controversial. On the one hand, Enes et al. (2011) reviewed dietary carbohydrate utilization by European sea bass and gilthead sea bream juveniles, concluding that more than 20% of digestible carbohydrates could diminish growth and feed utilization whereas other studies with more than 20% of starch had no effect (Fernández et al., 2007). On the other hand, in a previous study (García-Meilán et al., 2014) we found that sea bream fed with a commercial diet showed the same growth rate as sea bream fed in the morning with a diet that contained 40% of highly digestible carbohydrates from wheat and in the afternoon with commercial diet.

The effectiveness of a given diet is determined by digestion and absorption processes, which ultimately affect growth and food utilization (Debnath et al., 2007; Lemieux et al., 1999; Mohanta et al., 2008). Digestion begins in the stomach were pepsinogen is converted into active pepsin by HCl and proteins are denatured (Wu et al., 2009; Yúfera et al., 2004). When the chyme reaches the pyloric caeca, cholecystokinin stimulates pancreatic secretions which include, among other substances, proproteases, α-amylase and lipolytic enzymes, as well as DNAase and RNAase (Bakke et al., 2011). The activities of the main digestive enzymes differ between species and may be modified in relation to dietary composition and its digestibility, food quantity and the natural diet (Budington et al., 1997; Hidalgo et al., 1999; Pérez-Jiménez et al., 2009; Reimer, 1982; Santigosa et al., 2011a, 2011b, 2008; Zambonino-Infante & Cahu, 2007). The final step of digestion is carried out in the enterocyte brush border membrane where many enzymes, such as alkaline phosphatase, leucine aminopeptidase, maltase, saccharase, etc. that release molecules small enough for their absorption, are found (Bakke et al., 2011). In fish, absorption processes occur throughout the entire intestine by means of diffusion, facilitated transport or active transport (Mailliard et al., 1995). Storelli et al.(1989) described the presence of at least four Na^+-dependent amino acid transport systems (acidic, basic, neutral amino acids and a system for N-methylated amino acids and proline); alanine, glycine, glutamate, lysine and proline Na^+-independent transport systems have also been described (Storelli et al., 1989). The existence of peptide transporters, PepT1 and PepT2, was also demonstrated (Hakim et al., 2009; Sangaletti et al., 2009; Terova et al., 2009). Moreover, the intestinal fatty acid binding protein (I-FABP) may be involved in peptide or protein transport processes before endocytosis (Concha et al., 2002). D-glucose and D-galactose are transported by SGTL1 (Sala-Rabanal et al., 2004).

The optimum use of carbohydrates could help minimize production costs by sparing both protein and lipid sources, and might favour the maintenance of final product quality. The aim of this study was to test the effect of dietary carbohydrates on digestive and absorptive processes in sea bream. Fish were fed with diets containing 65% of plant-derived feed ingredients. Three different trials were performed. Diets in the first trial were isoproteic and isolipidic with increasing starch content; in the second trial they were isoproteic, with the lipid replaced with starch, and the third was isolipidic, with the protein replaced with starch.

2. Materials and methods

2.1. Diets

Eight diets (Table 1) were formulated by Skretting ARC (Norway). All diets contained 65% of plant ingredients (corn and wheat gluten, soy protein concentrate, cellulose and wheat starch). Three different trials were conducted simultaneously, differing in dietary composition (Table 1). In the first trial, three isoproteic and isolipidic diets (46% protein, 17% lipid) with different starch contents (5, 14 and 22%) were tested; in the second there were four isoproteic diets (46% protein) with increasing starch content as lipid content decreased (5, 11, 17, 22% and 23, 20, 19, 17%, respectively) and finally, the third trial tested three isolipidic diets (17% lipid) with decreasing protein content as starch increased (46, 42, 40% and 22, 29, 33%, respectively). Diet S22L17P46 was used in all three experimental trials. Diets were named according to their dietary composition: the subscript number indicates the percentage of dietary components that was the same within each group; the non-subscript number indicates the percentage difference in dietary composition between the tested diets.

2.2. Fish and sampling

Gilthead sea bream were acclimatized to the IRTA facilities (Sant Carles de la Ràpita, Spain) for two weeks. After that period, 600 sea bream, each weighing about 115 g, were randomly distributed in 24 fibreglass tanks (3 tanks per diet and 25 fish per tank). The water salinity was 35‰ and the oxygen content was 8 ppm. Animals were maintained at 22^{o}C with a 12L/12D photoperiod. Triplicate groups of fish were fed the corresponding diet ad libitum twice a day for a 14-week period (August–November).

At the end of the growth trial, nine animals per time and treatment were anaesthetized (phenoxyethanol 100 ppm), weighed and sacrificed by severing the spinal cord at 7 h and 24 h post-feeding. At 7 h post-feeding, the digestive tract was isolated and samples of pyloric caeca and proximal intestine, including the intestinal content, were collected. At 24 h post-feeding, the proximal intestine was isolated and was cut lengthwise, and washed in an isosmotic saline solution containing 0.1 M of protease inhibitor (phenyl-methyl-sulphonyl-fluoride). All samples were rapidly frozen in liquid nitrogen and maintained at -80ºC until required for the intestinal enzymatic studies and the nutrient absorption experiments.

All fish-handling procedures complied with European guidelines for animal care (Directive 2010/63/EU).

Table 1. Ingredient and nutrient composition of the experimental diets.

Diet (%)	$S_{22}L_{17}P_{46}$	$S5L_{17}P_{46}$	$S14L_{17}P_{46}$	$S5L23P_{46}$	$S11L20P_{46}$	$S17L19P_{46}$	$S29L_{17}P42$	$S33L_{17}P40$
Cellulose	0.0	17.1	7.7	11.5	6.9	2.4	0.0	0.0
Fish meal	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Corn gluten	13.1	13.1	13.1	13.0	13.0	13.0	14.7	9.8
Wheat gluten	20.0	20.0	20.0	20.0	20.0	20.0	20.0	21.0
Fish oil north atlantic	11.3	11.3	11.3	17.2	15.3	13.3	11.3	11.5
SPC 60%	11.5	11.5	11.5	11.5	11.5	11.5	2.7	2.9
Vitamin premix	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Yttrium premix	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Wheat starch	21.7	5.0	14.2	5.0	10.9	17.3	29.0	33.0
Dry matter	92.7	94.9	94.0	93.9	93.9	92.4	91.6	92.5
Moisture	7.3	5.1	6.0	6.1	6.1	7.6	8.4	7.5
Protein	46.5	45.7	46.2	45.5	46.3	47.3	42.1	39.6
Fat	17.0	16.4	17.3	22.8	20.3	19.1	17.0	17.2

SPC: Soybean Protein Concentrate. Vitamin premix is a proprietary formula of Skretting (Norway)

2.2.1. Zootechnical and morphological indexes

Total feed intake was calculated as [food intake (Kg)-food lost (Kg)]. Voluntary feed intake, regarding whole diets and weight gain, were expressed in: g BW (kg)$^{-0.70}$ day^{-1} to correct for the influence of fish size on feed consumption in gilthead sea bream (Lupatsch et al., 1998), where body weight (BW) was calculated from the initial and final body weight of fish as the geometric mean: BW=$(W_{ini} \cdot W_{fin})^{0.5}$. To evaluate the weight gain, and calculate the specific growth rate (SGR) and feed conversion ratio (FCR) during the experimental period, total biomass from all triplicate tanks was obtained from Skretting. The SGR was calculated as follows: $((\ln W_{fin}$ (g)-$\ln W_{ini}$ (g))/$t)\cdot 100$, where W_{fin} and W_{ini} represent the final and initial weights respectively, and t is the number of days of feeding. The FCR was calculated as g feed given/g live weight gain.

2.3. Intestinal pH and digestive enzyme analysis

The pH of the intestinal contents was measured (Crison, micro pH 2000) in the pyloric caeca (PC) and proximal intestine (PI). A modification of the procedure described by Santigosa et al. (2008) was used to measure alkaline protease and α-amylase activities in the intestinal samples, and to characterize trypsin-like and chymotrypsin-like activities, while lipase activity was determined following Santigosa et al. (2011a). Briefly, intestinal samples were individually homogenized (Politron 2000, Sorvall TC) at 4ºC in 50 mM TrisHCl buffer pH 7.5 to a final concentration of 250 mg mL^{-1}. Homogenates were centrifuged (1100 g, 15 min, 4ºC, Jouan CR 411) and supernatants were recovered and stored at -80ºC. For alkaline protease activity (TPA) determination, samples were reacted with 50 mM TrisHCl buffer containing 1% casein at the pH of the intestinal contents and 20 ºC. After 30 min, the addition of trichloroacetic acid 12% stopped the reaction and samples were kept at 4 ºC for one hour and then centrifuged (7500 g, 5 min, 4 ºC). Individual blanks were established for each sample. Supernatant absorbance was measured at 280 nm (UV-1603, Shimadzu). Alkaline protease activity was measured as BAEE units, and bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as the standard.

Trypsin-like and chymotrypsin-like activities were characterized by zymography in which pure trypsin and albumin were used as controls and a commercial weight marker RPN 800 (Amersham, GE Healthcare, UK, 12000-225000Da) was used to determine the molecular weight of protease active fractions. Samples were loaded in 12% polyacrylamide gels (10x10.5x0.1 cm) and electrophoresis was performed at a constant current of 15 mA per gel for 150 min (EPS 301 Power Supply, 4 ºC). The gels were incubated under agitation with TrisHCl buffer containing 2% casein at the pH of the intestinal content for 30 min at 4 ºC, and after that were shaken for 90 min at room temperature. Gels were washed and stained in a methanol:acetic acid:water solution (40:40:10) with 0.1% BBC R-250 (Coomassie Brilliant Blue R-250). As in previous studies in sea bream (Garcia-Meilán et al., 2013, 2014), six proteases were detected; three presented trypsin-like activity (90, 60 and 55 KDa) and the other three showed chymotrypsin-like activity (50, 30 and 25 KDa).

α-Amylase activity was measured using a potato starch covalently labelled with Remazol Brilliant Blue R dye, commonly known as amylose azure. The homogenate reacted with 0.5% tracer solution containing soybean trypsin inhibitor (SBTI 0.04 mg mL^{-1}) for 30 minutes, after which the reaction was stopped by the addition of 6% acetic acid and was centrifuged (13000 g; 10 min at 4 ºC). Supernatant absorbance was measured at 595 nm (UV-1603, Shimadzu). α-Amylase (Sigma Aldrich, Spain, 66U/mg solid) was used as the standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 and 20 ºC.

For lipase determination, homogenates were mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg L^{-1} colipase (pH 8.3), and the increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33944 U/mg protein, 22980 U/mg solid) was used as a standard. One unit hydrolyzes 1.0 micro equivalent of fatty acid from triacetin in 1 h at pH 7.4 and 20 ºC.

Intestinal transit was studied by comparing the PI/PC ratios of protease and α-amylase activities. The protein concentration of samples was measured according to the Bradford method (1976), using bovine albumin as a standard.

2.4. Brush border membrane enzyme activities

Samples taken from the proximal intestine at 24 h post-feeding were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100). Alkaline phosphatase activity (AP, EC 3.1.3.1) was analysed following Weiser (1973). Briefly, the homogenates reacted with a buffer solution that contained p-nitrophenylphosphate (p-NPP) (in mM: 500 TrisHCl, 0.3 $ZnCl_2$, 10 $MgCl_2 \cdot 6H_2O$, 0.5 p-NPP, pH 9.4). After 15 minutes, the reaction was stopped with NaOH 0.5 N. Supernatant absorbance was measured at 420 nm (UV-1603, Shimadzu). P-nitrophenol (p-NP) (Sigma Aldrich, Spain) was used as the standard. Units of alkaline phosphatase activity are calculated as the amount of enzyme activity liberating 1 mmol of p-nitrophenol per hour.

Leucine-amino peptidase activity (LAP, EC 3.4.11.1) was measured according to Maroux et al. (1973). L-Leu-p-nitroamilide (0.1 M) was mixed with phosphate buffer (in mM: 40 K_2HPO_4, 40 KH_2PO_4) and sample. The increase in absorbance was recorded at 410 nm in the linear zone for 10 minutes. Leucine amino peptidase (Sigma Aldrich, Spain, 25UN) was used as the standard. One unit of enzyme activity is equal to 1 micromole of L-Leucinamide hydrolyzed per minute at 25 ºC and pH 8.5.

2.5. Nutrient uptake analysis

Samples from the proximal intestine were processed to obtained brush border membrane vesicles (BBMVs) according to Sala-Rabanal et al. (2004). To separate mucosa from the muscular layers, samples were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100). Selective precipitation of basolateral and mitochondrial membranes was achieved through the addition of $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugation enabled the purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe. The enrichment in brush border membrane preparations was determined by measuring alkaline phosphatase activity, which has been described as a good marker of BBMVs (Weiser, 1973). Good enrichment was observed (8.84 ± 0.74) and based on Santigosa et al. (2011a,b) results, vesicular preparations were used when enrichment was higher than 4 times. At 20 ºC the absorption capacities of L-lysine, an essential amino acid, L-alanine, a non-essential one, and D-glucose were determined in vesicular suspensions according to the procedure described by Santigosa et al. (2011b). These suspensions were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient, Osm 320, pH 7.4) for 5 sec. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 320, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22-µm cellulose nitrate filters (Millipore, Bedford MA) previously soaked in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). Radiolabelled nutrients were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).

Dietary composition modifies the vesicular volume (Santigosa et al. 2011a, 2001b), and therefore the influx values have to be normalized. The vesicular volume was determined by the incubation of BBMV preparations with incubation buffer on ice and measuring the amount of L-alanine retained inside the vesicles at equilibrium (Sala-Rabanal et al., 2004). After 90 min, the reaction was stopped and the amount of L-alanine was measured following the procedure described above.

The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford method (1976).

2.6. Statistical analysis

Significant differences between groups were determined by one-way ANOVA followed by Duncan post hoc test. Differences between intestinal segments were determined using the Student’s *t*-test (P<0.05). The software used was SPSS Statistics 22.0 (SPSS Inc., EUA).

3. Results

Intestinal content pH and digestive enzyme activities (protease, α-amylase and lipase) from the pyloric caeca (PC) and proximal intestine (PI) (Figure 1), and protease alkaline characterization by zymography (Figure 2), were analysed at 7 h post-feeding. In addition, alkaline phosphatase (AP) and leucine aminopeptidase (LAP) activities (Figure 3) and nutrient absorption capacities (Figure 4) were analysed in the PI at 24 h post-feeding. Table 2 shows the SGR, total feed intake, voluntary feed intake and FCR. Data from the three trials are presented in the same graphs or tables.

Figure 1 shows that in general, intestinal content pH was higher in the PI than in the PC, being significantly different between the two intestinal regions for those animals fed diets containing less than 22% of dietary starch. Regarding total protease activity (TPA), the activity measured in the PC was lower than in the PI at 7 h post-feeding, except in S14$L_{17}P_{46}$, S29L_{17}P42 and S33L_{17}P40 fish. α-Amylase activity was high and did not differ between intestinal regions with any of the studied diets. Finally, lipase activity was lower in the PC than in the PI and showed higher individual variability, thus no differences were found between dietary treatments or intestinal segments except for S29L_{17}P42 and S33L_{17}P40. Lipase and α-amylase activities were lower than alkaline protease activity by a factor of 10^3, pointing to a limited ability to digest lipids and carbohydrates versus protein.

Brush border enzyme activities, alkaline phosphatase (AP) and leucine aminopeptidase (LAP) (Figure 3) were modulated differently by changes in dietary composition, with AP being more sensitive to changes than LAP.

Absorption capacity studies (Figure 4) showed that L-Lys uptake (76.8 ± 4.1 pmols.mg $prot^{-1}.s^{-1}$), an essential amino acid, was significantly higher than that found for a non-essential amino acid (L-Ala, 45.6 ± 2.2) and D-Glc (46.2 ± 3.3). Furthermore, D-Glc influx varied widely in response to dietary changes, unlike that of any of the studied amino acids.

3.1. Trial I: Effects of increasing starch in isoproteic and isolipidic diets

The pH of the PC increased with increasing starch content (Figure 1, Trial 1), whereas no differences were found in the PI.

S5, S14 and S22$L_{17}P_{46}$ showed baseline APT activity in the PC, and S22 animals showed higher activity (Figure 1, Trial 1). In the PI, the highest protease activity (Figure 1, Trial 1) was measured in sea bream fed the highest starch content (S22$L_{17}P_{46}$), indicating that protein digestion is still occurring in these animals 7 h post-feeding.

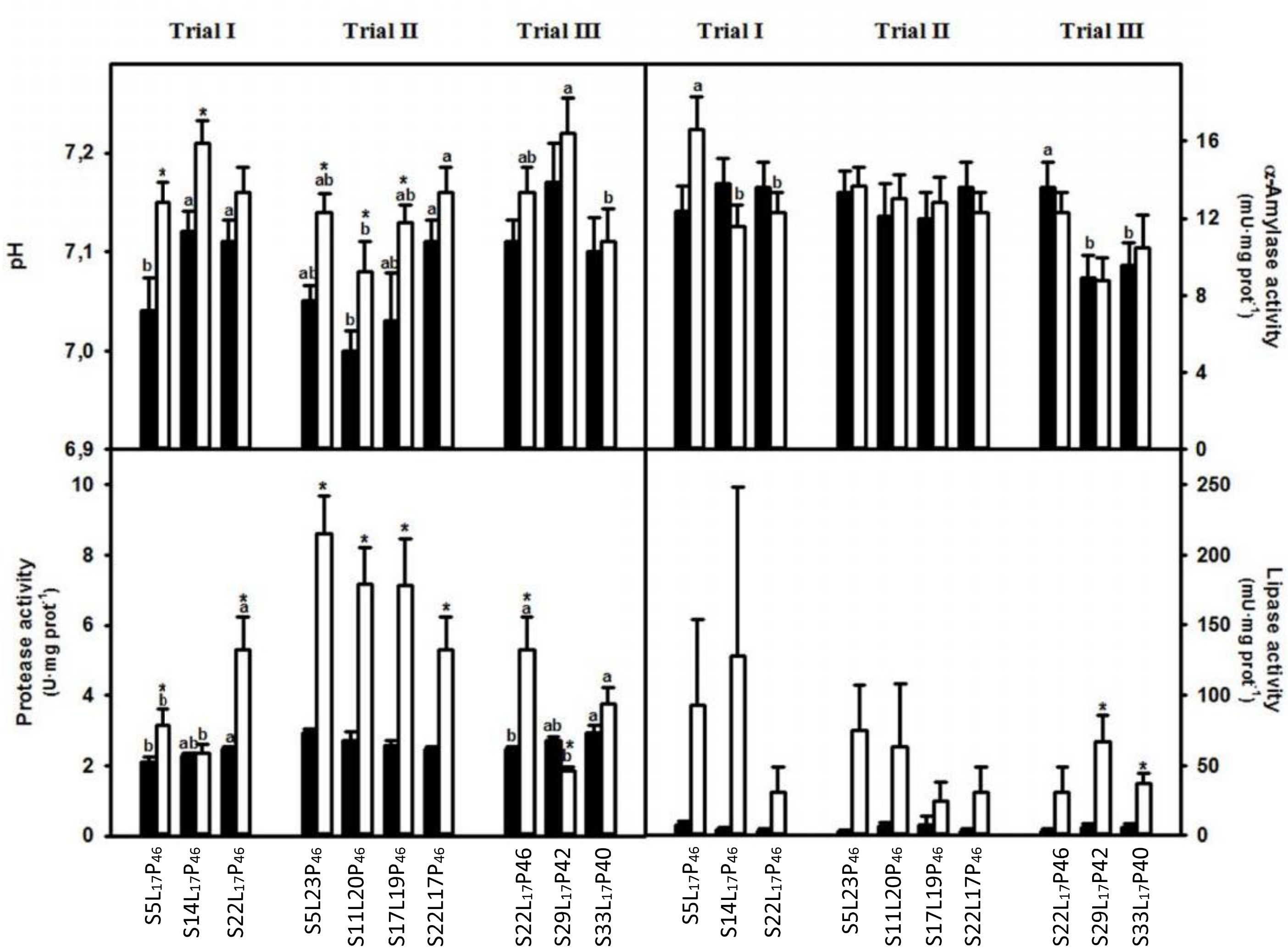

Figure 1. Intestinal pH content, alkaline protease, α-amylase and lipase activities in the pyloric caeca (black bars) and proximal intestine (white bars) of sea bream at 7 h post-feeding (n=9). Values are the mean ± SEM. Significant differences (P< 0.05) between conditions are shown by letters; significant differences (P< 0.05) between intestinal segments are shown by *.

Zymography from the PI (Figure 2, Trial 1) corroborated the TPA activity, showing that animals fed with low and intermediate starch diets (S5 and S14$L_{17}P_{46}$) had lower protease activity. No chymotrypsin activity (25, 30 and 50 KDa) was detected in these animals, whereas trypsin-like (55, 60K and 90 KDa) activity was lower than in S22$L_{17}P_{46}$ fish. Moreover, increasing the starch in the diet did not modify α-amylase activity in the PC (Figure 1, Trial 1). In contrast, in the PI the highest α-amylase activity (Figure 1, Trial 1) was measured in sea bream fed the lowest starch content (S5$L_{17}P_{46}$), possibly to optimize dietary starch digestion. Alkaline protease and α-amylase PI/PC ratios were calculated. No significant difference in this parameter was found for protease (1.37 ± 0.12); but the α-amylase PI/PC ratio showed significant differences between S5$L_{17}P_{46}$ and S14$L_{17}P_{46}$, being 1.51 ± 0.14 and 0.87 ± 0.09, respectively. S22$L_{17}P_{46}$ showed intermediate values (1.27 ± 0.23).

No differences between the alkaline protease PI/PC ratio and α-amylase PI/PC ratio were found. Nor was there any difference in lipase activity when the dietary starch content increased (Figure 1, Trial 1).

The effect of 5, 14 and 22% starch in the diet on alkaline phosphatase and leucine aminopeptidase activities, two brush border membrane enzymes, was measured (Figure 3, Trial 1). No significant differences were found, but AP tended to diminish and LAP to increase as starch content in the diet increased.

Regarding nutrient absorption capacities (Figure 4, Trial 1), no differences were found in L-Lys influx as dietary starch increased. However, high L-Ala and D-Glc uptake was detected in S14$L_{17}P_{46}$ fish in comparison with S5$L_{17}P_{46}$ and S22$L_{17}P_{46}$ animals.

No differences in SGR, voluntary feed intake or FCR were found as the starch content of the diet increased (Table 2, Trial 1), although S14$L_{17}P_{46}$ sea bream presented the lowest total feed intake and FCR and the highest voluntary feed intake.

3.2. **Trial II:** Lipid replacement by carbohydrates in isoproteic diets

No significant changes in α-amylase, protease and lipase activities (Figure 1, Trial 2) were detected when dietary lipids were replaced by starch; although the latter two activities tended to decrease, animals fed the diet with the highest starch and lowest lipid content (S22L17P_{46}) showed the most elevated intestinal pH (Figure 1, Trial 2). Consistent with alkaline protease activity, in PI zymography (Figure 2, Trial 2) there was a proportional reduction in all detected bands, mainly in 60 KDa trypsin-like activity, as dietary starch increased and lipid decreased. Moreover, significant differences in alkaline protease activity PI/PC ratios were found between animals fed extreme diets (S5L23P_{46} and S22L17P_{46}: 2.83 ± 0.32 and 1.68 ± 0.35, respectively).

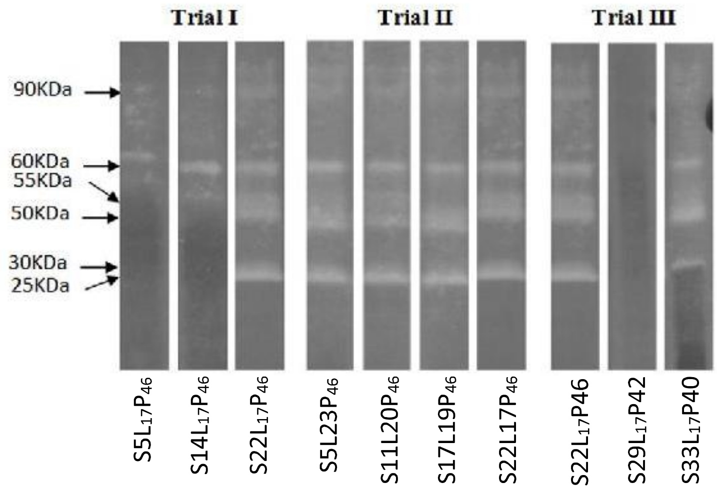

Figure 2. Model zymogram of alkaline protease activity in proximal intestine extracts was obtained at the corresponding intestinal pH. Figures show the molecular weight of bands with proteolytic activity. All samples were analysed individually; the figure shows a representative result.

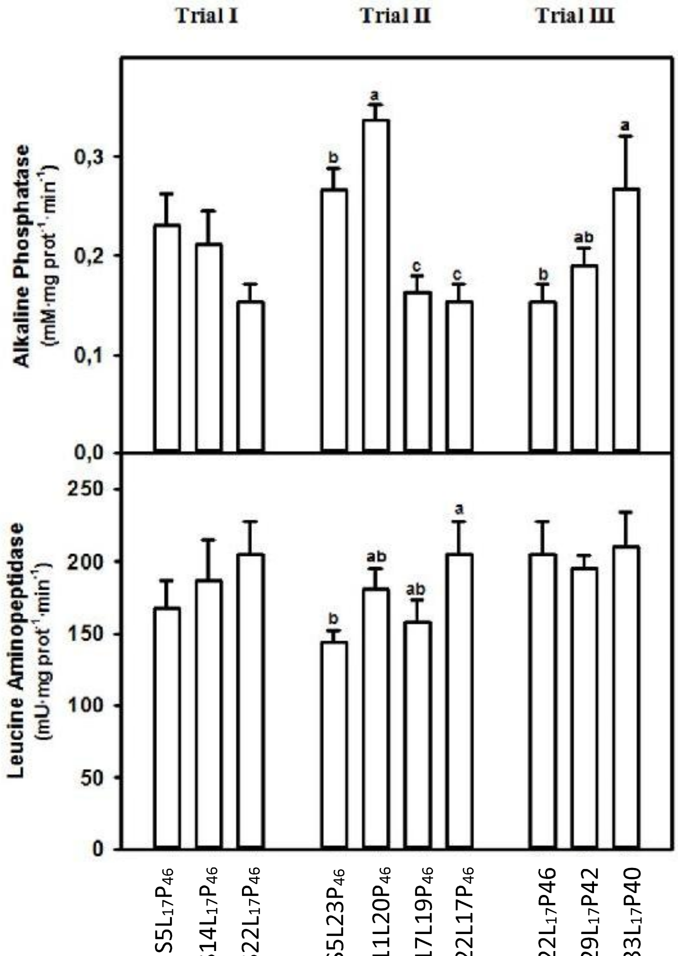

Figure 3. Alkaline phosphatase (AP) and leucine aminopeptidase (LAP) activities (n=9) measured in brush border membrane of sea bream proximal intestine 24 h post-feeding. Values are the mean ± SEM. Significant differences (P< 0.05) between dietary conditions are shown by letters.

S11L20P_{46} and S17L19P_{46} sea bream presented an intermediate APT PI/PC ratio (2.31 ± 0.28 and 2.72 ± 0.44, respectively). No significant difference in this parameter was found for α-amylase (1.31 ± 0.11). Finally, the alkaline protease PI/PC ratio and α-amylase PI/PC ratio differed in S5L23P_{46}, S11L20P_{46} and S17L19P_{46} animals, but not S22L17P_{46}; indicating differential timing of digestion of starch and protein in animals fed the three diets highest in fat and lowest starch.

The highest AP activities (Figure 3, Trial 2) were detected in fish fed the two diets with high lipid and low starch content (S5L23P_{46} and S11L20P_{46}). Moreover, a significant difference in LAP activity was found between animals fed extreme diets, being more elevated in S22L17P_{46} (Figure 3, Trial 2).

The L-Lys absorption capacity (Figure 4, Trial 2) tended to increase with the replacement of dietary lipids by starch from S5L23P_{46} to S17L19P_{46}. In contrast, the animals fed the diet with the highest starch and lowest lipid content (S22L17P_{46}) showed the lowest L-Lys uptake, and being significantly different from S17L19P_{46} sea bream. In addition, the absorption capacity of L-Ala and D-Glc tended to decrease with the substitution of dietary lipids by starch, being significantly different for glucose influx between S5L23P_{46} and S11L20P_{46} versus S17L19P_{46} animals.

No differences were found in SGR, total feed intake, voluntary feed intake or FCR when lipid was substituted for starch (Table 2, Trial 2).

3.3. **Trial III:** Protein sparing effect by carbohydrates in isolipidic diets

The substitution of dietary protein by starch provoked a significant decrease in protease and α-amylase activities (S22L_{17}P46 versus S29L_{17}P42 and S33L_{17}P40). In fact, the lowest activity of these enzymes was detected in S29L_{17}P42 animals; these fish also showed the highest pH of the intestinal content, probably as a compensatory mechanism (Figure 1, Trial 3). The TPA results were corroborated by zymography of the proximal intestine (Figure 2, Trial 3); indeed, no bands from protease were detected in S29L_{17}P42 animals. Alkaline protease and α-amylase PI/PC ratios were calculated. No significant difference in this parameter was found for α-amylase (1.32 ± 0.13), but the alkaline protease PI/PC ratio differed significantly between S22L_{17}P46 and S29L_{17}P42 and S33L_{17}P40, being 1.68 ± 0.35, 0.68 ± 0.03 and 1.11 ± 0.08, respectively. There were no differences between the alkaline protease PI/PC ratio and α-amylase PI/PC ratio, nor was there any difference in lipase activity when dietary protein was replaced by starch (Figure 1, Trial 3).

Replacement of dietary protein by starch also provoked a significant increase in AP activity (S22L_{17}P46 versus S33L_{17}P40) (Figure 3, Trial 3), and a significant decrease in D-Glc absorption capacity (S22L_{17}P46 versus S29L_{17}P42 and S33L_{17}P40) but had no effect on LAP activity (Figure 3, Trial 3) or L-Lys or L-Ala absorption capacity (Figure 4, Trial 3).

Finally, substitution of dietary protein by starch provoked a significant decrease in SGR and voluntary feed intake (S22L_{17}P46, S29L_{17}P42 versus S33L_{17}P40) and a significant increase in FCR (S22L_{17}P46, S29L_{17}P42 versus S33L_{17}P40). No differences were detected in total feed intake, although this parameter tended to be lower in S22L_{17}P46 animals.

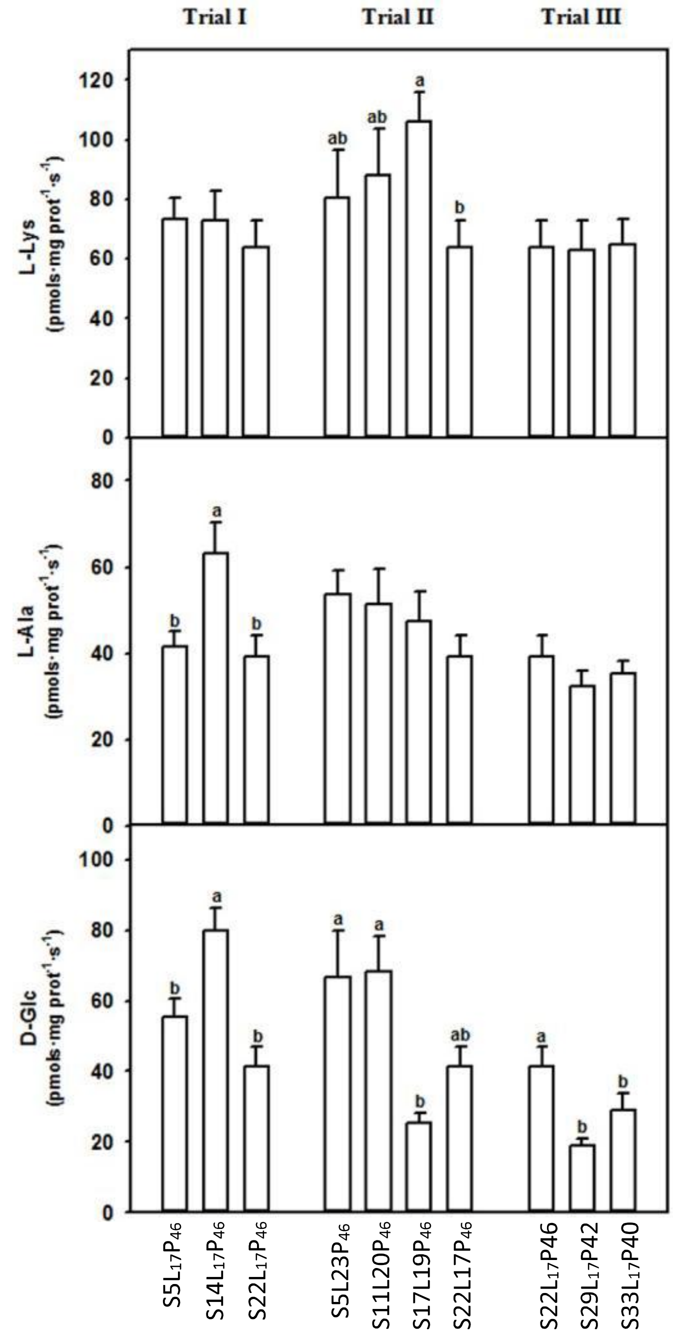

Figure 4. L-Lys, L-Ala and D-Glc absorption capacities (n=9) in brush border membrane vesicles from proximal intestine from sea bream 24 h post-feeding. Values are the mean ± SEM. Significant differences (P< 0.05) between dietary conditions are shown by letters.

Table 2. Total intake (n=3), FCR (n=3), SGR (n=75), and voluntary feed intake (n=3) for each experimental condition. Values are presented as mean ± SEM. Different letters show significant differences (P<0.05) between diets.

Diet	SGR (%)	Total Feed intake (Kg)	Voluntary Feed Intake ($g \cdot Kg^{-0.7} \cdot day^{-1}$)	FCR
$S5L_{17}P_{46}$	1.14 ± 0.010	11.55 ± 0.62a	9.44 ± 0.30	1.61 ± 0.07
$S14L_{17}P_{46}$	1.13 ± 0.030	9.68 ± 0.59b	10.61 ± 0.68	1.38 ± 0.14
$S22L_{17}P_{46}$	1.08 ± 0.010	10.90 ± 0.05ab	9.44 ± 0.05	1.63 ± 0.02
$S5L23P_{46}$	1.09 ± 0.026	10.89 ± 0.47	9.53 ± 0.33	1.62 ± 0.09
$S11L20P_{46}$	1.12 ± 0.001	11.16 ± 0.38	9.54 ± 0.22	1.59 ± 0.05
$S17L19P_{46}$	1.13 ± 0.018	10.72 ± 0.33	9.80 ± 0.17	1.50 ± 0.04
$S22L17P_{46}$	1.08 ± 0.010	10.90 ± 0.05	9.44 ± 0.05	1.63 ± 0.02
$S22L_{17}P46$	1.08 ± 0.010a	10.90 ± 0.05	9.44 ± 0.05a	1.63 ± 0.02b
$S29L_{17}P42$	1.05 ± 0.010a	11.09 ± 0.39	9.11 ± 0.18a	1.73 ± 0.06b
$S33L_{17}P40$	0.95 ± 0.010b	11.56 ± 0.30	8.28 ± 0.09b	2.15 ± 0.02a

4. Discussion

Vegetable ingredients are commonly used to replace fish meal and fish oil in aquaculture due to their availability and low price (SOFIA, 2014). They are a source of both protein and energy (García-Meilán et al., 2013; 2014), and the binding properties of starch are also useful (Krogdahl et al., 2005). The protein sparing effect of dietary lipid or carbohydrates has been studied in sea bream, being well established for the former (Company et al., 1999; Vergara et al., 1999); however, there is controversy over the effect of using carbohydrates (Enes et al., 2011; García-Meilán et al., 2014), probably due to the many factors that affect starch digestibility (Enes et al., 2011).

Previous studies in this species reported that at 5 h post-feeding the pH of the stomach was neutral and digestion was taking place between the PC and PI depending on dietary composition (García-Meilán et al., 2013, 2014; Santigosa et al., 2011a). Accordingly, in the present study, at 7 h post-feeding, a neutral pH was found in the stomach and the study of acid digestion was discarded because pepsin activity is linked to a pH of less than 4.0 for pepsin activation, with an optimum close to 2.0 or 3.0 (Cao et al., 2011; Nikolopoulou et al., 2011; Yúfera et al., 2004; 2012). Moreover TPA determination showed baseline values in the PC, whereas digestion was occurring in the PI. Therefore, the PC provides information about how animals adapt to the administered diet, while the PI provides information on the different factors affecting the digestive process.

In accordance with previously reported data for this species (Deguara et al., 2003; Yúfera et al., 2004; García-Meilán et al., 2013, 2014; Nikolopoulou et al., 2011), intestinal pH values measured in the PI were higher than in the PC and neutral or slightly alkaline (between 6.93 and 7.21). The pH of the intestinal content was affected by dietary ingredients due to their different acid binding capacities and bicarbonate secretion from the pancreas, pancreatic ducts, bile and intestine (Wilson and Grossel, 2003; Grossel and Genz, 2006). On the one hand, dietary ingredients that bind more acid in the stomach promote a higher gastric pH and consequently a higher intestinal pH (Lawlor et al., 2005). Cereals show lower acid binding capacities than fish meal and legumes such as soybean (Lawlor et al., 2005); in fact, a reduction in protein content was also associated with a lower acid binding capacity (Lawlor et al., 2005). Moreover, a low acid binding capacity was reported for fats, even lower than that of wheat (Lawlor et al., 2005), the cereal used in the present trials. On the other hand, the specific contribution of bicarbonate secretion from the pancreas, pancreatic ducts, bile and intestine is not known (Wilson and Grossel, 2003; Grossel and Genz, 2006), but buffering capacity could be affected by dietary composition. In this sense, results from the present study showed higher intestinal pH content as dietary starch increased in the PC but no differences were found in the PI during digestion. Moreover, when ingredients with low acid binding capacities (e.g. lipid) were included in the diet, the sea bream were able to up-regulate bicarbonate secretion to maintain the digestive process: a fall in pH would negatively affect digestive enzyme activity since their optimum pH is alkaline (Alarcón et al., 1998; Fernández et al., 2001; García Meilán et al., 2013). The same compensatory mechanism involving intestinal pH was found in sea bream fed diets with lower protein content.

It is known that alkaline protease activity can be modulated by diet in carnivorous species (Buddington et al., 1997; García-Meilán et al., 2014). Thus high levels of trypsin have been described in Atlantic salmon (Krogdahl et al., 2003), European sea bass (Pères et al., 1998) and sea bream (García-Meilán et al., 2013) as dietary protein increases. This up-regulation of TPA activity has been related to the stimulation of pancreatic secretion (Bakke et al., 2011). Accordingly, low levels of alkaline protease activity were detected in the present study when dietary protein was lower, whereas there were no effects on α-amylase or lipase activities. Moreover, some studies have reported that dietary starch and/or lipid levels could interfere with proteolytic activity (Fernández et al., 2001; Fountoulaki et al., 2005; García-Meilán et al., 2013; Hidalgo et al., 1999; Murashita et al., 2008), consistent with the up-regulation of proximal TPA in animals fed high starch and lipid diets in the present study.

The relationship between α-amylase and dietary starch level in carnivorous species is unclear (Krogdahl et al., 2005). However, up-regulation of this enzyme in the PI was found in sea bream fed with 19% lipid and moderate starch (less than 10%) diets (García-Meilán et al., 2013), which could be a way of counteracting enzyme inhibition by both lipid and starch, as described by Fountoulaki et al. (2005). Although this compensatory mechanism was not found in the present study in animals fed high lipid and 12 to 22% starch diets, they showed higher amylase activity than in other studies by our group that used diets containing a lower percentage of starch (Santigosa et al., 2008; García-Meilan, 2013); together these data indicate that α-amylase activity is more constitutive than protease in carnivorous species (Buddington et al., 1997; García-Meilán et al., 2014). Moreover, it is known that starch molecules can absorb α-amylase, thus inhibiting the enzyme (Enes et al., 2011; Fountoulaki et al., 2005), which could explain the lower α-amylase activity detected when animals were fed diet containing 29 and 33% starch.

Regarding lipase activity, some studies have shown that it could be modulated by dietary lipid content (García Meilán et al., 2014; Buchet et al., 2000; Li et al., 2012; Ma et al., 2014; Zambonino-Infante and Cahu, 1999). In this study higher lipase activity was detected in sea bream fed high lipid diets. However, as in this study, previous studies found that protein and carbohydrate content had no effect on lipase activity (García-Meilán et al., 2013; 2014;

Santigosa et al., 2011b). In contrast, a recent study, in an obscure puffer fish, reported a negative correlation between lipase activity and the amylose/amylopectin ratio (Liu et al., 2014).
Feeding carnivorous fish with diets in which protein and/or lipid have been partially replaced by vegetable ingredients could affect digestive enzyme activity (Francis et al., 2001; Krogdahl et al., 2010; Santigosa et al., 2011a, 2011b, 2008) and also alter the trypsin/chymotrypsin ratio (García-Meilán et al., 2013, 2014; Santigosa et al., 2008, 2011a), modifying the digestibility of the diet. Thus, the kind and quantity of ingredients and the presence of antinutritional factors (Francis et al., 2001; Krogdahl et al., 2010; Santigosa et al., 2011a, 2011b, 2008) cannot be ignored in the formulation of diets containing vegetable ingredients. In the present study, besides wheat starch, diets used in the first trial (effect of increased starch) differed in their cellulose content, diets in the second trial (lipid replacement by carbohydrates) differed in their percentage of cellulose and fish oil, and finally diets in the third trial (protein replacement by carbohydrates) differed in their content of corn gluten and SPC 60%. A negative effect on feed digestibility of non-starch polysaccharides such as cellulose has been described in fish species including rainbow trout and Atlantic salmon (Storebakken et al., 1985; Aslaksen et al., 2007). Their negative effects may be either due to non-specific binding to bile acids or the obstruction of digestive enzyme activity and movement of substrates in the intestine (Storebakken et al., 1998). In this study, S22$L_{17}P_{46}$ animals fed a cellulose-free diet showed higher alkaline protease activity versus animals fed S5$L_{17}P_{46}$ and S14$L_{17}P_{46}$ diets that contained 7.7 and 17.1% cellulose, respectively. Non-starch polysaccharides could trap water and form gum-like substances in the intestine, increasing the viscosity of the intestinal contents and inhibiting digestive enzyme activity (Francis et al., 2001). Moreover, cellulose increases the rate of intestinal transit, reducing the time available for nutrient absorption and impairing nutrient digestibility (Francis et al., 2001; Krogdahl et al., 2005). On the other hand, Lekva et al. (2010) described a high tolerance of cellulose inclusion (18%) in Atlantic cod that might be due to the elevated levels of chitin in their natural diet. The β-1, 4-glycosidic bonds between monomers are the same for α-cellulose and chitin. A similar situation may occur in gilthead sea bream, given that the natural diet is mainly based on molluscs, crustaceans and small fish (FAO, 2014), and also that protease, α-amylase and lipase activities were up-regulated when high levels of fibre were added to the diet, with no effects on final growth. On the other hand, the oxycarotenoids present in corn gluten meal could give the fillet a yellow pigmentation, reducing market acceptance (Skonberg et al., 1998). In present study, this ingredient accounted for 9.8 to 14.7% of the diet, and no visual changes in the colour of the fillet were observed. Finally, some authors have reported that the inclusion of high levels of SCP (15%) in the diet had no effect on growth and did not lead to

abnormalities in digestive morphology (Van der Ingh et al., 1991; Kaushik et al., 1995). SCP in this work accounted for 2.7 and 11.5% of the diet. Moreover, in the present study both trypsin and chymotrypsin activities were correlated with total protease activity and there were no differences in the trypsin/chymotrypsin ratio, as reported by García-Meilán et al. (2014) when comparing animals fed a diet containing high levels of digestible carbohydrates with a commercial one. In contrast, Santigosa et al. (2008) and García-Meilán et al. (2013) reported differential inhibition of chymotrypsin activity versus trypsin activity when using diets with different percentages of vegetable ingredients or in which protein was replaced with lipid and starch, leading to an increased trypsin/chymotrypsin ratio that was associated with the presence of anti-nutritional factors in the diet.

The transit rate is affected by dietary composition and consequently modifies the time in which digestion and absorption can occur. On the one hand, higher transit rates were detected following an increase in dietary starch (Spannhof and Plantikov, 1983) or a decrease in dietary protein (García-Meilán et al., 2013), and thus both may lead to digestion and absorption in more distal regions. Accordingly, sea bream fed high starch and low protein diets showed lower TPA and α-amylase activity. On the other hand, Fountoulaki et al. (2005) related high dietary lipid content to a lower intestinal transit rate, which result in elevated TPA activity in the proximal intestine 7 h post-feeding in animals fed diets containing 19, 20 and 23% dietary lipid.

Brush border enzymes carry out the final stages of food degradation and assimilation (Klein et al., 1998) and their activity is greatly modulated by the intraluminal presence of dietary substrates (Kimura et al., 1978; Kuperman and Kuz'mina, 1994; Cézard et al., 1983). Alkaline phosphatase activity is often used to evaluate intestinal membrane function and integrity (Silva et al., 2010) and is expressed by active and mature mucosal enterocytes (Harpaz et al., 1999). Several authors found an increase in AP activity with high dietary lipid levels (Gawlicka et al., 2002; Grewal and Mahmood, 2004; Krogdahl et al., 2003; Mozes et al., 2004; Stenson et al., 1989), reflecting the fact that this enzyme is thought to be involved in lipid absorption (Narisawa et al., 2003; Zhang et al., 1996). Accordingly, up-regulation of this membrane enzyme was detected in sea bream fed high lipid diets, and also in fish fed high starch and low protein, suggesting that it could be a compensatory mechanism for increasing lipid uptake in an attempt to maintain growth. Regarding leucine aminopeptidase activity, different dietary proteins had no effect in tilapia (Hakim et al., 2006), as found in the present study for sea bream. Moreover, the negative effect of high dietary lipid on this proteolytic enzyme may be associated with lower protein digestibility due to the presence of lipids, as reported by Fountoulaki et al. (2005).

Sea bream were able to modulate the capacity for amino acid and glucose transport in the pyloric caeca and proximal and distal intestine (Santigosa et al., 2011b) or whole intestine

(García-Meilán et al., 2013, 2014) by up- or down-regulation to meet their needs in response to dietary composition. L-Lys is an essential amino acid, and low availability could lead to poor growth (Rathore et al., 2010; Sánchez-Lozano et al., 2011; Tibaldi and Lanari, 1991; Zhou et al., 2011; Cheng et al., 2003). Fountoulaki et al. (2005) reported that protein digestibility was not affected by starch, fibre or protein when dietary lipid content was low. In this study, L-Lys absorption in the proximal intestine was not modified by either high starch or cellulose inclusion or protein replacement by starch when lipid comprised 17% of the diet. However, a more elevated dietary lipid content provoked the up-regulation of L-Lys influx, probably as a compensatory mechanism due to low proximal intestine L-Lys availability as a result of low protein digestibility that led to digestion in more distal intestinal regions. Secondly, up-regulation of D-Glc and L-Ala absorption capacities has been described as a compensatory mechanism in animals fed with highly substituted diets (Santigosa et al., 2011b) or high protein or starch diets (García-Meilán et al., 2013, 2014). It was also suggested that high levels monosaccharides could inhibit amino acid transport (Ferraris and Ahearn, 1984; Vinardell, 1990). In the present study, animals fed with protein, lipid and cellulose contents of at least 46, 20 and 6.9% respectively and starch levels below 14% showed the up-regulation of D-Glc and L-Ala absorption capacities mentioned above, indicating that the principal dietary components could modulate nutrient absorption to improve feed use, assure growth and spare protein. This could influence the subsequent use of these nutrients as an energy source (García-Meilán et al., 2013; Santigosa et al., 2011b).

When starch is incorporated into isoproteic and isolipidic diets or lipid is replaced by starch in the diet, compensatory mechanisms such as intestinal pH, changes in pancreatic and intestinal digestive enzymes and nutrient absorption capacities take place, allowing the maintenance of total feed intake, voluntary feed intake, FCR and growth, and suggesting that animals are able to modulate the digestive process when protein requirements are met. In contrast, when dietary protein was replaced with starch, digestive compensatory mechanisms were not sufficient and a significant increase in FCR and decrease in voluntary feed intake and SGR were found.

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CAPÍTULO II

Effects of dietary protein-to-lipid ratio on digestive and absorptive processes in sea bass fingerlings

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Abstract

Two 8-week trials, one in spring and the other in autumn-winter, were performed with sea bass fingerlings. After each growth period, pancreatic digestive enzyme synthesis in the pyloric caeca (PC), food anticipatory activity adaptation in the proximal intestine (PI) and nutrient absorption capacities in the distal intestine were measured at 24 h post-feeding.
In spring, five isoenergetic diets with different protein levels (40, 45, 50, 55 and 59%) were formulated by simultaneously changing lipid and starch content. Animals fed the low-protein diets showed reduced accumulation of alkaline protease (40 to 50% diet), lipase (40 to 45% diet) and α-amylase (40 to 55% diet) in the PC; however, the activities of digestive enzymes in the PI were high in fish fed both low and high dietary protein diets, being significant for alkaline protease (40 and 59% diet) and α-amylase (40 and 45% diet). Zymography of the PC identified four proteases with trypsin-like activity (23, 22, 20 and 17 KDa) and an 18 KDa chymotrypsin activity that were modulated differentially according to diet. The PC/PI ratios were highest for alkaline protease and lowest for α-amylase, showing different patterns of response to changes in dietary composition: higher for protease on 45, 50 and 55% animals and α-amylase on 59% sea bass. L-Lys and D-Glc uptake capacities were down-regulated as dietary protein content declined, while no changes were found in L-Met and L-Trp. Based on the above-mentioned data and the optimum growth of animals fed with 50% protein, a protein-to-energy ratio study was performed.
Sea bass were fed with five isoproteic (52%) diets that differed in dietary lipid content (12, 14, 16, 18 and 20%). Alkaline protease and lipase synthesis in the PC was up-regulated in low energy diets (12, 14 and 16% and 12 and 14%, respectively) and no changes were found in α-amylase. PC zymography was consistent with protease measurements. Only food anticipatory activity of lipase was up-regulated in animals fed 12% of lipid. L-Met and D-Glc were up-regulated by increasing dietary lipid and L-Lys and L-Trp were not modified. Dietary lipid content had no effect on final body weight.
Finally, seasonal effects were studied. Alkaline protease synthesis in the PC was significantly higher in spring and animals showed higher growth in this season than in the autumn-winter. However, adaptation of food anticipatory activity in the PI was higher in autumn-winter. No differences were found in nutrient absorption capacities between seasons.

Keywords: amylase, brush border membrane vesicles, fish, lipase, protease, protein-to-lipid ratio

1. Introduction

European sea bass is one of the main Mediterranean carnivorous fish species cultured in the sea aquaculture industry (SOFIA, 2014). To promote the production of this species, efforts should be made to develop more sustainable diets that allow, at each stage of development, the saving of protein and replacement of fish meal and fish oil in high percentages. The resulting reduction of nitrogen excretion and promotion of the use of alternative ingredients would make the aquaculture industry more sustainable (SOFIA, 2014). It is known that European sea bass growth is not altered by replacing fish meal (Messina et al., 2013) or fish oil (Mourente et al., 2005; Eroldogan et al., 2013) with vegetable ingredients.

Most studies on protein requirements in sea bass have been conducted in juveniles (Alliot et al., 1974, 1984; Ballestrazzi et al., 1994; Dias et al., 2003; Metailler et al., 1981), with there being limited information on fingerlings weighing less than 13 g (Alliot et al., 1974, 1979a; Dias et al., 1998; Peres and Oliva-Teles, 1999a; Pérez et al., 1997; Tibaldi et al., 1991). None of these studies have considered digestive and absorptive processes. As in other carnivorous fish, protein requirements for maximum growth in sea bass are high (New, 1986; Hidalgo and Alliot, 1988; Perez et al., 1997; Sánchez-Muros et al., 1998; Oliva-Teles, 2000), and have been estimated to account for 40 to 50% of the diet (Oliva-Teles, 2000; Dias et al., 1998, 2003). The data regarding dietary lipids are variable. Dias et al. (1998) reported better growth performance on diets containing 18 –19% lipid versus diets with a lower lipid content, whereas Peres and Oliva-Teles (1999b) found no differences in the growth of fish fed from 12 to 24% lipids. Other authors (Metailler et al., 1981; Garcia-Alcazar et al., 1994; Pérez et al., 1997) found no beneficial effects of a dietary lipid content above 12%.

Compared to salmonids, available data in marine fish indicates that their protein retention efficiency is lower whereas the protein-to-energy ratio is higher (Kaushik, 1997a), suggesting that conventional energy sources have a limited protein sparing effect. Despite this, the inclusion of lipids and/or digestible carbohydrate in the diet of European sea bass can allow protein sparing (Alliot et al., 1979b; Cho and Kaushik, 1990; Pérez et al., 1997; Dias et al., 1998; Peres and Oliva-Teles 1999a, b, 2002). Moreover, Pérez et al. (1997) suggested that the sparing effect of lipid is likely to be higher than that of carbohydrate. However, substitution of fish oil by vegetable oils modifies the fillet fatty acid profile, thus decreasing quality (Eroldogan et al., 2013; Mourente et al., 2005). On the other hand, carbohydrate metabolization depends on water temperature, ration, dietary content, molecular complexity, and technological treatments applied to the molecule (Hidalgo and Alliot, 1988; Kaushik and Médale, 1994; Pfeffer, 1995) and also on lipid inclusion (Fountoulaki et al., 2005). Enes et al. (2001) reviewed dietary carbohydrate utilization by this species, concluding that up to 20% carbohydrate inclusion did not affect growth or feed utilization in juveniles.

Digestion and nutrient uptake are key processes in optimizing the use of diet and differ between species, dietary composition and nutrient digestibility (Buddington et al., 1997). Digestion begins in the stomach where hydrochloric acid denatures protein and converts pepsinogen into active pepsin (Wu et al., 2009; Yúfera et al., 2004). When the chyme reaches the pyloric caeca, cholecystokinin is secreted, stimulating the gall bladder and pancreas to secrete electrolytes, bile and pancreatic enzymes (Pivnenko et al., 1997; Krogdahl et al., 1999), including proteases (e.g. trypsin and chymotrypsin), glucosidases (e.g. α-amylase), and lipolytic enzymes (e.g. lipase and colipase) (Bakke et al., 2011). Digestive enzymes hydrolyze protein, carbohydrates and lipids in metabolites small enough to be picked up by the membrane-bound enzymes found in the brush border membrane (Bakke et al., 2011). In sea bass, digestive proenzymes from the hepatopancreas (Nejedli and Tlak Gajger, 2013) and exocrine pancreatic tissue dispersed in the adipose tissue that surround the pyloric caeca (PC) (Kamaci et al., 2010) are released into the PC.

Unlike in mammals, nutrient uptake in fish occurs along the entire intestine by diffusion, facilitated or active transport (Maillard et al., 1995). Four Na^+-dependent amino acid transporter systems have been described in fish (Storelli et al., 1989): for acid, basic or neutral and proline and N-methylated amino acids. Moreover, alanine, glycine, glutamate, lysine and proline Na^+-independent transport systems have been described (Storelli et al., 1989). Absorption of di- and tripeptides is carried out by PepT1 and PepT2 transporters (Hakim et al., 2009; Sangaletti et al., 2009; Terova et al., 2009; Verri et al., 2010) and SGTL1 transports D-glucose and D-galactose by active transport and co-transport (Sala-Rabanal et al., 2004). Absorption capacities are affected by environmental factors such as temperature and water salinity and biotic factors such as species, natural diet, diet formulation, availability and digestibility (Buddington et al., 1997).

The aim of this study was to 1) test the protein sparing effect of lipids and carbohydrates and 2) determine the best protein-to-lipid ratio for digestion and absorption processes in small European sea bass juveniles fed with diets in which 38% of fish meal was replaced by vegetable sources.

2. Material and Methods

2.1 Diets, experimental trials and sampling

Two trials were performed at the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) in Sant Carles de la Ràpita (Catalonia, Spain). In both, sea bass fingerlings (± 5 g body weight) were acclimatized for 2 weeks and then randomly distributed in 15 fibreglass tanks with a capacity of 500 L (100 fish per tank). Triplicate groups were maintained between 21–22 ºC, in a 12L/12D photoperiod, with 34.5‰ salinity and 85% oxygen saturation for an 8-week period in spring (April to June) for the first trial, and in autumn-winter (December to January) for the second trial.

Experimental diets containing 38% of vegetable ingredients (wheat, wheat gluten and soybean concentrate or SPC60) were formulated by Skretting (Stavanger, Norway). In order to preserve fillet quality, only fish oil has been used in dietary formulations. Five isoenergetic (21.97 KJ/kg) diets that differed in protein content (40, 45, 50, 55 and 59%) were formulated in which lipid and highly digestible carbohydrates were used to replace protein. The diets were named P40, P45, P50, P55 and P59 (Table 1A) and were administered ad libitum twice a day to animals during the first trial. Based on the results of the first trial, five isoproteic diets (52%) were formulated that differed in dietary lipid content (12, 14, 16, 18 and 20%); these were named 12L, 14L, 16L, 18L and 20L (Table 1B). Sea bream were fed the corresponding diet ad libitum twice a day during the second trial. The study also compared spring versus autumn-winter growth in animals fed 51% protein, 18% lipid and 10.5% starch and their associated digestive and absorption capacity. At the end of the growth trials, specific growth rate (SGR) was calculated as follows: $((\ln W_{fin}\,(g) - \ln W_{ini}\,(g))/t) \cdot 100)$, where W_{fin} and W_{ini} represent the final and initial weights respectively, and t is the number of feeding days.

Table 1A. Dietary composition

Experiment I: Protein sparing effect by lipids and carbohydrates

%	P40	P45	P50	P55	P59
Ingredient					
Fish meal	49.9	51.5	53.0	54.5	56.3
Wheat	33.4	25.8	17.7	10.6	6.8
Wheat gluten	1.9	5.0	7.0	10.0	12.9
Soybean Concentrate	0.2	4.7	10.7	15.0	15.5
Fish oil	12.0	10.4	9.0	7.3	6.1

Vitamin Mix 2.5% and Itrium Oxide 0.1%.

After 24 h fasting, 20 fish from each condition were anaesthetized (100 ppm of phenoxyethanol), weighed and sacrificed by severing their spinal cord. Digestive tracts were isolated on ice and adherent tissue was removed. In this work the adipose tissue that surrounds the pyloric caeca was not removed, and therefore the activity detected in the PC at 24 h post-intake corresponded to synthesis by this pancreatic tissue. In contrast, enzyme activity detected in the PI corresponded to food-anticipatory activity and was a result of peristaltic movements.

Stomach, pyloric caeca (PC) and proximal intestine (PI) samples, including the intestinal content, were rapidly frozen in liquid nitrogen and kept at -80 ºC until intestinal enzymatic analysis. Distal intestine (DI) segments were opened lengthwise, washed in isosmotic saline solution containing protease inhibitor (phenylsulphonyl-methyl-fluoride, PMSF 0.1 M) and rapidly frozen in liquid nitrogen and kept at -80 ºC until nutrient absorption experiments were performed.

Table 1B. Dietary composition

Experiment II: Protein-to-energy ratio study

%	12L	14L	16L	18L	20L
Ingredient					
Wheat	19.9	17.6	15.2	12.8	10.5
Wheat gluten	4.2	4.5	4.9	5.2	5.5
Fish oil	3.2	5.3	7.3	9.4	11.5
Fish meal			50		
SPC 60%			20		

SPC: soybean protein concentrate; Vitamin Mix 2.5%; Itrium Oxide 0.1%.
Energy (MJ/Kg): 14, 20, 21.5, 22 and 22.5, respectively.

All fish-handling procedures complied with the European guidelines for animal care (Directive 2010/63/EU).

2.2. Intestinal pH content and digestive enzyme analysis

The pH of the intestinal content was measured (Crison, Micro pH 2000). Alkaline protease and α-amylase activities were measured according to a modification of Santigosa et al. (2008). Thus, intestinal samples were individually homogenized (Politron 2000, Sorvall TC) at 4 ºC in 50 mM TrisHCl buffer pH 7.5 to a final concentration of 250 mg·mL^{-1}. Homogenates were then centrifuged (1100 g, 15 min, 4 ºC, Jouan CR 411) and supernatants were recovered and stored at -80 ºC. For alkaline protease activity measurements, samples were reacted with 50 mM TrisHCl buffer containing 1% casein at intestinal pH. After 30 min at 20 ºC, the reaction was stopped by adding trichloroacetic acid (12%). The samples were kept at 4 ºC for 1 h and then centrifuged (7500 g, 5 min, 4 ºC). Supernatant absorbance was measured at 280 nm (Genesis2, Milton Roy). Each sample was analysed in triplicate and individual blanks were established. Bovine trypsin (Sigma-Aldrich, Spain, 12,100 BAEE U/mg protein, NC-IUB, 1979) was used as the standard.

Individual alkaline protease activities were characterized using zymograms according to Santigosa et al. (2008) and García-Meilán et al. (2013). Samples were diluted and loaded on 12% polyacrylamide gel (10x10.5x0.1 cm). Electrophoresis was performed at a constant current of 15 mA per gel for 150 min (EPS 301 Power Supply, 4 ºC). Protease-active fractions were visualized using the method described by García-Carreño et al. (1993). Thus, the gels were incubated at 4 ºC under agitation at intestinal pH with TrisHCl solution containing 2% casein. After 30 min, the temperature was raised to 25 ºC for 90 min with shaking. Gels were washed and stained in a methanol:acetic:water solution (40:10:40) containing 0.1% Blue Brilliant Coomassie R-250. Destaining was carried out using the same solution without colorant. Pure trypsin and albumin were used as controls and a RPN 800 commercial weight marker (Amersahm, GE Healthcare, Uk, 12,000–225,000 Da) was used to determine the molecular weight of protease fractions. Proteolytic activities were characterized by the combination of the homogenate with water or the corresponding inhibition solution for 30 min at 20 ºC. Inhibition solutions were selected following Alarcón et al. (1998): TLCK (10 mM in HCl 1 mM) was used to inhibit trypsin-like activities and TPCK (10 mM in methanol) and ZPCK (10mM in dioxane) to inhibit chymotrypsin-like activities.

For α-amylase measurement, homogenates were combined with 0.5% of tracer solution containing soybean trypsin inhibitor (0.04 mg·mL^{-1}) to prevent proteolysis. The reaction was stopped after 30 min by adding 6% acetic acid, and placing at 4 ºC for 30 min. Then, samples were centrifuged (13,000 g, 10 min at 4 ºC). Supernatant absorbance was recorded at 595 nm. Each sample was analysed in triplicate and individual blanks were established. α-Amylase (Sigma-Aldrich, Spain, 66 U/mg solid) was used as the standard. One unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 at 20 ºC.

Lipase activity was determined according to Santigosa et al. (2011a). Homogenate was combined with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycolate, 0.9 deoxycolate, 0.8 tartrate, 0.12 DGGR (1,2-o-dilauryl-racglycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg·L^{-1} colipase (pH 8.3), and the increase in absorbance was recorded at 580 nm between 10 and 20 min. Lipase (Sigma-Aldrich, Spain, 33,944 U/mg protein, 22,980 U/mg solid) was used as a standard. One unit hydrolyzes 1.0 microequivalent of fatty acid from triacetin in 1 h at pH 7.4 at 20 ºC.

The concentration of soluble protein homogenates was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.3. Nutrient uptake analysis

Brush-border membrane vesicles (BBMVs) from the distal intestine were obtained as described by Sala-Rabanal et al. (2004) and nutrient absorption capacities were measured according to Santigosa et al. (2011a, b). Tissue was homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES and pH 7.4) and mitochondrial and basolateral membranes were precipitated by addition of $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugation allowed the purification and concentration of apical enterocyte membranes that were vesiculated (in mM: 300 Mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H2O$, 4.08 LiN_3, and pH 7.4) using an insulin syringe. To ensure enrichment in BBMVs, alkaline phosphatase activity was measured in the initial homogenate and final BBMVs preparations following Weiser (1973).

To determine L-Lysine, L-Methionine, L-Tryptophan and D-Glucose absorption capacities BBMVs were mixed with incubation buffer (in mM: 250 NaSCN, 100 Mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient at 320 mOsm and pH 7.4). In the linear uptake zone (5 seconds), the reaction was stopped by adding cold stop buffer (in mM: 250 NaSCN, 100 Mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, at 320 mOsm and pH 7.4). Then, it was rapidly passed under negative pressure through 0.22-µm cellulose nitrate filters (Millipore, Bedford MA), previously wetted in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were done at 20 ºC. Radiolabelled nutrients were obtained from Amersham Pharmacia Biotech (Spain).

The vesicular volume of BBMV preparations was measured, following a modification of Sala-Rabanal et al. (2004), to normalize influx values. Thus, L-proline retained inside the vesicles in the steady state was measured by incubating the BBMV preparation with incubation buffer on ice. The reaction was then stopped after 90 minutes and measured following the protocol described above.

The protein concentration in BBMV suspensions was determined following the method described by Bradford (1976) using BIORAD® reagent.

2.4 Statistical analysis

Significant differences between groups were determined by one-way ANOVA followed by Tukey's post hoc test. Differences between intestinal segments and seasonality were determined using Student's *t*-test ($P<0.05$). The software used was SPSS Statistics 21.0 (IBM Corporation, USA).

3. Results

3.1. Protein sparing effect by lipids and carbohydrates

Five isoenergetic diets with different protein contents (from 40 to 59%) were used to study the protein sparing effect on digestive enzyme activities (Figure 1 and 2) and nutrient absorption capacity (Figure 3). Both lipid and highly digestible carbohydrates were used to replace protein in the experimental diets.

The pH of the stomach, PC and PI contents was measured (6.85 ± 0.06, 6.98 ± 0.02 and 7.17 ± 0.04, respectively) and no differences were found between animals fed different diets for any of the digestive segments studied. The neutral pH measured in the stomach indicated that acid digestion had finished. Moreover, the pH of the proximal intestine was significantly higher than that of the pyloric caeca.

Total protease activity, lipase and α-amylase activities measured in the PC and PI are shown in Figure 1. α-Amylase activity was lower ($mU \cdot mg^{-1}$ protein) than that of alkaline protease and lipase activity ($U \cdot mg^{-1}$ protein). Animals fed the lower protein diets (P40, P45 and P50) showed less alkaline protease activity in the PC (-16%) than P55 and P59 sea bass, indicating reduced protease pancreatic synthesis in the former group (Figure 1B).

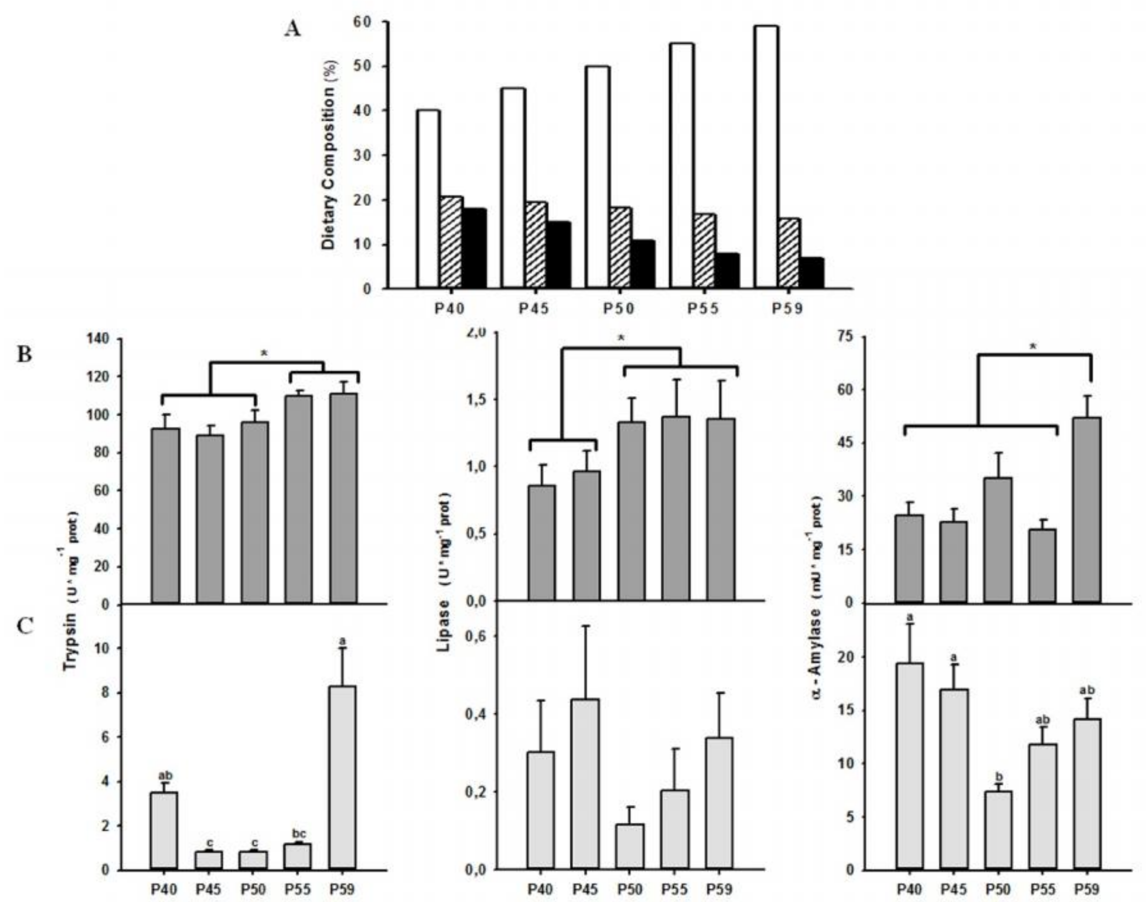

Figure 1. A) Dietary composition (%) of protein (white bars), lipid (striped bars) and carbohydrate (black bars), and pancreatic enzyme activities **B)** in the PC (dark grey bars) and **C)** in the PI (light grey bars). Values expressed as mean ± SEM (n = 10). Significant differences (P<0.05) between dietary treatments are shown by letters or by an * between grouped experimental conditions.

Zymography was conducted to characterize individual alkaline protease activities in the PC and their molecular weight (Figure 2). Five bands were characterized ranging from 17 KDa to 23 KDa. Figure 2A shows an inhibition zymogram in which reductions in band intensity can be seen when comparing the column of the homogenate treated with a specific trypsin (TLCK) or chymotrypsin (TPCK and ZPCK) inhibitor with the non-treated homogenate, allowing identification of the specific activity of a given protease. Thus, four bands corresponding to trypsin-like activity (17, 20, 22 and 23 KDa) and one band corresponding to chymotrypsin-like activity (18 KDa) were detected. Individual protease activity in the PC is shown in Figure 2B. The 23, 22 and 17 KDa trypsin-like activities increased from P45 to P59, being higher in P40 sea bass than in P45. Moreover, the 20 KDa trypsin band was slightly more intense in P55 to P59 sea bass. Finally, higher 18 KDa chymotrypsin-like activity was detected in P59 compared to the other groups. Thus, the band pattern detected by zymography was consistent with the alkaline protease measurements.

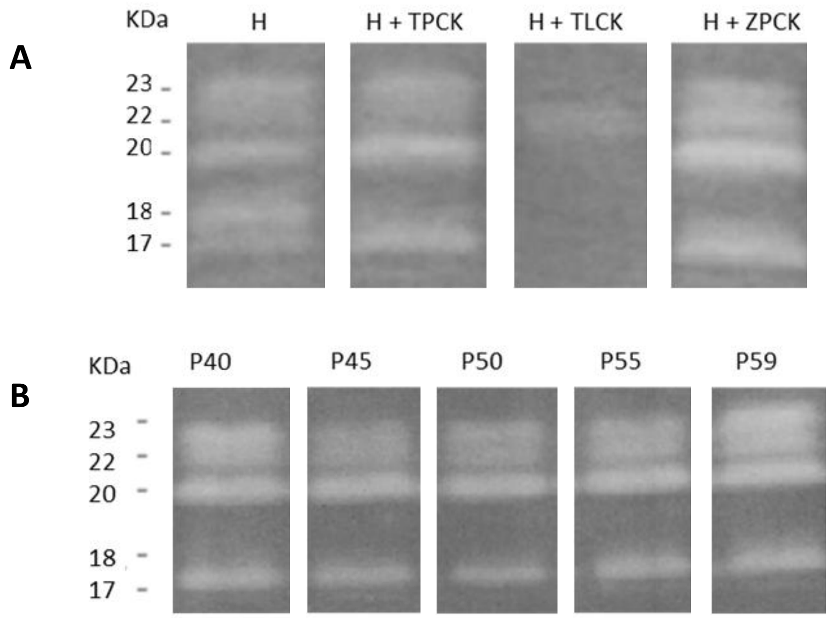

Figure 2. A) Inhibition zymogram of alkaline protease activity in the PC. Lane H = Homogenate; H+TPCK and H+ZPCK = Chymotrypsin inhibitors; H+TLCK = Trypsin inhibitor. **B)** Model zymogram of alkaline protease activity in PC extracts. Figure shows the molecular weight of each band with proteolytic activity. All samples were analysed individually; the figure shows a representative result.

In the PI the highest alkaline protease activity (Figure 1C) was detected in fish fed extreme diets (in P40 and P59 fish), denoting adaptation of food-anticipatory TPA activity to both low and high dietary protein contents. However, P59 animals showed more protease activity than P40 fish (58.3%). An increase in dietary lipid and starch resulted in a lower amount of enzyme stored in the pancreas (Figure 1B), in P40 and P45 animals for lipase (-32%) and P40, P45, P50 and P55 fish for α-amylase (-51%). In the PI, α-amylase activity was significantly higher in P40 and P45 fish than in P50 animals, whereas P59 and P55 showed intermediate activities, again pointing to a food-anticipatory α-amylase activity adaptation to both low and high dietary starch contents (Figure 1C). Lipase activity in the PI followed the same profile as α-amylase, but no significant changes were observed.

Table 2. Alkaline protease and a-amylase PC/PI ratio from the first trial. Significant differences ($P<0.05$) between dietary treatments are shown by letters.

PC/PI Ratio	Alkaline protease	α-Amylase
P40	25.78 ± 4.11b	1.89 ± 0.57b
P45	137 ± 21.91a	2.9 ± 0.78b
P50	128.9 ± 9.27a	3.42 ± 1.57b
P55	105.71 ± 12.24a	2.29 ± 0.55b
P59	41.57 ± 12.51b	8.58 ± 1.75a

The PC/PI ratios were calculated for alkaline protease, α-amylase (Table 2) and lipase; this ratio indicates the relationship between enzyme synthesized in the PC and adaptation to different nutritional conditions in the PI. This ratio was highest for protease (83.92 ± 7.93), intermediate for lipase (27.53 ± 8.67) and lowest for amylase (3.86 ± 0.59), with a significant difference between the three enzymes. Secondly, the PC/PI ratio was significantly higher for TPA in P45, P50 and P55 fish and for α-amylase in P59 sea bass. No changes were detected in lipase PC/PI ratio (27.5 ± 8.7).

L-Lys, L-Met, L-Trp and D-Glc absorption capacities in the DI were studied. As dietary protein content decreased and starch increased, L-Lys and D-Glc uptake capacity were down-regulated, being 2.6 times lower in P40 than P59 sea bass for L-Lys and 2 times lower for D-Glc (Figure 3). No significant differences were found in L-Trp (94.7 ± 6.4 pmols·$\mu l^{-1} \cdot s^{-1}$) or L-Met (19.5 ± 1.4 pmols·$\mu l^{-1} \cdot s^{-1}$) absorption capacities.

Final weight and SGR are shown in Table 3. P50 sea bream showed significantly higher growth than P40 (+29%), as confirmed by the SGR.

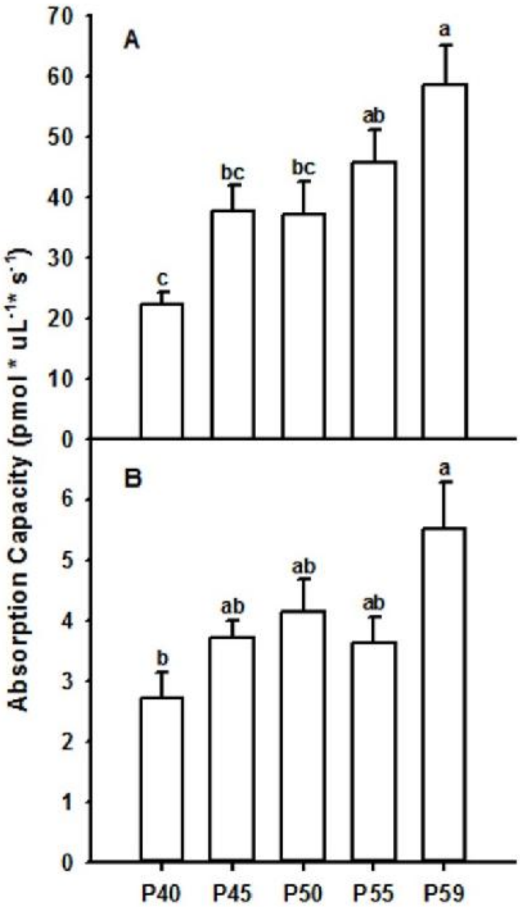

Figure 3. L-Lys (**A**) and D-Glc (**B**) absorption capacities in BBMVs of the DI. Values are represented as mean ± SEM (n = 4). Significant differences (P<0.05) between dietary treatments are shown by letters.

Table 3. Final weight and SGR (n=10) for each experimental condition in the first trial. Values are represented as mean ± SEM. Significant differences (P<0.05) between dietary treatments are shown by letters.

	Weight	SGR
P40	21.6 ± 1.2b	2.56 ± 0.10b
P45	24.9 ± 1.0ab	2.84 ± 0.07ab
P50	27.8 ± 1.2a	3.03 ± 0.07a
P55	24.5 ± 1.6ab	2.81 ± 0.11ab
P59	25.1 ± 1.2ab	2.84 ± 0.10ab

According to these data, the optimal dietary protein content was established as around 50 to 55% and a second trial to determine the best protein-to-energy ratio was carried out.

3.2. Protein-to-energy ratio study

Five isoproteic diets (52%) named 12L, 14L, 16L, 18L and 20L according to lipid inclusion in the diet were used to study the effect of protein-to-energy ratio on digestive enzymes activities (Figure 4 and 5) and nutrient absorption capacity (Figure 6). The diets' digestible energy was 14, 20, 21.5, 22 and 22.5 MJ/Kg, respectively.

The pH of the stomach, PC and PI contents was measured (7.06 ± 0.01, 6.97 ± 0.01 and 7.09 ± 0.01, respectively) and no differences were found between animals fed different diets for any of the digestive segments studied although pH was significantly higher in the PI than in the PC. Alkaline protease, lipase and α-amylase activities measured in the PC and PI are shown in Figure 4.

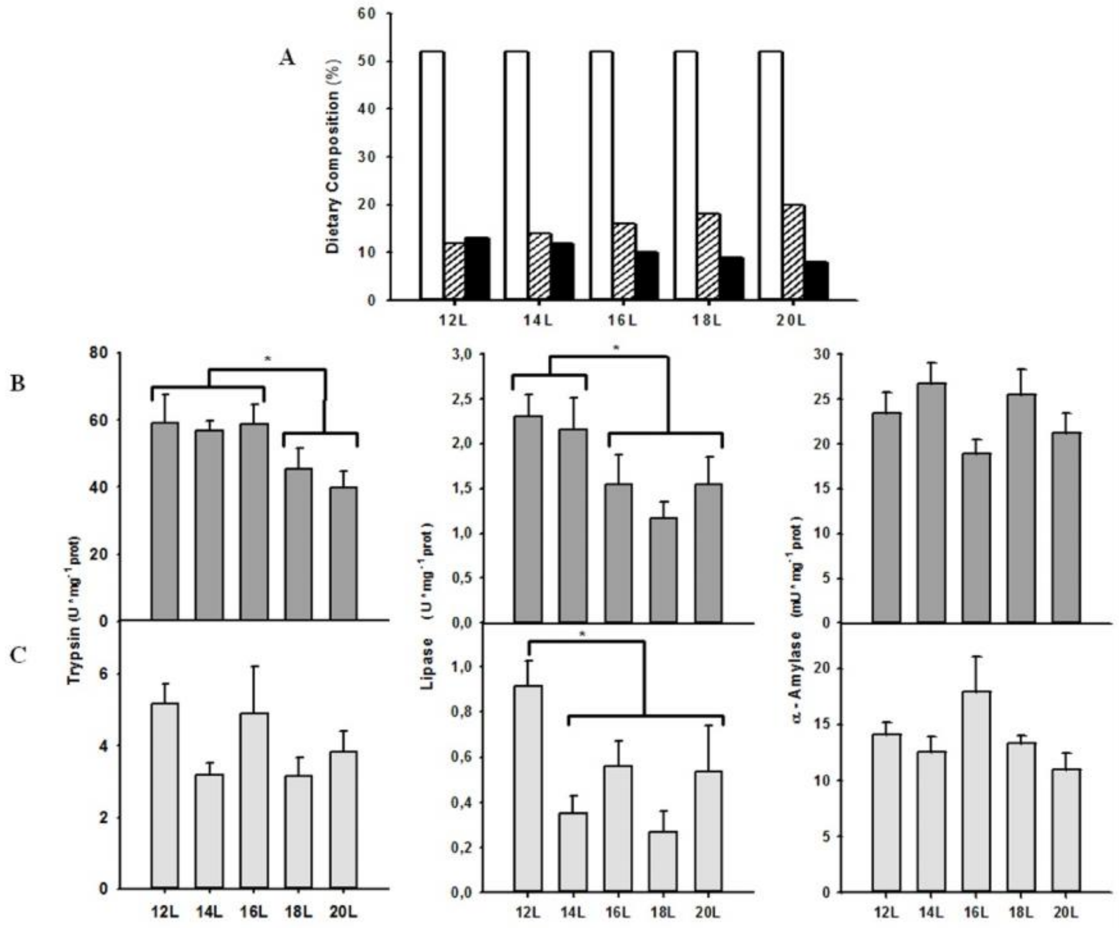

Figure 4. A) Dietary composition (%) of protein (white bars), lipid (striped bars) and carbohydrate (black bars), and pancreatic enzyme activities **B)** in the PC (dark grey bars) and **C)** in the PI (light grey bars). Values expressed as mean ± SEM (n = 10). Significant differences (P<0.05) between dietary treatments are shown by letters or by an * between grouped experimental conditions.

In the PC, animals fed the lower lipid diets showed higher alkaline protease (25.9% in 12L, 14L and 16L fish) and lipase (32.7% in 12L and 14L animals) activities than in the other diets, indicating up-regulation of protease and lipase pancreatic synthesis when the digestible energy of the diet was less than 21.5 and 20 MJ/Kg, respectively. No changes in α-amylase synthesis were found due to dietary lipid content (Figure 4B).

There was no evidence of adaptation of food-anticipatory TPA and α-amylase activities in the PI as the protein-to-energy ratio increased (Figure 4C); however, 12L sea bass showed a significant increase in PI lipase activity (47.25%) versus animals fed diets containing more energy. 12L and 14L sea bass showed similar individual protease activities, presenting lower activity for 17 and 18 KDa proteases and higher activity for 22 KDa proteases versus 16L sea bass. Animals fed diets containing higher levels of energy (18L and 20L) showed lower protease activity than 16L fish, 23, 22, 18 and 17 KDa alkaline proteases were affected. Moreover, the alkaline protease activity PC/PI ratio was significantly higher in 18L versus 12L sea bass (Table 4); however, no differences were found in the α-amylase and lipase PC/PI ratio (2.86 ± 0.52 and 5.94 ±1.04, respectively).

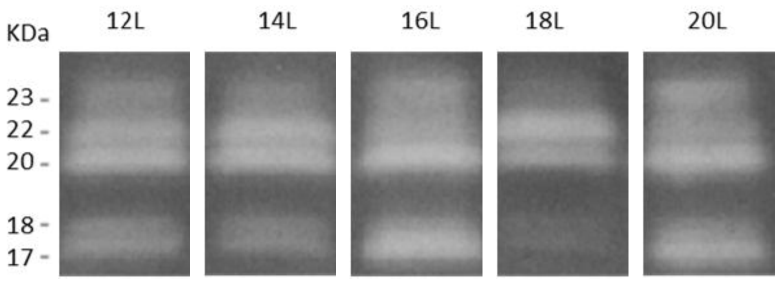

Figure 5. Model zymogram of alkaline protease activity in PC extracts. Figure shows the molecular weight of each band with proteolytic activity. All samples were analysed individually; the figure shows a representative result.

Table 4. Alkaline protease and a-amylase PC/PI ratio from the second trial. Significant differences (P<0.05) between dietary treatments are shown by letters.

PC/PI Ratio	Alkaline protease	α-Amylase
12L	6.6 ± 0.95b	1.98 ± 0.26
14L	15.3 ± 3.48ab	2.37 ± 0.12
16L	18.29 ± 3.69ab	4.86 ± 2.34
18L	29.43 ± 10.14a	1.9 ± 0.14
20L	18.5 ± 4.24b	3.34 ± 0.91

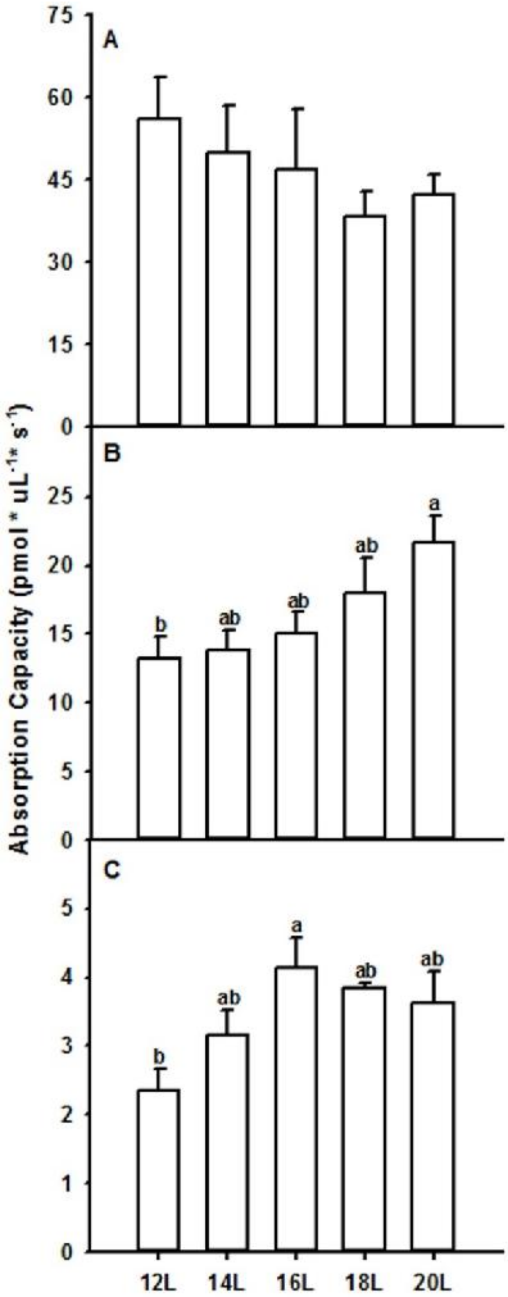

Figure 6. L-Lys (**A**), L-Met (**B**) and D-Glc (**C**) absorption capacities in BBMVs of the DI. Values are represented as mean ± SEM (n = 4). Significant differences (P<0.05) between dietary treatments are shown by letters.

As in the previous trial, L-Lys, L-Met, L-Trp and D-Glc absorption capacities in the DI were studied. No differences were found in L-Lys uptake due to dietary lipid inclusion (Figure 6A). Moreover, L-Met and D-Glc absorption capacities were significantly up-regulated as dietary lipid content increased (Figure 6B and C); however, L-Met increased gradually and significant differences were found between 12L and 20L sea bass, whereas D-Glc reached the maximum uptake for 16L sea bream and was at a similar level in 18L and 20L fish. Regarding L-Trp absorption capacity, no differences were found between dietary treatments (106.6 ± 5.1 pmols·µl^{-1}·s^{-1}).

No differences in final weight (24.0 ± 0.6g) nor SGR (2.75 ± 0.05) were found between dietary treatments.

3.3. Effect of seasonal variation

pH, pancreatic enzyme activities and nutrient absorption capacity were compared between P50 and 18L sea bass; in the first trial, animals were fed in spring and in the second in autumn-winter. The two diets were isoenergetic, had a similar principal composition (Figure 7A) and only differed in one of their ingredients: soybean concentrate for P50 and SPC60% for 18L.

There were no differences in the pH of the stomach, PC and PI (7.08 ± 0.01, 7.02 ± 0.01 and 7.11 ± 0.02, respectively) during spring and autumn-winter.

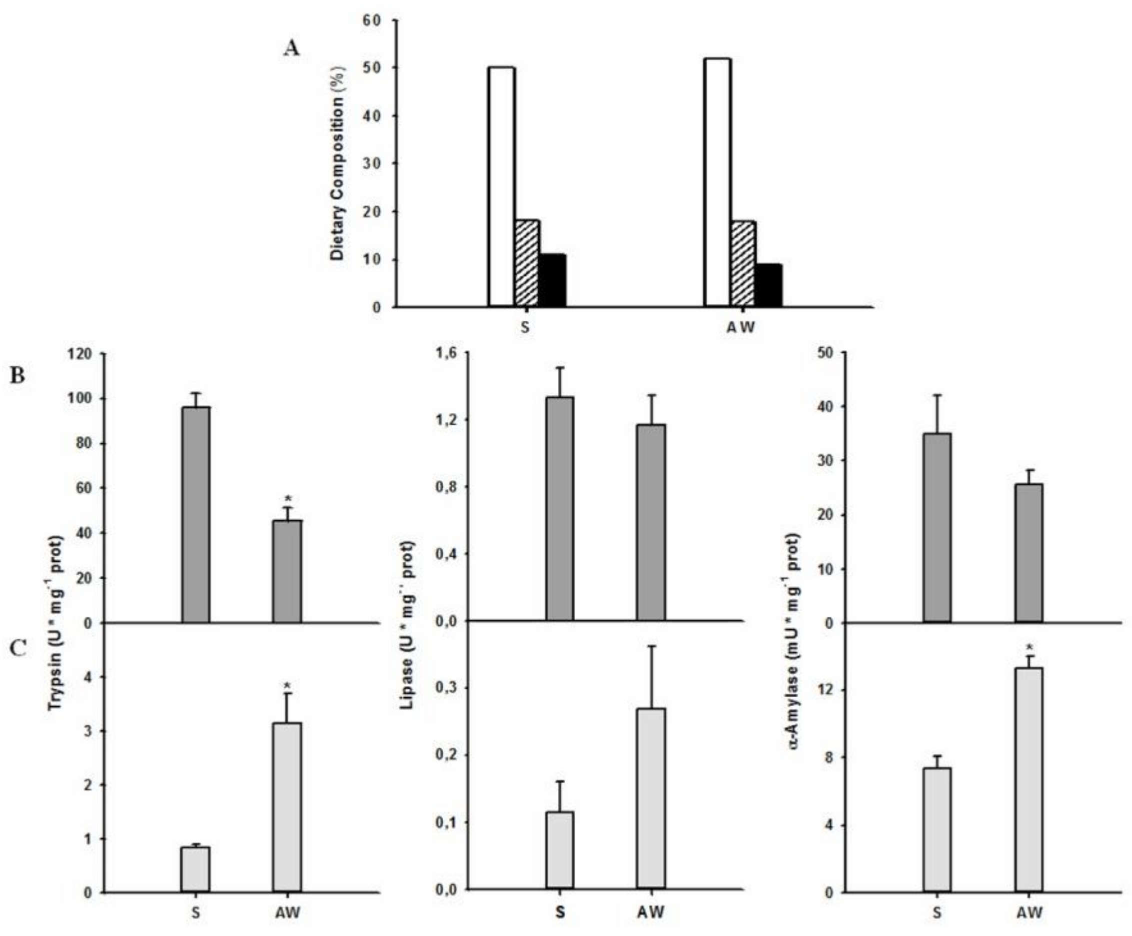

Figure 7. A) Dietary composition (%) of protein (white bars), lipid (striped bars) and carbohydrate (black bars) and pancreatic enzyme activities **B)** in the PC (dark grey bars) and **C)** in the PI (light grey bars), comparing seasonality between spring (S) and autumn-winter (AW). Values expressed as mean ± SEM. Significant differences (P<0.05) between seasons are shown by an asterisk.

Pancreatic enzyme activities from the PC and PI are shown in Figure 7. In the PC, alkaline protease activity was significantly higher in spring (52.7%), whereas no differences were found in lipase and α-amylase activities between seasons (Figure 7B). Digestive enzyme activities in the PI were higher in autumn-winter than in spring, being significantly higher for alkaline protease and α-amylase (Figure 7C). The PC/PI ratio for alkaline protease activity was significantly higher in spring-grown sea bass (Table 2 and 4), but no differences were found in the lipase or α-amylase PC/PI ratio. There were no differences in L-Lys, L-Met, L-Trp or D-Glc absorption capacities between fish grown in spring or autumn-winter (37.7 ± 3.3, 19.3 ± 2.2, 80.4 ± 10.1 and 3.9 ± 0.4 pmols·$\mu l^{-1}\cdot s^{-1}$, respectively).

SGR was significantly higher in spring (3.03 ± 0.07) than in autumn-winter sea bass (2.66 ± 0.11).

4. Discussion

The need to save protein and replace fish meal and fish oil to make the aquaculture industry more sustainable (SOFIA, 2014) and the limited information regarding digestive and absorptive processes in sea bass fingerlings, one of the main Mediterranean carnivorous fish currently being cultured, led us to undertake a study on the sparing effect of lipids and carbohydrates and the best protein-to-energy ratio in sea bass fingerlings fed diets in which 38% of fish meal had been replaced. Moreover, as there is a seasonal shift in the demand-feeding pattern and food anticipatory activity in sea bass (Azzaydi et al., 2007; Sánchez-Vázquez et al., 1998), the effects of seasonal variation were also studied.

The stomach pH of most teleosts is neutral when empty, being acidic after feeding (Yúfera et al., 2004; 2012). Moreover, pepsin activity is linked to lower pH, which must be less than 4.0 for pepsin activation with an optimum close to 2.0 or 3.0 (Cao et al., 2011; Nikolopoulou et al., 2011; Yúfera et al., 2004, 2012). Thus, according to the literature and the neutral gastric pH measured in the present study, which is expected at 24 h post-feeding, acid protease activity was not measured. The pH of the PI was significantly higher than that in the PC, as previously described by Nikolopoulou et al. (2011) for this species. Similar results were found for sea bream (Deguara et al., 2003; García-Meilán et al., 2013, 2014). Moreover, this study found no evidence of any effect of protein or lipid dietary percentage on intestinal pH, as also described by Nikolopoulou et al. (2011); there was no seasonal effect either.

Regarding digestive enzyme activities, the present study showed that alkaline protease was significantly higher than lipase or α-amylase activity, as described previously for carnivorous species (Buddington et al., 1997). In yellowtail king fish lipase activity was higher than α-amylase (Bowyer et al., 2012), as shown for sea bass in the present work; however in sea bream the levels of activity were variable, possibly due to differences in developmental stage, rearing conditions or dietary composition (García-Meilán et al., 2013, 2014; Santigosa et al., 2008, 2011a).

In sea bass, digestive proenzymes that are released into the PC have their origin in the hepatopancreas (Nejedli and Tlak Gajger, 2013) and the exocrine pancreatic tissue embedded within fat surrounding the PC (Kamaci et al., 2010). In the present study adipose tissue was not removed, therefore changes in enzyme activity detected at 24 h post-feeding in this intestinal segment should mainly reflect synthesis in the pancreas, as reported by Murashita et al. (2005) for yellowtail. In contrast, in the PI, the digestive food anticipatory activity that was detected was the result of secretion and peristaltic movements. Such anticipatory activity confers an adaptive advantage as food acquisition and utilization are improved (Comperatore and Stephan, 1987). Regarding nutrient absorption capacities, measurements were made in the distal intestine as in carnivorous species it seems that 5–10% of total absorption occurs in this intestinal region (Krogdahl et al., 1999); however, up-regulation of nutrient uptake was observed, likely as a way of compensating for the relatively small intestinal length and low transepithelial transport in the proximal intestine (Ferraris and Ahearn, 1984).

Dietary protein content affects pancreatic enzyme secretion (Bakke et al., 2011). Thus, an increase in alkaline protease activity was found as dietary protein inclusion increased in European sea bass (Péres et al., 1998), gilthead sea bream (García-Meilán et al., 2013), yellowtail (Kofuji et al., 2005) and Atlantic salmon (Krogdahl et al., 2003), which also secreted high levels of trypsin. Accordingly, the results of the current study showed an increase in alkaline protease synthesis as dietary protein increased, due to the increase in trypsin-like activities. Moreover, chymotrypsin was not detected in animals fed low protein diets. This changes the trypsin/chymotrypsin ratio and affects the digestive process. However, Rodiles et al. (2012) found no change in this ratio in Senegales sole when protein content was modified. Moreover, an up-regulation of lipase release following an increase in dietary protein was found in yellowtail (Murashita et al., 2008) and also in the present study. Meanwhile significantly higher α-amylase synthesis was found only in sea bass fingerlings fed the diet with higher protein and lower starch levels. In this sense, sea bass larvae were able to up-regulate α-amylase activity when fed diets with a high starch content but seem to lose this ability as they grow (Cahu and Zambonino-Infante, 1995; Peres et al., 1999). Moreover, Murashita et al. (2008) reported that α-amylase activity was not affected by dietary protein, lipid or starch in yellowtail. Thus, α-amylase activity seems more constitutive than trypsin activity in carnivorous species (Cahu and Zambonino-Infante, 1995, Buddington et al., 1997; Krogdahl et al., 2005), as shown in the present study.

Regarding food anticipatory activity in the PI, alkaline protease, lipase and α-amylase activities were up-regulated in extreme diets, showing the lowest values in P50 animals. Thus, animals fed both low protein and high lipid and starch diets or high protein and low lipid and starch diets attempt to improve nutrient digestion and assimilation. In this sense, various authors (Krogdahl et al., 1994; Olli et al., 1994; Haard et al., 1996; Francis et al., 2001; Sveier et al., 2001; Escaffe et al., 2007; Romarheim et al., 2007; Santigosa et al., 2008) have described changes in the intestinal distribution and activity of digestive enzymes as a compensatory mechanism for digestive processes.

With regards to protein-to-energy ratio, an increase in the synthesis of protease and lipase was detected as well as higher food anticipatory lipase activity with low lipid content diets, probably as a way of compensating for the low energy content in relation to energy requirements at fingerlings stage, as described by Kousoulaki et al. (2015). A negative effect of high dietary lipid on alkaline protease activity has been described in sea bream (Fountoulaki et al., 2005) and yellowtail (Murashita et al., 2007), and is thus another factor to consider. Despite this, Peres and Oliva-Teles (1999b) reported that protein digestibility was not affected by dietary lipid level in European sea bass. However more studies are needed to clarify this situation. On the other hand, dietary lipids appeared to have no effect on α-amylase synthesis or food anticipatory activity, as described previously for Japanese sea bass (Luo et al., 2010).

Amino acid and D-glucose transport capacities may be up-regulated to compensate for the nutritional deficit caused by dietary composition in trout and sea bream (Santigosa et al., 2011a, b) or short starvation periods (Golovanova, 1992; Santigosa et al., 2011b). In accordance with this, up-regulation of D-Glc absorption capacity was found in the distal intestine when dietary starch was low. Similar changes were found for α-amylase synthesis in sea bass fed high protein diets, but not in the protein-to-energy ratio study. Similar patterns were described in sea bream for α-amylase activity and L-Ala and D-Glc absorption capacities (García-Meilán et al., 2013, 2014; Santigosa et al., 2011b). L-Lys absorption capacity increases in the DI in animals fed high protein diets, probably as the amount of dietary protein leads to digestion in more distal regions (Santigosa et al., 2011b), despite the observed increase in synthesis and basal activity of proteases.

The optimum dietary protein level for sea bass juveniles was estimated to be around 50% (Alliot et al., 1979a; Hidalgo and Alliot, 1988; Peres and Oliva-Teles, 1999a). In sea bass fingerlings the best growth was also achieved by animals fed with P50 diet and these animals presented the lowest food anticipatory enzyme activities and intermediate L-Lys and D-glucose absorption capacity. In contrast, P40, P45, P55 and P59 animals showed compensatory mechanisms involving digestive pancreatic enzyme synthesis, food anticipatory protease, lipase and α-amylase activities and nutrient absorption capacity; although P45, P55 and P59 fingerlings showed a slightly lower final weight than P50 animals (-10.4, -11.9 and 9.7%, respectively), the

reduction was only significant in P40 sea bass (-22.3%). However, an increase in dietary lipid (from 12 to 20%) and energy levels (from 14 to 22.5 $MJ.Kg^{-1}$) did not affect growth performance, as previously described for this species (Metailler et al., 1981; Tibaldi et al., 1991; Morales and Oliva-Teles, 1995; Peres and Oliva-Teles, 1999b; Boujard et al., 2004).
Finally, differences in digestive enzyme synthesis and food anticipatory enzyme activities were found between spring and autumn-winter; with protease synthesis being higher in spring and food anticipatory protease and α-amylase activities higher in autumn-winter. Similar seasonal variations were found in trypsin and chymotrypsin activities in yellowtail, and might be a result of a slower transit speed of digesta during colder water temperature months as suggested by Kofuji et al. (2005). A reduction in digestive protease activities and subsequent low growth rates, with a direct correlation to water temperature, has also been reported in roach (Hofer, 1979), rainbow trout (McLeese and Stevens 1982), pike perch and bream (Gelman et al., 1984) and Atlantic salmon (Einarsson et al., 1997). However, sea bass fingerlings in the present study had the same rearing conditions in both seasons, and therefore these changes in digestive enzyme activities could be explained by the existence of an endogenous pacemaker (Sánchez-Vázquez al., 1995a, 1995b, 1998). Moreover, at the same growth temperature sea bass showed higher food intake in spring, coinciding with the lengthening of photoperiod, and a reduction in autumn associated with the shortening of photoperiod (Madrid, 2001), which could also explain the differences in growth in the present study. Unfortunately, no data on feed intake was available in this trial because the rearing tanks did not allow adequate collection of uneaten feed.

In conclusion, for sea bass fingerlings, the present study suggests that 52:16 is a good dietary protein-to-lipid ratio. Both higher protein and lipid levels extend digestion to more distal regions and lipid also interferes with proteolytic enzyme activities. In contrast, lower protein dietary levels negatively affect growth and lower lipid levels, thus triggering digestive compensatory mechanisms.

5. Acknowledgments

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CAPÍTULO III

Different protein to energy ratio diets for gilthead sea bream (*Sparus aurata*): Effects on digestive and absorptive processes

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Abstract

Sea bream juveniles were fed for a 12-week period with 7 isoenergetic diets with different levels of protein (35, 38, 41, 44, 47, 50 and 53%). The diets were formulated by changing simultaneously the levels of lipid (from 27 to 19%) and starch (from 21 to 10%). After the growth period, we studied the digestive enzyme activities at 5 h post-feeding and the nutrient absorption capacity at 24 h post-feeding. A progressive increase in the total protease activity (TPA) was found as the content of diet protein rose (from 35 to 41%) and a diminution of TPA activity was detected in sea bream fed high soybean concentrate content diets (50 and 53% dietary protein). Lipase activity did not change by dietary composition. An up-regulation of α-amylase activity, D-glucose and L-Ala absorption capacity was found in fish fed with the lowest carbohydrate diets, corresponding to 50 and 53% dietary protein. Moreover, the L-lysine absorption capacity was up-regulated in fish fed with low protein diets (35, 38, 41 and 44%). Thus, changes in diet composition can modulate enzymatic activities and nutrient absorption capacity, to improve food use and assure growth performance. In this sense, only sea bream fed with the lowest protein diet (35%) had a significantly lower SGR (< 1), although they had the highest voluntary feed intake. Sea bream fed with P_{44} and P_{47} diets need the minimal adaptive changes to diet and showed good growth.

Keywords: amylase, brush border membrane vesicles, fish, lipase, protease, substitution

1. Introduction

Several studies have been undertaken to establish the optimum dietary protein and protein to energy ratio for growing gilthead sea bream. Sabaut and Luquet (1973) estimated the optimum protein level for juvenile sea bream as 40%. This value was later revised to 45-46% (Santinha et al., 1996; Vergara et al., 1996). Regarding the optimal dietary lipid level for this species, the best growth performances were observed with 15-16% dietary lipids when fish oil was used as the lipid source (Vergara et al. 1996; Vergara and Jauncey, 1993). However, Lupatsch et al. (2001) stated that voluntary feed intake is maintained at least until 26% of dietary lipid; while growth and protein gain were optimal in diets with 48% of protein and 22.5% of lipid. The effect of dietary carbohydrates remains controversial in marine fish species (Enes et al., 2011; Peres et al., 1999), although it has been reported that sea bream can use starch without compromising growth when the diet contains up to 20% of carbohydrates (Fernández et al., 2007). The fish strain, water temperature and quality, oxygen availability, biomass density, and the biological value of dietary protein and non-protein energy substrates may influence the described requirements (NRC, 1983).

Digestion and absorption are key processes in the optimal use of a diet. Digestion processes begin in the stomach, where HCl denatures protein and converts pepsinogen to active pepsin (Wu et al., 2009; Yúfera et al., 2004). After the stomach, the chime passes to the pyloric caeca. When the pyloric caeca are full, cholecystokinin is secreted, and stimulates pancreatic secretions that include a) electrolytes, which neutralize the acidic pH of the chime coming from the stomach; b) bile acids that emulsify dietary lipids and fat-soluble vitamins, and c) pancreatic enzymes, including proproteases (Krogdahl et al., 1999; Pivnenko et al., 1997), α-amylase, lipolytic enzymes (Kurtovic et al., 2009), as well as DNAase and RNAase. The amounts and activities of the secreted pancreatic enzymes appear to vary with species and/or their dietary preferences; and with the nutrient composition and digestibility of the diet (Buddington et al., 1997).

Absorption processes occurs by diffusion, facilitated transport or active transport (Mailliard et al., 1995) and take place throughout the entire intestine, which differentiates fish from mammals. In fish, the presence of at least four Na^+-dependent amino acid transport systems (acidic, basic and neutral amino acids, and a system responsible for proline and N-methylated amino acids) have been described (Storelli et al., 1989). In addition, Na^+-independent transport systems have been found for alanine, glycine and lysine, proline and glutamate (Storelli et al., 1989), while L-histidine appears to be transported by a highly specific transporter (Glover and Wood, 2008). The transport of peptides by PepT1 and PepT2 transporters has been demonstrated recently (Goncalves et al., 2007; Hakim et al., 2009; Romano et al., 2006; Sangaletti et al., 2009; Terova et al., 2009). It has been suggested that intestinal fatty acid binding protein (I-FABP) could be involved in transport processes, by binding the peptides or proteins before endocytosis (Concha et al., 2002). Finally, D-glucose and D-galactose are transported by SGTL1, where uptake occurs by both facilitated and active transport (Sala-Rabanal et al., 2004). Nutrient absorption capacity can be affected by environmental differences such as temperature and water salinity, and by biotic factors such as the species, and/or their dietary preferences, diet formulation, digestibility and availability (Buddington et al., 1997).

The massive growth of aquaculture and the limited supply of fish meal (FM) and fish oil (SOFIA, 2010) means that alternative food sources need to be tested to make the industry more sustainable. In recent years, several studies have suggested that plant protein meals may be a good alternative (Bell and Waagbø, 2008; Turchini et al., 2009). It has been demonstrated that up to 75% of fish meal can be replaced without compromising the growth of sea bream (Albrektsen et al., 2006; Gómez-Requeni 2004). However, less is known about how the digestive and absorptive processes are affected by these dietary modifications (Santigosa et al., 2011a, 2011b, 2008; Venou et al, 2003). The replacement of FM by a blend of plant proteins decreases digestive enzyme activity. Nevertheless, animals can compensate for this reduction by increasing the relative intestinal length and the trypsin and chymotrypsin ratio of digestive proteases of pancreatic origin (upregulating trypsin activity) to achieve digestive balance (Santigosa et al., 2008). Nutrient absorption capacity is also modified in response to the inclusion of high levels of plant protein sources, probably because of distinct luminal nutrient availability due to supplementation with free amino acids, which leads to asynchronous utilization (Ambardekar et al., 2009; Santigosa et al., 2011b). Absorption patterns could be affected by changes in gut histology (Santigosa et al. 2008), as transporters are embedded in the lipid bilayer and affected by its characteristics (Houpe et al., 1997; Spector and Yorek, 1985) and by enterocyte development (Smith, 1993). In our knowledge, no studies have been carried out in sea bream on the effect of high lipid diets on digestive and absorptive processes.

Our aim was to find the best protein to energy ratio in a high lipid diet in which 50% of FM was replaced by plant protein sources; minimizing the protein content of the diet by including lipid and starch. Here, we studied the effect of diet on digestive and absorptive processes in sea bream.

2. Materials and methods

2.1 Diets

Seven isoenergetic diets (23 $MJ \cdot Kg^{-1}$, Table 1) were formulated (Skretting, Norway) with different levels of protein by changing the levels of lipid and starch simultaneously. Diets were named according to the protein content: P_{35}, P_{38}, P_{41}, P_{44}, P_{47}, P_{50} and P_{53}. As the protein increases, the dietary lipid and starch decreases. In all diets, 50% of fish meal (FM) was substituted by plant protein sources (wheat, wheat gluten and soybean protein concentrate). Fish oil was used as the only source of lipid. We used wheat as it has been described as good carbohydrate source for gilthead sea bream (Venou et al., 2003).

Table 1. Ingredient and nutrient composition of the experimental diets.

Ingredients (%)	P_{35}	P_{38}	P_{41}	P_{44}	P_{47}	P_{50}	P_{53}
Fish meal	26.9	26.7	29.4	32.0	34.7	37.3	40.0
Fish oil	21.0	19.0	17.6	16.2	14.7	13.3	11.9
Wheat gluten	6.0	10.7	11.8	12.8	13.9	14.9	16.0
SPC	9.0	10.7	11.8	12.8	13.9	14.9	16.0
Wheat meal	36.8	32.5	29.2	25.9	22.5	19.2	15.8
Vitamin Premix	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Composition (%) in wet matter							
Dry Matter	92.18	92.14	92.20	92.26	92.33	92.39	92.48
Crude Protein	35.00	37.99	40.99	43.99	47.00	50.00	53.00
Crude Fat	26.80	24.17	23.05	21.93	20.82	19.70	18.62
Estimated Starch	21.28	19.06	17.23	15.40	13.52	11.69	9.81

Fish meal: 66.8% crude protein, 13.1% crude fat.
Wheat gluten: 78.3% crude protein, 5% crude fat, 5% starch
SPC (Soybean protein concentrate): 61.5% crude protein; 1.8% crude fat.
Wheat meal: 14.9% crude protein; 3.0% crude fat; 57.0% starch
Vitamin premix is a proprietary formula of Skretting (Norway)

2.2 Fish and sampling

Gilthead sea bream from "Fauna Marina S.L." (San Fernando, Cádiz) were acclimatized for two weeks in 12m^3 fiberglass tanks at IRTA (Sant Carles de la Ràpita, Spain). Six hundred and thirty fish (of about 70 g) were randomly distributed into 21 fiberglass tanks (400 L; 30 fish per tank). The water salinity was 34.5‰ and the oxygen content in the outlet water was higher than 85% saturation.

During the 12-week trial (May-July), triplicate groups of fish were fed the corresponding diet twice a day (at 9:00 and 13:00) until visual satiety. The temperature (18 to 28ºC) and photoperiod (14 to 14:30 hours of daylight) were natural, according to the course of the trial.

At the end of the growth trial, ten animals per time and treatment were anaesthetized (phenoxyethanol 100 ppm), weighed and sacrificed by severing the spinal cord at 5 h and 24 h post-feeding. In the first sampling, the digestive tract was isolated and fish were classified according to their stomach contents (full versus empty). Samples of stomach, pyloric caeca and proximal intestine, including the intestinal content, were collected and rapidly frozen in liquid nitrogen and maintained at -80ºC until the enzymatic studies. In the second sampling, the whole intestine was isolated, measured and was cut lengthwise, washed in an isosmotic saline solution containing 0.1M of protease inhibitor (phenyl-methyl-sulfonyl-fluoride) and frozen in liquid nitrogen until the nutrient absorption experiments were performed.

All fish-handling procedures complied with European guidelines for animal care (Directive 2010/63/EU).

2.2.1 Zootechnical and morphological indexes

Total feed intake was calculated as [food intake (Kg)-food lost (Kg)]. Voluntary feed intake regarding whole diets and weight gain were expressed in: g BW $(kg)^{-0.70}$ day^{-1} to correct for the influence of fish size on feed consumption in gilthead sea bream (Lupatsch and Kissil, 1998), where body weight (BW) was calculated from the initial and final body weight of fish as the geometric mean: $BW=(W_{ini} \cdot W_{fin})^{0.5}$. Total biomass from all triplicate tanks was obtained from Skretting to evaluate the weight gain during the experimental period and used to calculate SGR and feed conversion ratio (FCR). The specific growth rate (SGR) was calculated as follows: $((\ln W_{fin}$ (g)- $\ln W_{ini}$ (g))/t)·100, where W_{fin} and W_{ini} represent the final and initial weights respectively, and t is the number of feeding days. The feed conversion ratio (FCR) was calculated as g feed given/g live weight gain. Relative intestinal length (RIL) was measured excluding pyloric caeca and is expressed in relation to each animal weight (Santigosa et al., 2008).

2.3 Gastric and intestinal pH content and intestinal digestive enzyme analysis

The gastric and intestinal pH content of the samples was measured (Crison, Micro pH 2000). Intestinal sample homogenization, total alkaline protease, trypsin and chymotrypsin activities and α-amylase were measured according to a modification of Santigosa et al. (2008). Lipase determination was carried out following Santigosa et al. (2011a). Briefly, intestinal samples were individually homogenized (Politron 2000, Sorvall TC) at 4ºC in 50 mM TrisHCl buffer pH 7.5 to a final concentration of 250 mg mL^{-1}. Homogenates were centrifuged (1100 g, 15 min, 4ºC. Jouan CR 411) and supernatants were recovered and stored at -80ºC. For the total alkaline protease activity (TPA) determination, samples were reacted with 50 mM TrisHCl buffer at the pH of the intestinal content containing 1% casein at 20ºC. After 30 min, the reaction was stopped by adding trichloroacetic acid 12%. The samples were at maintained 4ºC for an hour and were then centrifuged (7500 g, 5 min, 4ºC). Individual blanks were established for each sample. Supernatant absorbance was measured at 280 nm (UV-1603, Shimadzu). Bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as standard.

Zymograms were used to characterize trypsin-like and chymotrypsin-like activities. Samples were loaded using 12% polyacrylamide gels (10x10.5x0.1 cm) and electrophoresis was performed at a constant current of 15mA per gel for 150 min (EPS 301 Power Supply, 4ºC). The gels were incubated under agitation at the pH of the intestinal content in TrisHCl buffer containing 2% casein (30 min, 4ºC). They were shaken for 90 min at room temperature. Gels were washed and stained in a methanol: acetic acid: water solution (40:40:10) with 0.1% of BBC R-250 (Brilliant Blue Coomassie R-250). Destaining was carried out using the same solution without colorant. Pure trypsin was used as a control, and a RPN 800 commercial weight marker (Amersham, GE Healthcare, UK, 12000-225000Da) was used to determine the molecular weight of protease activity fractions. To characterize trypsin-like and chymotrypsin-like activities we used specific inhibition solutions (Alarcón et al., 1998).

α-Amylase activity was measured using the tracer amylose azure, a potato starch that is covalently labelled with Remazol Brilliant Blue R dye. The homogenate was combined with 0.5% tracer solution containing soybean trypsin inhibitor (SBTI 0.04 mg mL^{-1}) to prevent proteolysis. The reaction was stopped after 30 min by adding 6% acetic acid and the sample was centrifuged (13000 g; 10 min at 4ºC). Supernatant absorbance was measured at 595 nm. α-Amylase (Sigma Aldrich, Spain, 66U/mg solid) was used as standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 at 20ºC (Santigosa et al., 2008).

For the lipase determination, homogenate was mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6’-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg L^{-1} colipase (pH 8.3). The increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33944U/mg protein, 22980U/mg solid) was used as a standard. One unit hydrolyses 1.0 micro equivalent of FA from triacetin in 1 h at pH 7.4 and 20ºC (Santigosa et al., 2011a).

The protein concentration in homogenates was determined by the Bradford method (1976) using bovine serum albumin as a standard.

2.4 Nutrient uptake analysis

Brush border membrane vesicles (BBMVs) from whole intestine were obtained as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenated in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100) to separate mucosa from the muscle layers. A selective basolateral and mitochondrial membrane precipitation was carried out by adding $MgCl_2$ to a final concentration of 10mM. Subsequent selective centrifugations allowed the purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe. The enrichment in brush border membrane preparations was determined by measuring alkaline phosphatase activity following Weiser (1973), which was described as a good marker of BBMVs.

The capacity to absorb three essential amino acids (L-leucine, L-lysine and L-phenylalanine), three non-essential amino acids (L-alanine, L-glutamic and L- proline) and D-glucose was determined in vesicular suspensions according to Santigosa et al. (2011b). These were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient, Osm 320, pH 7.4) for 5 sec. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 320, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22-µm cellulose nitrate filters (Millipore, Bedford MA), previously wetted in cold stop buffer. The filters were then washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were made at 20ºC. Radiolabelled nutrients were obtained from Amersham Pharmacia Biotech (Barcelona, Spain).

The vesicular volume of BBMV preparations was measured following Sala-Rabanal et al. (2004) to normalise influx values. Briefly, L-alanine retained inside the vesicles at the equilibrium situation was measured by incubating BBMV preparation with incubation buffer on ice. After 90 min, the reaction was stopped and the amount of L-alanine was measured following the procedure describe above.
The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford method (1976).

2.5 Statistical analysis

Differences between the presence and absence of food in stomach were determined using a Student's *t*-test. Differences between dietary treatments were determined using one-way ANOVA and Tukey's test. The Student's *t*-test was used to establish significant differences between intestinal regions ($P<0.05$). The software used was PASW Statistics 17.0 (SPSS, Inc., EUA).

3. Results

The measure of gastric and intestinal content pH and digestive enzyme activity was determined at 5 hours post-feeding. At that time, 41.4% of the animals still had feed in their stomachs and 58.6% had empty stomachs. First, the results are presented according to the presence or absence of feed in the stomach. Figure 1 shows the pH of stomach, pyloric caeca (PC) and proximal intestine (PI) content. The pH of the gastric content was found to vary depending on the status of the stomach (full versus empty). The pH was lower (approximately 4) when the stomach was full, which indicates that acid protease activity occurs. This value was significantly different from the pH values of an empty stomach (6.6 ± 0.04). The pH values measured for the intestinal regions (PC and PI) were close to 7 and slightly lower for the pyloric caeca when the stomach was full.

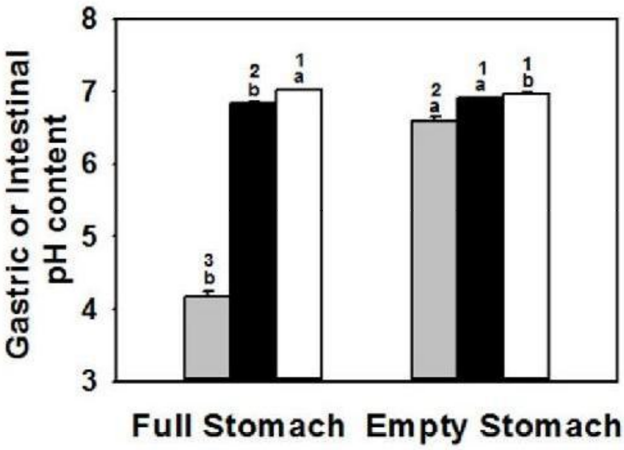

Figure 1. pH of gastric content (grey bars), pyloric caeca content (black bars) and proximal intestine content (white bars) in full (n=30) or empty stomach (n=40). Values are represented as the mean ± SEM. Significant differences (*P*< 0.05) between gastric and intestinal segments are shown by different numbers; significant differences between conditions (*P*< 0.05) are shown by different letters.

Figure 2 shows the total protease (Fig. 2A), lipase (Fig. 2B) and α-amylase activities (Fig. 2C) in PC and PI. Digestive enzyme activities were lower in pyloric caeca than in the proximal intestine, except for lipase activity when the stomach was empty. In the proximal intestine, total protease and lipase activities (Fig. 2A and 2B) were higher in the full versus the empty stomach. This pattern was not detected for proximal α-amylase activity (Fig. 2C) or pyloric caeca activities.

Animals fed with extreme diets had a lower frequency of feed content in stomach (30% for P_{35}, P_{50} and P_{53} animals and 20% for P_{38} fish). However, this frequency was higher (70, 50 and 60%, respectively) for sea bream fed with P_{41}, P_{44} and P_{47} diets.

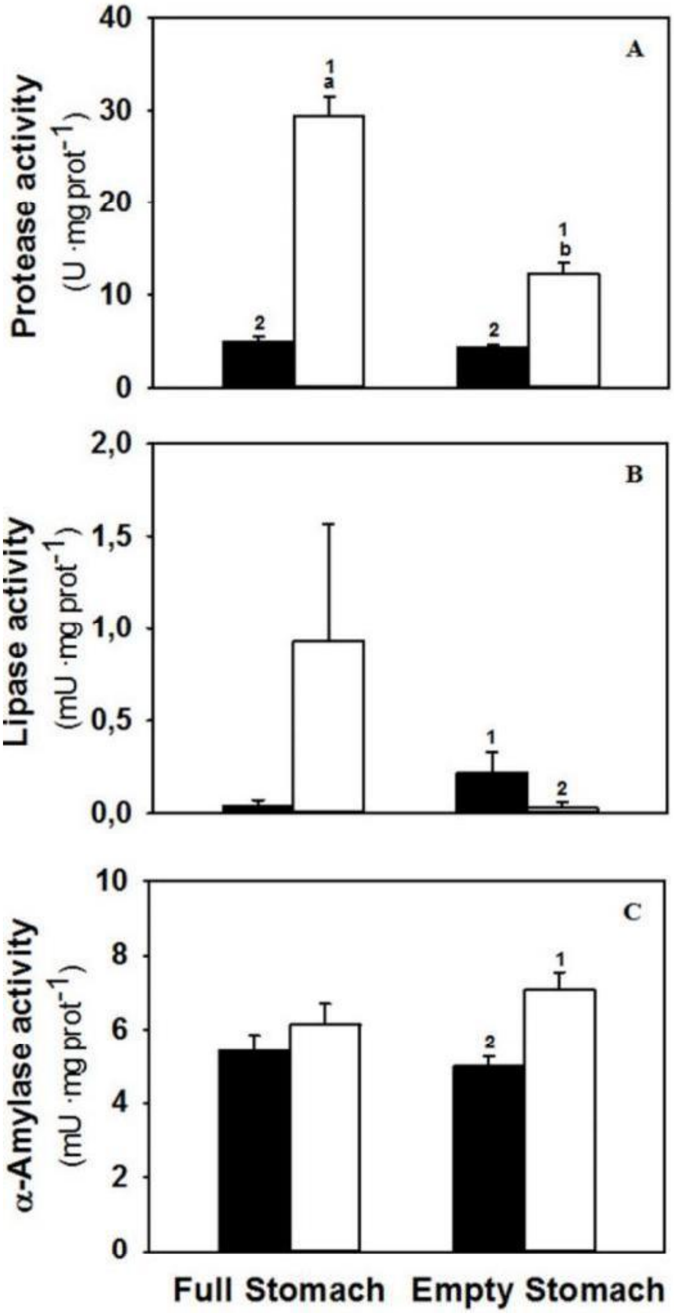

Figure 2 A) Total protease, **B)** lipase and **C)** α-amylase activity in pyloric caeca (black bars) and proximal intestine (white bars) in full (n=30) or empty stomach (n=40). Values are represented as the mean ± SEM. Significant differences (*P*< 0.05) between intestinal segments are shown by different numbers; significant differences between conditions (*P*< 0.05) are shown by different letters.

Second, we present the results according to the diet. Figure 3 shows the total protease activity (TPA). In pyloric caeca the values were higher for the animals fed with the lower protein content diets (P_{35} and P_{38}), while baseline activity was detected in the other groups. Proximal intestine TPA tended to be higher in gilthead sea bream fed with P_{41}, P_{44} and P_{47} diets. Fish fed with the diets P_{35} and P_{38} showed lower total protease activity. In these fish, the TPA PI/PC ratio was between 1-2. In contrast, in sea bream fed with P_{41}, P_{44} and P_{47} diets, the TPA PI/PC ratio was 7 to 9. In these fish, the TPA that was detected was significantly higher than in the rest of the experimental groups (Fig. 3). Finally, in fish fed P_{50} and P_{53} diets, the TPA PI/PC ratio measured was intermediate at between 3 and 7.

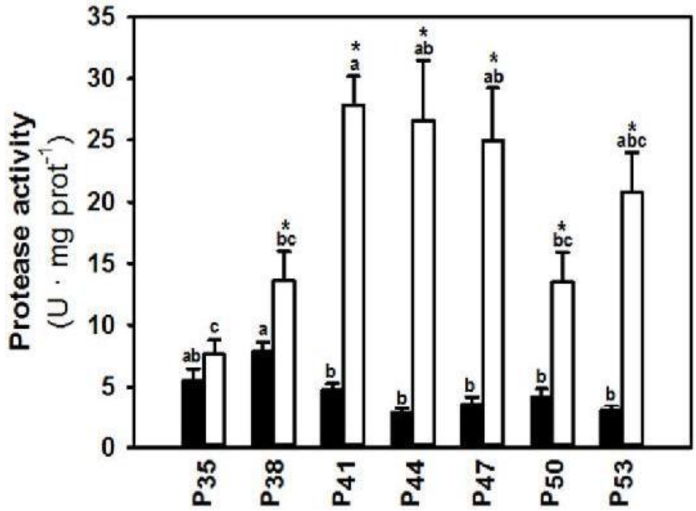

Figure 3. Total protease activity in pyloric caeca (black bars) and proximal intestine (white bars). Values are represented as the mean ± SEM of 10 fish. Significant differences (*P*< 0.05) between diets are shown by different letters; significant differences between intestinal segments (*P*< 0.05) are shown by *.

Figure 4 shows model zymograms from PC (4A) and PI (4B) obtained from each experimental group, these revealed the presence of six proteases at intestinal level. The band pattern found for each condition corresponds to the proteolytic profile measured in previously described intestinal segments (Fig. 3). Thus, the activity was maximal in pyloric caeca for P_{38} sea bream and faded to 55 KDa trypsin and chymotrypsin activity in other conditions. The proximal intestine band pattern (Fig. 4B) was modified in the extreme diets: in P_{35} fish chymotrypsin activity was not detected and 55 KDa and 90 KDa trypsin activities were attenuated. Similarly, chymotrypsin activity was not found in P_{50} fed-fish. Chymotrypsin was partially detected in P_{53} fed-fish, and specifically affected the 30 KDa band.

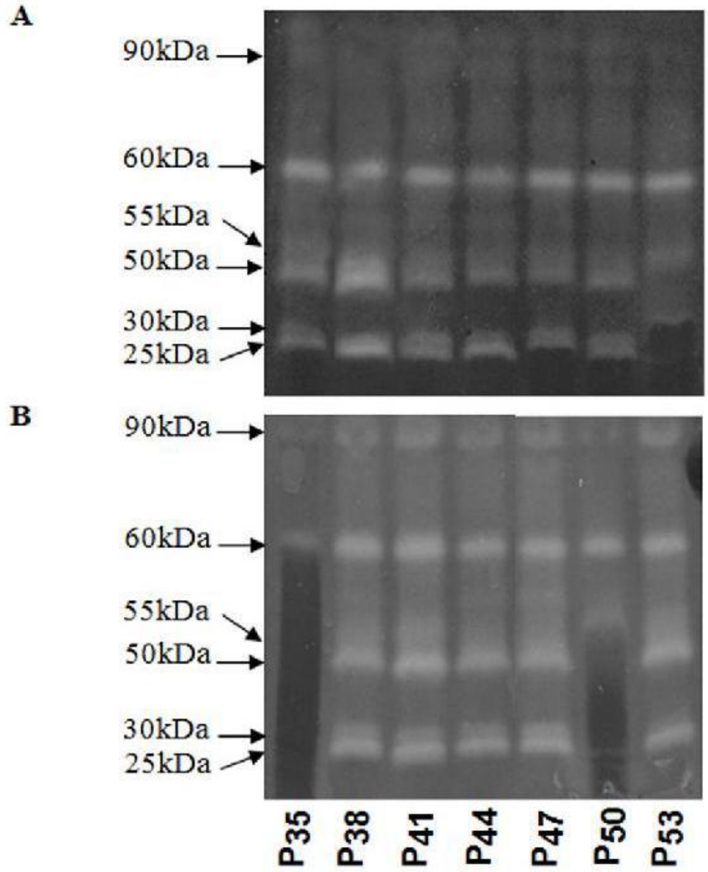

Figure 4. A) Model zymogram of the alkaline proteolytic activity in pyloric caeca extracts. **B)** Model zymogram of the alkaline proteolytic activity in proximal intestine extracts. C) Inhibition zymogram. Lane 1: homogenate (H), Lane 2: H+ZPCK (chymotrypsin inhibitor) and lane 3: H+TLCK (trypsin inhibitor). Figures show the molecular weight of bands with proteolytic activity.

Lipase and α-amylase activities were also measured in PC and PI. When lipase activity was measured no significant differences were found among experimental groups or intestinal segments. In PC lipase activity range from 0-2.6 mU lipase·mg protein^{-1} (mean 0.139 ± 0.061), while in PI range from 0-6 mU lipase·mg protein^{-1} (0.408 ± 0.27). In contrast, α-amylase activity 5 h after manual feeding (Figure 5) tended to increase as the starch content of the diet diminished, with the exception of the P_{35} fish, in which PI α-amylase activity was higher than in the other experimental groups.

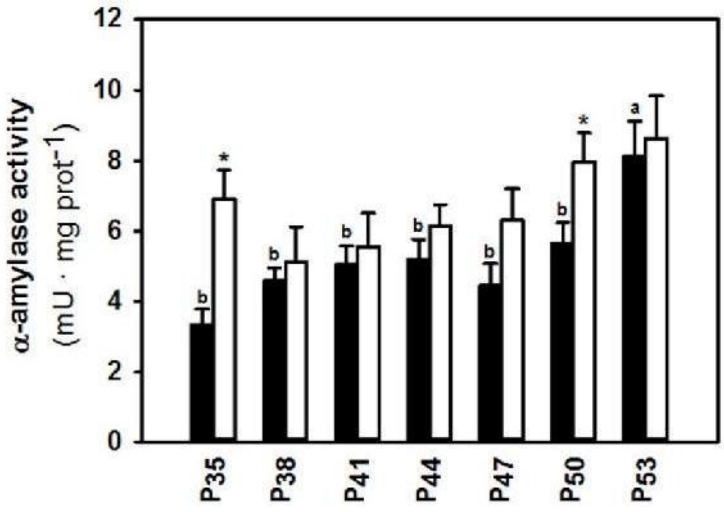

Figure 5. α-amylase activity in pyloric caeca (black bars) and proximal intestine (white bars).Values are represented as the mean ± SEM of 10 fish. Significant differences (P< 0.05) between diets are shown by different letters; significant differences between intestinal segments (P< 0.05) are shown by *.

The nutrient absorption capacity of the whole intestine 24 h post-intake was measured in brush border membrane vesicles (BBMVs) obtained from fish fed the experimental diets. To validate the vesiculation processes, the enrichment of alkaline phosphatase activity in BBMVs was determined to be between 4 and 6.6 times the adequate value, according to Santigosa et al. (2011a, 2011b). The capacity of the intestine to absorb essential and non-essential amino acids and D-glucose is shown in Figure 6 and Table 2. The L-Lys and L-Phe absorption capacities were higher than those of the other amino acids measured.

Sea bream fed with lower protein content diets tended to show higher lysine absorption capacity, whereas the absorption capacity of L-phenylalanine was higher in animals fed with the highest protein content diets (P_{50} and P_{53}). No differences were found for the L-Leu absorption capacity. D-glucose uptake (Figure 6) was significantly higher for the P_{50} and P_{53} groups, which may be due to the reduced contribution of diet carbohydrate, following the same profile as the α-amylase activity and the absorption capacity of L-Ala and L-Glu.

Table 2. Essential and non-essential amino acid intravesicular concentration in BBMVs (pmols·mm^{-2}·seg^{-1}). Values determinations. Different letters show significant differences (P < 0.05) between diets.

Essential	P_{35}	P_{38}	P_{41}	P_{44}	P_{47}
L-Phe	20.71 ± 3.65**ab**	18.25 ± 2.22**ab**	14.60 ± 0.48**b**	16.13 ± 1.07**ab**	15.39 ± 1.96**ab**
L-Leu	10.12 ± 1.48	9.14 ± 1.53	13.24 ± 0.26	12.16 ± 0.94	10.70 ± 1.28
Non-essential					
L-Pro	8.34 ± 0.41	8.86± 0.73	9.01 ± 1.26	9.05 ± 0.88	7.65 ± 0.46
L-Ala	4.45 ± 0.30**d**	5.13 ± 0.23**d**	5.40 ± 0.12**cd**	6.94 ± 0.37**b**	4.79 ± 0.21**d**
L-Glu	2.36 ± 0.11**b**	3.50 ± 0.36**ab**	2.34 ± 0.10**b**	3.19 ± 0.32**ab**	2.29 ± 0.27**b**

Table 3. Total intake (n=3), voluntary feed intake (n=3), SGR (n=3), FCR (n=3) and RIL (n=12) for each experimental con ± SEM. Different letters show significant differences (P < 0.05) between diets.

	P_{35}	P_{38}	P_{41}	P_{44}	P_{47}
Total Intake (Kg)	3.76 ± 0.10**d**	4.65 ± 0.10**bc**	4.37 ± 0.19**c**	4.82 ± 0.03**abc**	4.78 ± 0.08**abc**
Voluntary Feed Intake (g·kg$^{-0.7}$·day^{-1})	5.65 ± 0.04**a**	5.11 ± 0.07**b**	5.28 ± 0.07**b**	5.16 ± 0.01**b**	5.24 ± 0.03**b**
SGR	0.94 ± 0.03**c**	1.14 ± 0.03**b**	1.10 ± 0.02**b**	1.20 ± 0.02**ab**	1.22 ± 0.03**ab**
FCR	1.38 ± 0.008**a**	1.25 ± 0.041**abc**	1.26 ± 0.050**ab**	1.16 ± 0.014**bc**	1.16 ± 0.026**bc**
RIL (mm·g fish^{-1})	17.11 ± 1.73	16.45 ± 1.44	15.62 ± 1.27	14.77 ± 1.02	14.91 ± 0.98

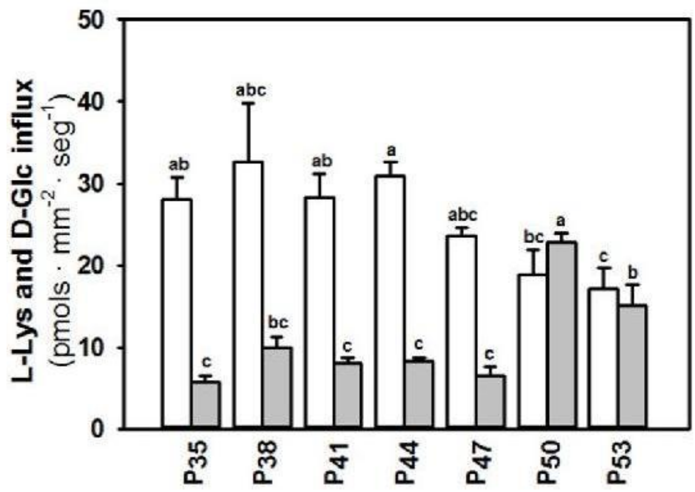

Figure 6. Intravesicular concentration of L-lysine (white bars) and D-glucose (grey bars) in brush border membrane vesicles obtained from whole intestine from sea bream. Results are represented as mean ± SEM of 6 determinations. Different letters show significant differences (P < 0.05) between diets.

Total intake, voluntary feed intake, SGR, FCR and RIL are shown in Table 3. The SGR was higher for gilthead sea bream fed the P_{50} and P_{53} diets, while the results were significantly lower for the P_{35}, P_{38} and P_{41} groups (-27.3, -12.3 and -15.4%, respectively, when compared to P_{50} fed-animals). Proportion to their size, the P_{35} animal's intake was superior to all other groups. A higher FCR was detected for sea bream P_{35}, P_{38} and P_{41}, and also a tendency to increase RIL in those groups.

4. Discussion

As fish meal is a limited primary source, it should be replaced by plant proteins, lipids or starch in fish diets (SOFIA, 2010). Therefore, the diets used in this trial contained 50% of plant origin protein. To improve growth, the diets used were also highly energetic, with a minimum content of 19% lipid. The study examines the effect of replacing protein by lipid and starch on digestive and absorptive processes.

Animals fed with P_{41}, P_{44} and P_{47} diets showed a higher frequency of feed content in stomach, suggesting a gastric emptying lower in these groups versus animals fed with extreme diets.

The protein content of a diet influences the secretion of pancreatic enzymes (Bakke et al., 2011). Some authors have stated that increased protein levels stimulate the pancreas to deliver a secretion that contains high levels of trypsin in Atlantic salmon (Krogdahl et al., 2003) and

European sea bass (Péres et al., 1998). Animals fed the P_{35}, P_{38} and P_{41} diets showed a progressive increase in intestinal proteolytic activity as the protein in the diet rose, reaching a plateau at 41% to 47% of protein. The lower protease activity measured for P_{35} and P_{38} fed-fish may also be due to the greater presence of lipid (27 and 24%) and starch (21 and 19%) in the diets. In studies using different experimental diets (Fountoulaki et al., 2005) or a single bolus of lipid (Murashita et al., 2008), high fat content was related with lower protease activity. High levels of starch affect the viscosity of digesta (Storebakken et al., 1987) and are considered to interfere with proteolytic activities (Fernández et al., 2001; Hidalgo et al., 1999; Munilla-Morán and Saborido-Rey, 1996). The lower TPA PI/PC ratio detected in P_{35} and P_{38} sea bream points to a slower intestinal digestive process. In contrast, P_{41}, P_{44} and P_{47} fish showed the highest PI/PC ratio, denoting that the digestion processes were taking part in PI. These could be related to the high lipid and starch content of both diets, as suggested by the results of Fountoulaki et al. (2005) in sea bream. In this trial, 50% of the protein in the diet was of plant origin, specifically from wheat gluten and soybean meal. The presence of anti-nutritional factors (ANFs) were not determined in the experimental diets, but it has been widely described that soybean meal could hinder the action of proteases by complexing with them and preventing their action, as previously described (Francis, 2001; Gatlin et al., 2007; Krogdahl et al., 2010). The possible ANFs' presence in these diets could inhibit chymotrypsin-like activities to a greater extent, and change the trypsin to chymotrypsin ratio, as Santigosa et al. (2008) suggested. The level of dietary protein can also alter the pattern of digestive protease secreted in the intestinal lumen (Rodiles et al., 2012). These changes in P_{50} and P_{53} sea bream could lead to asynchronous availability of amino acids and di-tri-peptides as a consequence of the specificity of proteases.

Amino acid transport capacity may be upregulated to compensate for the nutritional deficit caused by dietary composition (Santigosa et al., 2011a, b) or short starvation periods (Golovanova, 1992; Santigosa et al., 2011b). Thus, P_{35}, P_{38}, P_{41} and P_{44} gilthead sea bream showed a higher absorption capacity of L-Lys than animals fed with high protein diets. This compensation mechanism enabled the P_{44} animals to achieve similar growth rates to the P_{47}, P_{50} and P_{53} animals. Nevertheless, this was not enough for P_{41} and P_{38}, whose growth was slower (-15.4% and -12.3 respectively, versus P_{50}). In addition to the mechanism described above, sea bream P_{35} had significantly greater voluntary feed intake and FCR, although the latter did not differ from P_{38} and P_{41}. Nevertheless, growth fell by 27%. Another compensation mechanism to the lower protein content of diet was the tendency to increase RIL. Santigosa et al., (2008) described an increase in RIL as the plant protein content of diets rise.

The relationship between dietary nutrient levels of lipids and carbohydrates and corresponding enzyme secretions appears to be more complicated (Bogevik et al., 2009; Krogdahl and Sundby, 1999; Morais et al., 2007; Zambonino Infante and Cahu, 2007). In this trial, intestinal lipase activity was low and was not affected by dietary lipid content (Santigosa et al., 2011a). This suggests that there was limited digestion of oils in high lipid diets. In contrast, it is well known that high starch levels can provoke absorption of amylase onto starch molecules, thus inhibiting the enzyme's activity (Fountoulaki et al., 2005; Spannhof and Plantikov, 1983). In addition, amylase activity is inhibited by both dietary starch and lipid, as shown in Fountoulaki et al. (2005). This is in accordance with the results for P_{50} and P_{53} animals, which showed higher amylase activity. Similarly to the α-amylase activity, the upregulated capacity to absorb D-Glc in gilthead sea bream fed P_{50} was probably due to the lower availability of this nutrient, as Santigosa et al. (2011b) suggested. Both situations allow an improvement in D-Glc absorption. L-Ala showed the same absorption capacity profile as D-Glc, which suggests that this amino acid could be used as an energy source when diets have low starch content.

As described above, P_{35} sea bream presented significantly lower growth rates than all other fish, and increased their voluntary feed intake as a compensatory mechanism, which further increased dietary carbohydrate intake. These facts could be related to the higher proximal α-amylase activity detected in the proximal intestine as Fountoulaki et al. (2005) suggested in fish fed with high starch diets.

It is well known that fibre and the non-starch polysaccharides (NSPs) present in plant-based diets negatively affect enzymatic activities and increase transit rates (Francis 2001; Gatlin et al. 2007; Krogdahl et al., 2010). In this trial, the total amount of fibre and NSPs did not vary between diets, but their origin differed depending on the proportions of plant ingredients included. The kind of dietary ANFs could differentially affect the digestion processes and transit rates.

Finally, Murashita et al. (2007) suggested that pancreatic lipase and trypsin were released before α-amylase. The results were in accordance with this hypothesis when the enzymatic activities, measured in pyloric caeca and proximal intestine, were compared between fish with full or empty stomachs.

In summary, when dietary composition changes, intestinal enzymatic activities and nutrient absorptive capacity may be modulated in fish. Voluntary feed intake may also change to improve feed use and assure growth performance. Sea bream fed the P_{44} and P_{47} diets needed minimal adaptive changes and grew well.

5. Acknowledgments

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CAPÍTULO IV

Meal timing affects protein-sparing effect by carbohydrates in sea bream: Effects on digestive and absorptive processes

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Abstract

A commercial diet (C) (48% protein and 20% lipids) and a high-digestible carbohydrate diet (CH) (37% protein, 12.5% lipids and 40% high-digestible carbohydrates) were used to feed sea bream juveniles for an 8-week period. In the commercial diet, more than 60% of ingredients were of plant origin from various sources, whereas the only component of plant origin in the CH diet was wheat. To determine the best time to administer carbohydrates and the possible protein sparing effect, three different dietary regimes were established: C, CH-M and CH-A, and the corresponding diet was fed to sea bream in the morning (1.6% of bw) and in the afternoon (1% of bw), calculating quantities according to the amount of feed that fish ate during the acclimatization period. After the growth trial, specific growth rate (SGR), relative intestinal length, intestinal pH content, gastric and pancreatic digestive enzyme activities and nutrient absorption capacities were studied 5h post-feeding after each meal (morning and afternoon). The acid protease activity measured was anticipatory and was higher when the next meal would have more protein. No differences in relative intestinal length or feed buffering capacity were found. The smaller ration given to sea bream in the afternoon led to a lower pancreatic release of alkaline protease and α-amylase and an up-regulation of D-Glc and L-Ala absorption capacity. A higher transit rate was measured when sea bream were fed the CH diet. When high-digestible carbohydrates were administered in the morning and the commercial diet in the afternoon, we observed a better assimilation of both diets due to compensatory mechanisms such as an increase in L-Lys, D-Glc and L-Ala absorption capacity after the morning feed, and a higher pancreatic release of alkaline protease and amylase after the afternoon feed. In contrast, when high-digestible carbohydrates were given in the afternoon, only a significant up-regulation of the capacity to absorb L-Lys was detected. Thus, the inclusion of high-digestible carbohydrates in the diet improved digestion and absorption processes when administered in the morning, leading to a protein sparing effect that yielded growth comparable to that of fish fed an exclusively commercial diet.

Keywords: amylase, brush border membrane vesicles, lipase, protease, starch, substitution

1. Introduction

Fish meal provides an adequate balance of amino acids, but increased demand and prices, as well as uncertain supply, render it necessary to identify alternative protein sources (SOFIA, 2012). At present, over 50% of the fish meal in commercial diets is replaced by protein of plant origin; thus, the possible presence of anti-nutritional factors (ANFs) and the limitation of some amino acids, such as lysine or methionine (Francis et al., 2001, Gatlin et al., 2007; Krogdahl et al., 2010), may promote changes in digestive and absorptive capacities (Santigosa et al., 2011a; 2011b; 2008) in order to minimize the effects on metabolism (Metón et al., 1999) and compensate for those dietary changes.

The protein-sparing effect may possibly present a means to minimize the use of fish meal, by increasing the lipid and/or carbohydrate content of the feed. The use of dietary lipids instead of protein is well established in fish (Company et al., 1999; Vergara et al., 1999). A voluntary feed intake of at least 26% dietary lipids, and a diet of 48% protein and 22.5% lipids, has been established as providing optimal growth and protein gain (Lupatsch et al., 2001). However, despite these results, the high prices and limited supplies of fish oil (SOFIA, 2012) restrict its use, and it is often replaced by vegetable oils that diminish the quality of the final product (De Francesco et al., 2007; Fountoulaki et al., 2009; Izquierdo et al., 2005; Menoyo et al., 2004). On the other hand, the utilization of dietary carbohydrates for energy purposes in salmonids and other carnivorous species appears to be limited (Hemre et al., 2002; Stone et al., 2003; Wilson, 1994). Although several studies have indicated a protein-sparing effect by dietary carbohydrates in sea bream and sea bass (Couto et al., 2008; Dias et al., 1998; Enes et al., 2006; Fernández et al., 2007; Peres and Oliva-Teles, 2002), others have failed to demonstrate such an effect (Enes et al., 2008; Lanari et al., 1999; Moreira et al., 2008). Enes et al. (2011) have reported that European sea bass and gilthead sea bream juveniles can ingest up to 20% digestible carbohydrates without adverse effects on growth or feed utilization. Besides individual differences between species, the main factors affecting carbohydrate digestibility in fish are dietary inclusion level, molecular complexity, botanical origin, technological treatments applied, and water and temperature (Krogdahl et al., 2005; Stone, 2003; Venou et al., 2003; Wilson, 1994). In this respect, wheat has been described as a high-digestible carbohydrate source (Venou et al., 2003). Due to their lower amylose/amylopectin ratio and their structure, starch granules are more vulnerable to damage by industrial processing and attack by amylases (Bergot, 1993; Cousin et al., 1996).

Digestion and absorption processes are the key to successful utilization of a given diet and feed timing. Digestion processes begin in the stomach, where HCl converts pepsinogen into active pepsin (Wu et al., 2009; Yúfera et al., 2004). When the chyme passes into the pyloric caeca, cholecystokinin is secreted, in turn stimulating pancreatic secretions which include, among other substances, proproteases, α-amylase and lipolytic enzymes, as well as DNAase and RNAase (Bakke et al., 2011). The activity of pancreatic enzymes in fish has been studied in relation to the influence of diet composition, food quantity and the natural diet (Budington et al., 1997; Hidalgo et al., 1999; Pérez-Jiménez et al., 2009; Reimer, 1982; Santigosa et al., 2011a; 2011b; 2008; Zambonino-Infante and Cahu, 2007). The activity of the main digestive enzymes, such as proteases, lipase and amylase, may be one of the most important parameters that determine the effectiveness of a given diet, optimizing growth and food utilization (Debnath et al., 2007; Lemieux et al., 1999; Mohanta et al., 2008).

Absorption processes occur throughout the entire intestine by means of diffusion, facilitated transport or active transport (Mailliard et al., 1995). In fish, the presence has been described of at least four Na^+-dependent and Na^+-independent amino acid transport systems (Storelli et al., 1989) and two peptide transporters (Hakim et al., 2009; Sangaletti et al., 2009; Terova et al., 2009). The apical translocation of lipolytic products in fish is not well understood and seems to occur by diffusion or facilitated transport, while D-glucose and D-galactose are transported by SGTL1 (Sala-Rabanal et al., 2004). Santigosa et al. (2011a; 2011b) have described modifications in the absorption pattern after a short starvation period, or as a consequence of low availability of certain amino acids due to fish meal replacement.

Those processes are also affected by biological rhythms, which are driven by endogenous oscillators that affect physiological, behavioral, endocrine and metabolic variables, increasing the probability of success and optimizing energy use (Dardente and Cermakian, 2007; DeCoursey, 2004). The study of feeding behavior in several fish species has revealed that adjusting feeding times to match natural rhythms improves nutritional efficiency, feeding frequency and food conversion efficiency (Bolliet et al., 2001), and can also improve growth performance in many commercially cultured species such as rainbow trout (Boujard et al., 1995; Reddy et al., 1994), European sea bass (Azzaydi et al., 1999) and channel catfish (*Ictalurus punctatus*) (Noeske-Hallin et al., 1985). Food-anticipatory activity confers an adaptative advantage as it may improve food acquisition and utilization (Comperatore and Stephan, 1987).

In order to study the protein-sparing effect by carbohydrates and determine the best time for administration, we analysed the effect on digestive and absorptive processes in sea bream fed a commercial diet of including one feed of a high-digestible carbohydrate diet in the dietary regime, administered in the morning or in the afternoon.

2. Materials and methods

2.1 Diets

Two diets were used, a commercial diet (C) (D-2 EXCEL 1P Skretting, Spain) and a high-digestible carbohydrate diet (CH) (University of Valencia, Spain). C diet contained 48% protein, 20% lipids, 8% ashes and 3.2% fiber, and had as ingredients: fish meal, soybean meal, fish oil, soybean oil and wheat. “EXCEL” diets contained 27% fish meal and 9% fish oil (García et al., 2012). The proximate composition of CH diet was 37% protein, 12.5% lipids, 8.5% ashes, 1.8% fiber and 40% high-digestible carbohydrates (calculated according Venou et al., 2003). The ingredients of CH diet were fish meal, fish oil and gelatinized wheat starch; that was the only source from vegetable origin.

2.2 Fish and sampling

Gilthead sea bream juveniles from Cripesa (Tarragona, Spain) were acclimatized for two weeks to the facilities at the University of Barcelona. After that period, 180 sea bream (± 21g body weight) were randomly distributed in 9 fiberglass tanks (400L) equipped with a semi-closed recirculating system with physical and biological filters, ozone, and continuous aeration with a 35% weekly sea water renewal rate, and maintained at 24ºC with a 12L/12D photoperiod. Water parameters such as temperature, oxygen content, pH, nitrate and nitrite content were recorded daily. The animals were fed twice a day (10:00 am and 17:00 pm) for an 8-week period (November-January).

Three experimental groups were studied in triplicate: commercial diet (C), a high-digestible carbohydrate diet in the morning (CH-M) and a high-digestible carbohydrate diet in the afternoon (CH-A). The daily ration was adjusted to 2.6% of total body weight, 1.6% in the morning and 1.0% in the afternoon. The quantity of the morning and afternoon ration was calculated according to the amount of feed that fish ate during the acclimatization period.

At the end of the 8-week experimental trial, two samples were collected at 5h post-feeding, one after the morning and one after the afternoon meal. Fish were anaesthetized (MS222 0.1 g · L^{-1}), weighed and sacrificed by severing the spinal cord. The SGR was calculated as follows: $((\ln W_{fin}$ (g)- $\ln W_{ini}$ (g))/t)·100, where W_{fin} and W_{ini} were the final and initial weights respectively, and t was the number of feeding days. The digestive tract of eight fish per treatment was isolated, and relative intestinal length (RIL) was measured excluding pyloric caeca, expressed in relation to each animal's weight (Santigosa et al., 2008). Samples were also collected from stomach, pyloric caeca and proximal intestine, including the intestinal content. These were rapidly frozen in liquid nitrogen and maintained at -80ºC until enzymatic analyses. In addition, the intestinal tract of four animals per treatment was isolated and cut lengthwise, washed in an isosmotic saline solution containing 0.1M protease inhibitor (phenyl-methyl-sulfonyl-fluoride) and frozen in liquid nitrogen until nutrient absorption experiments were performed. All fish-handling procedures complied with European guidelines on animal care (Directive 2010/63/EU).

2.3 Acid protease activity

Stomach samples were collected in order to determine acid protease activity according to Alarcón et al. (1998). Briefly, samples were individually homogenized (Politron 2000, Sorvall TC) at 4ºC to a final concentration of 50 mg mL^{-1} in 50mM TrisHCl buffer pH 7.5; this was centrifuged for 15 minutes (1100g, 4ºC. Jouan CR 411) and supernatants were recovered and stored at

-80ºC. For acid protease activity determination, homogenates from stomach were reacted with 50mM GlicineHCl buffer at pH 2 containing 1% bovine hemoglobin at 20ºC for 30 minutes. After that, the reaction was stopped by adding 12% trichloroacetic acid. The samples were kept at 4ºC for an hour and centrifuged (7500 g, 5 min, 4ºC). Individual blanks for each sample were established. Supernatant absorbance was measured at 280nm (UV-1603, Shimadzu). Pepsin from porcine gastric mucosa (Sigma Aldrich, Spain, 3440 U/mg solid) was used as standard and acid protease activity was measured as BAEE units.

2.4 Intestinal pH and digestive enzyme analysis

The pH of intestinal content was measured (Crison, micro pH 2000) in pyloric caeca (PC) and proximal intestine (PI). The pH in PC was 7.08 ± 0.01, and 7.35 ± 0.02 in PI. Intestinal sample homogenization, alkaline protease activity, trypsin and chymotrypsin-like activities and α-amylase were determined using a modification of the procedure described by Santigosa et al. (2008), and lipase was determined following Santigosa et al. (2011a). Alkaline protease activity from intestinal segments was determined as described above, except that the incubation buffer containing 50mM TrisHCl buffer had the pH of the intestinal content and 1% casein. Bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as standard and alkaline protease activity was measured as BAEE units. Trypsin-like and chymotrypsin-like activities were characterized by zymography using 12% polyacrylamide gels (10x10.5x0.1 cm). Samples, a commercial weight marker RPN 800 (Amersham, GE Healthcare, UK, 12000-225000Da) and pure trypsin and albumin as controls, were loaded and electrophoresis was performed at a constant current of 15mA per gel for 150 min (EPS 301 Power Supply, 4ºC). The gels were incubated under agitation in intestinal content pH TrisHCl buffer containing 2% casein (30 min, 4ºC), and collected after 90 min of shaking at room temperature. Gels were washed and stained in a methanol:acetic acid:water solution (40:40:10) with 0.1% BBC R-250 (Coomassie Brilliant Blue R-250). Destaining was carried out using the same solution without colorant. Trypsin-like and chymotrypsin-like activities were characterized using specific inhibition solutions according to the procedure described by Alarcón et al. (1998). Six proteases were detected; three presented trypsin-like activity (90, 60K and 55KDa) and the other ones showed chymotrypsin-like activity (50, 30 and 25 KDa) (Figure 1).

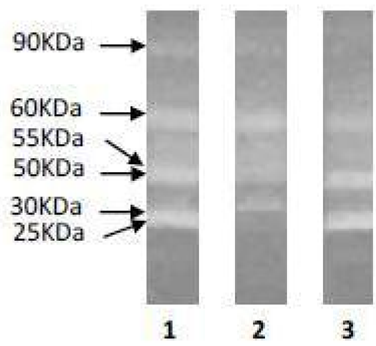

Figure 1. Inhibition zymogram. Lane 1: homogenate (H), Lane 2: H+ZPCK (chymotrypsin inhibitor) and lane 3: H+TLCK (trypsin inhibitor). Figure show the molecular weight of bands with proteolytic activity.

α-Amylase activity was measured using the tracer material, amylose azure, a potato starch covalently labeled with Remazol Brilliant Blue R dye. A 0.5% tracer solution containing soybean trypsin inhibitor (SBTI 0.04 mg mL^{-1}) to prevent proteolysis was reacted with the homogenate for 30 minutes, after which 6% acetic acid was added. The mix was centrifuged (13000 g; 10 min 4ºC) and supernatant absorbance was measured at 595 nm. α-Amylase (Sigma Aldrich, Spain, 66U/mg solid) was used as standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 and 20ºC. PI/PC ratios of protease and α-amylase activities were calculated in order to determine how intestinal transit was affected by the dietary treatment.

For lipase determination, homogenates were mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg L^{-1} colipase (pH 8.3), and the increase in absorbance was recorded at 580nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33944U/mg protein, 22980U/mg solid) was used as a standard. One unit hydrolyses 1.0 micro equivalent of fatty acid from triacetin in 1h at pH 7.4 and 20ºC.

The Bradford method (1976) was used to determine the homogenate protein concentration, using bovine albumin as a standard.

2.5 Nutrient uptake analysis

Brush border membrane vesicles (BBMVs) from whole intestine were obtained as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100) to separate mucosa from the muscular layers. Selective precipitation of basolateral and mitochondrial membranes was achieved through the addition of $MgCl_2$ to a final concentration of 10mM. Subsequent selective centrifugations enabled purification and concentration of apical enterocyte membranes, which were vesiculated (in mM:

300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe. Alkaline phosphatase activity, which has been described as a good marker of BBMVs, was determined following Weiser (1973) in order to determine enrichment in brush border membrane preparations. The capacity to absorb L-lysine, an essential amino acid, L-alanine, a non-essential one, and D-glucose was determined in vesicular suspensions according to the procedure described by Santigosa et al. (2011b). These were mixed with incubation buffer (in mM: 250NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabeled nutrient, 0.01 ^{3}H-nutrient, Osm 320, pH 7.4) for 5 sec. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 320, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22-µm cellulose nitrate filters (Millipore, Bedford MA) previously soaked in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were performed at 20ºC. Radiolabeled nutrients were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).
Changes in vesicular volume, due to diet composition (Santigosa et al. 2011a, 2001b), rendered it necessary to normalize influx values. In order to determine vesicular volume, L-alanine retained inside the vesicles at equilibrium was measured by incubating a BBMV preparation with incubation buffer on ice (Sala-Rabanal et al., 2004). After 90 min, the reaction was stopped and the amount of L-alanine was measured following the procedure describe above.
The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford method (1976).

2.6 Statistical analysis

Significant differences between groups were determined by one-way ANOVA followed by Tukey's post hoc test. Differences between meal times were determined using the Student's *t*-test (P<0.05). The software used was PASW Statistics 17.0 (SPSS Inc., EUA).

3. Results

Digestive enzyme activities were studied at 5h post-feeding after morning and afternoon meals in stomach, pyloric caeca and proximal intestine for each experimental condition.
Figure 2 shows the results for acid protease activity. At 5h post-feeding, the stomach will be empty, indicating that any activity detected will correspond to food-anticipatory activity related to the next feed. Lower acid proteolytic activity was measured for sea bream in the afternoon (26.68 ± 1.89 U pepsin · mg^{-1} prot) than in the morning (36.46 ± 2.13), consistent with the hours until the next meal (11h to the morning feeding and 3h to the afternoon one). No differences

were detected between experimental groups after the afternoon meal, whereas after the morning feed, sea bream fed the C diet in the afternoon showed higher anticipatory activity than those fed the CH diet. Significant differences for CH-M sea bream were observed between morning and afternoon feeding. These results suggest that when CH was included in the dietary regime, the pepsin profile changed depending on the time of administration.

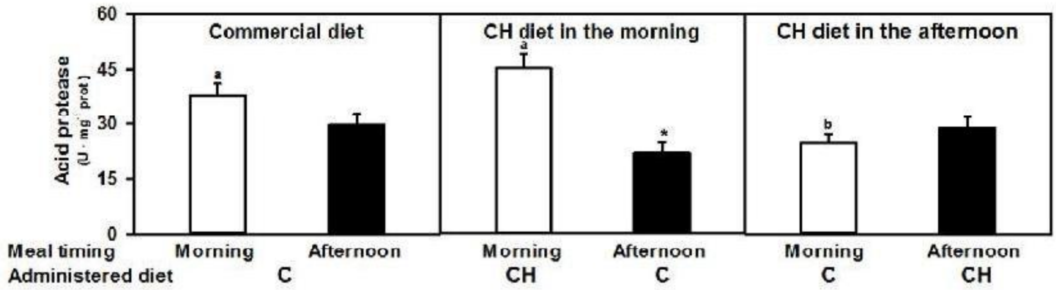

Figure 2. Stomach acid protease activity in the morning (white bars) and in the afternoon (black bars). Values are expressed as the mean ± SEM of 8 fish. Significant differences (*P*< 0.05) between diets are shown by different letters and significant differences between meal times are shown by an asterisk*.

Figure 3 shows alkaline protease, α-amylase and lipase activities measured in PC and PI 5h post-feeding after the morning or the afternoon meals, for each dietary regime. Alkaline protease activity was higher than α-amylase and lipase activities by a factor of 10^3, indicating a limited ability to digest lipids and carbohydrates compared with protein. Fish fed an exclusively commercial diet showed higher pancreatic enzyme activities (alkaline protease, α-amylase and lipase) after the morning meal, consistent with the ration administered (1.6%. in the morning versus 1% in the afternoon). Inclusion of the CH diet in the morning affected pancreatic enzyme activities in the afternoon meal. In this respect, when alkaline protease, α-amylase and lipase activities were measured 5h after the afternoon feed, CH-M sea bream showed significantly higher activities than C fish. Moreover, CH-M animals presented the highest detected protease activity (85.35 ± 13.39 U protease · mg^{-1} protein) of all conditions. However, when the CH diet was administered in the afternoon, no significant differences in digestive enzyme activities measured 5h after the morning feed were found between CH-A and C sea bream. Neither were any significant differences in lipase activity detected between dietary regimes, although higher activity was measured after the morning feed in C animals, whereas when the CH diet was included in the dietary regime, higher activity was detected after the afternoon meal.

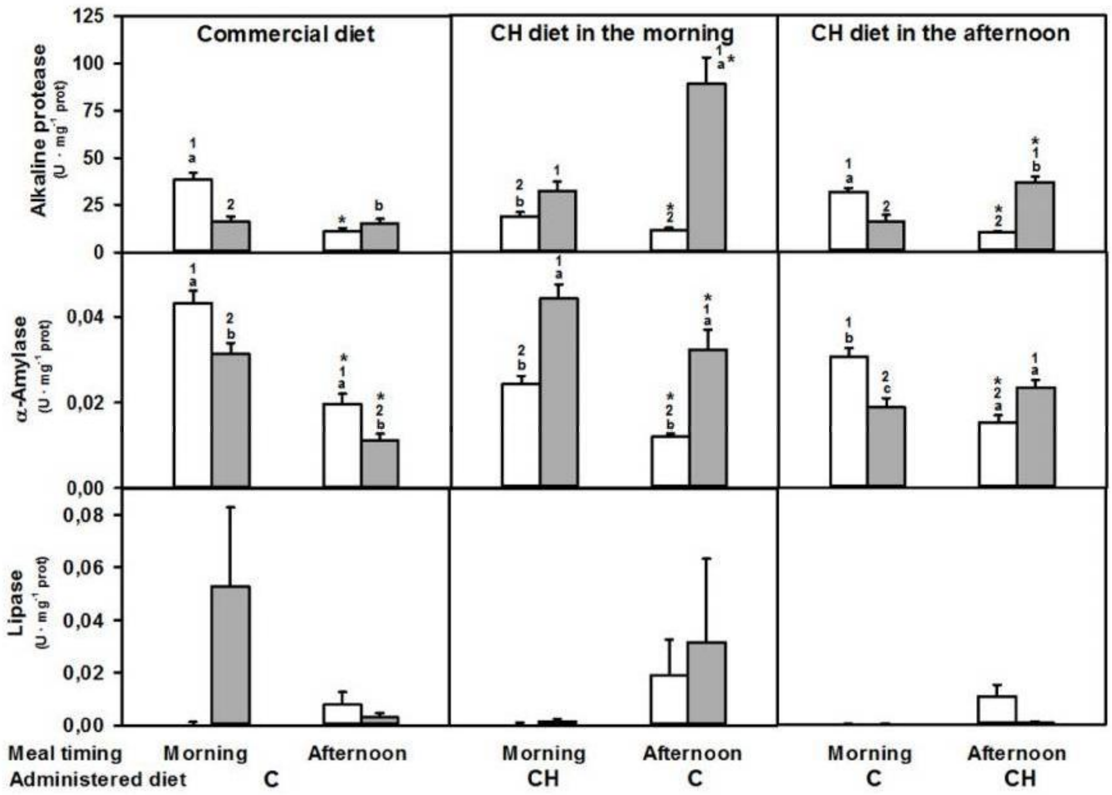

Figure 3. A) Total protease, **B)** α-amylase and **C)** lipase activity in pyloric caeca (white bars) and proximal intestine (grey bars). Values are expressed as the mean ± SEM of 8 fish. Significant differences (*P*< 0.05) between diets are shown by different letters; significant differences between intestinal segments (*P*< 0.05) are shown by different numbers and significant differences between meal times are shown by an asterisk*.

The ratio between proximal intestine and pyloric caeca enzymatic activities provides an indication of the intestinal transit rate; thus, higher activity in the PI versus the PC indicates higher intestinal transit rate. Sea bream fed the commercial diet in the morning showed significantly higher alkaline protease and α-amylase activity in PC than in PI. In contrast, when sea bream were fed the CH diet in the morning, higher activity of both enzymes was detected in PI, indicating an increase in the intestinal transit rate; this increase was also detected when the CH diet was given in the afternoon. C animals showed an increase in intestinal transit rate for protease activity when afternoon versus the morning feeding was compared, whereas no changes in this parameter were detected for α-amylase activity.

After the afternoon feed, C and CH-M sea bream showed significant differences in intestinal transit rate, although they were fed the same diet and ration, and the highest intestinal transit rate was observed when the CH diet was given in the morning. However, when the CH diet was given to fish in the afternoon, the transit rate detected 5h after the morning feed was that expected for the diet and ration administered.

Figure 4 shows model zymograms of PC and PI 5h after feeding for each experimental group. According to the alkaline protease measured, sea bream fed the CH diet in the morning showed little activity in PC (L5) of all the proteases detected compared with the other groups after the morning meal (L1 and L9), while no differences were found between conditions after the afternoon meal (L3, L7 and L11). Changes in PI zymography were also consistent with the alkaline protease measured, increasing in the afternoon when the CH diet was included in the feeding regime in the morning (L8 versus L6).

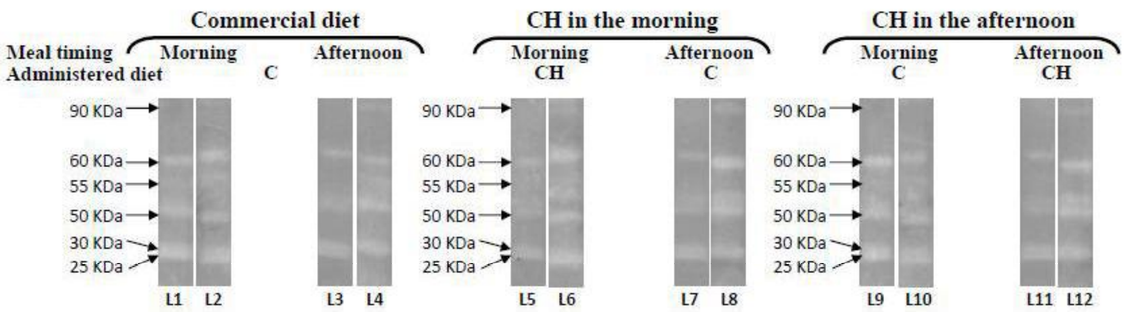

Figure 4. Model zymogram of alkaline proteolytic activity in pyloric caeca extracts, represented in odd numbers, and in proximal intestine, shown in even ones. Figures show the molecular weight of bands with proteolytic activity. All samples were analyzed individually; the Figure shows a representative result.

Nutrient absorption capacity was measured in BBMVs from whole intestine 5h post-feeding (Figure 5). When the CH diet was included in the dietary regime, L-Lys absorption capacity tended to be up-regulated after the morning feed, attaining statistical significance for CH-A animals. No differences were detected between the experimental conditions when L-Lys uptake capacity was analyzed after the afternoon feed. On the other hand, L-Ala and D-Glc absorption capacity was up-regulated when the CH diet was incorporated into the dietary regime of sea bream in the morning. An up-regulation in D-Glc and L-Ala absorption was also detected in animals fed the commercial diet in the afternoon versus morning.

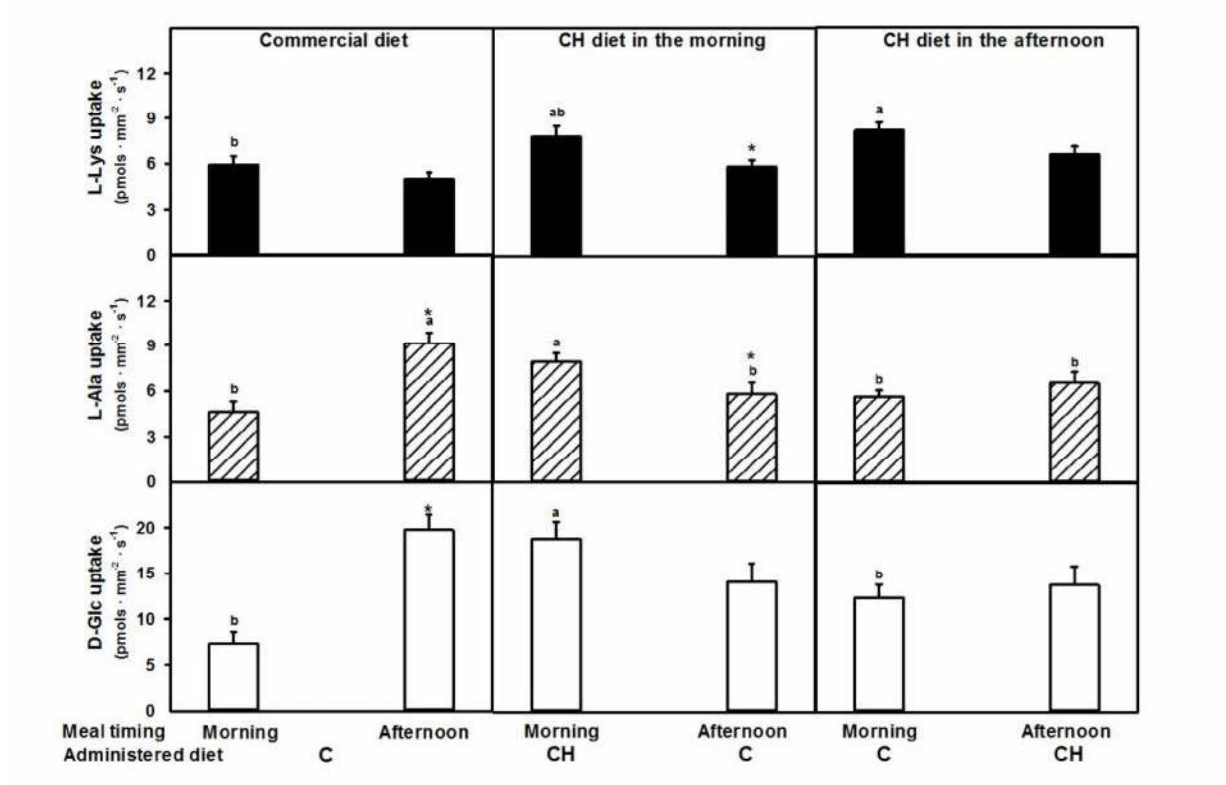

Figure 5. Absorption capacity of L-Lys (black bars), L-Ala (hatched bars) and D-Glc (white bars) in brush border membrane vesicles obtained from whole intestine from sea bream after the morning meal and afternoon meal. Results are expressed as the mean ± SEM of 4 determinations. Different letters indicate significant differences ($P < 0.05$) between diets and significant differences between meal times are shown by an asterisk*.

RIL was measured in order to determine intestinal adaptation to diet, but no differences were found for RIL between experimental conditions (25.6 ± 1.5; 22.6 ± 1.4 and 24.1 ± 2.0 for C, CH-M and CH-A, respectively).

Lastly, no significant differences were found in SGR, but a tendency towards lower growth was detected for CH-A animals (1.28 ± 0.06, 1.28 ± 0.06 and 1.12 ± 0.05 for C, CH-M and CH-A, respectively).

4. Discussion

Fish are capable of regulating their intake to meet energy and nutrient requirements according to their natural feeding rhythms (Boujard and Leatherland, 1992; Kaushik and Médale, 1994). Several studies have been conducted on the effects of daily rhythms on food-anticipatory activity, hormones and feeding frequency in sea bream (López-Olmeda et al., 2009; Sánchez et al., 2009). Furthermore, Madrid (1994) have reported different levels of nutrient assimilation when the meal times of a given diet were changed. Thus, synchronization of the biological rhythms of synthesis, activation or secretion of digestive and/or metabolic enzymes leads to a better diet improvement (Sánchez-Muros et al., 2003). Furthermore, Montoya et al., (2010) have described an increase in digestive enzyme activity some hours before meal time, indicating a strong effect of feeding. Based on these premises, in the present study we sought to determine the best time for carbohydrate administration in order to leverage the protein-sparing effect without compromising nutrient assimilation and/or fish growth.

It has been reported that fish size, growing temperature, ration and ingredients used in diet formulations can affect gastric content evacuation. Thus, Guillaume and Choubert (2001) reviewed that gastric evacuation time increased with fish size and ration and decreased with temperature. When plant-based ingredients are included in the diet, gastric evacuation time is higher, increasing more with legumes than with cereals (Adamidou et al., 2009; Storebakken et al., 1999). Moreover, Venou et al. (2003) have reported that differences in ingredient processing can also modify gastric evacuation time, which is higher for extruded cereals than for raw cereals. It has also been described that the amount of feed present in the stomach determines content pH (Márquez et al., 2012). In their study of sea bream fed replacement diets, García-Meilán et al. (2013) found that at 5h post-feeding, 57% of fish had an empty stomach with a pH close to 6.6, while 43% still had stomach content but with a pH (approximately 4) that was far from optimal for pepsinogen activation (Yúfera et al., 2012). In the present study, similar gastric evacuation at 5h post-feeding was observed in small sea bream fed a smaller ration and similar diet; thus, the acid protease activity measured reflected food-anticipatory activity. Irrespective of the diet administered in the morning, this activity was higher when the C diet was given to fish in the afternoon, indicating an adaptation to diet protein content to improve protein digestion. Lawlor et al. (2005) tested the buffering capacity of several ingredients present in animal diets, and showed that cereals had a lower buffering capacity than plant protein meals, and that these had a lower capacity than fish meal and gluten. Thus, the ingredients used in replacement diets modify feed buffering capacity, which could affect intestinal pH and consequently modulate digestive enzyme activities. In this study, no differences in intestinal content pH were detected between diets even though the plant replacement component was higher in the C than CH diet (over 60% versus 47%); in the C diet, this principally composed a blend of soybean meal, soybean oil, and wheat; whereas in the CH diet, it consisted exclusively of wheat. In both situations, intestinal pH was similar to that reported in literature for this species (García-Meilán et al., 2013), although it was far from optimal for intestinal enzyme performance (Alarcón et al., 1998; Fernández et al., 2001).

The pattern of feeding, 1.6% in the morning and 1% in the afternoon, was established according to the amount of feed that fish ate during the acclimatization period. This determines nutrient delivery to the intestinal lumen, which is the most important stimulus of exocrine pancreatic secretion of digestive enzymes in fish and mammals (Krogdahl and Sundby 1999) and also regulates nutrient absorption capacity (Santigosa et al., 2011b). Thus, in C animals, alkaline protease and α-amylase activities were higher after the morning feed compared with the afternoon one, probably due to the higher amount of protein and starch that fish received in the morning. Consistent with these results, several authors have described an increase in alkaline protease activity as dietary protein increases (Bakke et al., 2011; García-Meilán et al., 2013; Krogdahl et al., 2003; Péres et al., 1998). As regards amylase activity, Krogdahl et al. (2005) found that this was regulated in herbivorous and omnivorous species according to starch intake, whereas this relationship was unclear for carnivorous species. Furthermore, the intestinal transit rate showed that with a smaller ration (1%), there was a higher digestion of protein than starch. Similarly, Buddington et al. (1997) reported than protease activity can be modulated by diet, whereas α-amylase activity seems to be more constitutive. Moreover, low starch diets have been related to a higher capacity to absorb the nutrients D-Glc and L-Ala (García-Meilán et al., 2013; Santigosa et al., 2011b), and we observed an increase in the capacity to absorb both nutrients in C sea bream fed in the afternoon. Differences in diet composition, such as high lipid levels in the C diet or high starch content in the CH diet, may be involved in the differential transit rate found after feeding. In their study of sea bream, Fountoulaki et al. (2005) found that a low transit rate was related to a high lipid content, and Spannhof and Plantikow (1983) detected an increase in the intestinal transit rate of trout when a high amount of starch was included in the diet. We found a differential regulation in the intestinal transit rate of CH-M sea bream after afternoon feeding, associated with an increase in digestive enzyme activities. This increase led to improved intestinal nutrient availability, especially with regard to dietary protein content, in order to compensate for the lower protein content of the CH-M versus C diet (-14.1%). In contrast, CH-A sea bream (-8.8% dietary protein versus C animals) did not show this up-regulation of digestive enzyme activities. Moreover, fish fed the CH diet showed an up-regulation of the capacity to absorb L-Lys, presumably in order to compensate for lower amino acid availability. Up-regulation of the capacity to absorb essential amino acids has also been described in short starvation periods by Santigosa et al. (2011b). We also observed an up-regulation of the capacity to absorb D-Glc and L-Ala when sea bream were fed the CH diet in the morning, a better energy improvement that was not detected in CH-A animals. These

compensatory mechanisms described above enabled CH-M fish to achieve similar growth to C sea bream, indicating a protein-sparing effect when carbohydrates were fed to sea bream in the morning but not when CH were given in the afternoon.
Other possible factors that could affect digestive enzyme activities include anti-nutritional elements present in plant ingredients (Francis et al., 2001; Gatlin et al., 2007, Krogdahl et al., 2010; Turchini et al., 2009) or differences in chyme emulsification due to dietary lipid content (Santigosa et al., 2011a). Moreover, the α-amylase activity measured was higher than that found in other trials (García-Meilán et al., 2013; Santigosa et al., 2008; 2011a), probably due to the high presence of starch in diet. As Enes et al. (2011) have suggested, such carbohydrases saturation could affect starch digestibility as the amount included in the diet is increased. Lipase activity is not affected by high lipid content diets (Santigosa et al., 2011b; García-Meilán et al., 2013) or by high-digestible carbohydrates, as in the present case.
According to García-Meilán et al. (2013), six proteolytic bands were detected in intestinal zymograms. Thus, the three bands with higher molecular weight presented trypsin-like activity (90, 60 and 55 KDa) and the bands with lower molecular weight showed chymotrypsin-like activity (50, 30 and 25KDa). It is known that the pancreatic release of proteolytic enzymes and the trypsin/chymotrypsin ratio were affected by the percentage of dietary protein and also by the plant sources included in diet (García-Meilán et al., 2013, Santigosa et al., 2008 and 2011a). Furthermore, the incorporation of carbohydrates of diet modified the release of trypsin and chymotrypsin by not their ratio.

In conclusion, the inclusion of high-digestible carbohydrates in the diet improved digestion and absorption processes when administered in the morning, leading to a protein-sparing effect that yielded growth comparable to that of fish fed an exclusively commercial diet.

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CAPÍTULO V

Modulation of digestive and absorptive processes with age and/or after a lipid dietary change in gilthead sea bream

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Abstract

During a 19-week trial, sea bream juveniles were fed with two isonitrogenous and isoenergetic diets in which 75% of the fish meal was replaced by plant protein sources and that only differed in the lipid source: fish oil (FO) or vegetable oil (VO). After the growth period, intestinal pH content, digestive enzyme activities, nutrient absorption capacity and final weight were studied in the pre-feeding stage on the FO or VO diet and also after a dietary change at different animal weights (92 g, 147 g, 233 g), both in the short term and at the end of the trial.
Intestinal pH content, alkaline protease, α-amylase and lipase activities and nutrient absorption capacities decreased as the animals grew. Moreover, the VO group tended to have higher alkaline protease and α-amylase activities, whereas lipase activity was higher in the FO group; these changes were significant in small fish. The absorption capacities of essential amino acids were higher than those of non-essential amino acids and D-Glc. Moreover, absorption capacities were higher in small VO animals compared to FO. No changes in growth rate were found at the end of the trial. However, FO animals had a higher growth rate during the first half of the trial after which growth slowed, whereas VO animals grew at a lower but more steady rate throughout the trial.
Small animals were not able to adapt their digestive enzyme activities or nutrient absorption capacities to the new or original feeding condition after a short-term dietary change, whereas large animals generally adapted.
After 9–15 weeks on the FO diet, animals were able to adapt their protease activity and absorption capacity to the new situation; however, α-amylase activity declined. In contrast, after changing to the VO diet animals up-regulated their nutrient absorption capacities and α-amylase activity whereas alkaline protease activity tended to decrease. At the end of the trial final body weight was not affected by dietary treatment.

Keywords: amylase, brush border membrane vesicles, fish, lipase, protease, substitution

1. Introduction

The need for more sustainable and increased fish farming activity, together with the high prices and limited availability of fish oil (FO) (SOFIA, 2014), makes the identification of alternative lipid sources and the search for the best time during the growth period to incorporate these ingredients into the diet important. Some vegetable oils such as soybean oil and rapeseed oil are considered possible alternative lipid sources since they are rich in PUFA, especially linoleic (18:2n-6) and oleic acid (18:1n-9), but devoid of n-3 HUFA (Caballero et al., 2002; Fountoulaki et al., 2009; Izquierdo et al., 2005; Montero et al., 2005; Mourente and Bell, 2006).

Digestion and absorption processes are the key to successful utilization of a given diet and optimization of growth (Debnath et al., 2007; Lemieux et al., 1999; Mohanta et al., 2008). Digestion processes begin in the stomach when HCl converts pepsinogen into pepsin (Wu et al., 2009; Yúfera et al., 2004). As the chyme passes into the pyloric caeca, cholecystokinin is secreted, in turn stimulating the release of pancreatic enzymes including proproteases, α-amylase and lipase (Bakke et al., 2011). In fish, these processes have been studied in relation to the influence of dietary composition, food quantity and the natural diet (Budington et al., 1997; Hidalgo et al., 1999; Pérez-Jiménez et al., 2009; Reimer, 1982; Santigosa et al., 2011a; 2011b; 2008; Zambonino-Infante and Cahu, 2007). The final step of peptide and carbohydrate hydrolysis takes place at the brush border membrane of enterocytes and involves aminopeptidases and disaccharidases respectively, releasing molecules small enough for absorption (Bakke et al., 2011). Absorption processes occur throughout the entire intestine by means of diffusion and Na+-independent and Na+-dependent nutrient transport systems (Hakim et al., 2009; Sala-Rabanal et al., 2004; Sangaletti et al., 2009; Storelli et al., 1989; Terova et al., 2009). It has been reported that both postprandial nutrient availability and dietary nutrient deficit upregulate the capacity for absorption (García-Meilán et al., 2013; Santigosa et al., 2011a). However, only a few studies have compared enzyme digestive activities and nutrient absorption capacities at different developmental stages in fish (Refstie et al., 2006). It is known that the inclusion of vegetable oils (VOs) in the diets of carnivorous fish reduces the intestinal transit rate without comprising pancreatic enzyme activities (Santigosa et al., 2011b) and provokes a modification of the enterocyte membrane composition (Caballero et al., 2003; Sitjà-Bobadilla et al., 2005) that could change intestinal function by reducing lipid and protein digestibility (Francis et al. 2007; Geurden et al., 2009; Santigosa et al., 2011b) and nutrient absorption capacities (Santigosa et al., 2011b).

Most studies in which FO was replace by VOs have shown no or only minimal growth reduction (Roselund et al., 2001; Bell et al., 2003a, b; Tocher et al., 2001; Richard et al., 2006; Izquierdo et al., 2003, 2005; Figuereido-Silva et al., 2005; Martins et al., 2006; Wassef et al., 2007; Menoyo et al., 2004; Benedito-Palos et al., 2007); however Turchini et al. (2009) suggested that this could be due to the use of short feeding trial periods or the utilization of statistical tests with limited experimental power that could be responsible for type II errors. Moreover, the low capacity of marine fish to convert linoleic and linolenic acids into HUFA (Sargent et al., 2002) when vegetable oils are used leads to modifications in the fillet fatty acid profile (Izquierdo et al., 2003, 2005; Montero et al., 2005). In order to ensure growth, dietary improvement and fillet quality, two strategies are routinely used: 1) growing fish using low to moderate vegetable oil inclusion diets and 2) growing the animals first with a high vegetable oil inclusion diet, but covering the essential fatty acid requirements, and then with a fish oil diet. Neither of these two strategies achieve the fillet fatty acid profile of sea bream fed on fish oil as a source of lipid (Fountoulaki et al., 2009; Izquierdo et al., 2003, 2005).

Given that sea bream preferentially retain n-3 HUFA in muscle (Izquierdo et al., 2003; Ibarz et al., 2005), we decided to test another strategy i.e. growing animals first using a FO diet, and secondly with a high VO inclusion diet.

In this study sea bream were fed a FO or VO diet (75% of fish oil replaced with soybean and rapeseed oils) and the effects of these diets on digestive enzyme activities in the pyloric caeca and proximal intestine and nutrient absorption capacities in the distal intestine were determined: during growth on the FO or VO diet and after a dietary change at different animal weights, both short term and at the end of the trial.

2. Materials and methods

2.1. Diets

Two isonitrogenous and isoenergetic diets with the same principal composition were formulated by Skretting (Norway) (Table 1). Both diets had the same meal composition, with 75% of fish meal (FM) replaced by plant protein sources (wheat, wheat gluten and soybean protein concentrate). The diets only differed in the lipid source administered: only fish oil for the FO diet and 75% of fish oil replaced with a blend of soybean and rapeseed oil in the same proportions for the VO diet (Table 1 and 2).

Table 1. Ingredient and principal composition of the experimental diets.

Ingredients (%)	**FO diet**	**VO diet**
Fish meal	20	
Soybean concentrate	15	
Soybean extracted	15	
Wheat gluten	16.4	
Wheat	15.7	
Min Vit	2.0	
Ytrium oxide	0.1	
Fish oil	15.8	3.8
Soybean oil	-	6
Rapeseed oil	-	6

Principal composition: 45.0% Protein, 20.4% Fat, 6.9% Ash.

Table 2. Fatty acid composition of the experimental diets provided by Skretting (Norway).

Fatty Acids (%)	FO diet	VO diet	Fatty Acids (%)	FO diet	VO diet
Sum Saturated	26.1	17.1	**Sum n-6 FA**	9.0	25.7
C14:0	6.4	2.7	**C16:2n-6**	1.0	0.3
C16:0	16.4	11.4	**C18:2n-6**	6.3	24.5
C18:0	2.7	2.3	**C20:4n-6**	0.7	0.3

Fatty Acids (%)	FO diet	VO diet	Fatty Acids (%)	FO diet	VO diet
Sum Monoenes	24.6	33.7	**Sum n-3 FA**	29.0	15.9
C16:1n-7	7.0	2.8	**C18:3n-3**	1.1	4.8
C18:1n-9	10.3	24.0	**C18:4n3**	2.4	1.0
C18:1n-7	2.7	2.6	**C20:4n-3**	0.6	0.2
C20:1	2.1	1.9	**C20:5n-3**	14.5	5.3
C22:1	1.9	1.7	**C22:5n-3**	1.6	0.6
C24:1n-9	0.4	0.3	**C22:6n-3**	8.1	3.7

2.2. Fish and sampling

Gilthead sea bream from a fish farm in southern Spain were acclimatized for 15 days in IMIDA facilities (San Pedro del Pinatar, Murcia, Spain) in 2000 L circular tanks. After acclimatization, 361 sea bream each weighing about 62 g and fitted with a microchip, were randomly distributed into 10 cylindro-conical tanks (850 L) supplied with running sea water. The tanks were equipped with a recirculating system with biological filters, an ultraviolet lamp and a heat pump to control temperature. Water parameters such as temperature, oxygen content, pH, ammonia, nitrate and nitrite content were recorded daily.

During the 19-week trial (May–October), fish were fed the corresponding diet twice a day until visual satiety. Fish were subjected to a 12L/12D photoperiod and a temperature ranging between 23 ºC and 25 ºC.

Each tank followed a different feeding programme (Figure 1): two groups were maintained on a FO (white) or VO (black) diet throughout the trial, three groups started feeding on the VO diet and changed to FO at three different stages of growth (92, 147 and 233 g; 92VOFO, 147VOFO and 233VOFO, respectively), and the last three groups started feeding on the FO diet and then changed to VO (92FOVO, 147FOVO and 233FOVO). Sampling was conducted four times during the growth trial (Figure 1), and at each sampling point, five animals per treatment were anaesthetized in iced water, weighed and sacrificed by severing the spinal cord. Sampling took place in the pre-feeding stage: after one day of fasting and while waiting for the upcoming meal. The digestive tract was isolated and samples of pyloric caeca and proximal intestine, including the intestinal content, were collected and rapidly frozen in liquid nitrogen and maintained at -80ºC until required for enzymatic studies. The distal intestine was cut lengthwise, washed in an isosmotic saline solution containing 0.1 M protease inhibitor (phenyl-methyl-sulphonyl-fluoride) and frozen in liquid nitrogen until further analysis.

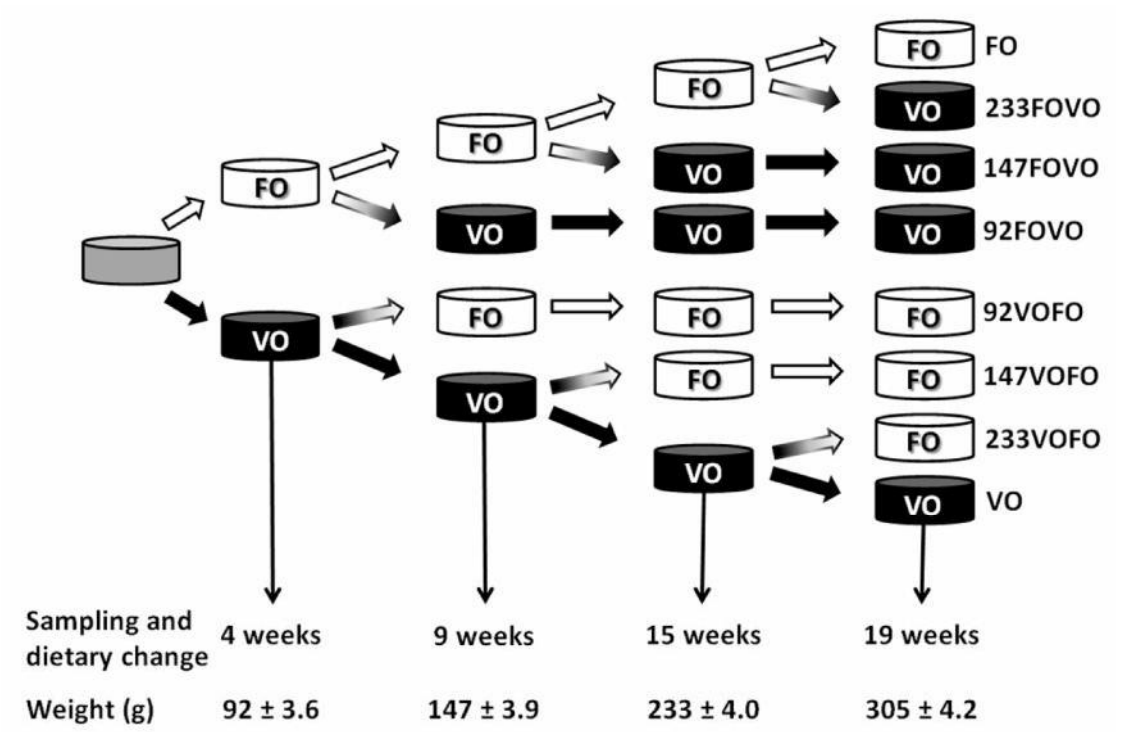

Figure 1. Scheme for the experimental design, sampling and dietary change, and mean weight of the animals at each sampling point.

All fish-handling procedures complied with European guidelines for animal care (Directive 2010/63/EU).

2.3. Intestinal pH content and digestive enzyme analysis

The intestinal pH content of the samples was measured (Crison, Micro pH 2000). Total alkaline protease and α-amylase were measured according to a modification of Santigosa et al. (2008). Lipase determination was carried out following Santigosa et al. (2011a). Briefly, intestinal samples were individually homogenized (Politron 2000, Sorvall TC) at 4ºC in 50 mM TrisHCl buffer pH 7.5 to a final concentration of 250 mg mL^{-1}. Homogenates were centrifuged (1100 g, 15 min, 4ºC, Jouan CR 411) and supernatants were recovered and stored at -80ºC. To measure total alkaline protease activity (TPA), samples were reacted with 50 mM TrisHCl buffer containing 1% casein at the pH of the intestinal content at 20ºC. After 30 min, the reaction was stopped by adding trichloroacetic acid 12%. The samples were maintained at 4ºC for one hour and then centrifuged (7500 g, 5 min, 4ºC). Individual blanks were established for each sample. Supernatant absorbance was measured at 280 nm (UV-1603, Shimadzu). Bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as the standard.

α-Amylase activity was measured using a 0.5% tracer solution containing amylose azure and soybean trypsin inhibitor (SBTI 0.04 mg mL^{-1}). The solution was reacted with samples for 30 minutes, after which 6% acetic acid was added. The mix was centrifuged (13000 g; 10 min, 4ºC) and supernatant absorbance was measured at 595 nm. α-Amylase (Sigma Aldrich, Spain, 66 U/mg solid) was used as the standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 and 20ºC.

For lipase determination, homogenate was mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg L^{-1} colipase (pH 8.3). The increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33944U/mg protein, 22980U/mg solid) was used as a standard. One unit hydrolyzes 1.0 micro equivalent of FA from triacetin in 1 h at pH 7.4 and 20ºC (Santigosa et al., 2011a).

The protein concentration in homogenates was determined by the Bradford method (1976) using bovine serum albumin as a standard.

2.4. Nutrient uptake analysis

Brush border membrane vesicles (BBMVs) from distal intestine (DI) were obtained as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100) to separate mucosa from the muscle layers. A selective basolateral and mitochondrial membrane precipitation was carried out by adding $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugation allowed the purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4{\cdot}7H_20$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe.

The enrichment in brush border membrane preparations was determined by measuring alkaline phosphatase activity following Weiser (1973), a method that has been described as being a good marker of BBMVs.

The capacity to absorb amino acids (L-alanine, L-lysine, L-methionine, L-phenylalanine, L-tryptophan) and D-glucose was determined in vesicular suspensions according to Santigosa et al. (2011b). The suspensions were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled nutrient, 0.01 ^3H-nutrient, Osm 320, pH 7.4) for 5 sec. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, Osm 320, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22-µm cellulose nitrate filters (Millipore, Bedford MA), previously wetted in cold stop buffer. The filters were then washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were made at 20ºC. Radiolabelled nutrients were obtained from Amersham Pharmacia Biotech (Barcelona, Spain).

The vesicular volume of BBMV preparations was measured following Sala-Rabanal et al. (2004) to normalize influx values. Briefly, L-alanine retained inside the vesicles in the equilibrium condition was measured by incubating BBMV preparation with incubation buffer on ice. After 90 min, the reaction was stopped and the amount of L-alanine was measured following the procedure described above.

The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford method (1976).

2.5. Statistical analysis

Differences between dietary treatments during the trial were determined using one-way ANOVA and the Duncan test. The Student *t*-test was used to establish significant differences between dietary treatments and intestinal regions at each sampling point (P<0.05). The software used was SPSS Statistics 22.0 (SPSS, Inc., EUA).

3. **Results**

3.1. Effects of growth on FO or VO diet on digestion and absorption processes

Intestinal pH content (Figure 2), digestive enzyme activities in the pyloric caeca (PC) and proximal intestine (PI) (Figure 3), nutrient absorption capacity of the distal intestine (Figure 4) and growth rate (Table 3) were analysed in sea bream fed FO or VO diets throughout growth. Figure 2 shows that regardless of the diet, the intestinal pH was higher in the PI than in the PC; this difference was greater in smaller fish. Comparison of animals fed FO and VO revealed that pH values were significantly higher in both intestinal regions for VO sea bream at 92 g and 305 g and at 147 g in the PI. The magnitude of increase in intestinal pH between the PI and PC, for VO versus FO animals, was 2 and 3.5 in fish weighing 147 g and 305 g respectively. No significant differences were found between dietary treatments in sea bream weighing 92 g and 233 g.

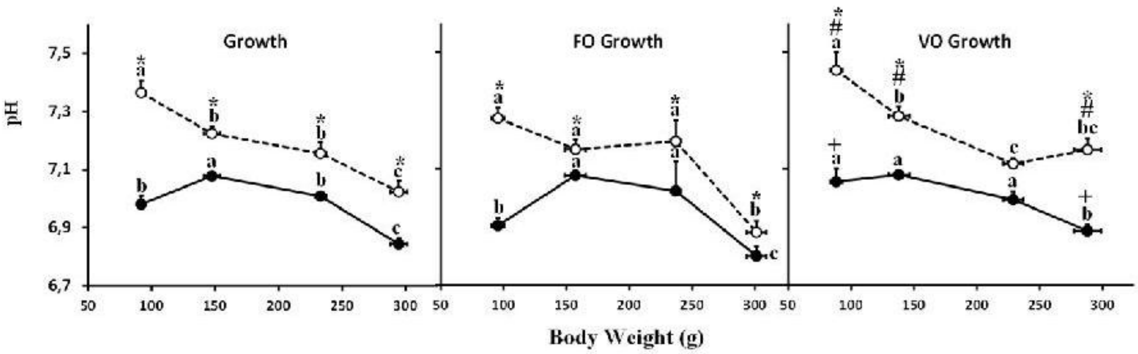

Figure 2. pH of pyloric caeca content (solid line) and proximal intestine content (dashed line) during growth in all animals, and sea bream on the FO diet and VO diet. Values are the mean ± SEM of five fish. Significant differences (P< 0.05) throughout growth are shown by different letters; significant differences between intestinal segments are shown by * and significant differences between dietary conditions (P< 0.05) are shown by + in the PC and # in the PI.

Figure 3 shows that in both intestinal segments, alkaline protease activity was the highest in sea bream weighing 92 g and 147 g; it was also higher in VO animals versus FO: 2.24 ± 0.5 and 4.30 ± 0.7 times higher for 92 g and 147 g fish in the PC and 1.36 ± 0.1 and 1.15 ± 0.1 times higher respectively in the PI. Moreover, in the PC, α-amylase activity was significantly higher in 147 g fish versus animals weighing 92 g, 233 g and 305 g; however, in the PI this activity was significantly higher in 305 g sea bream. Thus, significant differences in α-amylase activity were found between the PC and PI in 147 g fish (PC/PI ratio 2.7 ± 0.4 and 5.6 ± 1.1 for FO and VO animals, respectively) and 305 g fish (PC/PI ratio 0.57 ± 0.1 and 0.78 ± 0.2 for FO and VO animals, respectively).

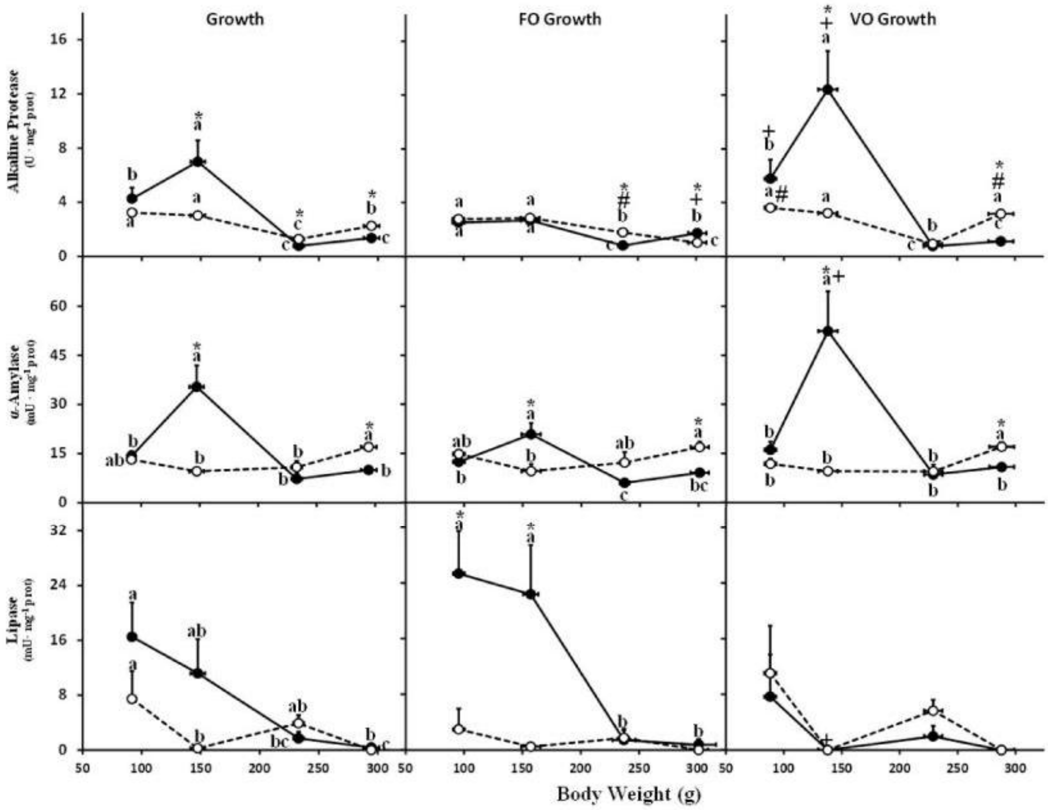

Figure 3. Total protease, lipase and α-amylase activity in pyloric caeca (solid line) and proximal intestine (dash line) during growth in all animals and in sea bream on the FO diet and VO diet. Values are the mean ± SEM of five fish. Significant differences (*P*< 0.05) during growth are shown by different letters; significant differences between intestinal segments are shown by * and significant differences between dietary conditions (*P*< 0.05) are shown by + in the PC and # in the PI.

Significant differences in α-amylase activity between feeding conditions were only detected in 147 g fish in the PC. As for alkaline protease activity, α-amylase activity was higher in 305 g fish than in 233 g fish. Lipase activity was highest in 92 g sea bream (Figure 3). While on the VO animals there were no significant differences in lipase activity either during growth stages or between intestinal segments. In contrast, this enzyme was significantly more active in the PC of FO animals at 92 g and 147 g than at 233 g and 305 g (21.7 ± 4.1 times); activity in the PI was low and no differences were detected during growth.

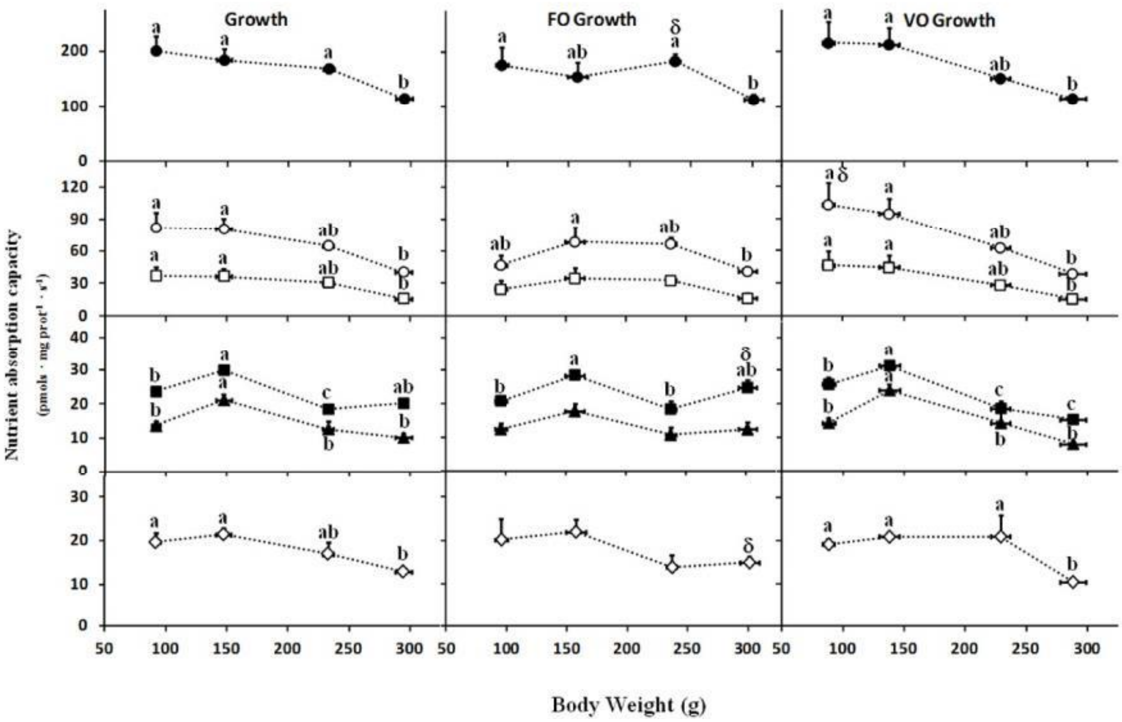

Figure 4. Intravesicular concentration of L-Trp (closed circle); L-Phe (open circle); L-Lys (open square); L-Met (closed square); L-Ala (closed triangle) and D-Glc (open diamond) in brush border membrane vesicles obtained from the DI from sea bream during growth in all animals, and on the FO diet and VO diet. Results are mean ± SEM of six measurements. Different letters show significant differences (P < 0.05) during growth, and significant differences between dietary conditions (*P*< 0.05) are shown by δ.

Amino acid and D-Glc uptake capacities were measured in the distal intestine. Figure 4 shows that the absorption capacities of essential amino acids were higher than those of non-essential amino acids: L-Trp showed the highest absorption capacity, followed by L-Phe, L-Lys, L-Met and finally L-Ala. D-glucose showed a similar absorption capacity to that measured for L-Met and L-Ala. The essential amino acids studied, for both FO and VO animals, showed elevated absorption capacities in 147 g fish, with the lowest values in 305 g fish. Essential amino acid uptake in the VO group and L-Trp uptake in the FO group were similar in 92 g and 147 g animals. Significant differences between FO and VO animals were found for L-Trp and L-Phe influx in 233 g and 92 g animals, respectively. The highest absorption capacities for L-Met and L-Ala were detected in 147 g animals. Both amino acids showed the same uptake profile throughout growth, which was modified according to dietary treatment. In this sense, when data were normalized relative to the absorption capacity of 147 g animals there were no differences between the L-Met and L-Ala uptake profile in 92 g FO fish, while a significantly higher absorption capacity was measured for L-Met (37.6%) in 92 g VO fish. Moreover, the influx of both amino acids tended to be higher in 305 g FO sea bream versus 233 g fish, being the highest for L-Met (34.5%). In contrast, in 305 g VO animals the uptake of both amino acids tended to decrease, the fall in L-Ala uptake being significantly higher (-43.3%). Small animals (92 g and 147 g) showed the highest absorption capacity for D-Glc and 305 g sea bream the lowest. When FO and VO feeding conditions were compared, 233 g FO fish tended to uptake less D-Glc than VO animals while in 305 g FO sea bream uptake was significantly higher than in VO fish.

Table 3 shows growth of sampled animals between 0–9 weeks, 9–15 weeks and 15–19 weeks. Specific growth rate (SGR) decreased significantly throughout growth in FO fish, showing significant differences between the first 9 weeks and 9–15 weeks and then remaining steady until the end of the trial (15–19 weeks). In VO animals tended to be lower but steady until 15 weeks and declined significantly from 15 to 19 weeks.

Table 3. SGR of sampled animals under FO and VO feeding condition at each sampling point (4, 9, 15, 19 weeks). No changes in SGR were found between 0–4 and 4–9 weeks, and data are presented in 0–9-week periods. Different letters show significant differences ($P < 0.05$) during growth.

	0-9 weeks	**9-15 weeks**	**15-19 weeks**
FO	1.46 ± 0.08a	1.00 ± 0.11b	0.82 ± 0.12b
VO	1.25 ± 0.09a	1.23 ± 0.15a	0.79 ± 0.11b

3.2. Short-term effects of dietary change at different animal weights

When fish weighed about 92, 147 and 233 g the diet was changed from VO to FO (92VOFO, 147VOFO and 233VOFO fish) or vice versa (92FOVO, 147FOVO and 233FOVO animals). Samples were taken at least 4 weeks later to study the short-term effects of dietary change on digestive and absorptive processes. Tables 4 and 5 show the data obtained. Smaller animals showed lower digestive adaptation after dietary change. In this sense, both 92VOFO and 92FOVO showed lower alkaline protease and amylase activities, although intestinal pH was maintained in the pyloric caeca and was significantly higher in the proximal intestine (Table 4). The 147 and 233 VOFO and FOVO sea bream showed a different response in digestive enzyme activities to dietary change than smaller animals; and this response differed for TPA and α-amylase.

Table 4. Pre-feeding pH and digestive enzyme activities a short time after dietary change. Values are mean ± SEM of five determinations. Significant differences between intestinal segments are represented by *. Significant differences regarding FO are represented by a or c (a for the original diet and c for the final diet). Significant differences regarding VO are represented by b or d (b for the original diet and d for the final diet). Uppercase letters indicate an increase, lowercase letters indicate a decrease.

Digestive enzymes

Dietary condition	Sampling weight (g)	pH		Alkaline Protease ($U \cdot mg\ prot^{-1}$)		α-Amylase ($mU \cdot mg\ prot^{-1}$)		Lipase ($mU \cdot mg\ prot^{-1}$)	
		Pyloric caeca	Proximal Intestine	Pyloric caeca	Proximal Intestine	Pyloric caeca	Proximal Intestine	Pyloric caeca	Proximal Intestine
FO	147	7.08 ± 0.02	7.17 ± 0.03	2.68 ± 0.15	2.85 ± 0.09	20.8 ± 3.5*	9.6 ± 2.1	22.36 ± 7.07*	0.49 ± 0.49
92VOFO		7.16 ± 0.04	7.48 ± 0.04C,D	0.59 ± 0.11b,c	1.24 ± 0.16b,c*	3.6 ± 0.5b,c	5.6 ± 1.1b	2.03 ± 0.98c	0.56 ± 0.41
92FOVO		7.09 ± 0.02	7.36 ± 0.06A	0.54 ± 0.04a,d	0.59 ± 0.03a,d	1.8 ± 0.4a,d	2.8 ± 0.5a,d	1.26 ± 1.04a	3.46 ± 1.61
VO		7.08 ± 0.02	7.28 ± 0.03	12.40 ± 2.87*	3.21 ± 0.20	52.4 ± 12.2*	9.6 ± 0.7	0.01 ± 0.01	0.01 ± 0.00
FO	233	7.02 ± 0.01	7.19 ± 0.07	0.81 ± 0.08	1.78 ± 0.09*	6.0 ± 0.7	12.2 ± 3.2	1.43 ± 1.43	1.73 ± 1.26
147VOFO		7.03 ± 0.02	7.28 ± 0.04B	1.16 ± 0.12B,C	2.10 ± 0.24B*	8.7 ± 1.4	14.1 ± 2.3	0.97 ± 0.62	6.14 ± 3.21
147FOVO		7.06 ± 0.02D	7.20 ± 0.02D	1.15 ± 0.06A,D	2.22 ± 0.21D*	12.2 ± 1.4A	8.7 ± 1.2	3.51 ± 2.33	9.01 ± 2.24A
VO		6.99 ± 0.03	7.12 ± 0.02	0.76 ± 0.09	0.93 ± 0.12	8.6 ± 1.1	9.5 ± 2.1	1.97 ± 1.49	5.65 ± 1.55
FO	305	6.80 ± 0.03	6.88 ± 0.04	1.73 ± 0.17*	1.02 ± 0.11	9.1 ± 1.0	16.9 ± 1.7*	0.77 ± 0.77	0.00 ± 0.00
233VOFO		6.74 ± 0.03d	6.96 ± 0.05d	2.26 ± 0.17B,C*	1.41 ± 0.11b,C	3.2 ± 0.3b,c	7.8 ± 1.0b,c*	0.00 ± 0.00	0.00 ± 0.00
233FOVO		6.74 ± 0.01d	6.84 ± 0.03d	3.01 ± 0.20A,D*	1.30 ± 0.16d	10.2 ± 1.0	11.7 ± 1.2a,d	0.00 ± 0.00	0.50 ± 0.50
VO		6.89 ± 0.02	7.17 ± 0.04	1.12 ± 0.09	3.16 ± 0.12*	10.9 ± 1.2	17.0 ± 1.4*	0.00 ± 0.00	0.00 ± 0.00

Table 5. Pre-feeding amino acid intravesicular concentration in BBMVs of the DI and weight a short time after dietary change. Values are mean ± SEM of six measurements. Significant differences regarding FO are represented by a or c (a for the original diet and c for the final diet). Significant differences regarding VO are represented by b or d (b for the original diet and d for the final diet). Uppercase letters indicate an increase, lowercase letters indicate a decrease.

Absorption capacity ($pmols \cdot mg^{-1}$ prot $\cdot s^{-1}$)						
Dietary condition	**Sampling weight (g)**	**L-Lys**	**L-Ala**	**D-Glc**	**L-Trp**	**Final Weight (g)**
FO	**147**	34.7 ± 9.6	17.9 ± 2.1	22.0 ± 2.9	154.9 ± 25.6	157.2 ± 8.2
92VOFO		19.6 ± 1.7	10.6 ± 0.9$_{b,c}$	18.4 ± 1.3	201.4 ± 9.0	143.9 ± 8.2
92FOVO		11.4 ± 1.0$_{a,d}$	7.8 ± 0.4$_{a,d}$	8.2 ± 0.4$_{a,d}$	166.9 ± 5.9	147.3 ± 6.9
VO		44.2 ± 11.4	24.0 ± 2.0	20.9 ± 1.0	213.2 ± 29.8	137.84 ± 8.34
FO	**233**	32.6 ± 3.9	10.9 ± 2.3	13.9 ± 2.7	183.3 ± 12.8	237.0 ± 4.2
147VOFO		10.0 ± 1.0$_{b,c}$	7.9 ± 0.6	12.3 ± 0.9	185.7 ± 25.5	235.6 ± 6.4
147FOVO		9.1 ± 1.0$_{a,d}$	10.0 ± 1.7	14.5 ± 1.4	153.4 ± 14.8	223.0 ± 20.8
VO		28.2 ± 2.1	14.3 ± 3.7	21.0 ± 5.0	151.8 ± 5.2	228.9 ± 7.9
FO	**305**	16.3 ± 4.5	12.5 ± 2.1	15.0 ± 1.3	113.5 ± 8.7	301.1 ± 7.8
233VOFO		9.4 ± 1.3$_{b}$	7.7 ± 1.2	8.7 ± 2.2$_{c}$	74.0 ± 8.3$_{b,c}$	312.8 ± 11.4
233FOVO		40.3 ± 12.7	12.3 ± 1.7	12.7 ± 1.1	161.1 ± 15.9$_{A,D}$	298.5 ± 13.9
VO		15.4 ± 2.0	8.1 ± 0.6	10.5 ± 0.9	114.9 ± 3.5	287.9 ± 10.8

In this sense, significantly higher alkaline protease activity was found in the PC and PI for 147VOFO and 147FOVO compared to FO and VO, indicating adaptation by the sea bream to the new condition; however, alkaline protease activity was significantly lower in the PI of 233FOVO fish. In contrast, there was only a tendency for higher α-amylase activity in 147VOFO and 147FOVO fish, and this activity was significantly lower in 233VOFO and 233FOVO animals, not reaching the expected values for the new diet. Lipase activity also differed during growth after short-term dietary change, declining significantly in the PC of 92VOFO and 92FOVO animals compared to FO animals, whereas for 147 and 233 FOVO and VOFO sea bream lipase activity did not differ, except for 147FOVO in PI versus FO.

Table 5 shows that nutrient uptake capacity was modified during growth after short-term dietary change. Thus, L-Lys, L-Ala and D-Glc absorption capacities tended to be lower respect FO and VO fish; being significantly lower for L-Lys in 92FOVO, 147FOVO and 147VOFO, for L-Ala in 92FOVO and 92VOFO and for D-Glc only in the former. In 233VOFO L-Lys absorption was significantly lower than in VO fish and D-Glc uptake was significantly lower than in FO animals, whereas in 233FOVO sea bream, L-Lys, L-Ala and D-Glc uptake was similar to that in FO and VO fish, indicating that animals were able to adapt to the new diet. L-Trp absorption capacity showed another pattern. In small animals, L-Trp absorption capacity decreased as animal weight increased (VO, 92VOFO, 92FOVO and FO). 147VOFO sea bream tended to have the same L-Trp uptake capacity as FO fish, whereas in 147FOVO animals it was similar to VO, indicating that fish were able to adapt their L-Trp absorption capacity to those values expected for the new diet. Finally 233VOFO fish down-regulated their L-Trp absorption capacity without reaching the expected FO values, and had even lower values than VO animals. In contrast, 233FOVO fish had higher L-Trp uptake than expected for the new and the origin diets.

3.3. Effects at the end of the trial of dietary change at different animal weights

Table 6 shows the pH and digestive enzyme activities in the PC and PI and Table 7 shows the nutrient absorption capacities in the DI and final weight at the end of the trial (19 weeks of growth). At this stage, the long-term (92 and 147 fish) and short-term (233 sea bream) effects of dietary change were compared. Alkaline protease activity (Table 6) tended to be higher in the PC than PI in animals fed FO (FO and VOFO) although the intestinal pH tended to be higher in the PI. Moreover, protease activity (Table 6) was higher in VOFO compared to FO sea bream, and was significantly higher in the PI of 92VOFO, in the PC in 147VOFO and for both intestinal segments in 233VOFO fish. In FOVO sea bream, intestinal pH was lower in both intestinal segments compared to VO fish, being significantly lower in 147 and 233FOVO animals.

Table 6. Pre-feeding pH and digestive enzyme activities at the end of the trial. Values are mean ± SEM of five measurements. Significant differences between intestinal segments are represented by *. Significant differences regarding FO are represented by c and significant differences regarding VO are represented by d. Uppercase letters indicate an increase, lowercase letters indicate a decrease.

Digestive enzymes

Dietary condition	Weeks FO/VO	pH		Alkaline Protease ($U \cdot mg\ prot^{-1}$)		α-Amylase ($mU \cdot mg\ prot^{-1}$)		Lipase ($mU \cdot mg\ prot^{-1}$)	
		Pyloric caeca	Proximal Intestine	Pyloric caeca	Proximal Intestine	Pyloric caeca	Proximal Intestine	Pyloric caeca	Proximal Intestine
FO	**19**	6.80 ± 0.03	6.88 ± 0.04	1.73 ± 0.17*	1.02 ± 0.11	9.1 ± 1.0	16.9 ± 1.7*	0.77 ± 0.77	0.00 ± 0.00
92VOFO	**15**	6.82 ± 0.02	7.10 ± 0.10	1.59 ± 0.16	1.49 ± 0.07C	12.4 ± 1.9	11.1 ± 1.1c	1.16 ± 1.16	0.00 ± 0.00
147VOFO	**9**	6.76 ± 0.03	7.05 ± 0.04C	3.41 ± 0.32C*	1.16 ± 0.10	11.3 ± 0.7	9.3 ± 0.9c	1.14 ± 1.14	1.83 ± 1.36
233VOFO	**4**	6.74 ± 0.03	6.96 ± 0.05	2.26 ± 0.17C*	1.41 ± 0.11C	3.2 ± 0.3c	7.8 ± 1.0c*	0.00 ± 0.00	0.00 ± 0.00
VO	**19**	6.89 ± 0.02	7.17 ± 0.04	1.12 ± 0.09	3.16 ± 0.12*	10.9 ± 1.2	17.0± 1.4*	0.00 ± 0.00	0.00 ± 0.00
92FOVO	**15**	6.86 ± 0.03	7.07 ± 0.07	1.26 ± 0.12	1.61 ± 0.11d*	13.4 ± 1.2	20.2 ± 1.8*	0.00 ± 0.00	1.02 ± 1.02
147FOVO	**9**	6.79 ± 0.03d	7.05 ± 0.04d	1.35 ± 0.12	1.45 ± 0.09d	14.5 ± 1.1D	14.3 ± 2.0	0.00 ± 0.00	1.48 ± 1.48
233FOVO	**4**	6.74 ± 0.01d	6.84 ± 0.03d	3.01 ± 0.20D*	1.30 ± 0.16d	10.2 ± 1.0	11.7 ± 1.2d	0.00 ± 0.00	0.50 ± 0.50

Table 7. Pre-feeding amino acid intravesicular concentration in BBMVs of the DI and final weight at the end of the trial. Values are mean ± SEM of six measurements. Significant differences regarding FO are represented by c and significant differences regarding VO are represented by d. Uppercase letters indicate an increase, lowercase letters indicate a decrease.

Absorption capacity ($pmols \cdot mg^{-1}$ $prot \cdot s^{-1}$)						
Dietary condition	**Weeks FO/VO**	**L-Lys**	**L-Ala**	**D-Glc**	**L-Trp**	**Final Weight (g)**
FO	**19**	16.3 ± 4.5	12.5 ± 2.1	15.0 ± 1.3	113.5 ± 8.7	301.1 ± 7.8
92VOFO	**15**	27.2 ± 4.5	22.2 ± 0.8C	15.5 ± 0.8	138.2 ± 0.7C	313.2 ± 18.6
147VOFO	**9**	23.9 ± 1.3	16.1 ± 1.6	10.9 ± 2.1	124.6 ± 6.0	297.8 ± 8.7
233VOFO	**4**	9.4 ± 1.3	7.7 ± 1.2	8.7 ± 2.2c	74.0 ± 8.3c	312.8 ± 11.4
VO	**19**	15.4 ± 2.0	8.1 ± 0.6	10.5 ± 0.9	114.9 ± 3.5	287.9 ± 10.8
92FOVO	**15**	23.6 ± 1.3D	24.0 ± 1.2D	12.1 ± 0.6	142.0 ± 3.8D	308.8 ± 15.8
147FOVO	**9**	19.2 ± 4.8	12.7 ± 1.2D	28.4 ± 2.2D	144.7 ± 11.9D	315.4 ± 12.9
233FOVO	**4**	40.3 ± 12.7	12.3 ± 1.7	12.7 ± 1.1	161.1 ± 15.9D	298.5 ± 13.9

This helped make the total alkaline protease activity significantly lower in the PI of FOVO versus VO sea breams, but not the TPA higher in the PC. α-Amylase activity showed a different pattern to the TPA in response to dietary change. FO and 233VOFO sea bream had significantly higher α-amylase activity in the PI than in the PC. There was a significant reduction in α-amylase activity in 233VOFO fish in the PC and all VOFO animals in the PI with respect to FO sea bream. However, in FOVO animals, α-amylase activity tended to be higher in 92FOVO sea bream and lower in 233FOVO fish. The activity of this enzyme was more negatively affected by short-term dietary change (233VOFO and 233FOVO). No changes in lipase activity were detected at the end of the growth trial in either intestinal region, regardless of dietary change.

Nutrient absorption capacities were also affected by dietary change (Table 7). 92VOFO and 147VOFO animals tended to up-regulate amino acid absorption versus FO fish, with uptake of L-Trp and L-Ala being significantly higher in 92VOFO. In contrast, 233VOFO sea bream tended to down-regulate amino acid uptake, with this being significant for L-Trp. D-Glc influx tended to decrease in 147 and 233VOFO fish, being significantly lower for the latter. In contrast, in FOVO animals, there was a tendency for amino acid and D-Glc absorption capacities to increase regardless of the timing of dietary change. Thus, there was significant up-regulation of amino acid absorption in 92FOVO sea bream; in 147FOVO fish there was a significant increase in L-Trp, L-Ala and D-Glc absorption capacity and for 233FOVO animals only L-Trp influx was significantly higher than in VO sea bream. Final body weight did not differ significantly throughout growth between any of the tested conditions (Table 7).

4. Discussion

The principal composition, the kind of ingredients and their quantities, and the ANFs present in the diet affect digestion and absorption processes (Francis et al., 2001; Fountoulaki et al., 2005; García-Meilán et al., 2013, 2014; Santigosa et al., 2008, 2011a, 2011b), and these determine the efficient use of diet, animal growth and the final product quality (Caballero et al., 2002; Fountoulaki et al., 2009; Izquierdo et al., 2005; Montero et al., 2005; Mourente and Bell, 2006). In sea bream, as in other fish species (Kurokawa and Suzuki, 1995), digestive proenzymes from the hepatopancreas (Elbal and Agulleiro, 1986) and from the islets of exocrine pancreatic tissue that are dispersed in the surrounding adipose tissue (Nejedli and Tlak Gajger, 2013) are released into the PC. In this work all the adipose tissue was removed, so the activity detected in the PC corresponded to the amount of enzyme released, while the activity found in the PI was a result of peristaltic movements.

Some studies on digestive enzyme activity post-prandially and after at least one day of fasting have been done in sea bream (García-Meilán et al., 2013, 2014; Santigosa et al., 2008, 2011a), providing information about adaptation to the administered diet. On the one hand, post-prandial enzyme activities are affected by the ingredients used for dietary formulation and their quantities, how the food supply is managed (force-feeding, satiety, controlled or restricted feeding) and the time after feeding (García-Meilán et al., 2013, 2014; Santigosa et al., 2008, 2011a, Bonaldo et al., 2010). In general, we found a peak of activity 5–6 h post-feeding in sea bream. The effect on the digestion of several ANFs including protease inhibitors, non-starch polysaccharides, etc. present in plant ingredients has also been well documented (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). On the other hand, studies involving at least 24 h of fasting have provided information on animal adaptation/pre-feeding stage in relation to the administered diet (Pérez-Jiménez et al., 2009; Rodiles et al., 2012; Santigosa et al., 2008, 2011b). In this regard, the negative effect of starch and lipids on α-amylase and protease activities has been described (Fountoulaki et al., 2005; Spannhof and Plantikov, 1983). In the present study, sea bream were in the pre-feeding stage, after a day of fasting and waiting for the upcoming meal, leading to the release of digestive enzymes in the PC.

Intestinal pH affects digestive enzyme activities (Alarcón et al., 1998; Fernández et al., 2001; García-Meilán et al., 2013). The pH values measured in the present work in the PI were higher than in the PC and slightly alkaline (between 7 and 7.6) regardless of the administered diet, in accordance with previously reported data for this species (García-Meilán et al., 2013, 2014; Yúfera et al., 2004). The contribution of bicarbonate secretion from the pancreas, pancreatic ducts, bile and intestine is not known (Wilson and Grosell, 2003; Grosell and Genz, 2006) and could change during growth. The results showed that regardless of the diet, intestinal pH decreased as the animal grew. Although there are studies on intestinal pH at different specific weights or growth stages (Deguara et al., 2003; García-Meilán et al., 2013, 2014; Márquez et al., 2012; Nikolopoulou et al., 2011), no studies have been performed over an extended period of growth. The data suggest that the intestinal pH in the pre-feeding stage was higher in VO animals than FO, as previously described for sea bream fed with a by-product of the production of galactomannan from carob seeds (Nikolopoulou et al., 2011). In contrast, when meal of plant origin is included in the diet, the buffering capacities of these ingredients tend to diminish and might affect the buffering capacity of the diet (Lawlor et al., 2005).

It is known that gastric evacuation time increases with fish size (Guillaume and Choubert, 2001), and that this parameter determines pancreatic enzyme secretion (Murashita et al., 2005); this fact could be related to the decline in digestive enzyme activities as the fish grows. In this sense, Kuz'mina et al. (1996) reported a reduction in α-amylase activity in wild adult fish. Studies on nutrient digestibility in sea bream fed the FO diet revealed that 50 g fish showed approximately the same digestibility as 100–130 g fish (Fountoulaki et al., 2005; Venou et al., 2006) probably due to differential enzymes activities associated with their developmental stage, as found in the present study. These results could also be related to the decline in protein requirements described with increasing fish size (Page and Andrews, 1973; Kaushik and Luquet, 1984; Masser et al., 1991).

Physiological adaptations to diet type can also be observed; thus, carnivorous fish have short intestines, whereas herbivorous species have longer intestinal tracts and higher amylase activity; despite this, no differences were detected in trypsin or lipase activities, possibly reflecting the efficient digestion of both nutrients (Dabrowski, 1993; Raubenheimer et al., 2009; German et al., 2010).

The presence of plant oils in diets, such as rapeseed oil, with a lower saturated fatty acid content may accelerate the transit rate compared to that in animals fed FO diets (Tortensen et al., 2005; Karalazos et al., 2007). This higher transit rate increases the length of time without food in the gastrointestinal tract in VO animals, and might involve more enzyme accumulation in the pyloric caeca-pancreatic tissue due to the higher restoration time (Murashita et al., 2005). This higher enzyme accumulation may lead to higher pancreatic enzyme release in the PC in the pre-feeding stage, as found in the present study. In this sense, alkaline protease and α-amylase activities in VO-fed sea bream tended to be higher than in FO fish.

In Atlantic salmon, dietary fatty acid digestibility increases with increasing desaturation and decreases with increasing chain length (Røsjø et al., 2000). In this sense, lipase activity was lower in VO versus FO sea bream, as was described in yellowtail fed soybean meal versus fish meal (Nguyen et al., 2015).

Intestinal length, morphology and histology are strongly influenced by feeding habits and diet (Ferraris and Ahearn, 1984; Buddington et al., 1997) and could influence transport throughout the intestine. In fish, absorption processes take place along the entire intestine, and nutrient transport systems change quantitatively (Sala-Rabanal et al., 2004; Buddington et al., 1997). Some authors found that the proximal region, which receives the highest luminal load of nutrients, has higher uptake capacity and lower affinity than the distal intestine (Ferraris and Ahearn, 1983; Collie, 1985; Buddington and Diamond, 1987; Reshkin and Ahearn, 1987). Despite this, nutrient absorption capacity in the distal region was higher in carnivorous species than in herbivorous and omnivorous species (Ferraris and Ahearn, 1984), and 5–10% of total absorption appeared to take place in this intestinal region (Krogdahl et al., 1999). This increase in nutrient uptake in the distal region seems to compensate for the small relative intestinal length and the low rates of transepithelial transport in the proximal intestine (Ferraris and Ahearn, 1984).

Ferraris and Ahearn (1984) described the lower intestine of fish as a scavenger that removes the remaining nutrients from the diet. Thus, regardless of the diet, 147 g sea bream showed the maximum nutrient uptake; this is probably due to the higher digestive enzymes activities in the anterior intestine that could lead to higher nutrient availability in the distal regions. Rates of sugar transport are lower than those for amino acids to match the composition of the natural diet (Ferraris and Ahearn, 1984; Budddinton et al., 1987) or the administered diet (Santigosa et al., 2011b; Rosas et al., 2008), as shown here for sea bream. In this regard, the intestine of carnivorous fish appears to be unable to adapt its digestive functions to digest carbohydrates (Buddington et al., 1997).

The difference in absorption rates during growth may reflect different nutrient requirements that could be influenced by different stages of development (Kaushik and Médale, 1994; Lupatsch et al., 2001; Sánchez-Muros et al., 2003), with the results showing higher absorption of essential amino acids than non-essential ones during growth.

Hormones play a key role during nutrient uptake in fish, and GH levels may be altered by food availability, time of feeding and diet composition (Mackenzie et al., 1998), thereby leading to increased plasma GH levels during nutritional stress (Duan and Plisetskaya, 1993). Indeed, significantly higher GH levels were detected in sea bream fed a low-protein or high-lipid diet; this could be related to a compensatory protein sparing response (Pérez-Sánchez, 1995, 2000; Martí-Palanca et al., 1996; Company et al., 1999). Moreover, it is known that the developmental stage of the fish can affect its responsiveness to a stressor (Barton, 2002). Thus, high sensitively to stress has been described in dentex during the larval and juvenile periods (Rueda and Martínez, 2001), and it is also known that as fish mature, stress responses can decrease in magnitude, possibly as a result of a reduced threshold for regulatory feedback with the onset of maturity (Pottinger et al., 1995). In agreement with this, the negative effects of dietary change at the digestive level were greater in smaller animals. Moreover, animals weighing below 150 g were not capable of adapting to changing diet in a month; unlike larger fish. Furthermore, the nutritional state of the fish may also affect the magnitude of the stress response (Barton et al., 1986); accordingly, at the end of the experimental period, both VOFO and FOVO animals showed compensation mechanisms to improve digestive and absorptive processes. These were most important in those animals which finished their growth with the VO diet. These compensation mechanisms allowed the FOVO, VOFO and VO fish to achieve similar final weights to the FO animals.

As conclusions, in gilthead sea bream: 1) intestinal pH, alkaline protease, α-amylase and lipase activities and nutrient absorption capacities decreased as the animals grew; 2) digestive and absorptive process can be modulated by the fatty acid profile of the dietary lipid source; 3) small animals were not capable of adapting their digestive enzyme activities and/or nutrient absorption capacities to the new feeding condition after a short-term dietary change, whereas large animals generally adapted; and 4) after a long-term dietary change, animals were capable of adapting their digestive and absorptive capacities, and final body weight was not affected by dietary treatment.

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Discusión

6. Discusión General

Tradicionalmente, se han empleado harinas y aceites de pescado en la formulación de piensos para peces, sobre todo en especies carnívoras, debido a su excelente perfil nutricional. Sin embargo, el crecimiento del sector, la limitada disponibilidad de harinas y aceites de pescado y sus fluctuantes y cada vez más elevados precios, hacen necesaria la sustitución de estas materias primas. Actualmente, los ingredientes de origen vegetal son utilizados de forma rutinaria con este fin debido a su elevada disponibilidad, haciendo posible el crecimiento del sector (SOFIA, 2014). La sustitución de las harinas de pescado conlleva una reducción de los costes de producción, al tratarse de uno de los ingredientes más caros de las dietas y al permitir ahorrar proteína al remplazarla parcialmente por lípidos o almidón (Enes et al., 2011). Sin embargo, hay que considerar para cada especie, la combinación más adecuada de harinas vegetales a utilizar en la formulación de la dieta, teniendo en cuenta los límites de factores antinutricionales que admite la especie en estudio; y también su actividad enzimática digestiva, tanto los niveles basales de proteasa, lipasa y amilasa, como su capacidad de regulación. En otro caso se puede producir una reducción del crecimiento, y por lo tanto disminución de la rentabilidad de la explotación. Además, la inclusión de aceites de origen vegetal proporciona efectos beneficiosos en la calidad del producto final como la reducción de dioxinas y PCBs, pero también la reduce al modificarse el perfil de ácidos grasos del filete (Turchini et al., 2009).

Considerando que los procesos de digestión y absorción son clave para la buena asimilación de los nutrientes aportados por una determinada dieta; afectando a su posterior utilización, al crecimiento del animal y en última instancia la calidad del producto final (Caballero et al., 2002; Izquierdo et al., 2005; Montero et al., 2005; Mourente et al., 2006; Fountoulaki et al., 2009); se han realizado los estudios presentados en esta tesis, que se centran en el estudio de las actividades enzimáticas digestivas, la actividad de las enzimas ligadas a membrana intestinal y la capacidad de captación de nutrientes en situaciones post-prandiales y 24h post-ingesta. Se realizaron experimentos de ahorro proteico por la inclusión de lípido, de carbohidrato o mediante la combinación de ambos nutrientes (Capítulos I, II, III) (Tabla resumen 2, 3, 4); la administración de una determinada dieta considerando los ritmos biológicos del pez (Capítulo IV) (Tabla resumen 5 y 6) y la diferencias al alimentar con aceite de pescado o vegetal en distintas etapas del crecimiento (Capítulo V) (Tabla resumen 5, 6 y 7) en dos especies comerciales muy importantes del área mediterránea, la dorada y la lubina.

Se realizan dos tipos principales de estudios sobre los efectos de la dieta en la actividad enzimática digestiva: post-prandiales o de actividad anticipatoria. En este sentido, la tabla 1 presenta todos los factores a tener en cuenta para la interpretación de los resultados obtenidos. Así, el tiempo de muestreo determina la información que nos proporciona el estudio; dependiendo de la composición principal de la dieta, los ingredientes utilizados para su formulación y su cantidad, y la forma de administración (force-feeding, alimentación a saciedad, controlada o restrictiva).

La actividad enzimática post-prandial permite el estudio del proceso de digestión propiamente dicho, que entre las 5h y 6h post-ingesta se sitúa en ciegos pilóricos e intestino proximal (Santigosa et al., 2011a). En estas condiciones, la actividad enzimática digestiva medida en ciegos pilóricos nos proporciona información de su perfil de liberación; mientras que la actividad medida en intestino proximal también está afectada por la presencia de factores antinutricionales y la capacidad tamponadora de la dieta (Capítulo III, IV). En cambio, a tiempos post-ingesta posteriores (7-8 horas) la digestión tiene lugar en la región proximal mayoritariamente, mientras que la actividad detectada en ciegos pilóricos es anticipatoria a la ingesta de la tarde (Capítulo I). Por otro lado, los estudios realizados a 24h post-ingesta (Capítulo II, V) proporcionan información sobre la actividad anticipatoria del alimento (Pérez-Jiménez et al. 2009; Santigosa et al. 2008; 2011b; Rodiles et al. 2012) para una determinada dieta administrada; que proporcionará una ventaja adaptativa al animal para la adquisición y utilización del alimento (Comperatore y Stephan, 1987). Considerando que los ciegos pilóricos son el lugar de liberación de los enzimas pancreáticos, la actividad detectada en esta región intestinal se corresponderá con la secreción enzimática anticipatoria al alimento; mientras que en intestino proximal la actividad determinada será debida a los movimientos peristálticos.

Las dos especies estudiadas presentan hepatopáncreas (Elbal y Agulleiro, 1986; Nejedli y Tlak Gajger, 2013) e islotes de tejido pancreático exocrino dispersos en el tejido adiposo que rodea los ciegos pilóricos (Kamaci et al., 2010; Nejedli y Tlak Gajger, 2013). Así, cuando se analiza la actividad de los ciegos pilóricos con su grasa adyacente, el tejido pancreático exocrino está presente, y por tanto la actividad determinada se corresponderá con la síntesis de enzimas pancreáticos como describió Murashita et al., (2005) para el medregal del Japón (*Seriola quinqueradiata*).

El estómago presenta valores de pH neutros cuando está vacío, y se acidifica tras la ingestión del alimento (Yúfera et al., 2004; 2012). En este sentido, Guillaume y Choubert (2001), revisaron los factores que afectaban al tiempo de vaciado gástrico y determinaron que el tiempo de evacuación incrementaba de forma proporcional al tamaño del pez y la ración administrada, y disminuía por efecto de la bajada de la temperatura. Además, la inclusión de ingredientes de origen vegetal contribuye a alargar la digestión ácida, siendo mayor el tiempo de evacuación para la inclusión de legumbres frente a cereales (Storebakken et al., 1999; Adamidou et al. 2009). Por otro lado, Venou et al., (2003) demostró que los diferentes sistemas de procesado de estos ingredientes vegetales contribuían a modificar el tiempo de evacuación gástrica; siendo más elevado para cereales extrusionados que crudos.

El pH estomacal medido a 5h post-ingesta era casi neutro (Capítulos III y IV), con algunas excepciones, debidas posiblemente a la diferente composición de ingredientes vegetales de la dieta. Sin embargo, incluso en estos casos (Capítulo III) el pH determinado dista del óptimo de activación descrito para la pepsina en varias especies (Yúfera et al., 2004; Cao et al., 2011; Nikolopoulou et al., 2011), y se consideró que la digestión ácida había finalizado; por este motivo, no se analizó. El pH de estómago no fue medido en otros capítulos, bien porque se muestreó a más de 5h post-ingesta (Capítulo I) o bien debido al previsible incremento del vaciado gástrico debido al menor tamaño de los animales (Capítulos II y IV). Así la actividad proteasa ácida sólo se midió en el capítulo IV como actividad anticipatoria.

Los resultados obtenidos para la actividad pepsina anticipatoria mostraron que está modulada por el contenido proteico de la dieta, siendo mayor cuanto mayor es el porcentaje de inclusión y la ración administrada. Además, se modula también en función del tiempo que exista entre comidas.

Los pHs del contenido intestinal determinados (próximos a 7.0) son ligeramente superiores en intestino proximal respecto a ciegos pilóricos y similares a los descritos para estas especies (Deguara et al. 2003; Yúfera et al. 2004; Nikolopoulou et al. 2011). De acuerdo con los resultados obtenidos por Nikolopoulou et al., (2011), la dorada mostró una regulación al alza del pH del contenido intestinal en aquellas dietas con menor capacidad tampón debido a los ingredientes utilizados y/o su composición (harinas o aceites de origen vegetal y distintos niveles de proteína o lípido en la dieta) (Lawlor et al., 2005). Mientras que en el caso de la lubina (Capítulo II) no se detectaron cambios del pH intestinal ni por efecto de la proteína ni por el contenido lipídico de acuerdo con lo descrito por Nikolopoulou et al., (2011). Se conoce el efecto que tiene el pH del contenido intestinal sobre la actividad enzimática digestiva (Alarcón et al. 1998; Fernández et al., 2001; Nikolopoulou et al., 2011); y la ausencia de cambios significativos en el pH del contenido intestinal entre los diferentes experimentos sugiere que los animales son capaces de regular al alza la secreción de bicarbonato, como mecanismo compensatorio, para contrarrestar los efectos que una dieta con baja capacidad tampón podría causar, manteniendo así la funcionalidad del proceso digestivo, ya que si el pH descendiera las actividades enzimáticas digestivas se verían afectadas negativamente, afectando a la digestión, posterior absorción y al crecimiento de los animales. De hecho, Lawlor et al., (2005) en su estudio sobre la capacidad tampón de los diferentes ingredientes comúnmente utilizados en la formulación de piensos, demostró que los cereales tenían una menor capacidad tampón que las proteínas de origen vegetal; y estas a su vez menor capacidad que el aceite de pescado y el gluten. Por otro lado, la disminución del contenido proteico o de aceite de pescado de la dieta también estaría asociada a una menor capacidad de unir ácido (Lawlor et al., 2005), y por lo tanto esa dieta presentaría una menor capacidad tampón. Demostró también, que los lípidos presentan una baja capacidad para unir ácidos, incluso menor que la del trigo, el cereal utilizado en los experimentos llevados a cabo en esta tesis.

A excepción de los distintos experimentos anteriormente mencionados, realizados en momentos puntuales del crecimiento, no tenemos conocimiento de que exista ningún estudio de pH del contenido intestinal realizado a lo largo del crecimiento. En este sentido, los resultados obtenidos en el capítulo V, muestran que existe una disminución del pH del contenido intestinal a medida que el animal crece, que podría estar relacionada con los cambios en la secreción de bicarbonato a lo largo del crecimiento (Wilson y Grossel, 2003; Grossel y Genz, 2006).

La cantidad de proteína de la dieta, y por lo tanto también la ración administrada, pueden modular la secreción de enzimas pancreáticas (Bakke et al., 2011). En este sentido, se ha detectado un incremento de la actividad proteasa alcalina, principalmente tripsina, en respuesta a niveles crecientes de proteína en dieta para distintas especies carnívoras (Buddington et al., 1997; Peres et al., 1998; Krogdahl et al., 2003; Kofuji et al., 2005), tal y como muestran los resultados obtenidos capítulos I, II, III; y en el capítulo IV la modulación de esta enzima por efecto de la ración. Las actividades proteasa alcalinas digestivas en doradas alimentadas con dietas de elevado contenido lipídico (Capítulo III) son más elevadas en intestino proximal si el porcentaje de proteína en dieta es moderado (41 a 47%) que si es más alto; posiblemente debido a la alteración de la relación tripsina/quimotripsina (Santigosa et al. 2008; Rodiles et al. 2012), que puede llevar a una disminución de la disponibilidad de aminoácidos, di- y tripéptidos y llegar a afectar negativamente al crecimiento (Rodiles et al., 2012). Estos resultados estarían reforzados por el incremento de actividad proteasa anticipatoria detectado en lubinas alimentadas con dietas de alto contenido proteico (Capítulo II). La mayor síntesis detectada en lubinas alimentadas con elevado contenido proteico (55 y 59%) respecto a la dorada podría ser debida a los mayores requerimientos propios de la especie y del momento del desarrollo. Por otro lado, ésta actividad enzimática digestiva es más elevada para las dietas de menor contenido proteico (Capítulo I y III), posiblemente como mecanismo compensatorio; pudiendo llegar a regular al alza incluso la actividad anticipatoria como ocurre para doradas alimentadas con elevados niveles de almidón (Capítulo I).

De acuerdo con varios estudios, los niveles de lípido y de almidón presentes en la dieta pueden afectar negativamente a la actividad proteolítica de los peces (Storebakken et al, 1987; Hidalgo et al., 1999; Fernández et al., 2001; Fountoulaki et al., 2005; Murashita et al., 2008). Así, en los animales alimentados con dietas de bajo contenido proteico y elevado almidón y/o lípido detectamos una menor actividad proteasa que podría afectar a la relación tripsina/quimotripsina (Capítulo III). Sin embargo, un elevado incremento del almidón en dieta modifica las actividades tripsina y quimotripsina, pero no su relación (Capítulo IV). En el caso de lubina, Peres y Oliva-Teles (1999) afirmaron que la digestibilidad de la proteína de la dieta no está afectada por el nivel de lípidos, de acuerdo con los resultados obtenidos en el capítulo II ya que no se encontraron cambios en la relación tripsina/quimotripsina.

La relación entre los niveles de lípido y almidón de la dieta y sus respectivas enzimas parece más complicada, sobre todo en especies carnívoras (Krogdahl y Sundby, 1999; Krogdahl et al., 2005; Morais et al., 2007; Zambonino-Infante y Cahu, 2007; Bogevik et al., 2009).

Se ha descrito que la actividad α-amilasa es más constitutiva que la actividad tripsina para las especies carnívoras (Cahu y Zambonino-Infante, 1995; Buddington et al., 1997; Krogdahl et al., 2005), tal y como muestran los resultados de estos experimentos donde la regulación de la síntesis y secreción de esta enzima tiene menor capacidad de respuesta que la proteasa frente a los cambios en la composición de la dieta. En este sentido la administración de una dieta con elevado contenido de carbohidratos altamente digeribles (Capítulo IV) hizo que su actividad fuera la más elevada de las que hasta ahora habíamos determinado, pero sin presentar diferencias frente a los animales alimentados con la dieta comercial. Por otro lado, un estudio en larvas de lubina pone de manifiesto que la capacidad de regular la secreción de α-amilasa se pierde a medida que crecen (Cahu y Zambonino-Infante, 1995; Peres et al., 1999), como demostró Kuz'mina et al., (1996) en peces salvajes adultos de acuerdo con los resultados obtenidos para la actividad anticipatoria de esta enzima en el Capítulo V. Además, los experimentos realizados en medregal del Japón por Murashita et al., (2008) muestran que esta actividad enzimática no está afectada ni por la proteína, ni por el lípido, ni por el almidón de la dieta cuando se administran estos tres nutrientes de forma independiente. Sin embargo, en dorada el mayor contenido de proteína de la dieta regula al alza su actividad. Los animales alimentados con dietas de bajo contenido de almidón y elevada proteína, muestran un incremento de la síntesis de α-amilasa para lubina (Capítulo II) y de la actividad digestiva para dorada (Capítulo IV); también en el caso de animales alimentados con elevado contenido de fibra (Capítulo I), posiblemente como mecanismo compensatorio debido a la baja disponibilidad de glucosa; y que generalmente se acompaña de un incremento de la capacidad de absorción de este nutriente. Además, la presencia de elevados niveles de lípido y almidón en dieta (Capítulo III), pueden llegar a saturar la actividad carbohidrasa (Spannhof y Plantikow, 1983; Fountoulaki et al., 2005; Enes et al., 2011), pudiendo esta ser la causa de la regulación al alza como mecanismo compensatorio, ya que desaparece cuando los niveles de almidón y lípido en dieta son más moderados (Capítulo I). Por otro lado, elevados niveles de almidón pueden provocar la adsorción de α-amilasa a las moléculas de almidón inhibiendo su actividad (Fountoulaki et al., 2005; Enes et al., 2011), de acuerdo con los resultados obtenidos en el capítulo I.

El contenido lipídico de la dieta puede modular la actividad lipasa (Zambonino-Infante y Cahu, 1999; Buchet et al. 2000; Li et al. 2012; Ma et al. 2014). En este sentido, la actividad del enzima incrementó para las doradas alimentadas con mayor contenido de lípido (Capítulos I y IV) y para aquellas que presentan un mayor contenido de fibra en dieta (Capítulo I), posiblemente intentando compensar el aumento del tránsito y por la unión de ácidos biliares (Storebakken et al., 1998). Sin embargo, ni en estudios previos (Santigosa et al., 2008) ni en el capítulo III, se detectaron cambios en la actividad lipasa por efecto de los lípidos, posiblemente debido a las diferencias en las condiciones de cultivo, la composición de la dieta y la etapa de crecimiento. También se ha descrito un incremento en su actividad por efecto de la proteína en medregal del Japón (Murashita et al., 2008) de acuerdo con los resultados obtenidos para lubinas alimentadas con dietas de alto contenido proteico (Capítulo II). En este caso además, los animales alimentados con dietas poco energéticas presentaron un incremento de la síntesis y la actividad lipasa anticipatoria como mecanismo compensatorio posiblemente debido al elevado requerimiento energético en esa etapa de desarrollo (Kousoulaki et al., 2015), idea que estaría reforzada por la tendencia al incremento detectada también en doradas alimentadas con un 12.5% de lípido (Capítulo IV).

La inclusión de harinas y aceites vegetales está ligada a la presencia de factores antinutricionales (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010), que dependerán del tipo de ingredientes utilizados y su cantidad (Francis et al., 2001; Krogdahl et al., 2010; Santigosa et al., 2008; 2011a; 2011b), y cuya relación determinará los efectos que puedan tener sobre los animales. En ninguno de los casos se determinó su presencia, pero se conoce que la soja, extensamente utilizada, podría afectar a la actividad de las proteasas, ya que presenta inhibidores que se acomplejan con ellas impidiendo su acción (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). En este sentido, la presencia de soja cercana al 15% en las doradas alimentadas con un 50 y un 53% de proteína (Capítulo III) podría causar principalmente una inhibición de la actividad quimotripsina, tal y como refleja la zimografía, aumentando la relación tripsina-quimotripsina, como sugirió (Santigosa et al., 2008; 2011a) y modificando la digestibilidad de la proteína. Sin embargo, la utilización de una dieta comercial que también contenía este ingrediente (Capítulo IV) y la inclusión de SPC60 en las dietas para dorada (Capítulo I) no modificaron la relación tripsina/quimotripsina; posiblemente porque no alcanzaban el 15% de inclusión en ninguno de los casos y no presentaron efectos negativos ni a nivel intestinal ni en crecimiento (Van der Ingh et al., 1991; Kaushik et al., 1995). Los polisacáridos distintos del almidón (NSPs), como la celulosa, también afectan negativamente a la actividad enzimática digestiva, inhibiendo su acción e uniéndose inespecíficamente a ácidos biliares (Storebakken et al. 1998; Francis et al. 2001). Incrementan además la velocidad de tránsito atrapando agua y aumentando la viscosidad del quimo (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010), disminuyendo así el tiempo de actuación de las enzimas y como consecuencia afectando a la posterior absorción de nutrientes (Francis et al., 2001; Krogdahl et al., 2005). Sus efectos negativos han sido descritos en especies como la trucha (Storebakken et al., 1985) y el salmón del Atlántico (Aslaksen et al., 2007). Las doradas alimentadas con dietas de moderado y elevado contenido de celulosa (7.7 y 17.1%) (Capítulo I), presentan una serie de mecanismos compensatorios tanto a nivel digestivo, incremento de la actividad α-amilasa y lipasa, como absortivo, mayor captación de L-Ala y D-Glc; que han permitido a los animales mantener el mismo crecimiento que aquellos cuya dieta carecía de fibra; a pesar que su actividad proteolítica era menor. Se han obtenido resultados similares en bacalao, donde niveles de inclusión de celulosa en dieta del 18% no han tenido efectos negativos sobre el crecimiento (Lekva et al. 2010) y se han relacionado con los elevados niveles de quitina que presenta su dieta natural; ya que la quitinasa podría actuar sobre los enlaces β-1, 4-glucosídicos de la celulosa. En dorada podríamos encontrarnos una situación similar, considerando que su dieta natural se basa principalmente en moluscos, crustáceos y pequeños peces (FAO, 2014).

La presencia de fibra no es el único factor capaz de modificar el tránsito intestinal; las proporciones que presenten los componentes principales de la dieta (proteína, lípido y almidón) también pueden provocar alteraciones de este ritmo; que como consecuencia modificaran el tiempo en el que tengan lugar los procesos digestivos y absortivos y por tanto afectaran a la disponibilidad de nutrientes. Se han detectado incrementos del tránsito intestinal debido a la inclusión de almidón en dieta (Spannhof y Plantikow, 1983). Así, doradas alimentadas con una dieta con un 40% de carbohidratos altamente digeribles presentan un incremento del tránsito al compararlas con peces alimentados con una dieta comercial con mayor porcentaje de lípido y proteína en dieta (Capítulo IV); lo mismo ocurre en animales alimentados con elevados porcentajes de almidón en dieta (29 y 33% versus 22%) y con niveles decrecientes de proteína en dieta (42 y 40% versus 46%) (Capítulo I); de hecho, ambos nutrientes podrían estar implicados en el mayor tránsito detectado. Los resultados obtenidos en el capítulo III, apoyarían esta hipótesis, ya que las doradas alimentadas con mayor contenido proteico (P_{50} y P_{53}) presentan un tránsito más lento que las alimentadas con porcentajes de proteína intermedios (P_{41}, P_{44} y P_{47}). Tanto en el capítulo I, como en el III los animales alimentados con un mayor contenido lípidico mostraron un menor tránsito intestinal coincidiendo con lo descrito por Fountoulaki et al., (2005).

Se ha demostrado que los aceites vegetales con bajo contenido de ácidos grasos saturados aceleran el tránsito intestinal respecto a los aceites de pescado (Tortensen et al., 2005; Karalazos et al., 2007). En este sentido, en el capítulo V, se detectó una tendencia al incremento en la secreción de proteasa y amilasa anticipatorias tras más de un día de ayuno para los animales alimentados con aceite vegetal (colza y soja al 50%) frente a los alimentados con aceite de pescado. Este incremento se podría relacionar con un mayor tiempo para la recuperación del nivel de enzimas digestivas previo al alimento de acuerdo con lo sugerido por Murashita et al., (2005). En experimentos previos de doble sustitución (Santigosa et al., 2011a) las doradas mostraron un enlentecimiento del tránsito con un 66% de sustitución, efecto posiblemente debido a la utilización de aceite de palma que contiene un 48% de ácidos grasos saturados frente al 14.2% de la soja y el 4.6% de la colza (Turchini et al., 2009).

Las enzimas ligadas a membrana están implicadas en la última fase de la digestión (Klein y Cohn, 1998) y su actividad está modulada por la presencia de nutrientes en el lumen intestinal (Kimura et al., 1978; Cézard et al., 1983; Kuperman y Kuz'mina, 1994). La actividad fosfatasa alcalina presentó una regulación al alza para las doradas alimentadas con elevado contenido lipídico (Capítulo I) de acuerdo con lo descrito por diversos autores (Stenson et al., 1989; Gawlicka et al., 2002; Krogdahl et al., 2003; Grewal y Mahmood, 2004; Mozes et al., 2004;). Se detectó también esta regulación en los peces alimentados con bajo contenido proteico y elevado almidón; sugiriendo que puede tratarse de un mecanismo compensatorio que podría estar involucrado en la absorción de lípido, tal y como se ha sugerido en mamíferos (Narisawa et al., 2003) y en bivalvos (Zhang et al., 1996). La leucina aminopeptidasa no presentó ningún tipo de regulación por efecto de la proteína de la dieta, de acuerdo con lo descrito para tilapia por Hakim et al., (2006); y se vio afectada negativamente por el elevado contenido lipídico de la dieta, pudiéndose relacionar con la menor digestibilidad de proteína provocada por la presencia de lípido (Fountoulaki et al., 2005).

La longitud, la morfología y la histología intestinal están fuertemente influenciadas por la dieta y los hábitos alimentarios (Ferraris y Ahearn, 1984; Buddington et al., 1997) y pueden afectar a los sistemas de transporte que se encuentran a lo largo de todo el intestino (Buddington et al., 1997; Sala-Rabanal et al., 2004). Además, la presencia de proteína de origen vegetal en la dieta puede llevar a un incremento de la longitud intestinal relativa (Santigosa et al., 2008). Varios autores han demostrado que la región proximal, donde se recibe la mayor cantidad de nutrientes, es la que presenta mayor capacidad de captación (Ferraris y Ahearn, 1983; Collie, 1985; Buddington y Diamond, 1987; Reskin y Ahearn, 1987); mientras que a nivel distal tiene lugar entre el 5-10% de la absorción total para especies carnívoras (Krogdahl et al., 1999). Así, la modulación de la capacidad de transporte de aminoácidos y de glucosa en ciegos pilóricos, intestino proximal e intestino distal en respuesta a la composición de la dieta para compensar un déficit nutricional (Santigosa et al., 2011a; b) o en periodos cortos de ayuno (Golovanova, 1992; Santigosa et al., 2011b) favorece la utilización del alimento, asegurando el crecimiento y ahorrando proteína siempre cuando sea posible.

Las regulaciones al alza o a la baja en la capacidad de captación son detectables tanto si se trabaja con el intestino íntegro como si se estudian las regiones intestinales por separado. El estudio a partir de intestino íntegro nos estaría aportando una visión mucho más fisiológica de lo que realmente está ocurriendo a nivel intestinal (Capítulos III y IV); mientras que los cambios a nivel distal (estudiados en los Capítulos I, II y V), pondrían de manifiesto los mecanismos compensatorios utilizados por el animal para intentar captar todas aquellos nutrientes que le sean necesarios (Ferraris y Ahearn, 1984). La capacidad de captación de aminoácidos esenciales es superior a la de no-esenciales y a la captación de glucosa, de acuerdo con la composición de la dieta administrada (Rosas et al., 2008; Santigosa et al., 2011b) y la baja capacidad de las especies carnívoras de peces para digerir los carbohidratos (Buddington et al., 1997). De hecho, un déficit de uno de estos aminoácidos esenciales, por ejemplo la L-Lys puede llevar a un menor crecimiento (Tibaldi and Lanari, 1991; Cheng et al., 2003; Rathore et al., 2010; Sánchez-Lozano et al., 2011; Zhou et al., 2011).

Se detectó una regulación al alza de la capacidad de absorción de lisina en aquellos animales alimentados con dietas con bajo contenido de proteína (del 35 al 44% en el capítulo III, y del 37% en el capítulo IV), posiblemente como mecanismo compensatorio por déficit de aminoácidos esenciales en periodos de ayuno cortos (Santigosa et al., 2001b); mientras que para la captación de L-Phe se detectó en los animales alimentados con las dietas extremas y la L-Leu no se modificó (Capítulo III). En el capítulo I, la capacidad de captación de L-Lys no está afectada por el contenido de almidón o celulosa de la dieta siempre y cuando la cantidad de lípido sea baja. Sin embargo, el incremento de éste último, implica la regulación al alza de la capacidad de captación, posiblemente como mecanismo compensatorio en respuesta a la disminución de la digestibilidad de proteína (Fountoulaki et al., 2005). Ésta hipótesis se ve reforzada por el incremento de actividad proteasa para estos animales. Este mismo mecanismo compensatorio se detectó en lubinas alimentadas con dietas de elevado contenido proteico (59%) (Capítulo II), que podría deberse a que el proceso digestivo es más largo y se desplaza hacia regiones más distales, de acuerdo con Santigosa et al., (2011b); además también se detecta un incremento tanto de la síntesis como de la actividad anticipatoria de proteasas para estos peces. La captación de L-Met se vio incrementada en lubinas alimentadas con dietas isoproteicas y con elevada presencia de lípido (Capítulo II). Por otro lado, se ha detectado un mecanismo compensatorio posiblemente relacionado con la obtención de energía. En este sentido, en dietas con altos niveles de proteína y generalmente bajos contenidos en almidón se ha detectado una regulación al alza de la capacidad de captación de la D-Glc y L-Ala, de acuerdo con los resultados obtenidos por Santigosa et al., (2011b) para dietas con elevados porcentajes de sustitución; que puede ir o no acompañado, como ya se ha comentado antes, de una regulación al alza de la actividad α-amilasa aumentando así la disponibilidad de glucosa para su absorción. Éste mismo mecanismo también se detectó en animales alimentados con dietas de elevados contenidos de almidón (40%) y bajos de proteína (37%) (Capítulo IV), puede que con la misma finalidad que el caso anterior, la obtención de energía.

Además de los mecanismos de regulación a nivel digestivo y absortivo, en situaciones de necesidad los animales son capaces de incrementar su ingesta voluntaria, como ocurre en doradas alimentadas con bajos contenidos de proteína y elevados contenidos de lípido y almidón en dieta (Capítulo III). También se ha descrito la situación contraria, una disminución de la ingesta voluntaria en los animales alimentados con dietas de elevados contenidos de almidón (33%), cuyo efecto se refleja en el mayor FCR y menor crecimiento (Capítulo I). En el caso de las lubinas (Capítulo II), el mejor crecimiento se alcanzó para los peces alimentados con un 50% de proteína, siendo éstos los que menos mecanismos compensatorios necesitaban activar para mantener su crecimiento; mientras que la alimentación con dietas isoproteicas de diferente contenido lipídico no afectó al crecimiento.

El capítulo IV demuestra que la dieta administrada se digiere de forma diferencial en función del momento de su administración, de acuerdo con lo descrito por Madrid (1994), permitiendo que las doradas alimentadas con la dieta rica en carbohidratos alcancen el mismo crecimiento que las alimentadas con la dieta comercial presentando un claro efecto sparing de proteína por carbohidrato, siempre y cuando se administre por la mañana; ya que su administración por la tarde afecta negativamente al crecimiento.

Siguiendo con esta línea, los efectos de la estacionalidad estudiados en lubina (Capítulo II) mostraron que los animales presentan un mayor crecimiento en primavera ya que la síntesis de proteasas es significativamente superior. Estos cambios estacionales en la secreción de enzimas también se detectaron en otras especies (Hofer, 1979; McLeese y Stevens 1982; Gelman et al., 1984; Einarsson et al., 1997; Kofuji et al., 2005) y se relacionaron con la menor velocidad del proceso digestivo debido a la baja temperatura del agua en los meses más fríos. Sin embargo, las condiciones de cultivo de las lubinas fueron las mismas en primavera que en otoño-invierno lo que pone de manifiesto la existencia de un reloj biológico interno (Sánchez-Vázquez et al., 1995a, b; 1998).

Por lo tanto, si la dieta administrada y los ritmos biológicos se sincronizan, los animales son capaces de aprovechar muchísimo mejor el alimento proporcionado (Sanchez-Muros et al., 2003), mediante la modulación de los procesos digestivos y absortivos, ya que además son capaces de regular su ingesta para cubrir sus requerimientos energéticos y nutricionales (Boujard y Leatherland, 1992; Kaushik y Médale, 1994).

Como hemos comentado previamente, el tiempo de vaciado gástrico incrementa con la talla del animal (Guillaume y Choubert, 2001) y la llegada del alimento a ciegos pilóricos determina la liberación de enzimas pancreáticas (Murashita et al., 2005); por lo tanto podría relacionarse con una menor actividad enzimática digestiva y absortiva a medida que el animal crece (Capítulo V); como se describió para α-amilasa (Kuz'mina et al., 1996). Estos resultados también pueden relacionarse con el menor requerimiento proteico al aumentar el tamaño del pez (Page y Andrews, 1973; Kaushik y Luquet, 1984; Masser et al., 1991). Además, muestra que los procesos digestivos y absortivos están modulados por el perfil de ácidos grasos de la dieta administrada, siendo superiores para los animales alimentados con la dieta VO pudiendo relacionarse con las diferencias existentes en los requerimientos del animal en función del momento del desarrollo (Kaushik y Médale, 1994; Lupatsch et al., 2001; Sánchez-Muros et al., 2003); aunque no podemos descartar que sea también debido al incremento del tránsito como hemos comentado anteriormente.

Por otro lado, el estrés que comporta un cambio de dieta a corto plazo en animales pequeños (92g) (Rueda y Martínez, 2001) frente a los de mayor tamaño (Pottinger et al., 1995) impide que los primeros se adapten a la nueva situación, mientras que los segundos generalmente sí se adaptan (capítulo V). El cambio de dieta a largo plazo (Capítulo V) activó más mecanismos compensatorios a nivel digestivo y absortivo en aquellos animales que pasaban a alimentarse con la dieta VO; sin embargo no se detectaron diferencias en el crecimiento respecto a las doradas alimentadas durante todo el experimento con FO.

Finalmente, dorada y lubina son capaces de modular sus procesos digestivos y absortivos para preservar su crecimiento cuando son alimentadas con dietas de sustitución de la proteína, tanto por proteínas vegetales como por lípidos o almidón, siempre y cuando la dieta cubra los requerimientos y los factores antinutricionales que presente sean tolerados por el animal. El presente trabajo describe tres niveles de regulación de los procesos digestivos y absortivos: 1) La modulación de la actividad digestiva intestinal y de la capacidad de absorción de nutrientes, 2) la regulación al alza de la síntesis y secreción de enzimas pancreáticos y 3) el incremento de la ingesta voluntaria. En dorada, la digestión y absorción de una dieta rica en carbohidratos altamente digeribles es mejor cuando se administra por la mañana, logrando un efecto sparing que permite un crecimiento comparable al obtenido con una dieta comercial. La sustitución de un alto porcentaje de aceite de pescado por aceite vegetal modula la actividad anticipatoria digestiva y la capacidad de absorción. Además, el cambio de dieta de FO a VO o viceversa afecta en mayor grado a los animales pequeños.

Tabla 1. Resumen de Material y Métodos

		Capítulo I			Capítulo II		Capítulo III	Capítulo IV	
	Experimento	FxS	LxS	PxS	Px(L y S)	Ratio Prot/Lip	Ratio Prot/Energía	C	
DIETAS	**Composición Principal**	$L_{17}P_{46}$ S_5 S_{14} S_{22}	P_{46} S_5L_{23} $S_{11}L_{20}$ $S_{17}L_{19}$ $S_{22}L_{17}$	L_{17} $S_{22}P_{46}$ $S_{29}P_{42}$ $S_{33}P_{40}$	P40 $P_{40}L_{21}S_{18}$ P45 $P_{45}L_{19}S_{15}$ P50 $P_{50}L_{18}S_{11}$ P55 $P_{55}L_{17}S_8$ P59 $P_{59}L_{16}S_7$	P_{52} L12 $L_{12}S_{13}$ L14 $L_{14}S_{12}$ L16 $L_{16}S_{10}$ L18 $L_{18}S_9$ L20 $L_{20}S_8$	P35 $P_{35}L_{27}S_{21}$ P38 $P_{38}L_{24}S_{19}$ P41 $P_{41}L_{23}S_{17}$ P44 $P_{44}L_{22}S_{15}$ P47 $P_{47}L_{21}S_{14}$ P50 $P_{50}L_{20}S_{12}$ P53 $P_{53}L_{19}S_{10}$	$P_{48}L_{20}$	P
	% Sustitución de harinas	65			38		50	60	
	% Sustitución de aceites	0			0		0	11	
	Materias primas de origen vegetal utilizadas en la formulación	Gluten de trigo y maíz			Gluten de trigo		Gluten de trigo		
		SPC 60%			Concentrado de soja	SPC 60%	SPC	Harina de soja	
		Almidón de trigo			Trigo		Harina de trigo	Trigo	Alr
								Aceite de soja	
		Celulosa							
CONDICIONES EXPERIMENTALESS	**Período del año**	Agosto-Noviembre			Abril-Junio	Diciembre-Enero	Mayo-Julio	Noviembre-En	
	Peso inicial (g)	115			5		70	21	
	Duración (semanas)	14			8		12	8	
	Tiempo muestreo post-ingesta	7h		24h	24h		5h / 24h	5h	
ANÁLISIS	**Zona intestinal estudiada**	CP	IP	IP	CP / IP	ID	CP / IP / Entero	E / CP / IP	E
	Enzimas de origen pancreático	X			X		X	X	
	Enzimas de Membrana			X	—		—	—	
	Capacidad de absorción			X		X	X		

F: fibra; S: almidón; L: lípido; P: proteína; C: dieta comercial; CH: dieta rica en carbohidratos altamente digeribles; FO: dieta con
estómago; CP: ciegos pilóricos; IP: intestino proximal; ID: intestino distal; Entero: intestino íntegro.

Tabla 2. Efectos de las dietas de sustitución sobre la actividad digestiva. Resumen de resultados.

		Capítulo I						**Capítulo II**		
Tiempo post-ingesta		7h						24h		
Región intestinal		CP			IP			CP y páncreas exocrino (difuso)		IP
Tipo de actividad		Anticipatoria			Digestiva			Síntesis + anticipatoria		Anticipato
Experimento		FxS	LxS	PxS	FxS	LxS	PxS	Px(LyS)	Prot/Lip	Px(LyS)
APT	+ –	S_{22} S_{14} S_{5}	S_{5} S_{11} S_{17} S_{22}	S_{33} S_{29} S_{22}	S_{22} S_{5} S_{14}	S_{5} S_{11} S_{17} S_{22}	S_{22} S_{33} S_{29}	P_{55} P_{59} P_{40} P_{45} P_{50}	L_{12} L_{14} L_{16} L_{18} L_{20}	P_{59} P_{40} P_{55} P_{45} P_{50}
α-amilasa	+ –	NS	NS	S_{22} S_{29} S_{33}	S_{5} S_{14} S_{22}	NS	NS	P_{59} P_{50} P_{40} P_{45} P_{55}	NS	P_{40} P_{45} P_{55} P_{59} P_{50}
Lipasa	+ –	NS	NS	NS	S_{5} S_{14} S_{22}	S_{5} S_{11} S_{17} S_{22}	NS	P_{50} P_{55} P_{59} P_{40} P_{45}	L_{12} L_{14} L_{16} L_{18} L_{20}	P_{40} P_{45} P_{59} P_{55} P_{50}
Aumento de la relación T/Q		-----------			S_{5} S_{14}	NS	NS	NS	NS	-------

Las cajas agrupan condiciones que no presentan diferencias significativas entre sí, y las cajas punteadas marcan tendencias. F
pilóricos; IP: intestino proximal; APT: actividad proteasa total; Relación T/Q: relación tripsina/quimotripsina; NS: ausencia de difer

Tabla 3. Efectos de las dietas de sustitución sobre la actividad de las enzimas de membrana y la capacidad de

			Capítulo I			Capítulo II		
Tiempo post-ingesta			24h			24h		
Región intestinal			IP			ID		
Experimento			FxS	LxS	PxS	Px(LyS)	Prot/Lip	
ENZIMAS DE MEMBRANA	AP	+ / -	S_{5} S_{14} S_{22}	S_{11} S_{5} S_{17} S_{22}	S_{33} S_{29} S_{22}	----------	----------	
	LAP	+ / -	S_{22} S_{14} S_{5}	S_{22} S_{11} S_{17} S_{5}	NS	----------	----------	
CAPACIDAD DE ABSORCIÓN	L-Trp		----------	----------	----------	NS	NS	
	L-Lys L-Phe L-Leu	+ / -	NS	S_{17} S_{5} S_{11} S_{22}	NS	P_{59} P_{55} P_{45} P_{50} P_{40}	NS	P_{44} P_{35} P_{41} P_{38} P_{47} P_{50} P_{53}
	L-Ala L-Met L-Glu L-Pro	+ / -	S_{14} S_{5} S_{22}	S_{5} S_{11} S_{17} S_{22}	S_{22} S_{29} S_{33}	NS	L_{20} L_{14} L_{16} L_{18} L_{12}	P_{50} P_{44} P_{53} P_{41} P_{35} P_{38} P_{47}
	D-Glc	+ / -	S_{14} S_{5} S_{22}	S_{5} S_{11} S_{22} S_{17}	S_{22} S_{33} S_{29}	P_{59} P_{45} P_{50} P_{55} P_{40}	L_{16} L_{14} L_{18} L_{20} L_{12}	

Las cajas agrupan condiciones que no presentan diferencias significativas entre sí, y las cajas punteadas marcan tendencias. F: proximal; ID: intestino distal; Entero: intestino íntegro; AP: Fosfatasa alcalina; LAP: Leucina aminopeptidasa; NS: ausencia de difer

Tabla 4. Efectos de las dietas de sustitución sobre el crecimiento y la ingesta. Resumen de resultados

		Capítulo I			Capítulo II	
Experimento		FxS	LxS	PxS	Px(LyS)	Prot/Lip
SGR	+ -	NS	NS	S_{22} S_{29} S_{33}	P_{50} P_{45} P_{55} P_{59} P_{40}	NS
Ingesta total	+ -	S_5 S_{22} S_{14}	NS	NS	----------	-----------
Ingesta de comida voluntaria	+ -	NS	NS	S_{22} S_{29} S_{33}	----------	-----------
FCR	+ -	NS	NS	S_{33} S_{22} S_{29}	----------	-----------

Las cajas agrupan condiciones que no presentan diferencias significativas entre sí, y las cajas punteadas marcan tendencias. F: f crecimiento específica; FCR: tasa de conversión del alimento; NS: ausencia de diferencias significativas.

Tabla 5. Efectos de los ritmos circadianos y el peso de los animales sobre la actividad enzimática digestiva. Re

		Capítulo IV						Capí	
Tiempo post-ingesta		5h						+2	
Región intestinal		E		CP		IP		CP	
Tipo de actividad		Anticipatoria		Digestiva				Antici	
Experimento		C / CH-M / CH-A						Efecto del p	
		Toma mañana	Toma Tarde	Toma mañana	Toma Tarde	Toma mañana	Toma Tarde	FO	VO
Pepsina	+ -	CH-M C CH-A	NS	---------	---------	---------	---------	---------	---------
APT	+ -	----------	----------	C CH-A CH-M	NS	NS	CH-M CH-A C	92g 147g **305g** 233g	**147 g** **92g** 233g 305 g
α-amilasa	+ -	----------	----------	C CH-A CH-M	C CH-A CH-M	C H-M C CH-A	CH-M CH-A C	147g 92g 305g 233g	**147g** 92g 233g 305g
Lipasa	+ -	----------	----------	NS	NS	C CH-M CH-A	CH-M C CH-A	92g 147 g 233g 305g	NS
Aumento de la relación T/Q		----------		NS	NS	NS	NS	----	

Las cajas agrupan condiciones que no presentan diferencias significativas entre sí, y las cajas punteadas marcan tendencias. Los los animales FO y VO, señalando en cada caso la condición que presenta mayor actividad. E: Estómago; CP: ciegos pilóricos carbohidratos por la mañana; CH-A: carbohidratos por la tarde; FO: dieta de aceite de pescado; VO: dieta con aceite vegetal; A tripsina/quimotripsina; NS: ausencia de diferencias significativas.

Tabla 6. Efecto de los ritmos circadianos y el peso de los animales sobre la capacidad de absorción. Resumen

			Capítulo IV		Capítulo V	
Tiempo post-ingesta			5h		+24h	
Región intestinal			Entero		ID	
Experimento			C / CH-M / CH-A		Efecto del peso del animal	
			Toma mañana	Toma Tarde	FO	
CAPACIDAD D DE ABSORCIÓN DE NUTRIENTES	**L-Trp**	+	———	———	92g **233g** 147g 305g	
	L-Lys / L-Phe	+ / -	CH-A CH-M C	NS	**NS** / 147g 233g 92g 305g	92g 14 233 305
	L-Ala / L-Met	+ / -	CH-M / C CH-A	C / CH-M CH-A	**NS** / 147g 305g 92g 233g	147 / 92g 2 305
	D-Glc	+ / -	CH-M / CH-A C	NS	**NS**	
SGR		+ / -	C CH-M CH-A		NS	

Las cajas agrupan condiciones que no presentan diferencias significativas entre sí, y las cajas punteadas marcan tendencias. Los los animales FO y VO, señalando en cada caso la condición que presenta mayor actividad. Entero: intestino íntegro; ID: intestino d mañana; CH-A: carbohidratos por la tarde; FO: dieta de aceite de pescado; VO: dieta con aceite vegetal; SGR: tasa de crecimiento

Tabla 7. Efecto del cambio de dieta a corto y largo plazo. Resumen de resultados

			Capítulo V																							
Tiempo post-ingesta			+24h																							
Experimento			Efecto del cambio de dieta a corto plazo																		Efecto del cambio						
			VO → FO									FO → VO									VO → FO						
Segmento intestinal			CP			IP			ID			CP			IP			ID			CP			IP			I
Respecto a dieta de			92 VOFO	147VOFO	233VOFO	92 VOFO	147VOFO	233VOFO	92 VOFO	147VOFO	233VOFO	92 FOVO	147FOVO	233FOVO	92 FOVO	147FOVO	233FOVO	92 FOVO	147FOVO	233FOVO	92 VOFO	147VOFO	233VOFO	92 VOFO	147VOFO	233VOFO	92 VOFO
ENZIMAS DIGESTIVOS	APT	Inicio	azul	rojo	rojo	azul	rojo	azul				azul	rojo	rojo	azul	gris	gris										
		Final	azul	rojo	rojo	azul	gris	rojo				azul	rojo	rojo	azul	rojo	azul				gris	rojo	rojo	rojo	gris	rojo	
	Amilasa	Inicio	azul	gris	azul	azul	gris	azul				azul	rojo	gris	azul	gris	azul										
		Final	azul	gris	azul	gris	gris	azul				azul	gris	gris	azul	gris	azul				gris	gris	azul	azul	azul	azul	
	Lipasa	Inicio	gris	gris	gris	gris	gris	gris				azul	gris	gris	gris	rojo	gris										
		Final	azul	gris	gris	gris	gris	gris				gris	gris	gris	gris	gris	gris				gris	gris	gris	gris	gris	gris	
CAPACIDAD DE ABSORCIÓN	L-Trp	Inicio							gris	gris	azul							gris	gris	rojo							
		Final							gris	gris	azul							gris	gris	rojo							rojo
	L-Lys	Inicio							gris	azul	azul							azul	azul	gris							
		Final							gris	azul	gris							azul	azul	gris							gris
	L-Ala	Inicio							azul	gris	gris							azul	gris	gris							
		Final							azul	gris	gris							azul	gris	gris							rojo
	D-Glc	Inicio							gris	gris	gris							azul	gris	gris							
		Final							gris	gris	azul							azul	gris	gris							gris
Peso final		Inicio				gris	gris	gris							gris	gris	gris							gris	gris	gris	
		Final				gris	gris	gris							gris	gris	gris							gris	gris	gris	

En rojo se presentan las regulaciones al alza, en azul a la baja y en gris la ausencia de cambios significativos. FO: dieta de aceite de
pilóricos; IP: intestino proximal; ID: intestino distal.

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Conclusiones

7. Conclusiones

1. El pH del contenido intestinal en dorada y lubina es próximo a 7, tiende a disminuir con el crecimiento del animal y a incrementar, como mecanismo compensatorio para permitir la digestión, con la inclusión de ingredientes de origen vegetal, la disminución de proteína o la mayor inclusión de lípido en la dieta.

2. Ambas especies presentaron proteasas con actividad tripsina y quimotripsina-like. En la dorada se caracterizaron 6 bandas con pesos moleculares entre los 25 y 90 KDa, tres de estas bandas presentaron actividad tripsina-like (90, 60, 55KDa) y tres quimotripsina-like (50, 30, 25KDa). Las proteasas detectadas, para la lubina, mostraron un menor peso molecular, cuatro de ellas presentaron actividad tripsina-like (23, 22, 20, 17Kda) y una actividad quimotripsina-like (18KDa).

3. La síntesis y la actividad proteasa están moduladas por la composición principal y de ingredientes de la dieta. Así, la alimentación con niveles bajos o moderados de proteína incrementa la actividad proteasa anticipatoria; pudiendo afectar a la relación tripsina/quimotripsina. Además, en este rango de niveles de proteína, la actividad proteasa digestiva está directamente relacionada con el nivel de proteína de la dieta. La alimentación con dietas de alto contenido proteico estimula la síntesis de proteasas del páncreas exocrino difuso y puede llegar a reducir su actividad digestiva y, si se utiliza en la formulación ingredientes vegetales como la soja, aumentar la relación tripsina/quimotripsina. Además, se ha relacionado la alimentación con dietas de un contenido de lípidos superior al 18% con una menor síntesis y actividad de las proteasas. La inclusión de aceites vegetales estimula la actividad anticipatoria proteasa, sobre todo en peces de pequeño tamaño. Por otro lado, la presencia de celulosa en la dieta lleva a la disminución de la actividad anticipatoria y digestiva proteasa, provocando un aumento de la relación tripsina/quimotripsina. El tamaño del pez también afecta a la actividad proteasa anticipatoria, disminuyendo a medida que el animal crece.

4. La síntesis y la actividad α-amilasa también están moduladas por la composición principal y de ingredientes de la dieta; aunque la actividad α-amilasa se muestra más constitutiva que la proteasa. Así, la alimentación con niveles bajos o moderados de proteína

incrementa la actividad α-amilasa anticipatoria. En cambio, la alimentación con dietas de muy elevado contenido proteico estimula la síntesis de α-amilasa del páncreas exocrino difuso y la actividad digestiva de la enzima. Además, se ha relacionado la alimentación con dietas de alto contenido en fibra con un aumento de la actividad digestiva α-amilasa. Finalmente, la actividad anticipatoria de la enzima está afectada negativamente por el remplazo de proteína por almidón y positivamente, en animales de hasta 150g, por la inclusión de aceites vegetales en la dieta. El tamaño del pez también afecta a la actividad α-amilasa anticipatoria, disminuyendo a medida que el animal crece.

5. La alimentación con elevados niveles de proteína o bajos niveles de lípidos incrementa la síntesis de lipasa del páncreas exocrino difuso. Además, bajos niveles de lípidos estimulan la actividad anticipatoria del enzima, bajos niveles de almidón tienden a aumentar la actividad lipasa digestiva y la inclusión de aceites de origen vegetal en animales pequeños, reduce la actividad anticipatoria lipasa. El tamaño del pez también afecta a la actividad lipasa anticipatoria, disminuyendo a medida que el animal crece.

6. En referencia a los enzimas de membrana, la actividad anticipatoria alcalina fosfatasa incrementa en animales alimentados con un nivel de lípidos en dieta superior al 19%; sugiriendo su posible implicación en la captación de lípidos. Además, la sustitución de proteína por almidón disminuye la actividad de este enzima. Por otro lado, la actividad anticipatoria leucina aminopeptidasa desciende en los animales alimentados con un nivel de lípidos en dieta superior al 20% y tiende a estar afectada negativamente por el nivel dietario de fibra.

7. La capacidad de absorción de los aminoácidos esenciales es superior a la de los no esenciales, y ambas están moduladas por la composición principal y de ingredientes de la dieta y tienden a disminuir con el crecimiento del animal. La alimentación con dietas de alto contenido proteico regula al alza, en intestino entero, las capacidades de absorción de fenilalanina, alanina y glutámico y a la baja la de lisina; en cambio, está ultima, con elevados niveles dietarios de proteína, está regulada al alza en intestino distal. Además, la alimentación con dietas de bajo contenido proteico, en intestino entero, modula al alza la capacidad de absorción de lisina y a la baja la de alanina y glutámico. Por otro lado, alimentar con bajo contenido en lípidos regula a la baja la captación de alanina en intestino proximal y distal. La inclusión de aceites de origen vegetal aumenta la capacidad de absorción de alanina y metionina en los animales de 147g.

8. La capacidad de absorción de D-Glucosa en intestino distal está regulada a la baja en animales alimentados con dietas de bajo contenido en proteína o lípido y al alza por dietas de alto contenido proteico tanto en intestino distal como entero. Además, la sustitución de proteína por almidón disminuye la capacidad de captación del monosacárido en intestino proximal, de hecho este parámetro presenta valores máximos con niveles dietarios de almidón de hasta el 14%. El tamaño del pez también afecta a la capacidad de absorción de glucosa, disminuyendo a medida que el animal crece.

9. En dorada, la digestión y absorción de una dieta rica en carbohidratos altamente digeribles es mejor cuando se administra por la mañana, logrando un efecto sparing que permite un crecimiento comparable al obtenido con una dieta comercial.

10. La sustitución del 75% del aceite de pescado de la dieta por aceites vegetales (soja y colza) modula al alza las actividades anticipatorias de las proteasas alcalinas y la α-amilasa y a la baja la de lipasa. Además incrementa la capacidad de absorción de L-Phe en los animales de menor tamaño. El cambio de dieta de FO a VO o viceversa afecta de manera diferente a los animales en función de su peso. Así, la modulación de las actividades digestivas y absortivas permite a los animales de más de 100g adaptarse a la nueva dieta tras un mes de alimentación, en cambio, los más pequeños necesitan más tiempo, pero se realice el cambio de dieta a los 92, 147 o 233 g, los animales llegan a la vez a pesar unos 300g.

11. Finalmente, dorada y lubina son capaces de modular sus procesos digestivos y absortivos para preservar su crecimiento cuando son alimentadas con dietas de sustitución de la proteína, tanto por proteínas vegetales como por lípidos o almidón, siempre y cuando la dieta cubra los requerimientos y los factores antinutricionales que presente sean tolerados por el animal. El presente trabajo describe tres niveles de regulación de los procesos digestivos y absortivos: 1) La modulación de la actividad digestiva intestinal y de la capacidad de absorción de nutrientes, 2) la regulación al alza de la síntesis y secreción de enzimas pancreáticos y 3) el incremento de la ingesta voluntaria.

Anexo

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Different protein to energy ratio diets for gilthead sea bream (*Sparus aurata*): Effects on digestive and absorptive processes

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Sea bream juveniles were fed for a 12-week period with 7 isoenergetic diets with different levels of protein (35, 38, 41, 44, 47, 50 and 53%). The diets were formulated by changing simultaneously the levels of lipid (from 27 to 19%) and starch (from 21 to 10%). After the growth period, we studied the digestive enzyme activities at 5 h post-feeding and the nutrient absorption capacity at 24 h post-feeding. A progressive increase in the total protease activity (TPA) was found as the content of diet protein rose (from 35 to 41%) and a diminution of TPA activity was detected in sea bream fed high soybean concentrate content diets (50 and 53% dietary protein). Lipase activity did not change by dietary composition. An up-regulation of α-amylase activity, and D-glucose and L-Ala absorption capacities was found in fish fed with the lowest carbohydrate diets, corresponding to 50 and 53% dietary protein. Moreover, the L-lysine absorption capacity was upregulated in fish fed with low protein diets (35, 38, 41 and 44%). Thus, changes in diet composition can modulate enzymatic activities and nutrient absorption capacity, to improve food use and assure growth performance. In this sense, only sea bream fed with the lowest protein diet (35%) had a significantly lower SGR (<1), although they had the highest voluntary feed intake. Sea bream fed with P_{44} and P_{47} diets need the minimal adaptive changes to diet and showed good growth.

1. Introduction

Several studies have been undertaken to establish the optimum dietary protein and protein to energy ratio for growing gilthead sea bream. Sabaut and Luquet (1973) estimated the optimum protein level for juvenile sea bream as 40%. This value was later revised to 45–46% (Santinha et al., 1996; Vergara et al., 1996). Regarding the optimal dietary lipid level for this species, the best growth performances were observed with 15–16% dietary lipids when fish oil was used as the lipid source (Vergara et al., 1996; Vergara and Jauncey, 1993). However, Lupatsch et al. (2001) stated that voluntary feed intake is maintained at least until 26% of dietary lipid; while growth and protein gain were optimal in diets with 48% of protein and 22.5% of lipid. The effect of dietary carbohydrates remains controversial in marine fish species (Enes et al., 2011; Peres et al., 1999), although it has been reported that sea bream can use starch without compromising growth when the diet contains up to 20% of carbohydrates (Fernández et al., 2007). The fish strain, water temperature and quality, oxygen availability, biomass density, and the biological value of dietary protein and non-protein energy substrates may influence the described requirements (NRC, 1983).

Digestion and absorption are key processes in the optimal use of a diet. Digestion processes begin in the stomach, where HCl denatures protein and converts pepsinogen to active pepsin (Wu et al., 2009; Yúfera et al., 2004). After the stomach, the chime passes to the pyloric caeca. When the pyloric caeca are full, cholecystokinin is secreted, and stimulates pancreatic secretions that include a) electrolytes, which neutralize the acidic pH of the chime coming from the stomach; b) bile acids that emulsify dietary lipids and fat-soluble vitamins, and c) pancreatic enzymes, including proproteases (Krogdahl et al., 1999; Pivnenko et al., 1997), α-amylase, lipolytic enzymes (Kurtovic et al., 2009), as well as DNAse and RNAse. The amounts and activities of the secreted pancreatic enzymes appear to vary with species and/or their dietary preferences; and with the nutrient composition and digestibility of the diet (Buddington et al., 1997).

Absorption processes occur by diffusion, facilitated transport or active transport (Mailliard et al., 1995) and take place throughout the entire intestine, which differentiates fish from mammals. In fish, the presence of at least four Na^+-dependent amino acid transport systems (acidic, basic and neutral amino acids, and a system responsible for proline and N-methylated amino acids) has been described (Storelli et al., 1989). In addition, Na^+-independent transport systems have been found for alanine, glycine and lysine, proline and glutamate (Storelli et al., 1989), while L-histidine appears to be transported by a highly specific transporter (Glover and Wood, 2008). The transport of peptides by PepT1 and PepT2 transporters has been demonstrated recently (Goncalves et al., 2007; Hakim et al., 2009; Romano et al., 2006; Sangaletti et al., 2009; Terova et al., 2009). It has been suggested that

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intestinal fatty acid binding protein (I-FABP) could be involved in transport processes, by binding the peptides or proteins before endocytosis (Concha et al., 2002). Finally, D-glucose and D-galactose are transported by SGTL1, where uptake occurs by both facilitated transport and active transport (Sala-Rabanal et al., 2004). Nutrient absorption capacity can be affected by environmental differences such as temperature and water salinity, and by biotic factors such as the species, and/or their dietary preferences, diet formulation, digestibility and availability (Buddington et al., 1997).

The massive growth of aquaculture and the limited supply of fish meal (FM) and fish oil (SOFIA, 2010) means that alternative food sources need to be tested to make the industry more sustainable. In recent years, several studies have suggested that plant protein meals may be a good alternative (Bell and Waagbø, 2008; Turchini et al., 2009). It has been demonstrated that up to 75% of fish meal can be replaced without compromising the growth of sea bream (Albrektsen et al., 2006; Gómez-Requeni et al., 2004). However, less is known about how the digestive and absorptive processes are affected by these dietary modifications (Santigosa et al., 2008, 2011a,b; Venou et al, 2003). The replacement of FM by a blend of plant proteins decreases digestive enzyme activity. Nevertheless, animals can compensate for this reduction by increasing the relative intestinal length and the trypsin and chymotrypsin ratio of digestive proteases of pancreatic origin (upregulating trypsin activity) to achieve digestive balance (Santigosa et al., 2008). Nutrient absorption capacity is also modified in response to the inclusion of high levels of plant protein sources, probably because of distinct luminal nutrient availability due to supplementation with free amino acids, which leads to asynchronous utilization (Ambardekara et al., 2009; Santigosa et al., 2011b). Absorption patterns could be affected by changes in gut histology (Santigosa et al., 2008), as transporters are embedded in the lipid bilayer and affected by its characteristics (Houpe et al., 1997; Spector and Yorek, 1985) and by enterocyte development (Smith, 1993). In our knowledge, no studies have been carried out in sea bream on the effect of high lipid diets on digestive and absorptive processes.

Our aim was to find the best protein to energy ratio in a high lipid diet in which 50% of FM was replaced by plant protein sources; minimizing the protein content of the diet by including lipid and starch. Here, we studied the effect of diet on digestive and absorptive processes in sea bream.

2. Materials and methods

2.1. Diets

Seven isoenergetic diets (23 MJ·kg^{-1}, Table 1) were formulated (Skretting, Norway) with different levels of protein by changing the levels of lipid and starch simultaneously. Diets were named according to the protein content: P_{35}, P_{38}, P_{41}, P_{44}, P_{47}, P_{50} and P_{53}. As the protein increases, the dietary lipid and starch decrease. In all diets, 50% of fish meal (FM) was substituted by plant protein sources (wheat, wheat gluten and soybean protein concentrate). Fish oil was used as the only source of lipid. We used wheat as it has been described as a good carbohydrate source for gilthead sea bream (Venou et al., 2003).

2.2. Fish and sampling

Gilthead sea bream from "Fauna Marina S.L." (San Fernando, Cádiz) were acclimatized for two weeks in 12 m^3 fiberglass tanks at IRTA (Sant Carles de la Ràpita, Spain). Six hundred and thirty fish (of about 70 g) were randomly distributed into 21 fiberglass tanks (400 L; 30 fish per tank). The water salinity was 34.5‰ and the oxygen content in the outlet water was higher than 85% saturation.

During the 12-week trial (May–July), triplicate groups of fish were fed the corresponding diet twice a day (at 9:00 and 13:00) until visual satiety. The temperature (18 to 28 °C) and photoperiod (14 to 14:30 h of daylight) were natural, according to the course of the trial.

Table 1
Ingredient and nutrient composition of the experimental diets.

	P_{35}	P_{38}	P_{41}	P_{44}	P_{47}	P_{50}	P_{53}
Ingredients (%)							
Fish meal	26.9	26.7	29.4	32.0	34.7	37.3	40.0
Fish oil	21.0	19.0	17.6	16.2	14.7	13.3	11.9
Wheat gluten	6.0	10.7	11.8	12.8	13.9	14.9	16.0
SPC	9.0	10.7	11.8	12.8	13.9	14.9	16.0
Wheat meal	36.8	32.5	29.2	25.9	22.5	19.2	15.8
Vitamin premix	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Composition (%) in wet matter							
Dry matter	92.18	92.14	92.20	92.26	92.33	92.39	92.48
Crude protein	35.00	37.99	40.99	43.99	47.00	50.00	53.00
Crude fat	26.80	24.17	23.05	21.93	20.82	19.70	18.62
Estimated starch	21.28	19.06	17.23	15.40	13.52	11.69	9.81

Fish meal: 66.8% crude protein, 13.1% crude fat.
Wheat gluten: 78.3% crude protein, 5% crude fat, 5% starch.
SPC (soybean protein concentrate): 61.5% crude protein; 1.8% crude fat.
Wheat meal: 14.9% crude protein; 3.0% crude fat; 57.0% starch.
Vitamin premix is a proprietary formula of Skretting (Norway).

At the end of the growth trial, ten animals per time and treatment were anesthetized (phenoxyethanol 100 ppm), weighed and sacrificed by severing the spinal cord at 5 h and 24 h post-feeding. In the first sampling, the digestive tract was isolated and fish were classified according to their stomach contents (full versus empty). Samples of the stomach, pyloric caeca and proximal intestine, including the intestinal content, were collected and rapidly frozen in liquid nitrogen and maintained at −80 °C until the enzymatic studies. In the second sampling, the whole intestine was isolated, measured and was cut lengthwise, washed in an isosmotic saline solution containing 0.1 M of protease inhibitor (phenyl-methyl-sulfonyl-fluoride) and frozen in liquid nitrogen until the nutrient absorption experiments were performed.

All fish-handling procedures complied with the European guidelines for animal care (Directive 2010/63/EU).

2.2.1. Zootechnical and morphological indexes

Total feed intake was calculated as [food intake (kg) − food lost (kg)]. Voluntary feed intake regarding whole diets and weight gain were expressed in: g BW (kg)$^{-0.7}$ day^{-1} to correct for the influence of fish size on feed consumption in gilthead sea bream (Lupatsch and Kissil, 1998), where body weight (BW) was calculated from the initial and final body weights of fish as the geometric mean: $BW = (W_{ini} \cdot W_{fin})^{0.5}$. Total biomass from all triplicate tanks was obtained from Skretting to evaluate the weight gain during the experimental period and used to calculate SGR and feed conversion ratio (FCR). The specific growth rate (SGR) was calculated as follows: $((\ln W_{fin}\ (g) - \ln W_{ini}\ (g))/t) \cdot 100$, where W_{fin} and W_{ini} represent the final and initial weights respectively, and t is the number of feeding days. The feed conversion ratio (FCR) was calculated as g feed given/g live weight gain. Relative intestinal length (RIL) was measured excluding the pyloric caeca and is expressed in relation to each animal weight (Santigosa et al., 2008).

2.3. Gastric and intestinal pH content and intestinal digestive enzyme analysis

The gastric and intestinal pH content of the samples was measured (Crison, Micro pH 2000). Intestinal sample homogenization, total alkaline protease, trypsin and chymotrypsin activities and α-amylase were measured according to a modification of Santigosa et al. (2008). Lipase determination was carried out following Santigosa et al. (2011a). Briefly, intestinal samples were individually homogenized (Polytron 2000, Sorvall TC) at 4 °C in 50 mM Tris–HCl buffer pH 7.5 to a final concentration of 250 mg mL^{-1}. Homogenates were centrifuged (1100 g, 15 min, 4 °C, Jouan CR 411) and supernatants were recovered and stored at −80 °C. For the total alkaline protease activity (TPA) determination, samples were reacted with 50 mM Tris–HCl buffer at the pH of the intestinal content containing 1% casein at 20 °C. After 30 min, the reaction was stopped by adding trichloroacetic acid 12%. The samples were at maintained 4 °C for an hour and were then centrifuged (7500 g, 5 min, 4 °C). Individual blanks were established for each sample. Supernatant absorbance was measured at 280 nm (UV-1603, Shimadzu). Bovine trypsin (Sigma Aldrich, Spain, 12,100 BAEE U/mg protein, NC-IUB, 1979) was used as standard.

Zymograms were used to characterize trypsin-like and chymotrypsin-like activities. Samples were loaded using 12% polyacrylamide gels ($10 \times 10.5 \times 0.1$ cm) and electrophoresis was performed at a constant current of 15 mA per gel for 150 min (EPS 301 Power Supply, 4 °C). The gels were incubated under agitation at the pH of the intestinal content in Tris–HCl buffer containing 2% casein (30 min, 4 °C). They were shaken for 90 min at room temperature. Gels were washed and stained in a methanol:acetic acid:water solution (40:40:10) with 0.1% of BBC R-250 (Brilliant Blue Coomassie R-250). Destaining was carried out using the same solution without colorant. Pure trypsin was used as a control, and a RPN 800 commercial weight marker (Amersham, GE Healthcare, UK, 12,000–225,000 Da) was used to determine the molecular weight of protease activity fractions. To characterize trypsin-like and chymotrypsin-like activities we used specific inhibition solutions (Alarcon et al., 1998).

α-Amylase activity was measured using the tracer amylose azure, a potato starch that is covalently labeled with Remazol Brilliant Blue R dye. The homogenate was combined with 0.5% tracer solution containing soybean trypsin inhibitor (SBTI 0.04 mg mL^{-1}) to prevent proteolysis. The reaction was stopped after 30 min by adding 6% acetic acid and the sample was centrifuged (13,000 *g*; 10 min at 4 °C). Supernatant absorbance was measured at 595 nm. α-Amylase (Sigma Aldrich, Spain, 66 U/mg solid) was used as standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 at 20 °C (Santigosa et al., 2008).

For the lipase determination, homogenate was mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg L^{-1} colipase (pH 8.3). The increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33,944 U/mg protein, 22,980 U/mg solid) was used as a standard. One unit hydrolyses 1.0 micro equivalent of FA from triacetin in 1 h at pH 7.4 and 20 °C (Santigosa et al., 2011a).

The protein concentration in homogenates was determined by the Bradford (1976) method using bovine serum albumin as a standard.

2.4. Nutrient uptake analysis

Brush border membrane vesicles (BBMVs) from whole intestine were obtained as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenated in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100) to separate mucosa from the muscle layers. A selective basolateral and mitochondrial membrane precipitation was carried out by adding $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugations allowed the purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe. The enrichment in brush border membrane preparations was determined by measuring alkaline phosphatase activity following Weiser (1973), which was described as a good marker of BBMVs.

The capacity to absorb three essential amino acids (L-leucine, L-lysine and L-phenylalanine), three non-essential amino acids (L-alanine, L-glutamic and L-proline) and D-glucose was determined in vesicular suspensions according to Santigosa et al. (2011b). These were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient, Osm 320, pH 7.4) for 5 s. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 320, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22-μm cellulose nitrate filters (Millipore, Bedford MA), previously wetted in cold stop buffer. The filters were then washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were made at 20 °C. Radiolabelled nutrients were obtained from Amersham Pharmacia Biotech (Barcelona, Spain).

The vesicular volume of BBMV preparations was measured following Sala-Rabanal et al. (2004) to normalize influx values. Briefly, L-alanine retained inside the vesicles at the equilibrium situation was measured by incubating BBMV preparation with incubation buffer on ice. After 90 min, the reaction was stopped and the amount of L-alanine was measured following the procedure describe above.

The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford (1976) method.

2.5. Statistical analysis

Differences between the presence and absence of food in the stomach were determined using a Student's *t*-test. Differences between dietary treatments were determined using one-way ANOVA and Tukey's test. The Student's *t*-test was used to establish significant differences between intestinal regions ($P < 0.05$). The software used was PASW Statistics 17.0 (SPSS, Inc., EUA).

3. Results

The measure of gastric and intestinal content pH and digestive enzyme activity was determined at 5 hours post-feeding. At that time, 41.4% of the animals still had feed in their stomachs and 58.6% had empty stomachs. First, the results are presented according to the presence or absence of feed in the stomach.

Fig. 1 shows the pH of the stomach, pyloric caeca (PC) and proximal intestine (PI) contents. The pH of the gastric content was found to vary depending on the status of the stomach (full versus empty). The pH was lower (approximately 4) when the stomach was full, which indicates that acid protease activity occurs. This value was significantly different from the pH values of an empty stomach (6.6 ± 0.04). The pH values measured for the intestinal regions (PC and PI) were close to 7 and slightly lower for the pyloric caeca when the stomach was full. Fig. 2 shows the total protease (Fig. 2A), lipase (Fig. 2B)

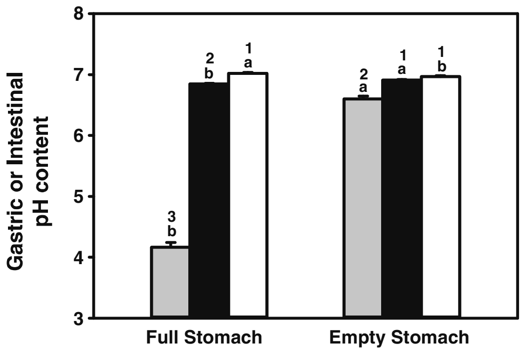

Fig. 1. pH of gastric content (gray bars), pyloric caeca content (black bars) and proximal intestine content (white bars) in full (n = 30) or empty stomach (n = 40). Values are represented as the mean ± SEM. Significant differences ($P < 0.05$) between gastric and intestinal segments are shown by different numbers; significant differences between conditions ($P < 0.05$) are shown by different letters.

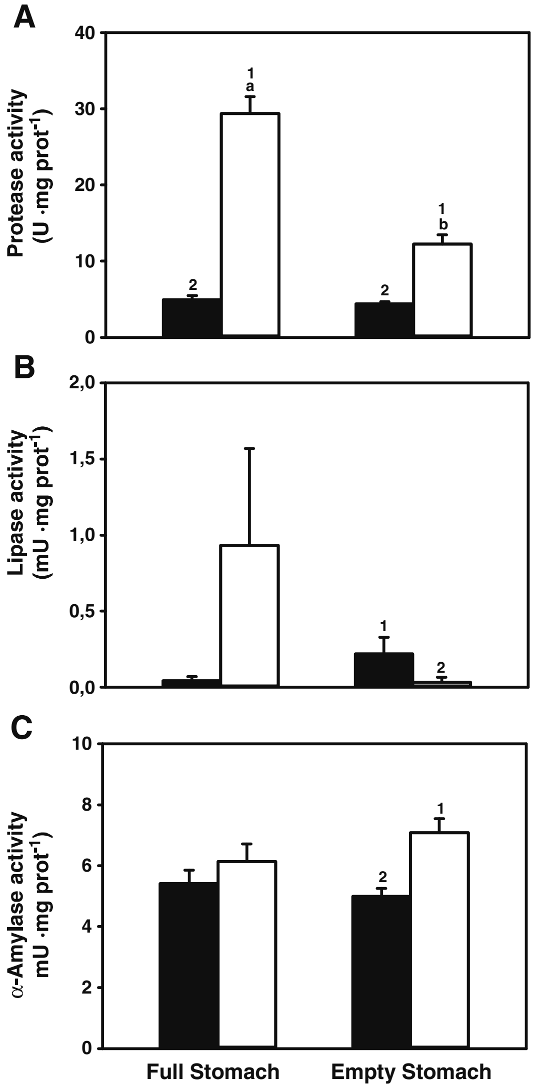

Fig. 2. A) Total protease, B) lipase and C) α-amylase activities in pyloric caeca (black bars) and proximal intestine (white bars) in full (n = 30) or empty stomach (n = 40). Values are represented as the mean ± SEM. Significant differences ($P < 0.05$) between intestinal segments are shown by different numbers; significant differences between conditions ($P < 0.05$) are shown by different letters.

and α-amylase activities (Fig. 2C) in PC and PI. Digestive enzyme activities were lower in the pyloric caeca than in the proximal intestine, except for lipase activity when the stomach was empty. In the proximal intestine, total protease and lipase activities (Fig. 2A and B) were higher in the full versus the empty stomach. This pattern was not detected for proximal α-amylase activity (Fig. 2C) or pyloric caeca activities.

Animals fed with extreme diets had a lower frequency of feed content in the stomach (30% for P_{35}, P_{50} and P_{53} animals and 20% for P_{38} fish). However, this frequency was higher (70, 50 and 60%, respectively) for sea bream fed with P_{41}, P_{44} and P_{47} diets.

Second, we present the results according to the diet. Fig. 3 shows the total protease activity (TPA). In pyloric caeca the values were higher for the animals fed with the lower protein content diets (P_{35} and P_{38}), while baseline activity was detected in the other groups. Proximal intestine TPA tended to be higher in gilthead sea bream fed with P_{41}, P_{44} and P_{47} diets. Fish fed with the diets P_{35} and P_{38} showed lower total protease activity. In these fish, the TPA PI/PC ratio was between 1 and 2. In contrast, in sea bream fed with P_{41}, P_{44} and P_{47} diets, the TPA PI/PC ratio was 7 to 9. In these fish, the TPA that was detected was significantly higher than in the rest of the experimental

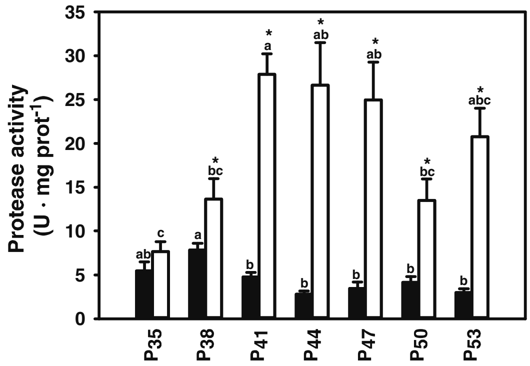

Fig. 3. Total protease activity in pyloric caeca (black bars) and proximal intestine (white bars). Values are represented as the mean ± SEM of 10 fish. Significant differences ($P < 0.05$) between diets are shown by different letters; significant differences between intestinal segments ($P < 0.05$) are shown by *.

groups (Fig. 3). Finally, in fish fed P_{50} and P_{53} diets, the TPA PI/PC ratio measured was intermediate at between 3 and 7.

Fig. 4 shows model zymograms from PC (4A) and PI (4B) obtained from each experimental group, these revealed the presence of six proteases at intestinal level. The band pattern found for each condition corresponds to the proteolytic profile measured in previously described intestinal segments (Fig. 3). Thus, the activity was maximal in pyloric caeca for P_{38} sea bream and faded to 55 kDa trypsin and chymotrypsin activity in other conditions. The proximal intestine band pattern (Fig. 4B) was modified in the extreme diets: in P_{35} fish chymotrypsin

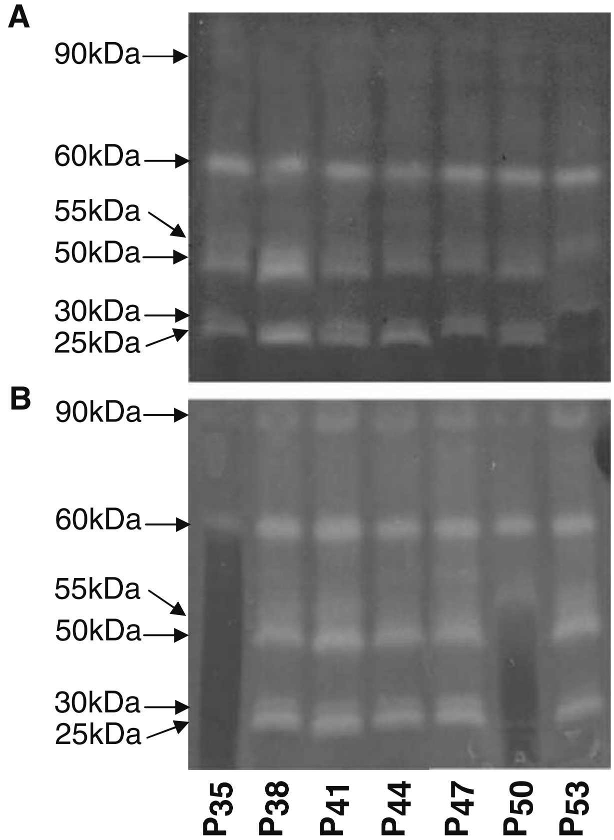

Fig. 4. A) Model zymogram of the alkaline proteolytic activity in pyloric caeca extracts was done at pH 6.9 ± 0.1. B) Model zymogram of the alkaline proteolytic activity in proximal intestine extracts was done at pH 7.0 ± 0.01. Figures show the molecular weight of bands with proteolytic activity (bands of 90, 60 and 55 kDa have trypsin-like activity and 50, 30 and 25 kDa have chymotrypsin activity). All samples were analyzed individually; the figure shows a representative result.

activity was not detected and 55 kDa and 90 kDa trypsin activities were attenuated. Similarly, chymotrypsin activity was not found in P_{50} fed-fish. Chymotrypsin was partially detected in P_{53} fed-fish, and specifically affected the 30 kDa band.

Lipase and α-amylase activities were also measured in PC and PI. When lipase activity was measured no significant differences were found among experimental groups or intestinal segments. In PC lipase activity ranges from 0 to 2.6 mU lipase·mg protein^{-1} (mean 0.139 ± 0.061), while in PI ranges from 0 to 6 mU lipase·mg protein^{-1} (0.408 ± 0.27). In contrast, α-amylase activity 5 h after manual feeding (Fig. 5) tended to increase as the starch content of the diet diminished, with the exception of the P_{35} fish, in which PI α-amylase activity was higher than in the other experimental groups.

The nutrient absorption capacity of the whole intestine 24 h post-intake was measured in brush border membrane vesicles (BBMVs) obtained from fish fed the experimental diets. To validate the vesiculation processes, the enrichment of alkaline phosphatase activity in BBMVs was determined to be between 4 and 6.6 times the adequate value, according to Santigosa et al. (2011a,b). The capacity of the intestine to absorb essential and non-essential amino acids and D-glucose is shown in Fig. 6 and Table 2. The L-Lys and L-Phe absorption capacities were higher than those of the other amino acids measured. Sea bream fed with lower protein content diets tended to show higher lysine absorption capacity, whereas the absorption capacity of L-phenylalanine was higher in animals fed with the highest protein content diets (P_{50} and P_{53}). No differences were found for the L-Leu absorption capacity. D-Glucose uptake (Fig. 6) was significantly higher for the P_{50} and P_{53} groups, which may be due to the reduced contribution of diet carbohydrate, following the same profile as the α-amylase activity and the absorption capacity of L-Ala and L-Glu.

Total intake, voluntary feed intake, SGR, FCR and RIL are shown in Table 3. The SGR was higher for gilthead sea bream fed the P_{50} and P_{53} diets, while the results were significantly lower for the P_{35}, P_{38} and P_{41} groups (−27.3, −12.3 and −15.4%, respectively, when compared to P_{50} fed-animals). Proportion to their size, the P_{35} animal's intake was superior to all other groups. A higher FCR was detected for sea bream P_{35}, P_{38} and P_{41}, and also a tendency to increase RIL in those groups.

4. Discussion

As fish meal is a limited primary source, it should be replaced by plant proteins, lipids or starch in fish diets (SOFIA, 2010). Therefore, the diets used in this trial contained 50% of plant origin protein. To improve growth, the diets used were also highly energetic, with a minimum content of 19% lipid. The study examines the effect of replacing protein by lipid and starch on digestive and absorptive processes.

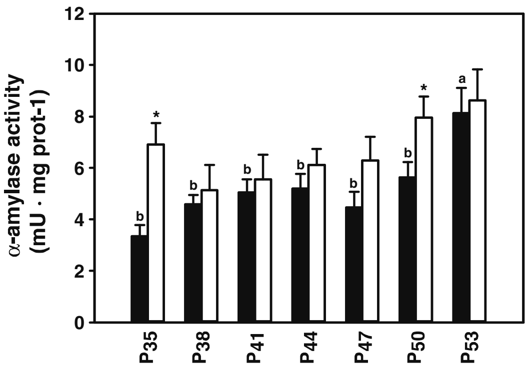

Fig. 5. α-Amylase activity in pyloric caeca (black bars) and proximal intestine (white bars). Values are represented as the mean ± SEM of 10 fish. Significant differences ($P < 0.05$) between diets are shown by different letters; significant differences between intestinal segments ($P < 0.05$) are shown by *.

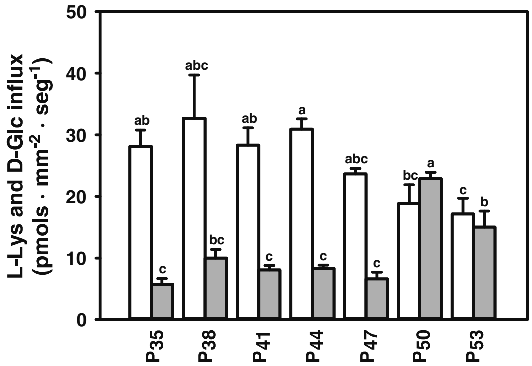

Fig. 6. Intravesicular concentration of L-lysine (white bars) and D-glucose (gray bars) in brush border membrane vesicles obtained from whole intestine from sea bream. Results are represented as mean ± SEM of 6 determinations. Different letters show significant differences ($P < 0.05$) between diets.

Animals fed with P_{41}, P_{44} and P_{47} diets showed a higher frequency of feed content in the stomach, suggesting a gastric emptying lower in these groups versus animals fed with extreme diets.

The protein content of a diet influences the secretion of pancreatic enzymes (Bakke et al., 2011). Some authors have stated that increased protein levels stimulate the pancreas to deliver a secretion that contains high levels of trypsin in Atlantic salmon (Krogdahl et al., 2003) and European sea bass (Péres et al., 1998). Animals fed the P_{35}, P_{38} and P_{41} diets showed a progressive increase in intestinal proteolytic activity as the protein in the diet rose, reaching a plateau at 41% to 47% of protein. The lower protease activity measured for P_{35} and P_{38} fed-fish may also be due to the greater presence of lipid (27 and 24%) and starch (21 and 19%) in the diets. In studies using different experimental diets (Fountoulaki et al., 2005) or a single bolus of lipid (Murashita et al., 2008), high fat content was related with lower protease activity. High levels of starch affect the viscosity of digesta (Storebakken and Austreng, 1987) and are considered to interfere with proteolytic activities (Fernández et al., 2001; Hidalgo et al., 1999; Munilla-Moran and Saborido-Rey, 1996). The lower TPA PI/PC ratio detected in P_{35} and P_{38} sea bream points to a slower intestinal digestive process. In contrast, P_{41}, P_{44} and P_{47} fish showed the highest PI/PC ratio, denoting that the digestion processes were taking part in PI. These could be related to the high lipid and starch content of both diets, as suggested by the results of Fountoulaki et al. (2005) in sea bream. In this trial, 50% of the protein in the diet was of plant origin, specifically from wheat gluten and soybean meal. The presence of anti-nutritional factors (ANFs) were not determined in the experimental diets, but it has been widely described that soybean meal could hinder the action of proteases by complexing with them and preventing their action, as previously described (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). The possible ANFs' presence in these diets could inhibit chymotrypsin-like activities to a greater extent, and change the trypsin to chymotrypsin ratio, as Santigosa et al. (2008) suggested. The level of dietary protein can also alter the pattern of digestive protease secreted in the intestinal lumen (Rodiles et al., 2012). These changes in P_{50} and P_{53} sea bream could lead to asynchronous availability of amino acids and di-tripeptides as a consequence of the specificity of proteases.

Amino acid transport capacity may be upregulated to compensate for the nutritional deficit caused by dietary composition (Santigosa et al., 2011a,b) or short starvation periods (Golovanova, 1992; Santigosa et al., 2011b). Thus, P_{35}, P_{38}, P_{41} and P_{44} gilthead sea bream showed a higher absorption capacity of L-Lys than animals fed with high protein diets. This compensation mechanism enabled the P_{44} animals to achieve similar growth rates to the P_{47}, P_{50} and P_{53} animals. Nevertheless, this was not enough for P_{41} and P_{38}, whose growth was slower (−15.4% and −12.3 respectively, versus P_{50}). In addition to the mechanism described above, sea bream P_{35} had significantly

Table 2
Essential and non-essential amino acid intravesicular concentrations in BBMVs ($pmol \cdot mm^{-2} \cdot seg^{-1}$). Values are represented as mean ± SEM of 6 determinations. Different letters show significant differences ($P < 0.05$) between diets.

	P_{35}	P_{38}	P_{41}	P_{44}	P_{47}	P_{50}	P_{53}
Essential							
L-Phe	20.71 ± 3.65ab	18.25 ± 2.22ab	14.60 ± 0.48b	16.13 ± 1.07ab	15.39 ± 1.96ab	22.24 ± 1.72ab	24.61 ± 3.24a
L-Leu	10.12 ± 1.48	9.14 ± 1.53	13.24 ± 0.26	12.16 ± 0.94	10.70 ± 1.28	12.23 ± 1.46	9.16 ± 0.73
Non-essential							
L-Pro	8.34 ± 0.41	8.86 ± 0.73	9.01 ± 1.26	9.05 ± 0.88	7.65 ± 0.46	8.32 ± 0.65	7.20 ± 0.24
L-Ala	4.45 ± 0.30d	5.13 ± 0.23d	5.40 ± 0.12cd	6.94 ± 0.37b	4.79 ± 0.21d	8.57 ± 0.41a	6.78 ± 0.54bc
L-Glu	2.36 ± 0.11b	3.50 ± 0.36ab	2.34 ± 0.10b	3.19 ± 0.32ab	2.29 ± 0.27b	3.12 ± 0.38ab	4.79 ± 0.94a

Table 3
Total intake (n = 3), voluntary feed intake (n = 3), SGR (n = 3), FCR (n = 3) and RIL (n = 12) for each experimental condition. Values are represented as mean ± SEM. Different letters show significant differences ($P < 0.05$) between diets.

	P_{35}	P_{38}	P_{41}	P_{44}	P_{47}	P_{50}	P_{53}
Total intake (kg)	3.76 ± 0.10d	4.65 ± 0.10ab	4.37 ± 0.19c	4.82 ± 0.03abc	4.78 ± 0.08abc	5.24 ± 0.09a	4.93 ± 0.14ab
Voluntary feed intake ($g \cdot kg^{-0.7} \cdot day^{-1}$)	5.65 ± 0.04a	5.11 ± 0.07b	5.28 ± 0.07b	5.16 ± 0.01b	5.24 ± 0.03b	5.08 ± 0.01b	5.15 ± 0.07b
SGR	0.94 ± 0.03c	1.14 ± 0.03b	1.10 ± 0.02b	1.20 ± 0.02ab	1.22 ± 0.03ab	1.30 ± 0.03a	1.26 ± 0.02a
FCR	1.38 ± 0.008a	1.25 ± 0.041abc	1.26 ± 0.050ab	1.16 ± 0.014bc	1.16 ± 0.026bc	1.14 ± 0.006bc	1.12 ± 0.015c
RIL ($mm \cdot g\ fish^{-1}$)	17.11 ± 1.73	16.45 ± 1.44	15.62 ± 1.27	14.77 ± 1.02	14.91 ± 0.98	13.53 ± 1.02	13.43 ± 1.13

greater voluntary feed intake and FCR, although the latter did not differ from P_{38} and P_{41}. Nevertheless, growth fell by 27%. Another compensation mechanism to the lower protein content of diet was the tendency to increase RIL. Santigosa et al. (2008) described an increase in RIL as the plant protein content of diets rise.

The relationship between dietary nutrient levels of lipids and carbohydrates and corresponding enzyme secretions appears to be more complicated (Bogevik et al., 2009; Krogdahl and Sundby, 1999; Morais et al., 2007; Zambonino Infante and Cahu, 2007). In this trial, intestinal lipase activity was low and was not affected by dietary lipid content (Santigosa et al., 2011a). This suggests that there was limited digestion of oils in high lipid diets. In contrast, it is well known that high starch levels can provoke absorption of amylase onto starch molecules, thus inhibiting the enzyme's activity (Fountoulaki et al., 2005; Spannhof and Plantikov, 1983). In addition, amylase activity is inhibited by both dietary starch and lipid, as shown in Fountoulaki et al. (2005). This is in accordance with the results for P_{50} and P_{53} animals, which showed higher amylase activity. Similar to the α-amylase activity, the upregulated capacity to absorb D-Glc in gilthead sea bream fed P_{50} was probably due to the lower availability of this nutrient, as Santigosa et al. (2011b) suggested. Both situations allow an improvement in D-Glc absorption. L-Ala showed the same absorption capacity profile as D-Glc, which suggests that this amino acid could be used as an energy source when diets have low starch content.

As described above, P_{35} sea bream presented significantly lower growth rates than all other fish, and increased their voluntary feed intake as a compensatory mechanism, which further increased the dietary carbohydrate intake. These facts could be related to the higher proximal α-amylase activity detected in the proximal intestine as Fountoulaki et al. (2005) suggested in fish fed with high starch diets.

It is well known that fiber and the non-starch polysaccharides (NSPs) present in plant-based diets negatively affect enzymatic activities and increase transit rates (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). In this trial, the total amount of fiber and NSPs did not vary between diets, but their origin differed depending on the proportions of plant ingredients included. The kind of dietary ANFs could differentially affect the digestion processes and transit rates.

Finally, Murashita et al. (2007) suggested that pancreatic lipase and trypsin were released before α-amylase. The results were in accordance with this hypothesis when the enzymatic activities, measured in pyloric caeca and proximal intestine, were compared between fish with full or empty stomachs.

In summary, when dietary composition changes, intestinal enzymatic activities and nutrient absorptive capacity may be modulated in fish. Voluntary feed intake may also change to improve feed use and assure growth performance. Sea bream fed the P_{44} and P_{47} diets needed minimal adaptive changes and grew well.

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Meal timing affects protein-sparing effect by carbohydrates in sea bream: Effects on digestive and absorptive processes

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ABSTRACT

A commercial diet (C) (48% protein and 20% lipids) and a high-digestible carbohydrate diet (CH) (37% protein, 12.5% lipids and 40% high-digestible carbohydrates) were used to feed sea bream juveniles for an 8-week period. In the commercial diet, more than 60% of ingredients were of plant origin from various sources, whereas the only component of plant origin in the CH diet was wheat. To determine the best time to administer carbohydrates and the possible protein-sparing effect, three different dietary regimes were established: C, CH-M and CH-A, and the corresponding diet was fed to sea bream in the morning (1.6% of bw) and in the afternoon (1% of bw), calculating quantities according to the amount of feed that fish ate during the acclimatization period. After the growth trial, specific growth rate (SGR), relative intestinal length, intestinal pH content, gastric and pancreatic digestive enzyme activities and nutrient absorption capacities were studied 5 h post-feeding after each meal (morning and afternoon). The acid protease activity measured was anticipatory and was higher when the next meal would have more protein. No differences in relative intestinal length or feed buffering capacity were found. The smaller ration given to sea bream in the afternoon led to a lower pancreatic release of alkaline protease and α-amylase and an up-regulation of D-Glc and L-Ala absorption capacity. A higher transit rate was measured when sea bream were fed the CH diet. When high-digestible carbohydrates were administered in the morning and the commercial diet in the afternoon, we observed a better assimilation of both diets due to compensatory mechanisms such as an increase in L-Lys, D-Glc and L-Ala absorption capacity after the morning feed, and a higher pancreatic release of alkaline protease and amylase after the afternoon feed. In contrast, when high-digestible carbohydrates were given in the afternoon, only a significant up-regulation of the capacity to absorb L-Lys was detected. Thus, the inclusion of high-digestible carbohydrates in the diet improved digestion and absorption processes when administered in the morning, leading to a protein-sparing effect that yielded growth comparable to that of fish fed an exclusively commercial diet.

1. Introduction

Fish meal provides an adequate balance of amino acids, but increased demand and prices, as well as uncertain supply, render it necessary to identify alternative protein sources (SOFIA, 2012). At present, over 50% of the fish meal in commercial diets is replaced by protein of plant origin; thus, the possible presence of anti-nutritional factors (ANFs) and the limitation of some amino acids, such as lysine or methionine (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010), may promote changes in digestive and absorptive capacities (Santigosa et al., 2008, 2011a,b) in order to minimize the effects on metabolism (Metón et al., 1999) and compensate for those dietary changes.

The protein-sparing effect may possibly present a means to minimize the use of fish meal, by increasing the lipid and/or carbohydrate content of the feed. The use of dietary lipids instead of protein is well established in fish (Company et al., 1999; Vergara et al., 1999). A voluntary feed intake of at least 26% dietary lipids, and a diet of 48% protein and 22.5% lipids, has been established as providing optimal growth and protein gain (Lupatsch et al., 2001). However, despite these results, the high prices and limited supplies of fish oil (SOFIA, 2012) restrict its use, and it is often replaced by vegetable oils that diminish the quality of the final product (De Francesco et al., 2007; Fountoulaki et al., 2009; Izquierdo et al., 2005; Menoyo et al., 2004). On the other hand, the utilization of dietary carbohydrates for energy purposes in salmonids and other carnivorous species appears to be limited (Hemre et al., 2002; Stone et al., 2003; Wilson, 1994). Although several studies have indicated a protein-sparing effect by dietary carbohydrates in sea bream and sea bass (Couto et al., 2008; Dias et al., 1998; Enes et al., 2006; Fernández et al., 2007; Peres and Oliva-Teles, 2002), others have failed to demonstrate such an effect (Enes et al., 2008; Lanari et al., 1999; Moreira et al., 2008). Enes et al. (2011) have reported that European sea bass and gilthead sea bream juveniles can ingest up to 20% digestible carbohydrates without adverse effects on growth or feed utilization. Besides individual differences between species, the main factors affecting carbohydrate digestibility in fish are dietary inclusion level, molecular

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complexity, botanical origin, technological treatments applied, and water and temperature (Krogdahl et al., 2005; Stone, 2003; Venou et al., 2003; Wilson, 1994). In this respect, wheat has been described as a high-digestible carbohydrate source (Venou et al., 2003). Due to their lower amylose/amylopectin ratio and their structure, starch granules are more vulnerable to damage by industrial processing and attack by amylases (Bergot, 1993; Cousin et al., 1996).

Digestion and absorption processes are the key to successful utilization of a given diet and feed timing. Digestion processes begin in the stomach, where HCl converts pepsinogen into active pepsin (Wu et al., 2009; Yúfera et al., 2004). When the chyme passes into the pyloric caeca, cholecystokinin is secreted, in turn stimulating pancreatic secretions which include, among other substances, proproteases, α-amylase and lipolytic enzymes, as well as DNAase and RNAase (Bakke et al., 2011). The activity of pancreatic enzymes in fish has been studied in relation to the influence of diet composition, food quantity and the natural diet (Buddington et al., 1997; Hidalgo et al., 1999; Pérez-Jiménez et al., 2009; Reimer, 1982; Santigosa et al., 2008, 2011a,b; Zambonino Infante & Cahu, 2007). The activity of the main digestive enzymes, such as proteases, lipase and amylase, may be one of the most important parameters that determine the effectiveness of a given diet, optimizing growth and food utilization (Debnath et al., 2007; Lemieux et al., 1999; Mohanta et al., 2008).

Absorption processes occur throughout the entire intestine by means of diffusion, facilitated transport or active transport (Mailliard et al., 1995). In fish, the presence has been described of at least four Na^+-dependent and Na^+-independent amino acid transport systems (Storelli et al., 1989) and two peptide transporters (Hakim et al., 2009; Sangaletti et al., 2009; Terova et al., 2009). The apical translocation of lipolytic products in fish is not well understood and seems to occur by diffusion or facilitated transport, while D-glucose and D-galactose are transported by SGTL1 (Sala-Rabanal et al., 2004). Santigosa et al. (2011a,b) have described modifications in the absorption pattern after a short starvation period, or as a consequence of low availability of certain amino acids due to fish meal replacement.

Those processes are also affected by biological rhythms, which are driven by endogenous oscillators that affect physiological, behavioral, endocrine and metabolic variables, increasing the probability of success and optimizing energy use (Dardente and Cermakian, 2007; DeCoursey, 2004). The study of feeding behavior in several fish species has revealed that adjusting feeding times to match natural rhythms improves nutritional efficiency, feeding frequency and food conversion efficiency (Bolliet et al., 2001), and can also improve growth performance in many commercially cultured species such as rainbow trout (Boujard et al., 1995; Reddy et al., 1994), European sea bass (Azzaydi et al., 1999) and channel catfish (*Ictalurus punctatus*) (Noeske-Hallin et al., 1985). Food-anticipatory activity confers an adaptative advantage as it may improve food acquisition and utilization (Comperatore and Stephan, 1987).

In order to study the protein-sparing effect by carbohydrates and determine the best time for administration, we analyzed the effect on digestive and absorptive processes in sea bream fed a commercial diet of including one feed of a high-digestible carbohydrate diet in the dietary regime, administered in the morning or in the afternoon.

2. Materials and methods

2.1. Diets

Two diets were used, a commercial diet (C) (D-2 EXCEL 1P Skretting, Spain) and a high-digestible carbohydrate diet (CH) (University of Valencia, Spain). C diet contained 48% protein, 20% lipids, 8% ashes and 3.2% fiber, and had as ingredients: fish meal, soybean meal, fish oil, soybean oil and wheat. "EXCEL" diets contained 27% fish meal and 9% fish oil (García, 2012). The proximate composition of CH diet was 37% protein, 12.5% lipids, 8.5% ashes, 1.8% fiber and 40% high-digestible carbohydrates (calculated according Venou et al., 2003). The ingredients of CH diet were fish meal, fish oil and gelatinized wheat starch; that was the only source from vegetable origin.

2.2. Fish and sampling

Gilthead sea bream juveniles from Cripesa (Tarragona, Spain) were acclimatized for 2 weeks to the facilities at the University of Barcelona. After that period, 180 sea bream (±21 g body weight) were randomly distributed in 9 fiberglass tanks (400 L) equipped with a semi-closed recirculating system with physical and biological filters, ozone, and continuous aeration with a 35% weekly sea water renewal rate, and maintained at 24 °C with a 12 L/12D photoperiod. Water parameters such as temperature, oxygen content, pH, nitrate and nitrite content were recorded daily. The animals were fed twice a day (10:00 am and 17:00 pm) for an 8-week period (November–January).

Three experimental groups were studied in triplicate: commercial diet (C), a high-digestible carbohydrate diet in the morning (CH-M) and a high-digestible carbohydrate diet in the afternoon (CH-A). The daily ration was adjusted to 2.6% of total body weight, 1.6% in the morning and 1.0% in the afternoon. The quantity of the morning and afternoon ration was calculated according to the amount of feed that fish ate during the acclimatization period.

At the end of the 8-week experimental trial, two samples were collected at 5 h post-feeding, one after the morning and one after the afternoon meal. Fish were anesthetized (MS222 0.1 g L^{-1}), weighed and sacrificed by severing the spinal cord. The SGR was calculated as follows: $((\ln W_{fin}\ (g) - \ln W_{ini}\ (g))/t) \cdot 100$, where W_{fin} and W_{ini} were the final and initial weights respectively, and t was the number of feeding days. The digestive tract of eight fish per treatment was isolated, and relative intestinal length (RIL) was measured excluding pyloric caeca, expressed in relation to each animal's weight (Santigosa et al., 2008). Samples were also collected from stomach, pyloric caeca and proximal intestine, including the intestinal content. These were rapidly frozen in liquid nitrogen and maintained at −80 °C until enzymatic analyses. In addition, the intestinal tract of four animals per treatment was isolated and cut lengthwise, washed in an isosmotic saline solution containing 0.1 M protease inhibitor (phenyl-methyl-sulfonyl-fluoride) and frozen in liquid nitrogen until nutrient absorption experiments were performed. All fish-handling procedures complied with European guidelines on animal care (Directive 2010/63/EU).

2.3. Acid protease activity

Stomach samples were collected in order to determine acid protease activity according to Alarcón et al. (1998). Briefly, samples were individually homogenized (Politron 2000, Sorvall TC) at 4 °C to a final concentration of 50 mg mL^{-1} in 50 mM Tris–HCl buffer pH 7.5; this was centrifuged for 15 min (1100 g, 4 °C, Jouan CR 411) and supernatants were recovered and stored at −80 °C. For acid protease activity determination, homogenates from stomach were reacted with 50 mM glicine–HCl buffer at pH 2 containing 1% bovine hemoglobin at 20 °C for 30 min. After that, the reaction was stopped by adding 12% trichloroacetic acid. The samples were kept at 4 °C for an hour and centrifuged (7500 *g*, 5 min, 4 °C). Individual blanks for each sample were established. Supernatant absorbance was measured at 280 nm (UV-1603, Shimadzu). Pepsin from porcine gastric mucosa (Sigma Aldrich, Spain, 3440 U/mg solid) was used as standard and acid protease activity was measured as BAEE units.

2.4. Intestinal pH and digestive enzyme analysis

The pH of intestinal content was measured (Crison, micro pH 2000) in pyloric caeca (PC) and proximal intestine (PI). The pH in PC was

7.08 ± 0.01, and 7.35 ± 0.02 in PI. Intestinal sample homogenization, alkaline protease activity, trypsin and chymotrypsin-like activities and α-amylase were determined using a modification of the procedure described by Santigosa et al. (2008), and lipase was determined following Santigosa et al. (2011a). Alkaline protease activity from intestinal segments was determined as described above, except that the incubation buffer containing 50 mM Tris–HCl buffer had the pH of the intestinal content and 1% casein. Bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as standard and alkaline protease activity was measured as BAEE units. Trypsin-like and chymotrypsin-like activities were characterized by zymography using 12% polyacrylamide gels ($10 \times 10.5 \times 0.1$ cm). Samples, a commercial weight marker RPN 800 (Amersham, GE Healthcare, UK, 12,000–225,000 Da) and pure trypsin and albumin as controls, were loaded and electrophoresis was performed at a constant current of 15 mA per gel for 150 min (EPS 301 Power Supply, 4 °C). The gels were incubated under agitation in intestinal content pH Tris–HCl buffer containing 2% casein (30 min, 4 °C), and collected after 90 min of shaking at room temperature. Gels were washed and stained in a methanol: acetic acid:water solution (40:40:10) with 0.1% BBC R-250 (Coomassie Brilliant Blue R-250). Destaining was carried out using the same solution without colorant. Trypsin-like and chymotrypsin-like activities were characterized using specific inhibition solutions according to the procedure described by Alarcón et al. (1998). Six proteases were detected; three presented trypsin-like activity (90, 60 and 55 kDa) and the other ones showed chymotrypsin-like activity (50, 30 and 25 kDa) (Fig. 1).

α-Amylase activity was measured using the tracer material, amylose azure, a potato starch covalently labeled with Remazol Brilliant Blue R dye. A 0.5% tracer solution containing soybean trypsin inhibitor (SBTI 0.04 mg mL^{-1}) to prevent proteolysis was reacted with the homogenate for 30 min, after which 6% acetic acid was added. The mix was centrifuged (13,000 g; 10 min 4 °C) and supernatant absorbance was measured at 595 nm. α-Amylase (Sigma Aldrich, Spain, 66U/mg solid) was used as standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 and 20 °C. PI/PC ratios of protease and α-amylase activities were calculated in order to determine how intestinal transit was affected by the dietary treatment.

For lipase determination, homogenates were mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester), 0.05 $CaCl_2$, 30 mannitol and 1 mg L^{-1} colipase (pH 8.3), and the increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33,944 U/mg protein, 22,980 U/mg solid) was used as a standard. One unit hydrolyzes 1.0 micro equivalent of fatty acid from triacetin in 1 h at pH 7.4 and 20 °C.

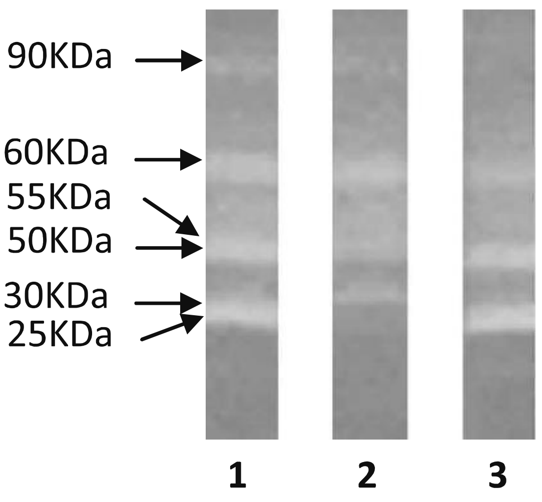

Fig. 1. Inhibition zymogram. Lane 1: homogenate (H), Lane 2: H + ZPCK (chymotrypsin inhibitor) and lane 3: H + TLCK (trypsin inhibitor). Figure shows the molecular weight of bands with proteolytic activity.

The Bradford method (1976) was used to determine the homogenate protein concentration, using bovine albumin as a standard.

2.5. Nutrient uptake analysis

Brush border membrane vesicles (BBMVs) from whole intestine were obtained as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, 100 Osm) to separate mucosa from the muscular layers. Selective precipitation of basolateral and mitochondrial membranes was achieved through the addition of $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugations enabled purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe. Alkaline phosphatase activity, which has been described as a good marker of BBMVs, was determined following Weiser (1973) in order to determine enrichment in brush border membrane preparations. The capacity to absorb L-lysine, an essential amino acid, L-alanine, a non-essential one, and D-glucose was determined in vesicular suspensions according to the procedure described by Santigosa et al. (2011b). These were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabeled nutrient, 0.01 ^3H-nutrient, 320 Osm, pH 7.4) for 5 s. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, 320 Osm, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22-μm cellulose nitrate filters (Millipore, Bedford MA) previously soaked in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were performed at 20 °C. Radiolabeled nutrients were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).

Changes in vesicular volume, due to diet composition (Santigosa et al., 2011a,b), rendered it necessary to normalize influx values. In order to determine vesicular volume, L-alanine retained inside the vesicles at equilibrium was measured by incubating a BBMV preparation with incubation buffer on ice (Sala-Rabanal et al., 2004). After 90 min, the reaction was stopped and the amount of L-alanine was measured following the procedure describe above.

The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford method (1976).

2.6. Statistical analysis

Significant differences between groups were determined by one-way ANOVA followed by Tukey's post hoc test. Differences between meal times were determined using the Student's *t*-test ($P < 0.05$). The software used was PASW Statistics 17.0 (SPSS Inc., EUA).

3. Results

Digestive enzyme activities were studied at 5 h post-feeding after morning and afternoon meals in stomach, pyloric caeca and proximal intestine for each experimental condition.

Fig. 2 shows the results for acid protease activity. At 5 h post-feeding, the stomach will be empty, indicating that any activity detected will correspond to food-anticipatory activity related to the next feed. Lower acid proteolytic activity was measured for sea bream in the afternoon (26.68 ± 1.89 U pepsin mg^{-1} prot) than in the morning (36.46 ± 2.13), consistent with the hours until the next meal (11 h to the morning feeding and 3 h to the afternoon one). No differences

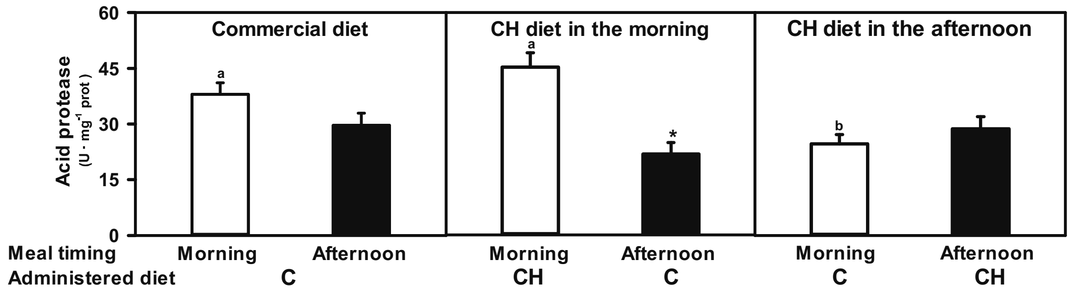

Fig. 2. Stomach acid protease activity in the morning (white bars) and in the afternoon (black bars). Values are expressed as the mean ± SEM of 8 fish. Significant differences ($P < 0.05$) between diets are shown by different letters and significant differences between meal times are shown by an asterisk.

were detected between experimental groups after the afternoon meal, whereas after the morning feed, sea bream fed the C diet in the afternoon showed higher anticipatory activity than those fed the CH diet. Significant differences for CH-M sea bream were observed between morning and afternoon feeding. These results suggest that when CH was included in the dietary regime, the pepsin profile changed depending on the time of administration.

Fig. 3 shows alkaline protease, α-amylase and lipase activities measured in PC and PI 5 h post-feeding after the morning or the afternoon meals, for each dietary regime. Alkaline protease activity was higher than α-amylase and lipase activities by a factor of 10^3, indicating a limited ability to digest lipids and carbohydrates compared with protein. Fish fed an exclusively commercial diet showed higher pancreatic enzyme activities (alkaline protease, α-amylase and lipase) after the morning meal, consistent with the ration administered (1.6% in the morning versus 1% in the afternoon). Inclusion of the CH diet in the morning affected pancreatic enzyme activities in the afternoon meal. In this respect, when alkaline protease, α-amylase and lipase activities were measured 5 h after the afternoon feed, CH-M sea bream showed significantly higher activities than C fish. Moreover, CH-M animals presented the highest detected protease activity (85.35 ± 13.39 U protease mg^{-1} protein) of all conditions. However, when the CH diet was administered in the afternoon, no significant differences in digestive enzyme activities measured 5 h after the morning feed were found between CH-A and C sea bream. Neither were any significant differences in lipase activity detected between dietary regimes, although higher activity was measured after the morning feed in C animals, whereas when the CH diet was included in the dietary regime, higher activity was detected after the afternoon meal.

The ratio between proximal intestine and pyloric caeca enzymatic activities provides an indication of the intestinal transit rate; thus, higher activity in the PI versus the PC indicates higher intestinal transit rate. Sea bream fed the commercial diet in the morning showed significantly higher alkaline protease and α-amylase activity in PC than in PI. In contrast, when sea bream were fed the CH diet in the morning, higher activity of both enzymes was detected in PI, indicating an increase in the intestinal transit rate; this increase was also detected when the CH diet was given in the afternoon. C animals showed an increase in intestinal transit rate for protease activity when afternoon versus the morning feeding was compared, whereas no changes in this parameter were

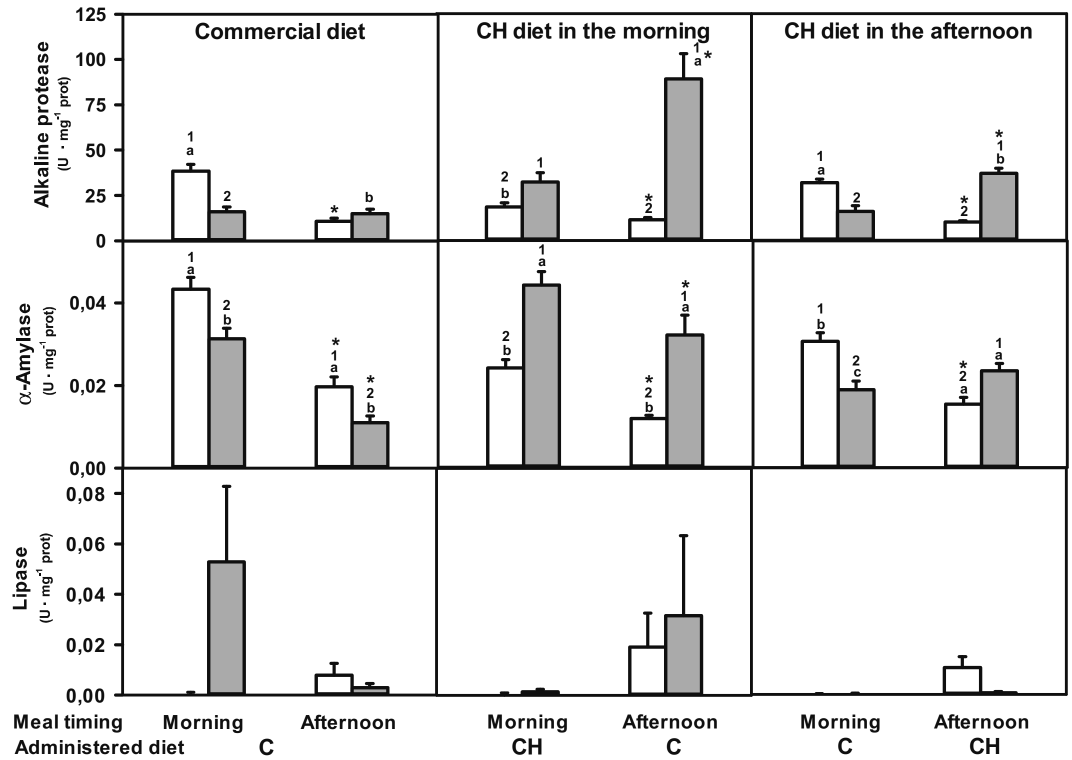

Fig. 3. (A) Total protease, B) α-amylase and C) lipase activity in pyloric caeca (white bars) and proximal intestine (gray bars). Values are expressed as the mean ± SEM of 8 fish. Significant differences ($P < 0.05$) between diets are shown by different letters; significant differences between intestinal segments ($P < 0.05$) are shown by different numbers and significant differences between meal times are shown by an asterisk.

detected for α-amylase activity. After the afternoon feed, C and CH-M sea bream showed significant differences in intestinal transit rate, although they were fed the same diet and ration, and the highest intestinal transit rate was observed when the CH diet was given in the morning. However, when the CH diet was given to fish in the afternoon, the transit rate detected 5 h after the morning feed was that expected for the diet and ration administered.

Fig. 4 shows model zymograms of PC and PI 5 h after feeding for each experimental group. According to the alkaline protease measured, sea bream fed the CH diet in the morning showed little activity in PC (L5) of all the proteases detected compared with the other groups after the morning meal (L1 and L9), while no differences were found between conditions after the afternoon meal (L3, L7 and L11). Changes in PI zymography were also consistent with the alkaline protease measured, increasing in the afternoon when the CH diet was included in the feeding regime in the morning (L8 versus L6).

Nutrient absorption capacity was measured in BBMVs from whole intestine 5 h post-feeding (Fig. 5). When the CH diet was included in the dietary regime, L-Lys absorption capacity tended to be up-regulated after the morning feed, attaining statistical significance for CH-A animals. No differences were detected between the experimental conditions when L-Lys uptake capacity was analyzed after the afternoon feed. On the other hand, L-Ala and D-Glc absorption capacity was up-regulated when the CH diet was incorporated into the dietary regime of sea bream in the morning. An up-regulation in D-Glc and L-Ala absorption was also detected in animals fed the commercial diet in the afternoon versus morning.

RIL was measured in order to determine intestinal adaptation to diet, but no differences were found for RIL between experimental conditions (25.6 ± 1.5; 22.6 ± 1.4 and 24.1 ± 2.0 for C, CH-M and CH-A, respectively).

Lastly, no significant differences were found in SGR, but a tendency towards lower growth was detected for CH-A animals (1.28 ± 0.06, 1.28 ± 0.06 and 1.12 ± 0.05 for C, CH-M and CH-A, respectively).

4. Discussion

Fish are capable of regulating their intake to meet energy and nutrient requirements according to their natural feeding rhythms (Boujard and Leatherland, 1992; Kaushik and Médale, 1994). Several studies have been conducted on the effects of daily rhythms on food-anticipatory activity, hormones and feeding frequency in sea bream (López-Olmeda et al., 2009; Sánchez et al., 2009). Furthermore, Madrid (1994) reported different levels of nutrient assimilation when the meal times of a given diet were changed. Thus, synchronization of the biological rhythms of synthesis, activation or secretion of digestive and/or metabolic enzymes leads to a better diet improvement (Sánchez-Muros et al., 2003). Furthermore, Montoya et al. (2010) described an increase in digestive enzyme activity some hours before meal time, indicating a strong effect of feeding. Based on these premises, in the present study we sought to determine the best time for carbohydrate administration in order to leverage the protein-sparing effect without compromising nutrient assimilation and/or fish growth.

It has been reported that fish size, growing temperature, ration and ingredients used in diet formulations can affect gastric content evacuation. Thus, Guillaume and Choubert (2001) reviewed that gastric evacuation time increased with fish size and ration and decreased with temperature. When plant-based ingredients are included in the diet, gastric evacuation time is higher, increasing more with legumes than with cereals (Adamidou et al., 2009; Storebakken et al., 1999). Moreover, Venou et al. (2003) reported that differences in ingredient processing can also modify gastric evacuation time, which is higher for extruded cereals than for raw cereals. It has also been described that the amount of feed present in the stomach determines content pH (Márquez et al., 2012). In their study of sea bream fed replacement diets, García-Meilán et al. (2013) found that at 5 h post-feeding, 57% of fish had an empty stomach with a pH close to 6.6, while 43% still had stomach content but with a pH (approximately 4) that was far from optimal for pepsinogen activation (Yúfera et al., 2012). In the present study, similar gastric evacuation at 5 h post-feeding was observed in small sea bream fed a smaller ration and similar diet; thus, the acid protease activity measured reflected food-anticipatory activity. Irrespective of the diet administered in the morning, this activity was higher when the C diet was given to fish in the afternoon, indicating an adaptation to diet protein content to improve protein digestion. Lawlor et al. (2005) tested the buffering capacity of several ingredients present in animal diets, and showed that cereals had a lower buffering capacity than plant protein meals, and that these had a lower capacity than fish meal and gluten. Thus, the ingredients used in replacement diets modify feed buffering capacity, which could affect intestinal pH and consequently modulate digestive enzyme activities. In this study, no differences in intestinal content pH were detected between diets even though the plant replacement component was higher in the C than CH diet (over 60% versus 47%); in the C diet, this principally is composed of a blend of soybean meal, soybean oil, and wheat; whereas in the CH diet, it consisted exclusively of wheat. In both situations, intestinal pH was similar to that reported in literature for this species (García-Meilán et al., 2013), although it was far from optimal for intestinal enzyme performance (Alarcón et al., 1998; Fernández et al., 2001).

The pattern of feeding, 1.6% in the morning and 1% in the afternoon, was established according to the amount of feed that fish ate during the acclimatization period. This determines nutrient delivery to the intestinal lumen, which is the most important stimulus of exocrine pancreatic secretion of digestive enzymes in fish and mammals (Krogdahl and Sundby, 1999) and also regulates nutrient absorption capacity (Santigosa et al., 2011b). Thus, in C animals, alkaline protease and α-amylase activities were higher after the morning feed compared with the afternoon one, probably due to the higher amount of protein and starch that fish received in the morning. Consistent with these results, several authors have described an increase in alkaline protease activity as dietary protein increases (Bakke et al., 2011; García-Meilán et al.,

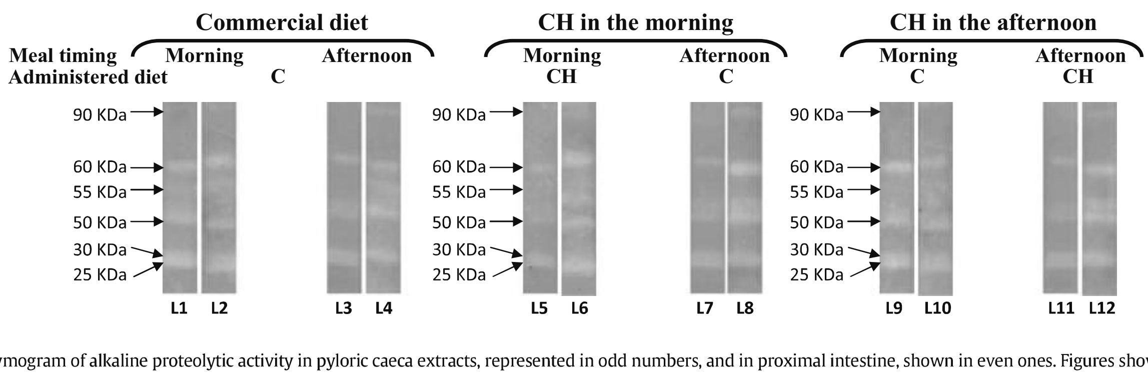

Fig. 4. Model zymogram of alkaline proteolytic activity in pyloric caeca extracts, represented in odd numbers, and in proximal intestine, shown in even ones. Figures show the molecular weight of bands with proteolytic activity. All samples were analyzed individually; the figure shows a representative result.

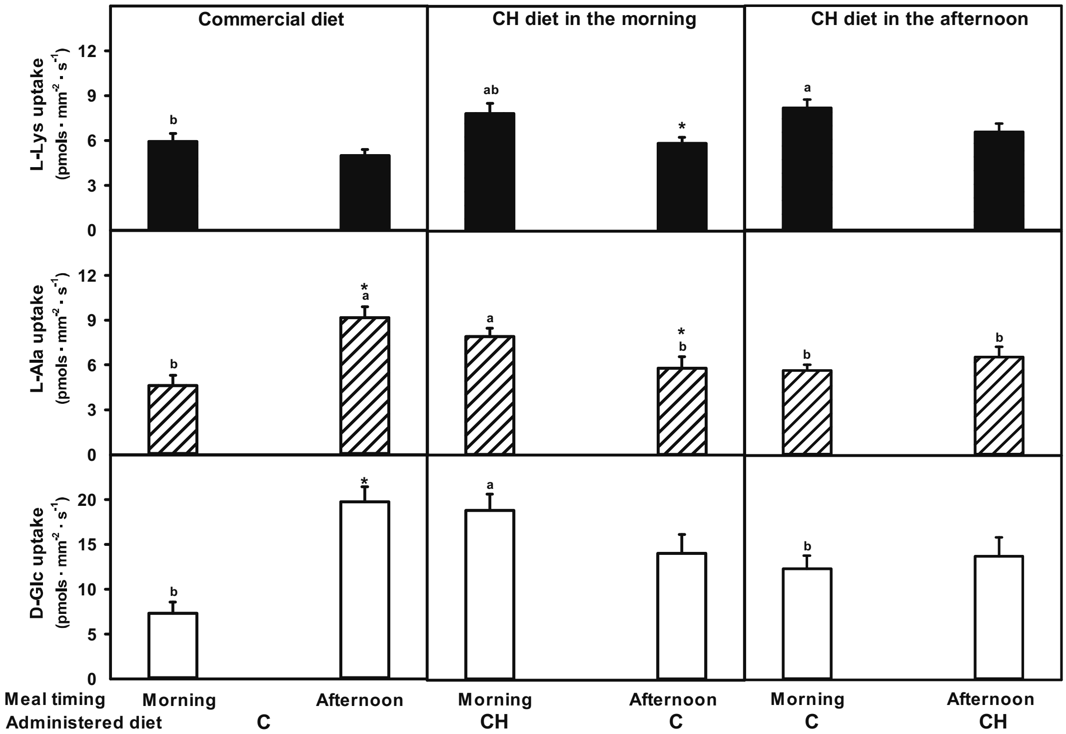

Fig. 5. Absorption capacity of L-Lys (black bars), L-Ala (hatched bars) and D-Glc (white bars) in brush border membrane vesicles obtained from whole intestine from sea bream after the morning meal and afternoon meal. Results are expressed as the mean ± SEM of 4 determinations. Different letters indicate significant differences ($P < 0.05$) between diets and significant differences between meal times are shown by an asterisk.

2013; Krogdahl et al., 2003; Péres et al., 1998). As regards amylase activity, Krogdahl et al. (2005) found that this was regulated in herbivorous and omnivorous species according to starch intake, whereas this relationship was unclear for carnivorous species. Furthermore, the intestinal transit rate showed that with a smaller ration (1%), there was a higher digestion of protein than starch. Similarly, Buddington et al. (1997) reported that protease activity can be modulated by diet, whereas α-amylase activity seems to be more constitutive. Moreover, low starch diets have been related to a higher capacity to absorb the nutrients D-Glc and L-Ala (García-Meilán et al., 2013; Santigosa et al., 2011b), and we observed an increase in the capacity to absorb both nutrients in C sea bream fed in the afternoon. Differences in diet composition, such as high lipid levels in the C diet or high starch content in the CH diet, may be involved in the differential transit rate found after feeding. In their study of sea bream, Fountoulaki et al. (2005) found that a low transit rate was related to a high lipid content, and Spannhof and Plantikov (1983) detected an increase in the intestinal transit rate of trout when a high amount of starch was included in the diet. We found a differential regulation in the intestinal transit rate of CH-M sea bream after afternoon feeding, associated with an increase in digestive enzyme activities. This increase led to improved intestinal nutrient availability, especially with regard to dietary protein content, in order to compensate for the lower protein content of the CH-M versus C diet (−14.1%). In contrast, CH-A sea bream (−8.8% dietary protein versus C animals) did not show this up-regulation of digestive enzyme activities. Moreover, fish fed the CH diet showed an up-regulation of the capacity to absorb L-Lys, presumably in order to compensate for lower amino acid availability. Up-regulation of the capacity to absorb essential amino acids has also been described in short starvation periods by Santigosa et al. (2011b). We also observed an up-regulation of the capacity to absorb D-Glc and L-Ala when sea bream were fed the CH diet in the morning, a better energy improvement that was not detected in CH-A animals. These compensatory mechanisms described above enabled CH-M fish to achieve similar growth to C sea bream, indicating a protein-sparing effect when carbohydrates were fed to sea bream in the morning but not when CH were given in the afternoon.

Other possible factors that could affect digestive enzyme activities include anti-nutritional elements present in plant ingredients (Francis et al., 2001; Gatlin et al., 2007, Krogdahl et al., 2010; Turchini et al., 2009) or differences in chyme emulsification due to dietary lipid content (Santigosa et al., 2011a). Moreover, the α-amylase activity measured was higher than that found in other trials (García-Meilán et al., 2013; Santigosa et al., 2008, 2011a), probably due to the high presence of starch in diet. As Enes et al. (2011) suggested, such carbohydrases saturation could affect starch digestibility as the amount included in the diet is increased. Lipase activity is not affected by high lipid content diets (García-Meilán et al., 2013; Santigosa et al., 2011b) or by high-digestible carbohydrates, as in the present case.

According to García-Meilán et al. (2013), six proteolytic bands were detected in intestinal zymograms. Thus, the three bands with higher molecular weight presented trypsin-like activity (90, 60 and 55 kDa) and the bands with lower molecular weight showed chymotrypsin-like activity (50, 30 and 25 kDa). It is known that the pancreatic release of proteolytic enzymes and the trypsin/chymotrypsin ratio were affected by the percentage of dietary protein and also by the plant sources included in diet (García-Meilán et al., 2013; Santigosa et al., 2008, 2011a). Furthermore, the incorporation of carbohydrates of diet modified the release of trypsin and chymotrypsin by not their ratio.

In conclusion, the inclusion of high-digestible carbohydrates in the diet improved digestion and absorption processes when administered in the morning, leading to a protein-sparing effect that yielded growth comparable to that of fish fed an exclusively commercial diet.

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